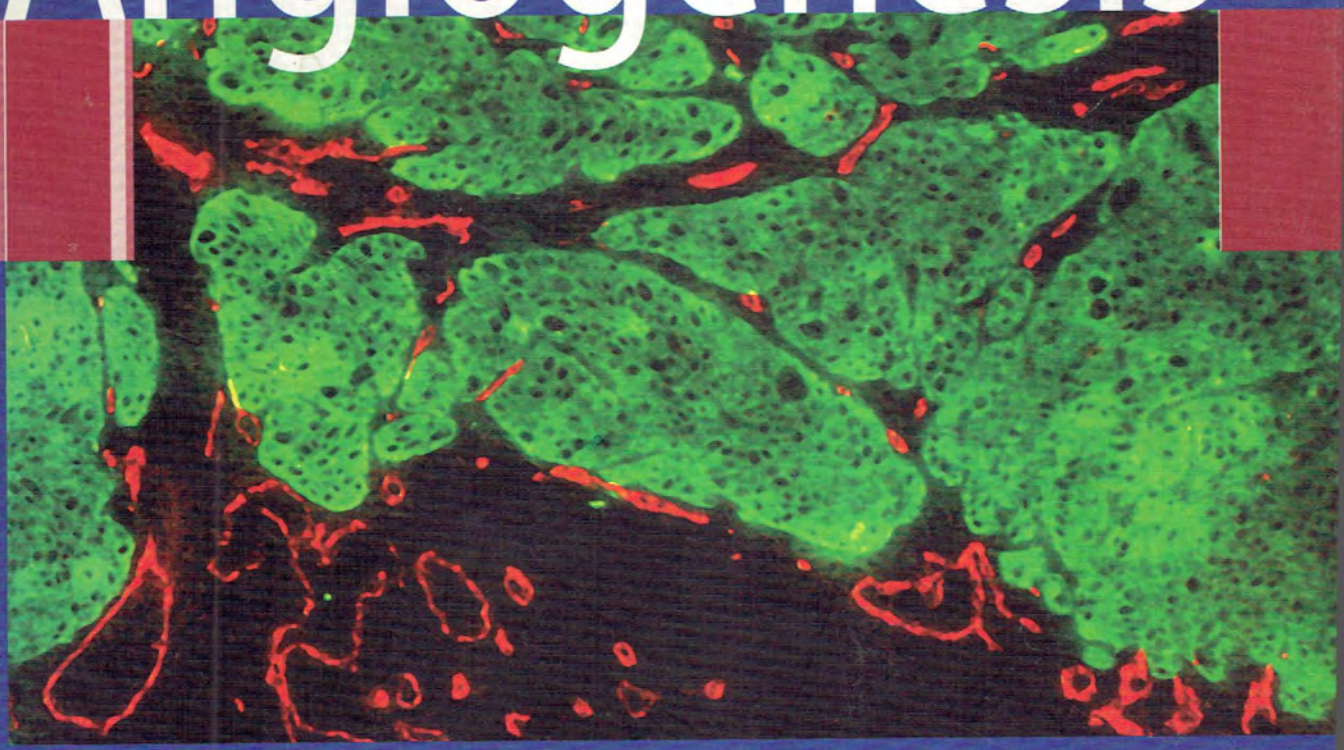
Dieter Marmé
Norbert Fusenig *Editors*

Tumor Angiogenesis



Basic Mechanisms and Cancer Therapy

Springer

Dieter Marmé · Norbert Fusenig (Editors)

Tumor Angiogenesis

Basic Mechanisms and Cancer Therapy

With Contributions by Numerous Experts

With 187 Figures in 338 Separate Illustrations in Color and 72 Tables

Springer

Dieter Marmé, PhD
Professor, Klinik für Tumorbiologie
Breisacher Str. 117
79106 Freiburg i. Br.
Germany

Norbert Fusenig, MD
Professor, former Head of Division of Carcinogenesis and Differentiation
DKFZ
Im Neuenheimer Feld 280
69120 Heidelberg
Germany

Library of Congress Control Number: 2007920488

ISBN 978-3-540-33176-6 Springer Berlin Heidelberg New York

Springer is part of Springer Science+Business Media

http//www.springer.com

Printed in Germany

Medical Editor: Dr. Ute Heilmann, Heidelberg
Desk Editor: Dörthe Mennecke-Bühler, Heidelberg
Production Editor: Kurt Teichmann, Mauer
Cover-Design: Frido Steinen-Broo, eStudio Calamar, Spain
Typesetting: Verlagsservice Teichmann, Mauer

Printed on acid-free paper - 21/3180xq - 5 4 3 2 1 0

Preface

Tumor development and progression occur as a result of cumulative acquisition of genetic alterations affecting oncogenes and tumor suppressor genes. As a consequence of these alterations the arising tumor gains some fatal properties such as increased cell proliferation and decreased apoptosis, resulting in a net accumulation of transformed cells. Once a critical volume is achieved, lack of oxygen and nutrients limits further growth. To overcome this obstacle, the tumor cells initiate a program focused on the formation of new blood vessels within the host tissue. This process is termed tumor angiogenesis and contributes to the progression of most solid tumors and the formation of metastases.

Since its discovery more than 30 years ago by Dr. Judah Folkman, tumor angiogenesis has been proposed as an ideal target for novel tumor therapies. Today the first anti-angiogenic compounds are available for the treatment of patients but their success in the clinic is rather limited when given as monotherapies. This is in contrast to many preclinical results which revealed a much higher efficacy of these therapeutics in appropriate animal models. The reasons for this discrepancy are manifold, one being the existence of more than one angiogenic signaling system capable of driving tumor angiogenesis. Therefore it is no surprise that the inhibition of just one system is not sufficient to block the formation of new blood vessels in patients. With these facts in mind, novel angiogenic targets have been identified and validated as a basis for the development of new anti-angiogenic drugs.

Because of the complexity of the angiogenic process and the need for novel strategies to evaluate anti-angiogenic therapeutics in the clinic, we decided to bring together the most renowned researchers and clinicians in this area to review the many facets of the problem and to explore modalities by which anti-angiogenic therapies could be made more effective for the patients. The message from their contributions is clear: Tumor angiogenesis is a very attractive target for novel cancer therapies. However, there are still many white areas on the map showing the entire process in human cancer.

A cooperative effort of researchers and clinicians in both academia and the pharmaceutical industry will be needed to fill the white areas with knowledge, to identify and validate the crucial targets, and to develop novel strategies to block their activi-

ties. Anti-angiogenic therapy is a cornerstone in cancer treatment. Now we have to learn how to combine these therapies with other targeted therapies for the greatest benefit of the individual patient.

We express our deepest gratitude to all our colleagues from academia and industry who have made this book the first comprehensive anthology covering all major aspects of tumor angiogenesis and bridging the gap between bench and bedside.

Freiburg — Dieter Marmé
Heidelberg — Norbert Fusenig

Contents

Contributors

Till Acker
Institute of Neurology (Edinger Institute)
Neuroscience Center, 4th&5th Floor
Heinrich-Hoffmann-Straße 7
60528 Frankfurt/Main
Germany

Dr. Kari Alitalo
Professor, University of Helsinki
Department of Pathology
Cancer Biology Laboratory
Haartmaninkatu 3
290 Helsinki 29
Finland

Zuleima Aguilar
Oncology Clinical Development
Pfizer Global Research and Development
Pfizer Inc.
10578 Science Center Drive (B95)
San Diego, CA 92121
USA

Hellmut G. Augustin, DVM, PhD
Aventis Foundation-endowed Chair for Vascular Biology
Medical Faculty Mannheim
University of Heidelberg, and
German Cancer Research Center Heidelberg (DKFZ)
Im Neuenheimer Feld 581
69120 Heidelberg
Germany

Monica Autiero
Center for Transgene Technology and Gene Therapy
Flanders Interuniversity Institute for Biotechnology
Herestraat 49
3000 Leuven
Belgium

Megan Baldwin, PhD
Genentech, Inc.
Dept. of Tumor Biology and Angiogenesis
1 DNA Way
South San Francisco, CA 94080
USA

Peter Baluk, PhD
Cardiovascular Research Institute
University of California
513 Parnassus Avenue
San Francisco, CA 94143-0130
USA

Alan Barge
Astra Zeneca Pharmaceuticals
F433 Alderley House
Alderley Park
Macclesfield, Cheshire, SK10 4TG
UK

Carlo Bello
Oncology Clinical Development
Pfizer Global Research and Development
Pfizer Inc.
10578 Science Center Drive (B95)
San Diego, CA 92121
USA

Francesco Bertolini
Division of Hematology-Oncology
Department of Medicine
European Institute of Oncology
Via Ripamonti 435
20141 Milano
Italy

Dr. Christer Betsholtz
Professor, Karolinska Institutet
Cancer Center Karolinska, R8:03
Dept. of Oncology-Pathology
Karolinska Institutet
SE-171 76 Stockholm
Sweden

Laurence M. Boon, MD, PhD
Professor, Centre for Vascular Anomalies
Department of Plastic Surgery
Cliniques universitaires St-Luc
Université catholique de Louvain
Avenue Hippocrate 10
B-1200 Brussels
Belgium

Dr. Peter Bohlen
2237 Cortina Circle
Escondido, CA 92029
USA

Dr. Karine Bouvrée
INSERM U36
11 Place Marcelin Berthelot
75005 Paris
France

M. Christiane Brahimi-Horn
Institute of Signaling
Developmental Biology and Cancer Research
CNRS UMR 6543
Centre A. Lacassagne
33 Avenue Valombrose
F-06189 Nice
France

Dana M. Brantley-Sieders
A-4323, MCN
Department of Medicine
Vanderbilt University School of Medicine
1161 21st Ave South
Nashville, TN 37232
USA

Dr. Peter Carmeliet
Professor, Center for Transgene Technology
and Gene Therapy
Flanders Interuniversity Institute for Biotechnology
Herestraat 49
3000 Leuven
Belgium

Ugo Cavallaro, PhD
IFOM – FIRC Institute of Molecular Oncology
Via Adamello 16
I-20139 Milano
Italy

Dr. David J. Chaplin
OXiGENE Inc.
Magdalen Centre
Robert Robinson Avenue
The Oxford Science Park
Oxford OX4 4GA
UK

Emmanouil Chavakis
University of Frankfurt
Molecular Cardiology
Theodor-Stern-Kai 7
60590 Frankfurt
Germany

Isan Chen, MD
Director, Oncology Clinical Development
Pfizer Global Research and Development
Pfizer Inc.
10578 Science Center Drive (B95)
San Diego, CA 92121
USA

Professor Jin Chen
A-4323, MCN
Department of Medicine
Vanderbilt University School of Medicine
Vanderbilt Ingram Cancer Center
1161 21st Ave South
Nashville, TN 37232
USA

Dr. Gerhard Christofori
Professor, Institute of Biochemistry and Genetics
Department of Clinical-Biological Sciences
University of Basel
Center for Biomedicine
Mattenstraße 28
4058 Basel
Switzerland

Dr. med. Marcus Czabanka
Department of Neurosurgery
Universitätsmedizin Charité Berlin
Campus Virchow-Klinikum
Augustenburger Platz 1
13353 Berlin
Germany

Dr. Patricia A. D'Amore
Schepens Eye Research Institute
20 Staniford Street
Boston, MA 02114
USA

Stefanie Dimmeler, PhD
University of Frankfurt
Molecular Cardiology
Theodor-Stern-Kai 7
60590 Frankfurt
Germany

PD Dr. Joachim Drevs
Medical Director
Tumorklinik SanaFontis
An den Heilquellen 2
79111 Freiburg i.Br.
Germany

Dan G. Duda, DMD, PhD
Steele Laboratory for Tumor Biology
Department of Radiation Oncology
Massachusetts General Hospital
100 Blossom Street
Cox-734
Boston, MA 02114
USA

J. Dumas
Bayer HealthCare - Pharmaceuticals
400 Morgan Lane
West Haven, CT 06516
USA

Dr. Anne Eichmann
INSERM U36
11 Place Marcelin Berthelot
75005 Paris
France

Michael Elkin, PhD
Department of Oncology
Hadassah-Hebrew University Medical Center
Jerusalem 91120
Israel

Urban Emmenegger
Sunnybrook Health Science Center
Department of Molecular and Cellular Biology Research
Reichmann Research Bldg.
2075 Bayview Avenue
Toronto, Ont M4N 3M5
Canada

Beverly L. Falcón
Cardiovascular Research Institute
University of California
513 Parnassus Avenue
San Francisco, California 94143-0130
USA

Dr. Napoleone Ferrara
Genentech, Inc.
Department of Molecular Oncology
1 DNA Way
South San Francisco, CA 94080
USA

Isaiah J. Fidler, DVM, PhD
Professor and Chairman
The University of Texas
M. D. Anderson Cancer Center
Department of Cancer Biology, Unit 173
1515 Holcombe Boulevard
Houston, TX 77030
USA

Ulrike Fiedler, PhD
ProQinase GmbH
Breisacher Str. 117
79106 Freiburg
Germany

Judah Folkman, MD
Children's Hospital and Harvard Medical School
Karp Family Research Laboratories 12.129
300 Longwood Avenue
Boston, MA 02115-5737
USA

Norbert Fusenig, MD
Professor, DKFZ
Im Neuenheimer Feld 280
69120 Heidelberg
Germany

Liat Fux, PhD
Cancer and Vascular Biology Research Center
The Rappaport Faculty of Medicine
Technion
Haifa 31096
Israel

Hans-Peter Gerber, PhD
Director Translational Biology
Seattle Genetics
21823 30th Drive, SE
Bothell, WA, 98021
USA

Giuseppe Giaccone, MD, PhD
VU Medical Center
Department of Medical Oncology
De Boelelaan 1117
1081 HV Amsterdam
The Netherlands

PD Dr. Axel Glasmacher
Medizinischer Direktor
Celgene GmbH
Joseph-Wild-Str. 20
D-81829 München
Germany

Dr. Hartmut Goldschmidt
Professor, Leiter der Sektion Multiples Myelom
Medizinische Klinik V und
Nationales Centrum für Tumorerkrankungen (NCT)
Im Neuenheimer Feld 410
D-69120 Heidelberg
Germany

Anne Goodwin, PhD
Department of Biology
Massachusetts College of Liberal Arts
375 Church St.
North Adams, MA 01247
USA

Dragan Grabulovski, CSO
Covagen AG
c/o ETH Zürich, Y17 M22
Winterthurstraße 190
8057 Zürich
Switzerland

Hiroya Hashizume
Cardiovascular Research Institute
University of California
513 Parnassus Avenue
San Francisco, California 94143-0130
USA

Daniel J. Hicklin
ImClone Systems
180 Varick Street
New York, NY 10014
USA

Dr. Jens Hillengass
Med. Klinik V
Universitätsklinikum Heidelberg
Im Neuenheimer Feld 410
69120 Heidelberg
Germany

Klaas Hoekman, MD, PhD
VU Medical Center
Department of Medical Oncology
De Boelelaan 1117
1081 HV Amsterdam
The Netherlands

Mark A. Horsfield
University Hospitals of Leicester NHS Trust
Leicester Royal Infirmary
Infirmary Square
Leicester LE1 5WW
UK

Paul E. Hughes
Oncology Research
Amgen Inc.
One Amgen Center Drive
Thousand Oaks, CA 91320
USA

Neta Ilan, PhD
Cancer and Vascular Biology Research Center
The Rappaport Faculty of Medicine
Technion
Haifa 31096
Israel

Dr. Luisa Iruela-Arispe
UCLA
Department of Molecular, Cell & Developmental Biology
611 Charles Young Drive East
Paul Boyer Hall
Room 559
Los Angeles, CA 90095-1570
USA

Alan Jackson
Imaging Science and Biomedical Engineering
University of Manchester
Oxford Road
Manchester M13 9PT
UK

Rakesh Jain, PhD
Steele Laboratory for Tumor Biology
Department of Radiation Oncology
Massachusetts General Hospital
100 Blossom Street
Cox-734
Boston, MA 02114
USA

Gordon Jayson, FRCP, PhD
Professor of Medical Oncology
Cancer Research UK
Department of Medical Oncology
Christie Hospital
Wilmslow Road
Withington
Manchester M20 4BX
UK

Dr. Juliane M. Jürgensmeier
Cancer and Infection Research
AstraZeneca
Mereside, Alderley Park
Macclesfield, Cheshire, SK10 4TG
UK

Dr. Raghu Kalluri
Associate Professor of Medicine
Harvard Medical School
Beth Israel Deaconess Medical Center
330, Brookline Ave (DANA 514)
Boston, MA 02215
USA

Dr. Chryso Kanthou
University of Sheffield
Academic Unit of Surgical Oncology
School of Medicine & Biomedical Sciences
Beech Hill Road
Sheffield S10 2RX
UK

Dr. Richard L. Kendall
Oncology Research
Amgen Inc.
One Amgen Center Drive
Thousand Oaks, CA 91320
USA

Dr. Robert S. Kerbel
Professor, Sunnybrook Health Science Center
Department of Molecular and
Cellular Biology Research
Reichmann Research Bldg.
2075 Bayview Avenue
Toronto, Ont M4N 3M5
Canada

PD Dr. Fabian Kiessling
DKFZ
Juniorgroup Molecular Imaging
Dept. Medical Physics in Radiology
Im Neuenheimer Feld 280
69120 Heidelberg
Germany

Sun-Jin Kim, MD, PhD
The University of Texas
M. D. Anderson Cancer Center
Department of Cancer Biology, Unit 173
1515 Holcombe Boulevard
Houston, TX 77030
USA

PD Dr. Oliver Kisker
Merck KGaA
Frankfurter Straße 250
64293 Darmstadt
Germany

PD Dr.med. Dipl.-Phys. Martin Krix
Bracco-Altana Pharma GmbH
Clinical Research
Max-Stromeyer-Str. 116
78467 Konstanz
Germany

Julie Laferrière
Institute of Signaling
Developmental Biology and Cancer Research
CNRS UMR 6543
Centre A. Lacassagne
33 Avenue Valombrose
06189 Nice
France

Robert R. Langley, PhD
The University of Texas
M. D. Anderson Cancer Center
Department of Cancer Biology, Unit 173
1515 Holcombe Boulevard
Houston, TX 77030
USA

Dr. Jack Lawler
Beth Israel Deaconess Medical Center
Department of Pathology
330 Brookline Ave.
Boston, MA 02215
USA

Nathan V. Lee
UCLA
Department of Molecular, Cell & Developmental Biology
611 Charles Young Drive East
Paul Boyer Hall
Room 559
Los Angeles, CA 90095-1570
USA

Marja Lohela, MSci
Molecular/Cancer Biology Laboratory
Biomedicum Helsinki
University of Helsinki
P.O.B. 63
00014 Helsinki
Finland

Shan Man
Sunnybrook Health Science Center
Department of Molecular and Cellular Biology Research
Reichmann Research Bldg.
2075 Bayview Avenue
Toronto, Ont M4N 3M5
Canada

Patrizia Mancuso
Division of Hematology-Oncology
Department of Medicine
European Institute of Oncology
Via Ripamonti 435
20141 Milan
Italy

Dieter Marmé, PhD
Professor, Klinik für Tumorbiologie
Breisacher Str. 117
79106 Freiburg i. Br.
Germany

Dr. Robert Mass
Genentech, Inc.
1 DNA Way
South San Francisco, CA 94080-4990
USA

Nathalie Mazure
Institute of Signaling
Developmental Biology and Cancer Research
CNRS UMR 6543
Centre A. Lacassagne
33 Avenue Valombrose
06189 Nice
France

Donald M. McDonald
Cardiovascular Research Institute
University of California
513 Parnassus Avenue
San Francisco, California 94143-0130
USA

PD Dr. Thomas M. Moehler
idrei research
Knesebeckstr. 30
10623 Berlin
Germany

Dr. Bruno Morgan
University Hospitals of Leicester NHS Trust
Leicester Royal Infirmary
Infirmary Square
Leicester LE1 5WW
UK

Dr. Margareta Mueller
Deutsches Krebsforschungszentrum (DKFZ)
Im Neuenheimer Feld 280
69120 Heidelberg
Germany

Raquel Munoz
Sunnybrook Health Science Center
Department of Molecular and Cellular Biology Research
Reichmann Research Bldg.
2075 Bayview Avenue
Toronto, Ont M4N 3M5
Canada

Dr. Dario Neri
Swiss Federal Institute of Technology Zürich
Institute of Pharmaceutical Sciences
Wolfgang-Pauli-Str. 10
8093 Zürich
Switzerland

Professor Dr. Gera Neufeld
Technion
Israel Institute of Technology
Cancer and Vascular Biology Research Center
P.O. Box 9697
1 Efron St.
31096 Haifa
Israel

James P.B. O'Connor
Cancer Research UK
Department of Medical Oncology
Christie Hospital
Wilmslow Road
Manchester M20 4BX
UK
and
Imaging Science and Biomedical Engineering
University of Manchester
Oxford Road
Manchester, M13 9PT
UK

Jonathan D. Oliner
Oncology Research
Amgen Inc.
One Amgen Center Drive
Thousand Oaks, CA 91320
USA

Arne Östman, PhD
Professor of Molecular Oncology
Cancer Center Karolinska, R8:03
Dept. of Oncology-Pathology
Karolinska Institutet
SE-171 76 Stockholm
Sweden

Dr. Luc Pardanaud
INSERM U833
Collège de France
11 Place Marcelin Berthelot
75005 Paris
France

Karl H. Plate, MD
Professor and Chairperson
Institute of Neurology (Edinger Institute)
Neuroscience Center, 4th&5th Floor
Heinrich-Hoffmann-Straße 7
60528 Frankfurt/Main
Germany

Anthony Polverino
Oncology Research
Amgen Inc.
1201 Amgen Court West
Seattle, WA 98119
USA

Dr. Jacques Pouysségur
Centre A Lacassagne
CNRS
Institute of Signaling
Developmental Biology and Cancer Research
UMR 6543
33 Avenue Valombrose
06189 Nice
France

Valentina Raia
Division of Hematology-Oncology
Department of Medicine
European Institute of Oncology
Via Ripamonti 435
20141 Milan
Italy

Nicole Revencu, MD
de Duve Institute
Université catholique de Louvain
Laboratory of Human Molecular Genetics (GEHU)
Avenue Hippocrate 74(+5), UCL 75.39
B-1200 Brussels
Belgium

Daniela D. Rosa
Cancer Research UK
Department of Medical Oncology
Christie Hospital
Wilmslow Road
Manchester M20 4BX
UK

Carmen Ruiz de Almodovar
The Centre for Transgene Technology and Gene Therapy
Flanders Institute for Biotechnology
University of Leuven
3000 Leuven
Belgium

Anderson J. Ryan
Astra Zeneca Pharmaceuticals
F433 Alderley House
Alderley Park
Macclesfield, Cheshire, SK10 4TG
UK

Barbara Sennino
Cardiovascular Research Institute
University of California
513 Parnassus Avenue
San Francisco, California 94143-0130
USA

Yuval Shaked
Division of Hematology-Oncology
Department of Medicine
European Institute of Oncology
20141 Milano
Italy

Farbod Shojaei, PhD
Genentech, Inc.
Department of Tumor Biology and Angiogenesis
1 DNA Way
South San Francisco, CA 94080
USA

PD Dr. Jonathan P. Sleeman
Universität Karlsruhe
Institut für Genetik
und
Forschungszentrum Karlsruhe
Institut für Toxikologie und Genetik
Postfach 3640
76021 Karlsruhe
Germany

Dr. Malin Sund
Department of Surgery
Umeå University
SE-90185 Umeå
Sweden

Gillian M. Tozer
Professor, University of Sheffield
Academic Unit of Surgical Oncology
School of Medicine & Biomedical Sciences
Beech Hill Road
Sheffield S10 2RX
UK

Dr. Clemens Unger
Professor, Medical Director
Tumorbiology Center
Breisacher Str. 117
79106 Freiburg i.Br.
Germany

Dr. Carmen Urbich
University of Frankfurt
Molecular Cardiology
Theodor-Stern-Kai 7
60590 Frankfurt
Germany

Professor Dr. med. Peter Vajkoczy
Department of Neurosurgery
Universitätsmedizin Charité Berlin
Campus Virchow-Klinikum
Augustenburger Platz 1
13353 Berlin
Germany

Hester van Cruijsen, MD
VU Medical Center
Department of Medical Oncology
De Boelelaan 1117
NL-1081 HV Amsterdam
The Netherlands

Miikka Vikkula, MD, PhD
Professor, de Duve Institute
Université catholique de Louvain
Laboratory of Human Molecular Genetics (GEHU)
Avenue Hippocrate 74(+5), UCL 75.39
B-1200 Brussels
Belgium

Israel Vlodavsky, PhD
Cancer and Vascular Biology Research Center
The Rappaport Faculty of Medicine
Technion
Haifa 31096
Israel

Dr. Dimitris Voliotis
Professor, Bayer AG Pharma Research Center
Director, Global Clinical Leader Oncology
BHC-PH-PD-GMD
Global Clinical Development
D-42096 Wuppertal
Germany

Dr. Steve Wedge
Cancer and Infection Research
AstraZeneca
Mereside, Alderley Park
Macclesfield, Cheshire, SK10 4TG
UK

Andreas Wicki
Institute of Biochemistry and Genetics
Department of Clinical-Biological Sciences
University of Basel
Centre of Biomedicine
Mattenstraße 28
4058 Basel
Switzerland

Serena Zacchigna
Center for Transgene Technology and Gene Therapy
Flanders Interuniversity Institute for Biotechnology
Herestraat 49
3000 Leuven
Belgium

Zhenping Zhu
ImClone Systems
180 Varick Street
New York, NY 10014

Historical Overview

Tumor Angiogenesis: from Bench to Bedside

JUDAH FOLKMAN

CONTENTS

1.1 Introduction

An association between cancer and blood vessels has been observed for more than a century. These reports dealt mainly with angioarchitecture of tumors, vascular patterns unique to tumors, effects of irradiation on tumor vasculature, alteration in tumor blood flow, increased vascularity of the peripheral shell of tumors, and the delivery of intravital dyes and anti-cancer drugs to the tumor bed (Thiersch 1865; Goldmann 1907; Thiessen 1936). In a few reports, experimental tumors were transplanted into transparent chambers in the mouse or rabbit. In some reports, the tumor and the host vascular bed were separated by a micropore filter to determine whether a diffusible substance was released from tumors that could stimulate blood vessel growth (Ide et al. 1939; Algire 1943; Algire and Legallais 1947; Toolan 1951; Greene 1952; Day et al. 1959; Zweifach 1961; Gullino and Grantham 1962; Goldacre and Sylvén 1962; Warner 1964; Greenblatt and Shubik 1968; Greenblatt et al 1969; Rubin and Casarett 1966; Tannock 1970). For a definitive historical review from 1865 to 1970, see Peterson (1979). Furthermore, surgeons often reported excessive bleeding from tumors, and "serpentiginous veins" on the surface of tumors. Numerous explanations for these findings were offered in the literature. The vascularity of tumors was attributed to vasodilation, inflammation, dying tumor cells, increased tumor metabolism, overproduction of lactic acid or uric acid, or hypoxia from "tumors outgrowing

their blood supply." There were no molecular mediators of angiogenesis in these reports. One of the most common assumptions prior to 1970 was that excessive vascularity of tumors was a *side effect* of growing tumors or dying tumor cells.

In this chapter I have set out to review: (1) the original concepts; (2) the development of bioassays; (3) the discovery of the first angiogenesis regulatory molecules; and (4) the genetic basis upon which modern research in tumor angiogenesis has been founded. I briefly outline how these studies in tumor angiogenesis evolved to a larger field of angiogenesis research and to clinical validation. Research in tumor angiogenesis itself is now a wide-ranging field described in more than 30 books and monographs. Angiogenesis inhibitors alone are the subject of more than 39,000 reports in the scientific literature. The very informative chapters in this book describe recent progress and new directions in tumor angiogenesis research.

In 1962, I perfused hemoglobin solutions into the carotid artery of rabbit and canine thyroid glands isolated in glass chambers during a study to develop blood transfusion substitutes for the US Navy (Fig. 1.1). When murine melanomas were implanted into the glands, tiny tumors grew up to ~1 mm^3, but did not undergo further expansion (Folkman et al. 1962, 1963, 1966; Folkman and Gimbrone 1971). When these microscopic tumors were transplanted to syngeneic mice, the tumors grew to more than 1,000 times their original volume in the perfused thyroid gland. Large tumors in mice were highly neovascularized, in contrast to tumors in the isolated organs, which were viable, but not vascularized. This difference suggested that in the absence of neovascularization, tumors would stop growing at a volume in the range of ~1 mm^3, most likely due to the limits of oxygen diffusion.

The hemoglobin solution was acellular; it did not contain red cells, leukocytes, or platelets. When we subsequently perfused isolated thyroid glands with platelet-rich medium, endothelial vascular integrity was preserved (Gimbrone et al. 1969). This result implied that absence of platelets was a possible mechanism for lack of neovessels in the earlier experiments of thyroid glands perfused with hemoglobin solution. Today it is known that platelets contain endothelial mitogens and survival factors such as bFGF and VEGF (Folkman et al. 2001).

1.1.1 Hypothesis That Tumor Growth is Angiogenesis-dependent

In 1971 I first published a hypothesis that "tumor growth is angiogenesis dependent" (Folkman 1971). This paper also: (1) predicted that most tumors would be unable to grow beyond a microscopic size of 1–2 mm^3 without recruiting new blood vessels; (2) introduced the concept that tumors would be found to secrete diffusible angiogenic molecules; (3) described a model of tumor dormancy due to blocked angiogenesis; (4) proposed the term antiangiogenesis to mean the prevention of new capillary sprouts from being recruited into an early tumor implant; (5) predicted the future discovery of angiogenesis inhibitors; and (6) advanced the idea that an antibody to a tumor angiogenic factor (TAF), could be an anti-cancer drug. The hypothesis itself was formulated not only from the 1962 experiments of restricted tumor growth in the absence of neovascularization in isolated organs, but also from experiments completed in my laboratory during 1971, and a year later. We had demonstrated tumor dormancy at a microscopic size due to blocked angiogenesis of tumors in the aqueous humor of the anterior chamber of the rabbit eye (Gimbrone et al. 1972). We also had demonstrated DNA synthesis by autoradiography, induced in endothelial cells of a tumor bed in vivo (Cavallo et al. 1972). The concept that tumor growth is angiogenesis-dependent was extended and supported in subsequent invited reviews (Folkman 1974a, 1974b, 1975, 1978; Folkman et al. 1974; Brem et al. 1975; Folkman and Gimbrone 1975; Folkman and Klagsbrun 1975; Folkman and Cotran 1976).

It has been stated that the 1971 *New England Journal of Medicine* paper initiated the field of angiogenesis research (Folkman 1971). However, this field

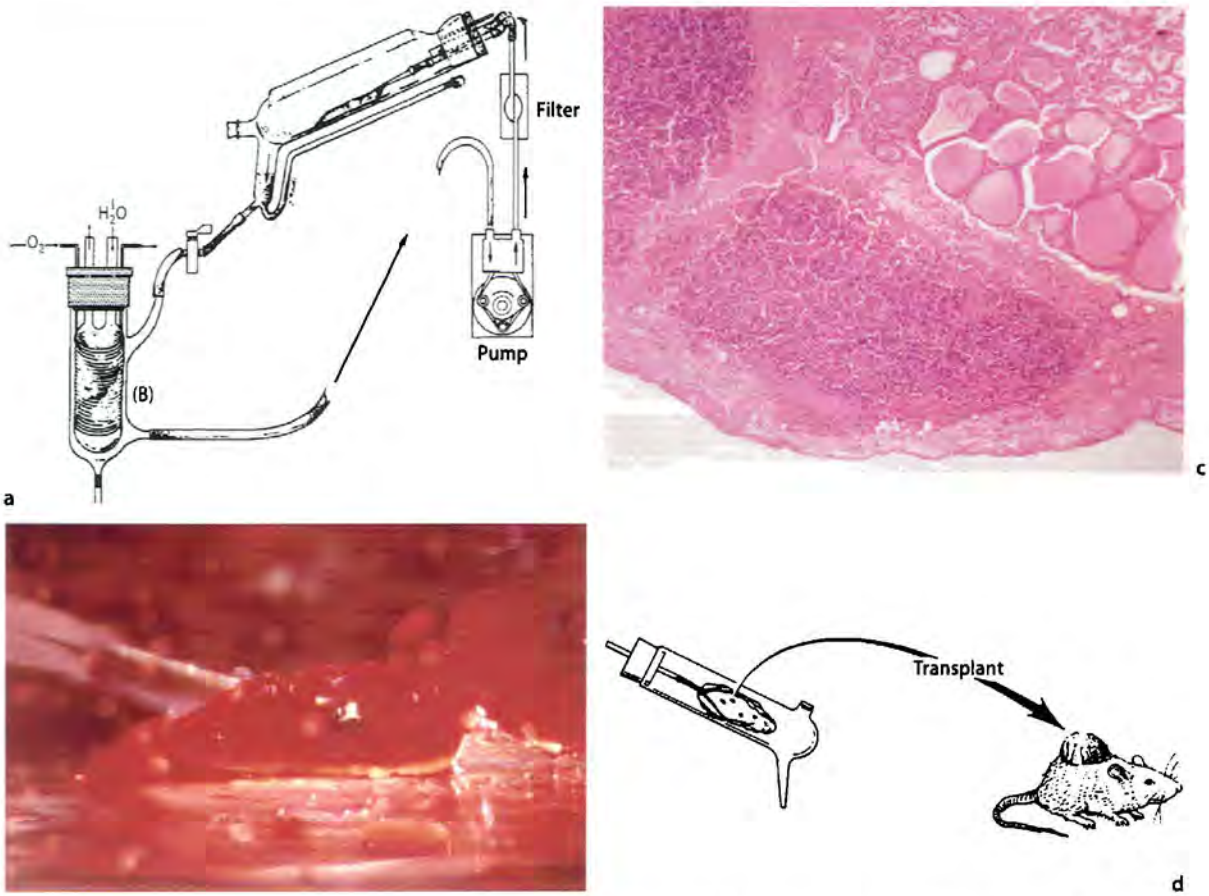


Fig. 1.1a–d. Perfusion of isolated canine thyroid gland through the carotid artery with hemoglobin solution. a The perfusion circuit includes a silicone rubber oxygenator and a roller pump with silicone rubber tubing. b Transilluminated canine thyroid gland in the perfusion chamber, containing a transplanted murine melanoma that grew to ~1 mm^3 and stopped expanding. c Histologic section of thyroid gland showing viable tumor embedded among viable thyroid follicles. d When the tiny, non-expanding tumor was transplanted to a syngeneic mouse, it grew to more than 1000 times its initial volume in the perfused thyroid gland. The large tumor in the mouse was highly neovascularized, in contrast to its precursor tumor which was not vascularized. The hemoglobin solution was acellular, i.e., it did not contain red cells, leukocytes, or platelets. Reprinted from Folkman (2007) with permission of the publisher. Also, see Folkman et al. (1962, 1963)

was slow to develop. Throughout the 1970s, very few scientists believed that tumors needed new blood vessels, and there were hardly any papers from other investigators (Fig. 1.2) (see also Folkman 2007). The conventional wisdom was that tumor vascularity was non-specific inflammation. Skeptics challenged the hypothesis that tumor growth depended on angiogenesis. Reviewers complained that the conclusions of the experiments reached beyond the data. At the time the hypothesis was published, there were no bioassays for angiogenesis, no endothelial cells in long-term culture, and no angiogenesis regulatory molecules. During the 1970s, we set out to remedy these deficiencies so that reagents and methods would eventually become available to isolate and purify proangiogenic and antiangiogenic factors. These advances would be needed to find supporting evidence for the beleaguered hypothesis.

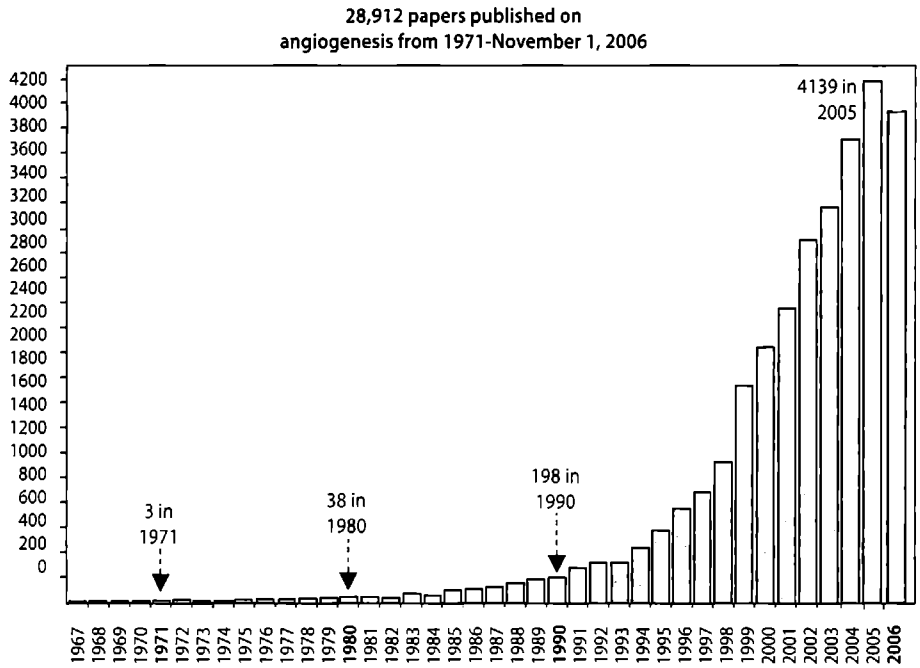


Fig. 1.2. Publications with "angiogenesis" in the title from 1967 to November 2006. There are almost 29,000 papers since the 1971 publication in the *New England Journal of Medicine*, but very few in the first 10 years thereafter

1.1.2 Development of Bioassays for Angiogenesis Research

1.1.2.1 Corneal Neovascularization

In the early 1970s, a challenging problem was how to maintain an in vivo tumor separate from its vascular bed in order to prove that tumors secreted diffusible "angiogenic" molecules. Methods for growing tumor implants on micropore filters in transparent chambers in animals had become more sophisticated (Greenblatt and Shubik 1968; Ehrmann and Knoth 1968), but it was difficult to rule out the possibility that pseudopodia of tumor cells had made contact with the underlying vascular bed. Young scientists who are beginning angiogenesis research today are incredulous at how hard it was in the 1970s to convince scientific colleagues that a diffusible angiogenic substance existed. Michael Gimbrone, a post-doctoral fellow, and I implanted tumors (of approximately 0.5 mm^3) into the stromal layers of the rabbit cornea at distances of up to 2 mm from the limbal edge (Fig. 1.3). New capillary blood vessels grew from the limbus, invaded the stroma of the avascular corneas, and reached the edge of the tumor over a period of approximately 8–10 days. When tumors were implanted beyond 3 mm from the limbus (or in the center of the rabbit cornea, which is approximately 12 mm in diameter), no neovascularization was observed (Gimbrone et al. 1974a, 1974b).

Vascularized tumors turned from white to red, grew exponentially in three dimensions, and became exophytic and protruded from the cornea within 2–3 weeks. Non-vascularized tumors in the center of the cornea expanded slowly in two dimensions, as thin, flat, translucent, intracorneal lesions until one edge extended to within ~2 mm of the limbus and recruited new blood vessels (Folkman 1978). This method demonstrated that a diffusible "angiogenic factor" existed, and that such a putative angiogenic molecule could possibly be isolated from tumors. However, when tumor extracts were

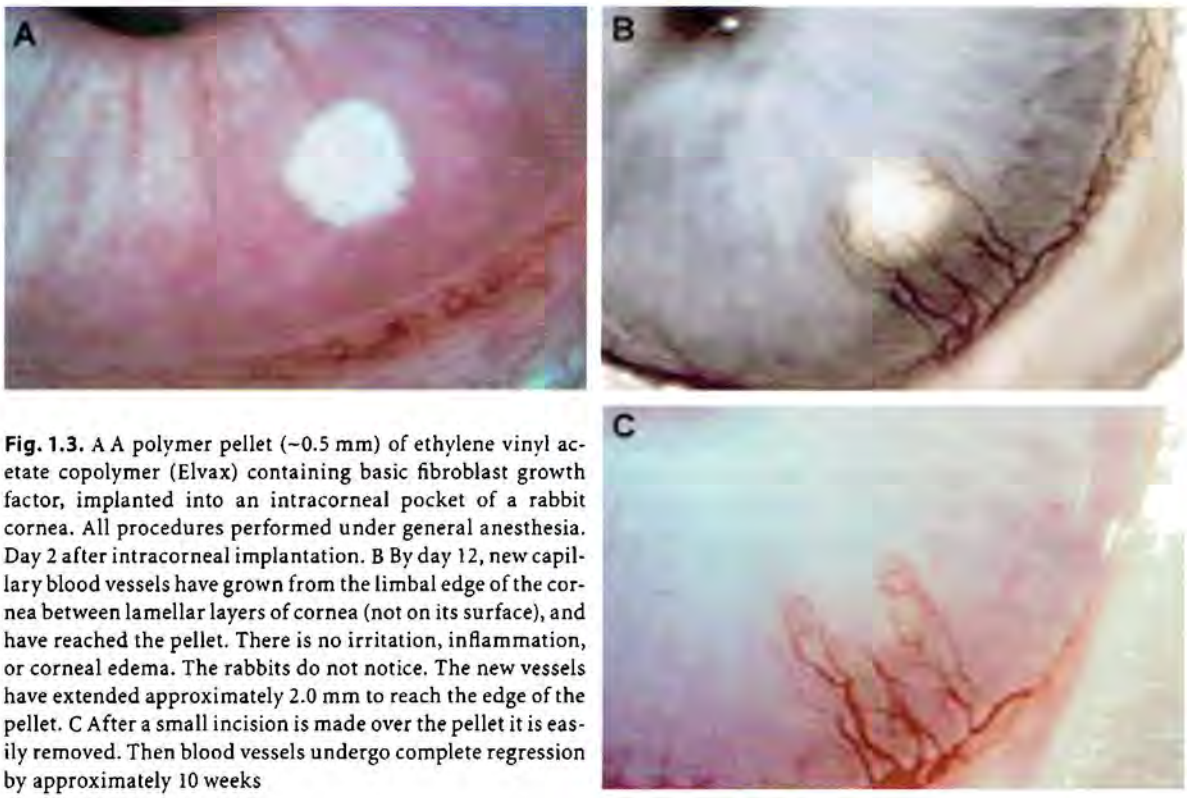


Fig. 1.3. A A polymer pellet (~0.5 mm) of ethylene vinyl acetate copolymer (Elvax) containing basic fibroblast growth factor, implanted into an intracorneal pocket of a rabbit cornea. All procedures performed under general anesthesia. Day 2 after intracorneal implantation. B By day 12, new capillary blood vessels have grown from the limbal edge of the cornea between lamellar layers of cornea (not on its surface), and have reached the pellet. There is no irritation, inflammation, or corneal edema. The rabbits do not notice. The new vessels have extended approximately 2.0 mm to reach the edge of the pellet. C After a small incision is made over the pellet it is easily removed. Then blood vessels undergo complete regression by approximately 10 weeks

implanted into the cornea to mimic a tumor implant, the extracts rapidly diffused away into the cornea. A focal steady-state concentration gradient of angiogenic activity, similar to a tumor implant, could not be established. Silicone rubber capsules that we had previously found to steadily release small molecules (< 500 Da) by diffusion through the polymer itself (Folkman and Long 1964), could not release proteins.

Robert Langer, a post-doctoral fellow, solved the problem. He dissolved the polymer polyhydroxy ethylmethacrylate (polyhema), into alcohol and added lyophilized protein. When the solvent was evaporated, the protein remained trapped in a rubbery polymeric pellet. When the pellet was implanted into the cornea, water diffused into the pellet. This caused the formation of microchannels around the protein. Protein diffused out from these channels at zero-order kinetics for weeks to months (Langer and Folkman 1976; Brown et al. 1983). Another polymer, ethylene vinyl acetate copolymer (Elvax) dissolved in ethylene chloride was also used. These polymers did not irritate the cornea. Robert Auerbach reported that the mouse cornea could also be implanted with tumors or polymer pellets (Muthukkaruppan and Auerbach 1979). This advance permitted genetic experiments, and mice are now routinely employed for corneal neovascularization bioassays.

The corneal neovascularization bioassay and the method of implanted sustained-release corneal implants have played an important role in elucidating the process of tumor angiogenesis (Fig. 1.4b).

In 1978, we reported that removal of an angiogenic stimulus from the cornea resulted in regression of neovasculature by a series of sequential morphologic events (Ausprunk et al. 1978). This finding demonstrated that newly induced neovasculature does not become "established," as was the conventional wisdom at the time. Regression of corneal neovascularization also predicted that future angiogenesis

inhibitors could possibly cause new blood vessels to regress. These results provided a compelling rationale for the future attempts to discover angiogenesis inhibitors, and for the efforts to develop them.

These results also helped in the debate with certain colleagues who argued that the search for an angiogenesis inhibitor was a "fruitless exercise." Lymphangiogenesis was first dissociated from angiogenesis in the cornea; the two processes were induced by different concentrations of bFGF, and inhibited by different mechanisms (Chang et al. 2004).

Recently, corneal avascularity has been shown to be due to soluble VEGF receptor-1 (sflt-1), which binds VEGF and is highly concentrated in the cornea (Ambati et al. 2006). In the cornea, sflt-1 is expressed predominantly by corneal epithelium. The corneas of *corn1* mice are deficient in slft-1 and are spontaneously neovascularized (Smith et al. 1996). These mice could possibly be used to test antiangiogenic activity of systemically administered molecules, or to develop a standardized unit of antiangiogenic activity to compare different inhibitors.

1.1.2.2 Vascular Endothelial Cells In Vitro

Before the 1970s, it was thought that vascular endothelial cells could not survive in vitro, to say nothing of their long-term passage. In 1973, Gimbrone in my laboratory (Gimbrone et al. 1973, 1974a, 1974b) and Eric Jaffe's laboratory at Cornell (Jaffe et al. 1973), were independently the first to report long-term passage of vascular endothelial cells (from human umbilical veins) in vitro. The first long-term passage of cloned capillary endothelial cells came later and was reported in 1979 (Folkman et al. 1979), followed by the demonstration of angiogenesis in vitro (Folkman and Haudenschild 1980) (Fig. 1.4d). Endothelial cultures also facilitated discovery of endothelial mitogens and suppressors of endothelial cell proliferation that could then be tested in vivo for pro- or antiangiogenic activity. However, endothelial cell cultures were still not useful for guiding purification of endothelial mitogens until we found that vascular endothelial cells become refractory to virtually any mitogen once the cells had reached confluence, in contrast to confluent fibroblasts, which still responded to mitogens (Haudenschild et al. 1976).

It became clear that vascular endothelial cells were among the most stringently regulated cells at high cell density. This report deserves emphasis because those who are unaware of it today risk being misled by their experiments as researchers were 30 years ago. Until the mid-1970s, it was conventional practice to guide the purification of growth factors with fibroblast cell cultures. Fibroblasts (3T3 cells) were grown to confluence. When a putative growth factor was added, one or two additional rounds of DNA synthesis ensued. However, when *endothelial* cells were used to guide purification of endothelial mitogens, confluent endothelial cells did *not* undergo additional DNA synthesis, and investigators assumed that their tumor extracts were inactive. Therefore, it was necessary to incubate endothelial cells with a putative mitogen when the cells were sparse, not when they were confluent – a critical detail – and just the opposite of employing 3T3 fibroblasts to purify a mitogen for fibroblasts.

Changes in cell shape during confluence in vitro were later found to be a central mechanism of suppression of DNA synthesis in endothelial cells (Folkman and Moscona 1978). Shape control of DNA synthesis (Folkman and Moscona 1978) appears to have eluded discovery until the advent of successful in vitro growth of vascular endothelial cells. This mechanism was further elucidated by Donald Ingber, who showed how changes in cell shape can signal through integrins to regulate gene expression and DNA synthesis. He went on to develop an entirely new field of investigation of cell biology based on the mechanisms by which mechanical forces modify DNA synthesis and gene expression (Ingber et al. 1987; Ingber and Folkman 1989a, 1989b, 1989c; Huang and Ingber 2005). The experiments of Mina Bissell on cell shape and differentiation of function also yielded information on the role of cell shape in cell growth (Bissell et al. 1977).

When angiogenesis in vitro was demonstrated (Folkman and Haudenschild 1980) it became possible to elucidate the morphologic and molecular events of lumen formation in microvessels (Kuo et al. 2001).

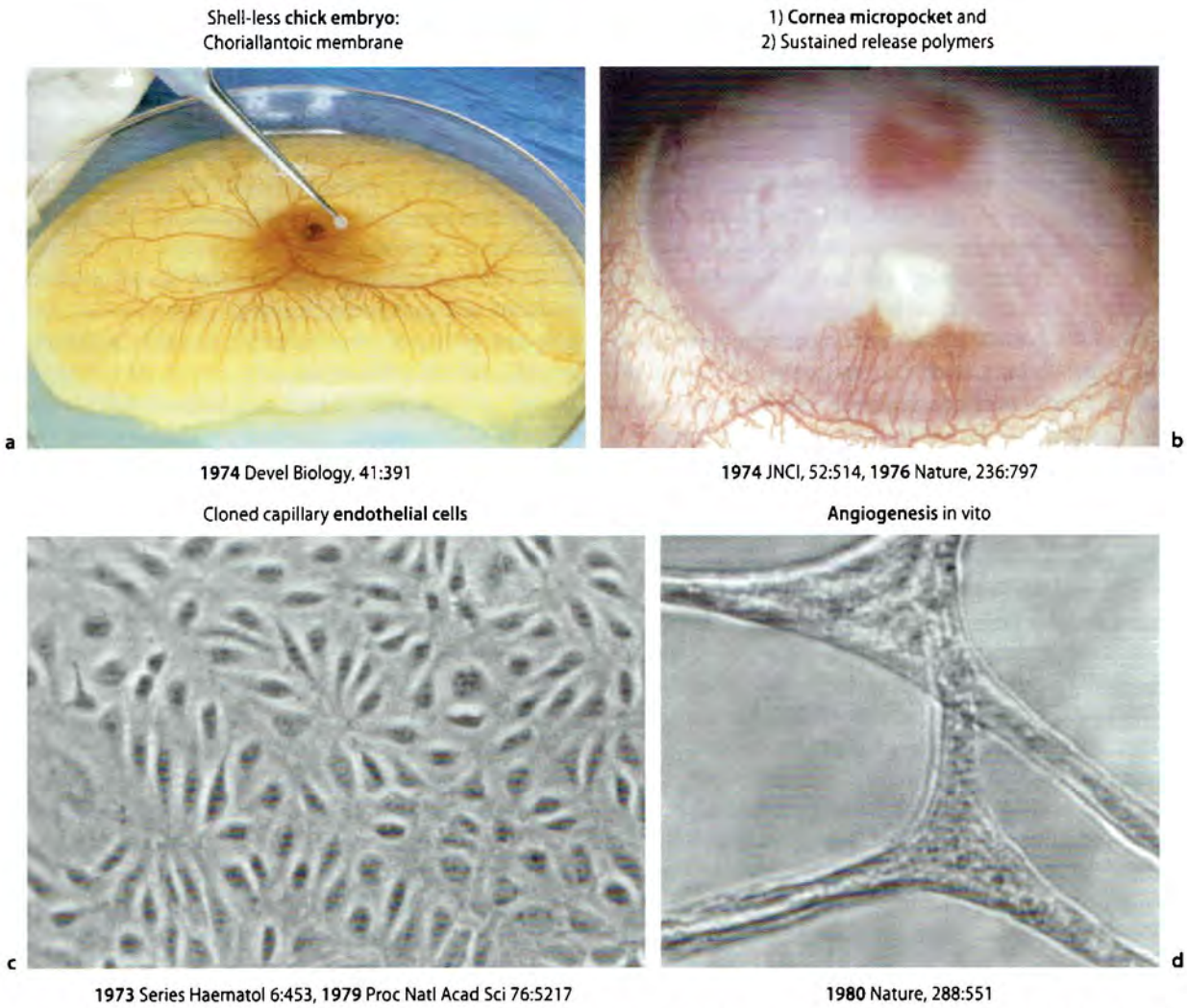


Fig. 1.4a–d. Bioassays for angiogenesis developed during the 1970s. a The chick embryo chorioallantoic membrane. b Corneal neovascularization stimulated by an implanted polymer releasing an angiogenic protein. c Capillary endothelial cells in vitro. d Angiogenesis in vitro

The development of the shell-less chick embryo that could be routinely cultured in large numbers from day 3 to at least day 18 permitted the growing chorioallantoic membrane to be employed as a vascularized substrate to test antiangiogenic and angiogenic molecules, starting from day 5 (Auerbach et al. 1974; Ausprunk et al. 1974) (Fig. 1.4a). It was demonstrated that normal and neoplastic tissues had quantitatively different mechanisms of vascularization after being grafted to the chorioallantoic membrane (Ausprunk et al. 1975). Therefore, 7 years before angiogenic molecules were discovered, these experiments showed that the angiogenic activity of tumor tissue was significantly greater than that of equivalent normal tissue. Today, this difference is understood in part by the ratio of expression or generation of pro- and antiangiogenic proteins by a tumor.

Ribatti et al. improved the chick chorioallantoic membrane bioassay (Ribatti et al. 1997a), and also studied the modification of angiogenesis on the chorioallantoic membrane, by heparin (Ribatti et al. 1995), bFGF (Ribatti et al. 1997b), and interferon alpha (Ribatti et al. 2002). Nguyen and Folkman (Nguyen et al. 1994), converted the chick chorioallantoic membrane to a quantitative angiogenesis bioassay by implanting the test protein in a white opaque gel sandwiched between two squares of nylon mesh. New microvessel sprouts grew *vertically* through the mesh. When separate sprouts protruded vertically through the mesh and anastomosed to form a capillary loop enveloping a nylon thread, this was compelling proof that the microvessels were new (and not just dilated vessels). They could be accurately quantified by the ratio of squares of mesh containing a microvessel to empty squares of mesh.

The chick embryo chorioallantoic membrane also made it possible to dissociate the early pre-angiogenic phase of tumor growth from the angiogenic growth phase (Knighton et al. 1977). This evidence further supported the concept that tumor growth was restricted in the absence of angiogenesis.

For recent reviews of in vivo models of angiogenesis see Murray (2001) and Norrby (2006).

1.1.3 Discovery of Angiogenic Molecules

It first became possible to isolate angiogenic molecules when the chick embryo chorioallantoic membrane was employed together with bioassays based on endothelial cell migration and proliferation in vitro, and with corneal neovascularization in vivo. At best, these were semiquantitative bioassays, but served the purpose of isolating and purifying angiogenic proteins.

In 1984, Yuen Shing and Michael Klagsbrun in my laboratory employed heparin-affinity chromatography to isolate and purify to homogeneity the first angiogenic endothelial cell mitogen from a tumor (Shing et al. 1984, 1985; Folkman and Klagsbrun 1987). When this protein was subsequently purified from bovine pituitary and sequenced by Esch et al. (1985), it proved to be basic fibroblast growth factor (bFGF). FGF had previously been isolated from the pituitary by Gospodarowicz and shown to be mitogenic for 3T3 fibroblasts and for vascular endothelial cells (Gospodarowicz 1974). It was also a survival factor for these cells (Gospodarowicz 1974; Gospodarowicz et al. 1976), but had not been completely purified.

By 1989, Rosalind Rosenthal in my laboratory had isolated and purified to homogeneity a second angiogenic protein from a different tumor that did not express bFGF. We had not yet sequenced this new protein, when we received a call from Napoleone Ferrara of Genentech, who had purified a novel angiogenic protein from pituitary cells. He had heard about the new angiogenic protein in our lab, and suggested that the two labs compare their proteins because Ferrara had already sequenced his protein. The two proteins were identical, and were named vascular endothelial growth factor (VEGF) by Ferrara. Ferrara's report was published in mid-1989 (Ferrara and Henzel 1989), and our paper reporting the first VEGF from a tumor was published in 1990, with Ferrara as a co-author (Rosenthal et al. 1990). In 1983, Senger and Dvorak had purified a vascular permeability factor (VPF) from tumor cells that promoted accumulation of ascites (Senger et al. 1983). By 1990, it was clear that VPF was also the same as VEGF. Thus, VEGF had an auspicious start, having been purified from three different sources, but first sequenced in Ferrara's laboratory.

Many other proangiogenic molecules have since been discovered and are discussed by other authors in this book. Recently, Klagsbrun discovered that neuropilin-1 is another receptor for VEGF and stimulates angiogenesis (Miao et al. 2000).

1.1.3.1 Storage of an Angiogenic Protein in Extracellular Matrix

In 1987, Israel Vlodavsky, Klagsbrun and Folkman reported that bFGF was stored in extracellular matrix, where it was bound to heparan sulfate proteoglycans (Vlodavsky et al. 1987; Folkman et al. 1988). This finding opened a new avenue of research.

Angiogenesis regulatory proteins were sequestered in extracellular matrix and basement membrane, where they were protected from degradation by their heparin affinity. This storage mechanism prevented endothelial cells in the vasculature from overexposure to these biologically active molecules. Because angiogenic molecules could be released at a site of extracellular membrane disruption, this provided a mechanism by which angiogenic activity was localized to a wound site. Angiogenesis regulatory proteins were also releasable by heparitinase and additionally by heparin-like molecules (Bashkin et al. 1989). Of interest is that Descemet's membrane in the cornea contains a high concentration of bFGF (Folkman et al. 1988).

1.1.4 Discovery of Angiogenesis Inhibitors

Throughout the 1970s, evidence that tumors were angiogenesis-dependent was based almost solely on blocking angiogenesis by mechanical separation of tumors from their nearest vascular bed. More compelling evidence would depend on pharmacologic blockade of angiogenesis. By 1980, the availability of bioassays and of angiogenic proteins facilitated the search for molecules with *anti*angiogenic activity.

Bruce Zetter in the Folkman lab reported that very low concentrations of interferon alpha specifically suppressed migration of endothelial cells in vitro (Brouty-Boye and Zetter 1980). Dvorak and Gresser (1989) and also Sidky and Borden (1987) then reported that interferon alpha inhibited angiogenesis in experimental animals. In 1988, I received a call from Carl White, a pulmonary specialist at Denver Jewish Hospital who was caring for a teenager who had progressive hemangiomatosis of both lungs and hemoptysis. The patient had failed all therapy for this disease, which up to that time was fatal.

I suggested a trial of frequent low-dose interferon alpha, based on our experimental elucidation of its antiendothelial properties and its antiangiogenic activity in animals (Dvorak and Gresser 1989), and because of its FDA approval. With very low doses of interferon alpha every second day, the patient made a complete recovery after several months and was treated for an additional 5 years (by subcutaneous self-injection), while he completed his education (White et al. 1989; Folkman 1989). He has a normal chest film and is in good health today, 18 years later. This is the first recorded case of antiangiogenic therapy. It was subsequently found in other patients that these low doses are antiangiogenic, but are neither cytotoxic nor immunosuppressive.

Stephanie Taylor and I showed that two antagonists of heparin, protamine and platelet factor 4, blocked angiogenesis in the chick embryo and produced dose-dependent avascular zones in the growing chorioallantoic membrane (Taylor and Folkman 1982).

Until the early 1980s, corticosteroids were classified as either glucocorticoid or mineralocorticoid. With Rosa Crum and Sandor Szabo, we discovered a third class of steroids, named "angiostatic steroids" (Crum et al. 1985). The most potent of these was tetrahydrocortisol, a pure angiogenesis inhibitor that had no glucocorticoid or mineralocorticoid activity. (Tetrahydrocortisol is currently in phase III clinical trials for the treatment of macular degeneration). In studies of the mechanism of action of angiostatic steroids, we found that they were potentiated by an arylsulfatase inhibitor (synthesized by Professor E.J. Corey), that inhibited desulfation of endogenous heparin (Chen et al. 1988). In 1994, after Fotsis et al. reported that the steroid 2-methoxyestradiol inhibited angiogenesis (Fotsis et al. 1994), Robert D'Amato in my lab reported that this angiostatic steroid inhibited tubulin polymerization by interacting at the colchicine site (D'Amato et al. 1994).

Eleven angiogenesis inhibitors were discovered in the Folkman laboratory (Table 1.1). Five of them were previously unknown molecules (i.e., TNP-470 [a synthetic analogue of fumagillin], angiostatin, endostatin, caplostatin, and cleaved antithrombin III). Of the known molecules in which antiangiogenic activity was a new function (i.e., platelet factor 4, interferon alpha, etc.), eight are *endogenous* angiogenesis inhibitors in the blood or in extracellular matrix. Many other laboratories joined this research effort (Auerbach and Auerbach 1994), and at the time of writing there are 28 known endogenous angiogenesis inhibitors (Folkman 2004; Nyberg et al. 2005).

1.1.4.1
Angiostatin and Endostatin: Their Role in Suppression of Metastasis by a Primary Tumor

Angiostatin (O'Reilly et al. 1994) and endostatin (O'Reilly et al. 1997; Boehm et al. 1997), are the first endogenous angiogenesis inhibitors found to be internal fragments of larger proteins which themselves do not regulate angiogenesis. Surgeons had long observed that the removal of certain primary tumors was followed by rapid growth of metastases at remote sites. This phenomenon was assumed to be due to release of tumor cells into the circulation during surgical removal of the primary tumor. This explanation, however, was inconsistent with the explosive growth of metastasis after removal of a primary tumor in humans and animals. An alternative hypothesis, that a primary tumor could suppress its remote metastasis, seemed counterintuitive, until the discovery that certain tumors could enzymatically cleave angiostatin (O'Reilly et al. 1994) from plasminogen, or endostatin from collagen XVIII (O'Reilly et al. 1997; Boehm et al. 1997). Expression of proangiogenic proteins, such as VEGF, within the primary tumor exceeded the generation of antiangiogenic proteins such as angiostatin or endostatin, resulting in vascularization and growth of the primary tumor (Roy et al. 2004; Cao et al. 1998). However, the angiogenesis inhibitors accumulate in the circulation because of their longer half-life.

Table 1.1. Molecules with antiangiogenic activity published from the Folkman laboratory between 1980 and 2005

Year	Molecule(s)	Reference
1980	Interferon α/β, new activity	Brouty-Boye and Zetter 1980
1982	Platelet factor 4, protamine	Taylor and Folkman 1982
1985	Angiostatic steroids	Crum et al. (Folkman) 1985
1990	TNP-470, a fumagillin analogue	Ingber et al. (Folkman) 1990
1994	Angiostatin	O'Reilly et al. (Folkman) 1994
1994	Thalidomide	D'Amato et al. (Folkman) 1994
1994	2-Methoxyestradiol (*see footnote*)	D'Amato et al. (Folkman) 1994
1997	Endostatin	O'Reilly et al. (Folkman) 1997
1999	Cleaved antithrombin III	O'Reilly et al. (Folkman) 1999
2002	3-Amino thalidomide	Lentzsch et al. (D'Amato) 2002
2003	DBP-maf (*see footnote*)	Kisker et al. 2003
2005	Caplostatin	Satchi-Fainaro et al. (Folkman) 2005

Ten of these molecules were discovered either as new molecules (e.g., angiostatin and endostatin), or as new functions of known molecules (e.g., interferon alpha and the angiostatic steroid tetrahydrocortisol). *2-Methoxyestradiol* was first found to be an angiogenesis inhibitor by Fotsis et al. (1994). Subsequently, D'Amato et al. (1994) reported its mechanism as an inhibitor of tubulin polymerization by acting at the colchicine site. *DBP-maf* was first discovered by Yamamoto and Kumashiro (2003) and found to be an angiogenesis inhibitor by Kisker et al. (2003). Eight of these are endogenous angiogenesis inhibitors (interferon alpha, platelet factor 4, angiostatic steroids (including 2-methoxyestradiol and tetrahydrocortisol), angiostatin, endostatin, cleaved antithrombin III, and DBP-maf (vitamin D binding protein-macrophage activating factor). (From Folkman J, *Nature Reviews Drug Discovery* 2007)

Removal of the primary tumor leads to a decrease in circulating inhibitor, over a period of about a week, as revealed by incubations of proliferating endothelial cells with serum samples taken each day after removal of the primary tumor (O'Reilly et al. 1994; Cao et al. 1998). Microscopic metastases become angiogenic and begin rapid growth within 4–5 days after removal of the primary tumor (Holmgren et al. 1995). Systemic replacement of the angiogenesis inhibitor lost after removal of the primary tumor, or after its regression by ionizing irradiation, prevents growth of remote metastases (Camphausen et al. 2001). Kalluri et al. have discovered other endogenous angiogenesis inhibitors that are internal fragments of collagens (Kamphaus et al. 2000; Hamano and Kalluri 2005).

1.1.5 The Switch to the Angiogenic Phenotype

Douglas Hanahan and I found that spontaneous murine tumors arising in his transgenic mice first appeared in a non-angiogenic state at a microscopic size of less than 1–2 mm^3. A small percentage (~10%) then switched to the angiogenic phenotype after a predictable time period (for example 6–7 weeks after birth for transgenic islet cell carcinomas) (Hanahan and Folkman 1996). The angiogenic tumors recruited new blood vessels and underwent rapid expansion of tumor mass. Subsequent studies revealed that the angiogenic switch resulted from a change in the net balance of positive and negative regulators of angiogenesis, i.e., increased expression of VEGF and other proangiogenic proteins accompanied by decreased expression of thrombospondin-1 and other antiangiogenic proteins.

1.1.5.1 The Angiogenic Switch in Human Tumors in Immunodeficient Mice

A similar switch to the angiogenic phenotype has been demonstrated in human cancers transplanted to SCID immunodeficient mice (Achilles et al. 2001; Udagawa et al. 2002; Almog et al. 2006; Naumov et al. 2006a, 2006b). Non-angiogenic and angiogenic tumor cells were cloned from human cancers obtained from discarded tumor specimens in the operating room, or from the American Tissue Culture Collection. These cells form either non-angiogenic or angiogenic tumors when injected subcutaneously, or into orthotopic sites such as the mammary fat pad in mice. Non-angiogenic tumors remain dormant at a microscopic size of less than 1 mm^3. They do not recruit blood vessels. The angiogenic tumors become highly neovascularized and grow rapidly. Non-angiogenic tumor cells proliferate at approximately the same rate as angiogenic tumor cells. In contrast, the apoptotic rate of tumor cells in the non-angiogenic tumors is significantly higher than that of tumor cells in angiogenic tumors. The non-angiogenic tumors remain at a microscopic size for a predictable period of time (from months to over a year), before switching to the angiogenic phenotype. The switch itself can be accurately quantified by bioluminescence if the tumor cells are infected with luciferase. For example, 95% of non-angiogenic human liposarcomas reproducibly switched to the angiogenic phenotype at a median of ~133 days ± 2 weeks (Almog et al. 2006). In contrast, approximately 60% of human breast cancers became angiogenic at a median of 80 days. A human osteogenic tumor did not switch to the angiogenic phenotype for more than a year, and then only 5% of tumors become angiogenic (Udagawa et al. 2002).

The initiating events that drive the angiogenic switch are unknown. However, a predictable, reproducible animal model now exists. Preliminary data have begun to fill in pieces of the puzzle. The angiogenic switch can be significantly accelerated by transfecting non-angiogenic human tumor cells with Ras (Udagawa et al. 2002). This induces a 38% increase in VEGF expression, and a 50% suppression of thrombospondin-1 expression within approximately 1 week in a human osteogenic sarcoma that in the absence of Ras transfection would not become angiogenic spontaneously for more than a year (Udagawa et al. 2002). Analyses of gene expressions before and after the angiogenic switch are under way for five different human cancers. At the time of writing, the mechanism of the angiogenic switch

in human cancer is unknown. If it were understood how to prevent or reverse the angiogenic switch, this could become a novel conceptual advance in controlling cancer.

1.1.6 U-shaped Dose–Efficacy Curve of Angiogenesis Inhibitors

Singh et al., in Isaiah Fidler's laboratory, first reported that low-dose interferon alpha inhibits angiogenesis by suppressing expression of bFGF from human cancer cells (Singh et al. 1995). Fidler and his associates further showed that dose–efficacy of the antiangiogenic activity of interferon alpha could be expressed as a biphasic curve that was U-shaped (Slaton et al. 1999). Higher doses are less effective than low doses (Fig. 1.5a).

Since these reports, other angiogenesis inhibitors have also shown a biphasic, U-shaped dose–response curve in experimental tumor models. These include rosiglitazone (Panigrahy et al. 2002), endostatin protein therapy (Celik et al. 2005) (Fig. 1.5b), and endostatin gene therapy (Tjin Tham Sjin et al. 2006). In fact, when endothelial cells are incubated in vitro with endostatin at increasing concentrations, gene expressions also reveal a U-shape (Abdollahi et al. 2004). Furthermore, anti-cancer cytotoxic chemotherapy, administered at frequent *low* doses, is more effective in tumor-bearing mice than high doses of chemotherapy administered less frequently. Low-dose frequent chemotherapy, also called 'antiangiogenic therapy' or 'metronomic therapy,' is more effective against mouse tumors that have become resistant to high-dose chemotherapy (Browder et al. 2000; Klement et al. 2000; Hanahan et al. 2000).

Conventional cytotoxic chemotherapy is generally administered on a linear dose–efficacy curve and is guided by the concept of "maximum tolerated dose." In contrast, if the experimental finding of a biphasic dose–response curve for angiogenesis inhibitors is validated in the clinic in the future, "maximum tolerated dose" (MTD) may become less useful for administering antiangiogenic therapy, and specific biomarkers of antiangiogenic activity may become more useful.

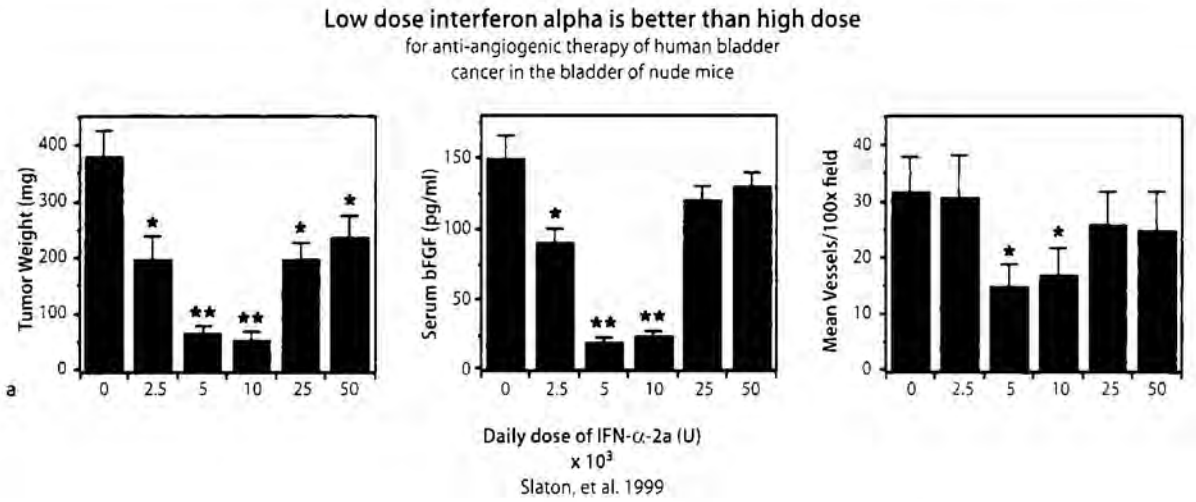
The mechanism of the biphasic response of vascular endothelium to different angiogenesis inhibitors is unclear, but its physiological function may be, in part, to protect vascular endothelium from surges in plasma concentrations of endothelial regulatory molecules.

1.1.7 The Platelet Angiogenesis Proteome

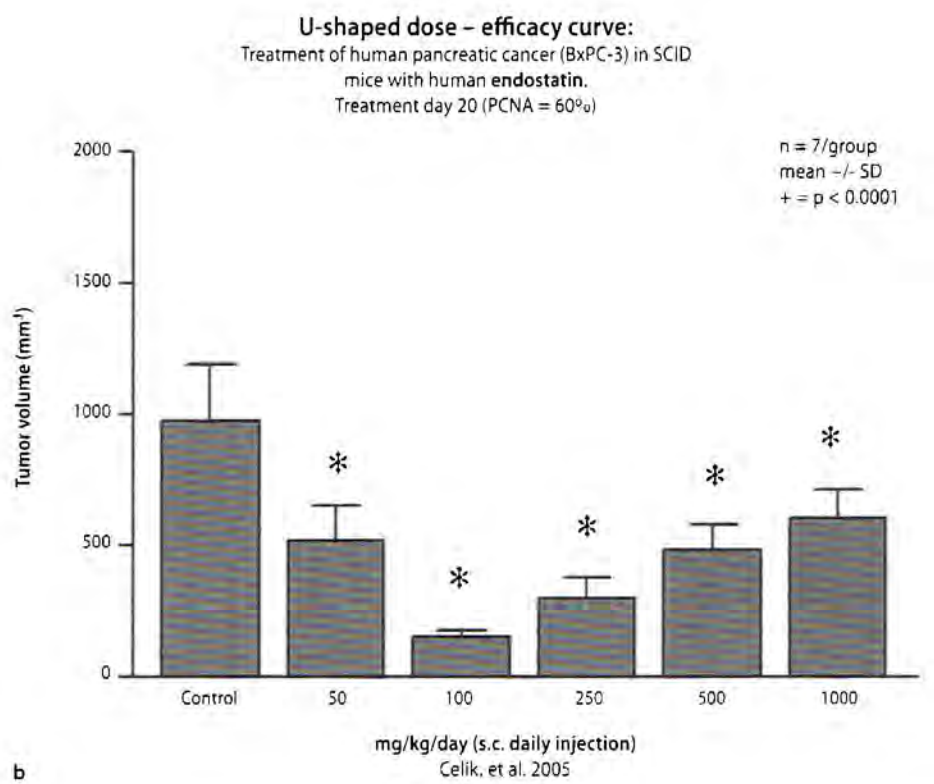
Platelets may also function as a circulating storage depot that protects vascular endothelium from surges in plasma concentrations of angiogenesis regulatory molecules. Platelets have been reported to be associated with both positive and negative regulators of angiogenesis (Folkman et al. 2001). Klement et al. reported that platelets can continuously scavenge angiogenesis regulatory molecules from plasma and sequester them within the alpha granules of platelets (Klement et al. 2004, 2006). Angiogenesis regulatory proteins sequestered by platelets are not completely released into serum during coagulation. Therefore, serum levels of angiogenesis regulatory proteins may not reflect the total content of these proteins in the blood. For example, Avastin (bevacizumab) administered to patients is taken up by platelets, where it binds VEGF and neutralizes its angiogenic activity (Verheul et al. 2006).

This new platelet biology also explains why pharmacodynamics of an angiogenic protein such as VEGF based only on serum may not always correlate with the burden of cancer in a patient. Furthermore, platelets exclude many other proteins, such as albumin, even though some of these proteins are more concentrated in plasma. Italiano et al, recently made the surprising discovery that proangiogenic proteins are contained together in one set of alpha granules and antiangiogenic proteins are contained in a different set of alpha granules (Italiano et al. 2006). Furthermore, the angiogenesis regulatory molecules that are segregated into two types of alpha granules in platelets may be released separately.

In preliminary studies, Klement et al. showed that platelets can scavenge angiogenesis regulatory proteins that are released into plasma from microscopic human tumors in SCID mice (Klement et al. 2004, 2006).



a



b

Fig. 1.5. a The interferon alpha suppression of bFGF expression by a human bladder cancer is revealed as a biphasic, U-shaped dose–efficacy curve. As a result, inhibition of tumor growth (tumor volume) and of microvessel density in the tumor are also U-shaped (adapted from Slaton et al. 1999). b A similar biphasic, U-shaped curve of efficacy is revealed when endostatin is administered systemically to SCID immunodeficient mice bearing human pancreatic cancer (from Celik et al. 2005)

1.1.8 Genetic Regulation of Angiogenesis

Soon after proangiogenic and antiangiogenic proteins were discovered, genes that regulate these proteins began to be elucidated. The genetic regulation of angiogenesis is itself a burgeoning field that can only briefly be discussed here. However, certain fundamental early reports deserve mention. Arbiser et al. immortalized vascular endothelial cells by transfection with the SV-40 large T oncogene (Arbiser et al. 1997). These cells formed microscopic, dormant, non-angiogenic tumors in vivo, but were not lethal to their host mice. After a second transfection, with the H-ras oncogene, the tumors became angiogenic, grew rapidly as angiosarcomas, and metastases killed the mice. This experiment, 10 years ago, revealed that the switch to the angiogenic phenotype, for at least one tumor type, was controlled by sequential transfection of two different oncogenes.

Rak et al. (2000) reported the sets of proangiogenic proteins (i.e., VEGF) up-regulated by oncogenes, and the antiangiogenic proteins down-regulated by oncogenes. D'Amato and colleagues reported a hierarchy of different mouse strains that have increasing angiogenic responsiveness to the same dose of a given angiogenic protein (Rohan et al. 2000). Interestingly, increasing angiogenic responsiveness correlated directly and precisely with increasing output of bone marrow-derived endothelial precursor cells (Udagawa et al. 2006; Shaked at al. 2005). Udagawa et al. showed that Ras transfection of human non-angiogenic tumor cells switched them to the angiogenic phenotype (Udagawa et al. 2002). Microscopic dormant tumors of which only 5% became angiogenic after ~1 year all became angiogenic at approximately 1 week after Ras transfection. The angiogenic switch was preceded by a 38% increased expression of VEGF and a 50% decreased expression of thrombospondin-1.

Folkman's laboratory in collaboration with Dean Felsher's laboratory reported that tumorigenesis by activated oncogenes is angiogenesis-dependent (Giuriato et al. 2006). The previous conventional wisdom was that inactivation of an oncogene responsible for tumorigenesis would lead to regression of the tumor (Folkman and Ryeom 2005; Chin et al. 1999; Jain et al. 2002). This phenomenon, called "oncogene addiction," implied that oncogene-driven tumor cell proliferation was necessary and sufficient to induce expansion of tumor mass, and that inactivation of the oncogene was necessary and sufficient to regress the tumor. However, Giuriato et al. showed that Myc inactivation alone did not lead to sustained tumor regression unless thrombospondin-1 expression was elevated, or p53 activity was normal. In both cases, elevated thrombospondin-1 expression is necessary to suppress angiogenesis, which then leads to tumor regression (Giuriato et al. 2006). In fact, Dameron et al. first showed that the tumor suppressor gene wild-type p53 inhibited tumorigenesis not only by inhibiting tumor cell proliferation, but also by maintaining up-regulated expression of thrombospondin-1 that inhibited angiogenesis (Dameron et al. 1994). Recently, Teodoro et al. reported an additional mechanism by which wild-type p53 could inhibit angiogenesis, and they demonstrated that p53 mobilized endostatin and tumstatin from their respective collagens XVIII and IV, through up-regulation of alpha(II) collagen prolyl-4-hydroxylase (Teodoro et al. 2006).

Endostatin controls perhaps the widest spectrum of genes that regulate angiogenesis. A gene array analysis of 90% of the human genome revealed that human endostatin down-regulates a broad spectrum of signaling pathways in human microvascular endothelium associated with proangiogenic activity including bFGF, bFGF receptors, HGF, EGFR, HIF-1 alpha, Id1, Id4, TNF-alpha receptor and others (Abdollahi et al. 2004; Folkman 2006). Endostatin simultaneously up-regulated many antiangiogenic genes including those encoding thrombospondin-1, maspin, APC (adenomatous polyposis coli) and others. In fact, endostatin can molecularly reset en masse the set of gene expressions underlying the angiogenic balance in tissues. This broad spectrum of antiangiogenic activity appears to be responsible, in part, for the lack of drug resistance observed with endostatin in experimental animals (Boehm et al. 1997).

These results may explain in part why individuals with Down syndrome are the most protected humans against cancer, having only one tenth the general incidence of all cancers except for testicular cancer and a megakaryocytic leukemia (Yang et al. 2002). Individuals with Down syndrome have a 1.6-fold circulating level of endostatin due to an extra copy of the gene for collagen XVIII on the trisomic chromosome 21 (Zorick et al. 2001).

Perhaps the most heuristic recent discovery of a genetic mechanism that regulates angiogenesis is the Notch ligand, delta-like 4, that negatively regulates formation of tip cells during angiogenesis (Ridgway et al. 2006; Noguera-Troise et al. 2006; Hellstrom et al. 2007; Lobov et al. 2007; Suchting et al. 2007). Delta-like 4 is a transmembrane ligand for Notch receptors that is expressed in arterial blood vessels and sprouting endothelium. VEGF stimulation of capillary sprouts induces delta-like 4 as a negative feed-back on endothelial tip cells (Ausprunk and Folkman 1977), which lead capillary sprout extension during angiogenesis.

Clinical Translation of Antiangiogenic Therapy

1.2.1 Low-Dose Interferon Alpha

The first use of antiangiogenic therapy in a human was in 1989 (see Sect. 1.1.4). A 12-year-old boy with life-threatening bilateral pulmonary hemangioendotheliomas was treated with low-dose daily interferon alpha for 7 months with complete regression of the pulmonary lesions and resolution of symptoms, after which the treatment was discontinued (White et al. 1989; Folkman 1989). The disease recurred within a month and the same treatment was resumed for the next 6 years. There was rapid resolution of the disease, and the patient has remained perfectly healthy and active during the past 12 years off therapy. High-grade giant cell tumors and angioblastomas, which failed all conventional therapy, have been treated with low-dose daily interferon and the results reported (Kaban et al. 1999, 2002; Marler et al. 2002; Folkman 2002). Two of 2 patients with refractory angioblastomas and 27 of 27 patients with high-grade giant-cell tumors remain off therapy and free of tumor for up to 8 years. The use of urinary bFGF as a biomarker (Nguyen et al. 1993) has been helpful in determining when to discontinue therapy (Marler et al. 2002).

1.2.2 Antiangiogenic Therapy of Cancer

Avastin (bevacizumab) is the first angiogenesis inhibitor that was tested in multi-center, placebo-controlled, double-blind clinical trials against cancer. It is also the first angiogenesis inhibitor that demonstrated significant prolongation of survival and significant prolongation of time to recurrence in patients with advanced colon cancer. At this writing eight drugs with antiangiogenic activity and varying degrees of other activities have received FDA approval for cancer therapy in the United States. Some of them have also been approved in more than 30 other countries, including the European Union and China (Table 1.2). Endostar is a modified form of endostatin approved in China for lung cancer. Twenty-two other drugs with antiangiogenic activity are in phase III clinical trials (Table 1.2). The majority of them target VEGF or one of its receptors. Therefore, currently most of the FDA-approved drugs as well as those in phase III clinical trials target a single proangiogenic protein. Approximately 30 drugs with antiangiogenic activity are in phase II clinical trials for the treatment of cancer (Table 1.3). Many of these are beginning to target more than one proangiogenic protein.

1.2.3 Mono-antiangiogenic Therapy Versus Broad-Spectrum Antiangiogenic Therapy

While approximately 60% of human cancers express VEGF, Avastin (bevacizumab) or other drugs that inhibit expression of VEGF or its receptors can be very effective against such tumors, especially if used in

combination with chemotherapy (Fig. 1.6). However, most human cancers, for example breast cancer, can also express up to five or six proangiogenic proteins (Relf et al. 1997). Therefore, eventually other angiogenic proteins (i.e., bFGF, PDGF, HGF or others) may be expressed by a tumor in which only VEGF is inhibited and give the clinical appearance of acquired "drug resistance." The "resistance" may in fact be a form of drug evasion (Casanovas 2005). Therefore, in the future, angiogenesis inhibitors may be administered in combination with other angiogenesis inhibitors, or in combination with antiangiogenic chemotherapy (metronomic, low-dose chemotherapy). Broad-spectrum angiogenesis inhibitors such as endostatin may be less susceptible to the development of "drug resistance" (Fig. 1.7). However, until these broad-spectrum angiogenesis inhibitors receive FDA approval, it is not clear whether they will encounter significantly less drug resistance. Jain has shown in both human and animals that certain angiogenesis inhibitors can "normalize" tumor blood vessels. This can lead to decreased vascular leakage, decreased intratumoral tissue pressure, temporarily increased blood flow (Jain 1988), and increased delivery of chemotherapy (Jain 1994; Carmeliet and Jain 2000). Also, other therapies delivered by the vasculature may be potentiated by antiangiogenic therapy (Jain 2001; Jain 2005; Batchelor et al. 2007).

Table 1.2. New drugs with antiangiogenic activity approved by the US Food and Drug Administration for clinical use in the USA, and by the appropriate regulatory agencies in more than 30 other countries. Velcade was approved as a proteasome inhibitor and subsequently was reported to be a potent angiogenesis inhibitor. (From Folkman J, *Nature Reviews Drug Discovery* 2007)

Date approved	Drug	Place	Disease
May 2003	Velcade (bortezomib)	USA (FDA)	Multiple myeloma
December 2003	Thalidomide	Australia	Multiple myeloma
February 2004	Avastin (bevacizumab)	USA (FDA)	Colorectal cancer
November 2004	Tarceva (erlotinib)	USA (FDA)	Lung cancer
December 2004	Avastin	Switzerland	Colorectal cancer
December 2004	Macugen	USA (FDA)	Macular degeneration
January 2005	Avastin	European Union (25 countries)	Colorectal cancer
September 2005	Endostatin (Endostar)	China (SFDA)	Lung cancer
November 2005	Tarceva	USA (FDA)	Pancreatic cancer
December 2005	Nexavar (sorafenib)	USA (FDA)	Kidney cancer
December 2005	Revlimid	USA (FDA)	Myelodysplastic syndrome
January 2006	Sutent (sunitinib)	USA (FDA)	Gastric (GIST); kidney cancer
June 2006	Lucentis	USA (FDA)	Macular degeneration
June 2006	Revlimid	USA (FDA)	Multiple myeloma
August 2006	Lucentis	Switzerland	Macular degeneration
September 2006	Lucentis	India	Macular degeneration
October 2006	Avastin	USA (FDA)	Lung cancer
November 2006	Lucentis	EU (provisional approval)	Macular degeneration

Table 1.3. Drugs with antiangiogenic activity and varying degrees of other activities in phase III trials for the treatment of cancer. (From Folkman J, *Nature Reviews Drug Discovery* 2007)

Agent	Target
AG3340 (Prinomastat) (Agouron Phamaceuticals)	MMP inhibitor
Avastin (Genentech)	VEGF
AZD2171 (AstraZeneca)	VEGFR-1, -2, -3, PDGFR
BMS-275291 (Bristol Myers Squibb)	MMP inhibitor
CCI-779 (Wyeth)	VEGFR, MTOR inhibitor
Ceflatonin (homoharringtonine) (ChemGenex)	Downregulates BEG in leukemic cells
Celebrex (celecoxib) (Pfizer)	Increases endostatin
GW786034 (pazopanib) (GlaxoSmithKline)	VEGFR
LY317615 (Enzastaurin) (Eli Lilly)	VEGF
Neovastat (Benefin/AE941) (Aetema Zentaris)	VEGFR-2, MMP inhibitor
Nexavar (sorafenib/BAY439006) (Bayer/Onyx)	VEGFR-2, PDGFR-beta
PTK787 (vatalanib) (Novartis)	VEGFR-1, -2, PDGFR
RAD001 (everolimus) (Novartis)	VEGFR, MTOR
Revlimid (lenalidomide/CC5013) (Celgene)	VEGF, precursor endothelial cells
Suramin (NCI)	IGF-1, EGFR, PDGFR, TGF-b; inhibits VEGF & bFGF
Sutent (SU11248) (Pfizer)	VEGFR-1, -2, -3, PDGFR
Tarceva (OSI774/erlotinib) (Genentech/OSI)	HER1, EGFR
Tetrathiomolybdate (TM) (Univ. of Michigan)	VEGF, Copper chelator
Thalidomide (Celgene Corporation)	VEGF, precursor endothelial cells
VEGF Trap (Regeneron Pharm.)	VEGF
Velcade (PS341/bortezomib) (Millennium Pharm.)	VEGF
ZD6474 (Zactima/vandetanib) (AstraZeneca)	VEGFR-2, EGFR

1.2.4 Treatment of Neovascular Age-related Macular Degeneration

Research in tumor angiogenesis became the basis in part for the current antiangiogenic therapy of age-related macular degeneration by two FDA-approved drugs, Macugen (pegaptanib) and Lucentis (ranibizumab), and off-label use of Avastin. In a series of publications beginning in 1993, Folkman and his collaborators Anthony Adamis, Patricia D'Amore, and Joan Miller demonstrated that VEGF was the major mediator of ocular neovascularization in the non-human primate and also reported correlative evidence for humans. In 1993 they reported that VEGF was synthesized and secreted by human retinal cells (Adamis et al. 1993). In 1994, they showed that iris neovascularization correlated spatially and temporally with intraocular VEGF levels (Miller et al. 1994). Moreover, retinal VEGF expression was shown to be upregulated in ischemic retina. Also in 1994, they reported that the vitreous of human eyes with

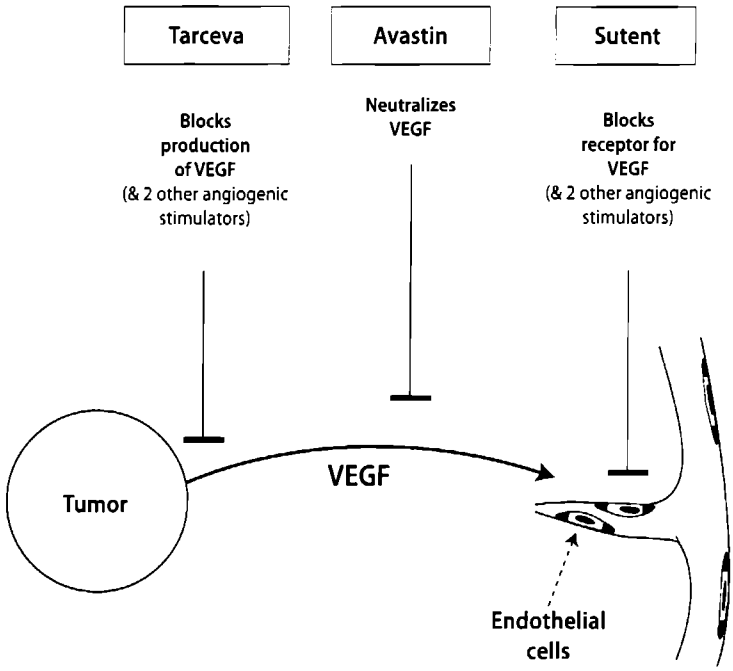


Fig. 1.6. The three general mechanisms of angiogenesis inhibitors that block VEGF: *left*, inhibition of tumor cell expression of VEGF; *center*, inhibition of the ligand; *right*, inhibition of the endothelial receptor(s) for VEGF. (From Folkman J, *Nature Reviews Drug Discovery* 2007)

proliferative diabetic retinopathy contained significantly increased VEGF levels (Adamis et al. 1994). Aiello et al., also reported high VEGF in diabetic vitreous. These were among the first papers in the scientific literature to characterize the role of VEGF in eye disease. In 1995, Folkman's laboratory, in collaboration with Napoleone Ferrara (of Genentech), reported that VEGF was the major endothelial mitogen made by hypoxic retinal cells (Shima et al. 1995). The Folkman laboratory then collaborated with Adamis, D'Amore, Miller, and Ferrara to prove that intravitreal injection of a neutralizing antibody to VEGF (the precursor to Avastin, produced by Ferrara at Genentech), inhibited retinal ischemia-associated neovascularization in a non-human primate (Adamis et al. 1996).

These findings led to the development of Macugen (pegaptanib), an anti-VEGF aptamer, by Eyetech and Lucentis, an anti-VEGF Fab fragment (ranibizumab), by Genentech. Macugen was approved by the FDA in 2004 for the treatment of age-related macular degeneration. Lucentis was approved in 2006. The very significant improvements in eyesight of patients with age-related macular degeneration have been described by Stone (2006).

Future Directions

As angiogenesis inhibitors come to be used in combinations, or together with other anti-cancer modalities, it is possible that cancer may eventually be treated as a "chronic manageable disease" (Folkman quoted in Ezzell 1998).

If biomarkers in blood or urine can be developed to achieve the accuracy of detecting recurrent cancer as early as serum calcitonin can detect recurrent medullary thyroid cancer, then it may eventually be possible to guide cancer therapy by biomarkers. For example, could angiogenesis-based biomarkers in blood (e.g., in platelets), or in urine (Roy et al. 2004), be used to detect recurrent cancer years before symptoms have appeared, or even before tu-

Table 1.4. Drugs with antiangiogenic activity and varying degrees of other activities in phase II trials for the treatment of cancer. (From Folkman J, *Nature Reviews Drug Discovery* 2007)

Agent	Target
A6 (Angstrom Pharmaceuticals)	Binds to uPA cell surface receptor
ABT-510 (Abbott Laboratories)	Thrombospondin-1 receptor CD36
Actimid (CC4047) (Celgene Corp.)	Downregulates TNF-α
AG-013736 (Pfizer)	VEGF, PDGFR
AMG706 (Amgen)	VEGF, PDGFR, KITR, RetR
AP23573 (Ariad Pharmaceuticals)	VEGF, MTOR inhibitor
AS1404 (Antisoma)	Vascular disrupting: releases TNF-α and vWF
Atiprimod (Callisto Pharm.)	VEGF, bFEGF, IL6
ATN-161 (Attenuon)	Alpha 5 beta 1 antagonist
BIBF1120 (Boehringer Ingelheim)	VEGF, PDGF, FGF receptor kinases
BMS-582664 (Bristol Myers Squibb)	VEGFR-2
CDP-791 (ImClone)	VEGFR-2, KDR
Combretastatin (Oxigene)	VE-Cadherin
E7820 (Elsai)	Inhibits integrin alpha 2 subunit on endothelium
EMD 121974 (cilengitide) (EMD)	Alpha v beta 3 and 5 antagonist
Genistein (McKesson Health Solutions)	Suppresses VEGF, neuropilin, and MMP-9
INGN 241 (Introgen Therapeutics)	VEGF, MDA-7
Interleukin-12 (NCI)	Upreregulates IP10
MEDI 522 (Abergrin) (Medimmune)	Antibody alpha V beta 3
MLN518 (tandutinib) (Millennium)	FLT3, PDGFR, cKit, CSF-1R
Panzem (2ME2) (EntreMed)	Inhibits tubulin polymerization
PI-88 (Progen Industries/Medigen)	bFGF, stimulates release of TSP1
PKC412 (Novartis)	VEGFR-2
PXD101 (CuraGen Corporation)	HDAC inhibitor
SUO14813 (Pfizer)	VEGFR-3, PDGFR-a, PDGFR-b, RET, FLT3
Tempostatin (Collard Biopharm.)	Extracellular matrix proteins
XL647 (Exelisis)	VEGFR, EGFR, HER2
XL784 (Exelisis)	ADAM-10, MMPs
XL880 (Exelisis)	VEGFR-2, C-met, RTK
XL999 (Exelisis)	VEGFR, PDGFR, FGFR, Flt-3, Scr

I	Blocks 1 major angiogenic protein	*Avastin* *VEGF Trap*	} *blocks VEGF*	
II	Blocks 2 or 3 angiogenic protein	*Sutent*	***Downregulates*** *VEGF receptor2* *PDGF receptor* *c-kit receptor*	
		Tarceva	*VEGF production* *bFGF production* *TGF-α by tumor cells*	
III	Blocks a **broad spectrum** of angiogenic regulators.	***Endostatin***	***Downregulates*** *VEGF* *bFGF* *bFGF receptor* *HIFI 1α* *EGF receptor*	***Upregulates*** *Thrombospondin-1* *Maspin* *HIFI 1α inhibitor* *TIMP-2* *Neuropilin*
		Caplostatin *(broad anti-cancer spectrum)*		

Fig. 1.7. Three types of angiogenesis inhibitors: *I* those that block a single proangiogenic protein; *II* those that can block two or three proangiogenic proteins; *III* those that are broad-spectrum angiogenesis inhibitors. (From Folkman J, *Nature Reviews Drug Discovery* 2007).

mors could be anatomically located? If this is possible, the management of cancer may eventually be liberated from dependency on determining anatomical location. This would be analogous to the history of treating infections. Before 1930, when very few drugs were available, many infections became abscesses. These had to be anatomically located for surgeons to drain them. Today, physicians treat infections guided mainly by blood biomarkers, such as the white blood cell count. For example, most patients with colon cancer are operated upon. At least 50–60% of colon cancers are cured by surgery. The rest will recur at approximately 5 years. If biomarkers could accurately detect the recurrence when it is still at a microscopic size, it could be possible to "treat the biomarker" with relatively non-toxic angiogenesis inhibitors, without having to know the anatomical location of the recurrent tumors.

It is also possible that the long-term management of age-related macular degeneration and of diabetic retinopathy by antiangiogenic therapy could be greatly improved if guided by angiogenesis-based biomarkers.

References

Abdollahi A, Hahnfeldt P, Maercker C, Gröne HJ, Debus J, Ansorge W, Folkman J, Hlatky L, Huber PE (2004) Endostatin's antiangiogenic signaling network. Molecular Cell 13:649–663

Achilles E-G, Fernandez A, Allred EN, Kisker O, Udagawa T, Beecken W-D, Flynn E, Folkman J (2001) Heterogeneity of angiogenic activity in a human liposarcoma: A proposed mechanism for 'no take' of human tumors in mice. J Natl Cancer Inst 93:1075–1081

Adamis, AP, Shima DT, Yeo K-T, Yeo T-K, Brown LF, Berse B, D'Amore PA, Folkman J (1993) Synthesis and secretion of vascular permeability factor/vascular endothelial growth factor by human retinal pigment epithelial cells. Biochem Biophys Res Commun 193:631–638

Adamis AP, Miller JW, Bernal M-T, D'Amico DJ, Folkman J, Yeo T-K, Yeo K-T (1994) Increased vascular endothelial growth factor levels in the vitreous of eyes with proliferative diabetic retinopathy. Am J Ophthal 118(4):445–450

Adamis AP, Shima DT, Tolentino MJ, Gragoudas ES, Ferrara N, Folkman J, D'Amore PA, Miller JW (1996) Inhibition of vascular endothelial growth factor prevents retinal ischemia-associated iris neovascularization in a nonhuman primate. Arch Ophthalmol 114:66–71

Aiello LP, Avery RL, Arrigg PG, Keyt BA, Jampel HD, Shah ST, Pasquale LR, Thieme H, Iwamoto MA, Park JE, Nguyen HV, Aiello LM, Ferrara N, King GA (1994) Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. N Engl J Med 331:1480–1487

Algire GH (1943) Microscopic studies of the early growth of

a transplantable melanoma of the mouse, using the transparent chamber technique. J Natl Cancer Inst 4:1

Algire GH, Legallais FY (1947) Growth rate of transplantable tumor in relation to latent period and host vascular reaction. Cancer Res 7:724

Almog N, Henke V, Flores L, Hlatky L, Kung AL, Wright RD, Berger R, Hutchinson L, Naumov GN, Bender E, Akslen LA, Achilles EG, Folkman J (2006) Prolonged dormancy of human liposarcoma is associated with impaired tumor angiogenesis. FASEB J 20:947–949

Ambati BK, Nozaki M, Singh N, Takeda A, Jani PD, Suthar T, Albuquerque RJ, Richter E, Sakurai E, Newcomb MT, Kleinman ME, Caldwell RB, Lin Q, Ogura Y, Orecchia A, Samuelson DA, Agnew DW, St Leger J, Green WR, Mahasreshti PJ, Curiel DT, Kwan D, Marsh H, Ikeda S, Leiper LJ, Collinson JM, Bogdanovich S, Khurana TS, Shibuya M, Baldwin ME, Ferrara N, Gerber HP, De Falco S, Witta J, Baffi JZ, Raisler BJ, Ambati J (2006) Corneal avascularity is due to soluble VEGF receptor-1. Nature 443:993–997

Arbiser JL, Moses MA, Fernandez CA, Ghiso N, Cao Y, Klauber N, Frank D, Brownlee M, Flynn E, Parangi S, Byers HR, Folkman J (1997) Oncogenic H-ras stimulates tumor angiogenesis by two distinct pathways. Proc Natl Acad Sci USA 94:861–866

Auerbach W, Auerbach R (1994) Angiogenesis inhibition: a review. Pharmacol Therap 63:265–311

Auerbach R, Kubai L, Knighton D, Folkman J (1974) A simple procedure for the long-term cultivation of chicken embryo. Dev Biol 41:391–394

Ausprunk DH, Folkman J (1977) Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumor angiogenesis. Microvasc Res 14:53–65

Ausprunk DH, Knighton DR, Folkman J (1974) Differentiation of vascular endothelium in the chick chorioallantois: a structural and autoradiographic study. Dev Biol 38:237–248

Ausprunk DH, Knighton DR, Folkman J (1975) Vascularization of normal and neoplastic tissues grafted to the chick chorioallantois. Am J Pathol 79(3):597–610

Ausprunk DH, Falterman K, Folkman J (1978) The sequence of events in the regression of corneal capillaries. Lab Invest 38:284–294

Bashkin P, Doctrow S, Klagsbrun M, Svahn CM, Folkman J, Vlodavsky I (1989) Basic fibroblast growth factor binds to subendothelial extracellular matrix and is released by heparitinase and heparin-like molecules. Biochem 28:1737–1743

Batchelor TT, Sorensen AG, di Tomaso E, Zhang WT, Duda DG, Cohen KS, Kozak KR, Cahill DP, Chen PJ, Zhu M, Ancukiewicz M, Mrugala MM, Plotkin S, Drappatz J, Louis DN, Ivy P, Scadden DT, Benner T, Loeffler JS, Wen PY, Jain RK (2007) AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor, normalizes tumor vasculature and alleviates edema in glioblastoma patients. Cancer Cell 11:83–95

Bissell MJ, Farson D, Tung AS (1977) Cell shape and hexose transport in normal and virus-transformed cells in culture. J Supramolecular Structure 6:1–12

Boehm T, Folkman J, Browder T, O'Reilly MS (1997) Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. Nature 390 404–7

Brem H, Arensman R, Folkman J (1975) Inhibition of tumor angiogenesis by a diffusible factor from cartilage. In: Slavkin HC, Greulich RC (eds) Extracellular matrix influences on gene expression. Academic Press, New York, pp 767–772

Brouty-Boye D, Zetter B (1980) Inhibition of cell motility by interferon. Science 208:516–518

Browder T, Butterfield CE, Kräling BM, Shi B, Marshall B, O'Reilly MS, Folkman J (2000) Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. Cancer Res 60:1878–1886

Brown LR, Wei CL, Langer R (1983) In vivo and in vitro release of macromolecules from drug delivery systems. J Pharm Sci 72:1181–1185

Cao Y, O'Reilly MS, Marshall B, Flynn E, Ji RW, Folkman J (1998) Expression of angiostatin cDNA in a murine fibrosarcoma suppresses primary tumor growth and produces long-term dormancy of metastases. J Clin Invest 101:1055–1063

Camphausen K, Moses MA, Beecken WD, Khan MK, Folkman J, O'Reilly MS (2001) Radiation therapy to a primary tumor accelerates metastatic growth in mice. Cancer Res 61:2207–2211

Carmeliet P, Jain R (2000) Angiogenesis in cancer and other diseases. Nature 407:249–257

Casanovas O, Hicklin DJ, Bergers G, Hanahan D (2005) Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. Cancer Cell 8:299–309

Cavallo T, Sade R, Folkman J, Cotran RS (1972) Tumor angiogenesis: rapid induction of endothelial mitoses demonstrated by autoradiography. J Cell Biol 54:408–420

Celik I, Surucu O, Dietz C, Heymach JV, Force J, Hoschele I, Becker CM, Folkman J, Kisker O (2005) Therapeutic efficacy of endostatin exhibits a biphasic dose–response curve. Cancer Res 65:11044–11050

Chang LK, Garcia-Cardena G, Farnebo F, Fannon M, Chen EJ, Butterfield C, Moses MA, Mulligan RC, Folkman J, Kaipainen A (2004) Dose-dependent response of FGF-2 for lymphangiogenesis. Proc Natl Acad Sci USA 101:11658–11663

Chen NT, Corey EJ, Folkman J (1988) Potentiation of angiostatic steroids by a synthetic inhibitor of arylsulfatase. Lab Invest 59:453–459

Chin L, Tam A, Pomerantz J, Wong M, Holash J, Bardeesy N, Shen Q, O'Hagan R, Pantginis J, Zhou H, Horner JW, Cordon-Cardo C, Yancopoulos GD, DePinho RA (1999) Essential role for oncogenic Ras in tumour maintenance. Nature 400:468–472

Crum R, Szabo S, Folkman J (1985) A new class of steroids inhibits angiogenesis in the presence of heparin or a heparin fragment. Science 230:1375–1378

Dameron KM, Volpert OM, Tainsky MA, Bouck N (1994) Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. Science 265:1582–1584

D'Amato RJ, Lin CM, Flynn E, Folkman J, Hamel E (1994) 2-Methoxyestradiol, an endogenous mammalian metabolite, inhibits tubulin polymerization by interacting at the colchicine site. Proc Natl Acad Sci USA 91:3964–3968

Day ED, Planinsek JA, Pressman D (1959) Localization of radio iodinated rat fibrinogen in transplanted rat tumors. J Natl Cancer Inst 23:799

Dvorak HF, Gresser I (1989) Microvascular injury in pathogenesis of interferon-induced necrosis of subcutaneous tumors in mice. J Natl Cancer Inst 81:497–502

Ehrmann RL, Knoth M (1968) Choriocarcinoma. Transfilter stimulation of vasoproliferation in the hamster cheek pouch. Studied by light and electron microscopy. J Natl Cancer Inst 41:1329–1341

Esch F, Esch F, Baird A, Ling N, Ueno N, Hill F, Denoroy L, Klepper R, Gospodarowicz D, Bohlen P, Guillemin R (1985) Primary structure of bovine pituitary basic fibroblast growth factor (FGF) and comparison with the amino-terminal sequence of bovine brain acidic FGF. Proc Natl Acad Sci USA 82:6507–6511

Ezzell C (1998) Starving tumors of their lifeblood. Sci Am 279:33–34

Ferrara N, Henzel WJ (1989) Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun 161:851–858

Folkman J (1971) Tumor angiogenesis: therapeutic implications. N Engl J Med 285:1182–1186

Folkman J (1974a) Tumor angiogenesis. In Klein G, Weinhouse S (eds) Advances in cancer research, vol 19. Academic Press, New York, pp 19:331–358

Folkman J (1974b) Tumor angiogenesis: role in regulation of tumor growth. In: Hay, ED, King TJ, Papaconstantinou J (eds) Macromolecules regulating growth and development. Academic Press, New York, pp 43–52

Folkman J (1975) Tumor angiogenesis. In: Becker FF (ed) Cancer: comprehensive treatise. Plenum Press, New York, pp 3:355–388

Folkman J (1978) Tumor angiogenesis and tumor immunity. In: Castro JE (ed) Immunological aspects of cancer. MTP Press, Lancaster, pp 267–282

Folkman J (1989) Successful treatment of an angiogenic disease. N Engl J Med 320:1211–1212

Folkman J (2002) Discussion. Antiangiogenic therapy with interferon alpha for giant cell lesions of the jaws. J Oral Maxillofac Surg 60:1111–1113

Folkman J (2004) Endogenous angiogenesis inhibitors. Acta Pathol Microbiol Immunol Scand 112:496–507

Folkman J (2006) Antiangiogenesis in cancer therapy – endostatin and its mechanisms of action. Exp Cell Res 312:594–607

Folkman J (2007) Angiogenesis: an organizing principle for drug discovery? Nature Reviews Drug Discovery 6:273–286

Folkman J, Cotran RS (1976) Relation of vascular proliferation to tumor growth. In: Richter GW (ed) International review of experimental pathology. Academic Press, New York, pp 16:207–248

Folkman J, Gimbrone MA Jr (1971) Perfusion of the thyroid. In: Diczfalusy E (ed) Karolinska Symposia on Research Methods in Reproduction Endocrinology, 4th Symposium: Perfusion Techniques. Stockholm: Karolinska Institutet 4:237–248

Folkman J, Gimbrone MA Jr (1975) Perfusion of the thyroid gland. In: Hardman JG, O'Malley BW (eds) Methods in enzymology. Hormone action, part D. Isolated cells, tissues and organ systems. Academic Press, New York, pp 39:359–364

Folkman J, Haudenschild C (1980) Angiogenesis in vitro. Nature 288:551–556

Folkman J, Klagsbrun M (1975) Tumor angiogenesis: effect on tumor growth and immunity. In: Gottlieb AA, Plescia OJ, Bishop DHL (eds) Fundamental aspects of neoplasia. Springer, New York, pp 401–412

Folkman J, Klagsbrun M (1987) Angiogenic factors. Science 235:442–447

Folkman J, Long DM (1964) The use of silicone rubber as a carrier for prolonged drug therapy. J Surg Res 4:139–142

Folkman J, Moscona A (1978) Role of cell shape in growth control. Nature 273:345–349

Folkman J, Ryeom S (2005) Is oncogene addiction angiogenesis-dependent? Cold Spring Harb Symp Quant Biol 70:389–97

Folkman MJ, Long DM, Becker FF (1962) Tumor growth in organ culture. Surg Forum 13:164–166

Folkman J, Long DM, Becker FF (1963) Growth and metastasis of tumor in organ culture. Cancer 16:453–467

Folkman J, Cole P, Zimmerman S (1966) Tumor behavior in isolated perfused organs: in vitro growth and metastases of biopsy material in rabbit thyroid and canine intestinal segment. Ann Surg 164:491–502

Folkman J, Hochberg M, Knighton D (1974) Self-regulation of growth in three dimensions: the role of surface area limitation. In: Clarkson B, Baserga R (eds) Cold Spring Harbor Symposium. Control of proliferation in animal cells. Cold Spring Harbor, New York, pp 833–842

Folkman J, Haudenschild CC, Zetter BR (1979) Long-term culture of capillary endothelial cells. Proc Natl Acad Sci USA 76:5217–5221

Folkman J, Klagsbrun M, Sasse J, Wadzinski M, Ingber D, Vlodavsky I (1988) A heparin-binding angiogenic protein – basic fibroblast growth factor – is stored within basement membrane. Am J Pathol 130:393–400

Folkman J, Browder T, Palmblad J (2001) Angiogenesis research: guidelines for translation to clinical application. Thromb Haemost 86:23–33

Fotsis T, Zhang Y, Pepper MS, Adlercreutz H, Montesano R, Nawroth PP, Schweigerer L (1994) The endogenous oestrogen metabolite 2-methoxyestradiol inhibits angiogenesis and suppresses tumor growth. Nature 368:237–239

Gimbrone MA Jr, Aster RH, Cotran RS, Corkery J, Jandl JH, Folkman J (1969) Preservation of vascular integrity in organs perfused in vitro with a platelet-rich medium. Nature 222:33–36

Gimbrone MA Jr, Leapman SB, Cotran RS, Folkman J (1972) Tumor dormancy in vivo by prevention of neovascularization. J Exp Med 136(2):261–276

Gimbrone MA Jr, Cotran RS, Folkman J (1973) Endothelial regeneration: studies with human endothelial cell cultures. Ser Haematol 6:453–455

Gimbrone MA Jr, Cotran RS, Leapman SB, Folkman J (1974a) Tumor growth and neovascularization: an experimental model using rabbit cornea. J Natl Canc Inst 52(2):413–427

Gimbrone MA Jr, Cotran RS, Folkman J (1974b) Human vascular endothelial cells in culture: growth and DNA synthesis. J Cell Biol 60:673–684

Giuriato S, Ryeom S, Fan AC, Bachireddy P, Lynch RC, Rioth MJ, van Riggelen J, Kopelman AM, Passegue E, Tang F, Folkman J, Felsher DW (2006) Sustained regression of tumors upon MYC inactivation requires p53 or thrombospondin-1 to reverse the angiogenic switch. Proc Natl Acad Sci USA 103:16266–71

Goldacre RJ, Sylvén B (1962) On the access of blood borne dyes to various tumor regions. Br J Cancer 16:306

Goldmann E (1907) Growth of malignant disease in man and the lower animals with special reference to vascular system. Proc R Soc Med 1:1

Gospodarowicz D (1974) Localization of a fibroblast growth factor and its effect with hydrocortisone on 3T3 cell growth. Nature 249:123–127

Gospodarowicz D, Moran J, Braun D, Birdwell C (1976) Clonal growth of bovine endothelial cells: fibroblast growth factor as a survival agent. Proc Natl Acad Sci USA 73:4120–4124

Greene HSN (1952) The significance of the heterologous transplantability of human cancer. Cancer 5:24

Greenblatt M, Shubik P (1968) Tumor angiogenesis: transfilter diffusion studies in the hamster by the transparent chamber technique. J Natl Cancer Inst 41:111–124

Greenblatt M, Choudari KVR, Sanders AG, Shubik P (1969) Mammalian microcirculation in the living animal: microcirculation of renal homograft transplants in hamsters. Am J Pathol 56:317

Gullino PM, Grantham FH (1962) Studies on the exchange of fluids between host and tumor. III. Regulation of blood flow in hepatomas and other rat tumors. J Natl Cancer Inst 28:211

Hamano Y, Kalluri R (2005) Tumstatin, the NC1 domain of alpha3 chain of type IV collagen, is an endogenous inhibitor of pathological angiogenesis and suppresses tumor growth. Biochem Biophys Res Commun 333:292–8

Hanahan D, Folkman J (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 86:353–364

Hanahan D, Bergers G, Bergsland E (2000) Less is more, regularly: metronomic dosing of cytotoxic drugs can target tumor angiogenesis in mice. J Clin Invest 105:1045–1047

Haudenschild CC, Zahniser D, Folkman J, Klagsbrun M (1976) Human vascular endothelial cells in culture. Lack of response to serum growth factors. Exp Cell Res 98:175–183

Hellstrom M, Phng LK, Hofmann JJ, Wallgard E, Coultas L, Lindblom P, Alva J, Nilsson AK, Karlsson L, Gaiano N, Yoon K, Rossant J, Iruela-Arispe ML, Kalen M, Gerhardt H, Betsholtz C (2007) Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. Nature 445:776–80

Holmgren L, O'Reilly MS, Folkman J (1995) Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. Nat Med 1:149–53

Huang S, Ingber DE (2005) Cell tension, matrix mechanics, and cancer development. Cancer Cell 8:175–176

Ide AG, Baker NH, Warren SL (1939) Vascularization of the Brown-Pearce rabbit epithelioma transplant as seen in the transparent ear chamber. Am J Roentgenol 42:891

Ingber DE, Folkman J (1989a) How does extracellular matrix control capillary morphogenesis? Cell 58:803–805

Ingber DE, Folkman J (1989b) Tension and compression as basic determinants of cell form and function: utilization of a cellular tensegrity mechanism. In: Stein W, Bronner F (eds) Cell shape: determinants, regulation, and regulatory role. Academic Press, New York, pp 3–31

Ingber DE, Folkman J (1989c) Mechanochemical switching between growth and differentiation during fibroblast growth factor – stimulated angiogenesis in vitro: role of extracellular matrix. J Cell Biol 109:317–330

Ingber DE, Madri JA, Folkman J (1987) Endothelial growth factors and extracellular matrix regulate DNA synthesis through modulation of cell and nuclear expansion. In Vitro Cell Dev Biol 23:387–394

Italiano J, Richardson JL, Folkman J, Klement G (2006) Blood platelets organize pro- and anti-angiogenic factors into separate, distinct alpha granules: implications for the regulation of angiogenesis. Blood 108:120a, abstract 393

Jaffe EA, Nachman RL, Becker CG, Minick CR (1973) Culture of human endothelial cells derived from umbilical veins. Ientification by morphologic and immunologic criteria. J Clin Invest 52:2745–2756

Jain RK (1988) Determinants of tumor blood flow: a review. Cancer Res 48:2641–2658

Jain RK (1994) Barriers to drug delivery. Sci Am 271:58–65

Jain RK (2001) Normalizing tumor vasculature with anti-angiogenic therapy: a new paradigm for combination therapy. Nat Med 7:987–989

Jain RK (2005) Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science 307:58–62

Jain M, Arvanitis C, Chu K, Dewey W, Leonhardt E, Trinh M, Sundberg CD, Bishop JM, Felsher DW (2002) Sustained loss of a neoplastic phenotype by brief inactivation of MYC. Science 297:102–104

Kaban LB, Mulliken JB, Ezekowitz RA, Ebb D, Smith PS, Folkman J (1999) Antiangiogenic therapy of a recurrent giant cell tumor of the mandible with interferon alfa-2a. Pediatrics 103:1145–1149

Kaban LB, Troulis MJ, Ebb D, August M, Hornicek FJ, Dodson TB (2002) Antiangiogenic therapy with interferon alpha for giant cell lesions of the jaws. J Oral Maxillofac Surg 60:1103–1111

Kamphaus GD, Colorado PC, Panka DJ, Hopfer H, Ramchandran R, Torre A, Maeshima Y, Mier JW, Sukhatme VP, Kalluri R (2000) Canstatin, a novel matrix-derived inhibitor of angiogenesis and tumor growth. J Biol Chem 275:1209–1215

Kisker O, Onizuka S, Becker CM, Fannon M, Flynn E, D'Amato R, Zetter B, Folkman J, Ray R, Swamy N, Pirie-Shepherd S (2003) Vitamin D binding protein-macrophage activating factor (DBP-maf) inhibits angiogenesis and tumor growth in mice. Neoplasia 5:32–40

Klement G, Baruchel S, Rak J, Man S, Clark K, Hicklin DJ, Bohlen P, Klerbel RS (2000) Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. J Clin Invest 105:R15–24

Klement G, Kikuchi L, Kieran M, Almog N, Yip TT, Folkman J (2004) Early tumor detection using platelets uptake of angiogenesis regulators. Proc 47th American Society of Hematology. Blood 104:239a, abstract 839

Klement G, Cervi D, Yip T, Folkman J, Italiano J (2006) Platelet PF-4 is an early marker of tumor angiogenesis. Blood 108:426a, abstract 1476

Knighton D, Ausprunk D, Tapper D, Folkman J (1977) Avascular and vascular phases of tumor growth in the chick embryo. Br J Canc 35:347–356

Kuo C, LaMontagne KR Jr, Garcia-Cardena G, Ackley BD, Kalman D, Parks S, Christofferson R, Kamihara J, Ding Y-H, Lo K-M, Gillies S, Folkman J, Mulligan RC, Javaherian K (2001) Oligomerization-dependent regulation of motility and morphogenesis by the collagen XVIII NC1/endostatin domain. J Cell Biol 152:1233–1246

Langer R, Folkman J (1976) Polymers for the sustained release of proteins and other macromolecules. Nature 263:797–800

Lobov IB, Renard RA, Papadopoulos N, Gale NW, Thurston G, Yancopoulos GD, Wiegand SJ (2007) Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. Proc Natl Acad Sci USA (in press)

Marler JJ, Rubin JB, Trede NS, Connors S, Grier H, Upton J, Mulliken JB, Folkman J (2002) Successful antiangiogenic therapy of giant cell angioblastoma with interferon alpha 2b: report of 2 cases. Pediatrics 109:E37

Miao HQ, Lee P, Lin H, Soker S, Klagsbrun M(2000) Neuropilin-1 expression by tumor cells promotes tumor angiogenesis and progression. FASEB J 14:2532–2539

Miller JW, Adamis AP, Shima DT, D'Amore PA, Moulton RS, O'Reilly MS, Folkman J, Dvorak HF, Brown LF, Berse B, Yeo T-K, Yeo K-T (1994) Vascular endothelial growth factor/vascular permeability factor is temporally and spatially correlated with ocular angiogenesis in a primate model. Am J Pathol 145:574–584

Murray JC (2001) Angiogenesis protocols, Humana Press, Totowa, New Jersey

Muthukkaruppan V, Auerbach R (1979) Angiogenesis in the mouse cornea. Science 205:1416–1418

Naumov GN, Bender E, Zurakowski D, Kang SY, Sampson D, Flynn E, Watnick RS, Straume O, Akslen LA, Folkman J, Almog N (2006a) A model of human tumor dormancy: an angiogenic switch from the nonangiogenic phenotype. J Natl Cancer Inst 98:316–325

Naumov GN, Akslen LA, Folkman J (2006b) Role of angiogenesis in human tumor dormancy. Cell Cycle 5:1779–1787

Noguera-Troise I, Daly C, Papadopoulos NJ, Coetzee S, Boland P, Gale NW, Lin HC, Yancopoulos GD, Thurston G (2006) Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. Nature 444:1032–1037

Norrby K (2006) In vivo models of angiogenesis. J Cell Mol Med 10:588–612

Nguyen M, Watanabe H, Budson AE, Richie JP, Folkman J (1993) Elevated levels of the angiogenic peptide basic fibroblast growth factor in urine of bladder cancer patients. J Natl Cancer Inst 85:241–2

Nguyen M, Shing Y, Folkman J (1994) Quantitation of angiogenesis and antiangiogenesis in the chick embryo chorioallantoic membrane. Microvasc Res 47:31–40

Nyberg P, Xie L, Kalluri R (2005) Endogenous inhibitors of angiogenesis. Cancer Res 65:3967–3979

O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, Lane WS, Cao Y, Sage EH, Folkman J (1994) Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell 79:315–28

O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR, Folkman J (1997) Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell 88:277–85

Panigrahy D, Singer S, Shen LQ, Butterfield CE, Freedman DA, Chen EJ, Moses MA, Kilroy S, Duensing S, Fletcher C, Fletcher JA, Hlatky L, Hahnfeldt P, Folkman J, Kaipainen A (2002) PPARγ ligands inhibit primary tumor growth and metastasis by inhibiting angiogenesis. J Clin Invest 110:923–932

Peterson H-I (ed) (1979) Tumor blood circulation: angiogenesis, vascular morphology, and blood flow of experimental and human tumors. CRC Press, West Palm Beach, Fla

Rak J; Yu JL; Klement G; Kerbel RS (2000) Oncogenes and angiogenesis: signaling three-dimensional tumor growth. J Investig Dermatol Symp Proc 5:24–33

Relf M, LeJeune S, Scott PA, Fox S, Smith K, Leek R, Moghaddam A, Whitehouse R, Bicknell R, Harris AL (1997) Expression of the angiogenic factors vascular endothelial cell growth factor, acidic and basic fibroblast growth factor, tumor growth factor beta-1, platelet-derived endothelial cell growth factor, placenta growth factor, and pleiotrophin in human primary breast cancer and its relation to angiogenesis. Cancer Res 57:963–9

Ribatti D, Locci P, Marinucci L, Lilli C, Roncali L, Becchetti E (1995). Exogenous heparin induces an increase in glycosaminoglycans of the chick embryo chorioallantoic membrane: its possible role in the regulation of angiogenic processes. Int J Microcirculation Clin Exp 15:181–185

Ribatti D, Gualandris A, Bastaki M, Vacca, A, Lurlaro M, Roncali L, Presta M (1997a) New model for the study of angiogenesis and antiangiogenesis in the chick embryo chorioallantoic membrane: the gelatin sponge/chorioallantoic membrane assay. J Vasc Res 34:455–463

Ribatti D, Nico B, Bertossi M, Roncali L, Presta M (1997b) Basic fibroblast growth factor-induced angiogenesis in the chick embryo chorioallantoic membrane: an electron microscopy study, Microvasc Res 53:187–190

Ribatti D, Crivellato E, Candussio L, Vacca A, Nico B, Benagiano V, Roncali L, Dammacco F (2002) Angiogenic activity of rat mast cells in the chick embryo chorioallantoic membrane is down-regulated by treatment with recombinant human a2a interferon and partly mediated by fibroblast growth factor-2. Haematologica 87:465–471

Ridgway J, Zhang G, Wu Y, Stawicki S, Liang WC, Chanthery Y, Kowalski J, Watts RJ, Callahan C, Kasman I, Singh M, Chien M, Tan C, Hongo JA, de Sauvage F, Plowman G, Yan M (2006) Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. Nature 444:1083–1087

Rohan RM, Fernandez A, Udagawa T, Yuan J, D'Amato RJ (2000) Genetic heterogeneity of angiogenesis in mice. FASEB J 14:871–876

Rosenthal RA, Megyesi JF, Henzel WJ, Ferrara N, Folkman J (1990) Conditioned medium from mouse sarcoma 180 cells contains vascular endothelial growth factor. Growth Factors 4:53–59

Roy R, Wewer UM, Zurakowski D, Pories SE, Moses MA (2004) ADAM 12 cleaves extracellular matrix proteins and correlates with cancer status and stage. J Biol Chem 279:51323–51330

Rubin R, Casarett G (1966) Microcirculation of tumors. I. Anatomy, function and necrosis. Clin Radiol 17:220

Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF (1983) Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 219:983–985

Shaked Y, Bertolini F, Man S, Rogers MS, Cervi D, Foutz T, Rawn K, Voskas D, Dumont DJ, Ben-David Y, Lawler J, Henkin J, Huber J, Hicklin DJ, D'Amato RJ, Kerbel RS (2005) Genetic heterogeneity of the vasculogenic phenotype parallels angiogenesis: implications for cellular surrogate marker analysis of antiangiogenesis. Cancer Cell 7:101–111

Shima DT, Adamis AP, Ferrara N, Yeo K-T, Yeo T-K, Allende R, Folkman J, D'Amore PA (1995) Hypoxic induction of endothelial cell growth factors in retinal cells: identification and characterization of vascular endothelial growth factor (VEGF) as the mitogen. Mol Med 1:182–193

Shing Y, Folkman J, Sullivan R, Butterfield C, Murray J, Klagsbrun M (1984) Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. Science 223:1296–1298

Shing Y, Folkman J, Haudenschild C, Lund D, Crum R, Klagsbrun M (1985) Angiogenesis is stimulated by a tumor-derived endothelial cell growth factor. J Cell Biochem 29:275–287

Sidky YA, Borden EC (1987) Inhibitors of angiogenesis by interferons: effects on tumor- and lymphocyte-induced vascular responses. Cancer Res 47:5155–5161

Singh RK, Gutman M, Bucana CD, Sanchez R, Llansa N, Fidler IJ (1995) Interferons alpha and beta down-regulate the expression of basic fibroblast growth factor in human carcinomas. Proc Natl Acad Sci USA 92:4562–4566

Slaton JW, Perrotte P, Inoue K, Dinney CP, Fidler IJ (1999) Interferon-alpha-mediated down-regulation of angiogenesis-related genes and therapy of bladder cancer are dependent on optimization of biological dose and schedule. Clin Cancer Res 5:2726–2734

Smith RS, Hawes NL, Kuhlmann SD, Heckenlively JR, Chang B, Roderick TH, Sundberg JP (1996) Corn1: a mouse model of corneal surface disease and neovascularization. Invest Ophthalmol Vis Sci 37:397–404

Stone EM (2006) A very effective treatment for neovascular macular degeneration. N Engl J Med 355:1493–1495

Suchting S, Freitas C, le Noble F, Benedito R, Breant C, Duarte A, Eichmann A (2007) The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. Proc Natl Acad Sci USA (in press)

Tannock IF (1970) Population kinetics of carcinoma cells, capillary endothelial cells, and fibroblasts in a transplanted mouse mammary tumor. Cancer Res 30:2470

Taylor S, Folkman J (1982) Protamine is an inhibitor of angiogenesis. Nature 297:307–12

Teodoro JG, Parker AE, Zhu X, Green MR (2006) P53-mediated inhibition of angiogenesis through up-regulation of a collaghen prolyl hydroxylase. Science 313:968–971

Thiersch C (1865) Der Epithelialkrebs namentlich der Haut mit Atlas. Leipzig. As cited in Rogers W, Edlich RF, Lewis DV, Aust JB. Surg Clin North Am 47:1473

Thiessen NW (1936) The vascularity of benign and malignant lesions of the stomach. Surg Gynecol Obstet 2:149

Tjin Tham Sjin RM, Naspinsku J, Birsner AE, Li C, Chan R, Lo K, Gillies S, Folkman J, Samulski J, Javaherian K

(2006) Endostatin therapy reveals a U-shaped curve for anti-tumor activity. Cancer Gene Ther 13:619–627

Toolan HW (1951) Proliferation and vascularization of adult human epithelium in subcutaneous tissues of x-irradiated heterologous hosts. Proc Soc Exp Biol Med 78:540

Udagawa T, Fernandez A, Achilles EG, Folkman J, D'Amato RJ (2002) Persistence of microscopic human cancers in mice: alterations in the angiogenic balance accompanies loss of tumor dormancy. FASEB J 16:1361–1370

Udagawa T, Puder M, Wood M, Schaefer BC, D'Amato RJ (2006) Analysis of tumor-associated stromal cells using SCID GFP transgenic mice: contribution of local and bone marrow-derived host cells. FASEB J 20:95–102

Verheul HMW, Lolkema MPJ, Qian DZ, Hilkes YHA, Liapi E, Pili R, Voest EE (2006) Uptake of bevacizumab by platelets blocks the biological activity of platelet-derived vascular endothelial growth actor (VEGF). Proceedings of the American Association of Cancer Research 47:5708 (abstract)

Vlodavsky I, Folkman J, Sullivan R, Fridman R, Ishai-Michaeli R, Sasse J, Klagsbrun M (1987) Endothelial cell-derived basic fibroblast growth factor: Synthesis and deposition into subendothelial extracellular matrix. Proc Natl Acad Sci USA 84:2292–2296

Warner L (1964) Frog renal adenocarcinoma: living vascular tree in ocular transplants. Anat Rec 148:425

White CW, Sondheimer HM, Crouch EC, Wilson H, Fan LL (1989) Treatment of pulmonary hemangiomatosis with recombinant interferon alfa-2a. N Engl J Med 320:1197–1200

Yamamoto N, Kumashiro R (1993) Conversion of vitamin D3 binding protein (group-specific component) to a macrophage activating factor by the stepwise action of beta-galactosidase of B cells and sialidase of T cells. J Immunol 151:2794–2802

Yang Q, Rasmussen SA, Friedman JM (2002) Mortality associated with Down's syndrome in the USA from 1983 to 1997: A population-based study. Lancet 359:1019–1025

Zorick TS, Mustacchi Z, Bando SY, Zatz M, Moreira-Filho CA, Olsen B, Passos-Bueno MR (2001) High serum endostatin levels in Down syndrome: implications for improved treatment and prevention of solid tumours. Eur J Hum Genet 9:811–814

Zweifach BW (1961) Functional behavior of the microcirculation. Thomas, Springfield, Ill

Mechanisms

Vasculogenesis and Angiogenesis in Development

2

ANNE EICHMANN, KARINE BOUVRÉE, LUC PARDANAUD

Abstract

We summarize here recent findings that provide novel insights into the mechanisms regulating the emergence of endothelial progenitors from the mesoderm, their coalescence into the primary vascular system, the remodeling into arteries and veins, the presence of neural guidance receptors on endothelial tip cells and the identification of circulating endothelial cells early in ontogeny. We presente candidate molecules involved in these different processes during vascular development.

CONTENTS

Introduction

The vertebrate vascular system is comprised of a highly organized, branching network of arteries, capillaries and veins that penetrates virtually all body tissues, enabling efficient exchange of oxygen and nutrients and removal of waste products. Blood, which is the carrier of oxygen, carbon dioxide and metabolic products, is pumped from the heart through the arterial system into the tissue capillary bed, where exchanges occur. The blood is then channeled through the venous system back into the heart. The blood–vascular system is affected by numerous pathologies, including atherosclerosis and cancer, the two major causes of death in developed countries (Folkman 1995; Cines et al. 1998; Ferrara and Alitalo 1999; Carmeliet and Jain 2000, for reviews). The lymphatic system drains extravasated fluid, the lymph, from the extracellular space and returns it to the venous circulation. The lymphatic vasculature is also essential for the immune defense, as lymph and

any foreign material present in it, such as microbial antigens, are filtered through the chain of lymph nodes (Alitalo et al. 2005).

The capillary bed, which comprises the largest surface of the vascular system, is composed solely of endothelial cells (EC), occasionally associated with external pericytes. These simple capillary tubes are surrounded by a basement membrane. Larger vessels have additional layers constituting the vessel wall, which are composed of a muscular layer, the tunica media, and an outer connective tissue layer, the tunica adventitia, containing vasa vasorum and nerves (Wheater et al. 1978). The size of the vessel wall varies according to the vessel size and type.

Blood vessel formation during embryonic development is achieved by two successive processes, called vasculogenesis and angiogenesis (Risau 1997; Coultas et al. 2005). The term vasculogenesis describes the de novo specification of endothelial precursor cells or angioblasts from the mesoderm (Fig. 2.1). These newly formed cells coalesce into lumenized tubes of the primary vascular plexus, which consists of the central axial vessels (i.e., the dorsal aortae and the cardinal veins), as well as of a meshwork of homogeneously sized capillaries. Lumenization of forming capillary tubes was studied almost 100 years ago by observations of living chick embryos cultured on glass coverslips (Sabin 1920) and was thought to involve 'liquefaction' of intracellular compartments of early EC. Recently, two-photon imaging of living transgenic zebrafish embryos expressing green fluorescent protein fused to the vacuolar protein cdc42 showed that lumen formation in the intersegmental vessels is indeed driven by formation and fusion of intraendothelial vacuoles (Kamei et al. 2006). The primary vascular plexus is established before the onset of heart beat and is ready to receive the first circulatory output. This primitive network subsequently expands via angiogenesis, i.e., sprouting, bridging and branching by intussusception of pre-existing vessels. Angiogenesis leads to remodeling of the primary vascular plexus into a highly branched hierarchical vascular tree, composed of arteries and veins (Fig. 2.1). Lymphatic vessels develop mainly by sprouting from embryonic veins, although existence of mesoderm-derived lymphangioblasts has been described as well (Wilting et al. 2001) (Fig. 2.1). Recruitment of mural cells (pericytes in medium-sized and smooth muscle cells in large vessels) around the endothelial layer completes the formation of a functional network. In this chapter, we will describe successively the main cellular and molecular events that occur during vasculogenesis and angiogenesis.

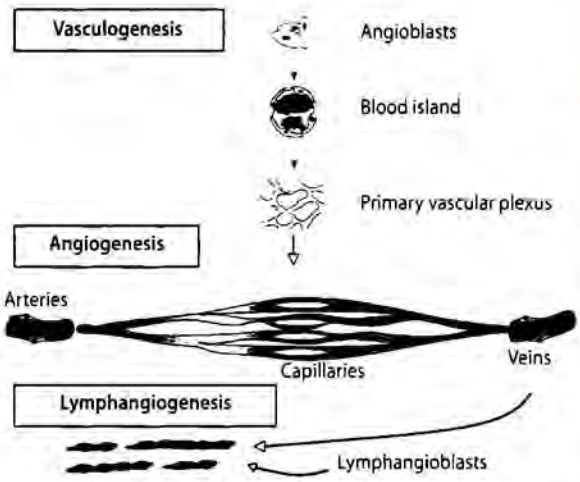


Fig. 2.1. Schematic diagram of the different steps in vascular development

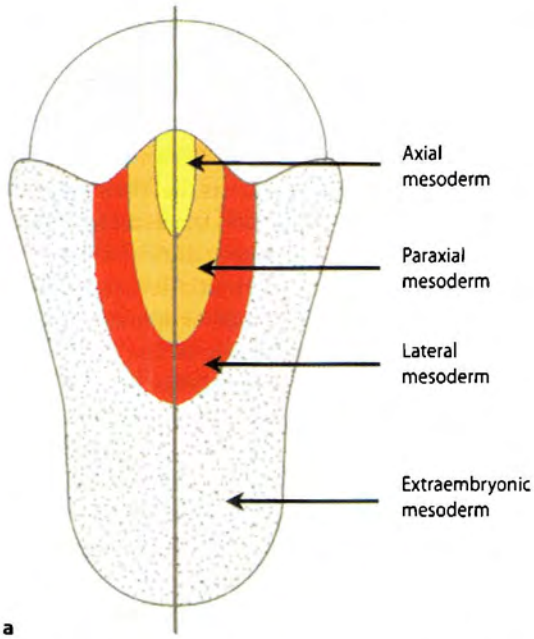
2.2 Vasculogenesis

As the diffusion distance of oxygen is limited (100–200 μm), the vascular system in any organ and tissue has to be established early during development. EC differentiation is first observed during gastrulation, when cells invaginate through the primitive streak to form the mesoderm. Newly formed mesodermal cells soon organize into axial mesoderm (notochord), paraxial mesoderm (somites), intermediate mesoderm (kidney and gonads) and lateral plate mesoderm (Fig. 2.2a). The lateral plate mesoderm will split into two layers after the formation of the coelom: a dorsal sheet, the somatopleural

mesoderm, and a ventral sheet, the splanchnopleural mesoderm. The dorsal sheet is in contact with the ectoderm and will form the body wall and limbs while the ventral sheet is in contact with the endoderm and will form the visceral organs. The posterior part of the mesoderm, which occupies about half of the embryo during early gastrulation stages, will give rise to the extraembryonic mesoderm.

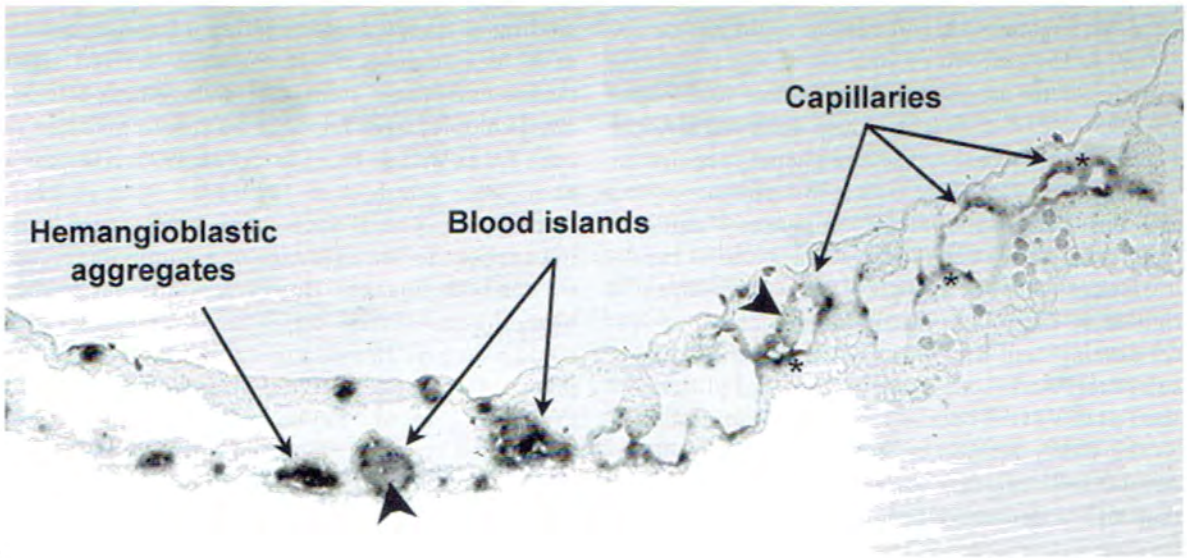
The first EC that form in the gastrulating embryo originate from lateral and posterior mesoderm (Murray 1932). These territories express the vascular endothelial growth factor receptor-2 (VEGFR2) (Eichmann et al. 1993) (Fig. 2.2b). Vascular endothelial growth factor (VEGF) and its receptor VEGFR2 are the most critical drivers of embryonic vessel formation (see Olsson et al. 2006 for review). VEGF is expressed in spatial and temporal association with almost all physiological events of vascular formation in vivo (Jakeman et al. 1993; Shweiki et al. 1993). VEGFR2 expression is already observed at very early stages of development (Fig. 2.2b) and subsequently becomes mainly restricted to EC of all types of blood vessels as well as lymphatic vessels (Eichmann et al. 1993; Yamaguchi et al. 1993; Wilting et al. 1997). Mice deficient in VEGFR2 ($VEGFR2^{-/-}$) die in utero between 8.5 and 9.5 days post-coitum, as a result of an early defect in the development of hematopoietic cells (HC) and EC. Yolk-sac blood islands were absent at 7.5 days, organized blood vessels could not be observed in the embryo or yolk sac at any stage, and hematopoietic progenitors were absent (Shalaby et al. 1995). VEGF-deficient mouse embryos also die at E8.5–E9.5 and exhibit severe phenotypes similar to that of the $VEGFR2^{-/-}$ mice; this phenotype was also observed in the $VEGF^{+/-}$ embryos (Carmeliet et al. 1996; Ferrara et al. 1996). The lethality resulting from the loss of a single allele is indicative of a tight dose-dependent regulation of embryonic vessel development by VEGF. Taken together, the results described above confirm the major position of the VEGF/VEGFR2 system in vascular formation.

The newly formed lateral and posterior mesodermal cells migrate toward the yolk sac, where they will differentiate to blood island EC and HC. During their migration, the precursors aggregate to clusters, termed hemangioblastic aggregates (Sabin 1920). The peripheral cells of these aggregates subsequently flatten and differentiate to EC, while the centrally located cells differentiate to HC (Fig. 2.2c). The simultaneous emergence of EC and HC in the blood islands led to the hypothesis that they were derived from a common precursor, the hemangioblast (Sabin 1920). VEGFR2 expression during successive stages of hemangioblast differentiation shows that gastrulating precursors as well as hemangioblastic aggregates are positive, while in the differentiated islands only the EC express this gene and no expression is detected in HC (Fig. 2.2c). These observations are compatible with the hypothesis that VEGFR2 labels a bipotent progenitor and that after lineage diversification, only one of the two daughter cells maintains expression of this gene. In support of this idea, isolated $VEGFR2^{+}$ cells from posterior territories of chick embryos at the gastrulation stage cultured in semi-solid medium in vitro differentiated to HC of different lineages. In the presence of VEGF, EC differentiation of the $VEGFR2^{+}$ precursors was induced (Eichmann et al. 1997). These experiments showed that $VEGFR2^{+}$ precursors could indeed give rise to EC as well as HC, consistent with the hypothesis that this receptor is expressed by a common precursor. However, at the single cell level, an individual $VEGFR2^{+}$ cell would either differentiate to an EC or an HC, but not both, precluding a direct demonstration of the existence of a 'hemangioblast'. In cultures derived from mouse embryonic stem (ES) cells, single-cell-derived colonies were found to be able to give rise to both EC as well as HC (Choi et al. 1998; Nishikawa et al. 1998; Schuh et al. 1999; Fehling et al. 2003; Huber et al. 2004; D'Souza et al. 2005), again lending support to the existence of a common precursor for both lineages. However, additional studies have shown that ES cell-derived $VEGFR2^{+}$ cells could also give rise to smooth muscle cells in the presence of platelet-derived growth factor (PDGF) (Yamashita et al. 2000), indicating that rather than being strictly committed to only the EC and the HC lineages, these cells may be pluri- or multipotent progenitors. Cell-tracking experiments in zebrafish embryos have recently revealed bipo-



a

b



c

Fig. 2.2a–c. VEGFR2 expression during early chick vascular development. a Fate map of gastrulating chick embryo. b, c VEGFR2 in situ hybridization. b VEGFR2 is expressed in presumptive extraembryonic mesoderm in gastrulating chick embryos. c Transverse section of a 15-somite chick embryo shows that VEGFR2 is expressed by hemangioblastic aggregates while VEGFR2 is downregulated in HC in both blood islands and capillaries (*arrowheads*). In contrast, VEGFR2 expression is maintained in EC (*asterisks*)

tential hemangioblastic precursors present in the ventral mesoderm of gastrulation-stage embryos (Vogeli et al. 2006). Interestingly, the data published by Vogeli et al. suggest that hemangioblasts represent a distinct subpopulation of endothelial and hematopoietic precursors, and that not all EC and HC are derived from common precursors in zebrafish embryos. Recently in the mouse, Ueno and Weissman (2006) showed a polyclonal origin of yolk sac blood islands using genetic cell tracking approaches.

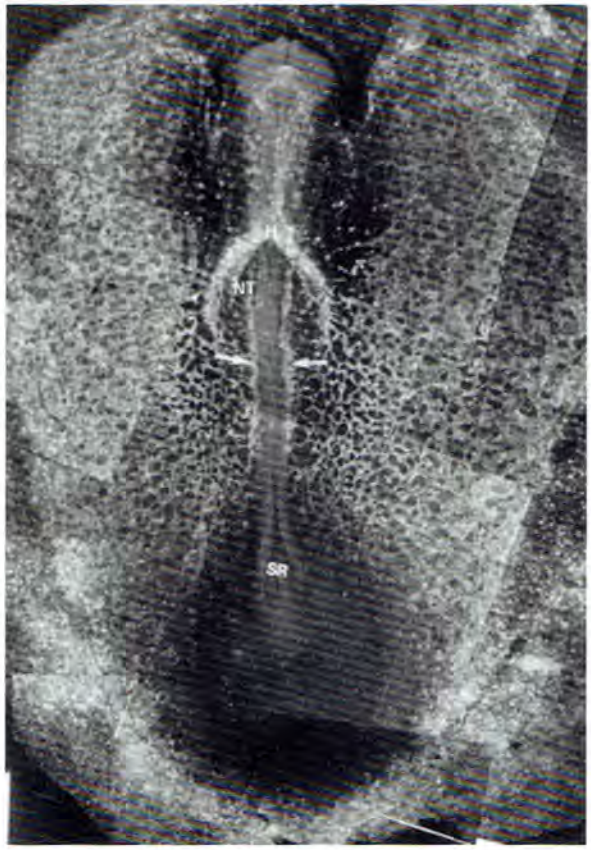
Formation of the Primary Capillary Plexus and Remodeling into Arteries and Veins

Following the differentiation of the yolk sac blood islands, EC surrounding these blood islands rapidly anastomose to form a capillary meshwork, which serves as a scaffold for the beginnings of circulation. Figure 2.3a shows the structure of the primary capillary plexus revealed by immunohistochemistry with the QH1 antibody, specific for EC in the quail (Pardanaud et al. 1987). The embryo has thus laid down the rudiments of its vascular system before the onset of heart beat around the 10-somite stage in the chick. Inside the embryo proper, one major vessel, the dorsal aorta, and numerous capillaries have differentiated, while a meshwork of homogeneously sized capillaries is present in the yolk sac (Fig. 2.3a). EC differentiation in the embryo proper during this developmental time window occurs in the absence of associated hematopoiesis.

After the onset of heartbeat and of blood flow, the yolk sac capillary plexus is rapidly remodeled into arteries and veins and a circulatory loop essential for survival is established. Figure 2.3b shows a chick embryo about 24 h older than the embryo in Fig. 2.3a injected with FITC-dextran: it is obvious that the primary capillary plexus has been remodeled into a functional circulatory system containing arteries, capillaries and veins. This remodeling step is critical for the embryo's survival and indeed, many mouse mutants for genes involved in vascular development die during this 'remodeling' phase (see Coultas et al. 2005 for review). The newly formed blood island HC are channeled through this primitive circulation. These yolk sac hematopoietic precursors mostly differentiate into primitive erythrocytes, which are replaced, as development proceeds, by definitive hematopoietic precursors generated in the embryo proper (Dieterlen-Lièvre 1975; Cumano et al. 2001). These definitive precursors are again observed to develop in close association with the ventral endothelium of the dorsal aorta (Pardanaud et al. 1996; Jaffredo et al. 1998).

Molecular Markers Specific for Arteries and Veins

Based on classic studies, it was believed that EC of the primary capillary plexus constitute a rather homogeneous group of cells and that differentiation into arteries and veins occurred due to the influence of hemodynamic forces (Thoma 1893). Over the past few years, however, several signaling molecules have been discovered that label arterial or venous EC from early developmental stages onward, prior to the onset of flow and the assembly of a vascular wall. Interestingly, most of these molecules are also expressed in the nervous system, where they regulate cell fate decisions and guidance of migration of neuronal precursors as well as of developing axons (Carmeliet and Tessier-Lavigne 2005; Eichmann et al. 2005). It is thus tempting to speculate that these molecules regulate similar cell behaviors in the developing vascular system. Arterial EC in chick, mouse and zebrafish selectively express ephrin-B2, neuropilin-1 (NRP-1) and members of the Notch pathway, including Notch receptors, ligands and downstream effectors (Adams 2003; Alva and Iruela-Arispe 2004; Shawber and Kitajewski 2004). Other molecules are specifically expressed in the venous system, including EphB4, the recep-



a

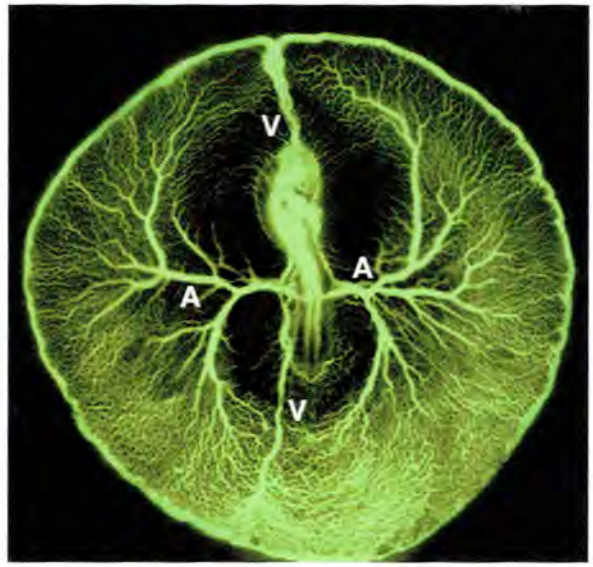


Fig. 2.3a,b. Remodeling of the primary vascular plexus during avian development. a Primary capillary plexus of a 12-somite quail embryo revealed by QH1 whole-mount immunostaining. Arrows point to dorsal aorta. b Functional circulation visualized by injection of FITC-dextran in a chick embryo 24 h older than the embryo shown in a (courtesy of J. Favier and G. Sihn). *NT*, Neural tube; *SR*, sinus rhomboidalis; *A*, arteries; *V*, veins

tor for arterial ephrin-B2 (Gerety et al. 1999) and COUPTFII (You et al. 2005). The neuropilin-2 (NRP-2) receptor is expressed by veins and, at later developmental stages, becomes restricted to lymphatic vessels in chick and mice (Herzog et al. 2001; Yuan et al. 2002). In chick embryos, NRP-1 and -2 receptors are expressed on separate, but mixed populations of cells in the yolk sac blood islands. They become segregated prior to the onset of flow to arterial (NRP-1, posterior) and venous (NRP-2, anterior) poles of the embryo (Herzog et al. 2005). Based on these specific expression patterns and on lineage studies in the zebrafish embryo (Zhong et al. 2001), it was proposed that arterial and venous fates are genetically pre-determined. A possible role for these signaling molecules in arterial–venous differentiation was suggested by the phenotypes of mouse and zebrafish mutants: ephrinB2 and EphB4 knockout mouse embryos displayed arrested remodeling of the primary vascular plexus into arteries and veins during early development, leading to death around E9.5 (Wang et al. 1998; Adams et al. 1999; Gerety et al. 1999). Endothelial-specific nrp-1 mouse mutants failed to express arterial markers in the arteries of the embryonic dermis, although these vessels were positioned properly (Gu et al. 2003; Mukouyama et al. 2005).

Zebrafish mutant studies have shown a requirement for Notch signaling to repress venous fate in arteries: inhibition of the Notch signaling pathway using a dominant negative form of suppressor of hairless (SuH), a downstream effector of Notch, leads to decreased expression of arterial markers and ectopic expression of venous markers in arteries (Lawson et al. 2001). Disruption of the Notch signaling pathway in mice also leads to significant

vascular defects, ascribed to defective arterial–venous differentiation. Mutation of *dll4*, a Notch ligand selectively expressed in arteries, but not in veins, leads to defective development of the dorsal aorta and cardinal veins, with formation of arterial–venous shunts (Duarte et al. 2004; Gale et al. 2004; Krebs et al. 2004). Interestingly, these defects are already apparent when a single *dll4* allele is lost. Arterial markers such as ephrinB2 are downregulated and venous markers are ectopically expressed in the dorsal aorta of *dll4* mutants and of several other mutants of genes in the Notch pathway, including double mutants of Notch1 and Notch4, endothelial knockout of RBP, the SuH ortholog and double mutants of the downstream targets Hes and Hey (Fischer et al. 2004; Krebs et al. 2004). Conversely, endothelial-specific mutation of the nuclear receptor COUPTFII, expressed in veins, leads to ectopic activation of arterial markers in veins (You et al. 2005). Taken together, these studies suggest that the specification of angioblasts into arterial or venous lineages is genetically determined and occurs already before the onset of blood circulation. Failure in the specification of arterial and venous identities or in the establishment of the artery–vein boundaries leads to vascular fusions and dysplasia.

Role of Hemodynamic Forces in Remodeling

The presence of blood flow is known to be essential for remodeling of the primary vascular plexus into arteries and veins to occur. Nearly 100 years ago, Chapman showed by surgically removing the heart of chick embryos before the onset of circulation that the peripheral vasculature formed, but failed to remodel, without blood flow and pressure (Chapman 1918). Remodeling of the vasculature also did not occur after surgical removal of the heart of young chicken embryos and incubation of the embryos in high levels of oxygen to remove the effects of hypoxia (Manner et al. 1995).

Using in vivo time-lapse imaging of developing chick embryos, we analyzed the effects of hemodynamics on arterial–venous differentiation (le Noble et al. 2004). We observed that prior to the onset of flow, EC expressing arterial or venous markers were localized in a posterior arterial and an anterior venous pole. After the onset of cardiac activity and the start of perfusion, the vitelline artery forms in the posterior arterial pole by flow-driven fusion of pre-existing capillaries branching from the aorta at the level of somite 21 (Fig. 2.3b). During the subsequent stages, the arterial network expands and some small capillary branches are selectively disconnected from the arterial network. These segments do not regress or apoptose; instead, the disconnected vessels reconnect to the venous plexus. The disconnected segments lose their arterial identity and start to express venous markers. Detailed high-magnification intravital imaging shows that the disconnected segments, which are filled with blood and pressurized due to connections with more distal parts of the arterial network, grow and extend over the arteries, before reconnecting to the veins of the primary network (le Noble et al. 2004). The disconnected segments lack tip cells or filopodia, and are instead driven by their luminal pressure and guided by the strain fields generated by the surrounding large-caliber vessels (Nguyen et al. 2006). The relatively high pressure in the arteries repels the expanding disconnected segments, which avoid the arteries and can only reconnect to lower-pressure veins. Such avoidance of the arterial segments is also observed in the zebrafish parachordal vessel, which sprouts from the posterior cardinal vein and crosses the intersegmental artery without fusing to it (Isogai et al. 2003).

Arterial and venous identity can also be altered experimentally by changing the flow environment of the EC. Rerouting flow by artificially obstructing the vitelline artery (Stéphan 1952) results in perfusion of the arterial tree with blood of venous origin, which transforms the arteries into veins, both morphologically (Fig. 2.4) and genetically. Perfusing veins with arterial blood can likewise transform them into arteries (le Noble et al. 2004). Thus, while it is clear that there must be blood flow in an embryo for remodeling and arterial–venous differentiation to occur, the essential signal that flow imparts is not known. Blood flow

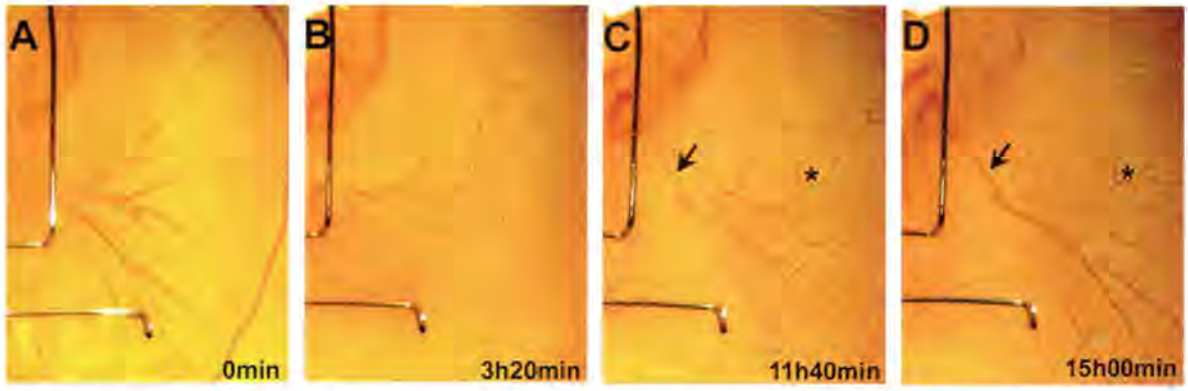


Fig. 2.4A–D. Transformation of embryonic arteries into veins by flow manipulation. Ligation of the right vitelline artery (see Fig. 2.3b) of a 30-somite chick embryo in ovo by insertion of a metal clip underneath the artery. A–D Images of time-lapse videomicroscopy taken at the indicated time points. The future major venous branch can be seen in C (*arrow*). Note incorporation of an entire branch of the vitelline artery into this vein (*arrows*, C, D). Asterisks in c and d indicate another arterial segment that is becoming venularized

carries nutrients, oxygen and signaling molecules to the vessels and creates physical forces acting on the EC and cells of the forming vessel wall. Therefore, the initiation of blood flow brings many different signals to the embryo.

2.6 Guidance of Capillaries by Endothelial Tip Cells

Despite the crucial role of hemodynamic forces in shaping vascular pattern, the gross vascular anatomy of developing mouse, chick or zebrafish embryos is characterized by highly reproducible branching patterns, suggesting the existence of additional patterning mechanisms. Indeed, during development, blood vessels navigate along stereotyped paths towards their targets – similar to axonal growth cones (Carmeliet 2003). The mechanisms regulating vessel navigation remain incompletely understood. It was only recently discovered that specialized EC termed 'tip cells' are located at the leading front of growing vessels. These tip cells respond to chemoattractant and repellent guidance cues that act over short or long range (Ruhrberg et al. 2002; Gerhardt et al. 2003), similar to axonal growth cones. The existence of such endothelial "growth cones" highlights the anatomical similarities between the nervous and vascular systems (Carmeliet 2003). Several receptors for axon guidance cues are expressing on growing vessels and were shown to regulate vessel pathfinding, including PlexinD1, Robo4 and the Netrin receptor UNC5B (Gitler et al. 2004; Torres-Vazquez et al. 2004; Bedell et al. 2005 ; Lu et al. 2004). These molecules are reviewed in detail by Carmeliet et al. in this book (Chap. 3).

Endothelial tip cells extend numerous thin filopodia that explore their environment and regulate extension of capillary sprouts. Using multiphoton time-lapse imaging of transgenic Tg(fli1:EGFP)y1 zebrafish, specifically expressing enhanced green fluorescent protein in EC, Isogai et al. 2003 documented the dynamic assembly of the intersegmental vessels (ISVs) in embryos. ISV formation is initiated by angioblast migration from the dorsal aorta into the intersomitic space (Childs et al. 2002). These angioblasts form sprouts that grow dorsally between the somites and the neural tube, tracking along vertical myotomal boundaries. The sprouts grow in a saltatory fashion with numerous active filopodia extending and retracting, particularly in the dorsal-

most leading extension. ISVs are formed before perfusion and filopodial movement of tip cells ceases as perfusion of these vessels is initiated. Endothelial tip cells are also seen at the front of the growing postnatal retinal vasculature in mice (Fig. 2.5) and in the early chick embryo yolk sac prior to the onset of flow (Fig. 2.6a). Similar to zebrafish, tip cells are far less numerous in perfused vascular beds (Figs. 2.5, 2.6b), suggesting a correlation between flow and filopodial extension that remains to be fully explored. However, it is clear that tip cell guidance of growing blood vessels is a general phenomenon in vascular development that is currently being intensely studied in pathological angiogenesis as well.

Endothelial tip cells express VEGFR-2 (Gerhardt et al. 2003), a high-affinity receptor of VEGF (Ferrara et al. 2003). VEGF exists as several alternatively spliced isoforms, VEGF120, 164 and 188 in mice, which differ in their matrix- and receptor-binding affinities. The shorter VEGF120 isoform is freely diffusible, as it lacks the heparin-binding domain necessary for interaction with the extracellular matrix, whereas the VEGF188 isoform remains bound to the extracellular matrix and the VEGF164 isoform has intermediate properties. In the postnatal mouse retina, tip-cell filopodia follow a gradient of matrix-bound VEGF produced by retinal astrocytes (Gerhardt et al. 2003). Alteration of the VEGF gradient by injection of soluble VEGFR-1 or by blocking antibodies against VEGFR-2 but not VEGFR-1 led to loss of tip-cell filopodia; conversely, increased branching of hyaloid vessels was observed in transgenic mice overexpressing VEGF164 under the control of the A-crystallin promoter. Endothelial tip cells primarily migrate but proliferate only minimally, in contrast to their subjacent EC, termed the "stalk cells", which do proliferate. Thus, these two types of EC interpret the VEGF signal differently: tip cells extend filopodia and stalk cells proliferate. The molecular regulation of these two distinct behaviors is currently not understood. Evidence for a role of VEGF in tip cell guidance is also deduced from the analysis of mouse mutants selectively expressing different VEGF isoforms (Ruhrberg et al. 2002). Vascular development is normal in mice expressing only VEGF164, indicating that this isoform alone is sufficient to ensure proper vascular patterning (Stalmans et al. 2002; Stalmans et al. 2003). By contrast, mice expressing only VEGF120 or VEGF188 exhibited vessel navigation defects. In VEGF120 mice, EC become incorporated into existing vessels and increase vessel size rather than forming new branches. As a result, vessels are enlarged, stunted and hypobranched. In contrast, VEGF188 mice showed the opposite phenotype, that is, hyperbranched and thin vessels (Ruhrberg et al. 2002). Thus, sequestration of VEGF isoforms as gradients in the matrix is crucial for the balance between capillary branching and enlargement of vessel size. Application of VEGF to early chick embryos also leads to dramatic alteration of tip cell behavior and vascular hyperfusion, while sequestration of VEGF by soluble VEGFR-1 inhibited tip cell protrusions and proper vessel formation (Drake et al. 2006). Collectively, these experiments provide evidence for a positive role of VEGF in tip cell guidance.

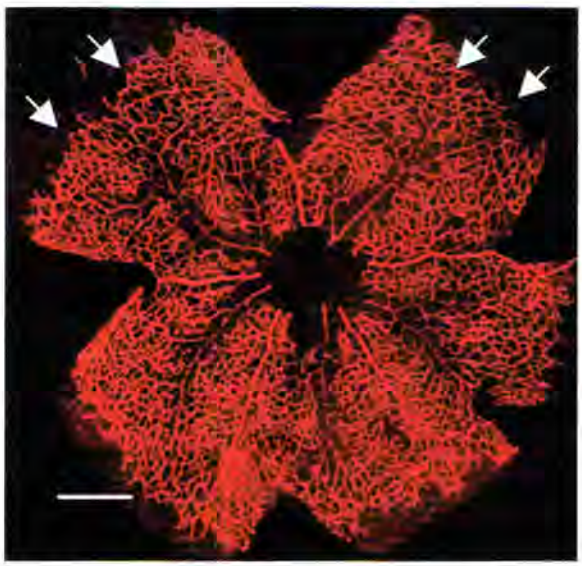


Fig. 2.5. Retinal vascular development. Vascular plexus of a postnatal day 5 mouse revealed by isolectin B4. Tip cells extending filopodia can be visualized at the edges of the vascular front (*arrows*), but are far less numerous in remodeled, perfused areas of the vascular plexus behind the vascular front

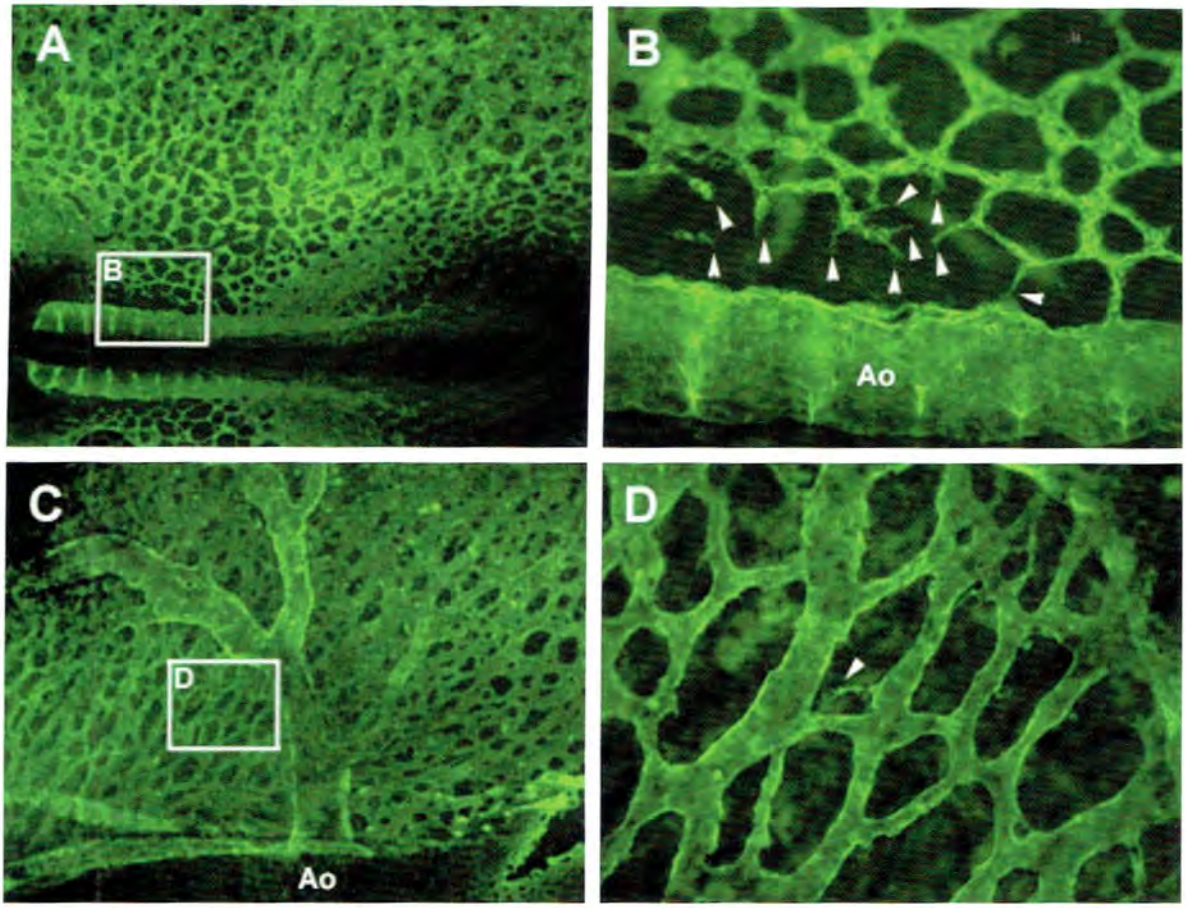


Fig. 2.6A–D. Tip cells in the vascular plexus of quail embryos. Whole-mount QH1 staining of a 12-somite quail embryo (A, B) and a 22-somite quail embryo (C, D). B, D Higher magnifications of boxed areas in A, C. Numerous tip cells extending filopodia can be observed in the primary vascular plexus before the onset of flow (B, *arrowheads*), in contrast to later stages when flow is already established (D, *arrowhead*). Ao, Aorta

2.7 Circulating Endothelial Cells or Progenitors in the Embryo

In the adult, once the definitive vascular network is established, EC remain essentially quiescent with neovascularization only occurring during physiological or pathological events. For a long time, adult neovascularization was thought to be exclusively achieved by angiogenesis. However, the existence of adult circulating EC (CEC) or endothelial progenitor cells (EPC) is now well established (see Urbich and Dimmeler 2004 for review). These cells have important potential therapeutic applications, as their administration could stimulate blood vessel growth in conditions of hypovascularization (hind-limb ischemia, myocardial infarction, stroke, wound healing). Genetic manipulation of CEC/EPC could also allow inhibition of blood vessel growth in conditions of hypervascularization (diabetic retinopathy and tumorigenesis).

The origin of CEC/EPC was recently investigated in the avian embryo, using the quail–chick para-

biosis model in which a quail embryo is added into a chick egg during the 2nd day of development (Pardanaud and Eichmann 2006). From the 8th day, the chorioallantoic membranes (CAM) of the two embryos fused, vascular anastomes were established, and cells could travel from one species to the other. CEC/EPC colonizing the chick embryos could be recognized using the QH1 monoclonal antibody specific for quail cells. The emergence of CEC/EPC was observed early in ontogeny, at day 2 of development, long before the formation of the bone marrow, although the precise territory generating these cells is still unknown. CEC/EPC could colonize all tissues of the chick but their number always remained low (Fig. 2.7a, b). However, CEC/EPC could efficiently be mobilized by wounding or grafting of an organ on the chick CAM, resulting in a significant participation of QH1+ CEC/EPC in the endothelial network of the grafted organs (Fig. 2.7c). Interestingly, only a minority of CEC/EPC (±5%) were integrated in chick endothelia (Fig. 2.7b) while the majority were located interstitially as isolated cells or integrated into chick endothelial cords. It is possible that these cells serve a structural bridging role or, alternatively, that they secrete paracrine growth factors. In adult ischemic tissues, Urbich and Dimmeler (2004) reported that interstitially located EPC could influence neovascularization in a paracrine manner by releasing pro-angiogenic factors.

Interestingly, when a chick CAM from a parabiosis was stimulated with VEGF for 2 days, while the vascular density was upgraded by comparison with PBS-treated CAM, the mobilization of QH1+ CEC/EPC did not occur. While this result seemed

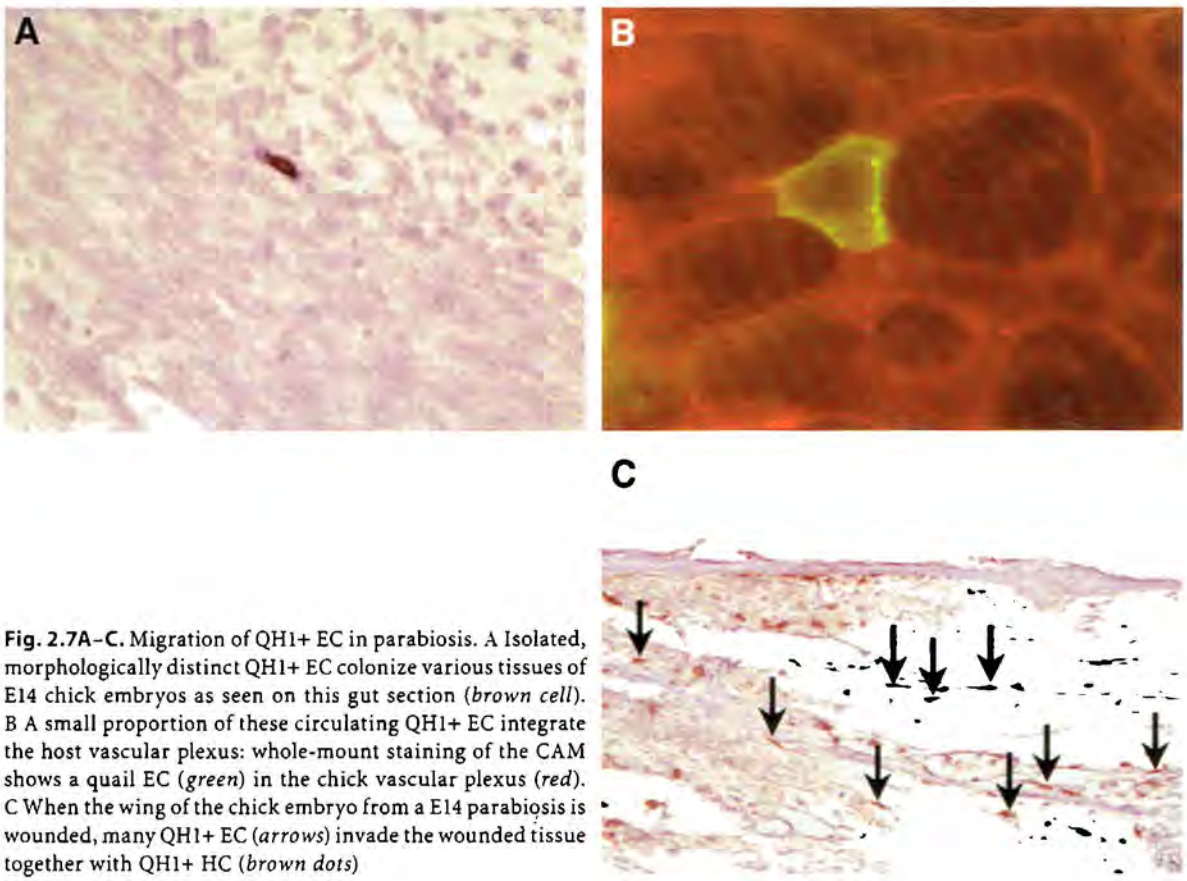


Fig. 2.7A–C. Migration of QH1+ EC in parabiosis. A Isolated, morphologically distinct QH1+ EC colonize various tissues of E14 chick embryos as seen on this gut section (*brown cell*). B A small proportion of these circulating QH1+ EC integrate the host vascular plexus: whole-mount staining of the CAM shows a quail EC (*green*) in the chick vascular plexus (*red*). C When the wing of the chick embryo from a E14 parabiosis is wounded, many QH1+ EC (*arrows*) invade the wounded tissue together with QH1+ HC (*brown dots*)

unexpected, VEGF did not always mobilize adult EPC either (De Palma et al. 2003; Ruzinova et al. 2003). VEGF-stimulated EPC did not systematically increase the formation of vessels (Young et al. 2002) but seemed to act indirectly on angiogenesis via the recruitment of bone marrow-derived circulating cells (Grunewald et al. 2006; Zentilin et al. 2006). In our model, CEC/EPC appeared to participate preferentially in angiogenic responses related to ischemia rather than in sprouting angiogenesis.

Perspectives

Research carried out over the past decade has provided major insights into the mechanisms regulating the emergence of endothelial progenitors from the mesoderm, their coalescence into the primary vascular system, and the remodeling of this system into arteries and veins. The molecules implicated in these different developmental processes are also essential for the maintenance of the adult vascular system. Elucidation of the precise function and interaction of the different molecular players will thus certainly lead to the development of novel treatments for vascular disorders.

The observation that arterial–venous differentiation is a flow-driven highly dynamic process that exhibits a high degree of EC plasticity is an important finding, and understanding the regulation of EC plasticity with respect to vessel identity has obvious important implications for the use of veins in coronary bypass surgery, restenoses and therapeutic arteriogenesis.

A particularly interesting aspect of recent research carried out on the vascular system is the identification of neural guidance receptors on blood vessels, in particular on endothelial tip cells. Identification of factors able to 'guide' developing blood vessels has obvious implications for pro- and antiangiogenic therapies that remain to be fully explored in the future. The close relation between the nervous and the vascular system is, moreover, highlighted by the finding that the patterning of developing arteries in the limb skin of mouse embryos has been shown to depend on interactions with nerves (Mukouyama et al. 2002). Future studies will be directed at exploring the precise interactions between blood vessels and nerves during development as well as in pathologies, in particular degenerative diseases like diabetic neuropathy that affect both nerves and microvessels.

References

Adams RH (2003) Molecular control of arterial–venous blood vessel identity. J Anat 202(1):105–112

Adams RH, Wilkinson GA, Weiss C, Diella F, Gale NW, Deutsch U, Risau W, Klein R (1999) Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. Genes Dev 13(3):295–306

Alitalo K, Tammela T, Petrova TV (2005) Lymphangiogenesis in development and human disease. Nature 438(7070):946–953

Alva JA, Iruela-Arispe ML (2004) Notch signaling in vascular morphogenesis. Curr Opin Hematol 11(4):278–283

Bedell VM, Yeo SY, Park KW, Chung J, Seth P, Shivalingappa V, Zhao J, Obara T, Sukhatme VP, Drummond IA, Li DY, Ramchandran R (2005) roundabout4 is essential for angiogenesis in vivo. Proc Natl Acad Sci U S A 102(18):6373–6378

Carmeliet P (2003) Blood vessels and nerves: common signals, pathways and diseases. Nat Rev Genet 4(9):710–720

Carmeliet P, Jain RK (2000) Angiogenesis in cancer and other diseases. Nature 407(6801):249–257

Carmeliet P, Tessier-Lavigne M (2005) Common mechanisms of nerve and blood vessel wiring. Nature 436(7048):193–200

Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A (1996) Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 380(6573):435–439

Chapman WB (1918) The effect of the heart-beat upon the development of the vascular system in the chick. Am J Anat 23:175–203

Childs S, Chen J, Garrity D, Fishman M (2002) Patterning of angiogenesis in the zebrafish embryo. Development 129(4):973–982

Choi K, Kennedy M, Kazarov A, Papadimitriou JC, Keller G (1998) A common precursor for hematopoietic and endothelial cells. Development 125(4):725–732

Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, McEver RP, Pober JS, Wick TM, Konkle BA, Schwartz BS, Barnathan ES, McCrae KR, Hug BA, Schmidt AM, Stern DM (1998) Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood 91(10):3527–3561

Coultas L, Chawengsaksophak K, Rossant J (2005) Endothelial cells and VEGF in vascular development. Nature 438(7070):937–945

Cumano A, Ferraz JC, Klaine M, Di Santo JP, Godin I (2001) Intraembryonic, but not yolk sac hematopoietic precursors, isolated before circulation, provide long-term multilineage reconstitution. Immunity 15(3):477–485

D'Souza SL, Elefanty AG, Keller G (2005) SCL/Tal-1 is essential for hematopoietic commitment of the hemangioblast but not for its development. Blood 105(10):3862–3870

De Palma M, Venneri MA, Roca C, Naldini L (2003) Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells. Nat Med 9(6):789–795

Dieterlen-Lièvre F (1975) On the origin of haemopoietic stem cells in the avian embryo: an experimental approach. J Embryol Exp Morphol 33(3):607–619

Drake CJ, Wessels A, Trusk T, Little CD (2006) Elevated vascular endothelial cell growth factor affects mesocardial morphogenesis and inhibits normal heart bending. Dev Dyn 235(1):10–18

Duarte A, Hirashima M, Benedito R, Trindade A, Diniz P, Bekman E, Costa L, Henrique D, Rossant J (2004) Dosage-sensitive requirement for mouse Dll4 in artery development. Genes Dev 18(20):2474–2478

Eichmann A, Marcelle C, Breant C, Le Douarin NM (1993) Two molecules related to the VEGF receptor are expressed in early endothelial cells during avian embryonic development. Mech Dev 42(1–2):33–48

Eichmann A, Corbel C, Nataf V, Vaigot P, Breant C, Le Douarin NM (1997) Ligand-dependent development of the endothelial and hemopoietic lineages from embryonic mesodermal cells expressing vascular endothelial growth factor receptor 2. Proc Natl Acad Sci U S A 94(10):5141–5146

Eichmann A, Makinen T, Alitalo K (2005) Neural guidance molecules regulate vascular remodeling and vessel navigation. Genes Dev 19(9):1013–1021

Fehling HJ, Lacaud G, Kubo A, Kennedy M, Robertson S, Keller G, Kouskoff V (2003) Tracking mesoderm induction and its specification to the hemangioblast during embryonic stem cell differentiation. Development 130(17):4217–4227

Ferrara N, Alitalo K (1999) Clinical applications of angiogenic growth factors and their inhibitors. Nat Med 5(12):1359–1364

Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW (1996) Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 380(6573):439–442

Ferrara N, Gerber HP, LeCouter J (2003) The biology of VEGF and its receptors. Nat Med 9(6):669–676

Fischer A, Schumacher N, Maier M, Sendtner M, Gessler M (2004) The Notch target genes Hey1 and Hey2 are required for embryonic vascular development. Genes Dev 18(8):901–911

Folkman J (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med 1(1):27–31

Gale NW, Dominguez MG, Noguera I, Pan L, Hughes V, Valenzuela DM, Murphy AJ, Adams NC, Lin HC, Holash J, Thurston G, Yancopoulos GD (2004) Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. Proc Natl Acad Sci U S A 101(45):15949–15954

Gerety SS, Wang HU, Chen ZF, Anderson DJ (1999) Symmetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand ephrin-B2 in cardiovascular development. Mol Cell 4(3):403–414

Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, Jeltsch M, Mitchell C, Alitalo K, Shima D, Betsholtz C (2003) VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. J Cell Biol 161(6):1163–1177

Gitler AD, Lu MM, Epstein JA (2004) PlexinD1 and semaphorin signaling are required in endothelial cells for cardiovascular development. Dev Cell 7(1):107–116

Grunewald M, Avraham I, Dor Y, Bachar-Lustig E, Itin A, Jung S, Chimenti S, Landsman L, Abramovitch R, Keshet E (2006) VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. Cell 124(1):175–189

Gu C, Rodriguez ER, Reimert DV, Shu T, Fritzsch B, Richards LJ, Kolodkin AL, Ginty DD (2003) Neuropilin-1 conveys semaphorin and VEGF signaling during neural and cardiovascular development. Dev Cell 5(1):45–57

Herzog Y, Kalcheim C, Kahane N, Reshef R, Neufeld G (2001) Differential expression of neuropilin-1 and neuropilin-2 in arteries and veins. Mech Dev 109(1):115–119

Herzog Y, Guttmann-Raviv N, Neufeld G (2005) Segregation of arterial and venous markers in subpopulations of blood islands before vessel formation. Dev Dyn 232(4):1047–1055

Huber TL, Kouskoff V, Fehling HJ, Palis J, Keller G (2004) Haemangioblast commitment is initiated in the primitive streak of the mouse embryo. Nature 432(7017):625–630

Isogai S, Lawson ND, Torrealday S, Horiguchi M, Weinstein BM (2003) Angiogenic network formation in the developing vertebrate trunk. Development 130(21):5281–5290

Jaffredo T, Gautier R, Eichmann A, Dieterlen-Lievre F (1998) Intraaortic hemopoietic cells are derived from endothelial cells during ontogeny. Development 125(22):4575–4583

Jakeman LB, Armanini M, Phillips HS, Ferrara N (1993) Developmental expression of binding sites and messenger ribonucleic acid for vascular endothelial growth factor suggests a role for this protein in vasculogenesis and angiogenesis. Endocrinology 133(2):848–859

Kamei M, Saunders WB, Bayless KJ, Dye L, Davis GE, Weinstein BM (2006) Endothelial tubes assemble from intracellular vacuoles in vivo. Nature 442(7101):453–456

Krebs LT, Shutter JR, Tanigaki K, Honjo T, Stark KL, Gridley T (2004) Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. Genes Dev 18(20):2469–2473

Lawson ND, Scheer N, Pham VN, Kim CH, Chitnis AB, Campos-Ortega JA, Weinstein BM (2001) Notch signaling is required for arterial-venous differentiation during embryonic vascular development. Development 128(19):3675–3683

le Noble F, Moyon D, Pardanaud L, Yuan L, Djonov V, Matthijsen R, Breant C, Fleury V, Eichmann A (2004) Flow regulates arterial-venous differentiation in the chick embryo yolk sac. Development 131(2):361–375

Lu X, Le Noble F, Yuan L, Jiang Q, De Lafarge B, Sugiyama D, Breant C, Claes F, De Smet F, Thomas JL, Autiero M, Carmeliet P, Tessier-Lavigne M, Eichmann A (2004) The netrin receptor UNC5B mediates guidance events controlling morphogenesis of the vascular system. Nature 432(7014):179–186

Manner J, Seidl W, Steding G (1995) Formation of the cervical flexure: an experimental study on chick embryos. Acta Anat 152:1–10

Mukouyama YS, Shin D, Britsch S, Taniguchi M, Anderson DJ (2002) Sensory nerves determine the pattern of arterial differentiation and blood vessel branching in the skin. Cell 109(6):693–705

Mukouyama YS, Gerber HP, Ferrara N, Gu C, Anderson DJ (2005) Peripheral nerve-derived VEGF promotes arterial differentiation via neuropilin 1-mediated positive feedback. Development 132(5):941–952

Murray P (1932) The development "in vitro" of blood of the early chick embryo. Proc R Soc 111:497–521

Nguyen TH, Eichmann A, Le Noble F, Fleury V (2006) Dynamics of vascular branching morphogenesis: the effect of blood and tissue flow. Phys Rev E Stat Nonlin Soft Matter Phys 73(6 Pt 1):061907

Nishikawa SI, Nishikawa S, Hirashima M, Matsuyoshi N, Kodama H (1998) Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hemopoietic lineages. Development 125(9):1747–1757

Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L (2006) VEGF receptor signalling – in control of vascular function. Nat Rev Mol Cell Biol 7(5):359–371

Pardanaud L, Eichmann A (2006) Identification, emergence and mobilization of circulating endothelial cells or progenitors in the embryo. Development 133(13):2527–2537

Pardanaud L, Altmann C, Kitos P, Dieterlen-Lievre F, Buck CA (1987) Vasculogenesis in the early quail blastodisc as studied with a monoclonal antibody recognizing endothelial cells. Development 100(2):339–349

Pardanaud L, Luton D, Prigent M, Bourcheix LM, Catala M, Dieterlen-Lievre F (1996) Two distinct endothelial lineages in ontogeny, one of them related to hemopoiesis. Development 122(5):1363–1371

Risau W (1997) Mechanisms of angiogenesis. Nature 386(6626):671–674

Ruhrberg C, Gerhardt H, Golding M, Watson R, Ioannidou S, Fujisawa H, Betsholtz C, Shima DT (2002) Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. Genes Dev 16(20):2684–2698

Ruzinova MB, Schoer RA, Gerald W, Egan JE, Pandolfi PP, Rafii S, Manova K, Mittal V, Benezra R (2003) Effect of angiogenesis inhibition by Id loss and the contribution of bone-marrow-derived endothelial cells in spontaneous murine tumors. Cancer Cell 4(4):277–289

Sabin FR (1920) Studies on the origin of blood-vessels and of red blood corpuscules as seen in the living blastoderm of chicks during the second day of incubation. Carnegie Contrib Embryol 272:214–262

Schuh AC, Faloon P, Hu QL, Bhimani M, Choi K (1999) In vitro hematopoietic and endothelial potential of flk-1(-/-) embryonic stem cells and embryos. Proc Natl Acad Sci U S A 96(5):2159–2164

Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 376(6535):62–66

Shawber CJ, Kitajewski J (2004) Notch function in the vasculature: insights from zebrafish, mouse and man. Bioessays 26(3):225–234

Shweiki D, Itin A, Neufeld G, Gitay-Goren H, Keshet E (1993) Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis. J Clin Invest 91(5):2235–2243

Stalmans I, Ng YS, Rohan R, Fruttiger M, Bouche A, Yuce A, Fujisawa H, Hermans B, Shani M, Jansen S, Hicklin D, Anderson DJ, Gardiner T, Hammes HP, Moons L, Dewerchin M, Collen D, Carmeliet P, D'Amore PA (2002) Arteriolar and venular patterning in retinas of mice selectively expressing VEGF isoforms. J Clin Invest 109(3):327–336

Stalmans I, Lambrechts D, De Smet F, Jansen S, Wang J, Maity S, Kneer P, von der Ohe M, Swillen A, Maes C, Gewillig M, Molin DG, Hellings P, Boetel T, Haardt M, Compernolle V, Dewerchin M, Plaisance S, Vlietinck R, Emanuel B, Gittenberger-de Groot AC, Scambler P, Morrow B, Driscol DA, Moons L, Esguerra CV, Carmeliet G, Behn-Krappa A, Devriendt K, Collen D, Conway SJ, Carmeliet P (2003) VEGF: a modifier of the del22q11 (DiGeorge) syndrome? Nat Med 9(2):173–182

Stéphan F (1952) Contribution expérimentale à l'étude du developpement du système circulatoire chez l'embryon de poulet. Bull Biol France Belg. Paris, L.P.U. de France. LXXXVI:218–310

Thoma R (1893) Untersuchungen über due Histogenese und Histomechanik des Gefäßsystems. Enke, Stuttgart

Torres-Vazquez J, Gitler AD, Fraser SD, Berk JD, Van NP, Fishman MC, Childs S, Epstein JA, Weinstein BM (2004) Semaphorin–plexin signaling guides patterning of the developing vasculature. Dev Cell 7(1):117–123

Ueno H, Weissman IL (2006) Clonal analysis of mouse development reveals a polyclonal origin for yolk sac blood islands. Dev Cell 11(4):519-533

Urbich C, Dimmeler S (2004) Endothelial progenitor cells functional characterization. Trends Cardiovasc Med 14(8):318–322

Vogeli KM, Jin SW, Martin GR, Stainier DY (2006) A common progenitor for haematopoietic and endothelial lineages in the zebrafish gastrula. Nature 443(7109):337–339

Wang HU, Chen ZF, Anderson DJ (1998) Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. Cell 93(5):741–753

Wheater PR, Burkitt HG, Daniels VG (1978) Functional histology: a text and colour atlas. Churchill Livingstone, Edinburgh

Wilting J, Eichmann A, Christ B (1997) Expression of the avian VEGF receptor homologues Quek1 and Quek2 in blood-vascular and lymphatic endothelial and non-endothelial cells during quail embryonic development. Cell Tissue Res 288(2):207–223

Wilting J, Papoutsi M, Othman-Hassan K, Rodriguez-Niedenfuhr M, Prols F, Tomarev SI, Eichmann A (2001) Development of the avian lymphatic system. Microsc Res Tech 55(2):81–91

Yamaguchi TP, Dumont DJ, Conlon RA, Breitman ML, Rossant J (1993) flk-1, an flt-related receptor tyrosine kinase is an early marker for endothelial cell precursors. Development 118(2):489–498

Yamashita J, Itoh H, Hirashima M, Ogawa M, Nishikawa S, Yurugi T, Naito M, Nakao K (2000) Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. Nature 408(6808):92–96

You LR, Lin FJ, Lee CT, DeMayo FJ, Tsai MJ, Tsai SY (2005) Suppression of Notch signalling by the COUP-TFII transcription factor regulates vein identity. Nature 435(7038):98–104

Young PP, Hofling AA, Sands MS (2002) VEGF increases engraftment of bone marrow-derived endothelial progenitor cells (EPCs) into vasculature of newborn murine recipients. Proc Natl Acad Sci U S A 99(18):11951–11956

Yuan L, Moyon D, Pardanaud L, Breant C, Karkkainen MJ, Alitalo K, Eichmann A (2002) Abnormal lymphatic vessel development in neuropilin 2 mutant mice. Development 129(20):4797–4806

Zentilin L, Tafuro S, Zacchigna S, Arsic N, Pattarini L, Sinigaglia M, Giacca M (2006) Bone marrow mononuclear cells are recruited to the sites of VEGF-induced neovascularization but are not incorporated into the newly formed vessels. Blood 107(9):3546–3554

Zhong TP, Childs S, Leu JP, Fishman MC (2001) Gridlock signalling pathway fashions the first embryonic artery. Nature 414(6860):216–220

Guidance of Vascular and Neuronal Network Formation

3

CARMEN RUIZ DE ALMODOVAR, SERENA ZACCHIGNA, MONICA AUTIERO, PETER CARMELIET

Abstract

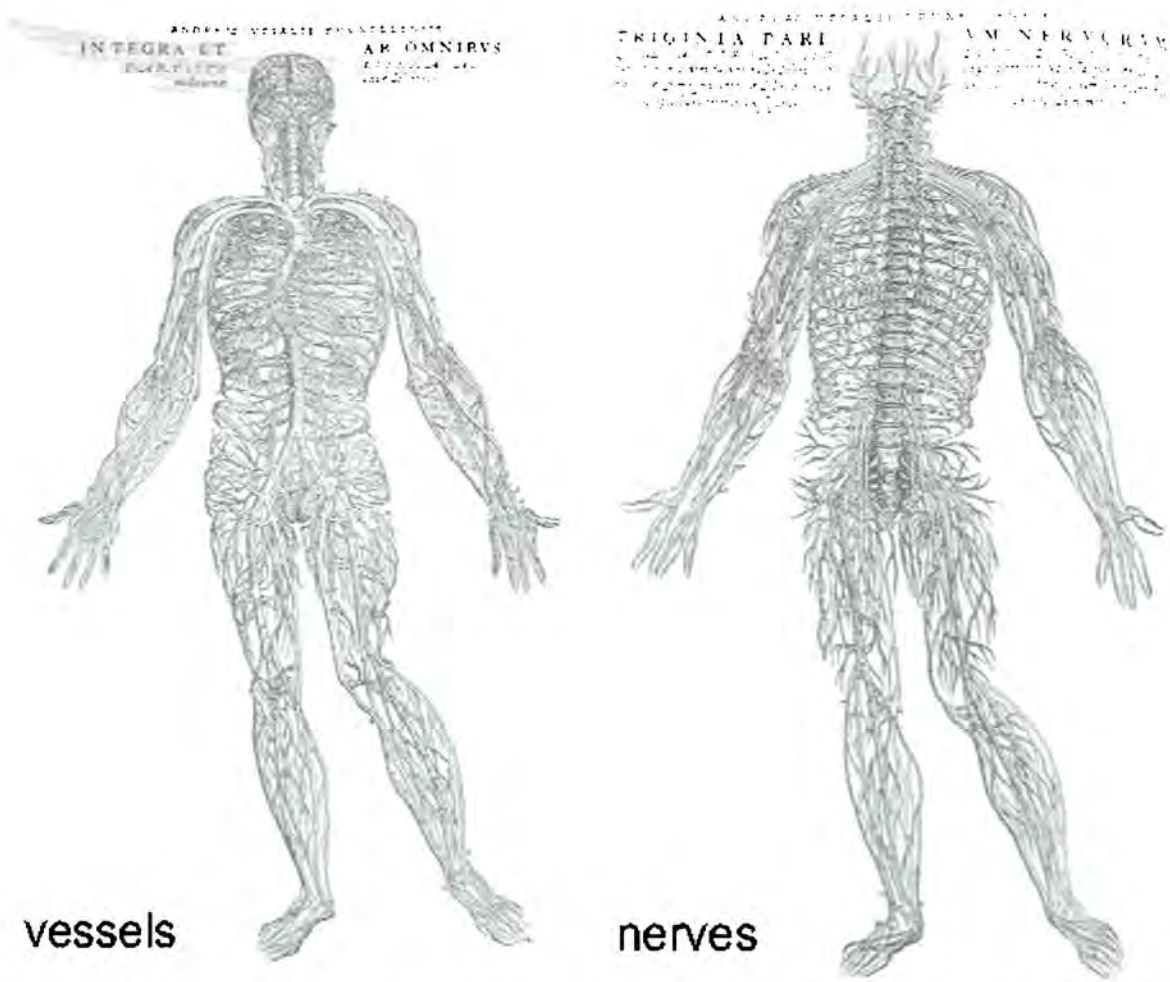
During evolution animals had to evolve in order to perform more and more complex tasks. For this, they developed two main specialized tissues: a highly branched vascular system to ensure adequate blood supply, and an intricate nervous system to transmit electrical signals to peripheral organs. During development, the patterning of both the vascular and the nervous system is achieved through the coordinated action of a variety of guidance cues that direct the growing nerves and vessels to specific targets. Emerging evidence suggests that the same set of attractive and repulsive molecules drives the migration of both axons and endothelial tip cells, thus playing a central role in the development of an ordered and stereotyped network. In this chapter we will discuss the similarities between axon and blood vessel guidance as well as the evidence showing that molecules first identified as axon guidance cues also play a role in blood vessel guidance.

CONTENTS

General Introduction

During the course of evolution, vertebrates have learned to perform more complex and sophisticated tasks – this challenge could only be met by the coincident development of two intertwined anatomic systems, blood vessels and nerves: the former providing nutrients, the latter transmitting electrical signals required for coordination. Although functionally distinct, these two systems are architecturally similar, structured into ramifying and hierarchically ordered networks (Fig. 3.1). The nervous and vascular systems share a remarkable conservation in their overall anatomical architecture, among individuals of the same

species and across the vertebrate phyla as well. They often display similar patterns in peripheral tissues, with nerve fibers and blood vessels following parallel routes, suggesting the existence of developmental links between the two systems (Carmeliet 2003). Both systems are composed of largely separate efferent and afferent networks (i.e. motor and sensory nerves in the nervous system and arteries and veins in the vasculature). To follow the same path, axons and vessels often take advantage of one another. In some cases, vessels produce signals (such as artemin and neurotrophin-3) that attract axons to track alongside a pioneer vessel (Kuruvilla et al. 2004; Honma et al. 2002), and conversely, nerves release signals to guide blood vessels (such as vascular endothelial growth factor, VEGF; Mukouyama et al. 2002). In addition, the coordinated morphogenesis of the two networks suggests that they are directed by common genetically programmed mechanisms. In fact, evidence is now emerging that blood vessels, which arose later in evolution than nerves, co-opted several of the or-



a

Fig. 3.1a,b. Parallelism in the stereotyped branching patterns of vessels and nerves. Already five centuries ago, the Belgian anatomist Andreas Vesalius illustrated the striking similarity between the arborization pattern of vessels (a) and nerves (b) throughout the human body (reproduced from Vesalius 1543).

ganizational principles and molecular mechanisms that evolved to wire up the nervous system.

The correct wiring of the nervous system relies on the ability of axons and dendrites to locate and recognize their appropriate target. Neurons send out a cable-like axon that migrates over considerable distances to reach its final destination. To help it find its way in the developing embryo, the axon is provided with a highly motile and sensitive structure, the growth cone (Huber et al. 2003; Dickson 2002) (Fig. 3.2a). Through dynamic cycles of extension and retraction of filopodia, the growth cone continually explores and responds to the appropriate set of cues, reassessing its spatial environment and accurately selecting a correct trajectory among the maze of possible routes.

Two successive processes underlie blood vessel formation during embryonic development: *vasculogenesis* is the differentiation of endothelial precursor cells from the mesoderm and their coalescence into tubes of the primary vascular plexus, which consists of the central axial vessels in addition to a meshwork of homogeneously sized capillaries. Through the process of *angiogenesis*, this primitive network expands by sprouting, bridging and branching from preexisting vessels and leads to a highly branched hierarchical vascular tree composed of arteries and veins. The recruitment of mural cells (pericytes and smooth muscle cells) around the endothelial layer completes the formation of a functional vascular network (Carmeliet 2000).

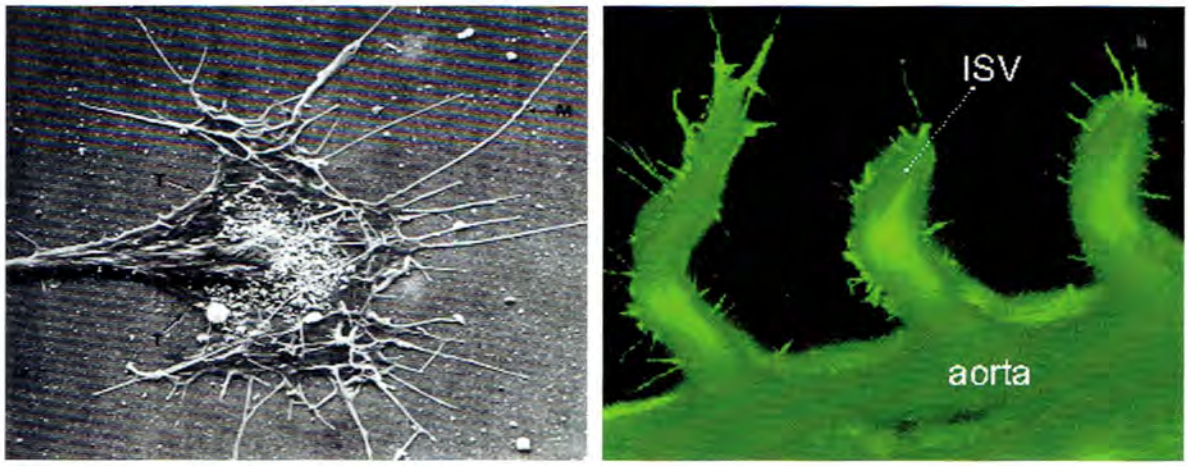
Although much knowledge of the molecular mechanisms of vasculogenesis and angiogenesis has been generated in the past decade, critical questions on how the vessels choose and follow specific paths to reach their targets remain unanswered. Understanding this process may have implications not only for vascular biology but also for the development of future strategies aimed at inducing or inhibiting angiogenesis.

Recent findings have shown that, like neurons and their axons, new vessels are guided along the correct path by integrating attractive and repulsive cues from the environment. Only 3 years ago, specialized endothelial cells (ECs) in the leading forefront of capillaries, called "tip cells" (Gerhardt et al. 2003) (Fig. 3.2b), were found to perform function analogous to that of growth cones in axons, continuously extending and retracting numerous filopodia to explore their environment and functioning to define the direction in which the new vascular sprout grows (Gerhardt et al. 2003). These tip cells seem to 'pave the path' for the subjacent 'stalk' ECs and proliferate minimally, whereas stalk cells proliferate extensively while migrating in the wake of the tip cells, permitting extension of the nascent vessel (Gerhardt et al. 2003).

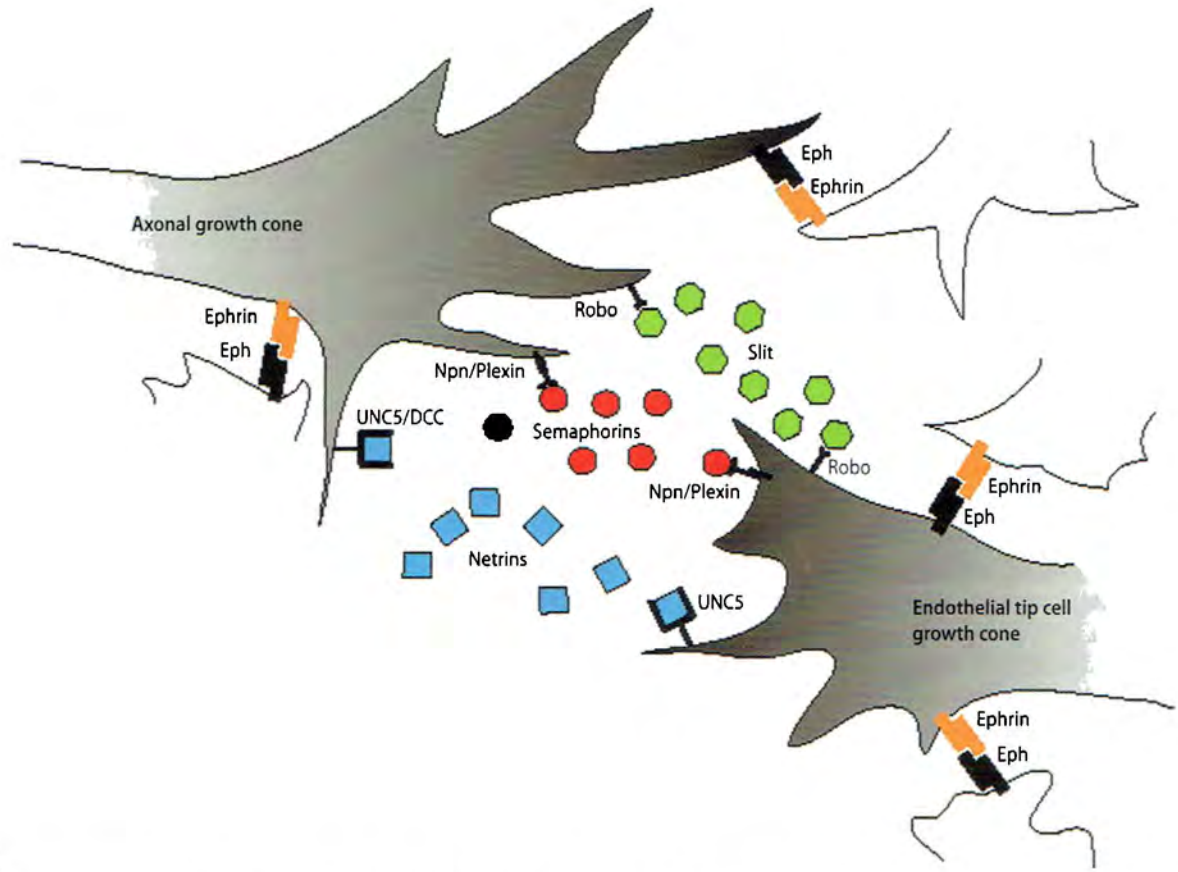
The biological and molecular similarities between the nervous and vascular systems have drawn a lot of attention lately, since guidance cues, first discovered in the neuronal system, have also been shown to guide blood vessels to their targets using similar mechanisms. In particular, four families of neuronal guidance ligands and their cognate receptors (Fig. 3.2c) have now been implicated in angiogenesis: members of the UNC-5 family, which bind netrins; roundabouts (Robo), which bind Slits; neuropilins (Npns), which bind semaphorins and members of the VEGF family; and Ephs, which bind ephrins. In this chapter we will first discuss the similarities between the angiogenic sprouting mechanisms and the ones that regulate axonal terminal arborization. We will then discuss the angiogenic properties of axon guidance cues and their possible role in tumor angiogenesis.

Parallelism Between Terminal Axon Arborization and Vessel Sprouting

Angiogenic and axon sprouting ensure the coverage of a target tissue by blood vessels and nerves: the former provide the required nutrients and remove the toxic waste products, while the latter release the necessary biochemical and electrical signals. In each case, target cells regulate the release of growth factors to direct sprouting. For axon terminals, target cells lacking a synaptic input secrete growth factors, such as nerve growth factor (NGF), that



a



c

Fig. 3.2a–c. Morphological and molecular similarities between axonal growth cones and endothelial tip cells. a Scanning electron micrograph of an axonal growth cone, terminating with numerous filopodial extensions (reproduced from Wessells and Nuttall 1978). b Multiphoton imaging of tip cell filopodia extending from the dorsal aorta in a 22-h-old zebrafish embryo. c The guidance of both axons and endothelial cells is directed by four major classes of ligands and their cognate receptors.

induce their innervation and which are downregulated when the target receives appropriate electrical stimulation (Goodman and Shatz 1993). For vessels, a hypoxic tissue secretes regulators of angiogenesis, such as VEGF, thus inviting its vascularization. Later on, when the target cells receive appropriate oxygen supply, VEGF expression is downregulated (Carmeliet 2003). In both systems sprouting requires the presence of these growth factors forming an appropriate gradient.

Specific gradients of neurotrophic factors are involved in terminal arborization of axons. For instance, target innervation of sympathetic and some sensory connections is mediated by a gradient of NGF (Davies 2000). In NGF mutant mice, lacking Bax (to prevent cell death), axons stall outside the target (Glebova and Ginty 2004), indicating that NGF-responsive axons invade target organs only when a NGF gradient is released by the target tissue. Similar results were obtained when the NGF gradient was reversed by autocrine expression of a NGF transgene in neurons (Glebova and Ginty 2004). Finaly, local gradients of NGF within the target organs were also shown to regulate the degree of axon terminal arborization (Diamond et al. 1992).

VEGF stimulates division and migration of ECs, and is critical for vascular development (Ferrara et al. 1996; Carmeliet et al. 1996). Similar to NGF in axon target innervation and arborization, VEGF is critical for the guidance of ECs to their targets. In mammals, different VEGF isoforms are generated from alternative splicing of a single gene (Ferrara et al. 2003). In humans the three main isoforms are VEGF121, VEGF165 and VEGF189 while in mice the same isoforms have one aminoacid less. All isoforms bind the tyrosine kinase receptors VEGFR-1 and -2, but, of the soluble isoforms, only $VEGF_{165}$ binds Npn-1 and -2 (Ferrara et al. 2003; Neufeld et al. 2002a). The shorter $VEGF_{121}$ is freely diffusible, lacks the heparin-binding domain necessary for the interaction with the extracellular matrix, and thereby acts over a long range. In contrast, the $VEGF_{189}$ isoform acts over a short range because it remains bound to the extracellular matrix, while the $VEGF_{165}$ isoform has intermediate properties Ferrara et al. 2003). By virtue of their different affinities for the extracellular matrix, these isoforms produce a gradient extending from the target tissue to the tip cell of the growing vessel, thus providing long-range and short-range guidance cues for correct vessel patterning. Evidence for a role of VEGF gradients in tip cell guidance was deduced from the analysis of three mouse lines, each engineered to express a single VEGF isoform. Thus, in these transgenic mice the VEGF gradient is disrupted by the lack of two of the VEGF isoforms. $VEGF_{164}$ mice are normal, indicating that this isoform alone is sufficient to ensure proper vascular patterning, but $VEGF_{120}$ and $VEGF_{188}$ mice exhibit serious vascular remodeling defects (Carmeliet et al. 1999; Stalmans et al. 2002). In $VEGF_{120}$ mice, ECs become incorporated in existing vessels and increase vessel size rather than forming new branches. As a consequence, vessels in these mutants are enlarged, stunted and hypobranched (Stalmans et al. 2002; Ruhrberg et al. 2002). $VEGF_{188}$ mice, by contrast, show the opposite phenotype with hyperbranched and thin vessels (Stalmans et al. 2002). Thus, a long-range VEGF gradient (provided by the diffusible isoforms $VEGF_{120}$ and $VEGF_{164}$) allows ECs to maintain their directional course to the target cell, whereas short-range matrix-bound VEGF (due to $VEGF_{164}$ and $VEGF_{188}$) guideposts are necessary for ECs to migrate step-by-step along the journey.

Collectively, similar principles that govern axon target innervation and the appropriate pattern of terminal arborization are also used for blood vessel formation and patterning.

Common Cues in Axon and Blood Vessel Guidance

Another similarity between the nervous and vascular system is the requirement of common molecular cues for their correct wiring. These cues are responsible for the highly stereotyped pattern of axons and vessels. To reach their target, axons have to travel

long distances; they simplify this task by breaking up their long trajectory into short segments and navigating from one "intermediate target" or "choice point" to the next (Tessier-Lavigne and Goodman 1996)(Fig. 3.3). Guidance cues can either be attractant or repellent and depending on their diffusible properties, they may act at short-range (being cell- or matrix-associated) or long-range (being diffusible) (Tessier-Lavigne and Goodman 1996). Axons are usually attracted to a choice point by long-range attracting signals produced by the intermediate target. Once there, they are expelled by short or long-range repellents, also produced by cells at the choice point, to continue on their path (Tessier-Lavigne and Goodman 1996). Like axons, ECs have to migrate over short distances (similar in length to the shorter segments navigated by axons) to reach their final destination. Between choice points, both axons and vessels are guided through tissue corridors by combinations of attractive cues made by cells along the corridors and repulsive signals expressed in the surrounding tissues. As mentioned above, recent findings have shown that molecules that were originally identified as axon guidance cues also play a role in blood vessel guidance (Carmeliet and Tessier-Lavigne 2005). We will now discuss the emerging evidence for the role of four families of classical axon guidance cues in vessel guidance.

3.3.1 Netrins and Their DCC and UNC-5 Receptors

The netrins are a family of proteins that are highly conserved from *Caenorhabditis elegans* to mammals. Their structure shares similarities with the short arms of laminin-γ (Netrin-1 and -3) or β-chains (Netrin-4). They contain a laminin VI domain, three EGF-like repeats and a carboxy-terminal domain that can bind heparin, heparan sulfate proteogly-

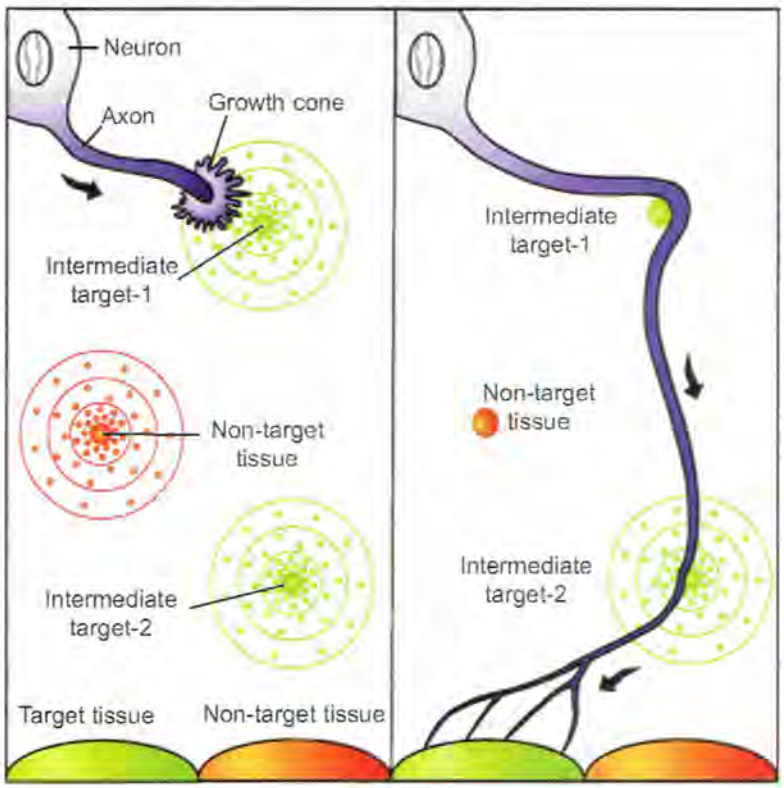


Fig. 3.3. Model of axonal growth cone navigation. To reach its final target, the axon first moves towards intermediate choice point, depending on the action of specific molecular cues, which act by either attracting or repelling its growth cone (from Barallobre et al. 2005).

cans or membrane glucolipids, thereby allowing interaction with components of the extracellular matrix or the cell surface (Barallobre et al. 2005). The extent of their diffusion is determined by both their expression level and the concentration of binding sites in the surrounding tissue. They bind two families of receptors, each with a single transmembrane domain. In vertebrates, the deleted colorectal cancer (DCC) receptor family comprises DCC and Neogenin, which share homology to UNC-40 in *C. elegans* and Frazzled in *Drosophila* (Barallobre et al. 2005). The other family of receptors that binds netrins is the uncoordinated 5 (UNC-5) family. Vertebrates have four homologues, UNC-5A, -B, -C and -D, which are orthologs of UNC-5 in *C. elegans* (Barallobre et al. 2005). Similar to other guidance cues, netrins have a dual role in axon guidance, i.e. they can act as either attractant or repellent molecules (Barallobre et al. 2005). Signaling of netrins through DCC receptors induces axon attraction, while a repulsion effect is generated by the binding of netrins to UNC-5 receptors (short-range repulsion) or to a combination of UNC-5 and DCC receptors (long-range repulsion) (Hong et al. 1999; Keleman and Dickson 2001).

In species with bilateral symmetry, neurons connect from one side of the central nervous system (CNS) to the other by projecting axons across the midline via commissures. In this way, a proper and coordinated function of the brain is ensured. Netrins are expressed in midline cells of the CNS. Netrin-1, the most studied member of the family, is expressed in the floor plate and in neuroepithelial cells of the ventral region of the spinal cord during development (Barallobre et al. 2005). Netrin-1 attracts commissural axons to the midline by binding DCC expressed on the axon surface (Fig. 3.4a). In Netrin-1- and DCC-deficient mice, commissural axons start growing towards the floor plate but most of them do not reach it; instead, they stall or are misrouted on their trajectory (Fazeli et al. 1997; Serafini et al. 1996). In wild-type embryos, once axons have reached the midline, their response to the chemoattractant activity of Netrin-1 is silenced to avoid stalling at the midline. This is achieved by the Commissureless (Comm) receptor in *Drosophila* and by Robo-3 in mouse (see below), whose expression levels become higher in postcrossing commissural axons, by forming a complex with DCC that inactivates Netrin-1 attractant activity (Stein and Tessier-Lavigen 2001) (Fig. 3.4b).

Genetic evidence for a role for Netrin-1 in vessel guidance has been provided recently. Lu et al. have shown that Netrin-1 can act as a repellent in blood vessel guidance via the UNC-5B receptor (Lu et al. 2004). UNC-5B, which is the only receptor expressed in the vascular system during mouse early development, is specifically expressed in arteries. Genetic deletion of this receptor in mice (embryonic E12.5 lethal) results in excessive vessel branching and increased extension of the tip-cell filopodia (Lu et al. 2004). A role for *UNC-5B* and *Netrin-1* in mediating endothelial repulsion was also shown in a study of the development of intersomitic vessels (ISV) in Unc-5b and Netrin-1a knockdown zebrafish embryos (Lu et al. 2004). ISVs in zebrafish follow a very stereotyped pattern. ISVs sprout from the dorsal aorta and grow dorsally between the somites and neural tube; eventually, they elongate and fuse with vessels from the adjacent segments to form the dorsolateral anastomotic vessel (DLAV) (Lawson and Weinstein 2002) (Fig. 3.5a). Secondary sprouts then come out from the posterior cardinal vein (PCV) and migrate dorsally up to the horizontal myoseptum to form the parachordal vessel (PAV) (Lawson and Weinstein 2002) (Fig. 3.5b). ISV formation occurs before perfusion and proceeds independently of oxygen levels, thus suggesting that a genetic program regulates the growth of these vessels. In *Unc-5b* or *Netrin-1a* morphants, while the initial sprouting of the ISVs into the intersegmental space is unaffected, aberrant pathfinding occurs at the level of either the horizontal myoseptum or the floor plate (which normally express Netrin-1a), where ISVs in both morphants deviate laterally instead of extending dorsally (Fig. 3.5c). Capillary branching was increased and ISVs were misguided, therefore, resembling the phenotype observed in Unc-5b-deficient mice (Lu et al. 2004). In vitro migration assays demonstrated that Netrin-1 inhibited migration of ECs expressing UNC-5B, consistent with a possible negative role of Netrin-1 on filopodial extension. Moreover, when injecting recombinant Netrin-1 into hindbrains of E10.5 wild-type

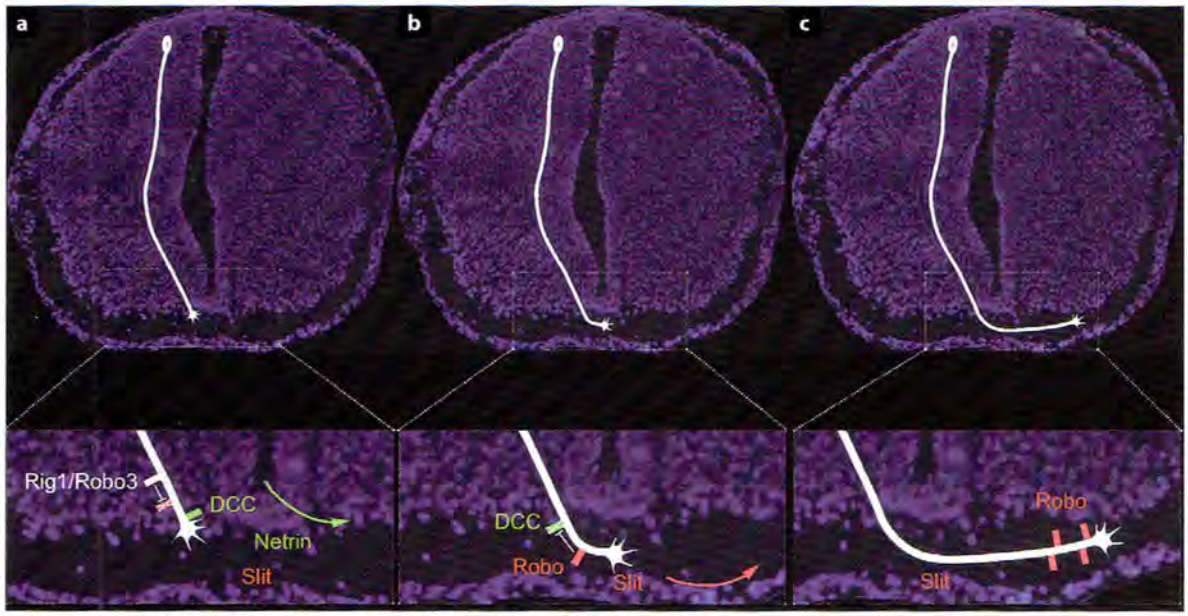


Fig. 3.4a–c. Mechanisms of axon guidance at the spinal cord midline in mammals. a Before crossing, netrins expressed at the midline attract commissural axons through the DCC receptor. At the same time, Rig1/Robo3 silences Robo, which would otherwise repel axons from entering the midline. b At the midline, Slit activates Robo, which in turn silences DCC, thereby preventing axon stalling and promoting axon expulsion from the midline. c After crossing, high levels of Robo expression on the axons prevent them from recrossing the midline.

embryos a marked retraction of the tip cell filopodia occurred compared to BSA-injected control. In addition, this effect was abolished in *Unc-5b* knockout mice (Lu et al. 2004). Taken together, these results suggest that Netrin-1, by binding to UNC-5B, inhibits vessel branching at specific "signaling points".

Another study reported that Netrin-1 stimulates the proliferation and migration of ECs and vascular smooth muscle cells (SMCs) in vitro (Park et al. 2004). The Neogenin receptor seems to be responsible for transducing these effects in SMCs, but the receptor through which Netrin-1 is signaling in ECs remains undefined (Park et al. 2004). A possible candidate is the adenosine A2b receptor, which is expressed in ECs and binds Netrin-1 (Corset et al. 2000), but its functional role remains to be determined. Besides Netrin-1, Netrin-4 also stimulates EC proliferation and tube formation (Wilson et al. 2006); in this case, the responsible receptor was not identified since Netrin-4 did not bind any of the known netrin receptors (Wilson et al. 2006). After knockdown of *Netrin-1a* in zebrafish embryos, the ISVs and the DLAVs formed normally but the formation of the PAVs was inhibited, presumably because Netrin-1a is required to induce EC migration along the muscle pioneer cells when forming the PAV (Wilson et al. 2006). Netrins also promoted neovascularization and reperfusion in a murine model of peripheral vascular disease (hindlimb ischemia) (Wilson et al. 2006). Therefore, it remains to be established why Netrin-1 has been reported to have repulsive and attractive activities. One possible, but outstanding, reconciling hypothesis is that Netrin-1 may act as a repulsive or attractant cue for ECs depending on the receptor type to which it binds. Obviously, additional studies will be required to better understand the intricate mechanisms of this vessel-guidance system.

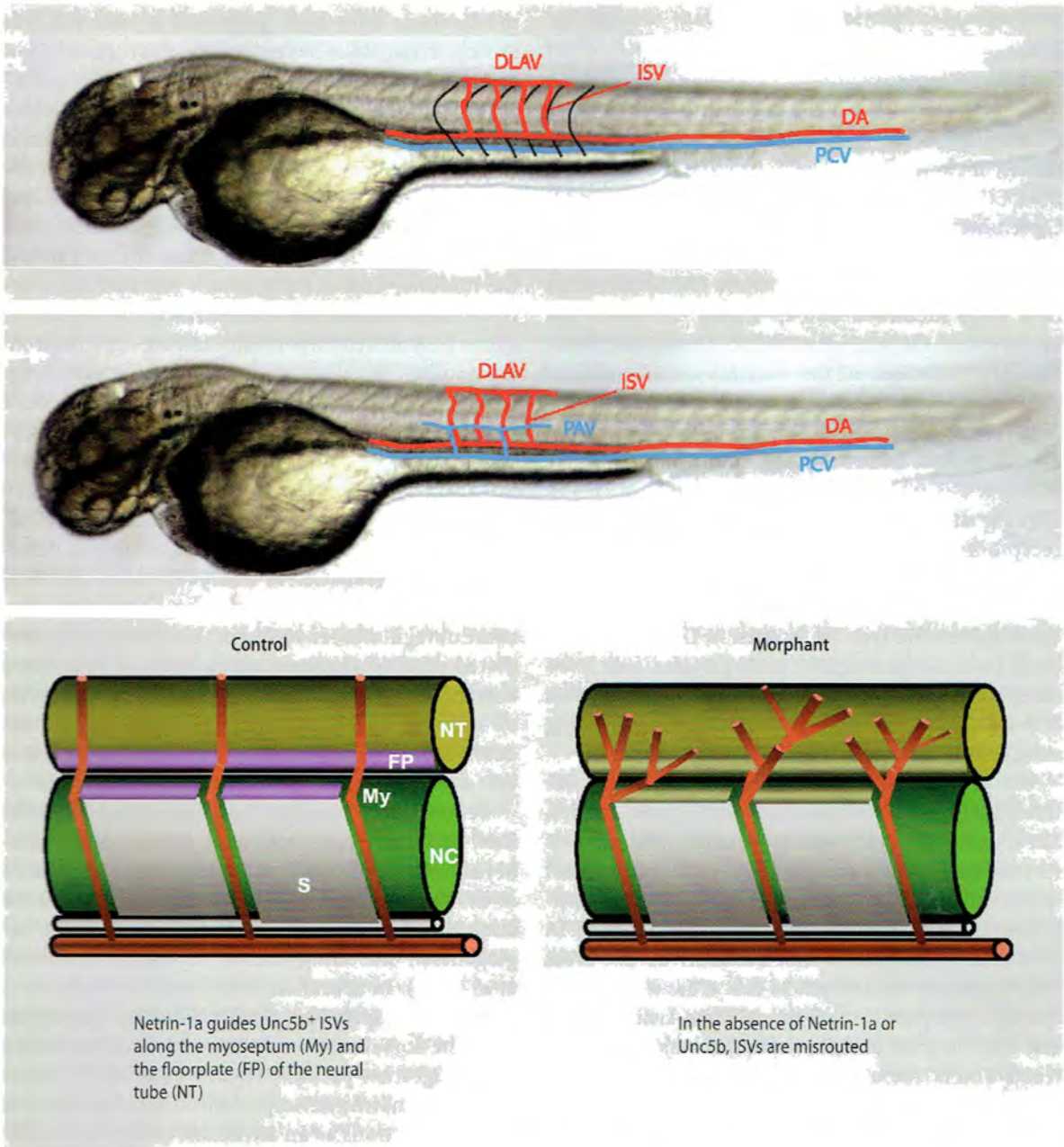


Fig. 3.5a–c. Role of netrins in the regulation of vascular development in zebrafish. a Lateral view of a 36-h-old zebrafish embryo, showing primary sprouting of ISVs from the DA et each myoseptal boundary, elongating dorsally and interconnecting to form the DLAV. b Later in development, a secondary set of vascular sprouts emerge from the PCV, often alongside the nearest primary vessels, contributing to the formation of the PAV. c Schematic representation of ISV guidance defects observed in zebrafish embryos after morpholino knockdown of Netrin-1a or Unc-5B. The left panel shows normal ISV development, which is severely impaired in the absence of the guidance molecules Netrin-1a or Unc-5B (right panel). FP, floor plate; My, myoseptum; NC, notochord; S, somites, NT, neutral tube.

3.3.2 Slits and Roundabouts

Slits are proteins that have multiple binding domains, including four leucine-rich repeats (LRRs), nine EGF-like repeats (seven in *Drosophila*) and a C-terminal cystenin knot. They are highly conserved from C. elegans to vertebrates (Brose and Tessier-Lavigne 2000). In mammals, three family members have been identified (Slit-1, -2 and -3), which are orthologs of the one identified in *Drosophila* (Slit). Slits are expressed in the nervous system midline (Brose and Tessier-Lavigne 2000) and may have a dual role, as they repel certain axons but, conversely, also stimulate branching and elongation of others (Kidd et al. 1999; Li et al. 1999; Wang et al. 1999). Slits signal through binding single transmembrane receptors of the roundabout (Robo) family (Kidd et al. 1998). These receptors contain an extracellular region with five immunoglobulin (Ig) domains and three fibronectin type III repeats. In Drosophila, Slit binds to one Robo receptor; in vertebrates, four Robo receptors (Robo-1, -2, -3 and -4) are known, with Robo-4 (also known as magic roundabout) being structurally divergent from the other Robos.

Slit proteins have been shown to regulate midline guidance in *Drosophila* and vertebrates. In flies, Slit is expressed at the ventral midline, where it acts (through Robo) as a short-range repellent to prevent ipsilateral axons from crossing the midline and commissural axons from recrossing (Kidd et al. 1999). In flies lacking Slit, axons that normally do not cross the midline do so, and axons that cross it only once can then cross it several times. Mice lacking Slit-1 and Slit-2 display midline defects in major forebrain tracts and at the optic chiasm, yet spinal commissural axons appear unaffected (Plump et al. 2002). Analysis of a triple mouse Slit knockout showed that Slits, by binding to Robo-1 and -2, also repel commissural axons after they have crossed the midline (Long et al. 2004). How can commissural axons, which are attracted to the midline by Netrin-1, cross the midline if they are also repelled by Slits? A controlled switch ensures that Slits expel crossing axons only after they cross the midline, not before. Two mechanisms have been proposed to underlie this switch. First, Robo receptors are expressed at low levels in precrossing commissural axons (Kidd et al. 1998; Long et al. 2004). In *Drosophila*, the regulatory protein Commissureless (Comm) keeps the Robo receptor intracellularly away from the plasma membrane, thereby lowering Robo surface expression in precrossing commissural axons (Keleman et al. 2002, 2005). Once commissural axons have crossed the midline, Comm repression is lost and Robo becomes expressed at the axon surface. Consequently, axons become sensitive to Slits and are expelled from the midline. In mammals, a different mechanism for this switch has been described. In this case, Robo-1 and Robo-3 are expressed in precrossing commissural axons. Robo-3 functions as an "anti-Robo" in that it plays a role similar to that of Comm in *Drosophila*: it silences Robo-1 and blocks the binding of Slits to Robo-1, thereby eliminating its repulsive activity (Sabatier et al. 2004). After crossing, Robo-3 is downregulated and Robo-1 and Robo-2 become upregulated, ensuring in this case that Slit-mediated repulsion in axons starts only after they have crossed the midline (Sabatier et al. 2004) (Fig. 3.4b, c). Together with the silencing of midline attraction described above for Netrin-1, this mechanism assures that axons cross and leave the midline efficiently.

Several recent studies have implicated Slits and their receptors in angiogenesis. During mouse embryonic development, Robo-4 is selectively expressed in developing blood vessels (Park et al. 2003). In the adult, Robo-4 is expressed only at sites of active angiogenesis, including tumor vessels (Huminiecki et al. 2002). In zebrafish, knockdown of Robo-4 resulted in ISV sprouting defects: ISVs failed to sprout from the dorsal aorta or were arrested midway along their migration pathway (Bedell et al. 2005). These findings do not, however, provide insight into whether Robo-4 functions as an attractant or repellent guidance cue in ISV guidance. Although one study failed to detect binding of Slit-2 to Robo-4 (Suchting et al. 2005), in vitro experiments in another study showed that Robo-4 on human ECs bound soluble Slit-2 and that this binding inhibited EC migration, suggesting a repulsive role for Robo-4/Slit-2 in angiogenesis (Park et al. 2003).

A role for the binding of Robo-1/Slit-2 axis in tumor angiogenesis has also been described (Wang et al. 2003). In vitro studies with human umbilical vein endothelial cells (HUVECs), which express Robo-1, showed that their exposure to a Slit-2 source stimulated their chemotaxis. In addition, Slit-2 was found to be expressed in many tumor cell lines and biopsies around the central necrotic area. In vivo experiments also supported an attractive role for Slit-2 on Robo-1 expressing vessels. These results are thus in disagreement with the documented repulsive activity of Slit-2, as mentioned above (Park et al. 2003). Additional studies will help to clarify the role of Slit-2 in developmental and pathological angiogenesis and explain whether it has opposite effects depending on the Robo receptor subtype to which it binds.

3.3.3 Semaphorins, Neuropilins and Plexins

Semaphorins belong to a large family of both membrane and secreted proteins, characterized by the presence of a highly conserved 500-amino-acid extracellular domain (Sema domain) that mediates the binding to multimeric receptor complexes, mainly composed of plexins and neuropilins, but often including additional molecules (Suchting et al. 2005). To date, more than 20 semaphorins have been identified, categorized into eight classes according to sequence similarities and structural properties. Generally, membrane-associated semaphorins bind directly to plexins, while class 3 secreted semaphorins (Sema3A–F) require neuropilins, which do not signal themselves but act as coreceptors for plexin signaling. Originally, genetic studies in Drosophila and mice implied semaphorins as major cues in axon guidance and neuronal cell migration. In general, they are considered to act as repellents, though Sema3A can also function as a chemoattractant, depending on the intracellular levels of cyclic nucleotides (Carmeliet and Tessier-Lavigne 2005). More recently, a large series of sophisticated loss of function approaches in different animal models have highlighted the importance of semaphorins in a variety of neuronal wiring processes. Collectively, these studies indicate that insufficient semaphorin signaling results in major axon projection defects, including axon defasciculation, overshooting, abnormal trajectories, misrouting and ectopic termination. Finally, semaphorins regulate the pruning of pre-existing axon branches in the hippocampus, but it is likely that they play a similar role in other regions of the brain as well (Carmeliet and Tessier-Lavigne 2005).

As mentioned above, the main signaling receptors for semaphorins in the nervous system are plexins, either alone or complexed with members of the neuropilin family. In mammals, there are at least nine plexins, most of which were originally described in mediating neuronal cell adhesion and contact, axon guidance and fasciculation (Tamagnone et al. 1999). However, more recently, plexin–semaphorin interactions have been implicated in a large series of other biological processes, including loss of cell–cell contacts in epithelia, regulation of angiogenesis, tumor growth and metastasis, and immune response (Kruger et al. 2005). The plexin family was originally identified via homology in the extracellular domain with the scatter factor receptors, the prototype of which is c-Met, the main receptor for HGF (hepatocyte growth factor, also called scatter factor-1). c-Met also regulates branching morphogenesis and axonal guidance in neuronal tissues (Zhang and Vande Woude 2003). In addition to sharing structural homology, plexins and scatter factor receptors mediate similar responses in target tissues, by inducing survival of sensory neurons, outgrowth of motoneuron axons, cell migration, proliferation and branching morphogenesis (known as the "scatter phenotype") in the epithelium. Furthermore, there are some hints that the two systems (class 3 semaphorins/plexins and HGF/c-Met) might be functionally linked, because Plexin-B1, upon binding Sema4D, interacts with c-Met via its extracellular domain; furthermore, c-Met phosphorylation seems to be essential for proper Plexin-B1 signaling (Giordano et al. 2002). In general, secreted class 3 semaphorins signal through Plexin-A, although, as already mentioned, they bind a member of the neuropilin family, acting as a co-receptor. An exception to this rule is the secreted Sema3E, which binds Plexin-D1 directly (Carmeliet and Tessier-Lavigne 2005).

The neuropilin (Npn) receptor family comprises two members, Npn-1 and Npn-2, with about 50% sequence homology and similar domain structure (Neufeld et al. 2002b). As the intracellular domain of neuropilins is extremely short, it is assumed that signaling through these receptors requires the association with other signaling moieties. Evidence for the importance of neuropilin's function was obtained almost a decade ago, when Npn-1 was shown to act as a coreceptor for Sema3A, inducing repulsion of the growth cone during development of the CNS (He and Tessier-Lavigne 1997). While Sema3A binds only Npn-1, other members of the family, such as Sema3B, Sema3C and Sema3F, bind both Npn-1 and Npn-2 (Chen et al. 1998; Takahashi et al. 1998).

It has been now established that both Npn-1 and Npn-2 are expressed by ECs and associate with VEGFR-1 and VEGFR-2 (Fig. 3.6). This unexpected finding was one of the first hints that molecules involved in semaphorin signaling in the nervous system might also play a role in vessel guidance. The expression of Npn-1 in ECs increases the affinity of $VEGF_{164}$ for VEGFR-2, thus enhancing VEGFR-2 signaling, leading to EC chemotaxis and other angiogenic steps (Miao et al. 1999). In contrast, when complexed with VEGFR-1, Npn-1 seems to prevent the binding of VEGF to this receptor (Fuh et al. 2000), but the general relevance of this finding remains to be determined. The heparin-binding form of PlGF (PlGF-2) and VEGF-B, two additional members of the VEGF family, also bind Npn-1 (Makinen et al. 1999; Migdal et al. 1998). Therefore, neuropilins have the unusual property of acting as receptors for two disparate ligand families, the semaphorin family of axonal guidance mediators and the VEGF family of angiogenic factors, suggesting the existence of common molecular mechanisms in these two biological processes (Fig. 3.6).

During embryogenesis, Npn-1 and Npn-2 display overlapping but distinct expression patterns in the nervous system. In the vascular system, both neuropilins are expressed in yolk sac ECs during vasculogenesis. At later stages, Npn-1 is preferentially expressed in arterial endothelium, whereas Npn-2 labels venous and lymphatic vessels (Eichmann et al. 2005). Interestingly, *npn-1* mutant mice exhibit defects in projections of the spinal and cranial nerves, and die at the embryonic stage due to severe cardiovascular dysfunction (Kawasaki et al. 1999). In contrast, mice lacking functional Npn-2 receptors are viable, with no evidence of cardiovascular defects, although small lymphatic vessels and capillaries fail to form (Chen et al. 2000).

The fact that both neurons and ECs express neuropilins, plexins, and VEGF receptors suggests a functional interplay between VEGF and semaphorins in the regulation of both vessel and nerve guidance and branching. In support of such a neural–vascular link, VEGF antagonizes the pro-apoptotic and collapsing effect of Sema3A on axons (Gu et al. 2002), while ECs respond to Sema3A by decreasing their migratory capacity, as well as microvessel and lamellipodia formation – effects that are reversed by VEGF (Miao et al. 1999). Moreover, VEGF induces the proliferation of different tumor cell lines, while Sema3A exerts a pro-apoptotic effect on most of the same cells (Guttmann-Raviv et al. 2006). The opposing effect of VEGF and Sema3A might imply that these two factors compete for overlapping binding sites in the extracellular domain of a series of shared receptors. An alternative mechanism is that they provide independent, opposite intracellular signals to their target cells. In support of the latter hypothesis, class 3 semaphorins participate in vascular morphogenesis by promoting an autocrine chemorepulsive signal, independent of VEGF (Serini et al. 2003; Bates et al. 2003; Bielenberg et al. 2006). However, the precise role of Sema3A in vascular morphogenesis in vivo remains to be elucidated, as others have not detected vessel defects in the same Sema3A-deficient mice (C. Ruhrberg, personal communication). Furthermore, additional genetic studies revealed that neural – but not vascular – morphogenesis was severely affected in mice, expressing a mutant Npn-1 that could no longer bind Sema3A, while still binding VEGF (*npn-1*^{Sema}-mice), indicating that Sema3A/Npn-1 is dispensable for vascular development (Gu et al. 2003). In addition, conditional silencing of Npn-1 in endothelial cells caused vascular malformations. As these defects were not present in npn-1^{Sema}-mice, they may have been caused by impaired VEGF/Npn-1 signaling in ECs – consistent with the concept

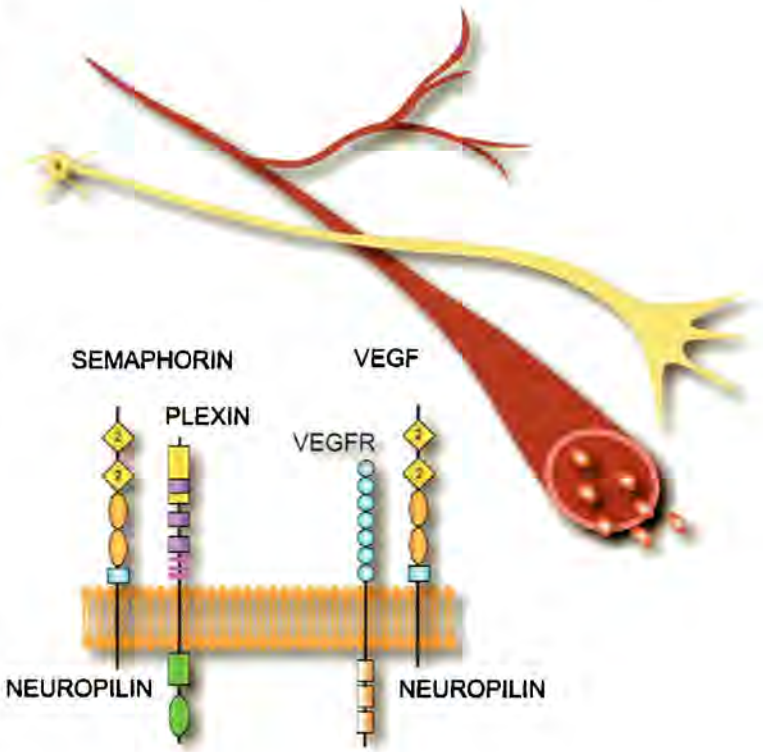


Fig. 3.6. Interplay between semaphorin and VEGF signaling during vessel and nerve growth. Semaphorins and VEGFs share the neuropilin receptors. Semaphorin signaling is traditionally considered to require neuropilin association with a transmembrane plexin, whereas the formation of a neuropilin/VEGFR complex promotes the binding of VEGF, thereby enhancing its activity. Although semaphorin and VEGF binding to neuropilins has been originally described in neurons and in ECs, respectively, it is likely that the two signaling events play a key role in the development of both vessels and nerves.

that VEGF signaling through Npn-1 is essential for proper vessel morphogenesis.

Sema4D has also recently been implicated in the regulation of EC biology, probably via binding Plexin-B1. The latter receptor is widely expressed in the nervous tissues, where it induces repulsion in developing axons and plays a part in maintenance of established neural pathways in the adult (Kruger et al. 2005). More recent studies now reveal that Plexin-B1 is also expressed in adult ECs and that Sema4D, via activation of Plexin-B1, induces tubulogenesis and migration of ECs, and angiogenesis in vivo (Basile et al. 2004).

Other plexins have been similarly implicated in vessel morphogenesis. Indeed, a series of genetic studies in zebrafish and mice revealed that Plexin-D1 mediates the vessel guidance activity of semaphorins (Weinstein 2005). Plexin-D1 is expressed nearly exclusively in ECs during development and is required for proper patterning of ISVs in zebrafish embryos. Class 3 semaphorins are expressed in somites in a complementary fashion, acting as a repulsive cue for ECs, thereby allowing them to select the appropriate ISV branching site (Weinstein 2005). Interestingly, Sema3E is expressed in the caudal region of the somites and binds Plexin-D1, independently of neuropilins (Gu et al. 2005). Moreover, Sema3E and Plexin-D1 mouse mutant embryos exhibit similar vascular phenotypes, suggesting that Sema3E signals through Plexin-D1 to restrict blood vessel growth to the intersomitic boundaries. Of note, however, Sema3E-deficient mice are viable, while Plexin-D1-deficient mice die shortly after birth due to major defects in the cardiac outflow tract, suggesting that different ligands, other than Sema3E, are required for proper cardiovascular patterning. Indeed, a model has been proposed according to which morphogenesis of the outflow tract requires coordinated signaling of VEGF through VEGFR-2/Npn-1, and of Sema3A and Sema3C through Plexin-D1/Npn-1 and PlexinD1/Npn-2, respectively (Eichmann et al. 2005).

Additional recent data have implicated semaphorins and neuropilins in tumor development (Bielenberg et al. 2006). Tumors produce a naturally occurring secreted soluble form of Npn-1 (sNpn-1), which may function as a VEGF trap, thus inhibiting tumor angiogenesis and growth (Guttmann-Raviv et al. 2006). However, the precise role of sNpn-1 remains unknown, as dimeric forms of sNpn-1 enhance the activity of VEGF and would therefore be expected to stimulate VEGF-induced tumor angiogenesis (Guttmann-Raviv et al. 2006). A role for Sema3F as a metastasis suppressor has been recently shown in different animal models, based on its ability to block the sprouting of peritumoral vessels, and to inhibit tumor cell adhesion and migration (Bielenberg et al. 2006).

3.3.4 Ephrins and Eph Receptors

The Eph receptors and their ligands, the ephrins, were first identified as repellent axon guidance molecules in the retino-tectal projection system (Carmeliet et al. 2005). Subsequently, they were recognized to act as guidance cues in a variety of developmental processes, including cell migration and positioning, axonal outgrowth and pathfinding, axon fasciculation and angiogenesis (O'Leary and Wilkinson 1999). Eph receptors and ephrins are membrane-bound proteins that function as a receptor–ligand pair, with 16 Eph receptors (the largest family of protein-tyrosine kinases) and nine ephrins identified so far in mammals. Both Eph receptors and their ephrin ligands are classified into A and B subfamilies, according to distinct structural properties of the ephrin ligands. Ephrin-A ligands are GPI-anchored peripheral membrane molecules, while ephrin-B ligands are transmembrane proteins. Although there is considerable crosstalk between A and B family members, type A ephrins preferentially bind EphA receptors and type B ephrins bind EphB receptors (Heroult et al. 2006).

The mechanisms via which Eph receptors are activated by ephrins have been mainly investigated by studying the establishment of topographically organized neuronal connections in many regions of the CNS. Although Ephs are generally described as receptors and ephrins as ligands, their interaction initiates bi-directional signals in both the Eph- and the ephrin-expressing cell (forward and reverse signaling, respectively; Kullander and Klein 2002). In addition, interactions between Eph receptors and ephrins on adjacent cells result in clustering of these molecules at the site of cell contact. This clustering appears to be crucial for signal initiation and the subsequent cellular response, which often results in a profound rearrangement of the assembly state of the local actin cytoskeleton. In fact, soluble monomeric ephrins cannot activate Eph receptors and often function as Eph receptor antagonists. Finally, both proteolysis and endocytosis of Eph–ephrin complexes constitute mechanisms to precisely regulate cell adhesion at sites of cell–cell contact. All these properties place Ephs and ephrins in a key position for processing information at the cell–cell interface. Depending on the cell type and the members of the Eph/ephrin family involved, the outcome of the interaction can be either increased adhesion (attraction) or decreased adhesion (repulsion).

At the level of the whole organism, the role of these molecules has been mainly investigated by loss-of-function studies that abolish both forward and reverse signaling, or by the use of "signaling" mutant forms of the proteins, which impair either one or the other of the signaling cascades. Phenotypic analysis of mice, expressing a mutant form of EphB2, lacking the tyrosine kinase intracellular domain, revealed that only the extracellular domain of EphB2, but not its signaling activity, is required for proper projection of anterior commissural axons (Henkemeyer et al. 1996). Because ephrins were detected in the anterior commissure tract, EphB2 might induce reverse signaling in ephrin-expressing axons (Henkemeyer et al. 1996). Ephrins also regulate the topographic projections of the retinal ganglion cell axons to the midbrain superior colliculus. Indeed, axons from the nasal retina, expressing low EphA levels, project to the posterior colliculus, containing abundant levels of the repellent ephrin-A. In contrast, axons from the temporal retina, which express a high density of EphA receptors, project to the anterior colliculus, where expression of the ephrin-A repellent is low (Carmeliet

and Tessier-Lavigne 2005). Overall, it has become clear that the direct interaction between ephrins and Eph receptors provides adhesive forces between cells, whereas more complex interactions and coupling with intracellular signaling molecules translate such contacts into both repulsive signals between adjacent cells and attractive guidance cues for cell migration, two events of paramount importance in both nerve and vessel development (Janes et al. 2005; Zimmer et al. 2003).

Ephrins and their receptors are widely expressed during embryonic development, playing versatile roles in morphogenesis – including vessel assembly and differentiation. For instance, ephrin-B2-null mice die at E10.5 as a consequence of impaired vascular differentiation and arterio–venous remodeling, which results in a failure to form a properly branched capillary network (Wang et al. 1998). EphB4-null mice essentially phenocopy ephrin-B2-null mice, thus defining EphB4/ephrin-B2 as principal regulators of vascular morphogenesis (Gerety et al. 1999) (Fig. 3.7). Notably, these loss of function studies in mice also highlighted that repulsive ehphrin-B2/EphB4 signaling is a key event in preventing the mixing of venous and arterial ECs and in demarcating arterial–venous cell boundaries. Consistent with their role in these processes, ephrin-B2 and EphB4 are selectively expressed in arteries and veins, respectively, in mouse embryos and this pattern seems to persist in the adult mouse as well (Gale et al. 2001; Shin et al. 2001). However, in humans, ephrin-B2 and EphB2 are mainly expressed by arterial endothelium, while EphB4 is expressed by both arterial and venous ECs. Moreover, expression analysis in human embryos indicates that the pattern of ephrin-B2/EphB4 is not strictly arterial–venous, suggesting that local environmental cues might also influence the expression of arterial and venous markers (Heroult et al. 2006). Indeed, mechanical forces, such as shear stress, have also been shown to control ephrin-B2 expression in arterial ECs, as demonstrated by flow manipulation experiments in the chick embryo (le Noble et al. 2004). Ephrin-B2 is also expressed by proliferating endothelium in different settings of physiological and

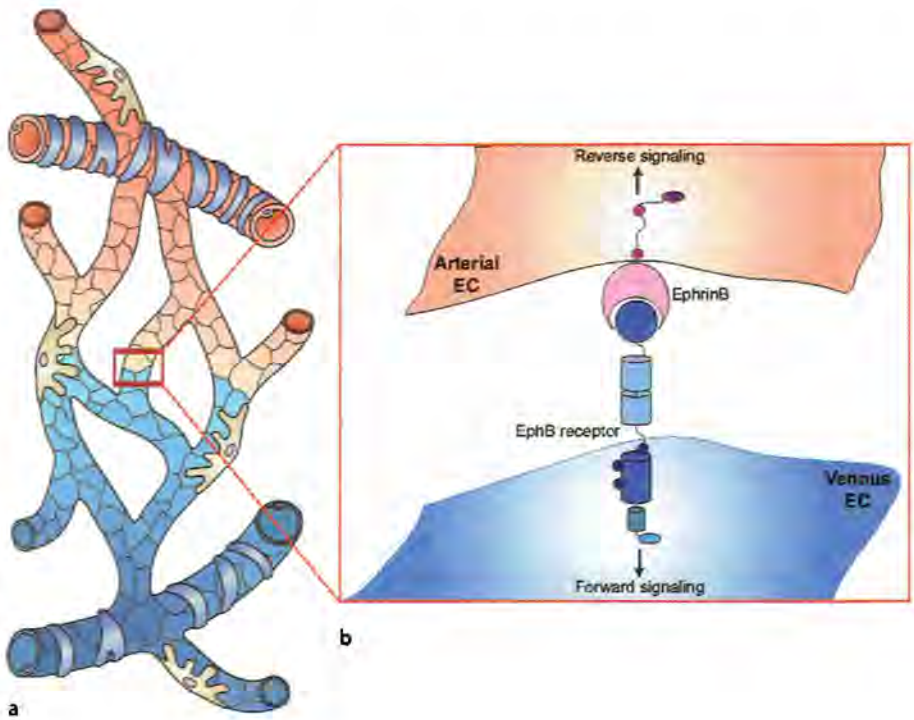


Fig. 3.7a,b. Role of ephrin–Eph signaling in the specification of artery–vein boundaries. a A blood vessel network is composed of arteries, ramifying into capillaries, which drain into veins. b Repulsion between EphB-expressing venous endothelial cells (forward signaling in blue) and Ephrin-B-expressing arterial endothelial cells (reverse signaling in red) controls the demarcation between arteries and veins, preventing the intermixing of the two vessel types.

pathological angiogenesis, including tumor growth, reproductive uterine cycle, and neovascularization following tissue ischemia (Gale et al. 2001).

The repulsive activities of EphB4 and ephrin-B2 suggest an "artery-to-vein push and pull" model of angiogenesis, reminiscent of the repulsive function of Eph receptors in neurons (Pasquale 2005). Neuronal activation of Eph receptors generally inhibits axonal growth and leads to growth cone collapse, as a consequence of either crosstalk with integrins or cytoskeletal reorganization involving the Rho pathway (Pasquale 2005). EC mixing experiments support a model whereby signaling via ephrin-B2 and EphB4 leads to propulsive and repulsive effects on ECs, respectively (Hamada et al. 2003). Expression of ephrin-B2 and EphB4 in ECs not only controls the positioning of ECs relative to each other; they also seem to play an important role in the complex morphogenic interactions of ECs with their neighboring stromal and mural cells (Heroult et al. 2006). Ephrins also have direct guidance functions in vascular development. For instance, ephrin-B2 is expressed in somites, where it prevents EphB3-/EphB4-expressing ISVs from entering somites, thus providing short-range guidance cues for vessels to navigate through tissue boundaries (Carmeliet and Tessier-Lavigne 2005). More recently, it has been proposed that Eph receptors might act in a bimodal manner, being capable of transmitting both pro-adhesive as well as anti-adhesive signals. In particular, reverse ephrin-B signaling has been implicated in both attractive and repulsive functions (Kullander and Klein 2002), suggesting that EphB receptors are able to transmit both propulsive and repulsive signals on EphB/ephrin-B interacting cells in the nervous, and likely also in the vascular system.

Similar Eph/ephrin events are likely to affect tumor angiogenesis and growth as well. Indeed, EphA1 was the first Eph receptor family member to be identified in an erythropoietin-producing human hepatocellular carcinoma cell line (Hirai et al. 1987). Since then, several members of the ephrin family have been recognized to be overexpressed or dysregulated in different tumor types (Surawska et al. 2004). The best-studied Eph receptor involved in cancer is EphA2, the expression levels of which correlate with tumor stage and tumor progression. Moreover, overexpression of EphA2 induces malignant transformation and confers tumorigenic potential to mammary epithelial cells. On the basis of these findings, EphA2 is currently considered a promising target for anti-tumorigenic and anti-angiogenic interventions. Indeed, interference with EphA2 signaling by different approaches has been invariably shown to decrease cell adhesion, to increase anoikis, and to inhibit tumor growth, tumor angiogenesis and metastasis (Heroult et al. 2006). Interestingly, perturbation of bi-directional EphB/ephrin-B signaling by overexpression of a soluble form of EphB4 inhibits tumor growth and tumor-associated angiogenesis in a melanoma model, probably as a result of interference with tumor–stromal cell interactions.

Perspectives

Emerging evidence has highlighted the importance of the neural–vascular link. Not only is there a clear parallelism in how blood vessels and nerves develop and branch topographically, but also how they share common mechanisms for cell signaling and pathfinding. First, there are striking similarities between the growth cone of axons and the endothelial tip cell in blood vessels. Both play a similar role in exploring the environment and function to define the direction in which the axon or the new vascular sprout grows. Second, increasing evidence reveals an angiogenic role for molecular cues previously described as axon guidances. Initial observations suggest that netrins or Slits may act as attractant or as repellent cues for ECs, but the underlying molecular mechanisms remain to be further resolved: Is this dual function dependent on the cellular context or the receptor type to which they bind? In addition, more research is required to elucidate the intracellular pathways linking guidance receptor activation to cytoskeletal changes in ECs: Are the signaling cascades in neuronal guidance systems similar in ECs? Another exciting new development is that at least some of

the molecules involved in vessel guidance similarly regulate axon guidance. For instance, VEGF, the key angiogenic factor, has been recognized to be involved in many neurobiological processes, including growth cone movement, neuronal survival and maintenance of neuronal circuitries (Carmeliet and Tessier-Lavigne 2005).

From a therapeutic perspective, the discovery of this neural–vascular link might also pave the way for the development of novel pro- and anti-angiogenic therapeutic strategies. The increasing evidence that several of the molecules involved in the pathfinding of vessels and nerves are also expressed by different tumor cells and regulate tumor cell growth, motility and invasion (Klagsbrun and Eichmann 2005) offers new therapeutic concepts. Initial evidence that interfering with Robo, semaphorin or ephrin signaling inhibits tumor angiogenesis in different animal models provides a first glimpse of this therapeutic potential (Wang et al. 2003; Bielenberg et al. 2006; Heroult et al. 2006). It also remains to be determined whether some of these molecules will be useful for stimulating the reperfusion of ischemic tissues in the clinic, an unmet medical need to date.

References

Barallobre MJ, Pascual M, Del Rio JA, Soriano E (2005) The Netrin family of guidance factors: emphasis on Netrin-1 signalling. Brain Res Brain Res Rev 49:22–47

Basile JR, Barac A, Zhu T, Guan KL, Gutkind JS (2004) Class IV semaphorins promote angiogenesis by stimulating Rho-initiated pathways through plexin-B. Cancer Res 64:5212–5224

Bates Det al (2003) Neurovascular congruence results from a shared patterning mechanism that utilizes Semaphorin3A and Neuropilin-1. Dev Biol 255:77–98

Bedell VM et al (2005) roundabout4 is essential for angiogenesis in vivo. Proc Natl Acad Sci USA 102:6373–6378

Bielenberg DR, Pettaway CA, Takashima S, Klagsbrun M (2006) Neuropilins in neoplasms: expression, regulation, and function. Exp Cell Res 312:584–593

Brose K, Tessier-Lavigne M (2000) Slit proteins: key regulators of axon guidance, axonal branching, and cell migration. Curr Opin Neurobiol 10:95–102

Carmeliet P (2000) Mechanisms of angiogenesis and arteriogenesis. Nat Med 6:389–395

Carmeliet P (2003) Blood vessels and nerves: common signals, pathways and diseases. Nat Rev Genet 4:710–20

Carmeliet P, Tessier-Lavigne M (2005) Common mechanisms of nerve and blood vessel wiring. Nature 436:193–200

Carmeliet P et al (1996) Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 380:435–439

Carmeliet P et al (1999) Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. Nat Med 5:495–502

Chen H, He Z, Bagri A, Tessier-Lavigne M (1998) Semaphorin-neuropilin interactions underlying sympathetic axon responses to class III semaphorins. Neuron 21:1283–1290

Chen H et al (2000) Neuropilin-2 regulates the development of selective cranial and sensory nerves and hippocampal mossy fiber projections. Neuron 25:43–56

Corset V et al (2000) Netrin-1-mediated axon outgrowth and cAMP production requires interaction with adenosine A2b receptor. Nature 407:747–750

Davies AM (2000)Neurotrophins: more to NGF than just survival. Curr Biol 10:R374–R376

Diamond J, Holmes M, Coughlin M (1992)Endogenous NGF and nerve impulses regulate the collateral sprouting of sensory axons in the skin of the adult rat. J Neurosci 12:1454–1466

Dickson BJ (2002) Molecular mechanisms of axon guidance. Science 298:1959–1964

Eichmann A, Le Noble F, Autiero M, Carmeliet P (2005) Guidance of vascular and neural network formation. Curr Opin Neurobiol 15:108–115

Fazeli A et al (1997)Phenotype of mice lacking functional Deleted in colorectal cancer (Dcc) gene. Nature 386:796–804

Ferrara Net al (1996)Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 380:439–442

Ferrara N, Gerber HP, LeCouter J (2003) The biology of VEGF and its receptors. Nat Med 9:669–676

Fuh G, Garcia KC, de Vos AM (2000) The interaction of neuropilin-1 with vascular endothelial growth factor and its receptor flt-1. J Biol Chem 275:26690–26695

Gale NW et al (2001) Ephrin-B2 selectively marks arterial vessels and neovascularization sites in the adult, with expression in both endothelial and smooth-muscle cells. Dev Biol 230:151–160

Gerety SS, Wang HU, Chen ZF, Anderson DJ (1999) Symmetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand ephrin-B2 in cardiovascular development. Mol Cell 4:403–414

Gerhardt H et al (2003) VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. J Cell Biol 161:1163–1177

Giordano S et al (2002) The semaphorin 4D receptor controls invasive growth by coupling with Met. Nat Cell Biol 4:720–724

Glebova NO, Ginty DD (2004) Heterogeneous requirement of NGF for sympathetic target innervation in vivo. J Neurosci 24:743–751

Goodman CS, Shatz CJ (1993) Developmental mechanisms that generate precise patterns of neuronal connectivity. Cell 72 [Suppl]:77–98

Gu C et al (2002) Characterization of neuropilin-1 structural features that confer binding to semaphorin 3A and vascular endothelial growth factor 165. J Biol Chem 277:18069–18076

Gu C et al (2003) Neuropilin-1 conveys semaphorin and VEGF signaling during neural and cardiovascular development. Dev Cell 5:45–57

Gu C et al (2005)Semaphorin 3E and plexin-D1 control vascular pattern independently of neuropilins. Science 307:265–268

Guttmann-Raviv N et al (2006) The neuropilins and their role in tumorigenesis and tumor progression. Cancer Lett 231:1–11

Hamada Ket al (2003) Distinct roles of ephrin-B2 forward and EphB4 reverse signaling in endothelial cells. Arterioscler Thromb Vasc Biol 23:190–197

He Z, Tessier-Lavigne M (1997) Neuropilin is a receptor for the axonal chemorepellent Semaphorin III. Cell 90:739–751

Henkemeyer M et al (1996) Nuk controls pathfinding of commissural axons in the mammalian central nervous system. Cell 86:35–46

Heroult M, Schaffner F, Augustin HG (2006) Eph receptor and ephrin ligand-mediated interactions during angiogenesis and tumor progression. Exp Cell Res 312:642–650

Hirai H, Maru Y, Hagiwara K, Nishida J, Takaku F (1987) A novel putative tyrosine kinase receptor encoded by the eph gene. Science 238:1717–1720

Hong K et al (1999) A ligand-gated association between cytoplasmic domains of UNC5 and DCC family receptors converts netrin-induced growth cone attraction to repulsion. Cell 97:927–941

Honma Y et al (2002) Artemin is a vascular-derived neurotropic factor for developing sympathetic neurons. Neuron 35:267–282

Huber AB, Kolodkin AL, Ginty DD, Cloutier JF (2003) Signaling at the growth cone: ligand-receptor complexes and the control of axon growth and guidance. Annu Rev Neurosci 26:509–563

Huminiecki L, Gorn M, Suchting S, Poulsom R, Bicknell R (2002) Magic roundabout is a new member of the roundabout receptor family that is endothelial specific and expressed at sites of active angiogenesis. Genomics 79:547–552

Janes PW et al (2005) Adam meets Eph: an ADAM substrate recognition module acts as a molecular switch for ephrin cleavage in trans. Cell 123:291–304

Kawasaki T et al (1999) A requirement for neuropilin-1 in embryonic vessel formation. Development 126:4895–4902

Keleman K, Dickson BJ (2001) Short- and long-range repulsion by the Drosophila Unc5 netrin receptor. Neuron 32:605–617

Keleman K et al (2002) Comm sorts robo to control axon guidance at the Drosophila midline. Cell 110:415–427

Keleman K, Ribeiro C, Dickson BJ (2005) Comm function in commissural axon guidance: cell-autonomous sorting of Robo in vivo. Nat Neurosci 8:156–163

Kidd T et al (1998) Roundabout controls axon crossing of the CNS midline and defines a novel subfamily of evolutionarily conserved guidance receptors. Cell 92:205–215

Kidd T, Bland KS, Goodman CS (1999) Slit is the midline repellent for the robo receptor in Drosophila. Cell 96:785–794

Klagsbrun M, Eichmann A (2005) A role for axon guidance receptors and ligands in blood vessel development and tumor angiogenesis. Cytokine Growth Factor Rev 16:535–548

Kruger RP, Aurandt J, Guan KL (2005) Semaphorins command cells to move. Nat Rev Mol Cell Biol 6:789–800

Kullander K, Klein R (2002) Mechanisms and functions of Eph and ephrin signalling. Nat Rev Mol Cell Biol 3:475–486

Kuruvilla R et al (2004) A neurotrophin signaling cascade coordinates sympathetic neuron development through differential control of TrkA trafficking and retrograde signaling. Cell 118:243–255

Lawson ND, Weinstein BM (2002) In vivo imaging of embryonic vascular development using transgenic zebrafish. Dev Biol 248:307–318

Le Noble F et al (2004) Flow regulates arterial-venous differentiation in the chick embryo yolk sac. Development 131:361–375

Li HS et al (1999) Vertebrate slit, a secreted ligand for the transmembrane protein roundabout, is a repellent for olfactory bulb axons. Cell 96:807–818

Long H et al (2004) Conserved roles for Slit and Robo proteins in midline commissural axon guidance. Neuron 42:213–223

Lu X et al (2004) The netrin receptor UNC5B mediates guidance events controlling morphogenesis of the vascular system. Nature 432:179–186

Makinen T et al (1999) Differential binding of vascular endothelial growth factor B splice and proteolytic isoforms to neuropilin-1. J Biol Chem 274:21217–21222

Miao HQ et al (1999) Neuropilin-1 mediates collapsin-1/semaphorin III inhibition of endothelial cell motility: functional competition of collapsin-1 and vascular endothelial growth factor-165. J Cell Biol 146:233–242

Migdal M et al (1998) Neuropilin-1 is a placenta growth factor-2 receptor. J Biol Chem 273:22272–22278

Mukouyama YS, Shin D, Britsch S, Taniguchi M, Anderson DJ (2002) Sensory nerves determine the pattern of arterial differentiation and blood vessel branching in the skin. Cell 109:693–705

Neufeld G, Kessler O, Herzog Y (2002a) The interaction of Neuropilin-1 and Neuropilin-2 with tyrosine-kinase receptors for VEGF. Adv Exp Med Biol 515:81–90

Neufeld G et al (2002b) The neuropilins: multifunctional semaphorin and VEGF receptors that modulate axon guidance and angiogenesis. Trends Cardiovasc Med 12:13–19

O'Leary DD, Wilkinson DG (1999) Eph receptors and ephrins in neural development. Curr Opin Neurobiol 9:65–73

Park KW et al (2003) Robo4 is a vascular-specific receptor that inhibits endothelial migration. Dev Biol 261:251–267

Park KW et al (2004) The axonal attractant Netrin-1 is an angiogenic factor. Proc Natl Acad Sci USA 101:16210–16215

Pasquale EB (2005)Eph receptor signalling casts a wide net on cell behaviour. Nat Rev Mol Cell Biol 6:462–475

Plump AS et al (2002) Slit1 and Slit2 cooperate to prevent premature midline crossing of retinal axons in the mouse visual system. Neuron 33:219–232

Ruhrberg C et al (2002) Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. Genes Dev 16:2684–2698

Sabatier C et al (2004) The divergent Robo family protein rig-1/Robo3 is a negative regulator of slit responsiveness required for midline crossing by commissural axons. Cell 117:157–169

Serafini T et al (1996) Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. Cell 87:1001–1014

Serini G et al (2003) Class 3 semaphorins control vascular morphogenesis by inhibiting integrin function. Nature 424:391–397

Shin D et al (2001) Expression of ephrinB2 identifies a stable genetic difference between arterial and venous vascular smooth muscle as well as endothelial cells, and marks subsets of microvessels at sites of adult neovascularization. Dev Biol 230:139–150

Stalmans I et al (2002) Arteriolar and venular patterning in retinas of mice selectively expressing VEGF isoforms. J Clin Invest 109:327–336

Stein E, Tessier-Lavigne M (2001) Hierarchical organization of guidance receptors: silencing of netrin attraction by slit through a Robo/DCC receptor complex. Science 291:1928–1938

Suchting S, Heal P, Tahtis K, Stewart LM, Bicknell R (2005) Soluble Robo4 receptor inhibits in vivo angiogenesis and endothelial cell migration. FASEB J 19:121–123

Surawska H, Ma PC, Salgia R (2004) The role of ephrins and Eph receptors in cancer. Cytokine Growth Factor Rev 15:419–433

Takahashi T, Nakamura F, Jin Z, Kalb RG, Strittmatter SM (1998) Semaphorins A and E act as antagonists of neuropilin-1 and agonists of neuropilin-2 receptors. Nat Neurosci 1:487–493

Tamagnone L et al (1999) Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. Cell 99:71–80

Tessier-Lavigne M, Goodman CS (1996) The molecular biology of axon guidance. Science 274:1123–1133

Vesalius A (1543) De Humani Corporis Fabrica (The Fabric of the Human Body) (Oporinus, Basel)

Wang B et al (2003) Induction of tumor angiogenesis by Slit-Robo signaling and inhibition of cancer growth by blocking Robo activity. Cancer Cell 4:19–29

Wang HU, Chen ZF, Anderson DJ (1998) Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. Cell 93:741–753

Wang KH et al (1999) Biochemical purification of a mammalian slit protein as a positive regulator of sensory axon elongation and branching. Cell 96:771–784

Weinstein BM (2005) Vessels and nerves: marching to the same tune. Cell 120:299–302

Wessells NK, Nuttall RP (1978) Normal branching, induced branching, and steering of cultured parasympathetic motor neurons. Exp Cell Res 115:111–122

Wilson BD et al (2006) Netrins promote developmental and therapeutic angiogenesis. Science 313:640–644

Zhang YW, Vande Woude GF (2003) HGF/SF-met signaling in the control of branching morphogenesis and invasion. J Cell Biochem 88:408–417

Zimmer M, Palmer A, Kohler J, Klein R (2003) EphB-ephrinB bi-directional endocytosis terminates adhesion allowing contact mediated repulsion. Nat Cell Biol 5:869–878

The Angiogenic Switch in Tumorigenesis

4

ANDREAS WICKI and GERHARD CHRISTOFORI

Abstract

Rapidly growing cancer tissue necessitates an increased blood supply. This is provided mainly by angiogenesis (blood vessel formation from pre-existing vessels) and vasculogenesis (de novo formation of vessels). Vascular co-option and vasculogenic mimicry may also play a role. The transition from a pre-vascular to a vascularized tumor phenotype is called the angiogenic switch. This switch is controlled by a balance between pro- and anti-angiogenic factors, which are secreted by the tumor cells themselves or by cells of the tumor microenvironment (in particular stromal cells and immune cells). The most prominent pro-angiogenic factors are vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF). Conversely, proteolytic fragments of the extracellular matrix (ECM) can act as potent angiogenesis inhibitors (e.g., endostatin). Other anti-angiogenic factors include cleaved derivatives of plasminogen (angiostatin) or antithrombin III (C-terminal antithrombin-fragment). The expression of pro- and anti-angiogenic factors by cancer cells is controlled directly by oncogenes, tumor suppressor genes and transcription factors, but also indirectly by environmental factors (such as oxygen or glucose supply). An important aspect of the angiogenic switch is the susceptibility of endothelial cells to pro-angiogenic stimuli. Genetic and epigenetic changes can modulate the response of the endothelial cells to VEGF and FGF and thus influence the angiogenic balance. Transgenic mouse models have been instrumental in elucidating the angiogenic switch and its effect on tumor progression. In patients, the angiogenic switch has been shown to occur in a number of cancer types, most prominently in breast and cervical cancer.

CONTENTS

The Angiogenic Switch Helps the Tumor to Overcome Growth Limitations Imposed by an Insufficient Blood Supply

With the induction of cellular hyperproliferation during tumor development, tumor outgrowth is usually restricted to no more than 1–2 mm in diameter. This phase is often called the avascular phase, in which the tumor is nourished by diffusion of oxygen and nutrients provided by nearby blood vessels, and tumor-associated formation of new blood vessels is not observed. Avascular tumors can reach a steady state, where tumor cell proliferation and apoptosis are in balance and a net increase in tumor volume does not occur. Such occult or dormant lesions may be found upon autopsy of individuals who have died of causes other than cancer. In order to exceed the size limit, tumors must gain access to an increased supply of oxygen and nutrients. These growing needs of enlarging tumors are usually met by angiogenesis, the formation of new blood vessels from existing vessels, such as capillaries or venules. The transition from the avascular phase of tumor development to the angiogenic phase is often referred to as the "angiogenic switch". The angiogenic switch, and the subsequent increase in tumoral blood vessel density induced by it, is the most important mechanism allowing tumors to overcome growth limitations imposed by insufficient blood supply.

When diffusion from nearby pre-existing vessels is no longer sufficient to sustain the tumor, there are at least four ways for a tumor to solve this dilemma and to continue to grow by gaining access to an enhanced supply of oxygen and nutrients (see Table 4.1) (Bergers and Benjamin 2003; Ribatti et al. 2003). The first and most important strategy is to induce the angiogenic switch and to initiate the process of angiogenesis. The tumor achieves this goal by secreting pro-angiogenic factors and/or by suppressing anti-angiogenic effectors resulting in the induction of endothelial cell proliferation and migration, vessel sprouting and tube formation. In addition, vessels may also increase in numbers by intussusception, i.e. the division of one vessel into two by the formation of a new vessel wall in the lumen of the original vessel (Burri et al. 2004).

Table 4.1. Tumor strategies to enhance blood supply

1. Angiogenesis
– Vascular sprouting from pre-existing capillaries or venules
– Vascular intussusception of pre-existing vessels
2. Vascular co-option
3. Vasculogenesis
– Mobilization, expansion and integration of vascular and hematological progenitor cells for de novo vessel formation
–Formation of mosaic vessels by integrating both tumor cells and vascular as well as hematological progenitor cells in de novo vessels
4. Vasculogenic mimicry
– Formation of vasculogenic networks by tumor cells without the contribution of vascular cells

Using a second strategy, tumors grow along pre-existing blood vessels and use these vessels for their own needs. Such co-option of existing blood vessels makes additional angiogenesis superfluous. A classical example of tumors using this strategy is provided by astrocytomas, which are often found to grow along blood vessels without forming a tumor capsule (Vajkoczy et al. 2002).

The third strategy is vasculogenesis, i.e. the de novo formation of blood vessels from bone marrow-derived precursor cells (Rafii et al. 2002). The formation of mosaic tumor blood vessels made up from both recruited vascular cells and non-vascular tumor cells may be viewed as a specific subtype of vasculogenesis.

The fourth strategy, whose relevance is still under debate, is the formation of a luminal network that instead of being lined by endothelial cells is generated by tumor cells themselves, a process called "vascular mimicry". Vascular mimicry has been

observed in highly aggressive melanomas, where tumor cells form PAS- and laminin-positive luminal networks that morphologically mimic blood vessels. However, the functional contribution of this type of vascular network to tumor progression has remained elusive (Hendrix et al. 2003).

More than one strategy may be found in the same tumor, and tumors might adopt different strategies according to their stage and grade of malignancy. In this chapter we will discuss the role of the angiogenic switch in inducing tumor angiogenesis and tumor progression.

Does Tumor Progression Depend on the Angiogenic Switch?

Since the first description of the angiogenic switch, its requirement for the outgrowth of many if not all types of solid tumors has been established (Folkman 1990). Strong evidence that the angiogenic switch is required for tumor outgrowth in vivo has been obtained by transplanting tumor cells into the avascular cornea of rabbits (Gimbrone et al. 1974). The transplanted tumor did not grow before vessels sprouting from the limbus reached the implant. If angiogenesis was inhibited, tumor growth was dramatically impaired, restricting the tumor nodule to a diameter of approximately 0.4 mm. Non-tumor tissue was not able to attract vessels to grow into the cornea. These findings were confirmed by experiments employing the chicken embryo chorioallantoic membrane (CAM) assay (Ausprunk et al. 1975). Tumors implanted into a CAM regressed within the first 3 days. However, when new vessels formed from pre-existing vessels and perfused the tumor tissue, tumors started to grow again.

As described above, the implantation of tumors into either an avascular compartment, such as the cornea, or into a vascularized surface, such as the chicken CAM, can elicit the ingrowth of new capillaries, suggesting that tumors release diffusible factors able to activate angiogenesis. Subsequent experiments showed that in the absence of adequate vasculature, tumors become necrotic and/or apoptotic. These observations underline the fact that proliferating tumors depend on sufficient access to the vasculature in order to thrive (Holmgren et al. 1995). In the past years, numerous results from different experimental systems and from first clinical trials have clearly demonstrated that angiogenesis, and hence the angiogenic switch, is required for tumor outgrowth (see also several chapters of this book).

Visualizing the Angiogenic Switch

Comparable to many human cancers, in some mouse models tumors develop in temporally and histologically distinct stages. Thereby, the experimental amenability and reproducibility afforded by the genetic predisposition to specific cancers in these mouse models allow a highly detailed investigation of the individual stages of tumorigenesis. Particularly well studied is the Rip1Tag2 transgenic mouse model of pancreatic β cell carcinogenesis. In these mice SV40 large-T antigen (Tag) is expressed under the control of the rat insulin promoter (Rip) (Hanahan 1985), resulting in the reproducible development of islet cell cancer in histologically distinct stages. Initially, all β cells in all islets of Langerhans express the oncogene, while the morphology of the islets remains normal. Subsequently, focal proliferation occurs in individual islets, which become hyperplastic (Teitelman et al. 1988). Hanahan and Folkman have identified a distinct stage, referred to as an angiogenic islet, which appears to be an intermediate stage between the hyperplastic islet and solid insulinoma (Folkman et al. 1989). By co-culturing capillary endothelial cells with hyperplastic islets isolated from Rip1Tag2 mice in a three-dimensional collagen gel, they observed that a subset of hyperplastic islets was able to induce the chemotactic migration, proliferation and tube formation of endothelial cells (angiogenic

islets), while other islets did not exert any effect on the co-cultured endothelial cells (non-angiogenic islets) (Fig. 4.1). These experiments clearly demonstrated that a switch to angiogenesis has occurred during the transition from non-angiogenic to angiogenic hyperplastic islets and that soluble pro-angiogenic factors are released to induce in vitro angiogenesis in this assay.

Subsequently, the angiogenic switch was also demonstrated in a number of other mouse models of carcinogenesis. The expression of bovine papillomavirus oncogenes elicits the formation of dermal fibrosarcomas in mice (Lacey et al. 1986). In this model, first angiogenic activities became evident in a late, pre-malignant stage (aggressive fibromatosis), for example by an increase in tumoral microvessel density, before the tumors progressed to highly vascularized end-stage fibrosarcomas (Kandel et al. 1991). In a third transgenic mouse model, papillomavirus type 16 (HPV-16) E6/E7 oncogenes were expressed in the epidermis to instigate the development of squamous cell carcinoma of the skin (K14HPV16; Arbeit et al. 1994). Again, weak angiogenic activity could be detected in the hyperplastic stage of the disease by a modest increase of vascularization in the underlying dermis. In a next step, progressive dysplasia led to an abundant formation of capillaries in the vicinity of the basement membrane between dermis and epidermis. A high density of vessels persisted in invasive squamous cancers that resulted from such dysplastic lesions (Smith-McCune et al. 1997).

4.4 The Angiogenic Switch in Human Cancer

The results from the mouse models suggest that the initiation of angiogenesis is indeed a rate-limiting step in tumor progression. Quantitative analysis of microvessel densities in many different cancer types in patients revealed that the angiogenic switch and the initiation of angiogenesis also occur during the growth of human cancers. Hyperproliferative lesions in humans, such as carcinomas in situ (CIS), have been screened for the occurrence of an angiogenic switch. In contrast to the comprehensive temporal and histological analysis in the mouse models, these studies initially concentrated on staining biopsy or autopsy

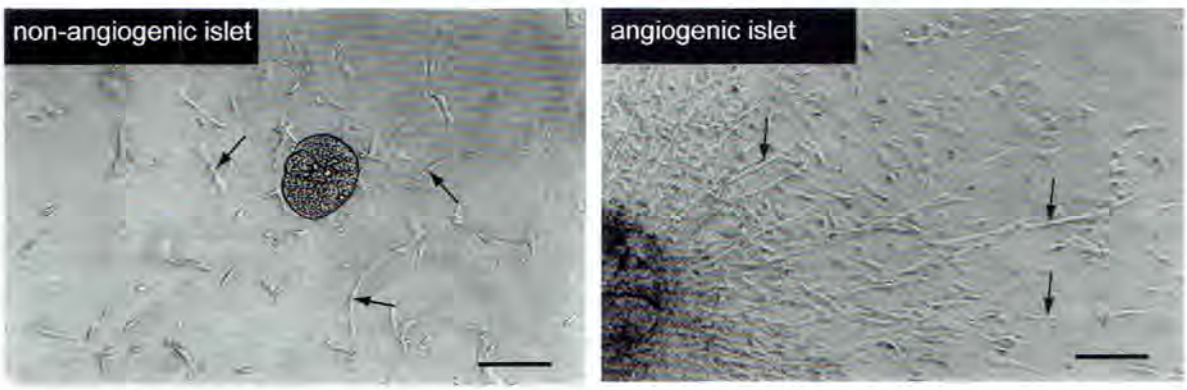


Fig. 4.1. Visualizing the angiogenic switch. Hyperplastic islets of Rip1Tag2 mice are co-cultured with endothelial cells in a three-dimensional collagen gel matrix. Islets that have not undergone the angiogenic switch (left panel) do not influence the distribution of the endothelial cells in the gel. In contrast, an islet that has undergone the angiogenic switch attracts endothelial cells, which converge upon the islet (right panel). The arrows indicate endothelial cells forming tubes in the collagen gel. Apparently, the proliferation and orientation of the endothelial cells is induced by pro-angiogenic factors, which are secreted by the angiogenic islet. Scale bar 50 μm

samples with antibodies against von Willebrand factor (vWF) and CD31 to localize and quantify vessels in pre-cancerous lesions. First evidence for the existence of an angiogenic switch in human cancers was obtained from histological stainings of pre-neoplastic lesions of the breast, including hyperplastic mammary gland ducts, dysplastic ducts and CIS. Using anti-vWF antibodies, a subset of CIS was considered angiogenic by its increased microvessel density (Weidner et al. 1992). Another human cancer that has been analyzed for its angiogenic status is cervical squamous cell carcinoma, a cancer type which also has been characterized extensively through the routine gynecological collection of Papanicolaou-stained cervix smears (Pap smears). Cervical dysplasia is graded as cervical in situ neoplasia (CIN) I–III, which is followed by the invasive cancer. When biopsies of cervical lesions were analyzed by staining with anti-vWF antibodies to quantify capillaries, an angiogenic switch was apparent in CIN II and CIN III lesions, with a marked increase of blood vessel density under the basement membrane underlying the dysplastic epithelium of CIN II and III lesions (Smith-McCune and Weidner 1994; Guidi et al. 1995). Moreover, histological analysis of benign squamous mucosa of the cervix in the neighborhood of CIN lesions revealed initial stages of angiogenesis, and expression of the pro-angiogenic vascular endothelial growth factor (VEGF-A, see below), while rare in normal cervical epithelium, could be detected in more than 90% of samples of benign cervix epithelium adjacent to CIN (Smith-McCune et al. 1998).

The angiogenic switch is executed by two different types of tissue: on one hand, tumor cells themselves, stromal cells or tumor-infiltrating immune cells begin to release soluble factors, whose main targets are endothelial and other vascular cells in the tumor microenvironment. On the other hand, the targeted endothelial and vascular cells are susceptible to the secreted factors by expressing the cognate receptors. The interplay between both pro- and anti-angiogenic factors and their respective receptors on cells of the vascular compartment is a key to understanding the angiogenic switch.

Pro-Angiogenic Factors

Angiogenesis is regulated by a tightly controlled balance between pro- and anti-angiogenic factors, which are secreted by the tumor cells themselves, by stromal cells or by cells of the immune system. The main targets of these factors are the endothelial cells of nearby vessels. Normal endothelial cells are the longest-lived cells outside of the nervous system. Only one in 10,000 endothelial cells of the body participates in the cell cycle, the others are quiescent (Engerman et al. 1967). An increase in endothelial cell proliferation and migration is an indicator that angiogenesis has been turned on by the angiogenic switch.

Many in vitro and in vivo assays have been developed to monitor the process of angiogenesis and to identify pro- and anti-angiogenic effectors (Cockerill et al. 1995). Most important are assays that quantitatively measure endothelial cell proliferation and migration. In the proliferation assay, the expansion of cultured capillary endothelial cells can be measured by counting the cells, by assessing the incorporation of radiolabeled nucleotides or by photometrically measuring mitochondrial activity. To quantify cell migration, two-chamber migration assays are often employed. Cells are seeded in an upper chamber separated by a porous membrane from a lower chamber. The lower chamber is filled with medium containing a chemoattractant, whereupon the cells migrate through the pores following the chemotactic gradient.

The prototype pro-angiogenic factor involved in many different processes of physiological and pathological angiogenesis is vascular endothelial growth factor A (VEGF-A). VEGF is a homodimeric glycoprotein with a molecular weight of approximately 45 kDa. Five main VEGF isoforms (121, 145, 165, 189 and 206 amino acids long) exist as a result of alternative splicing. The larger isoforms (VEGF 145, 165, 189 and 206) display basic surface charges and therefore are easily sequestered by heparin and heparin proteoglycans of the cell surface and the extracellular matrix (ECM). In contrast, VEGF 121 is

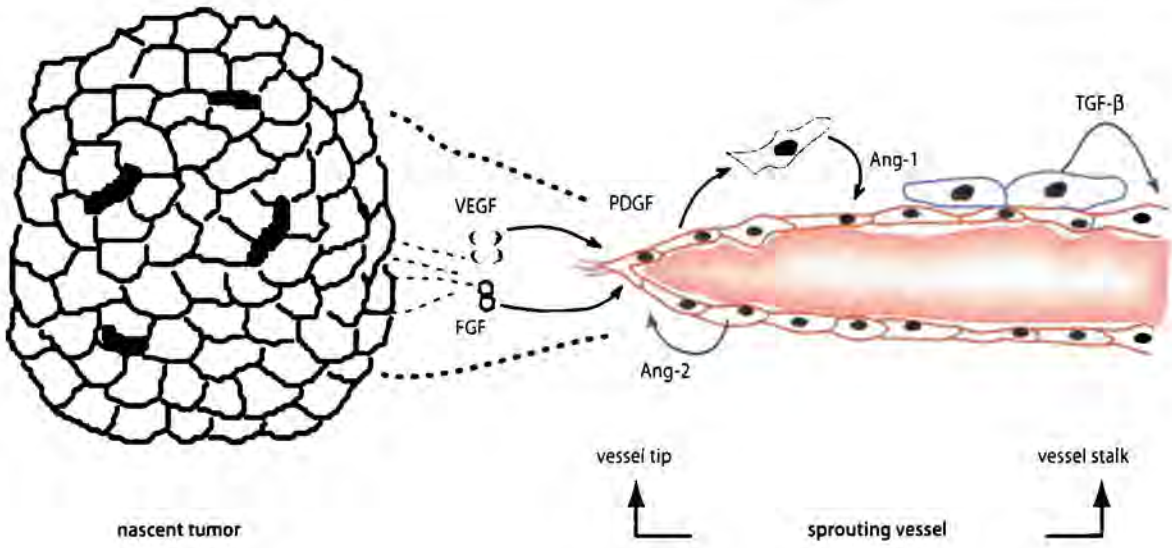
acidic and more freely diffusible. Heparin-binding VEGFs have been shown to play a role in vascular branching at the earliest stages of angiogenic invasion in several organs (Ruhrberg et al. 2002). The heparin-bound forms of VEGF can be released and thus activated by heparinases, plasmin, urokinase plasminogen activator (uPA) and matrix metalloproteases (MMPs). VEGF induces both endothelial cell proliferation and migration. More details concerning the function and regulation of VEGF are discussed in a separate chapter of this book. A second important group of pro-angiogenic factors is the fibroblast growth factor (FGF) family, in particular basic fibroblast growth factor (bFGF, FGF2) and acidic fibroblast growth factor (aFGF, FGF1) (Friesel and Maciag 1995; Folkman and Shing 1992; Christofori 1996). Both proteins belong to a family of growth factors that bind with high affinity to heparin and lack a typical consensus sequence for secretion. Nevertheless, they can be secreted from cells under certain circumstances and induce angiogenesis (Christofori 1996). Whereas the expression of VEGF-R is restricted to endothelial cells on blood and lymphatic vessels, FGF and FGF-R are expressed widely in the organism. Another factor reported to have pro-angiogenic activity is platelet-derived growth factor B (PDGF-B). PDGF-B induces tube formation, cell sprouting and proliferation of endothelial cells in vitro (Battegay et al. 1994). It is also mitogenic for smooth muscle cells and pericytes and induces the expression of VEGF and VEGF-R2 in cardiac endothelial cells (Edelberg et al. 1998). An additional group of pro-angiogenic factors are the angiopoietins. Although angiopoietins cannot elicit endothelial cell proliferation per se, they play an important role in the development of newly formed vessels. Angiopoietin-1 (Angl) acts as a maturation factor and promotes the recruitment of pericytes and smooth muscle cells to the developing vessel. Angiopoietin-2 (Ang2) seems to antagonize Angl activity, and its expression is often upregulated prior to vessel sprouting (Tait and Jones 2004). Both Angl and Ang2 are upregulated in a number of human cancers, yet Ang2 upregulation is more frequent. The shift of the Angl/Ang2 balance in favor of Ang2 makes the vasculature more plastic and amenable to sprouting. Another large group of pro-angiogenic effectors consists of cytokines. Growth-related oncogenes (Gro) α, β and γ, interleukin-8 (IL8), granulocyte chemoattractant protein-2 (GCP2) and epithelial neutrophil-activating protein 78 (ENA78) share three amino acids (Glu-Leu-Arg), the so-called ELR motif. Members of this subgroup of cytokines are chemotactic for endothelial cells in vitro and induce angiogenesis in the cornea pocket model in vivo (Belperio et al. 2000). Two other inflammation-associated factors, TGFβ and TNFα, have a dose-dependent impact on angiogenesis: low doses of TGFβ and TNFα stimulate proliferation of endothelial cells and tube formation in vitro, whereas higher doses exert the opposite effect (Frater-Schroder et al. 1987). Finally, MMP2 and MMP9 participate in the angiogenic switch. They clear the path for the migrating and proliferating endothelial cells by enzymatically degrading the matrix. MMP9 also renders hyperplastic islets and the epidermis angiogenic by releasing latent sequestered VEGF, as shown in the Rip1Tag2 and K14HPV16 mouse models (Bergers et al. 2000; Fang et al. 2000; Coussens et al. 2000). This dual action of activating endothelial cells by releasing latent pro-angiogenic factors and by degrading components of the ECM in the path of migrating endothelial cells is central to the pro-angiogenic effect of MMPs.

The expression of these pro-angiogenic effectors is regulated by upstream signals that have been partially characterized. Pro-angiogenic factors can be induced by cellular stress [in particular by hypoxia, glucose deprivation, formation of reactive oxygen species (ROS), cellular acidosis and iron deficiency], by the activation of oncogenes or by the loss of function of tumor suppressor genes (reviewed by Stein et al. 1995; North et al. 2005; Verheul et al. 2004; Kerbel and Folkman 2002). Table 4.2 summarizes the different factors capable of triggering the angiogenic switch and initiating angiogenesis. The combined action of the aforementioned pro-angiogenic factors initiates and sustains the tumor-associated angiogenesis. In this process, the pro-angiogenic factors VEGF, FGF, PDGF, Ang and TGFβ play distinct roles. The various functions of these pro-angiogenic factors are schematically depicted in Fig. 4.2.

Table 4.2. Induction of the angiogenic switch

1. Environmental and metabolic factors	Pathway (effectors)
Hypoxia / HIF-1α	VEGF-A↑[1]
Hypoxia / HIF-2α	VEGF-R2↑[2]
Glucose deprivation	VEGF↑[3], bFGF↑[3]
ROS / NF-κB	VEGF↑[4], IL-8↑[5]
Cellular acidosis	VEGF↑[6]
Iron deficiency	VEGF↑[7]
2. Gain of function: oncogenes	**Pathway (effectors)**
Phosphatidyl-inositol-3-kinase (PI3K)	VEGF↑[8]
Human papilloma virus (HPV)	VEGF↑[9], FGF-BP↑[10]
H-Ras	VEGF↑[11], TSP-1↓[12], MMPs[11]↑
EGFR	VEGF↑[13, 15], bFGF↑[14, 15], IL-8↑[15]
Erb/B2	VEGF↑[16]
Bcl2	VEGF↑[17]
Src	VEGF↑[18], TSP-1↓[19]
c-Myb	TSP-2↓[20]
Polyoma virus middle T (PyMT)	TSP-1↓[21]
Fos	VEGF expression[22]
Pituitary tumor-derived transforming gene 1 (PTTG1)	VEGF↑[23], bFGF↑[23]
3. Loss of function: tumor suppressor genes	**Pathway (effectors)**
p53	VEGF↑[24], HIF-1α↑[24], TSP-1↓[25]
PTEN	VEGF↑[26]
4. Transcription factors/repressors	**Pathway (effectors)**
Id1	TSP-1↓[27]

References: [1] = Blouw B et al., Cancer Cell, 2003, Semenza GL, Nat Rev Cancer, 2003; [2] = Elvert G et al., J Biol Chem, 2003; [3] = Shweiki D et al., Proc Natl Acad Sci, 1995, Stein I et al., Mol Cell Biol, 1995; [4] = Carmeliet P et al., Nature, 2000, Schafer T et al., J Biol Chem, 2003; [5] = Shono T et al., Mol Cell Biol, 1996; [6] = Shi Q et al., Oncogene, 2001; [7] = Beerepoot LV et al., Cancer Res, 1996; [8] = Jiang B et al., Proc Natl Acad Sci, 2000; [9] = LeBuanec H et al., Biomed Pharmacother, 1999, Lopez-Ocejo O et al., Oncogene, 2000; [10] = Stoppler H et al., Oncogene, 2001; [11] = Arbiser JL et al., Proc Natl Acad Sci, 1997; Rak J et al., Cancer Res, 1995; Grugel S et al., J Biol Chem, 1995; [12] = Zabrenetzky V et al., Int J Cancer, 1994; [13] = Petit AM et al., Am J Pathol, 1997, [14] = Inoue K et al., Clin Cancer Res, 2000; [15] = Perrotte P et al., Clin Cancer Res, 1999; [16] = Petit AM et al., Am J Pathol, 1997; [17] = Fernandez A et al., Natl Cancer Inst., 2001; [18] = Mukhopadhyay D et al., Cancer Res, 1995, Rak J et al., Cancer Res, 1995; [19] = Slack JL et al., Cell Growth Differ, 1994; [20] = Bein K et al., J Biol Chem, 1998; [21] = Sheibani N et al., Cancer Lett, 1996; [22] = Saez E et al., Cell, 1995; [23] = Heaney AP et al., Nature Med, 1999; [24] = Ravi R et al., Genes Dev, 2000; [25] = Dameron KM et al., Science, 1994; [26] = Koul D et al., Int J Oncol, 2002; [27] = Volpert OV et al., Cancer Cell, 2002.



VEGF	→	VEGF-R2
FGF	→	FGF-R1-4
PDGF-B	→	PDGF-Rβ
Angiopoietin -1, -2	→	Tie-2 receptor
TGFβ	→	ALK-5, ALK-1 (two types of TGFβ receptor I)

Fig. 4.2. Pro-angiogenic factors and the angiogenic switch. Pro-angiogenic factors that are released by tumor cells act on endothelial cells of a nearby vessel. VEGF mainly activates endothelial cell proliferation in the sprout stalk and migration of endothelial cells at the tip of the forming vessel. FGF appears to be important for maintaining angiogenesis. PDGF, produced by tumor or endothelial cells, is a potent mitogen and chemoattractant for precursors of pericytes and smooth muscle cells. Angiopoietins, secreted by either tumor or vascular cells, modulate vessel formation and maturation. Angiopoietin-1 stimulates endothelial cell differentiation and maturation, while angiopoietin-2 enhances vessel sprouting and vessel plasticity. Smooth muscle cells can secrete TGF-β, which modulates endothelial cell proliferation, differentiation, and maturation

4.6 Pro-Angiogenic Factors Possess Distinct Functions in the Initiation and Maintenance of the Angiogenic Switch

Different stages of the angiogenic switch, such as initiation and maintenance of angiogenesis, are regulated by different pro-angiogenic effectors. An example for this phenomenon is the temporally distinct action of VEGF and FGF. Both VEGF and FGF act directly on endothelial cells, stimulating proliferation and migration. To dissect the action of VEGF and FGF, soluble forms of the VEGF receptor (sVEGF-R) and the FGF receptor (sFGF-R) were cloned into an adenoviral vector and injected into RipTag mice. These soluble receptors then trapped the free forms of VEGF and FGF, thus selectively inhibiting their action. Whereas adenoviral expression of sVEGF-R predominantly affected the initiation of angiogenesis, sFGF-R impaired the maintenance of tumor angiogenesis (Compagni et al. 2000).

This phenomenon is also clinically important, since it has been shown that resistance to pharmaceutical VEGF blockade can be mediated by the action of other pro-angiogenic factors, in particular FGF (Casanovas et al. 2005).

Anti-angiogenic Factors

Anti-angiogenic factors, which can be produced by stromal cells or cells of the immune system, play a central role in controlling the fragile balance between initiation and termination of angiogenesis. There are two groups of endogenous anti-angiogenic factors: (1) proteins and protein-fragments of naturally occurring extra-cellular matrix and basement membrane components, and (2) growth factors, cytokines and other non-matrix-derived proteins that directly repress endothelial cell proliferation and migration. The first non-matrix-related anti-angiogenic factors to be identified were interferon-α and -β (IFNα, IFNβ). Interferons have been shown to inhibit tumor cell-induced angiogenesis in mice (Mitsuyasu 1991). IFNα is also capable of inhibiting MMP9 activity and expression (Ma et al. 2001). Furthermore, it reduces the secretion of pro-angiogenic IL8 (Lingen et al. 1998). Another important non-matrix-derived inhibitor of angiogenesis is angiostatin. Angiostatin has been reported to bind to a variety of cell surface molecules, including cell membrane ATPase, angiomotin, integrin αvβ3, annexin II, angiostatin-binding-sequence protein, c-met and NG2 proteoglycan (reviewed in Wahl et al. 2005). It inhibits both endothelial cell proliferation and migration and thus hinders tumor-associated angiogenesis. Furthermore, angiostatin can directly inhibit tumor growth and metastasis formation (O'Reilly et al. 1994).

The first matrix-related angiogenesis inhibitor to be characterized was thrombospondin 1 (TSP1), a large multifunctional ECM glycoprotein, which, apart from its anti-angiogenic properties, modulates cell adhesion, cell proliferation and survival, TGFβ activation and protease activation (Chen et al. 2000). A truncated and highly anti-angiogenic form of TSP1 was purified from a hamster cell line that had become tumorigenic concomitant with a mutation of p53 (Rastinejad et al. 1989; Good et al. 1990). Another matrix-related anti-angiogenic factor is endostatin, a proteolytic cleavage product of collagen XVIII. Endostatin specifically inhibits endothelial cell proliferation and potently inhibits angiogenesis and tumor growth (O'Reilly et al. 1997). Endostatin probably acts by blocking VEGF-R2 and suppressing Wnt signaling (reviewed by Dixelius et al. 2003). Canstatin and tumstatin, proteolytic cleavage products of collagen IV, have also been show to exhibit an anti-angiogenic effect. While canstatin promotes apoptosis of endothelial cells (Magnon et al. 2005), tumstatin acts as a specific inhibitor of endothelial cell protein synthesis (Maeshima et al. 2002). Table 4.3 presents a list of matrix-derived and non-matrix-derived anti-angiogenic factors. Their exact function in tumor angiogenesis is discussed in detail elsewhere in this book.

Obviously, the regulation of the expression and activities of anti-angiogenic factors directly affects the onset and maintenance of angiogenesis. In principle, the regulation of matrix-derived anti-angiogenic factors can take place either at the level of gene expression and cell secretion or at the level of proteolytic activation in the extracellular matrix ECM. The emerging concept is that expression and release of matrix-related anti-angiogenic factors by cancer cells can be regulated by both loss of function of tumor suppressor genes and activation of proto-oncogenes. Table 4.4 summarizes the influence of several signaling pathways on the expression of pro- and anti-angiogenic effectors. Expression of TSP1 has been shown to be directly regulated by wild-type p53 in fibroblasts and mammary epithelial cells (Dameron et al. 1994). Upon loss of p53, the levels of TSP1 drop dramatically in these cells and the surrounding ECM. Moreover, the cellular proto-oncogene Ras can modulate Myc activity to repress TSP1 expression (Watnick et al. 2003). The mechanism by which TSP1 and other matrix-related anti-angiogenic factors are then activated by proteases, either inside the producer cell or

Table 4.3. Anti-angiogenic effectors (reviewed by Nyberg et al., 2005)

Precursor molecule	*A/N*	Fragment/domain
Matrix-derived anti-angiogenic factors		
α1-chain of collagen IV	*N*	Arrestin
α2-chain of collagen IV	*N*	Canstatin
α3-chain of collagen IV	*N*	Tumstatin
Perlecan	*N*	Endorepellin
Collagen XVIII	*N*	Endostatin
Collagen XV	*N*	Endostatin-fragment
Fibronectin	*N*	Anastellin
Basement membrane preparations	*N*	Fibulin
TSP1	*A*	TSP1 fragments
TSP2	*A*	TSP2 fragments
Non-matrix-derived anti-angiogenic factors		
Plasminogen	*A*	Angiostatin
Antithrombin III	*A*	C-terminal antithrombin fragment
Prothrombin	*A*	Prothrombin kringle 2
Chondromodulin I	*A*	
VEGF-R1	*N*	Soluble VEGF-R1
Interferon α, interferon β	*A*	
IL4, IL12, IL18	*A*	
2-Methoxyestradiol	*A*	
Pigment epithelium derived factor (PEDF)	*A*	
Matrix-metalloprotease-2	*N*	Hemopexin-like domain (PEX) of MMP-2
Platelet factor 4 (PF4)	*A*	
Prolactin (PRL)	*N*	Prolactin fragment
Tissue inhibitors of MMPs (TIMPs)	*A*	
Troponin I	*A*	
Calreticulin	*N*	Vasostatin

A, The precursor molecule exhibits anti-angiogenic properties; *N*, an anti-angiogenic effect of the precursor molecule has not been observed.

Table 4.4. Inhibition of the angiogenic switch

Regulatory factor	Pathway (effectors)
Akt1	TSP-1↑[1], TSP-2↑[1], NO↑[1]
p53	TSP-1↑[2,3], BAI-1↑[4], EphA2↑[5], MMP-2↑[6], VEGF-A↓[7], HIF-1α↓[8], Cox2↑[9], MMP-1[10]↓
Rb2/p130	VEGF↓[11]
TrkB	VEGF↓[12]
VHL	HIF-1α↓[13], VEGF↓[13]

BAI, brain-specific angiogenesis inhibitor.

References: [1] = Chen J et al., Nat Med, 2005; [2] = Dameron et al., Science, 1994; [3] = Volpert OV, Breast Cancer Res Treat, 1995; [4] = Nishimori H et al., Oncogene, 1997; [5] = Dohn M et al., Oncogene, 2001, Brantley DM et al., Oncogene, 2002; Bian J et al., Mol Cell Biol, 1997; Pal S et al., Cancer Res, 2001; [8] = Ravi R et al., Genes Dev, 2000; [9] = Subbaramaiah K et al., J Biol Chem, 1999; [10] = Sun Y et al., Ann NY Acad Sci, 1999, Zhou Z et al., Proc Natl Acad Sci, 2000; [11] = Claudio PP et al., Cancer Res, 2001; [12] = McGregor LM et al., Proc Natl Acad Sci, 1999; [13] = Leung SK et al., J Biomed Biotechnol, 2002.

in the ECM after secretion, has remained elusive. In SUIT-2 cells, derived from human pancreatic cancer, proteolytic cleavage of collagen XVIII by cathepsin D, cathepsin L, several MMPs and elastase resulted in the formation of the anti-angiogenic protein endostatin (Brammer et al. 2005a). Canstatin and tumstatin are also activated through proteolytic cleavage of collagen residues. This underlines the polyvalent regulation of matrix-derived anti-angiogenic factors both at the level of production and secretion and at the level of activation and inactivation through different proteases.

Anti-angiogenic factors that are not derived from the extracellular matrix are also regulated at the level of gene expression and/or activation through proteolytic cleavage. The factors that are regulated at the expression level include interferons and the anti-angiogenic interleukins 4, 12 and 18, which are released from cells of the immune system. The release itself is regulated by inflammatory signals (Naldini and Carraro 2005). An example of a non-matrix-derived anti-angiogenic factor that is regulated at the level of proteolytic cleavage is angiostatin. Angiostatin is derived from plasminogen by various proteolytic cleavages and represents kringle domains 1–5 of plasminogen. Proteases that release angiostatin from plasminogen include several MMPs (MMP2, MMP3, MMP7, MMP9, MMP12), elastase, 13-kDa serine peptidase, 24-kDa endopeptidase, cathepsin D and prostate-specific antigen (PSA) (reviewed Cao and Xue 2004). Furthermore, plasmin auto-digestion also results in the formation of angiostatin. Such conundrum of activating proteases adds considerably to the complexity of the regulation of anti-angiogenic factors by proteolytic cleavage.

The Angiogenic Switch: A Balance Between Pro- and Anti-angiogenic Factors

Hanahan and Folkman hypothesized that the balance between pro-angiogenic factors and anti-angiogenic factors governs the angiogenic switch (Hanahan and Folkman 1996). In that model, the angiogenic switch tips the balance in favor of angiogenesis by either promoting the activities of pro-angiogenic factors or by inhibiting anti-angiogenic factors (Fig. 4.3). This hypothesis is supported by a number of experimental evidence. For example, adding increasing amounts of TSP1 to FGF2- and VEGF-stimulated endothelial cells abrogates their proliferation and migration (Good et al. 1990). Moreover, the restoration of p53 in tumor cells leads to an enhanced expression of TSP1, which in turn overrules the activities of pro-angiogenic factors either produced by the cells themselves or added exogenously (Dameron et al. 1994).

There are several ways to influence the balance between pro- and anti-angiogenic effector molecules and, thus, the angiogenic switch. Increased and decreased production and secretion of pro- and anti-angiogenic factors, respectively, will tip the balance to the angiogenic state, as described above. However, other mechanisms may also regulate the angiogenic balance. For instance, the specific secre-

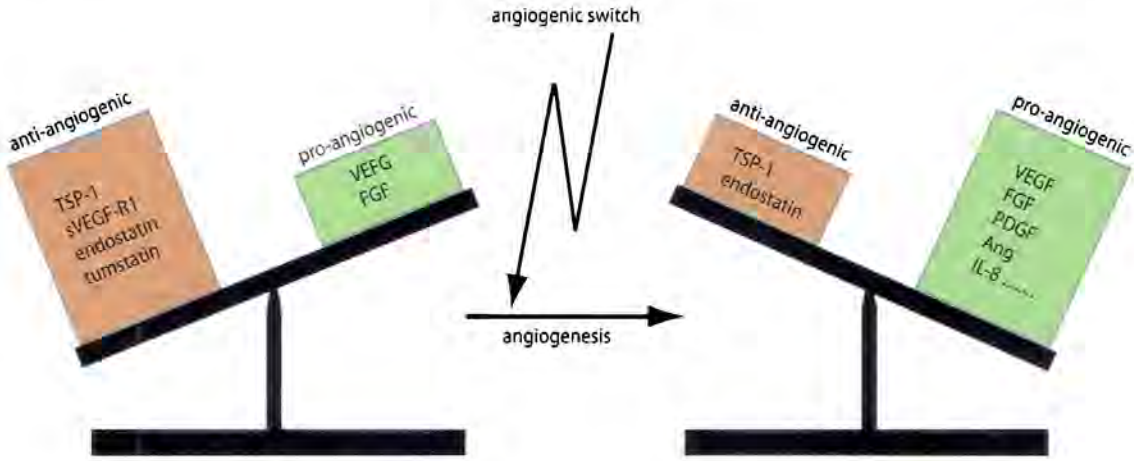


Fig. 4.3. The angiogenic balance. The angiogenic switch is governed by the balance between pro- and anti-angiogenic factors. Angiogenesis can be induced by upregulation of pro-angiogenic factors, downregulation of anti-angiogenic factors or both. The angiogenic switch tips the balance in favor of angiogenesis

tion of signal peptide-less FGF1 and FGF2 by tumor cells but not by non-transformed cells may contribute to the angiogenic switch (Christofori and Luef 1997; Kandel et al. 1991).

Also, the angiogenic activities of pro-angiogenic VEGF are controlled very tightly by several mechanisms. An interesting regulatory process is the inhibition of VEGF-A by soluble VEGF-R1 (sVEGF-R1), which is shed by endothelial cells. Upon binding of the ligand, sVEGF-R1 dimerizes and forms a dominant-negative complex with the mitogenically competent full-length VEGF-R2 (Kendall et al. 1996; Kendall and Thomas 1993). Moreover, shed VEGF-R1 also directly sequesters VEGF-A and prevents it from binding to VEGF-R2 on endothelial cells. Investigations in human breast cancer patients demonstrated that with increasing tumor size the ratio of VEGF-A to sVEGF-R1 shifts in favor of VEGF-A, thereby favoring the pro-angiogenic activity of VEGF-A (Hoar et al. 2004).

Different splice variants add an additional level of complexity to the regulation of VEGF-A. As mentioned above, VEGF-A exists in five main isoforms of different size, whereby the larger isoforms are sequestered at the cell surface or the ECM and the shorter isoforms are diffusible. The proteolytic cleavage and release of heparin-binding isoforms (latent VEGF) from the ECM represents a further important mechanism of angiogenesis control, as has been nicely demonstrated in the Rip1Tag2 transgenic mouse model of pancreatic β-cell carcinogenesis. Expression analysis of VEGF mRNA and protein during β-cell tumorigenesis in these mice revealed a constitutive expression of VEGF-A in normal islets and only a moderate upregulation of its expression during the late stages of tumorigenesis (Christofori et al. 1995). Yet, genetic ablation of VEGF-A gene function by knockout technology or repression of VEGF-A activity by the expression of soluble VEGF-R1 efficiently repressed the angiogenic switch and thus tumor outgrowth, indicating that VEGF-A plays an important role during the angiogenic switch in this model (Compagni et al. 2000; Inoue et al. 2002). Crossing Rip1Tag2 mice with MMP9 knockout mice also repressed the onset of angiogenesis, and detailed functional analysis revealed that MMP9 is required for the release of latent VEGF-A during the angiogenic switch (Bergers et al. 2000). Similar experiments in the K14-HPV16 mouse model of squamous cell carcinoma showed that MMP9 is actually supplied by infiltrating mast cells and monocytes, suggesting an important role of immune cells in the angiogenic switch (see below) (Coussens et al. 1999, 2000).

The importance of MMPs in manipulating the angiogenic balance has also been investigated in a modified chick CAM assay, where a nylon mesh coated with fibrillar type 1 collagen was implanted into the CAM. Expression of both FGF2 and VEGF resulted in a strong angiogenic response with migration of endothelial cells into the chick CAM implant. This response could be blocked by MMP inhibitors (Seandel et al. 2001). In addition, angiogenesis was markedly reduced if the implant was coated with collagenase-resistant collagen instead of wild-type fibrillar collagen. Interestingly, the migration of fibroblasts and monocytes into the modified implant was not reduced, indicating a specific role of MMP-mediated collagen cleavage in growth-factor-stimulated angiogenesis.

Feedback loops provide a further mechanism by which the angiogenic balance is modulated. Pro-angiogenic signals can induce the stimulation of anti-angiogenic signals to terminate angiogenesis and vice versa. For example, the generation of anti-angiogenic endostatin through elastase-mediated cleavage of collagen type XVIII is promoted by stimulation of human pancreatic cancer cells (SUIT-2 cells) with TNFα (Brammer et al. 2005b). Since TNF itself is known to increase the secretion of VEGF and thus to be pro-angiogenic, this exemplifies the interplay between several regulatory circuits governing the availability and the activities of pro- and anti-angiogenic factors.

The Tumor Microenvironment

It is now well appreciated that the tumor microenvironment exerts an important role in tumor progression. It not only contributes to the onset of tumor angiogenesis but also affects the malignant phenotype of tumor cells. Thereby, pro- and anti-angiogenic factors are not exclusively produced by tumor cells, they are also derived from stromal cells of the tumor microenvironment, including endothelial cells themselves, pericytes and smooth muscle cells, myoepithelial cells, fibroblasts and myofibroblasts and infiltrating cells of the immune system, such as macrophages, mast cells, dendritic cells, NK cells, and T and B lymphocytes (Coussens and Werb 2001; Egeblad and Werb 2002). Tumor-associated immune cells can modify and contribute to the angiogenic switch by secreting pro-angiogenic factors and proteases that are comparable to those secreted by the tumor cells themselves (Yu and Rak 2003; Bingle et al. 2002). Macrophages and monocytes seem to play a distinct role in tumor arteriogenesis, i.e., dilatation of primary blood vessels and formation of an arterial cell wall (Scholz et al. 2001). However, as with most aspects of tumor angiogenesis, inflammatory cells have a dual function and can also mediate a repertoire of anti-angiogenic factors and angiogenesis-inhibiting proteases. Table 4.5 gives an

Table 4.5. Immune cells and the angiogenic switch

Cell type	Function	Effectors
Macrophages[1]	Pro-angiogenic	VEGF, TGFα, IL8, FGF2, PDGF, substance P, prostaglandins, angiogenin
	Anti-angiogenic	TSP-1, IFNα, IFNγ
	Surface proteases	cathepsin D, tPA, uPA, MMP1, 2, 3, 7, 9, 12
Mast cells[2]	Pro-angiogenic	VEGF, FGF2, TGFβ, TNFα, IL8, histamine, NGF
	Surface proteases	Chymase, tryptase, MMP2, 9, heparanase
Neutrophils[3]	Pro-angiogenic	VEGF, IL8
	Anti-angiogenic	IL12, IP10
	Surface proteases	MMP9, uPA, elastase
Eosinophils[4]	Pro-angiogenic	VEGF, FGF2, TNFα, GM-CSF, NGF, IL8, eotaxin

PA, plasminogen activator (tissue or urokinase type); NGF, nerve growth factor; IP, interferon inducible protein; GM-CSF, granulocyte and macrophage colony-stimulating factor. Depending on the type of the expressed surface protease, angiogenesis can be enhanced or inhibited

References: [1] = reviewed by Yu JL et al., Breast Cancer Res, 2003 and Bingle L, J Pathol, 2002; [2] = reviewed by Yu JL et al., Breast Cancer Res, 2003 and Ribatti D et al., Clin Exp Allergy, 2004; [3] = reviewed by Yu JL et al., Breast Cancer Res, 2003; [4] = Puxeddu I et al., IJBCB, 2005

overview on the different pro- and anti-angiogenic factors contributed by inflammatory or other cells of the immune system.

Notably, cells of the immune system appear to modulate both tumor growth and tumor angiogenesis: they stimulate the adaptive immune response against tumor-specific antigens to repress tumor growth, yet at the same time they can promote tumor angiogenesis and progression by supporting innate and inflammatory responses. While the role of tumor-associated macrophages (TAMs) has been extensively addressed (reviewed in Pollard 2004), other cells of the immune system seem to contribute to tumor progression as well. Examples are mast cells providing MMPs for the activation of matrix-bound latent VEGF-A (see above; Coussens et al. 1999) or B cells which also appear to contribute to tumor progression (de Visser et al. 2005).

Tumors, i.e. tumor cells themselves and/or cells of the tumor microenvironment, respond to tumor hypoxia, necrosis, tissue repair and general inflammation and release a number of cytokines and chemokines that are chemoattractive for monocytes and macrophages, including CSF1, GM-CSF, MSP, TGFβ, CCL2, CCL7, CCL3, CCL4, and MIF. In turn, infiltrating macrophages secrete growth factors that affect tumor cells or tumor endothelium, including VEGF-A and -C, FGF2, TNFα, HGF, EGF family members, and PDGF (Balkwill et al. 2005; Pollard 2004). These cytokines and chemokines are also critical in the recruitment of secondary inflammatory cells, such as mast cells and neutrophils, which in turn support tumor progression by secreting pro-inflammatory, pro-angiogenic and pro-tumorigenic cytokines and proteases. Thus, depending on the chemokine milieu in the tumor environment, TAMs can be programmed to support an immune response against the tumor or rather repress adaptive immune responses and induce trophic activities of the tumor environment (Pollard 2004). Consistent with this notion, clinical studies have shown that in most, though not all, cancer types the presence of TAMs correlates with advanced tumor progression and poor prognosis (Bingle et al. 2002). Upon ablation of macrophages, for example by crossing MMTV-PyMT with CSF-1 KO mice, or by other means, tumor progression was slowed (Aharinejad et al. 2002; Lin et al. 2001). With regard to tumor angiogenesis, it is important to note that TAMs play an important role in the angiogenic switch, for instance by the secretion of VEGF-A, FGF2, NO, MMP7, MMP9, and uPA, which all support tumor cell proliferation and invasion or support angiogenesis by the release of bioactive VEGF-A from its latent matrix-bound forms (see above; Bergers et al. 2000; Hiratsuka et al. 2002). TAMs appear to concentrate in avascular hypoxic hot spots, where they are induced to express VEGF-A (Eubank et al. 2003; Harris 2002). Altogether, the data show that inflammation creates a microenvironment that is highly supportive of tumor angiogenesis and tumor progression. Yet, the roles of and the interplay among the various inflammatory cytokines and chemokines in the angiogenic switch are still only poorly understood.

Endothelial Cell Signaling

The fact that endothelial cells express a wide variety of growth factor and cytokine/chemokine receptors, and thus respond to numerous different growth factors, cytokines and chemokines, raises the important question of how the various signals are integrated within the cell to respond by increased or decreased angiogenesis. While it is now well established that certain pro-angiogenic factors can make endothelial cells more responsive to additional pro-angiogenic activities and that anti-angiogenic factors reduce this responsiveness, the molecular processes that underlie the integration of such various signaling pathways and determine the net outcome are only poorly understood. First experiments towards the unraveling of the interplay between angiogenic factors have revealed that, for instance, FGF2 stimulation of endothelial cells results in the upregulated expression of VEGF-R2, and VEGF-A can upregulate FGF-R expression. In addition, VEGF-A and FGF2 can synergistically promote angiogenesis through enhancement of endogenous PDGF-B signaling in

endothelial cells, also explaining the observed synergistic effects between these two prototype angiogenic factors (Kano et al. 2005). Moreover, Ang-2 is known to keep endothelial cells responsive to additional angiogenic factors; in fact, Ang-2-treated endothelial cells require additional pro-angiogenic signals, for instance provided by VEGF-A, not only to induce proliferation, migration and tube formation, but also to prevent apoptosis. Ang-1, in contrast, has been found to induce maturation and to inhibit leakiness of the endothelium by reducing its responsiveness to angiogenic factors (Lobov et al. 2002; Thurston et al. 2000). In a similar manner, TGFβ induces maturation and tube formation in endothelial cells cultured in three-dimensional matrices, yet induces apoptosis when cultured on plastic dishes, further underlining the importance of endothelial cell responsiveness to angiogenic signals (Sankar et al. 1996).

Subsequent efforts have addressed the susceptibility of endothelial and other vascular cells to angiogenic effectors by studying signaling pathways involved in the angiogenic process. For example, ablation of PKB/Akt-1 kinase activity in PKB/Akt-1 knockout mice resulted in a 40% increase of vascular density upon stimulation of endothelial cells with VEGF-A. However, the vessels in these mice appeared immature and leaky (Chen et al. 2005). In contrast, short-term inactivation of PKB/Akt-1 can result in impaired angiogenesis, as shown in a model of hindlimb ischemia, indicating different roles of PKB/Akt-1 at different stages of angiogenesis (Ackah et al. 2005). In addition, PKB/Akt-1 is also thought to modulate TSP1 expression in the endothelial cells themselves (Chen et al. 2005). The susceptibility of endothelial cells to pro-angiogenic signaling has also been studied in a mouse model carrying a PTEN knockout mutation specifically in endothelial cells. Heterozygous PTEN deficiency in endothelial cells was found to enhance tumor angiogenesis by rendering the cells more responsive to VEGF-A. A similar mechanism may be important in human Cowden disease, a hereditary syndrome of cancer susceptibility caused by a heterozygous PTEN mutation (Hamada et al. 2005). These experimental findings indicate that the angiogenic switch is accompanied by an endothelial switch, a change in signaling pathways and regulatory circuits which increases the susceptibility of endothelial cells to pro-angiogenic stimuli.

Splitting Vessels in Two: Vascular Intussusception

The intussusception of vessels is a mechanism of increasing vascular density by forming a new longitudinal vessel wall in the lumen of a pre-existing vessel and thus doubling the vessel number (Burri et al. 2004). This process may play a role in the angiogenic switch during tumor progression. For example, although VEGF mainly induces sprouting angiogenesis and increased vascular permeability, the treatment of chicken CAM with VEGF also results in a decreased intercapillary space due to enhanced vessel intussusception (Hagedorn et al. 2004). The exact pathways responsible for this phenomenon are not known yet, although an implication of VEGF and other pro-angiogenic factors seems plausible. Moreover, the role of intussusception in tumor angiogenesis needs to be studied in more detail.

Vascular Stem Cells and the Angiogenic Switch

So far, we have discussed angiogenesis and its function in tumor progression. However, there are alternative mechanisms of generating new vessels, and Fig. 4.4 gives an overview of the different pathways in which the angiogenic switch and/or its effectors are involved. One major way to generate new vessels, besides angiogenesis, is the de novo generation of blood vessels by vasculogenesis. Tumors are capable of mobilizing bone marrow-derived endothelial precursor cells, which then

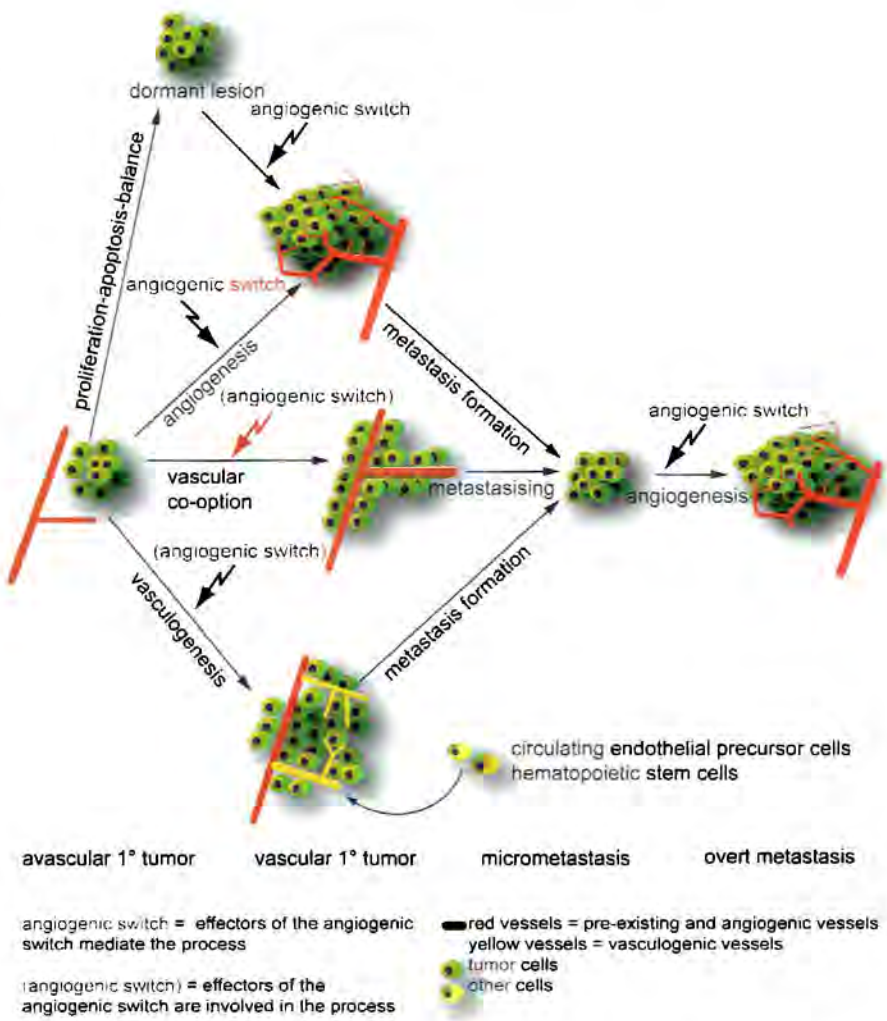


Fig. 4.4. The angiogenic switch in tumor progression. The angiogenic switch plays an important role in several steps of tumor progression. It promotes (1) the progression of a small, avascular primary tumor to a large, vascular tumor, (2) the activation of a dormant lesion and (3) the progression of micrometastases to overt macrometastases. Effector molecules of the angiogenic switch also take part in vascular co-option and vasculogenesis

migrate to the tumor and become incorporated into the developing new tumor vasculature (Rafii et al. 2002). Such circulating endothelial precursor cells (CEPs) were first discovered 30 years ago and they were shown to possess the ability to repair injured denuded vasculature. CEPs express different cell surface markers, including CD133, CXCR4, CD146, c-kit and VEGF-R2 (Gill et al. 2001; Peichev et al. 2000; Solovey et al. 1997). The finding that CEPs express VEGF-R2 instigated the search for a role of VEGF in the recruitment of CEPs from the bone marrow. Interestingly, a subset of hematopoietic stem cells expresses another member of the VEGF-R family, namely VEGF-R1. VEGF-A has been shown to be able to mobilize both kinds of stem cells involved in tumor vasculogenesis (Hattori et al. 2001). Blocking the action of VEGF-A on both stem cells by inhibiting VEGF-R1 and VEGF-R2 resulted in an impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells (Lyden et al. 2001). This activity of VEGF-A was shown to be ablated in three different knockout mouse models (Id1+/-Id3-/-, MMP-9-/-, p130-/-p27+/Δ51), resulting in a defect of VEGF-induced expansion of VEGF-R1-positive myeloid cells and VEGF-R2-positive endothelial precursor cells. This underlines

the importance of VEGF-mediated mobilization and expansion of hematopoietic and endothelial precursor cells in vasculogenesis (Heissig et al. 2002; Vidal et al. 2005). Placental growth factor (PlGF), a VEGF family member and specific ligand for VEGF-R1 (see corresponding chapter), has been shown to play a major role in the recruitment of CEPs to tumor tissue (Hattori et al. 2002).

The collaboration of co-recruited CEPs and hematopoietic stem cells is thought to facilitate the integration of CEPs into the rapidly growing tumor vasculature. In particular, a Tie2-expressing lineage of macrophages (TEM) has been identified which appears to be important for tumor vessel formation (De Palma et al. 2005). Various experiments have been performed to investigate the contribution of CEPs to tumor vasculogenesis. One approach was to induce endothelial differentiation of human adult multipotential progenitor cells (MAPCs) in vitro and to inject them into an immunocompromised mouse that carried a mouse Lewis lung carcinoma. After 5 days, 30% of the endothelial cells of newly formed tumor-associated vessels originated from MAPC-derived human endothelial cells. MAPC-derived endothelial cells could also be detected in spontaneously forming lymphomas of the same mice (Reyes et al. 2002). This and similar experiments underline the significant contribution of both vascular and hematopoietic stem cells in the formation of tumor-associated vessels. Together with other components of the tumor environment, vasculogenesis in general and CEPs in particular are targets for anti-tumor therapy.

Conclusions

Proliferating tumors need adequate blood perfusion to achieve sustainable growth. Tumors gain access to sufficient oxygen and nutrient supply by using one or more of four strategies: angiogenesis, vascular co-option, vasculogenesis and/or vascular mimicry. The angiogenic switch is the mechanism by which angiogenesis is induced and sustained. Thereby, the onset of angiogenesis and its maintenance is governed by a finely tuned balance between pro-angiogenic and anti-angiogenic factors, which in turn depends on the regulation of tumor suppressor genes, oncogenes, transcriptional repressors and genes involved in cellular response to environmental and metabolic factors. Such effectors of the angiogenic switch also play a role in the processes of vascular co-option and vasculogenesis, although more experimental insights are certainly warranted. Moreover, new vessels that are induced by the angiogenic switch are often immature and therefore functionally different from non-tumor-associated vessels, and, hence, the molecular mechanisms underlying the interplay between the various pro- and anti-angiogenic factors requires future attention. Finally, the molecular pathways determining the responsiveness of endothelial cells to pro- and anti-angiogenic factors during the angiogenic switch need to be investigated in more detail.

In summary, the importance of the angiogenic switch in the onset of angiogenesis has been documented in experimental animal models and in human cancer. Together, the results underline the notion that the angiogenic switch is a general mechanism of tumor progression and therefore an important target for the development of innovative cancer therapies.

References

Ackah, E, Yu J, Zoellner S, Iwakiri Y, Skurk C, Shibata R, Ouchi N, Easton RM, Galasso G, Birnbaum MJ, Walsh K, Sessa WC (2005) Akt1/protein kinase Balpha is critical for ischemic and VEGF-mediated angiogenesis. J Clin Invest 115(8):2119–2127

Aharinejad S, Abraham D, Paulus P, Abri H, Hofmann M, Grossschmidt K, Schafer R, Stanley ER, Hofbauer R (2002) Colony-stimulating factor-1 antisense treatment suppresses growth of human tumor xenografts in mice. Cancer Res 62(18):5317–5324

Arbeit JM, Munger K, Howley PM, Hanahan D (1994) Progressive squamous epithelial neoplasia in K14-human papillomavirus type 16 transgenic mice. J Virol 68(7):4358–4368

Arbiser JL, Moses MA, Fernandez CA, Ghiso N, Cao Y, Klauber N, Frank D, Brownlee M, Flynn E, Parangi S, Byers HR, Folkman J (1997) Oncogenic H-ras stimulates tumor angiogenesis by two distinct pathways. Proc Natl Acad Sci USA 94(3):861–866

Ausprunk DH, Knighton DR, Folkman J (1975) Vascularization of normal and neoplastic tissues grafted to the chick chorioallantois. Role of host and preexisting graft blood vessels. Am J Pathol 79(3):597–628

Balkwill F, Charles KA, Mantovani A (2005) Smoldering and polarized inflammation in the initiation and promotion of malignant disease. Cancer Cell 7(3):211–217

Battegay EJ, Rupp J, Iruela-Arispe L, Sage EH, Pech M (1994) PDGF-BB modulates endothelial proliferation and angiogenesis in vitro via PDGF beta-receptors. J Cell Biol 125(4):917–928

Beerepoot LV, Shima DT, Kuroki M, Yeo KT, Voest EE (1996) Up-regulation of vascular endothelial growth factor production by iron chelators. Cancer Res 56(16):3747–3751

Bein K, Ware JA, Simons M (1998) Myb-dependent regulation of thrombospondin 2 expression. Role of mRNA stability. J Biol Chem 273(33):21423–21429

Belperio JA, Keane MP, Arenberg DA, Addison CL, Ehlert JE, Burdick MD, Strieter RM (2000) CXC chemokines in angiogenesis. J Leukoc Biol 68(1):1–8

Bergers G, Benjamin LE (2003) Tumorigenesis and the angiogenic switch. Nat Rev Cancer 3(6):401–410

Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, Tanzawa K, Thorpe P, Itohara S, Werb Z, Hanahan D (2000) Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat Cell Biol 2(10):737–744

Bian J, Sun Y (1997) Transcriptional activation by p53 of the human type IV collagenase (gelatinase A or matrix metalloproteinase 2) promoter. Mol Cell Biol 17(11):6330–6338

Bingle L, Brown NJ, Lewis CE (2002) The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. J Pathol 196(3):254–265

Blouw B, Song H, Tihan T, Bosze J, Ferrara N, Gerber HP, Johnson RS, Bergers G (2003) The hypoxic response of tumors is dependent on their microenvironment. Cancer Cell 4(2):133–146

Brammer RD, Bramhall SR, Eggo MC (2005) Endostatin expression in pancreatic tissue is modulated by elastase. Br J Cancer 92(1):89–93

Brammer RD, Bramhall SR, Eggo MC (2005) Endostatin expression in a pancreatic cell line is modulated by a TNF alpha-dependent elastase. Br J Cancer 93(9) 2005:1024–1028

Brantley DM, Cheng N, Thompson EJ, Lin Q, Brekken RA, Thorpe PE, Muraoka RS, Cerretti DP, Pozzi A, Jackson D, Lin C, Chen J (2002) Soluble Eph A receptors inhibit tumor angiogenesis and progression in vivo. Oncogene 21(46):7011–7026

Burri PH, Hlushchuk R, Djonov V (2004) Intussusceptive angiogenesis: its emergence, its characteristics, and its significance. Dev Dyn 231(3):474–488

Cao Y, Xue L (2004) Angiostatin. Semin Thromb Hemost 30(1):83–93

Carmeliet P, Jain RK (2000) Angiogenesis in cancer and other diseases. Nature 407(6801):249–257

Casanovas O, Hicklin DJ, Bergers G, Hanahan D (2005) Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. Cancer Cell 8(4):299–309

Chen H, Herndon ME, Lawler J (2000) The cell biology of thrombospondin-1. Matrix Biol 19(7):597–614

Chen J, Somanath PR, Razorenova O, Chen WS, Hay N, Bornstein P, Byzova TV (2005) Akt1 regulates pathological angiogenesis, vascular maturation and permeability in vivo. Nat Med 11(11):1188–1196

Christofori G (1996) The role of fibroblast growth factors in tumour progression and angiogenesis. In: Bicknell R, Lewis CE, Ferrara N (eds) Tumour angiogenesis. Oxford University Press, Oxford, UK

Christofori G, Luef S (1997) Novel forms of acidic fibroblast growth factor-1 are constitutively exported by beta tumor cell lines independent from conventional secretion and apoptosis. Angiogenesis 1(1):55–70

Christofori G, Naik P, Hanahan D (1995) Vascular endothelial growth factor and its receptors, flt-1 and flk-1, are expressed in normal pancreatic islets and throughout islet cell tumorigenesis. Mol Endocrinol 9(12):1760–1770

Claudio PP, Stiegler P, Howard CM, Bellan C, Minimo C, Tosi GM, Rak J, Kovatich A, De Fazio P, Micheli P, Caputi M, Leoncini L, Kerbel R, Giordano GG, Giordano A (2001) RB2/p130 gene-enhanced expression down-regulates vascular endothelial growth factor expression and inhibits angiogenesis in vivo. Cancer Res 61(2):462–468

Cockerill GW, Gamble JR, Vadas MA (1995) Angiogenesis: models and modulators. Int Rev Cytol 159:113–160

Compagni A, Wilgenbus P, Impagnatiello MA, Cotten M, Christofori G (2000) Fibroblast growth factors are required for efficient tumor angiogenesis. Cancer Res 60(24):7163–7169

Coussens LM, Raymond WW, Bergers G, Laig-Webster M, Behrendtsen O, Werb Z, Caughey GH, Hanahan D (1999) Inflammatory mast cells up-regulate angiogenesis during squamous epithelial carcinogenesis. Genes Dev 13(11):1382–1397

Coussens LM, Tinkle CL, Hanahan D, Werb Z (2000) MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. Cell 103(3):481–490

Coussens LM, Werb Z (2001) Inflammatory cells and cancer: think different! J Exp Med 193(6):F23–6

Dameron KM, Volpert OV, Tainsky MA, Bouck N (1994) Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. Science 265(5178):1582–1584

De Palma M, Venneri MA, Galli R, Sergi LS, Politi LS, Sampaolesi M, Naldini L (2005) Tie2 identifies a hematopoi-

etic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. Cancer Cell 8(3):211–226

de Visser KE, Korets LV, Coussens LM (2005) De novo carcinogenesis promoted by chronic inflammation is B lymphocyte dependent. Cancer Cell 7(5):411–423

Dixelius J, Cross MJ, Matsumoto T, Claesson-Welsh L (2003) Endostatin action and intracellular signaling: beta-catenin as a potential target? Cancer Lett 196(1):1–12

Dohn M, Jiang J, Chen X (2001) Receptor tyrosine kinase EphA2 is regulated by p53-family proteins and induces apoptosis. Oncogene 20(45):6503–6515

Edelberg JM, Aird WC, Wu W, Rayburn H, Mamuya WS, Mercola M, Rosenberg RD (1998) PDGF mediates cardiac microvascular communication. J Clin Invest 102(4):837–843

Egeblad M, Werb Z (2002) New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer 2(3):161–174

Elvert G, Kappel A, Heidenreich R, Englmeier U, Lanz S, Acker T, Rauter M, Plate K, Sieweke M, Breier G, Flamme I (2003) Cooperative interaction of hypoxia-inducible factor-2alpha (HIF-2alpha) and Ets-1 in the transcriptional activation of vascular endothelial growth factor receptor-2 (Flk-1). J Biol Chem 278(9):7520–7530

Engerman RL, Pfaffenbach D, Davis MD (1967) Cell turnover of capillaries. Lab Invest 17(6):738–743

Eubank TD, Galloway M, Montague CM, Waldman WJ, Marsh CB (2003) M-CSF induces vascular endothelial growth factor production and angiogenic activity from human monocytes. J Immunol 171(5):2637–2643

Fang J, Shing Y, Wiederschain D, Yan L, Butterfield C, Jackson G, Harper J, Tamvakopoulos G, Moses MA (2000) Matrix metalloproteinase-2 is required for the switch to the angiogenic phenotype in a tumor model. Proc Natl Acad Sci USA 97(8):3884–3889

Fernandez A, Udagawa T, Schwesinger C, Beecken W, Achilles-Gerte E, McDonnell T, D'Amato R (2001) Angiogenic potential of prostate carcinoma cells overexpressing bcl-2. J Natl Cancer Inst 93(3):208–213

Folkman J (1990) What is the evidence that tumors are angiogenesis dependent? J Natl Cancer Inst 82(1):4–6

Folkman J, Shing Y (1992) Angiogenesis. J Biol Chem 267(16):10931–10934

Folkman J, Watson K, Ingber D, Hanahan D (1989) Induction of angiogenesis during the transition from hyperplasia to neoplasia. Nature 339(6219):58–61

Frater-Schroder M, Risau W, Hallmann R, Gautschi P, Bohlen P (1987) Tumor necrosis factor type alpha, a potent inhibitor of endothelial cell growth in vitro, is angiogenic in vivo. Proc Natl Acad Sci USA 84(15):5277–5281

Friesel RE, Maciag T (1995) Molecular mechanisms of angiogenesis: fibroblast growth factor signal transduction. FASEB J 9(10):919–925

Gill M, Dias S, Hattori K, Rivera ML, Hicklin D, Witte L, Girardi L, Yurt R, Himel H, Rafii S (2001) Vascular trauma induces rapid but transient mobilization of VEGFR2(+)AC133(+) endothelial precursor cells. Circ Res 88(2):167–174

Gimbrone MAJ, Cotran RS, Leapman SB, Folkman J (1974) Tumor growth and neovascularization: an experimental model using the rabbit cornea. J Natl Cancer Inst 52(2):413–427

Good DJ, Polverini PJ, Rastinejad F, Le Beau MM, Lemons RS, Frazier WA, Bouck NP (1990) A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. Proc Natl Acad Sci USA 87(17):6624–6628

Grugel S, Finkenzeller G, Weindel K, Barleon B, Marme D (1995) Both v-Ha-Ras and v-Raf stimulate expression of the vascular endothelial growth factor in NIH 3T3 cells. J Biol Chem 270(43):25915–25919

Guidi AJ, Abu-Jawdeh G, Berse B, Jackman RW, Tognazzi K, Dvorak HF, Brown LF, (1995) Vascular permeability factor (vascular endothelial growth factor) expression and angiogenesis in cervical neoplasia. J Natl Cancer Inst 87(16):1237–1245

Hagedorn M, Balke M, Schmidt A, Bloch W, Kurz H, Javerzat S, Rousseau B, Wilting J, Bikfalvi A (2004) VEGF coordinates interaction of pericytes and endothelial cells during vasculogenesis and experimental angiogenesis. Dev Dyn 230(1):23–33

Hamada K, Sasaki T, Koni PA, Natsui M, Kishimoto H, Sasaki J, Yajima N, Horie Y, Hasegawa G, Naito M, Miyazaki J, Suda T, Itoh H, Nakao K, Mak TW, Nakano T, Suzuki A (2005) The PTEN/PI3K pathway governs normal vascular development and tumor angiogenesis. Genes Dev 19(17):2054–2065

Hanahan D (1985) Heritable formation of pancreatic beta-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. Nature 315(6015):115–122

Hanahan D, Folkman J (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 86(3):353–364

Harris AL (2002) Hypoxia–a key regulatory factor in tumour growth. Nat Rev Cancer 2(1):38–47

Hattori K, Dias S, Heissig B, Hackett NR, Lyden D, Tateno M, Hicklin DJ, Zhu Z, Witte L, Crystal RG, Moore MA, Rafii S (2001) Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells. J Exp Med 193(9):1005–1014

Hattori K, Heissig B, Wu Y, Dias S, Tejada R, Ferris B, Hicklin DJ, Zhu Z, Bohlen P, Witte L, Hendrikx J, Hackett NR, Crystal RG, Moore MA, Werb Z, Lyden D, Rafii S (2002) Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. Nat Med 8(8):841–849

Heaney AP, Horwitz GA, Wang Z, Singson R, Melmed S (1999) Early involvement of estrogen-induced pituitary tumor transforming gene and fibroblast growth factor expression in prolactinoma pathogenesis. Nat Med 5(11):1317–1321

Heissig B, Hattori K, Dias S, Friedrich M, Ferris B, Hackett NR, Crystal RG, Besmer P, Lyden D, Moore MA, Werb Z, Rafii S (2002) Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. Cell 109(5):625–637

Hendrix MJ, Seftor EA, Hess AR, Seftor RE (2003) Vasculogenic mimicry and tumour-cell plasticity: lessons from melanoma. Nat Rev Cancer 3(6):411–421

Hiratsuka S, Nakamura K, Iwai S, Murakami M, Itoh T, Kijima H, Shipley JM, Senior RM, Shibuya M (2002) MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis. Cancer Cell 2(4):289–300

Hoar FJ, Lip GY, Belgore F, Stonelake PS (2004) Circulating levels of VEGF-A, VEGF-D and soluble VEGF-A receptor (sFlt-1) in human breast cancer. Int J Biol Markers 19(3):229–235

Holmgren L, O'Reilly MS, Folkman J (1995) Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. Nat Med 1(2):149–153

Inoue K, Perrotte P, Wood CG, Slaton JW, Sweeney P, Dinney CP (2000) Gene therapy of human bladder cancer with adenovirus-mediated antisense basic fibroblast growth factor. Clin Cancer Res 6(11):4422–4431

Inoue M, Hager JH, Ferrara N, Gerber HP, Hanahan D (2002) VEGF-A has a critical, nonredundant role in angiogenic switching and pancreatic beta cell carcinogenesis. Cancer Cell 1(2):193–202

Jiang BH, Zheng JZ, Aoki M, Vogt PK (2000) Phosphatidylinositol 3-kinase signaling mediates angiogenesis and expression of vascular endothelial growth factor in endothelial cells. Proc Natl Acad Sci USA 97(4):1749–1753

Kandel J, Bossy-Wetzel E, Radvanyi F, Klagsbrun M, Folkman J, Hanahan D (1991) Neovascularization is associated with a switch to the export of bFGF in the multistep development of fibrosarcoma. Cell 66(6):1095–1104

Kano MR, Morishita Y, Iwata C, Iwasaka S, Watabe T, Ouchi Y, Miyazono K, Miyazawa K (2005) VEGF-A and FGF-2 synergistically promote neoangiogenesis through enhancement of endogenous PDGF-B-PDGFRbeta signaling. J Cell Sci 118(Pt16):3759–3768

Kendall RL, Thomas KA (1993) Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. Proc Natl Acad Sci USA 90(22):10705–10709

Kendall RL, Wang G, Thomas KA (1996) Identification of a natural soluble form of the vascular endothelial growth factor receptor, FLT-1, and its heterodimerization with KDR. Biochem Biophys Res Commun 226(2):324–328

Kerbel R, Folkman J (2002) Clinical translation of angiogenesis inhibitors. Nat Rev Cancer 2(10):727–739

Koul D, Shen R, Garyali A, Ke LD, Liu TJ, Yung WK (2002) MMAC/PTEN tumor suppressor gene regulates vascular endothelial growth factor-mediated angiogenesis in prostate cancer. Int J Oncol 21(3):469–475

Lacey M, Alpert S, Hanahan D (1986) Bovine papillomavirus genome elicits skin tumours in transgenic mice. Nature 322(6080):609–612

Le Buanec H, D'Anna R, Lachgar A, Zagury JF, Bernard J, Ittele D, d'Alessio P, Hallez S, Giannouli C, Burny A, Bizzini B, Gallo RC, Zagury D (1999) HPV-16 E7 but not E6 oncogenic protein triggers both cellular immunosuppression and angiogenic processes. Biomed Pharmacother 53(9):424–431

Leung SK, Ohh M (2002) Playing Tag with HIF: The VHL Story. J Biomed Biotechnol 2(3):131–135

Lin EY, Nguyen AV, Russell RG, Pollard JW (2001) Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. J Exp Med 193(6):727–740

Lingen MW, Polverini PJ, Bouck NP (1998) Retinoic acid and interferon alpha act synergistically as antiangiogenic and antitumor agents against human head and neck squamous cell carcinoma. Cancer Res 58(23):5551–5558

Lobov IB, Brooks PC, Lang RA (2002) Angiopoietin-2 displays VEGF-dependent modulation of capillary structure and endothelial cell survival in vivo. Proc Natl Acad Sci USA 99(17):11205–11210

Lopez-Ocejo O, Viloria-Petit, Bequet-Romero M, Mukhopadhyay D, Rak J, Kerbel RS (2000) Oncogenes and tumor angiogenesis: the HPV-16 E6 oncoprotein activates the vascular endothelial growth factor (VEGF) gene promoter in a p53 independent manner. Oncogene 19(40):4611–4620

Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L, Chadburn A, Heissig B, Marks W, Witte L, Wu Y, Hicklin D, Zhu Z, Hackett NR, Crystal RG, Moore MA, Hajjar KA, Manova K, Benezra R, Rafii S (2001) Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat Med 7(11):1194–1201

Ma Z, Qin H, Benveniste EN (2001) Transcriptional suppression of matrix metalloproteinase-9 gene expression by IFN-gamma and IFN-beta: critical role of STAT-1alpha. J Immunol 167(9):5150–5159

Maeshima Y, Sudhakar A, Lively JC, Ueki K, Kharbanda S, Kahn CR, Sonenberg N, Hynes RO, Kalluri R (2002) Tumstatin, an endothelial cell-specific inhibitor of protein synthesis. Science 295(5552):140–143

Magnon C, Galaup A, Mullan B, Rouffiac V, Bouquet C, Bidart JM, Griscelli F, Opolon P, Perricaudet M (2005) Canstatin acts on endothelial and tumor cells via mitochondrial damage initiated through interaction with alphavbeta3 and alphavbeta5 integrins. Cancer Res 65(10):4353–4361

McGregor LM, McCune BK, Graff JR, McDowell PR, Romans KE, Yancopoulos GD, Ball DW, Baylin SB, Nelkin BD (1999) Roles of trk family neurotrophin receptors in

medullary thyroid carcinoma development and progression. Proc Natl Acad Sci USA 96(8):4540–4545

Mitsuyasu RT (1991) Interferon alpha in the treatment of AIDS-related Kaposi's sarcoma. Br J Haematol 79(Suppl 1):69–73

Mukhopadhyay D, Tsiokas L, Sukhatme VP (1995) Wild-type p53 and v-Src exert opposing influences on human vascular endothelial growth factor gene expression. Cancer Res 55(24):6161–6165

Naldini A, Carraro F (2005) Role of inflammatory mediators in angiogenesis. Curr Drug Targets Inflamm Allergy 4(1):3–8

Nishimori H, Shiratsuchi T, Urano T, Kimura Y, Kiyono K, Tatsumi K, Yoshida S, Ono M, Kuwano M, Nakamura Y, Tokino T (1997) A novel brain-specific p53-target gene, BAI1, containing thrombospondin type 1 repeats inhibits experimental angiogenesis. Oncogene 15(18):2145–2150

North S, Moenner M, Bikfalvi A (2005) Recent developments in the regulation of the angiogenic switch by cellular stress factors in tumors. Cancer Lett 218(1):1-14

Nyberg P, Xie L, Kalluri R (2005) Endogenous inhibitors of angiogenesis. Cancer Res 65(10):3967–3979

O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR, Folkman J (1997) Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell 88(2):277–285

O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, Lane WS, Cao Y, Sage EH, Folkman J (1994) Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell 79(2):315–328

Pal S, Datta K, Mukhopadhyay D (2001) Central role of p53 on regulation of vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) expression in mammary carcinoma. Cancer Res 61(18):6952–6957

Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M, Oz MC, Hicklin DJ, Witte L, Moore MA, Rafii S (2000) Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. Blood 95(3):952–958

Perrotte P, Matsumoto T, Inoue K, Kuniyasu H, Eve BY, Hicklin DJ, Radinsky R, Dinney CP (1999) Anti-epidermal growth factor receptor antibody C225 inhibits angiogenesis in human transitional cell carcinoma growing orthotopically in nude mice. Clin Cancer Res 5(2):257–265

Petit AM, Rak J, Hung MC, Rockwell P, Goldstein N, Fendly B, Kerbel RS (1997) Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells in vitro and in vivo: angiogenic implications for signal transduction therapy of solid tumors. Am J Pathol 151(6):1523–1530

Pollard JW (2004) Tumour-educated macrophages promote tumour progression and metastasis. Nat Rev Cancer 4(1):71–78

Puxeddu I, Alian A, Piliponsky AM, Ribatti D, Panet A, Levi-Schaffer F (2005) Human peripheral blood eosinophils induce angiogenesis. Int J Biochem Cell Biol 37(3):628–636

Rafii S, Lyden D, Benezra R, Hattori K, Heissig B (2002) Vascular and haematopoietic stem cells: novel targets for anti-angiogenesis therapy? Nat Rev Cancer 2(11):826–835

Rak J, Mitsuhashi Y, Bayko L, Filmus J, Shirasawa S, Sasazuki T, Kerbel RS (1995) Mutant ras oncogenes upregulate VEGF/VPF expression: implications for induction and inhibition of tumor angiogenesis. Cancer Res 55(20):4575–4580

Rastinejad F, Polverini PJ, Bouck NP (1989) Regulation of the activity of a new inhibitor of angiogenesis by a cancer suppressor gene. Cell 56(3):345–355

Ravi R, Mookerjee B, Bhujwalla ZM, Sutter CH, Artemov D, Zeng Q, Dillehay LE, Madan A, Semenza GL, Bedi A (2000) Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. Genes Dev 14(1):34–44

Reyes M, Dudek A, Jahagirdar B, Koodie L, Marker PH, Verfaillie CM (2002) Origin of endothelial progenitors in human postnatal bone marrow. J Clin Invest 109(3):337–346

Ribatti D, Crivellato E, Roccaro AM, Ria R, Vacca A (2004) Mast cell contribution to angiogenesis related to tumour progression. Clin Exp Allergy 34(11):1660–1664

Ribatti D, Vacca A, Dammacco F (2003) New non-angiogenesis dependent pathways for tumour growth. Eur J Cancer 39(13):1835–1841

Ruhrberg C, Gerhardt H, Golding M, Watson R, Ioannidou S, Fujisawa H, Betsholtz C, Shima DT (2002) Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. Genes Dev 16(20):2684–2698

Saez E, Rutberg SE, Mueller E, Oppenheim H, Smoluk J, Yuspa SH, Spiegelman BM (1995) c-fos is required for malignant progression of skin tumors. Cell 82(5):721–732

Sankar S, Mahooti-Brooks N, Bensen L, McCarthy TL, Centrella M, Madri JA (1996) Modulation of transforming growth factor beta receptor levels on microvascular endothelial cells during in vitro angiogenesis. J Clin Invest 97(6):1436–1446

Schafer T, Zentgraf H, Zehe C, Brugger B, Bernhagen J, Nickel W (2004) Unconventional secretion of fibroblast growth factor 2 is mediated by direct translocation across the plasma membrane of mammalian cells. J Biol Chem 279(8):6244–6251

Scholz D, Cai WJ, Schaper W (2001) Arteriogenesis, a new concept of vascular adaptation in occlusive disease. Angiogenesis 4(4):247–257

Seandel M, Noack-Kunnmann K, Zhu D, Aimes RT, Quigley JP (2001) Growth factor-induced angiogenesis in vivo requires specific cleavage of fibrillar type I collagen. Blood 97(8):2323–2332

Semenza GL (2003) Targeting HIF-1 for cancer therapy. Nat Rev Cancer 3(10):721–732

Sheibani N, Frazier WA (1996) Repression of thrombospondin-1 expression, a natural inhibitor of angiogenesis, in polyoma middle T transformed NIH3T3 cells. Cancer Lett 107(1):45–52

Shi Q, Le X, Wang B, Abbruzzese JL, Xiong Q, He Y, Xie K (2001) Regulation of vascular endothelial growth factor expression by acidosis in human cancer cells. Oncogene 20(28):3751–3756

Shono T, Ono M, Izumi H, Jimi SI, Matsushima K, Okamoto T, Kohno K, Kuwano M (1996) Involvement of the transcription factor NF-kappaB in tubular morphogenesis of human microvascular endothelial cells by oxidative stress. Mol Cell Biol 16(8):4231–4239

Shweiki D, Neeman M, Itin A, Keshet E (1995) Induction of vascular endothelial growth factor expression by hypoxia and by glucose deficiency in multicell spheroids: implications for tumor angiogenesis. Proc Natl Acad Sci USA 92(3):768–772

Slack JL, Bornstein P (1994) Transformation by v-src causes transient induction followed by repression of mouse thrombospondin-1. Cell Growth Differ 5(12):1373–1380

Smith-McCune KK, Zhu YH, Hanahan D, Arbeit J (1997) Cross-species comparison of angiogenesis during the premalignant stages of squamous carcinogenesis in the human cervix and K14-HPV16 transgenic mice. Cancer Res 57(7):1294–1300

Smith-McCune KK, Weidner N (1994) Demonstration and characterization of the angiogenic properties of cervical dysplasia. Cancer Res 54(3):800–804

Smith-McCune KK, Zhu Y, Darragh T (1998) Angiogenesis in histologically benign squamous mucosa is a sensitive marker for nearby cervical intraepithelial neoplasia. Angiogenesis 2(2):135–142

Solovey A, Lin Y, Browne P, Choong S, Wayner E, Hebbel RP (1997) Circulating activated endothelial cells in sickle cell anemia. N Engl J Med 337(22):1584–1590

Stein I, Neeman M, Shweiki D, Itin A, Keshet E (1995) Stabilization of vascular endothelial growth factor mRNA by hypoxia and hypoglycemia and coregulation with other ischemia-induced genes. Mol Cell Biol 15(10):5363–5368

Stoppler H, Malerczyk C, Block K, Aigner A, Czubayko F (2001) The human papillomavirus (HPV) 16 E6 oncoprotein leads to an increase in gene expression of the angiogenic switch molecule FGF-BP in non-immortalized human keratinocytes. Oncogene 20(50):7430–7436

Subbaramaiah K, Altorki N, Chung WJ, Mestre JR, Sampat A, Dannenberg AJ (1999) Inhibition of cyclooxygenase-2 gene expression by p53. J Biol Chem 274(16):10911–10915

Sun Y, Sun Y, Wenger L, Rutter JL, Brinckerhoff CE, Cheung HS (1999) Human metalloproteinase-1 (collagenase-1) is a tumor suppressor protein p53 target gene. Ann N Y Acad Sci 878:638–641

Tait CR, Jones PF (2004) Angiopoietins in tumours: the angiogenic switch. J Pathol 204(1):1–10

Teitelman G, Alpert S, Hanahan D (1988) Proliferation, senescence, and neoplastic progression of beta cells in hyperplasic pancreatic islets. Cell 52(1):97–105

Thurston G, Rudge JS, Ioffe E, Zhou H, Ross L, Croll SD, Glazer N, Holash J, McDonald DM, Yancopoulos GD (2000) Angiopoietin-1 protects the adult vasculature against plasma leakage. Nat Med 6(4):460–463

Vajkoczy P, Farhadi M, Gaumann A, Heidenreich R, Erber R, Wunder A, Tonn JC, Menger MD, Breier G (2002) Microtumor growth initiates angiogenic sprouting with simultaneous expression of VEGF, VEGF receptor-2, and angiopoietin-2. J Clin Invest 109(6):777–785

Verheul HM, Voest EE, Schlingemann RO (2004) Are tumours angiogenesis-dependent? J Pathol 202(1):5–13

Vidal A, Zacharoulis S, Guo W, Shaffer D, Giancotti F, Bramley AH, de la Hoz C, Jensen KK, Kato D, MacDonald DD, Knowles J, Yeh N, Frohman LA, Rafii S, Lyden D, Koff A (2005) p130Rb2 and p27kip1 cooperate to control mobilization of angiogenic progenitors from the bone marrow. Proc Natl Acad Sci USA 102(19):6890–6895

Volpert OV, Pili R, Sikder HA, Nelius T, Zaichuk T, Morris C, Shiflett CB, Devlin MK, Conant K, Alani RM (2002) Id1 regulates angiogenesis through transcriptional repression of thrombospondin-1. Cancer Cell 2(6):473–483

Volpert OV, Stellmach V, Bouck N (1995) The modulation of thrombospondin and other naturally occurring inhibitors of angiogenesis during tumor progression. Breast Cancer Res Treat 36(2):119–126

Wahl ML, Kenan DJ, Gonzalez-Gronow M, Pizzo SV (2005) Angiostatin's molecular mechanism: aspects of specificity and regulation elucidated. J Cell Biochem 96(2):242–261

Watnick RS, Cheng YN, Rangarajan A, Ince TA, Weinberg RA (2003) Ras modulates Myc activity to repress thrombospondin-1 expression and increase tumor angiogenesis. Cancer Cell 3(3):219–231

Weidner N, Folkman J, Pozza F, Bevilacqua P, Allred EN, Moore DH, Meli S, Gasparini G (1992) Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. J Natl Cancer Inst 84(24):1875–1887

Yu JL, Rak JW (2003) Host microenvironment in breast cancer development: inflammatory and immune cells in tumour angiogenesis and arteriogenesis. Breast Cancer Res 5(2):83–88

Zabrenetzky V, Harris CC, Steeg PS, Roberts DD (1994) Expression of the extracellular matrix molecule thrombospondin inversely correlates with malignant progression in melanoma, lung and breast carcinoma cell lines. Int J Cancer 59(2):191–195

Zhou Z, Apte SS, Soininen R, Cao R, Baaklini GY, Rauser RW, Wang J, Cao Y, Tryggvason K (2000) Impaired endochondral ossification and angiogenesis in mice deficient in membrane-type matrix metalloproteinase I. Proc Natl Acad Sci USA 97(8):4052–4057

Pathophysiology and Clinical Implications of Vascular Endothelial Growth Factor

5

NAPOLEONE FERRARA

Abstract

Inhibiting angiogenesis is a promising strategy to treat cancer and several other disorders, including intraocular neovascular syndromes. It is now well established that vascular endothelial growth factor (VEGF)-A is a major regulator of normal and pathological angiogenesis. VEGF-A is part of a gene family that also includes PlGF, VEGF-B, VEGF-C and VEGF-D. Targeting VEGF-A resulted in a therapeutic benefit in a variety of models of cancer and intraocular neovascular syndromes. VEGF inhibitors have been recently approved by the U.S. Food and Drug Administration for the treatment of cancer and the neovascular form of age-related macular degeneration. This chapter summarizes the basic biology of VEGF-A and illustrates the clinical progress in targeting this molecule.

CONTENTS

Introduction

The observation that tumor growth can be accompanied by increased vascularity was reported more than a century ago (for review, see Ferrara 2002). In 1971, Folkman proposed that anti-angiogenesis may be a valid strategy to treat human cancer and a search for regulators of angiogenesis that may also represent therapeutic targets began (Folkman 1971).

Neovascularization is essential also for physiological processes such as embryogenesis, tissue repair and reproductive functions (Folkman 1995). The development of the vascular tree initially occurs by "vasculogenesis", the in situ differentiation of endothelial cell precursors, the angioblasts, from the hemangioblasts (Risau and Flamme 1995). The juvenile vascular system then evolves from the primary capillary plexus by subsequent pruning and reorganization of endothelial cells in a process called "angiogenesis" (Risau 1997). Several studies suggest that incorporation of bone marrow-derived endothelial progenitor cells in the growing vessels complements the sprouting of resident endothelial cells (Asahara et al. 1997; Rafii et al. 2002; de Palma et al. 2003; Lyden et al. 2001; Ruzinova et al. 2003). Additionally, a subset of perivascular monocytes seems to be particularly important for new vessel growth (de Palma et al. 2005).

Numerous potential angiogenic factors have been described over the past two decades (Klagsbrun and d'Amore 1991; Yancopoulos et al. 2000). Much evidence indicates that vascular endothelial growth factor (VEGF) is a particularly important regulator of angiogenesis (Ferrara 2002). While new vessel growth and maturation are highly complex and coordinated processes, requiring the sequential activation of a series of receptors (e.g. Tie1, Tie2 and PDGFR-β) by numerous ligands in endothelial and mural cells (for recent reviews see Carmeliet 2003; Jain 2003), VEGF signaling often represents a rate-limiting step in angiogenesis. VEGF, referred to also as VEGF-A, is the prototype member of a gene family that includes placenta growth factor (PlGF) (Maglione et al. 1991, 1993), VEGF-B (Olofsson et al. 1996), VEGF-C (Joukov et al. 1996; Lee et al. 1996), and VEGF-D (Orlandini et al. 1996; Achen et al. 1998). The term VEGF will be used throughout this manuscript to refer to VEGF-A. Also, homologs of VEGF have been identified in the genome of the parapoxvirus Orf virus (Lyttle et al. 1994) and shown to have VEGF-like activities (Ogawa et al. 1998; Wise et al. 1999). VEGF-C and VEGF-D regulate lymphangiogenesis (Karkkainen et al. 2002; Stacker et al. 2002).

Identification of VEGF

Independent lines of research contributed to the discovery of VEGF, emphasizing the biological complexity of this molecule (Ferrara 2002).

In 1983, Senger et al. described the partial purification from the conditioned medium of a guinea-pig tumor cell line of a protein able to induce vascular leakage in the skin, which was named "tumor vascular permeability factor" (VPF) (Senger et al. 1983). The authors proposed that VPF could be a mediator of the high permeability of tumor blood vessels. However, these efforts did not yield the full purification of the VPF protein. The lack of amino acid sequence information precluded cDNA cloning and elucidation of the identity of VPF. Therefore, very limited progress in understanding the role of VPF took place over the following several years.

In 1989, Ferrara and Henzel reported the isolation of a diffusible endothelial mitogen from medium conditioned by bovine pituitary follicular cells, which they named "vascular endothelial growth factor" (VEGF) (Ferrara and Henzel 1989). NH2-terminal amino acid sequencing of purified VEGF proved that this protein was distinct from the known endothelial cell mitogens and indeed did not match any known protein in available databases (Ferrara and Henzel 1989). Subsequently, Connolly et al. independently reported the isolation and sequencing of VPF (Connolly et al. 1998). cDNA cloning of VEGF (Leung et al. 1989) and VPF (Keck et al. 1989) reported also in 1989 revealed that VEGF and VPF were surprisingly the same molecule.

Biological Activities of VEGF-A

VEGF-A promotes the growth the of vascular endothelial cells derived from arteries, veins and lymphatics (for review Ferrara and Davis-Smyth 1997). VEGF induces angiogenesis in tridimensional in

vitro models, inducing confluent microvascular endothelial cells to invade collagen gels and form capillary-like structures (Pepper et al. 1992, 1994). VEGF-A also induces angiogenesis in a variety of in vivo model systems (Ferrara 2004).

VEGF-A is a survival factor for endothelial cells (Alon et al. 1995; Gerber et al. 1998a,b; Benjamin et al. 1999; Yuan et al. 1996). In vitro, VEGF prevents endothelial apoptosis induced by serum starvation. Such activity is mediated by the PI 3′ kinase/Akt pathway (Gerber et al. 1998b; Fujio and Walsh 1999). Also, VEGF induces expression of the anti-apoptotic proteins Bcl-2, A1 (Gerber et al. 1998a), and survivin (Tran et al. 2002) in endothelial cells In vivo, VEGF's pro-survival effects are developmentally regulated. VEGF inhibition results in extensive apoptotic changes in the vasculature of neonatal, but not adult mice (Gerber et al. 1999). Furthermore, VEGF dependence has been demonstrated in endothelial cells of newly formed but not of established vessels within tumors (Benjamin et al. 1999; Yuan et al. 1996). Coverage by pericytes is one of the key events resulting in loss of VEGF-A dependence (Benjamin et al. 1999).

Endothelial cells are the primary targets of VEGF-A, but several studies have reported mitogenic and non-mitogenic effects of VEGF-A also on certain non-endothelial cell types, including retinal pigment epithelial cells (Guerrin et al. 1995), pancreatic duct cells (Oberg-Welsh et al. 1997) and Schwann cells (Sondell et al. 1999).

The earliest evidence that VEGF-A can affect myeloid cells came from a report describing its ability to promote monocyte chemotaxis (Clauss et al. 1990). Subsequently, VEGF-A was reported to have hematopoietic effects, inducing colony formation by mature subsets of granulocyte–macrophage progenitor cells (Broxmeyer et al. 1995). Also, VEGF increased production of B cells and the generation of immature myeloid cells (Khattori et al. 2001). Conditional gene-knockout technology has been employed to achieve selective VEGF gene ablation in bone marrow cell isolates and hematopoietic stem cells (HSCs) (Gerber et al. 2002). VEGF deficient HSCs and bone marrow mononuclear cells failed to repopulate lethally irradiated hosts, despite co-administration of a large excess of wild-type cells (Gerber et al. 2002).

VEGF Isoforms

The human VEGF-A gene is organized in eight exons, separated by seven introns (Houck et al. 1991; Tischer et al. 1991) and is localized in chromosome 6p21.3 (Vincenti et al. 1996). Alternative exon splicing results in the generation of four different isoforms, having respectively 121, 165, 189 and 206 amino acids following signal sequence cleavage ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$) (Houck et al. 1991; Tischer et al. 1991). $VEGF_{165}$, the predominant isoform, lacks the residues encoded by exon 6, while $VEGF_{121}$ lacks the residues encoded by exons 6 and 7. Less frequent splice variants have been also reported, including $VEGF_{145}$ (Poltorak et al. 1997), $VEGF_{183}$ (Jingjing et al. 1999), VEGF162 (Lange et al. 2003) and $VEGF_{165b}$, a variant reported to have an inhibitory effect on VEGF-induced mitogenesis (Bates et al. 2002).

Native VEGF is a heparin-binding homodimeric glycoprotein of 45 kDa (Ferrara and Henzel 1989). Such properties closely correspond to those of $VEGF_{165}$, which is now recognized as being the major VEGF isoform (Houck et al. 1992).

$VEGF_{121}$ is an acidic polypeptide that fails to bind to heparin (Houck et al. 1992). $VEGF_{189}$ and $VEGF_{206}$ are highly basic and bind to heparin with high affinity (Houck et al. 1992). $VEGF_{121}$ is a freely diffusible protein. In contrast, $VEGF_{189}$ and $VEGF_{206}$ are almost completely sequestered in the extracellular matrix (ECM). $VEGF_{165}$ has intermediate properties, since it is secreted but a significant fraction remains bound to the cell surface and ECM (Park et al. 1993). The ECM-bound isoforms may be released in a diffusible form by heparin or heparinase, which displaces them from their binding to heparin-like moieties, or by plasmin cleavage at the COOH terminus which generates a bioactive fragment consisting of the first 110 NH_2-terminal amino acids (Houck et al. 1992). Given the important role of plasminogen activation during physiological and pathological angiogenesis processes (Pepper 2001), this proteolytic mechanism can be particularly important in regulating locally the activity and bioavailability of

VEGF. More recent studies have shown that matrix metalloproteinase (MMP)-3 can also cleave $VEGF_{165}$ to generate non-heparin-binding, bioactive proteolytic fragments (Lee et al. 2005).

Regulation of VEGF Gene Expression

5.5.1 Oxygen Tension

Oxygen tension plays a key role in regulating the expression of a variety of genes including VEGF (Safran and Kaelin 2003). HIF-1 is a key mediator of hypoxic responses. HIF-1 is a basic, heterodimeric, helix–loop–helix protein consisting of two subunits, HIF-1α and aryl hydrocarbon receptor nuclear translocator (ARNT), known also as HIF-1β (Wang and Semenza 1995). In response to hypoxia, HIF-1 binds to specific enhancer elements, resulting in increased gene transcription. Recent studies have demonstrated the critical role of the product of the von Hippel–Lindau (VHL) tumor suppressor gene in HIF-1-dependent hypoxic responses (Mole et al. 2001). The VHL gene is inactivated in patients with von Hippel–Lindau disease and in most sporadic clear cell renal carcinomas (Lonser et al. 2003). Earlier studies indicated that a function of the VHL protein is to provide negative regulation of VEGF and other hypoxia-inducible genes (Iliopoulos et al. 1996). Recent studies demonstrated that one of the functions of VHL is to be part of a ubiquitin ligase complex that targets HIF subunits for proteasomal degradation following covalent attachment of a polyubiquitin chain (Jaakkola et al. 2001; Ivan et al. 2001). Oxygen promotes the hydroxylation of HIF at a proline residue, a requirement for the association with VHL (Jaakkola et al. 2001; Ivan et al. 2001). Recently, a family of prolyl hydroxylases related to Egl-9 gene product of *Caenorhabditis elegans* were identified as HIF prolyl hydroxylases (Safran and Kaelin 2003; Maxwell and Ratcliffe 2002; Pugh and Ratcliffe 2003).

5.5.2 Growth Factors, Hormones and Oncogenes

Several growth factors, including EGF, TGF-α, TGF-β, KGF, IGF-1, FGF and PDGF, upregulate VEGF mRNA expression (Frank et al. 1995; Pertovaara et al. 1994; Warren et al. 1996), suggesting that paracrine or autocrine release of such factors cooperates with local hypoxia in regulating VEGF release in the microenvironment. Also, inflammatory cytokines such as interleukin (IL)-1α and IL-6 induce expression of VEGF in several cell types, including synovial fibroblasts (Ben-Av et al. 1995; Cohen et al. 1996).

Hormones are also important regulators of VEGF gene expression. Thyroid-stimulating hormone has been shown to induce VEGF expression in several thyroid carcinoma cell lines (Soh et al. 1996). Shifren et al. have also shown that adrenocorticotropic hormone (ACTH) is able to induce VEGF expression in cultured human fetal adrenal cortical cells, suggesting that VEGF may be a local regulator of adrenal cortical angiogenesis and a mediator of the tropic action of ACTH (Shifren et al. 1998). Gonadotropins have been shown to be inducers of VEGF transcription in the ovary, both in vivo (Shweiki et al. 1993; Ferrara et al. 1998) and in vitro (Christenson and Stouffer 1997).

A variety of transforming events also result in induction of VEGF gene expression. Oncogenic mutations or amplification of ras lead to VEGF upregulation (Grugel et al. 1995; Okada et al. 1998). Mutations in the wnt-signaling pathway, which are frequently associated with pre-malignant colonic adenomas, result in upregulation of VEGF (Zhang et al. 2001). Interestingly, VEGF is upregulated in polyps of Apc knockout [Apc(Delta716)] mice, a model for human familial adenomatous polyposis (Seno et al. 2002). In both benign and malignant mouse intestinal tumors, stromal expression of COX-2 results in elevated PGE2 levels, which stimulate in turn cell surface receptor EP2, followed by induction of VEGF and angiogenesis (Seno et al. 2002; Williams et al. 2000; Sonoshita et al. 2001).

VEGF Receptors

VEGF binding sites were originally identified on the cell surface of vascular endothelial cells, in vitro (Plouet and Moukadiri 1990; Vaisman et al. 1990) and in vivo (Jakeman et al. 1992, 1993). Subsequently, VEGF receptors were shown to exist also in bone marrow-derived cells such as monocytes and macrophages (Shen et al. 1993). VEGF binds two highly related receptor tyrosine kinases (RTKs), VEGFR-1 and VEGFR-2. Both VEGFR-1 and VEGFR-2 have seven immunoglobulin (Ig)-like domains in the extracellular domain, a single transmembrane region, and a consensus tyrosine kinase sequence which is interrupted by a kinase-insert domain (Shibuya et al. 1990; Terman et al. 1991).

A member of the same family of RTKs is VEGFR-3 (Flt-4) (Pajusola et al. 1993), which, however, is not a receptor for VEGF-A, but binds the lymphangiogenic factors VEGF-C and VEGF-D (Karkkainen et al. 2002). In addition, as described below, VEGF interacts with a family of coreceptors, the neuropilins.

5.6.1 VEGFR-1(Flt-1)

Although Flt-1 (fms-like tyrosine kinase) was the first RTK to be identified as a VEGF receptor over a decade ago (de Vries et al. 1992), the precise function of this molecule is still subject to debate. Recent evidence indicates that the conflicting reports may be due, at least in part, to the fact that VEGFR-1 functions and signaling properties can differ depending on the developmental stage and the cell type, e.g. endothelial versus non-endothelial cells. VEGFR-1 expression is upregulated by hypoxia via a HIF-1-dependent mechanism (Gerber et al. 1997). VEGFR-1 binds not only VEGF-A but also PlGF (Park et al. 1994) and VEGF-B (Olofsson et al. 1998), which fail to bind VEGFR-2. An alternatively spliced soluble form of VEGFR-1 (sFlt-1) has been shown to be an inhibitor of VEGF activity (Kendall and Thomas 1993). The binding site for VEGF (and PlGF) has been mapped primarily to the second Ig-like domain (Davis-Smyth et al. 1996, 1998; Barlon et al. 1997). Flt-1 reveals a weak tyrosine autophosphorylation in response to VEGF (de Vries et al. 1992; Waltenberger et al. 1994). Park et al. initially proposed that VEGFR-1 may be not primarily a receptor transmitting a mitogenic signal, but rather a "decoy" receptor, able to regulate in a negative fashion the activity of VEGF on the vascular endothelium, by sequestering this factor and rendering it less available to VEGFR-2 (Park et al. 1994). Thus, the observed potentiation of the action of VEGF by PlGF could be explained, at least in part, by displacement of VEGF from VEGFR-1 binding (Park et al. 1994). Recent studies have shown that indeed synergy exists between VEGF and PlGF in vivo, especially during pathological situations, as evidenced by impaired tumorigenesis and vascular leakage in $Plgf^{-/-}$ mice (Carmeliet et al. 2001). Gille et al. have identified a repressor motif in the juxtamembrane region of VEGFR-1 that impairs PI-3 kinase activation and endothelial cell migration in response to VEGF (Gille et al. 2000). Regardless of the conflicting evidences on the role of VEGFR-1 as a signaling receptor, gene targeting studies have demonstrated the essential role of this molecule during embryogenesis. $VEGFR\text{-}1^{-/-}$ mice die in utero between day 8.5 and day 9.5 (Fong et al. 1995, 1999). Endothelial cells develop but fail to organize in vascular channels. Excessive proliferation of angioblasts has been reported to be responsible for such disorganization and lethality (Fong et al. 1999), indicating that, at least during early development, VEGFR-1 is a negative regulator of VEGF action. It appears that VEGFR-1 has a dual function in angiogenesis, acting in a positive or negative manner in different circumstances. Recently, VEGFR-1 signaling has been also linked to the induction of MMP-9 in lung endothelial cells and to the facilitation of lung metastases (Hiratsuka et al. 2002).

Several studies have emphasized the effects of VEGFR-1 in hematopoiesis and recruitment of bone marrow-derive angiogenic cells. VEGFR-1 activation by PlGF reconstitutes hematopoiesis by re-

cruiting VEGFR-1$^+$ HSC (Hattori et al. 2002). In addition, VEGFR-1 activation by enforced expression of PlGF rescues survival and ability to repopulate in VEGF$^{-/-}$ HSC (Gerber et al. 2002). LeCouter et al. recently provided evidence for a novel function of VEGFR-1 in liver sinusoidal endothelial cells (LSECs). VEGFR-1 activation achieved with a receptor-selective VEGF mutant or PlGF resulted in the paracrine release of HGF, IL-6 and other hepatotrophic molecules by LSECs, to the extent that hepatocytes were stimulated to proliferate when co-cultured with LSECs (LeCouter et al. 2003).

5.6.2
VEGFR-2 (KDR, Human; Flk-1, Mouse)

VEGFR-2 binds VEGF-A (kd 75–250 pM vs 25 pM) (Terman et al. 1992; Quinn et al. 1993; Millauer et al. 1993). The key role of this receptor in developmental angiogenesis and hematopoiesis is evidenced by lack of vasculogenesis and failure to develop blood islands and organized blood vessels in Flk-1 null mice, resulting in death in utero between day 8.5 and day 9.5 (Shalaby et al. 1995). There is now agreement that VEGFR-2 is the major mediator of the mitogenic, angiogenic and permeability-enhancing effects of VEGF.

VEGFR-2 undergoes dimerization and strong ligand-dependent tyrosine phosphorylation in intact cells, resulting in a mitogenic, chemotactic and pro-survival signal. Several tyrosine residues have been shown to be phosphorylated (for review see Matsumoto and Claesson-Welsh 2001). Takahashi et al. have shown that Y1175 and Y1214 are the two major VEGF-A-dependent autophosphorylation sites in VEGFR-2. However, only autophosphorylation of Y1175 is crucial for VEGF-dependent endothelial cell proliferation (Takahashi et al. 2001). VEGFR-2 activation induces endothelial cell growth by activating the Raf–Mek–Erk pathway. An unusual feature of VEGFR-2 activation of this pathway is the requirement for protein kinase C but not ras (Takahashi et al. 1999; Wu et al. 2000). VEGF mutants that bind selectively to VEGFR-2 are fully active endothelial cell mitogens, chemoattractants and permeability-enhancing agents, whereas mutants specific for VEGFR-1 are devoid of all three activities (Gille et al. 2001). Also, VEGF-E, a homologue of VEGF identified in the genome of the parapoxvirus Orf virus (Lyttle et al. 1994), which shows VEGF-like mitogenic and permeability-enhancing effects, binds and activates VEGFR-2 but fails to bind VEGFR-1 (Ogawa et al. 1998; Wise et al. 1999).

5.6.3
Neuropilin 1 and Neuropilin 2

Certain tumor and endothelial cells express cell-surface VEGF binding sites distinct from the two known VEGF RTKs (Soker et al. 1996). $VEGF_{121}$ fails to bind these sites, indicating that exon-7-encoded basic sequences are required for binding to this putative receptor (Soker et al. 1996). Soker et al. (1998) identified such isoform-specific VEGF receptor as neuropilin 1 (NRP1), a molecule that had been previously shown to bind the collapsin/semaphorin family and was implicated in neuronal guidance (for review see Neufeld et al. 2002). When co-expressed in cells with VEGFR-2, NRP1 enhanced the binding of $VEGF_{165}$ to VEGFR-2 and $VEGF_{165}$-mediated chemotaxis (Soker et al. 1998). NRP1 appears to present $VEGF_{165}$ to the VEGFR-2 in a manner that enhances the effectiveness of VEGFR-2-mediated signal transduction (Soker et al. 1998). Binding to NRP1 may contribute to explain the greater mitogenic potency of $VEGF_{165}$ relative to $VEGF_{121}$. So far there is no clear evidence that NRP1 or the related NRP2 signals subsequent VEGF binding (Neufeld et al. 2002). In contrast, in response to semaphorin binding, NRP1 and NRP2 signals axon repulsion. The formation of complexes with plexins is a requirement for NRP signaling in neurons (Tamagnone et al. 1999; Bagri and Tessier-Lavigne 2002). The role of NRP1 in the development of the vascular system has been demonstrated by gene targeting studies, showing embryonic lethality in null mice (Kawasaki et al. 1999). Also, other studies have linked NRP2 to lymphatic vessel development (Yuan et al. 2002). More recent studies show that

NRP1 and NRP2 are expressed on the cell surface of several tumor cell lines which bind $VEGF_{165}$ and display a chemotactic response to this ligand, suggesting a pro-tumor activity of NRPs, with or without the involvement of VEGF RTK signaling (Klagsbrun and Eichmann 2005).

Role of VEGF in Physiological Angiogenesis

Two studies have demonstrated an essential role of VEGF-A in embryonic vasculogenesis and angiogenesis in the mouse (Carmeliet et al. 1996; Ferrara et al. 1996). Inactivation of a single VEGF allele resulted in embryonic lethality between day 11 and 12. The $vegf^{+/-}$ embryos exhibited a number of developmental anomalies, defective vascularization in several organs and a reduced number of nucleated red blood cells within the blood islands in the yolk sac, indicating that VEGF regulates both vasculogenesis and early hematopoiesis. These findings indicate a critical VEGF-A gene-dosage dependence during development. Among the other members of the VEGF gene family, only VEGF-C plays an essential role in development; its inactivation results in embryonic lethality following defective lymphatic development and fluid accumulation in tissues (Karkkainen et al. 2003).

VEGF-A plays an important role in early postnatal life. Administration of a soluble VEGFR-1 chimeric protein (Gerber et al. 1999) results in growth arrest when the treatment is initiated at day 1 or day 8 postnatally. Such treatment is also accompanied by lethality, primarily due to inhibition of glomerular development and kidney failure (Gerber et al. 1999). The key role of VEGF in kidney development was also demonstrated by a very recent study showing that selective VEGF deletion in podocytes, using a Nephin promoter-driven Cre recombinase, leads to glomerular disease in a gene dosage-dependent fashion (Eremina et al. 2003). However, VEGF neutralization in fully developed normal mice (Gerber et al. 1999) or rats (Ostendorf et al. 1999) had no marked effects on glomerular function. In contrast, VEGF inhibition in adult rats with mesangioproliferative nephritis led to a reduction of glomerular endothelial regeneration and an increase in endothelial cell death, indicating that VEGF may be important for glomerular endothelial cell repair following injury, but not for endothelial survival in a healthy animal (Ostendorf et al. 1999).

Endochondral bone formation is a fundamental mechanism for longitudinal bone growth. Cartilage, an avascular tissue, is replaced by bone in a process named endochondral ossification (Poole 1991). VEGF-A mRNA is expressed by hypertrophic chondrocytes in the epiphyseal growth plate, suggesting that a VEGF gradient is needed for directional growth and cartilage invasion by metaphyseal blood vessels (Gerber et al. 1999; Carlevaro et al. 2000). Following VEGF blockade, blood vessel invasion is almost completely suppressed, concomitant with impaired trabecular bone formation, in developing mice and primates (Gerber et al. 1999; Ryan et al. 1999). Although proliferation, differentiation and maturation of chondrocytes were apparently normal, resorption of hypertrophic chondrocytes was inhibited, resulting in a marked expansion of the hypertrophic chondrocyte zone.

Angiogenesis is a key aspect of normal cyclical ovarian function. Follicular growth and the development of the corpus luteum (CL) are dependent on the proliferation of new capillary vessels (Bassett 1943). After blood vessel growth, the blood vessels regress, suggesting the coordinated action of inducers as well as inhibitors of angiogenesis in the course of the ovarian cycle (Goede et al. 1998; Maisonpierre et al. 1997).

Previous studies have shown that the VEGF-A mRNA expression is temporally and spatially related to the proliferation of blood vessels in the ovary (Phillips et al. 1990; Ravindranath et al. 1992). Administration of VEGF inhibitors delays follicular development (Zimmermann et al. 2001a) and suppresses luteal angiogenesis in rodents (Ferrara et al. 1998; Zimmermann et al. 2001b) as well as in primates (Ryan et al. 1999; Fraser et al. 2000). These studies have established that VEGF is a key regulator of reproductive angiogenesis.

Role of VEGF in Pathologic Conditions

5.8.1 Tumor Angiogenesis

5.8.1.1 Preclinical Studies

A wide variety of tumor cell lines secrete VEGF-A in vitro (Ferrara et al. 1992). In situ hybridization studies have shown that the VEGF mRNA is expressed in the majority of human tumors, including carcinomas of the lungs (Volm et al. 1997), breast (Brown et al. 1995; Yoshiji et al. 1996), gastrointestinal tract (Suzuki et al. 1996; Ellis et al. 1998; Uchida et al. 1998), kidney (Brown et al. 1993; Nicol et al. 1997; Tomisawa et al. 1999), bladder (Brown et al. 1993), ovary (Sowter et al. 1997; Yamamoto et al. 1997) and endometrium (Guidi et al. 1996) and intracranial tumors such as glioblastoma multiforme (Shweiki et al. 1992; Plate et al. 1992; Phillips et al. 1993) and capillary hemangioblastoma (Berkman et al. 1993; Wizigmann Voos et al. 1995).

In 1993, it was reported that a monoclonal antibody targeting VEGF-A inhibited the growth of several tumor cell lines in nude mice, while it had no effect on the proliferation of tumor cells in vitro (Kim et al. 1993). Subsequently, other tumor cell lines were found to be inhibited in vivo by anti-VEGF monoclonal antibodies (Warren et al. 1995; Melnyk et al. 1996; Asano et al. 1995; Borgstrom et al. 1996, 1998, 1999; Mesiano et al. 1998). For a recent review, see Gerber et al. (2005). Tumor growth inhibition was demonstrated also with other anti-VEGF treatments, including a retrovirus-delivered dominant negative VEGFR-2 mutant (Millauer et al. 1994), small-molecule inhibitors of VEGFR-2 signaling (Wood et al. 2000; Wedge et al. 2000), anti-VEGFR-2 antibodies (Prewett et al. 1999) and soluble VEGF receptors (Goldman et al. 1998; Gerber et al. 2000; Kuo et al. 2001; Holash et al. 2002).

While tumor cells usually represent the major source of VEGF, tumor-associated stroma is also an important site of VEGF production (Gerber et al. 2000; Tsuzuki et al. 2000; Kishimoto et al. 2000). Recent studies have shown that tumor-derived PDGF-A may be important for the recruitment of an angiogenic stroma which produces VEGF-A (Dong et al. 2004; Tejada et al. 2006).

Cre-*LoxP*-mediated gene targeting has been used to show that VEGF inactivation suppresses tumor angiogenesis in the Rip-Tag model, a well-established genetic model of insulinoma (Inoue et al. 2002).

Several studies have shown that combining anti-VEGF treatment with chemotherapy (Klement et al. 2000) or radiation therapy (Lee et al. 2000; (Lee et al. 2000; Kozin et al. 2001) results in a greater antitumor effect than either treatment alone. Various hypotheses have been put forward regarding the mechanism of such potentiation. Klement et al. proposed that chemotherapy, especially when delivered at close regular intervals using relatively low doses, with no prolonged drug-free periods ("metronomic therapy"), preferentially damages endothelial cells in tumor blood vessels and that the simultaneous blockade of VEGF-A blunts a survival signal for endothelial cells, thus amplifying the endothelial cell targeting effects of chemotherapy (Klement et al. 2000). A similar process, in principle, may take place when combining more conventional maximum tolerated dose chemotherapy regimens.

An alternative hypothesis has been proposed by Jain (2005). Anti-angiogenic agents would "normalize" the abnormal vasculature that is characteristic of many vessels in tumors, resulting in pruning of excessive endothelial and perivascular cells, in a drop in the normally high interstitial pressures detected in solid tumors, and temporarily improved oxygenation and delivery of chemotherapy to tumor cells (Jain 2005).

5.8.1.2 Clinical Trials in Cancer Patients with VEGF Inhibitors

Several VEGF inhibitors are in clinical development as anti-cancer agents. These include a humanized anti-VEGF monoclonal antibody (bevacizumab; Avastin™) (Presta et al. 1997), an anti-VEGFR-2 an-

tibody (Prewett et al. 1999), various small molecules inhibiting VEGFR-2 signal transduction (Wood et al. 2000; Wedge et al. 2000) and a VEGF receptor chimeric protein (Holash et al. 2002). For recent reviews, see Gasparini et al. (2005), Ferrara and Kerbel (2005) and Jain et al. (2006).

Based on a survival advantage conferred by the addition of bevacizumab to irinotecan, 5-fluorouracil and leucovorin (IFL) to patients with previously untreated metastatic colorectal cancer (Hurwitz et al. 2004), bevacizumab was approved by the FDA on 26 February 2004 as a first-line treatment for metastatic colorectal cancer in combination with 5-fluorouracil-based chemotherapy regimens (Ellis 2005). The role of bevacizumab in other tumor types and settings is currently under investigation, and phase III clinical trials in non-small cell lung cancer (NSCLC), renal cell cancer and metastatic breast cancer are ongoing. An interim analysis of a phase III study of women with previously untreated metastatic breast cancer treated with bevacizumab in combination with weekly paclitaxel chemotherapy showed that the study met its primary efficacy endpoint of improving progression-free survival compared to paclitaxel alone.

Also, administration of bevacizumab in combination with paclitaxel and carboplatin to patients with NSCLC resulted in increased response rate and time to progression relative to chemotherapy alone in a randomized phase II trial (Johnson et al. 2004). The most significant adverse event was serious hemoptysis. This was primarily associated with centrally located tumors with squamous histology, cavitation and central necrosis and proximity of disease to large vessels (Johnson et al. 2004).

Besides bevacizumab, several other types of VEGF inhibitors are being developed (reviewed in Gasparini et al. 2005 and Ferrara and Kerbel 2005). Among these, a variety of small-molecule RTK inhibitors targeting the VEGF receptors are at different stages of clinical development. The most advanced are SU11248 and Bay 43-9006 (sorafenib). SU11248 inhibits VEGFRs, PDGFR, c-kit and Flt-3 (Smith et al. 2004) and has been reported to have efficacy in imatinib-resistant gastrointestinal stromal tumor. SU11248 has been approved by the FDA for the treatment of Gleevec-resistant gastrointestinal stromal tumor (GIST) and metastatic renal cell carcinoma (Motzer et al. 2006). In 2006, sorafenib was approved by the FDA for the treatment of metastatic renal cell carcinoma.

5.8.2 Intraocular Neovascular Syndromes

The expression of VEGF mRNA is spatially and temporally correlated with neovascularization in several animal models of retinal ischemia (Alon et al. 1995; Miller et al. 1994; Pierce et al. 1996). This is consistent with the fact that VEGF-A gene expression is upregulated by hypoxia, via HIF-dependent transcriptional activation (Safran and Kaelin 2003). In 1994 it was reported that VEGF-A are elevated in the aqueous and vitreous humor of human eyes with proliferative retinopathy secondary to diabetes and other conditions (Aiello et al. 1994; Malecaze et al. 1994). Subsequently, animal studies using various VEGF inhibitors, including soluble VEGF receptor chimeric proteins (Aiello et al. 1995), monoclonal antibodies (Adamis et al. 1996), antisense oligonucleotides (Robinson et al. 1996) and small molecule VEGFR-2 kinase inhibitors (Ozaki et al. 2000), have directly demonstrated the role of VEGF as a mediator of ischemia-induced intraocular neovascularization.

Age-related macular degeneration (AMD) is the most common cause of severe, irreversible vision loss in older adults (Congdon et al. 2004). AMD is classified into nonexudative (dry) and exudative (wet or neovascular) disease. Although the exudative form accounts for approximately 10–20% of cases, it is responsible for 80–90% of the visual loss associated with AMD (Ferris et al. 1984). Neovascular lesions in AMD are classified using fluorescein angiography (Barbazetto et al. 2003). Pharmacologic therapies for neovascular AMD currently available in the US include verteporfin (Visudyne®) photodynamic therapy (Photodynamic Therapy of Subfoveal Choroidal Neovascularization 1999), which is approved only for predominantly classic lesions (50% or more of the lesion consists of classic choroidal neovascular-

ization), and pegaptanib sodium (Macugen®) (Gragoudas et al. 2004), which is approved for all types of neovascular AMD. Although both treatments can slow the progression of vision loss, only a small proportion of the treated patients experience any improvement in visual acuity.

5.8.2.1 Clinical Studies of Anti-VEGF Therapy for Neovascular AMD

Pegaptanib sodium and ranibizumab (Lucentis®) are the first ocular anti-VEGF treatments evaluated in large, randomized, controlled clinical trials for the treatment of neovascular AMD. Both are administered locally by intravitreal injection.

Pegaptanib sodium is a pegylated oligonucleotide aptamer that binds to and inactivates $VEGF_{165}$, and was approved in 2004 for the treatment of all angiographic subtypes of neovascular AMD (211). In a combined analysis of the VISION trials – two identical, large, controlled, double-masked, randomized, multicenter clinical trials involving patients with all CNV lesion types – 70% of subjects treated with the dose of 0.3 mg lost <15 letters at 1 year (the primary endpoint) compared with 55% for the sham injection group ($P<0.001$) (Gragoudas et al. 2004). Patients in the 0.3 mg pegaptinib sodium group on average lost approximately 8 letters at 1 year, compared with a loss of approximately 15 letters in the sham injection group ($P<0.002$).

Ranibizumab is a recombinant, humanized monoclonal antibody fragment (Fab) that binds to and neutralizes the biological activities of all known human VEGF isoforms, as well as the proteolytic cleavage product $VEGF_{110}$ (Houck et al. 1992; Chen et al. 1999). Ranibizumab is currently being evaluated in two large, phase III, multicenter, randomized, double-masked, controlled pivotal trials in different neovascular AMD patient populations (Ferrara et al. 2006).

The MARINA trial randomized 716 patients in the USA with minimally classic or occult without classic subfoveal CNV to one of three treatment arms: monthly sham injections, monthly intravitreal injections of 0.3 mg ranibizumab, or monthly intravitreal injections of 0.5 mg ranibizumab (Rosenfeld et al. 2006). In the primary analysis at 1 year, the study met its primary endpoint, with significantly fewer ranibizumab subjects than sham-injected patients losing <15 letters. On average at 1 year, visual acuity scores for ranibizumab-treated subjects were greater than at baseline while visual acuity scores for sham-injection subjects were worse. A significantly larger proportion of subjects treated with ranibizumab gained 15 letters at 1 year than in the sham-injection group. Key serious ocular adverse events occurring more frequently in ranibizumab-treated subjects included uveitis and endophthalmitis and were uncommon. Regarding key non-ocular serious adverse events, the frequency of myocardial infarctions was similar among the groups. Results through complete 2-year follow-up show that the visual acuity benefits observed at 1 year were maintained through the 2nd year and that the cumulative 2-year safety profile was similar to that observed at 1 year (Rosenfeld et al. 2006).

5.8.2.2 Other Anti-VEGF Therapies in Clinical Development

A number of other molecules targeting VEGF and its signaling pathway are in early stages of clinical development for the treatment of neovascular AMD. VEGF-Trap is a fusion protein combining the extracellular domains of VEGF receptors 1 and 2 with the Fc portion of an immunoglobulin to bind and inactivate VEGF (Holash et al. 2002). Also, bevacizumab is not being developed for the treatment of neovascular AMD but is nevertheless being used off-label for therapy of neovascular AMD (Michels et al. 2005; Rosenfeld et al. 2005).

Perspectives

Research conducted over the past 15 years has clearly established that the VEGF family plays an essential role in the regulation of embryonic and

postnatal physiologic angiogenesis processes, such as normal growth processes (Gerber et al. 1999a,b) and cyclical ovarian function (Ferrara et al. 1998). Furthermore, VEGF inhibition has been shown to suppress pathological angiogenesis in a wide variety of preclinical models, including genetic models of cancer, leading to the clinical development of a variety of VEGF inhibitors. Definitive clinical studies have provided proof that VEGF inhibition, using bevacizumab in combination with chemotherapy, may provide a significant clinical benefit, including increased survival, in patients with previously untreated metastatic colorectal cancer (Hurwitz et al. 2004). Ongoing clinical studies are testing the hypothesis that bevacizumab may have efficacy in other tumor types as well.

It would be of great importance to have reliable markers to monitor the activity of anti-angiogenic drugs. So far, the absence of such biomarkers may have impaired clinical development of various anti-angiogenic drugs. Potential candidates include circulating endothelial cells and their progenitor subset, as well as MRI dynamic measurement of vascular permeability/flow in response to angiogenesis inhibitors, although the long-term predictive value of these approaches remains to be established (Morgan et al. 2003; Willett et al. 2004; Shaked et al. 2005). Finally, therapeutic angiogenesis constitutes a promising approach for the treatment of ischemic disorders such as coronary or limb ischemia. However, in spite of extensive preclinical and clinical studies, there is still no proof of clinical efficacy from any pro-angiogenic treatment (Simons 2005). Therefore, it appears that numerous issues need to be resolved before this field may advance in a significant manner.

References

Achen MG, Jeltsch M, Kukk E, Makinen T, Vitali A, Wilks AF, Alitalo K, Stacker SA (1998) Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). Proc Natl Acad Sci USA 95:548–553

Adamis AP, Shima DT, Tolentino MJ, Gragoudas ES, Ferrara N, Folkman J, D'Amore PA, Miller JW (1996) Inhibition of vascular endothelial growth factor prevents retinal ischemia-associated iris neovascularization in a nonhuman primate. Arch-Ophthalmol 114:66–71

Aiello LP, Avery RL, Arrigg PG, Keyt BA, Jampel HD, Shah ST, Pasquale LR, Thieme H, Iwamoto MA, Park JE, Nguyen H, Aiello LM, Ferrara N, King GL (1994) Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders (see comments). N Engl J Med 331:1480–1487

Aiello LP, Pierce EA, Foley ED, Takagi H, Chen H, Riddle L, Ferrara N, King GL, Smith LE (1995) Suppression of retinal neovascularization in vivo by inhibition of vascular endothelial growth factor (VEGF) using soluble VEGF-receptor chimeric proteins. Proc Natl Acad Sci USA 92:10457–10461 issn: 10027–18424

Alon T, Hemo I, Itin A, Pe'er J, Stone J, Keshet E (1995) Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. Nat Med 1:1024–1028 issn: 1078–8956

Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM (1997) Isolation of putative progenitor endothelial cells for angiogenesis. Science 275:964–967

Asano M, Yukita A, Matsumoto T, Kondo S, Suzuki H (1995) Inhibition of tumor growth and metastasis by an immunoneutralizing monoclonal antibody to human vascular endothelial growth factor/vascular permeability factor121. Cancer Res 55:5296–5301

Bagri A, Tessier-Lavigne M (2002) Neuropilins as Semaphorin receptors: in vivo functions in neuronal cell migration and axon guidance. Adv Exp Med Biol 515:13–31

Barbazetto I, Burdan A, Bressler NM, Bressler SB, Haynes L, Kapetanios AD, Lukas J, Olsen K, Potter M, Reaves A, Rosenfeld P, Schachat AP, Strong HA, Wenkenstern A (2003) Photodynamic therapy of subfoveal choroidal neovascularization with verteporfin: fluorescein angiographic guidelines for evaluation and treatment–TAP and VIP report No. 2. Arch Ophthalmol 121:1253–1268

Barleon B, Totzke F, Herzog C, Blanke S, Kremmer E, Siemeister G, Marme D, Martiny-Baron G (1997) Mapping of the sites for ligand binding and receptor dimerization at the extracellular domain of the vascular endothelial growth factor receptor FLT-1. J Biol Chem 272:10382–10388

Bassett DL (1943) The changes in the vascular pattern of the ovary of the albino rat during the estrous cycle. Am J Anat 73:251–278

Bates DO, Cui TG, Doughty JM, Winkler M, Sugiono M, Shields JD, Peat D, Gillatt D, Harper SJ (2002) VEGF(165)b, an inhibitory splice variant of vascular endothelial growth factor, is down-regulated in renal cell carcinoma. Cancer Res 62:4123–4131

Ben-Av P, Crofford LJ, Wilder RL, Hla T (1995) Induction of

vascular endothelial growth factor expression in synovial fibroblasts. FEBS Lett 372:83–87

Benjamin LE, Golijanin D, Itin A, Pode D, Keshet E (1999) Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal (see comments). J Clin Invest 103:159–165

Berkman RA, Merrill MJ, Reinhold WC, Monacci WT, Saxena A, Clark WC, Robertson JT, Ali IU, Oldfield EH (1993) Expression of the vascular permeability factor/vascular endothelial growth factor gene in central nervous system neoplasms. J Clin Invest 91:153–159

Borgstrom P, Hillan KJ, Sriramarao P, Ferrara N (1996) Complete inhibition of angiogenesis and growth of microtumors by anti-vascular endothelial growth factor neutralizing antibody: novel concepts of angiostatic therapy from intravital videomicroscopy. Cancer Res 56:4032–4039

Borgstrom P, Bourdon MA, Hillan KJ, Sriramarao P, Ferrara N (1998) Neutralizing anti-vascular endothelial growth factor antibody completely inhibits angiogenesis and growth of human prostate carcinoma micro tumors in vivo. Prostate 35:1–10

Borgstrom P, Gold DP, Hillan KJ, Ferrara N (1999) Importance of VEGF for breast cancer angiogenesis in vivo: implications from intravital microscopy of combination treatments with an anti-VEGF neutralizing monoclonal antibody and doxorubicin. Anticancer Res 19:4203–4214

Brown LF, Berse B, Jackman RW, Tognazzi K, Manseau EJ, Dvorak HF, Senger DR (1993) Increased expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in kidney and bladder carcinomas. Am J Pathol 143:1255–1262 issn: 0002–9440

Brown LF, Berse B, Jackman RW, Tognazzi K, Guidi AJ, Dvorak HF, Senger DR, Connolly JL, Schnitt SJ (1995) Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in breast cancer. Hum Pathol 26:86–91 issn: 0046–8177

Broxmeyer HE, Cooper S, Li ZH, Lu L, Song HY, Kwon BS, Warren RE, Donner DB (1995) Myeloid progenitor cell regulatory effects of vascular endothelial cell growth factor. Int J Hematol 62:203–215 issn: 0925–5710

Carlevaro MF, Cermelli S, Cancedda R, Descalzi Cancedda F (2000) Vascular endothelial growth factor (VEGF) in cartilage neovascularization and chondrocyte differentiation: auto-paracrine role during endochondral bone formation. J Cell Sci 113:59–69

Carmeliet P (2003) Angiogenesis in health and disease. Nat Med 9:653–660

Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A (1996) Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 380:435–439 issn: 0028–0836

Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, de Mol M, Wu Y, Bono F, Devy L, Beck H, Scholz D, Acker T, DiPalma T, Dewerchin M, Noel A, Stalmans I, Barra A, Blacher S, Vandendriessche T, Ponten A, Eriksson U, Plate KH, Foidart JM, Schaper W, Charnock-Jones DS, Hicklin DJ, Herbert JM, Collen D, Persico MG (2001) Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat Med, 7:575–583

Chen Y, Wiesmann C, Fuh G, Li B, Christinger HW, McKay P, de Vos AM, Lowman HB (1999) Selection and analysis of an optimized anti-VEGF antibody: crystal structure of an affinity-matured Fab in complex with antigen. J Mol Biol 293:865–881

Christenson LK, Stouffer RL (1997) Follicle-stimulating hormone and luteinizing hormone/chorionic gonadotropin stimulation of vascular endothelial growth factor production by macaque granulosa cells from pre- and periovulatory follicles. J Clin Endocrinol Metab 82:2135–2142

Clauss M, Gerlach M, Gerlach H, Brett J, Wang F, Familletti PC, Pan YC, Olander JV, Connolly DT, Stern D (1990) Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration. J Exp Med 172:1535–1545

Cohen T, Nahari D, Cerem LW, Neufeld G, Levi BZ (1996) Interleukin 6 induces the expression of vascular endothelial growth factor. J Biol Chem 271:736–741 issn: 0021–9258

Congdon N, O'Colmain B, Klaver CC, Klein R, Munoz B, Friedman DS, Kempen J, Taylor HR, Mitchell P (2004) Causes and prevalence of visual impairment among adults in the United States. Arch Ophthalmol 122:477–485

Connolly DT, Olander JV, Heuvelman D, Nelson R, Monsell R, Siegel N, Haymore BL, Leimgruber R, Feder J (1989) Human vascular permeability factor. Isolation from U937 cells. J Biol Chem 264:20017–20024

Davis-Smyth T, Chen H, Park J, Presta LG, Ferrara N (1996) The second immunoglobulin-like domain of the VEGF tyrosine kinase receptor Flt-1 determines ligand binding and may initiate a signal transduction cascade. EMBO J 15:4919–4927

Davis-Smyth T, Presta LG, Ferrara N (1998) Mapping the charged residues in the second immunoglobulin-like domain of the vascular endothelial growth factor/placenta growth factor receptor Flt-1 required for binding and structural stability. J Biol Chem 273:3216–3222

De Palma M, Venneri MA, Roca C, Naldini L (2003) Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells. Nat Med 9:789–795

De Palma M, Venneri MA, Galli R, Sergi LS, Politi LS, Sampaolesi M, Naldini L (2005) Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. Cancer Cell 8:211–226

De Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Wil-

liams LT (1992) The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science 255:989–991

Dong J, Grunstein J, Tejada M, Peale F, Frantz G, Liang WC, Bai W, Yu L, Kowalski J, Liang X, Fuh G, Gerber HP, Ferrara N (2004) VEGF-null cells require PDGFR alpha signaling-mediated stromal fibroblast recruitment for tumorigenesis. EMBO J 23:2800–2810

Ellis LM (2005) Bevacizumab. Nat Rev Drug Discov [Suppl] S8–S9

Ellis LM, Takahashi Y, Fenoglio CJ, Cleary KR, Bucana CD, Evans DB (1998) Vessel counts and vascular endothelial growth factor expression in pancreatic adenocarcinoma. Eur J Cancer 34:337–340

Eremina V, Sood M, Haigh J, Nagy A, Lajoie G, Ferrara N, Gerber HP, Kikkawa Y, Miner JH, Quaggin SE (2003) Glomerular-specific alterations of VEGF-A expression lead to distinct congenital and acquired renal diseases. J Clin Invest 111:707–716

Ferrara N (2002) VEGF and the quest for tumour angiogenesis factors. Nat Rev Cancer 2:795–803

Ferrara N (2004) Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev 25:581–611

Ferrara N, Davis-Smyth T (1997) The biology of vascular endothelial growth factor. Endocr Rev 18:4–25

Ferrara N, Henzel WJ (1989) Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun 161:851–858

Ferrara N, Kerbel RS (2005) Angiogenesis as a therapeutic target. Nature 438:967–974

Ferrara N, Houck K, Jakeman L, Leung DW (1992) Molecular and biological properties of the vascular endothelial growth facmily of proteins. Endocr Rev 18:32

Ferrara N, Carver Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell Braxton L, Hillan KJ, Moore MW (1996) Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 380:439–442 issn: 0028–0836

Ferrara N, Chen H, Davis-Smyth T, Gerber H-P, Nguyen T-N, Peers D, Chisholm V, Hillan KJ, Schwall RH (1998) Vascular endothelial growth factor is essential for corpus luteum angiogenesis. Nat Med 4:336–340

Ferrara N, Damico LA, Shams N, Lowman H, Kim R (2006) Developmemt of Ranibizumab, an anti-VEGF Fab for the therapy of neovascular age-related macular degeneration. Retina 26:859–870

Ferris FL 3rd, Fine SL, Hyman L (1984) Age-related macular degeneration and blindness due to neovascular maculopathy. Arch Ophthalmol 102:1640–1642

Folkman J (1971) Tumor angiogenesis: therapeutic implications. N Engl J Med 285:1182–1186

Folkman J (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med 1:27–31 issn: 1078–8956

Fong GH, Rossant J, Gertsenstein M, Breitman ML (1995) Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature 376:66–70

Fong GH, Zhang L, Bryce DM, Peng J (1999) Increased hemangioblast commitment, not vascular disorganization, is the primary defect in flt-1 knock-out mice. Development 126:3015–3025

Frank S, Hubner G, Breier G, Longaker MT, Greenhalg DG, Werner S (1995) Regulation of VEGF expression in cultured keratinocytes. Implications for normal and impaited wound healing. J Biol Chem 270:12607–12613

Fraser HM, Dickson SE, Lunn SF, Wulff C, Morris KD, Carroll VA, Bicknell R (2000) Suppression of luteal angiogenesis in the primate after neutralization of vascular endothelial growth factor. Endocrinology 141:995–1000

Fujio Y, Walsh K (1999) Akt mediates cytoprotection of endothelial cells by vascular endothelial growth factor in an anchorage-dependent manner. J Biol Chem 274:16349–16354

Gasparini G, Longo R, Toi M, Ferrara N (2005) Angiogenic inhibitors: a new therapeutic strategy in oncology. Nat Clin Pract Oncol 2:562–577

Gerber HP, Ferrara N (2005) Pharmacology and pharmacodynamics of bevacizumab as monotherapy or in combination with cytotoxic therapy in preclinical studies. Cancer Res 65:671–680

Gerber HP, Condorelli F, Park J, Ferrara N (1997) Differential transcriptional regulation of the two VEGF receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. J Biol Chem 272:23659–23667

Gerber HP, Dixit V, Ferrara N (1998a) Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. J Biol Chem 273:13313–13316

Gerber HP, McMurtrey A, Kowalski J, Yan M, Keyt BA, Dixit V, Ferrara N (1998b) VEGF regulates endothelial cell survival by the PI3-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. J Biol Chem 273:30336–30343

Gerber HP, Hillan KJ, Ryan AM, Kowalski J, Keller G-A, Rangell L, Wright BD, Radtke F, Aguet M, Ferrara N (1999a) VEGF is required for growth and survival in neonatal mice. Development 126:1149–1159

Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N (1999b) VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat Med 5:623–628

Gerber HP, Kowalski J, Sherman D, Eberhard DA, Ferrara N (2000) Complete inhibition of rhabdomyosarcoma xenograft growth and neovascularization requires blockade of both tumor and host vascular endothelial growth factor. Cancer Res 60:6253–6258

Gerber H-P, Malik A, Solar GP, Sherman D, Liang X-H, Meng G, Hong K, Marsters J, Ferrara N (2002) Vascular endothelial growth factor regulates hematopoietic stem cell

survival by an internal autocrine loop mechanism. Nature 417:954–958

Gille H, Kowalski J, Yu L, Chen H, Pisabarro MT, Davis-Smyth T, Ferrara N (2000) A repressor sequence in the juxtamembrane domain of Flt-1 (VEGFR-1) constitutively inhibits VEGF-dependent PI 3 kinase activation and endothelial cell migration. EMBO J 19:4064–4073

Gille H, Kowalski J, Li B, LeCouter J, Moffat B, Zioncheck T, Pelletier N, Ferrara N (2001) Analysis of biological effects and signaling properties of Flt-1 (VEGFR-1) and KDR (VEGFR-2). A reassessment using novel receptor-specific VEGF mutants. J Biol Chem 276:3222–3230

Goede V, Schmidt T, Kimmina S, Kozian D, Augustin HG (1998) Analysis of blood vessel maturation processes during cyclic ovarian angiogenesis. Lab Invest 78:1385–1394

Goldman CK, Kendall RL, Cabrera G, Soroceanu L, Heike Y, Gillespie GY, Siegal GP, Mao X, Bett AJ, Huckle WR, Thomas KA, Curiel DT (1998) Paracrine expression of a native soluble vascular endothelial growth factor receptor inhibits tumor growth, metastasis, and mortality rate. Proc Natl Acad Sci USA 95:8795–8800

Gragoudas ES, Adamis AP, Cunningham ET Jr, Feinsod M, Guyer DR (2004) Pegaptanib for neovascular age-related macular degeneration. N Engl J Med 351:2805–2816

Grugel S, Finkenzeller G, Weindel K, Barleon B, Marme D (1995) Both v-Ha-Ras and v-Raf stimulate expression of the vascular endothelial growth factor in NIH 3T3 cells. J Biol Chem 270:25915–25919 issn: 20021–29258

Guerrin M, Moukadiri H, Chollet P, Moro F, Dutt K, Malecaze F, Plouet J (1995) Vasculotropin/vascular endothelial growth factor is an autocrine growth factor for human retinal pigment epithelial cells cultured in vitro. J Cell Physiol 164:385–394

Guidi AJ, Abu Jawdeh G, Tognazzi K, Dvorak HF, Brown LF (1996) Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in endometrial carcinoma. Cancer 78:454–460 issn: 0008–0543x

Hattori K, Dias S, Heissig B, Hackett NR, Lyden D, Tateno M, Hicklin DJ, Zhu Z, Witte L, Crystal RG, Moore MA, Rafii S (2001) Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells. J Exp Med 193:1005–1014

Hattori K, Heissig B, Wu Y, Dias S, Tejada R, Ferris B, Hicklin DJ, Zhu Z, Bohlen P, Witte L, Hendrikx J, Hackett NR, Crystal RG, Moore MA, Werb Z, Lyden D, Rafii S (2002) Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. Nat Med 8:841–849

Hiratsuka S, Nakamura K, Iwai S, Murakami M, Itoh T, Kijima H, Shipley JM, Senior RM, Shibuya M (2002) MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis. Cancer Cell 2:289–300

Holash J, Davis S, Papadopoulos N, Croll SD, Ho L, Russell M, Boland P, Leidich R, Hylton D, Burova E, Ioffe E, Huang T, Radziejewski C, Bailey K, Fandl JP, Daly T, Wiegand SJ, Yancopoulos GD, Rudge JS (2002) VEGF-Trap: a VEGF blocker with potent antitumor effects. Proc Natl Acad Sci USA 99:11393–11398

Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung DW (1991) The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. Mol Endocrinol 5:1806–1814

Houck KA, Leung DW, Rowland AM, Winer J, Ferrara N (1992) Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. J Biol Chem 267:26031–26037

Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth H, Helm W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R, Kabbinavar F (2004) Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 350:2335–2342

Iliopoulos O, Levy AP, Jiang C, Kaelin WG Jr, Goldberg MA (1996) Negative regulation of hypoxia-inducible genes by the von Hippel-Lindau protein. Proc Natl Acad Sci USA 93:10595–10599

Inoue M, Hager JH, Ferrara N, Gerber HP, Hanahan D (2002) VEGF-A has a critical, nonredundant role in angiogenic switching and pancreatic beta cell carcinogenesis. Cancer Cell 1:193–202

Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin WG Jr (2001) HIF-alpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science 292:464–468

Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ (2001) Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science 292:468–472

Jain RK (2003) Molecular regulation of vessel maturation. Nat Med 9:685–693

Jain RK (2005) Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science 307:58–62

Jain RK, Duda DG, Clark JW, Loeffler JS (2006) Lessons from phase III clinical trials on anti-VEGF therapy for cancer. Nat Clin Pract Oncol 3:24–40

Jakeman LB, Winer J, Bennett GL, Altar CA, Ferrara N (1992) Binding sites for vascular endothelial growth factor are localized on endothelial cells in adult rat tissues. J Clin Invest 89:244–253

Jakeman LB, Armanini M, Philips HS, Ferrara N (1993) Developmental expression of binding sites and mRNA for vascular endothelial growth factor suggests a role for this protein in vasculogenesis and angiogenesis. Endocrinology 133:848–859

Jingjing L, Xue Y, Agarwal N, Roque RS (1999) Human Muller cells express VEGF183, a novel spliced variant of vascular endothelial growth factor. Invest Ophthalmol Vis Sci 40:752–759

Johnson DH, Fehrenbacher L, Novotny WF, Herbst RS, Nemunaitis JJ, Jablons DM, Langer CJ, DeVore RF 3rd, Gaudreault J, Damico LA, Holmgren E, Kabbinavar F (2004) Randomized phase II trial comparing bevacizumab plus carboplatin and paclitaxel with carboplatin and paclitaxel alone in previously untreated locally advanced or metastatic non-small-cell lung cancer. J Clin Oncol 22:2184–2191

Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, Saksela O, Kalkkinen N, Alitalo K (1996) A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. EMBO J 15:1751 issn: 0261–4189

Karkkainen MJ, Makinen T, Alitalo K (2002) Lymphatic endothelium: a new frontier of metastasis research. Nat Cell Biol 4:E2–E5

Karkkainen MJ, Haiko P, Sainio K, Partanen J, Taipale J, Petrova TV, Jeltsch M, Jackson DG, Talikka M, Rauvala H, Betsholtz C, Alitalo K (2004) Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. Nat Immunol 5:74–80

Kawasaki T, Kitsukawa T, Bekku Y, Matsuda Y, Sanbo M, Yagi T, Fujisawa H (1999) A requirement for neuropilin-1 in embryonic vessel formation. Development 126:4895–4902

Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, Connolly DT (1989) Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science 246:1309–1312

Kendall RL, Thomas KA (1993) Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. Proc Natl Acad Sci USA 90:10705–10709

Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS, Ferrara N (1993) Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumor growth in vivo. Nature 362:841–844

Kishimoto J, Ehama R, Ge Y, Kobayashi T, Nishiyama T, Detmar M, Burgeson RE (2000) In vivo detection of human vascular endothelial growth factor promoter activity in transgenic mouse skin. Am J Pathol 157:103–110

Klagsbrun M, D'Amore PA (1991) Regulators of angiogenesis. Annu Rev Physiol 53:217–239

Klagsbrun M, Eichmann A (2005) A role for axon guidance receptors and ligands in blood vessel development and tumor angiogenesis. Cytokine Growth Factor Rev 16:535–548

Klement G, Baruchel S, Rak J, Man S, Clark K, Hicklin DJ, Bohlen P, Kerbel RS (2000) Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity (see comments). J Clin Invest 105:R15–R24

Kozin SV, Boucher Y, Hicklin DJ, Bohlen P, Jain RK, Suit HD (2001) Vascular endothelial growth factor receptor-2-blocking antibody potentiates radiation-induced long-term control of human tumor xenografts. Cancer Res 61:39–44

Kuo CJ, Farnebo F, Yu EY, Christofferson R, Swearigen RA, Carter R, von Recum HA, Yuan J, Kumihara J, Flynn E, D'Amato R, Folkman J, Mulligan RC (2001) Comparative evaluation of the antitumor activity of antiangiogenic proteins delivered by gene transfer. Proc Natl Acad Sci USA 98:4605–4610

Lange T, Guttmann-Raviv N, Baruch L, Machluf M, Neufeld G (2003) VEGF162, a new heparin-binding vascular endothelial growth factor splice form that is expressed in transformed human cells. J Biol Chem 278:17164–17169

LeCouter J, Moritz DR, Li B, Phillips GL, Liang XH, Gerber HP, Hillan KJ, Ferrara N (2003) Angiogenesis-independent endothelial protection of liver: role of VEGFR-1. Science 299:890–893

Lee CG, Heijn M, di Tomaso E, Griffon-Etienne G, Ancukiewicz M, Koike C, Park KR, Ferrara N, Jain RK, Suit HD, Boucher Y (2000) Anti-vascular endothelial growth factor treatment augments tumor radiation response under normoxic or hypoxic conditions. Cancer Res 60:5565–5570

Lee J, Gray A, Yuan J, Luoh SM, Avraham H, Wood WI (1996) Vascular endothelial growth factor-related protein: a ligand and specific activator of the tyrosine kinase receptor Flt4. Proc Natl Acad Sci USA 93:1988–1992 issn: 0027–8424

Lee S, Jilani SM, Nikolova GV, Carpizo D, Iruela-Arispe ML (2005) Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. J Cell Biol 169:681–691

Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. Science, 246:1306–1309

Lonser RR, Glenn GM, Walther M, Chew EY, Libutti SK, Linehan WM, Oldfield EH (2003) Von Hippel-Lindau disease. Lancet 361:2059–2067

Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L, Chadburn A, Heissig B, Marks W, Witte L, Wu Y, Hicklin D, Zhu Z, Hackett NR, Crystal RG, Moore MA, Hajjar KA, Manova K, Benezra R, Rafii S (2001) Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat Med 7:1194–1201

Lyttle DJ, Fraser KM, Flemings SB, Mercer AA, Robinson AJ (1994) Homologs of vascular endothelial growth factor are encoded by the poxvirus orf virus. J Virol 68:84–92

Maglione D, Guerriero V, Viglietto G, Delli-Bovi P, Persico MG (1991) Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. Proc Natl Acad Sci USA 88:9267–9271

Maglione D, Guerriero V, Viglietto G, Ferraro MG, Aprelikova O, Alitalo K, del Vecchio S, Lei KJ, Chou JY, Persico MG (1993) Two alternative mRNAs coding for the angiogenic factor, placenta growth factor (PlGF), are transcribed from a single gene of chromosome 14. Oncogene 8:925–931

Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegend SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopulos N, Daly TJ, Davis S, Sato TN, Yancopoulos GD (1997) Angiopoietin-2, a natural antagonist for Tie-2 that disrupts in vivo angiogenesis. Science 277:55–60

Malecaze F, Clemens S, Simorer-Pinotel V, Mathis A, Chollet P, Favard P, Bayard F, Plouet J (1994) Detection of vascular endothelial growth factor mRNA and vascular endothelial growth factor-like activity in proliferative diabetic retinopathy. Arch Ophthalmol 112:1476–1482

Matsumoto T, Claesson-Welsh L (2001) VEGF receptor signal transduction. Science's STKE 112 (RE21):1–17

Maxwell PH, Ratcliffe PJ (2002) Oxygen sensors and angiogenesis. Semin Cell Dev Biol 13:29–37

Melnyk O, Shuman MA, Kim KJ (1996) Vascular endothelial growth factor promotes tumor dissemination by a mechanism distinct from its effect on primary tumor growth. Cancer-Res, 56:921–924 issn: 0008–5472

Mesiano S, Ferrara N, Jaffe RB (1998) Role of vascular endothelial growth factor in ovarian cancer: inhibition of ascites formation by immunoneutralization. Am J Pathol 153:1249–1256

Michels S, Rosenfeld PJ, Puliafito CA, Marcus EN, Venkatraman AS (2005) Systemic bevacizumab (Avastin) therapy for neovascular age-related macular degeneration twelve-week results of an uncontrolled open-label clinical study. Ophthalmology 112:1035–1047

Millauer B, Shawver LK, Plate KH, Risau W, Ullrich A (1994) Glioblastoma growth inhibited in vivo by a dominant-negative Flk-1 mutant. Nature 367:576–579 issn: 0028–0836

Millauer B, Wizigmann Voos S, Schnurch H, Martinez R, Moller NP, Risau W, Ullrich A (1993) High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. Cell, 72:835–846 issn: 0092–8674

Miller JW, Adamis AP, Shima DT, D'Amore PA, Moulton RS, O'Reilly MS, Folkman J, Dvorak HF, Brown LF, Berse B, Yeo TK, Yeo KT (1994) Vascular endothelial growth factor/vascular permeability factor is temporally and spatially correlated with ocular angiogenesis in a primate model. Am J Pathol 145:574–584 issn: 0002–9440

Mole DR, Maxwell PH, Pugh CW, Ratcliffe PJ (2001) Regulation of HIF by the von Hippel-Lindau tumour suppressor: implications for cellular oxygen sensing. IUBMB Life 52:43–47

Morgan B, Thomas AL, Drevs J, Hennig J, Buchert M, Jivan A, Horsfield MA, Mross K, Ball HA, Lee L, Mietlowski W, Fuxuis S, Unger C, O'Byrne K, Henry A, Cherryman GR, Laurent D, Dugan M, Marme D, Steward WP (2003) Dynamic contrast-enhanced magnetic resonance imaging as a biomarker for the pharmacological response of PTK787/ZK 222584, an inhibitor of the vascular endothelial growth factor receptor tyrosine kinases, in patients with advanced colorectal cancer and liver metastases: results from two phase I studies. J Clin Oncol 21:3955–3964

Motzer RJ, Dror Michaelson M, Redman BG, Hudes GR, Wilding G, Figlin RA, Ginsberg MS, Kim ST, Baum CM, Deprimo SE, Li JZ, Bello CL, Theuer CP, George DJ, Rini BI (2006) Activity of SU11248, a multitargeted inhibitor of vascular endothelial growth factor receptor and platelet-derived growth factor receptor, in patients with metastatic renal cell carcinoma. J Clin Oncol 24:16–24

Neufeld G, Cohen T, Shraga N, Lange T, Kessler O, Herzog Y (2002) The neuropilins: multifunctional semaphorin and VEGF receptors that modulate axon guidance and angiogenesis. Trends Cardiovasc Med 12:13–19

Nicol D, Hii SI, Walsh M, Teh B, Thompson L, Kennett C, Gotley D (1997) Vascular endothelial growth factor expression is increased in renal cell carcinoma. J Urol 157:1482–1486

Oberg-Welsh C, Sandler S, Andersson A, Welsh M (1997) Effects of vascular endothelial growth factor on pancreatic duct cell replication and the insulin production of fetal islet-like cell clusters in vitro. Mol Cell Endocrinol 126:125–132

Ogawa S, Oku A, Sawano A, Yamaguchi S, Yazaki Y, Shibuya M (1998) A novel type of vascular endothelial growth factor, VEGF-E (NZ-7 VEGF), preferentially utilizes KDR/Flk-1 receptor and carries a potent mitotic activity without heparin-binding domain. J Biol Chem 273:31273–31282

Okada F, Rak JW, Croix BS, Lieubeau B, Kaya M, Roncari L, Shirasawa S, Sasazuki T, Kerbel RS (1998) Impact of oncogenes in tumor angiogenesis: mutant K-ras upregulation of vascular endothelial growth factor/vascular permeability factor is necessary, but not sufficient for tumorigenicity of human colorectal carcinoma cells. Proc Natl Acad Sci USA 95:3609–3614

Olofsson B, Pajusola K, Kaipainen A, von Euler G, Joukov V, Saksela O, Orpana A, Pettersson RF, Alitalo K, Eriksson U (1996) Vascular endothelial growth factor B, a novel growth factor for endothelial cells. Proc Natl Acad Sci USA 93:2576–2581 issn: 0027–8424

Olofsson B, Korpelainen E, Pepper MS, Mandriota SJ, Aase K, Kumar V, Gunji Y, Jeltsch MM, Shibuya M, Alitalo K, Eriksson U (1998) Vascular endothelial growth factor B (VEGF-B) binds to VEGF receptor-1 and regulates plasminogen activator activity in endothelial cells. Proc Natl Acad Sci USA 95:11709–11714

Orlandini M, Marconcini L, Ferruzzi R, Oliviero S (1996) Identification of a c-fos-induced gene that is related to the platelet-derived growth factor/vascular endothelial

growth factor family [corrected; erratum to be published]. Proc Natl Acad Sci USA 93:11675–11680

Ostendorf T, Kunter U, Eitner F, Loos A, Regele H, Kerjaschki D, Henninger DD, Janjic N, Floege J (1999) VEGF(165) mediates glomerular endothelial repair. J Clin Invest 104:913–923

Ozaki H, Seo MS, Ozaki K, Yamada H, Yamada E, Okamoto N, Hofmann F, Wood JM, Campochiaro PA (2000) Blockade of vascular endothelial cell growth factor receptor signaling is sufficient to completely prevent retinal neovascularization. Am J Pathol 156:697–707

Pajusola K, Aprelikova O, Korhonen J, Kaipainen A, Pertovaara L, Alitalo R, Alitalo K (1992) FLT4 receptor tyrosine kinase contains seven immunoglobulin-like loops and is expressed in multiple human tissues and cell lines [published erratum appears in Cancer Res 1993, 53:3845]. Cancer Res 52:5738–5743 issn: 0008–5472

Park JE, Keller G-A, Ferrara N (1993) The vascular endothelial growth factor isoforms (VEGF): differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. Mol Biol Cell 4:1317–1326

Park JE, Chen HH, Winer J, Houck KA, Ferrara N (1994) Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. J Biol Chem 269:25646–25654 issn: 20021–29258

Pepper MS (2001) Extracellular proteolysis and angiogenesis. Thromb Haemost 86:346–355

Pepper MS, Ferrara N, Orci L, Montesano R (1992) Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis in vitro. Biochem Biophys Res Commun 189:824–831

Pepper MS, Wasi S, Ferrara N, Orci L, Montesano R (1994) In vitro angiogenic and proteolytic properties of bovine lymphatic endothelial cells. Exp Cell Res 210:298–305

Pertovaara L, Kaipainen A, Mustonen T, Orpana A, Ferrara N, Saksela O, Alitalo K (1994) Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells. J Biol Chem 269:6271–6274

Phillips HS, Hains J, Leung DW, Ferrara N (1990) Vascular endothelial growth factor is expressed in rat corpus luteum. Endocrinology 127:965–967

Phillips HS, Armanini M, Stavrou D, Ferrara N, Westphal M (1993) Intense focal expression of vascular endothelial growth factor mRNA in human intracranial neoplasms: association with regions of necrosis. Int J Oncol 2:913–919

Photodynamic Therapy of Subfoveal Choroidal Neovascularization in Age-related Macular Degeneration With Verteporfin (1999) One-year results of 2 randomized clinical trials - TAP report 1. Arch Ophthalmol 117:1329–1345

Pierce EA, Foley ED, Smith LE (1996) Regulation of vascular endothelial growth factor by oxygen in a model of retinopathy of prematurity (see comments). Arch Ophthalmol 114:1219–1228

Plate KH, Breier G, Weich HA, Risau W (1992) Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. Nature 359:845–848

Plouet J, Moukadiri HJ (1990) Characerization of the receptors for vasculotropin on bovine adrenal cortex-derived capillary endothelial cells. J Biol Chem 265:22071–22075

Poltorak Z, Cohen T, Sivan R, Kandelis Y, Spira G, Vlodavsky I, Keshet E, Neufeld G (1997) VEGF145, a secreted vascular endothelial growth factor isoform that binds to extracellular matrix. J Biol Chem 272:7151–7158

Poole AR (ed) (1991) Cartilage: molecular aspects. CRC Press, Boca Raton, FL, pp 179–211

Presta LG, Chen H, O'Connor SJ, Chisholm V, Meng YG, Krummen L, Winkler M, Ferrara N (1997) Humanization of an anti-VEGF monoclonal antibody for the therapy of solid tumors and other disorders. Cancer Res 57:4593–4599

Prewett M, Huber J, Li Y, Santiago A, O'Connor W, King K, Overholser J, Hooper A, Pytowski B, Witte L, Bohlen P, Hicklin DJ (1999) Antivascular endothelial growth factor receptor (fetal liver kinase 1) monoclonal antibody inhibits tumor angiogenesis. Cancer Res 59:5209–5218

Pugh CW, Ratcliffe PJ (2003) Regulation of angiogenesis by hypoxia: role of the HIF system. Nat Med 9:677–684

Quinn TP, Peters KG, de Vries C, Ferrara N, Williams LT (1993) Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium. Proc Natl Acad Sci USA 90:7533–7537

Rafii S, Meeus S, Dias S, Hattori K, Heissig B, Shmelkov S, Rafii D, Lyden D (2002) Contribution of marrow-derived progenitors to vascular and cardiac regeneration. Semin Cell Dev Biol 13:61–67

Ravindranath N, Little-Ihrig L, Phillips HS, Ferrara N, Zeleznik AJ (1992) Vascular endothelial growth factor messenger ribonucleic acid expression in the primate ovary. Endocrinology 131:254–260

Risau W (1997) Mechanisms of angiogenesis. Nature 386:671–674

Risau W, Flamme I (1995) Vasculogenesis. Annu Rev Cell Dev Biol 11:73–91

Robinson GS, Pierce EA, Rook SL, Foley E, Webb R, Smith LE (1996) Oligodeoxynucleotides inhibit retinal neovascularization in a murine model of proliferative retinopathy. Proc Natl Acad Sci USA 93:4851–4856 issn: 0027–8424

Rosenfeld PJ, Moshfeghi AA, Puliafito CA (2005) Optical coherence tomography findings after an intravitreal injection of bevacizumab (avastin) for neovascular age-related macular degeneration. Ophthalmic Surg Lasers Imaging 36:331–335

Rosenfeld PJ, Brown DM, Heier JS, Boyer DS, Kaiser PK, Chung CY, Kim RY; MARINA Study Group (2006) Ra-

nibizumab for neovascular age-related macular degeneration. N Engl J Med 355:1419–1431

Ruzinova MB, Schoer RA, Gerald W, Egan JE, Pandolfi PP, Rafii S, Manova K, Mittal V, Benezra R (2003) Effect of angiogenesis inhibition by Id loss and the contribution of bone-marrow-derived endothelial cells in spontaneous murine tumors. Cancer Cell 4:277–289

Ryan AM, Eppler DB, Hagler KE, Bruner RH, Thomford PJ, Hall RL, Shopp GM, O'Neill CA (1999) Preclinical safety evaluation of rhuMAbVEGF, an antiangiogenic humanized monoclonal antibody. Toxicol Pathol 27:78–86

Safran M, Kaelin WJ Jr (2003) HIF hydroxylation and the mammalian oxygen-sensing pathway. J Clin Invest 111:779–783

Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF (1983) Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 219:983–985

Seno H, Oshima M, Ishikawa TO, Oshima H, Takaku K, Chiba T, Narumiya S, Taketo MM (2002) Cyclooxygenase 2- and prostaglandin E(2) receptor EP(2)-dependent angiogenesis in Apc(Delta716) mouse intestinal polyps. Cancer Res 62:506–511

Shaked Y, Bertolini F, Man S, Rogers MS, Cervi D, Foutz T, Rawn K, Voskas D, Dumont DJ, Ben-David Y, Lawler J, Henkin J, Huber J, Hicklin DJ, D'Amato RJ, Kerbel RS (2005) Genetic heterogeneity of the vasculogenic phenotype parallels angiogenesis; implications for cellular surrogate marker analysis of antiangiogenesis. Cancer Cell 7:101–111

Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 376:62–66

Shen H, Clauss M, Ryan J, Schmidt AM, Tijburg P, Borden L, Connolly D, Stern D, Kao J (1993) Characterization of vascular permeability factor/vascular endothelial growth factor receptors on mononuclear phagocytes. Blood 81:2767–2773

Shibuya M, Yamaguchi S, Yamane A, Ikeda T, Tojo A, Matsushime H, Sato M (1990) Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase (flt) closely related to the fms family. Oncogene 8:519–527

Shifren JL, Mesiano S, Taylor RN, Ferrara N, Jaffe RB (1998) Corticotropin regulates vascular endothelial growth factor expression in human fetal adrenal cortical cells. J Clin Endocrinol Metab 83:1342–1347

Shweiki D, Itin A, Soffer D, Keshet E (1992) Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 359:843–845

Shweiki D, Itin A, Neufeld G, Gitay-Goren H, Keshet E (1993) Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally-mediated angiogenesis. J Clin Invest 91:2235–2243

Simons M (2005) Angiogenesis: where do we stand now? Circulation 111:1556–1566

Smith JK, Mamoon NM, Duhe RJ (2004) Emerging roles of targeted small molecule protein-tyrosine kinase inhibitors in cancer therapy. Oncol Res 14:175–225

Soh EY, Sobhi SA, Wong MG, Meng YG, Siperstein AE, Clark OH, Duh QY (1996) Thyroid-stimulating hormone promotes the secretion of vascular endothelial growth factor in thyroid cancer cell lines. Surgery 120:944–947

Soker S, Fidder H, Neufeld G, Klagsbrun M (1996) Characterization of novel vascular endothelial growth factor (VEGF) receptors on tumor cells that bind VEGF165 via its exon 7-encoded domain. J Biol Chem 271:5761–5767 issn: 0021–9258

Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M (1998) Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell 92:735–745

Sondell M, Lundborg G, Kanje M (1999) Vascular endothelial growth factor has neurotrophic activity and stimulates axonal outgrowth, enhancing cell survival and Schwann cell proliferation in the peripheral nervous system. J Neurosci 19:5731–5740

Sonoshita M, Takaku K, Sasaki N, Sugimoto Y, Ushikubi F, Narumiya S, Oshima M, Taketo MM (2001) Acceleration of intestinal polyposis through prostaglandin receptor EP2 in Apc(Delta 716) knockout mice. Nat Med 7:1048–1051

Sowter HM, Corps AN, Evans AL, Clark DE, Charnock-Jones DS, Smith SK (1997) Expression and localization of the vascular endothelial growth factor family in ovarian epithelial tumors. Lab Invest 77:607–614

Stacker SA, Achen MG, Jussila L, Baldwin ME, Alitalo K (2002) Lymphangiogenesis and cancer metastasis. Nat Rev Cancer 2:573–583

Suzuki K, Hayashi N, Miyamoto Y, Yamamoto M, Ohkawa K, Ito Y, Sasaki Y, Yamaguchi Y, Nakase H, Noda K, Enomoto N, Arai K, Yamada Y, Yoshihara H, Tujimura T, Kawano K, Yoshikawa K, Kamada T (1996) Expression of vascular permeability factor/vascular endothelial growth factor in human hepatocellular carcinoma. Cancer Res 56:3004–3009

Takahashi T, Ueno H, Shibuya M (1999) VEGF activates protein kinase C-dependent, but Ras-independent Raf-MEK-MAP kinase pathway for DNA synthesis in primary endothelial cells. Oncogene 18:2221–2230

Takahashi T, Yamaguchi S, Chida K, Shibuya M (2001) A single autophosphorylation site on KDR/Flk-1 is essential for VEGF-A-dependent activation of PLC-gamma and DNA synthesis in vascular endothelial cells. Embo J 20:2768–2778

Tamagnone L, Artigiani S, Chen H, He Z, Ming GI, Song H, Chedotal A, Winberg ML, Goodman CS, Poo M, Tessier-Lavigne M, Comoglio PM (1999) Plexins are a large family

of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. Cell 99:71–80

Tejada M, Yu L, Dong J, Jung K, Meng G, Peale FV, Frantz GD, Hall L, Liang X, Gerber HP, Ferrara N (2006) Tumor-driven paracrine PDGF receptor -a signaling is a key determinent of stromal cell recruitment in a model of human lung carcinoma. Clin Cancer Res 12:2676–2688

Terman BI, Carrion ME, Kovacs E, Rasmussen BA, Eddy RL, Shows TB (1991) Identification of a new endothelial cell growth factor receptor tyrosine kinase. Oncogene 6:1677–1683 issn: 0950-9232

Terman BI, Dougher Vermazen M, Carrion ME, Dimitrov D, Armellino DC, Gospodarowicz D, Bohlen P (1992) Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. Biochem Biophys Res Commun 187:1579–1586 issn: 0006-1291x

Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC, Abraham JA (1991) The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. J Biol Chem 266:11947–11954

Tomisawa M, Tokunaga T, Oshika Y, Tsuchida T, Fukushima Y, Sato H, Kijima H, Yamazaki H, Ueyama Y, Tamaoki N, Nakamura M (1999) Expression pattern of vascular endothelial growth factor isoform is closely correlated with tumour stage and vascularisation in renal cell carcinoma. Eur J Cancer 35:133–137

Tran J, Master Z, Yu JL, Rak J, Dumont DJ, Kerbel RS (2002) A role for survivin in chemoresistance of endothelial cells mediated by VEGF. Proc Natl Acad Sci USA 99:4349–4354

Tsuzuki Y, Fukumura D, Oosthuyse B, Koike C, Carmeliet P, Jain RK (2000) Vascular endothelial growth factor (VEGF) modulation by targeting hypoxia-inducible factor-1alpha->hypoxia response element->VEGF cascade differentially regulates vascular response and growth rate in tumors. Cancer Res 60:6248–6252

Uchida S, Shimada Y, Watanabe G, Tanaka H, Shibagaki I, Miyahara T, Ishigami S, Imamura M (1998) In oesophageal squamous cell carcinoma vascular endothelial growth factor is associated with p53 mutation, advanced stage and poor prognosis. Br J Cancer 77:1704–1709

Vaisman N, Gospodarowicz D, Neufeld G (1990) Characterization of the receptors for vascular endothelial growth factor. J Biol Chem 265:19461–19466

Vincenti V, Cassano C, Rocchi M, Persico G (1996) Assignment of the vascular endothelial growth factor gene to human chromosome 6p21.3. Circulation 93:1493–1495 issn: 0009-7322

Volm M, Koomagi R, Mattern J (1997) Prognostic value of vascular endothelial growth factor and its receptor Flt-1 in squamous cell lung cancer. Int J Cancer 74:64–68

Waltenberger J, Claesson Welsh L, Siegbahn A, Shibuya M, Heldin CH (1994) Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. J Biol Chem 269:26988–26995 issn: 20021-29258

Wang GL, Semenza GL (1995) Purification and characterization of hypoxia-inducible factor 1. J Biol Chem 270:1230–1237 issn: 0021-9258

Warren RS, Yuan H, Matli MR, Gillett NA, Ferrara N (1995) Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. J Clin Invest 95:1789–1797 issn: 0021-9738

Warren RS, Yuan H, Matli MR, Ferrara N, Donner DB (1996) Induction of vascular endothelial growth factor by insulin-like growth factor 1 in colorectal carcinoma. J Biol Chem 271:29483–29488

Wedge SR, Ogilvie DJ, Dukes M, Kendrew J, Curwen JO, Hennequin LF, Thomas AP, Stokes ES, Curry B, Richmond GH, Wadsworth PF (2000) ZD4190: an orally active inhibitor of vascular endothelial growth factor signaling with broad-spectrum antitumor efficacy. Cancer Res 60:970–975

Willett CG, Boucher Y, Di Tomaso E, Duda DG, Munn LL, Tong RT, Chung DC, Sahani DV, Kalva SP, Kozin SV, Mino M, Cohen KS, Scadden DT, Hartford AC, Fischman AJ, Clark JW, Ryan DP, Zhu AX, Blaszkowsky LS, Chen HX, Shellito PC, Lauwers GY, Jain RK (2004) Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. Nat Med 10:145–147

Williams CS, Tsujii M, Reese J, Dey SK, DuBois RN (2000) Host cyclooxygenase-2 modulates carcinoma growth. J Clin Invest 105:1589–1594

Wise LM, Veikkola T, Mercer AA, Savory LJ, Fleming SB, Caesar C, Vitali A, Makinen T, Alitalo K, Stacker SA (1999) Vascular endothelial growth factor (VEGF)-like protein from orf virus NZ2 binds to VEGFR2 and neuropilin-1. Proc Natl Acad Sci USA 96:3071–3076

Wizigmann Voos S, Breier G, Risau W, Plate KH (1995) Upregulation of vascular endothelial growth factor and its receptors in von Hippel-Lindau disease-associated and sporadic hemangioblastomas. Cancer Res 55:1358–1364 issn: 0008-5472

Wood JM, Bold G, Buchdunger E, Cozens R, Ferrari S, Frei J, Hofmann F, Mestan J, Mett H, O'Reilly T, Persohn E, Rosel J, Schnell C, Stover D, Theuer A, Towbin H, Wenger F, Woods-Cook K, Menrad A, Siemeister G, Schirner M, Thierauch KH, Schneider MR, Drevs J, Martiny-Baron G, Totzke F (2000) PTK787/ZK 222584, a novel and potent inhibitor of vascular endothelial growth factor receptor tyrosine kinases, impairs vascular endothelial growth factor-induced responses and tumor growth after oral administration. Cancer Res 60:2178–2189

Wu LW, Mayo LD, Dunbar JD, Kessler KM, Baerwald MR, Jaffe EA, Wang D, Warren RS, Donner DB (2000) Utilization of distinct signaling pathways by receptors for vascular endothelial cell growth factor and other mitogens

in the induction of endothelial cell proliferation. J Biol Chem 275:5096–5103

Yamamoto S, Konishi I, Mandai M, Kuroda H, Komatsu T, Nanbu K, Sakahara H, Mori T (1997) Expression of vascular endothelial growth factor (VEGF) in epithelial ovarian neoplasms: correlation with clinicopathology and patient survival, and analysis of serum VEGF levels. Br J Cancer 76:1221–1227

Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J (2000) Vascular-specific growth factors and blood vessel formation. Nature 407:242–248

Yoshiji H, Gomez DE, Shibuya M, Thorgeirsson UP (1996) Expression of vascular endothelial growth factor, its receptor, and other angiogenic factors in human breast cancer. Cancer Res 56:2013–2016 issn: 0008–5472

Yuan F, Chen Y, Dellian M, Safabakhsh N, Ferrara N, Jain RK (1996) Time-dependent vascular regression and permeability changes in established human tumor xenografts induced by an anti-vascular endothelial growth factor/vascular permeability factor antibody. Proc Natl Acad Sci USA 93:14765–14770

Yuan L, Moyon D, Pardanaud L, Breant C, Karkkainen MJ, Alitalo K, Eichmann A (2002) Abnormal lymphatic vessel development in neuropilin 2 mutant mice. Development 129:4797–4806

Zhang X, Gaspard JP, Chung DC (2001) Regulation of vascular endothelial growth factor by the Wnt and K-ras pathways in colonic neoplasia. Cancer Res 61:6050–6054

Zimmermann RC, Hartman T, Bohlen P, Sauer MV, Kitajewski J, (2001a) Preovulatory treatment of mice with anti-VEGF receptor 2 antibody inhibits angiogenesis in corpora lutea. Microvasc Res 62:15–25

Zimmermann RC, Xiao E, Husami N, Sauer MV, Lobo R, Kitajewski J, Ferin M (2001b) Short-term administration of antivascular endothelial growth factor antibody in the late follicular phase delays follicular development in the rhesus monkey. J Clin Endocrinol Metab 86:768–772

Regulation of Angiogenesis and Vascular Homeostasis Through the Angiopoietin / Tie System

6

HELLMUT G. AUGUSTIN and ULRIKE FIEDLER

Abstract

Following the VEGF/VEGFR system, the interaction of angiopoietin ligands (Ang) with their corresponding Tie receptor was identified in 1996/97 as the second vascular-specific receptor tyrosine kinase signaling system (Suri et al. 1996; Davis et al. 1996; Maisonpierre et al. 1997). Ang/Tie signaling is believed to have important translational therapeutic prospects, and screening programs aimed at identifying low-molecular-weight Tie-2 inhibitors are being widely pursued in the pharmaceutical industry. Nevertheless, even after almost 10 years of angiopoietin research, the Ang/Tie system is molecularly and functionally poorly understood and therapeutic applications are far from being solidly validated.

CONTENTS

6.1 The Tie Receptors

The Tie receptors, Tie1 and Tie2, were identified in 1992 and 1993 as transmembrane orphan receptors. Both Tie1 and Tie2 are almost exclusively expressed by endothelial cells and hematopoietic stem cells (Partanen et al. 1992; Dumont et al. 1992; Iwama et al. 1993; Sato et al. 1993a; Sato et al. 1993b; Schnurch and Risau 1993; Korhonen et al. 1994). Tie2 expression is largely constitutive, whereas Tie1 expression appears to be regulated by shear forces and other microenvironmental milieu factors. Tie1 expression is upregulated in vivo at sites of disturbed flow and atherosclerotic plaques, suggesting that Tie1 may play a critical role in the regulation of endothelial cell functions in response

to changes in flow (Chen-Konak et al. 2003; Porat et al. 2004).

Tie1 and Tie2 share a similar overall structure consisting of an extracellular domain with 33% similarity and an intracellular split tyrosine kinase domain with 76% similarity (Schnurch and Risau 1993). Tie is the acronym for "*t*yrosine kinase with *i*mmunoglobulin and *E*GF homology domains," which reflects the typical extracellular domain structure. The extracellular domain consists of two immunoglobulin-like loops (Ig-like loops) flanking three epidermal growth factor (EGF)-like domains followed by three fibronectin type III repeats. The cytoplasmic domain is a split tyrosine kinase domain containing a 14-amino-acid linker, followed by a C-terminal tail (Schnurch and Risau 1993) (Fig. 6.1). Five phosphorylated tyrosine residues have been identified in Tie2 upon activation, each interacting with a distinct set of downstream signaling molecules and pathways (Eklund and Olsen 2006). Tie2 receptor phosphorylation occurs by autophosphorylation following ligand-mediated receptor clustering. The mechanisms mediating Tie1 activation are poorly understood. Tie1 is not phosphorylated in vivo and autophosphorylation cannot be stimulated, suggesting that Tie1 activation may primarily not occur in a ligand-dependent manner (Marron et al. 2000a). Interestingly, Tie1 can be proteolytically cleaved following stimulation of endothelial cells with VEGF, TNFα or PMA (Yabkowitz et al. 1999; Marron et al. 2000b). Moreover, high shear stress induces Tie1 shedding. Shedding of the extracellular domain leaves a membrane-bound endodomain consisting of the transmembrane domain and the cytoplasmic tyrosine kinase domain. This endodomain is baseline autophosphorylated and can induce PI3-kinase signaling, suggesting that Tie1 is capable of inducing signal transduction upon shedding (Chen-Konak et al. 2003). However, the mechanisms inducing Tie1 shedding in vivo have not been uncovered. Similarly, the ectodomain of Tie2 is cleaved and released into the supernatant upon stimulation of cultured endothelial cells with PMA. Correspondingly, soluble Tie2 (sTie2) can be detected in the blood (Reusch et al. 2001). Multiple studies have shown that sTie2 levels drop following anti-angiogenic therapy (Harris et al. 2001). Moreover, sTie2 itself is a potent angiogenesis inhibitor (Lin et al. 1998; Siemeister et al. 1999; Hangai et al. 2001).

The Angiopoietins

The ligands of Tie2, Angiopoietin-1 (Ang-1) and Angiopoietin-2 (Ang-2), were identified several years after the discovery of the Tie receptors (Davis et al. 1996; Maisonpierre et al. 1997). More recently, two additional ligands, Ang-3 and Ang-4, have been identified. Ang-3 is the mouse ortholog of Ang-4 (Valenzuela et al. 1999). Surprisingly, both are supposed to act as species-specific Tie2 antagonist (Ang-3) and agonist (Ang-4), respectively (Kim et al. 1999; Lee et al. 2004). The angiopoietins are secreted glycoproteins consisting of an N-terminal coiled-coil domain required for protein oligomerization and a C-terminal fibrinogen-like domain required for receptor binding (Fig. 6.1). Ang-1 and Ang-2 share 69% similarity in the coiled-coil domain and 63% similarity in the fibrinogen-like domain (Ward and Dumont 2002). Both form oligomers of different sizes. Ang-1 is predominantly multimeric (trimer or hexamer), and Ang-2 is a dimer (Ward and Dumont 2002; Davis et al. 2003). This suggests that regions within the coiled-coil domain determine the oligomerization status of the proteins. Both ligands bind to Tie2 with similar affinity (Kd, 3 nM) and they share the same binding sites within the first Ig-like loop and the EGF-like repeats of Tie2 (Maisonpierre et al. 1997; Fiedler et al. 2003; MacDonald et al. 2006).

Ang-1 is expressed by many cell types. Expression has been found in pericytes, smooth muscle cells and fibroblasts and also by several tumor cell lines (Fig. 6.2). Ang-1 has been reported to be transcriptionally regulated in some tumors and during inflammation (Stratmann et al. 1998; Sugimachi et al. 2003). Yet, transcriptional regulation is rather

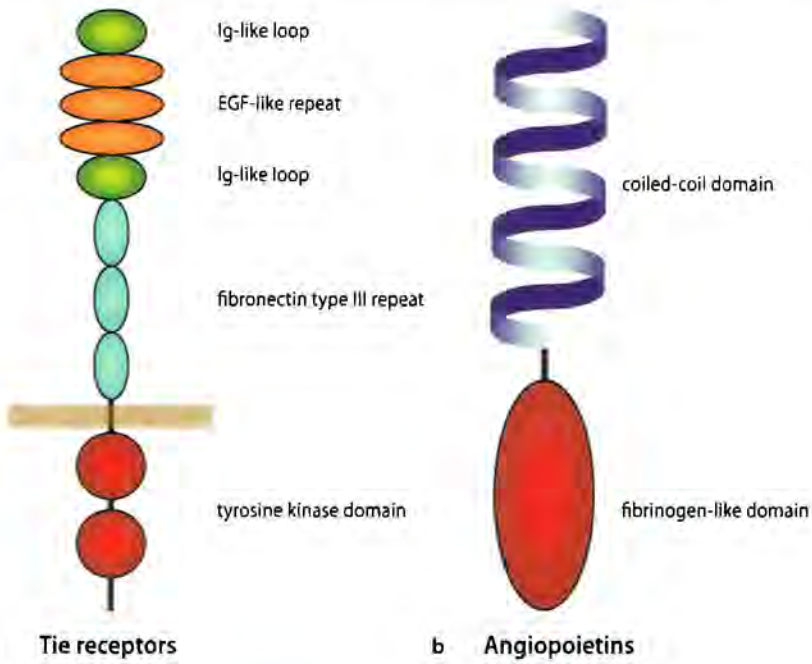


Fig. 6.1a,b. Modular structure of the Tie receptors and the Angiopoietins. a Tie receptors are receptor tyrosine kinases consisting of an N-terminal angiopoietin-binding domain and a C-terminal split tyrosine kinase domain. The extracellular domain is composed of two Ig-like loops flanking EGF-like repeats followed by fibronectin type III repeats. b The angiopoietins are secreted proteins consisting of an N-terminal coiled-coil domain and a C-terminal fibrinogen-like domain. The molecules oligomerize by the coiled-coil domain, which contains additional subclustering sequences and bind to Tie2 via the fibrinogen-like domain

moderate compared to the transcriptional regulation of other angiogenesis- and inflammation-regulating molecules, including VEGF, the selectins, VCAM-1 and Ang-2, indicating that Ang-1 is essentially constitutively expressed. In contrast, Ang-2 expression is tightly controlled and almost restricted to endothelial cells (Fig. 6.2) (Stratmann et al. 1998; Fiedler et al. 2006). Ang-2 mRNA expression is almost absent in the resting, quiescent vasculature, yet it is dramatically upregulated at sites of angiogenesis (Stratmann et al. 1998). Analysis of the Ang-2 promoter revealed that expression of Ang-2 is tightly controlled by positive and negative regulatory elements (Hegen et al. 2004). Strong positive regulators of Ang-2 promoter activity and mRNA expression are the pro-angiogenic cytokines VEGF and FGF-2. It has also been shown that Ang-2 mRNA expression is strongly induced by hypoxia (Mandriota and Pepper 1998; Oh et al. 1999). Moreover, high glucose can interfere with repression of Ang-2 expression and induce Ang-2 expression in Müller cells and endothelial cells in the diabetic retina (Hammes et al. 2004; Yao et al. 2006; Kruse and Fiedler, unpublished data).

6.3 Role of the Angiopoietin/Tie System During Embryonic Development

Both Tie receptors are critical for vascular development. Tie1-deficient mice die between E13.5 and shortly after birth due to hemorrhage, edema and poor vascular integrity (some variability in the phenotype is seen in different mouse strains) (Sato et al. 1995; Puri et al. 1995). Tie2-deficient mice die earlier during embryogenesis, between E9.5 and E10.5. Tie2 null embryos cannot survive as a consequence of poor vascular integrity which results in hemorrhage (Sato et al. 1995). Blood vessels fail to remodel and are poorly covered by mural cells. Moreover, Tie2-deficient mice have fewer endothelial cells and an underdeveloped heart. Ang-1-deficient mice die somewhat later than Tie2-deficient mice, between E11.5 and E12.5 (Suri et al. 1996). However, the phenotype of Ang-1 null embryos largely phenocopies Tie2-deficient embryos. Mice have poor vascular integrity with a loose association of pericytes to the endothelial lining. Ang-1-deficient mice display also

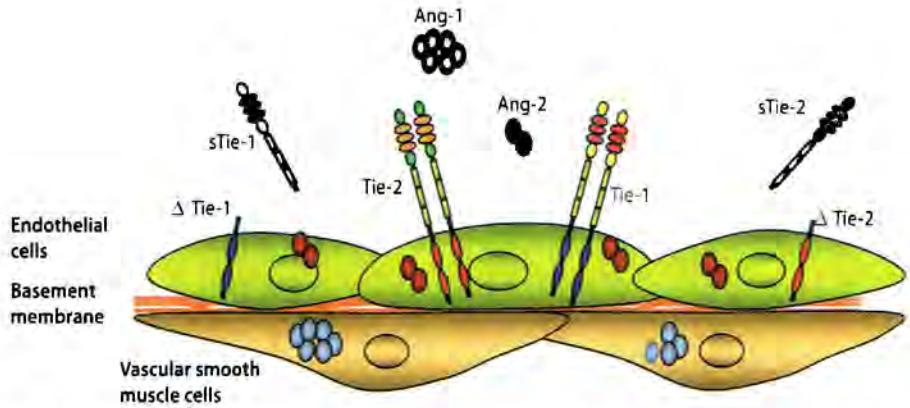


Fig. 6.2. Spatial expression and localization of Tie receptors and angiopoietins. Tie receptors are primarily expressed by endothelial cells. Angiopoietin-1 (Ang-1) is expressed by mural cells and some tumor cells. Thus, Ang-1 acts in paracrine manner on endothelial cells. Ang-2 is almost exclusively produced by endothelial cells. Ang-2 is stored in endothelial Weibel–Palade bodies from where it can be rapidly released upon stimulation. Thus, Ang-2 acts in autocrine fashion on endothelial cells. Both Tie receptors may be shedded under certain conditions, resulting in a soluble ectodomain and a membrane-anchored endodomain

growth retardation of the heart. Taken together, the complementarity of Ang-1-deficient mice and Tie2-deficient mice indicates that Ang-1 is the single non-redundant agonist of Tie2 and that Tie2 signaling regulates remodeling of the developing vasculature and vascular integrity.

In contrast to the embryonic lethal phenotypes of Ang-1- and Tie2-deficient mice, Ang-2 null mice have no overt vascular defects (Gale et al. 2002). Mice are born normally but develop chylous ascites within a few days after birth. Depending on the genetic background of the mouse strain, mice die within the first 14 days after birth (C129 background) or develop normally to adulthood with little postnatal lethality (C57/B6) (Gale et al. 2002; Fiedler et al. 2006). The blood vascular system of Ang-2 null mice appears to be normal, with subtle changes being detectable in the vasculature of the eye. The hyaloid vessels that nourish the lens during eye development fail to regress in Ang-2-deficient mice, indicating improper vessel remodeling (Hackett et al. 2002; Gale et al. 2002). Likewise, the lymphatic vasculature appears to be abnormal in Ang-2-deficient mice. Lymphatic vessels of the intestine are less branched and disorganized. This may actually be the cause of the chylous ascites of Ang-2-deficient mice bred in the C129 background. Interestingly, a genetic knock-in of Ang-1 into the Ang-2 locus rescues the lymphatic defect but not the perturbed hyaloid vessel regression in the eye, suggesting that Ang-2 may act as an agonist on the lymphatic vasculature and an antagonist in the blood vascular system (Gale et al. 2002).

Strong systemic overexpression of Ang-2, under control of either the CMV promoter (global expression) or the K14 promoter (skin expression with systemic Ang-2 effects) results in death of the mice around E10.5. These mice have a phenotype similar to that of Ang-1- and Tie2-deficient mice, indicating that Ang-2 is antagonizing Ang-1-mediated Tie2 signaling (Maisonpierre et al. 1997; Veikkola and Alitalo 2002).

Surprisingly, Ang-1 overexpression in mice results in the formation of enlarged, leakage-resistant blood vessels which are well covered by pericytes (Suri et al. 1998). Moreover, Ang-1 overexpression induces lymphangiogenesis and supports tumor growth (Tammela et al. 2005; Morisada et al. 2005). This suggests that a tight balance of Tie2 signaling is required to regulate vascular homeostasis and quiescence.

Angiopoietin/Tie-Induced Vascular Signaling

Binding of Ang-1 to Tie2 induces rapid receptor autophosphorylation in endothelial cells. In contrast, Ang-2 binding to Tie2 does not induce rapid receptor autophosphorylation in endothelial cells and it is able to inhibit Ang-1-induced receptor phosphorylation. Surprisingly, both ligands are capable of inducing Tie2 phosphorylation if Tie2 is expressed in non-endothelial cells (Maisonpierre et al. 1997). Thus, an endothelial cell innate mechanism controls agonistic Ang-1 functions and antagonistic Ang-2 functions. However, high concentrations or prolonged stimulation of endothelial cells with Ang-2 can induce Tie2 autophosphorylation, suggesting that Ang-2 may act as an agonist under these conditions (Kim et al. 2000; Teichert-Kuliszewska et al. 2001).

Tie2 activation in endothelial cells induces cell migration, sprouting and capillary-like tube formation. In turn, Tie2 activation promotes endothelial cell survival and blood vessel integrity. Tie2 becomes autophosphorylated at five different tyrosine residues within the intracellular C-terminal domain upon Ang-1 binding to Tie2 (Murray et al. 2001). Based on the current understanding of Tie2 signaling, each of the phosphorylated tyrosine residues activates a different signaling pathway by interacting with distinct signaling molecules. The PI3-kinase signaling pathway is activated upon interaction of p85, the regulatory subunit of PI3-kinase, with phosphorylated Tie2. As a consequence, Akt becomes activated and promotes endothelial cell survival and NO synthesis by activation of eNOS (Fujikawa et al. 1999). Moreover, pAkt inactivates the Forkhead transcription factor FKHR-1, which is a potent activator of Ang-2 expression (Daly et al. 2004). Another molecule that interacts with autophosphorylated Tie2 and becomes phosphorylated and activated is ABIN-2 (Hughes et al. 2003). ABIN-2 also protects endothelial cells from apoptosis by inhibiting NF-κB activation. Moreover, ABIN-2 activation by Tie2 signaling also inhibits the expression of molecules involved in inflammatory responses and thrombosis.

Other molecules recruited to phosphorylated Tie2 are Grb2, Grb7, Grb14, Dok-R and Shp-2, which are all involved in Tie2-mediated endothelial cell migration (Jones et al. 2003). Interestingly, the endothelial cell-specific phosphatase VE-PTP forms a complex with Tie2 and promotes dephosphorylation (Fachinger et al. 1999). It has recently been shown that VE-PTP is not critical for the initiation of blood vessel formation but essential for the maintenance and remodeling of the blood vascular system. This indicates that VE-PTP is a critical modulator of balanced Tie2 signaling (Baumer et al. 2006; Dominguez et al. 2007).

There is increasing evidence suggesting that Ang-1 does not just signal through Tie2 but also by affecting integrin signaling. It has been shown that Tie2 interacts with α5β1 integrin and that there is crosstalk between Tie2 and integrin signaling (Cascone et al. 2005). Furthermore, it has been reported that Ang-1 is also capable of binding to integrins directly and to induce integrin signaling (Carlson et al. 2001).

Surprisingly little is know about Ang-2-mediated Tie2 signaling. Genetic experiments in mice and advanced cell culture experiments have shown that Ang-2 is capable of acting as a Tie2 antagonist (Maisonpierre et al. 1997; Scharpfenecker et al. 2005). Likewise, Ang-2 is capable of inhibiting Ang-1-mediated Tie2 phosphorylation and activation in endothelial cells. Nevertheless, Ang-2 is also able to stimulate Tie2 phosphorylation and to induce tube formation under certain conditions in vitro (Kim et al. 2000; Teichert-Kuliszewska et al. 2001). This suggests that endothelial cell innate mechanisms control antagonist Ang-2 functions or that Ang-2 is capable to exert agonistic and antagonistic functions in a context-dependent manner.

A bona fide ligand for Tie1 has hitherto not been identified. There is evidence that Ang-1 binds at high concentrations to Tie1 to induce Tie1 autophosphorylation (Saharinen et al. 2005). However, Tie1 activation may also be induced by a direct interaction of pTie2 with Tie1 and subsequent cross-phosphorylation (Yuan et al. 2007). The shed version of Tie1 is constitutively autophosphorylated and induces the activation of the PI3-kinase pathway, suggesting that it is not ligand binding that activates Tie1 signal transduction (Marron et al. 2000a; Chen-Konak et al. 2003).

The Angiopoietin/Tie System in the Adult Vasculature

Cell biological experiments and the phenotypes of Ang-1- and Tie2-deficient mice suggest that Tie2 activation is primarily involved in the regulation of endothelial cell survival and in promoting vascular maturation and quiescence. Indeed, Tie2 is found to be phosphorylated and thereby constitutively activated in the resting vasculature (Wong et al. 1997; Fiedler, unpublished data). Moreover, Ang-1 exerts a vessel sealing effect (Thurston et al. 2000), acts in anti-inflammatory fashion (Gamble et al. 2000; Jeon et al. 2003; Ramsauer and D'Amore 2002), protects against cardiac allograft atherosclerosis (Nykanen et al. 2003) and radiation-induced endothelial cell damage (Cho et al. 2004b), and promotes wound-healing (Cho et al. 2006). In addition, Ang-1 is capable of inhibiting VEGF- and Ang-2-induced vessel formation (Asahara et al. 1998). This indicates that Ang-1-mediated Tie2 signaling prevents activation of the endothelium and controls vascular homeostasis. Surprisingly, Ang-1 induces angiogenesis in some experimental animal models upon overexpression. For example, Ang-1 overexpression enhances VEGF-induced angiogenesis in the cornea micropocket assay (Asahara et al. 1998). It also induces increased vascularization in the skin (Suri et al. 1998) as well as lymphangiogenesis (Tammela et al. 2005). This suggests that Tie2 activation needs to be tightly controlled and balanced. This conclusion is also supported by the observations that (1) Ang-1 induces pulmonary hypertension (Sullivan et al. 2003), (2) Tie2 overexpression in the skin results in a psoriasis-like phenotype (Voskas et al. 2005), and (3) an activating Tie2 mutation causes venous malformations that are composed of dilated endothelial channels (Vikkula et al. 1996).

A potent regulator balancing Ang-1/Tie2 signaling in vivo is Ang-2. Yet, surprisingly little is known about Ang-2 functions in vivo. Strong systemic embryonic overexpression of Ang-2 leads to lethality and essentially phenocopies Ang-1- and Tie2-deficient mice, which is the most compelling genetic evidence that Ang-2 acts as an antagonist of Ang-1/Tie2 signaling (Maisonpierre et al. 1997). Conceptually, this also implies that Ang-2 is a potentially dangerous molecule whose dosage and spatiotemporal availability needs to be tightly regulated. Supporting this concept is also the observation that local overexpression of Ang-2 in the heart is compatible with life (Visconti et al. 2002).

Endogenous Ang-2 expression is tightly controlled. Ang-2 mRNA expression is almost absent in the quiescent vasculature and dramatically upregulated upon angiogenic activation of endothelial cells (Stratmann et al. 1998). Moreover, Ang-2 protein is not just produced selectively by its own target cells, the vascular endothelium. Instead, it is stored in endothelial cell Weibel–Palade bodies (WPB), from where it can be rapidly released upon stimulation by WPB secretagogues such as PMA, histamine, or thrombin (Fiedler et al. 2004). Spheroidal co-culture experiments of smooth muscle cells and endothelial cells have shown that the release of Ang-2 from endothelial cells is able to destabilize the endothelium in an autocrine manner. This vascular destabilizing effect can be counteracted by Ang-1 and VEGF, indicating that Ang-2 interferes with Tie2 signaling (Scharpfenecker et al. 2005). Moreover, these findings are in line with the concept that Ang-2 induces blood vessel regression in the absence of VEGF and that it promotes angiogenesis in the presence of VEGF (Hanahan 1997). The hypothesis that Ang-2 interferes with blood vessel integrity is further supported by the finding that Ang-2 is a critical regulator of inflammatory responses. Ang-2-deficient mice are not able to elicit rapid inflammatory responses and do not induce adhesion molecule expression in response to TNFα challenge (Fiedler et al. 2006). This suggests that Ang-2 sensitizes the endothelium towards inflammatory and angiogenic cytokines. The underlying mechanisms are not fully unraveled, but it appears likely that Ang-2 interferes with Ang-1-mediated Tie2 signaling in vivo, thereby activating the endothelium. Thus, the ratio of Ang-1 to Ang-2 is critical to balance Tie2 signaling and regulate vascular homeostasis and responsiveness.

Therapeutic Potential of the Angiopoietin/Tie System

Therapeutic intervention with Angiopoietin/Tie signaling is one of the driving forces of current angiopoietin research. Large-scale screening programs focus on the development of low-molecular-weight Tie2 inhibitors that are capable to either selectively interfere with Tie2 signaling or that are part of the target portfolio of a multi-targeted tyrosine kinase inhibitor. The therapeutic potential of single or combinatorial Tie2 inhibitors has not yet been explored in great detail. Given the critical role of Tie2 for vascular maintenance and homeostasis, systemic Tie2 inhibitors may cause unwanted side effects by interfering with vascular stability and endothelial cell quiescence.

Soluble receptors and neutralizing ligand antibodies have been studied extensively to interfere with ligand/RTK interactions. Soluble Tie2 receptor derivatives (sTie2) have been employed as ligand traps and used in tumor experiments as well as in the corneal angiogenesis assay. Soluble Tie2 inhibits tumor growth and the growth of corneal blood vessels (Lin et al. 1998; Siemeister et al. 1999; Singh et al. 2005). In turn, the single treatment of retinal angiogenesis with sTie2 in the retinopathy of prematurity model is not sufficient to block newborn retinopathy (Agostini et al. 2005), suggesting that sTie2 is either not a very potent angiogenesis inhibitor or that it needs sustained long-term treatment for therapeutic efficacy. Another disadvantage of sTie2 is its property to interfere with both Ang-1 and Ang-2. It would be more rational for therapeutic applications to inhibit activation of the endothelium in pathological settings by specifically targeting Ang-2 and to support Ang-1-mediated vascular protection. Both approaches have been pursued experimentally and appear to be promising avenues for future clinical developments.

Ang-2 has therapeutically been selectively inhibited by single-chain-neutralizing Ang-2 antibodies and peptide-Fc fusion proteins that selectively bind Ang-2 but not Ang-1 (Oliner et al. 2004). This Ang-2 trap inhibits tumor growth in several experimental tumor models. Alternatively, Ang-2 production has been successfully blocked with an Ang-2 specific aptamer (White et al. 2003). Inhibition of Ang-2 synthesis is able to inhibit FGF-2-induced rat corneal angiogenesis. Thus, inhibition of Ang-2 inhibits neo-angiogenesis and may result in tumor regression. These findings are in line with a study showing that Ang-2 is critically involved in priming the vasculature towards inflammatory cytokines and that a lack of Ang-2 keeps the vascular bed quiescent (Fiedler et al. 2006). Surprisingly, though, tumors grow in Ang-2-deficient mice with essentially the same growth and vascularization kinetics as in wild-type mice, suggesting that Ang-2 may be dispensable for tumor angiogenesis in genetically engineered Ang-2-deficient mice (Nassare et al. unpublished data). The apparent discrepancy between Ang-2-neutralizing therapies in wild-type mice and tumor growth experiments in Ang-2-deficient mice indicates that local versus systemic Ang-2 functions as well as short-term versus long-term Ang-2 effects need to be mechanistically studied in different experimental animal models in order to more rationally develop Ang-2 manipulatory therapies.

The potential of therapeutic Ang-1 administration has been studied intensively during the past few years. The objectives of Ang-1 therapy are prevention of endothelial cell apoptosis, protection of the vasculature from activation and stabilization of the quiescent endothelial cell layer. Ang-1 and Ang-1 derivatives such as COMP-Ang-1 have been applied by adenoviral gene transfer in mice or by injection of the recombinant protein. COMP-Ang-1 inhibits endothelial cell apoptosis in an irradiation therapy model in mice (Cho et al. 2004c; Cho et al. 2004a). Furthermore, Ang-1 is a potent inhibitor of inflammation in different experimental settings (Gamble et al. 2000; Jeon et al. 2003; Ramsauer and D'Amore 2002). Ang-1 inhibits vascular permeability induced by VEGF and it inhibits the development of atherosclerosis and sepsis (Thurston et al. 2000; Nykanen et al. 2003). Ang-1 also acts in anti-thrombotic fashion and supports wound healing (Cho et al. 2006; Kim et al. 2002). This array of different applications impressively demonstrates the significant

therapeutic potential of Ang-1. However, Ang-1 also induces angiogenesis and vascular remodeling (Suri et al. 1998; Tammela et al. 2005). Moreover, Ang-1 is upregulated in patients with pulmonary hypertension and has been shown to promote pulmonary hypertension (Thistlethwaite et al. 2001; Sullivan et al. 2003; Chu et al. 2004). Thus, it remains to be seen whether the prospects of Ang-1 therapies are limited by uncontrollable side effects.

Conclusion

The angiopoietin/Tie system has, in the past 10 years, been solidly established as a key regulator of vascular maturation, quiescence, and remodeling. Yet, the molecular mechanisms underlying angiopoietin function are still poorly understood. The situation is also puzzling since some manipulatory experiments in vivo yield conflicting results which are not compatible with the simple Ang-1/Ang-2 agonist/antagonist model. What is solidly established, however, is that proper Tie2 signaling homeostasis depends on the ratio of Ang-1 and Ang-2 and their spatiotemporal availability. Constitutive Tie2 phosphorylation by Ang-1 controls the quiescent resting phenotype of the vasculature. In turn, the release of Ang-2 from the Ang-2 stores or the induced expression of Ang-2 shifts the balance locally in favor of Ang-2 and facilitates the responsiveness of the vascular bed to other stimuli. Yet, the molecular mechanisms by which Ang-2 affects endothelial cell function are not satisfactorily understood. It is not solidly established that Ang-2 acts solely by inhibiting Tie2 signaling. Furthermore, there is evidence that Tie2 hyperactivation may also lead to endothelial destabilization, suggesting that too much Ang-1 and subsequent Tie2 signaling may have the same deleterious effects as too little Tie2 signaling. Supporting this concept, there are several reports demonstrating that Ang-1 overexpression has pro-angiogenic effects. Conversely, there is evidence that Ang-2 can substitute Ang-1, depending on the spatiotemporal availability and concentration. Ang-2 induces endothelial cell sprouting and acts anti-apoptotically in vitro and can promote wound healing to the same extend as Ang-1. Furthermore, Ang-2 seems to have agonistic effects on the lymphatic vasculature, as supported by the phenotype of Ang-2-deficient mice.

In conclusion, the angiopoietin/Tie system is controlled by the well-balanced spatiotemporal availability of the ligands that control Tie2 signaling. As such, Tie2 signaling appears to follow a similarly tight dosage regulation as the VEGF/VEGFR system. Much needs to be learned about caliber, organ, and regional differences in Ang/Tie signaling and whether the ligands exert different functions in different vascular beds. Ongoing efforts aimed at therapeutic interference with the Ang/Tie system will benefit from further study of the complex biology of the angiopoietins and support the rational development and clinical implementation of Ang/Tie manipulatory therapies for different applications ranging from inflammation, psoriasis, retinopathies and atherosclerosis to the growth of tumors.

References

Agostini H, Boden K, Unsold A, Martin G, Hansen L, Fiedler U, Esser N, Marme D (2005) A single local injection of recombinant VEGF receptor 2 but not of Tie2 inhibits retinal neovascularization in the mouse. Curr Eye Res 30:249–257

Asahara T, Chen D, Takahashi T, Fujikawa K, Kearney M, Magner M, Yancopoulos GD, Isner JM (1998) Tie2 receptor ligands, angiopoietin-1 and angiopoietin-2, modulate VEGF-induced postnatal neovascularization. Circ Res 83:233–240

Baumer S, Keller L, Holtmann A, Funke R, August B, Gamp A, Wolburg H, Wolburg-Buchholz K, Deutsch U, Vestweber D (2006) Vascular endothelial cell-specific phosphotyrosine phosphatase (VE-PTP) activity is required for blood vessel development. Blood 107:4754–4762

Carlson TR, Feng Y, Maisonpierre PC, Mrksich M, Morla AO (2001) Direct cell adhesion to the angiopoietins mediated by integrins. J Biol Chem 276:26516–26525

Cascone I, Napione L, Maniero F, Serini G, Bussolino F (2005) Stable interaction between alpha5beta1 integrin and Tie2 tyrosine kinase receptor regulates endothelial cell response to Ang-1. J Cell Biol 170:993–1004

Chen-Konak L, Guetta-Shubin Y, Yahav H, Shay-Salit A, Zil-

berman M, Binah O, Resnick N (2003) Transcriptional and post-translation regulation of the Tie1 receptor by fluid shear stress changes in vascular endothelial cells. FASEB J 17:2121–2123

Cho CH, Kammerer RA, Lee HJ, Steinmetz MO, Ryu YS, Lee SH, Yasunaga K, Kim KT, Kim I, Choi HH, Kim W, Kim SH, Park SK, Lee GM, Koh GY (2004a) COMP-Angl: a designed angiopoietin-1 variant with nonleaky angiogenic activity. Proc Natl Acad Sci USA 101:5547–5552

Cho CH, Kammerer RA, Lee HJ, Yasunaga K, Kim KT, Choi HH, Kim W, Kim SH, Park SK, Lee GM, Koh GY (2004b) Designed angiopoietin-1 variant, COMP-Angl, protects against radiation-induced endothelial cell apoptosis. Proc Natl Acad Sci USA 101:5553–5558

Cho CH, Kammerer RA, Lee HJ, Yasunaga K, Kim KT, Choi HH, Kim W, Kim SH, Park SK, Lee GM, Koh GY (2004c) Designed angiopoietin-1 variant, COMP-Angl, protects against radiation-induced endothelial cell apoptosis. Proc Natl Acad Sci USA 101:5553–5558

Cho CH, Sung HK, Kim KT, Cheon HG, Oh GT, Hong HJ, Yoo OJ, Koh GY (2006) COMP-angiopoietin-1 promotes wound healing through enhanced angiogenesis, lymphangiogenesis, and blood flow in a diabetic mouse model. Proc Natl Acad Sci USA 103:4946–4951

Chu D, Sullivan CC, Du L, Cho AJ, Kido M, Wolf PL, Weitzman MD, Jamieson SW, Thistlethwaite PA (2004) A new animal model for pulmonary hypertension based on the overexpression of a single gene, angiopoietin-1. Ann Thorac Surg 77:449–456

Daly C, Wong V, Burova E, Wei Y, Zabski S, Griffiths J, Lai KM, Lin HC, Ioffe E, Yancopoulos GD, Rudge JS (2004) Angiopoietin-1 modulates endothelial cell function and gene expression via the transcription factor FKHR (FOXO1). Genes Dev 18:1060–1071

Davis S, Aldrich TH, Jones PF, Acheson A, Compton DL, Jain V, Ryan TE, Bruno J, Radziejewski C, Maisonpierre PC, Yancopoulos GD (1996) Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. Cell 87:1161–1169

Davis S, Papadopoulos N, Aldrich TH, Maisonpierre PC, Huang T, Kovac L, Xu A, Leidich R, Radziejewska E, Rafique A, Goldberg J, Jain V, Bailey K, Karow M, Fandl J, Samuelsson SJ, Ioffe E, Rudge JS, Daly TJ, Radziejewski C, Yancopoulos GD (2003) Angiopoietins have distinct modular domains essential for receptor binding, dimerization and superclustering. Nat Struct Biol 10:38–44

Dominguez MG, Hughes VC, Pan L, Simmons M, Daly C, Anderson K, Noguera-Troise I, Murphy AJ, Valenzuela DM, Davis S, Thruston G, Yancopoulos GD, Gale NW (2007) Vascular endothelial tyrosine phosphatase (VE-PTP)-null mice undergo vasculogenesis but die embryonically because of defects in angiogenesis. Proc Natl Acad Sci USA 104: 3243–3248

Dumont DJ, Yamaguchi TP, Conlon RA, Rossant J, Breitman ML (1992) Tek, a novel tyrosine kinase gene located on mouse chromosome 4, is expressed in endothelial cells and their presumptive precursors. Oncogene 7:1471–1480

Eklund L, Olsen BR (2006) Tie receptors and their angiopoietin ligands are context-dependent regulators of vascular remodeling. Exp Cell Res 312:630–641

Fachinger G, Deutsch U, Risau W (1999) Functional interaction of vascular endothelial-protein-tyrosine phosphatase with the angiopoietin receptor Tie-2. Oncogene 18:5948–5953

Fiedler U, Krissl T, Koidl S, Weiss C, Koblizek T, Deutsch U, Martiny-Baron G, Marme D, Augustin HG (2003) Angiopoietin-1 and angiopoietin-2 share the same binding domains in the Tie-2 receptor involving the first Ig-like loop and the epidermal growth factor-like repeats. J Biol Chem 278:1721–1727

Fiedler U, Scharpfenecker M, Koidl S, Hegen A, Grunow V, Schmidt JM, Kriz W, Thurston G, Augustin HG (2004) The Tie-2 ligand angiopoietin-2 is stored in and rapidly released upon stimulation from endothelial cell Weibel-Palade bodies. Blood 103:4150–4156

Fiedler U, Reiss Y, Scharpfenecker M, Grunow V, Koidl S, Thurston G, Gale NW, Witzenrath M, Rosseau S, Suttorp N, Sobke A, Herrmann M, Preissner KT, Vajkoczy P, Augustin HG (2006) Angiopoietin-2 sensitizes endothelial cells to TNF-alpha and has a crucial role in the induction of inflammation. Nat Med 12:235–239

Fujikawa K, de AS, I, Jain SK, Presman E, Christensen RA, Varticovski L (1999) Role of PI3-kinase in angiopoietin-1-mediated migration and attachment-dependent survival of endothelial cells. Exp Cell Res 253:663–672

Gale NW, Thurston G, Hackett SF, Renard R, Wang Q, McClain J, Martin C, Witte C, Witte MH, Jackson D, Suri C, Campochiaro PA, Wiegand SJ, Yancopoulos GD (2002) Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by Angiopoietin-1. Dev Cell 3:411–423

Gamble JR, Drew J, Trezise L, Underwood A, Parsons M, Kasminkas L, Rudge J, Yancopoulos G, Vadas MA (2000) Angiopoietin-1 is an antipermeability and anti-inflammatory agent in vitro and targets cell junctions. Circ Res 87:603–607

Hackett SF, Wiegand S, Yancopoulos G, Campochiaro PA (2002) Angiopoietin-2 plays an important role in retinal angiogenesis. J Cell Physiol 192:182–187

Hammes HP, Lin J, Wagner P, Feng Y, Vom HF, Krzizok T, Renner O, Breier G, Brownlee M, Deutsch U (2004) Angiopoietin-2 causes pericyte dropout in the normal retina: evidence for involvement in diabetic retinopathy. Diabetes 53:1104–1110

Hanahan D (1997) Signaling vascular morphogenesis and maintenance. Science 277:48–50

Hangai M, Moon YS, Kitaya N, Chan CK, Wu DY, Peters KG, Ryan SJ, Hinton DR (2001) Systemically expressed soluble Tie2 inhibits intraocular neovascularization. Hum Gene Ther 12:1311–1321

Harris AL, Reusch P, Barleon B, Hang C, Dobbs N, Marme D (2001) Soluble Tie2 and Flt1 extracellular domains in serum of patients with renal cancer and response to anti-angiogenic therapy. Clin Cancer Res 7:1992–1997

Hegen A, Koidl S, Weindel K, Marme D, Augustin HG, Fiedler U (2004) Expression of angiopoietin-2 in endothelial cells is controlled by positive and negative regulatory promoter elements. Arterioscler Thromb Vasc Biol 24:1803–1809

Hughes DP, Marron MB, Brindle NP (2003) The antiinflammatory endothelial tyrosine kinase Tie2 interacts with a novel nuclear factor-kappaB inhibitor ABIN-2. Circ Res 92:630–636

Iwama A, Hamaguchi I, Hashiyama M, Murayama Y, Yasunaga K, Suda T (1993) Molecular cloning and characterization of mouse TIE and TEK receptor tyrosine kinase genes and their expression in hematopoietic stem cells. Biochem Biophys Res Commun 195:301–309

Jeon BH, Khanday F, Deshpande S, Haile A, Ozaki M, Irani K (2003) Tie-ing the antiinflammatory effect of angiopoietin-1 to inhibition of NF-kappaB. Circ Res 92:586–588

Jones N, Chen SH, Sturk C, Master Z, Tran J, Kerbel RS, Dumont DJ (2003) A unique autophosphorylation site on Tie2/Tek mediates Dok-R phosphotyrosine binding domain binding and function. Mol Cell Biol 23:2658–2668

Kim I, Kim JH, Moon SO, Kwak HJ, Kim NG, Koh GY (2000) Angiopoietin-2 at high concentration can enhance endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Oncogene 19:4549–4552

Kim I, Kwak HJ, Ahn JE, So JN, Liu M, Koh KN, Koh GY (1999) Molecular cloning and characterization of a novel angiopoietin family protein, angiopoietin-3. FEBS Lett 443:353–356

Kim I, Oh JL, Ryu YS, So JN, Sessa WC, Walsh K, Koh GY (2002) Angiopoietin-1 negatively regulates expression and activity of tissue factor in endothelial cells. FASEB J 16:126–128

Korhonen J, Polvi A, Partanen J, Alitalo K (1994) The mouse tie receptor tyrosine kinase gene: expression during embryonic angiogenesis. Oncogene 9:395–403

Lee HJ, Cho CH, Hwang SJ, Choi HH, Kim KT, Ahn SY, Kim JH, Oh JL, Lee GM, Koh GY (2004) Biological characterization of angiopoietin-3 and angiopoietin-4. FASEB J 18:1200–1208

Lin P, Buxton JA, Acheson A, Radziejewski C, Maisonpierre PC, Yancopoulos GD, Channon KM, Hale LP, Dewhirst MW, George SE, Peters KG (1998) Antiangiogenic gene therapy targeting the endothelium-specific receptor tyrosine kinase Tie2. Proc Natl Acad Sci USA 95:8829–8834

MacDonald PR, Progias P, Ciani B, Patel S, Mayer U, Steinmetz MO, Kammerer RA (2006) Structure of the extracellular domain of Tie receptor tyrosine kinases and localization of the angiopoietin-binding epitope. J Biol Chem 281:28408–28414

Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, Yancopoulos GD (1997) Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 277:55–60

Mandriota SJ, Pepper MS (1998) Regulation of angiopoietin-2 mRNA levels in bovine microvascular endothelial cells by cytokines and hypoxia. Circ Res 83:852–859

Marron MB, Hughes DP, McCarthy MJ, Beaumont ER, Brindle NP (2000a) Tie-1 receptor tyrosine kinase endodomain interaction with SHP2: potential signalling mechanisms and roles in angiogenesis. Adv Exp Med Biol 476:35–46

Marron MB, Hughes DP, Edge MD, Forder CL, Brindle NP (2000b) Evidence for heterotypic interaction between the receptor tyrosine kinases TIE-1 and TIE-2. J Biol Chem 275:39741–39746

Morisada T, Oike Y, Yamada Y, Urano T, Akao M, Kubota Y, Maekawa H, Kimura Y, Ohmura M, Miyamoto T, Nozawa S, Koh GY, Alitalo K, Suda T (2005) Angiopoietin-1 promotes LYVE-1-positive lymphatic vessel formation. Blood 105:4649–4656

Murray BW, Padrique ES, Pinko C, McTigue MA (2001) Mechanistic effects of autophosphorylation on receptor tyrosine kinase catalysis: enzymatic characterization of Tie2 and phospho-Tie2. Biochemistry 40:10243–10253

Nykanen AI, Krebs R, Saaristo A, Turunen P, Alitalo K, Yla-Herttuala S, Koskinen PK, Lemstrom KB (2003) Angiopoietin-1 protects against the development of cardiac allograft arteriosclerosis. Circulation 107:1308–1314

Oh H, Takagi H, Suzuma K, Otani A, Matsumura M, Honda Y (1999) Hypoxia and vascular endothelial growth factor selectively up-regulate angiopoietin-2 in bovine microvascular endothelial cells. J Biol Chem 274:15732–15739

Oliner J, Min H, Leal J, Yu D, Rao S, You E, Tang X, Kim H, Meyer S, Han SJ, Hawkins N, Rosenfeld R, Davy E, Graham K, Jacobsen F, Stevenson S, Ho J, Chen Q, Hartmann T, Michaels M, Kelley M, Li L, Sitney K, Martin F, Sun JR, Zhang N, Lu J, Estrada J, Kumar R, Coxon A, Kaufman S, Pretorius J, Scully S, Cattley R, Payton M, Coats S, Nguyen L, Desilva B, Ndifor A, Hayward I, Radinsky R, Boone T, Kendall R (2004) Suppression of angiogenesis and tumor growth by selective inhibition of angiopoietin-2. Cancer Cell 6:507–516

Partanen J, Armstrong E, Makela TP, Korhonen J, Sandberg M, Renkonen R, Knuutila S, Huebner K, Alitalo K (1992) A novel endothelial cell surface receptor tyrosine kinase with extracellular epidermal growth factor homology domains. Mol Cell Biol 12:1698–1707

Porat RM, Grunewald M, Globerman A, Itin A, Barshtein G, Alhonen L, Alitalo K, Keshet E (2004) Specific induction of tie1 promoter by disturbed flow in atherosclerosis-prone vascular niches and flow-obstructing pathologies. Circ Res 94:394–401

Puri MC, Rossant J, Alitalo K, Bernstein A, Partanen J (1995) The receptor tyrosine kinase TIE is required for integ-

rity and survival of vascular endothelial cells. EMBO J 14:5884–5891

Ramsauer M, D'Amore PA (2002) Getting Tie(2)d up in angiogenesis. J Clin Invest 110:1615–1617

Reusch P, Barleon B, Weindel K, Martiny-Baron G, Godde A, Siemeister G, Marme D (2001) Identification of a soluble form of the angiopoietin receptor TIE-2 released from endothelial cells and present in human blood. Angiogenesis 4:123–131

Saharinen P, Kerkela K, Ekman N, Marron M, Brindle N, Lee GM, Augustin H, Koh GY, Alitalo K (2005) Multiple angiopoietin recombinant proteins activate the Tie1 receptor tyrosine kinase and promote its interaction with Tie2. J Cell Biol 169:239–243

Sato N, Beitz JG, Kato J, Yamamoto M, Clark JW, Calabresi P, Raymond A, Frackelton AR, Jr. (1993a) Platelet-derived growth factor indirectly stimulates angiogenesis in vitro. Am J Pathol 142:1119–1130

Sato TN, Qin Y, Kozak CA, Audus KL (1993b) Tie-1 and tie-2 define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system. Proc Natl Acad Sci USA 90:9355–9358

Sato TN, Tozawa Y, Deutsch U, Wolburg-Buchholz K, Fujiwara Y, Gendron-Maguire M, Gridley T, Wolburg H, Risau W, Qin Y (1995) Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. Nature 376:70–74

Scharpfenecker M, Fiedler U, Reiss Y, Augustin HG (2005) The Tie-2 ligand angiopoietin-2 destabilizes quiescent endothelium through an internal autocrine loop mechanism. J Cell Sci 118:771–780

Schnurch H, Risau W (1993) Expression of tie-2, a member of a novel family of receptor tyrosine kinases, in the endothelial cell lineage. Development 119:957–968

Siemeister G, Schirner M, Weindel K, Reusch P, Menrad A, Marme D, Martiny-Baron G (1999) Two independent mechanisms essential for tumor angiogenesis: inhibition of human melanoma xenograft growth by interfering with either the vascular endothelial growth factor receptor pathway or the Tie-2 pathway. Cancer Res 59:3185–3191

Singh N, Macnamara E, Rashid S, Ambati J, Kontos CD, Higgins E, Ambati BK (2005) Systemic soluble Tie2 expression inhibits and regresses corneal neovascularization. Biochem Biophys Res Commun 332:194–199

Stratmann A, Risau W, Plate KH (1998) Cell type-specific expression of angiopoietin-1 and angiopoietin-2 suggests a role in glioblastoma angiogenesis. Am J Pathol 153:1459–1466

Sugimachi K, Tanaka S, Taguchi K, Aishima S, Shimada M, Tsuneyoshi M (2003) Angiopoietin switching regulates angiogenesis and progression of human hepatocellular carcinoma. J Clin Pathol 56:854–860

Sullivan CC, Du L, Chu D, Cho AJ, Kido M, Wolf PL, Jamieson SW, Thistlethwaite PA (2003) Induction of pulmonary hypertension by an angiopoietin 1/TIE2/serotonin pathway. Proc Natl Acad Sci USA 100:12331–12336

Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, Sato TN, Yancopoulos GD (1996) Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. Cell 87:1171–1180

Suri C, McClain J, Thurston G, McDonald DM, Zhou H, Oldmixon EH, Sato TN, Yancopoulos GD (1998) Increased vascularization in mice overexpressing angiopoietin-1. Science 282:468–471

Tammela T, Saaristo A, Lohela M, Morisada T, Tornberg J, Norrmen C, Oike Y, Pajusola K, Thurston G, Suda T, Yla-Herttuala S, Alitalo K (2005) Angiopoietin-1 promotes lymphatic sprouting and hyperplasia. Blood 105:4642–4648

Teichert-Kuliszewska K, Maisonpierre PC, Jones N, Campbell AI, Master Z, Bendeck MP, Alitalo K, Dumont DJ, Yancopoulos GD, Stewart DJ (2001) Biological action of angiopoietin-2 in a fibrin matrix model of angiogenesis is associated with activation of Tie2. Cardiovasc Res 49:659–670

Thistlethwaite PA, Lee SH, Du LL, Wolf PL, Sullivan C, Pradhan S, Deutsch R, Jamieson SW (2001) Human angiopoietin gene expression is a marker for severity of pulmonary hypertension in patients undergoing pulmonary thromboendarterectomy. J Thorac Cardiovasc Surg 122:65–73

Thurston G, Rudge JS, Ioffe E, Zhou H, Ross L, Croll SD, Glazer N, Holash J, McDonald DM, Yancopoulos GD (2000) Angiopoietin-1 protects the adult vasculature against plasma leakage. Nat Med 6:460–463

Valenzuela DM, Griffiths JA, Rojas J, Aldrich TH, Jones PF, Zhou H, McClain J, Copeland NG, Gilbert DJ, Jenkins NA, Huang T, Papadopoulos N, Maisonpierre PC, Davis S, Yancopoulos GD (1999) Angiopoietins 3 and 4: diverging gene counterparts in mice and humans. Proc Natl Acad Sci USA 96:1904–1909

Veikkola T, Alitalo K (2002) Dual role of Ang2 in postnatal angiogenesis and lymphangiogenesis. Dev Cell 3:302–304

Vikkula M, Boon LM, Carraway KL, III, Calvert JT, Diamonti AJ, Goumnerov B, Pasyk KA, Marchuk DA, Warman ML, Cantley LC, Mulliken JB, Olsen BR (1996) Vascular dysmorphogenesis caused by an activating mutation in the receptor tyrosine kinase TIE2. Cell 87:1181–1190

Visconti RP, Richardson CD, Sato TN (2002) Orchestration of angiogenesis and arteriovenous contribution by angiopoietins and vascular endothelial growth factor (VEGF). Proc Natl Acad Sci USA 99:8219–8224

Voskas D, Jones N, Van Slyke P, Sturk C, Chang W, Haninec A, Babichev YO, Tran J, Master Z, Chen S, Ward N, Cruz M, Jones J, Kerbel RS, Jothy S, Dagnino L, Arbiser J, Kleement G, Dumont DJ (2005) A cyclosporine-sensitive psoriasis-like disease produced in Tie2 transgenic mice. Am J Pathol 166:843–855

Ward NL, Dumont DJ (2002) The angiopoietins and Tie2/Tek: adding to the complexity of cardiovascular development. Semin Cell Dev Biol 13:19–27

White RR, Shan S, Rusconi CP, Shetty G, Dewhirst MW, Kontos CD, Sullenger BA (2003) Inhibition of rat corneal angiogenesis by a nuclease-resistant RNA aptamer specific for angiopoietin-2. Proc Natl Acad Sci USA 100:5028–5033

Wong AL, Haroon ZA, Werner S, Dewhirst MW, Greenberg CS, Peters KG (1997) Tie2 expression and phosphorylation in angiogenic and quiescent adult tissues. Circ Res 81:567–574

Yabkowitz R, Meyer S, Black T, Elliott G, Merewether LA, Yamane HK (1999) Inflammatory cytokines and vascular endothelial growth factor stimulate the release of soluble tie receptor from human endothelial cells via metalloprotease activation. Blood 93:1969–1979

Yao D, Taguchi T, Matsumura T, Pestell R, Edelstein D, Giardino I, Suske G, Ahmed N, Thornalley PJ, Sarthy VP, Hammes HP, Brownlee M (2006) Methylglyoxal modification of mSin3A links glycolysis to angiopoietin-2 transcription. Cell 124:275–286

Yuan HT, Venkatesha S, Chan B, Deutsch U, Mammoto T, Sukhatme VP, Woolf AS, Karumanchi SA (2007) Activation of the orphan endothelial receptor Tie1 modifies Tie2-mediated intracellular signaling and cell survival. FASEB J May 15 [Epub ahead of print]

Eph Receptors and Ephrins: Role in Vascular Development and Tumor Angiogenesis

7

DANA M. BRANTLEY-SIEDERS and JIN CHEN

Abstract

The Eph family of receptor tyrosine kinases and their membrane-bound ligands, the ephrins, play a crucial role in vascular remodeling during embryogenesis. More recently, these molecules have been shown to regulate postnatal vascular remodeling, particularly tumor neovascularization. This chapter provides an overview of Eph receptors and ephrins in vascular development and tumor angiogenesis. Recent advances in our understanding of how these molecules function in both tumor tissue and host vasculature suggest that several Eph/ephrin family members will make excellent new targets for anticancer therapy.

CONTENTS

7.1 Eph Receptors and Ephrin Ligands: Structure and Function

The Eph family of receptor tyrosine kinases (RTKs) is the largest family of RTKs in the genome, consisting of at least 16 receptors and 9 ligands identified in multiple species (reviewed in: Brantley-Sieders and Chen 2004; Cheng et al. 2002a; Pasquale 2005). Unlike typical RTKs that bind to soluble ligands, Eph RTKs bind to ephrin ligands that are tethered to the cell membrane. The family is subdivided into two classes based on homology and binding to two distinct classes of ephrins (Fig. 7.1). Class A Eph receptors generally bind to A class ligands that are tethered to the cell membrane by a glycosyl-phosphatidyl inositol (GPI) linkage. Class B Eph receptors generally bind to class B ligands that are anchored to the cell membrane by a transmem-

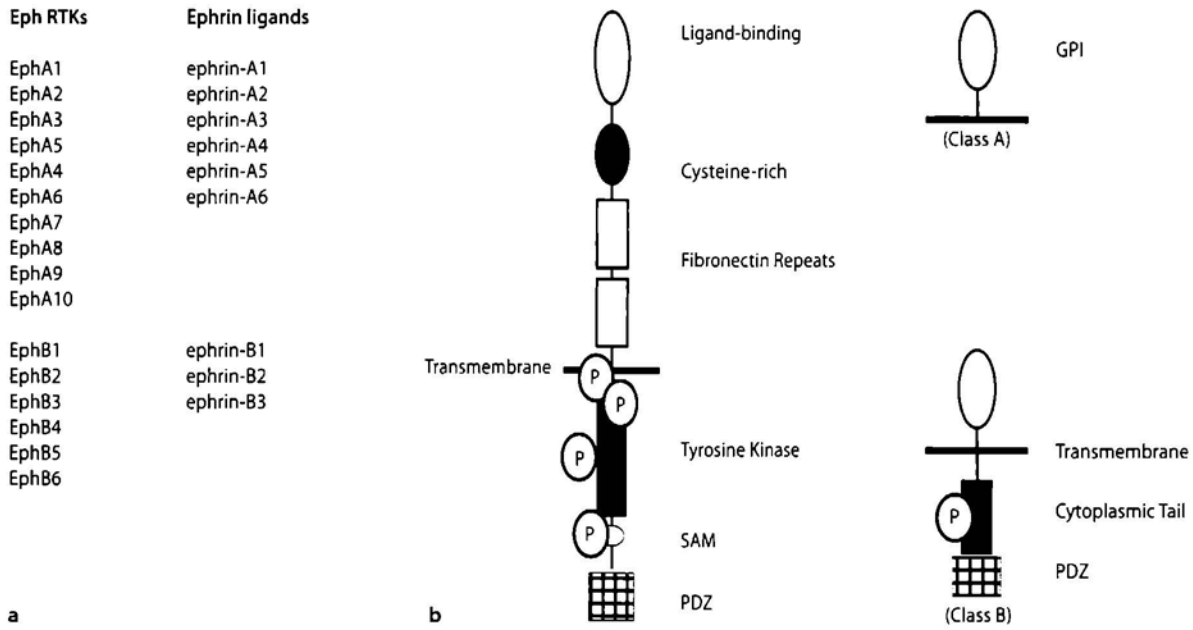


Fig. 7.1a,b. Eph family members: common structural features and signaling domains. a Sixteen Eph receptors and 9 ephrin ligands comprise the Eph family. b Structure and signaling domains common to receptors and ligands. The extracellular portion of Eph receptors share a ligand-binding domain followed by a cysteine-rich region and fibronectin type III repeats. A transmembrane spanning region is followed by a juxtamembrane region that harbors conserved tyrosine residues important in the activation of the receptor upon ligand engagement. This is followed by a kinase domain, which also contains tyrosine substrates that are targets for autophosphorylation. The kinase domain is followed by a sterile alpha motif (SAM), which is thought to regulate receptor oligomerization, and a PDS-95 postsynaptic density protein, discs large, zona occludens tight junction protein (PDZ)-binding domain, which binds to PDZ-containing proteins to create a scaffold for assembly of signaling molecules close to the cellular membrane. Ephrin ligands share an extracellular receptor-binding domain. A class ligands are anchored to the cellular membrane by a glycosyl-phosphatidyl inositol (GPI) linkage. Class B ligands are tethered to the cell membrane by a transmembrane spanning domain, followed by a cytoplasmic tail with conserved tyrosine residues that are targets for intracellular kinases. These ligands also contain a PDZ-binding domain

brane-spanning domain. In addition, class B ephrins possess a short cytoplasmic domain containing several tyrosine residues that can be phosphorylated by intracellular kinases, thereby facilitating "bi-directional" signaling through the ephrin as well as the Eph RTK. Bi-directional signaling is also thought to occur through class A ephrins via clustering and co-localization with intracellular signaling molecules in lipid raft membrane microdomains. Another relatively unique feature of this family relates to their effects on cell behavior. Rather than inducing proliferation upon activation like typical growth factor RTKs, Eph RTKs do not generally regulate cell growth responses. Instead, these receptors modulate signaling cascades that regulate cell shape, cellular adhesion, and cell motility (reviewed in: Brantley-Sieders and Chen 2004; Cheng et al. 2002a; Pasquale 2005).

Structurally, Eph RTKs consist of an amino-terminal ephrin-binding domain, immunoglobulin-like motifs, a cysteine-rich region containing an epidermal growth factor (EGF)-like motif, and two fibronectin type III repeats in the extracellular portion (Fig. 7.1). These domains are followed by a transmembrane domain and the intracellular portion of the receptor, which includes a juxtamembrane region, a kinase domain, a sterile α-motif (SAM), and a PDS-95 postsynaptic density protein,

Discs large, Zona occludens tight junction protein (PDZ)-binding domain (Fig. 7.1). The juxtamembrane region contains tyrosine residues that are involved in kinase domain activation upon phosphorylation and subsequent receptor conformational change. Activation of the kinase leads to transphosphorylation of clustered Eph RTKS upon ligand binding, creating phosphotyrosine-docking sites to initiate downstream signaling cascades by binding to SH2-containing proteins. In addition, kinase-independent signaling has also been reported for several Eph RTKS (Pasquale 2005). The SAM domain is thought to regulate receptor dimerization/clustering, and the PDZ-binding domain binds to other PDZ-containing proteins and is thought to create scaffolds for assembly of signaling complexes proximal to the cell membrane (reviewed in: Brantley-Sieders and Chen 2004; Cheng et al. 2002a; Pasquale 2005). These functional domains modulate forward signaling through the receptor upon ligand stimulation, and downstream signaling pathways relevant to vascular remodeling are discussed in more detail in the following sections.

Eph Receptors and Ephrins in Embryonic Vascular Development

Embryonic vascular morphogenesis involves remodeling of primitive, relatively homogeneous networks of embryonic and extraembryonic blood vessels, or primitive capillary plexus, into a branching network of large and small mature, interconnected vessels (Risau and Flamme 1995). Primary capillary plexus form through de novo differentiation and coalescence of endothelial progenitors, or hemangioblasts, through the process of vasculogenesis. Subsequent angiogenic remodeling occurs through sprouting of new branches, retraction of existing branches, joining of some capillaries and splitting of others. Pruning and remodeling occur through endothelial cell proliferation, apoptosis, and migration (Patan 2000; Yancopoulos et al. 2000). Vessels fully mature and become functional by recruitment of mural supporting cells from surrounding mesenchyme (Folkman and D'Amore 1996; Yancopoulos et al. 2000).

Several Eph family members participate in vascular remodeling in the embryo. Expression of Eph RTKs and ephrins has been detected in embryonic vasculature, particularly B class receptors and ligands. In mice, *Xenopus*, and chick, ephrin-B2 is expressed in arterial endothelial cells, including the extraembryonic yolk sac primary capillary plexus, large arteries within the embryo, and in the endocardium of the developing heart. The principal receptor for ephrin-B2, EphB4, displays a reciprocal expression pattern in embryonic veins in the yolk sac, larger veins including the anterior cardinal vein and vitelline vein, and also in endocardium (reviewed in: Brantley-Sieders and Chen 2004). This was the first evidence for a molecular differences between arterial and venous endothelial cells. Targeted disruption of either ephrin-B2 or EphB4 results in death of the embryo at E11 and E9.5–10, respectively, due to similar defects in angiogenic remodeling of both arteries and veins, as well as patterning defects in myocardium (Gerety et al. 1999; Wang et al. 1998). Differentiation of endothelial cells and vascular morphogenesis was normal in homozygous null embryos, with the formation of primitive capillary network structures. These networks, however, fail to remodel and branch into large and small vessels. Reverse signaling through ephrin-B2 also regulates angiogenesis in mice, as demonstrated by replacement of the endogenous ephrin-B2 gene with a cytoplasmic deletion mutant. Ephrin-B2$^{\Delta C/\Delta C}$ "knock-in" mutants display similar defects in remodeling of vessels in the yolk sac and in the embryo, and in heart morphogenesis (Adams et al. 2001). These data demonstrate that bi-directional signaling through both EphB4 and ephrin-B2 facilitates embryonic vascular remodeling and may distinguish arterial and venous differentiation in vivo. In addition, recent results from mice in which endogenous ephrin-B2 was replaced with a PDZ-binding domain deletion (ephrin-B2$^{\Delta V/\Delta V}$) or cytoplasmic tyrosine to phenylalanine replacement mutant (ephrin-B2$^{5F/5F}$) knock-in demonstrate the function of ephrin-B2 in lymphatic vessel remodeling (Makinen et al. 2005). While vas-

Table 7.1. Vascular phenotypes of Eph and ephrin mutant animals

Mouse strain	Vascular phenotype	Reference
EphA2$^{-/-}$<?1>	Impaired ephrin-mediated subcutaneous vessel remodeling; Impaired tumor angiogenesis	Brantley-Sieders et al. 2004, 2005
EphB2$^{-/-}$ / EphB3$^{-/-}$ (double knockout)	30% embryonic lethality E11; defective vascular remodeling, sprouting, and heart defects	Adams et al. 1999
EphB4$^{-/-}$	100% embryonic lethal E11.5; defective vascular remodeling, sprouting, heart defects	Gerety et al. 1999
Ephrin-B2$^{-/-}$	100% embryonic lethal E9.5–10; defects similar to those observed in EphB4–/–	Wang et al. 1998
[a]Endothelial ephrin-B2–/–	100% embryonic lethal E10.5; defects similar to those observed in conventional ephrin-B2 knockout	Gerety et al. 2002
[b]Ephrin-B2$^{\Delta C/\Delta C}$	100% embryonic lethal E10.5; similar defects to those observed in conventional ephrin-B2 knockout	Adams et al. 2001
[b]Ephrin-B2$^{\Delta V/\Delta V}$	No embryonic vascular phenotype; major defects in lymphatic vascular remodeling	Makinen et al. 2005
[b]Ephrin-B2$^{5F/5F}$	No embryonic vascular phenotype; mild defects in lymphatic vascular remodeling	Makinen et al. 2005
[c]CAG-ephrin-B2	Embryonic to early postnatal lethality; abnormal patterning intersomitic vessels; defective capillary-sized arterial–venous boundary formation; postnatal death from aortic aneurysms due to lack of vascular smooth muscle cells	Oike et al. 2002
[c]Tie2-ephrin-B2	Defective capillary-sized arterial–venous boundary formation	Oike et al. 2002

[a] Tissue-specific knockout of ephrin-B2 in endothelial cells by mating Floxed ephrin-B2 mice with Tie2-Cre mouse model system.

[b] "Knock in" animals in which the endogenous gene is replaced with a mutant version. $^{\Delta C/\Delta C}$, replacement with ephrin-B2 lacking C-terminal intracellular domain; $^{\Delta V/\Delta V}$, replacement with ephrin-B2 lacking C-terminal PDZ-binding domain; $^{5F/5F}$, replacement with ephrin-B2 in which five conserved tyrosine residues in the cytoplasmic tail were replaced with phenylalanine.

[c] Transgenic animals in which gene produce is overexpressed in specific tissues. CAG-ephrin-B2 transgenics overexpress ephrin-B2 ubiquitously under the control of the CMV enhancer-β-actin promoter-β-globin splicing acceptor. Tie2-ephrin-B2 transgenics overexpress ephrin-B2 specifically in endothelial cells.

cular remodeling defects observed in the conventional ephrin-B2-deficient embryos was not observed in either of these lines, they did present a mild (ephrin-B2$^{5F/5F}$) to severe (ephrin-B2$^{\Delta V/\Delta V}$) phenotype in terms of failure to remodel the primitive lymphatic plexus into large and small vessels, hyperplasia, and lack of valve formation (Makinen et al. 2005). These data demonstrate that signaling through distinct domains within the same Eph family member can elicit very different biological processes in vivo.

Ephrin-B2 is also expressed in mesenchyme surrounding some blood vessels, and becomes extended to smooth muscle cells and pericytes surrounding vessels as development proceeds (reviewed in: Brantley-Sieders and Chen 2004; Cheng

et al. 2002a). Experiments in which ephrin-B2 was overexpressed ubiquitously or in endothelial cells specifically suggest that this ligand also affects the mesenchymal component of the vasculature. Defects in intersomitic vessel patterning and outgrowth of venous vasculature was observed in the head region of transgenic embryos in which ephrin-B2 overexpression was ubiquitous. These embryos die shortly after birth from aortic aneurysms that occur due to lack of perivascular support surrounding the aorta. By contrast, transgenic embryos expressing ephrin-B2 specifically in endothelium did not display such defects (Oike et al. 2002). Tissue-specific deletion of ephrin-B2 in endothelium and endocardium, however, was sufficient to recapitulate angiogenic remodeling defects observed in conventional knockout animals (Gerety and Anderson 2002). Since the full complement of vascular defects is produced by deletion of ephrin-B2 in endothelium, while mesenchymal expression remained intact, these data suggest that mesenchymal ephrin-B2 is not sufficient for vessel remodeling. Mesenchymal expression of ephrin-B2 might, however, be necessary for proper remodeling, as demonstrated by several in vitro studies. For example, mesenchymal expression of ephrin-B2 was shown to enhance differentiation of paraaortic splanchnopleuric mesoderm and endothelial precursor-enriched cell populations within this tissue into endothelium, whereas overexpression of EphB4 was inhibitory (Zhang et al. 2001). Differentiation induced by mesenchymal ephrin-B2 was accompanied by morphogenesis into cord-like tubules and enhanced smooth muscle cell recruitment, demonstrating the importance of mesenchymal ephrin-B2 in vascular morphogenesis and maturation. Generation of mice in which ephrin-B2 is deleted only in mesenchymal cells could shed light on the function of ephrin-B2 in this tissue type.

Ephrin-B2 and EphB4 are not the only Eph family members that regulate embryonic vessel patterning. Ephrin-A1 is expressed in the developing vasculature, and promotes angiogenesis in vitro and in vivo (reviewed in: Brantley-Sieders and Chen 2004; Cheng et al. 2002a). Though no data are yet available concerning the role of ephrin-A1 in embryonic angiogenesis, this ligand and its principal receptor, EphA2, are known to regulate postnatal angiogenesis as discussed in the sections below. Ephrin-B1 is also expressed in embryonic vasculature, in both arteries and veins, as is EphB3 RTK. In addition, EphB2 RTK is expressed in vascular-associated mesenchyme (Adams et al. 1999). Although targeted disruption of EphB2 or EphB3 alone produced no discernable vascular phenotype, approximately 30% of double mutants die at E11 due to vascular remodeling defects in the head, heart, and intersomitic regions of the embryo, demonstrating that these EphB RTKs also participate in developmental angiogenesis (Adams et al. 2001). While ephrin-B1 expression cannot compensate for the loss of ephrin-B2 in null mutants, in vitro studies have demonstrated that this ligand can induce angiogenic responses in cultured endothelial cells, as can reverse signaling through ephrin-B1 (reviewed in: Brantley-Sieders and Chen 2004; Cheng et al. 2002a). These data suggest that the ephrin-B1 ligand might also be necessary, though not sufficient, for vascular remodeling during embryogenesis. Vascular phenotypes of Eph and ephrin mutants are summarized in Table 7.1

Eph Receptors and Ephrins in Postnatal Vascular Remodeling

In addition to embryonic vascular patterning, angiogenic processes also regulate tissue homeostasis in mature organisms. Postnatal vascular remodeling promotes healing in wounds, reperfusion of tissues after ischemic injury, and cyclic angiogenic remodeling that occurs in the female reproductive tract. Postnatal angiogenesis is also regulated by many Eph family members. Ephrin-B2 expression persists in adult arterial endothelium and vascular smooth muscle cells surrounding arteries, while EphB4 expression persists in adult venous endothelium, suggesting that this ligand–receptor pair may regulate boundary maintenance and/or vascu-

lar remodeling in mature tissues (Gale et al. 2001; Shin et al. 2001). Indeed, soluble ephrin-B2 facilities adhesion and migration of endothelial cells in culture, processes critical for angiogenic remodeling (Vindis et al. 2003). Soluble ephrin-A1, ephrin-B2, and the ectodomain of EphB1 induce corneal angiogenesis in adult mice, demonstrating that these mature endothelial cells have the capacity to respond to ephrin and Eph RTK signals (reviewed in: Brantley-Sieders and Chen 2004; Cheng et al. 2002a). More recently, upregulated ephrin-B2 expression was observed in arterial vessels following ischemic injury in vivo, as well as in response to VEGF, bFGF, and hepatocyte growth factor (HGF) in cultured endothelial cells in vitro (Hayashi et al. 2005). Interestingly while both VEGF and ephrin-B2 stimulated corneal neovascularization in vivo, ephrin-B2 treatment promoted venous angiogenesis with limited arterial angiogenesis (Hayashi et al. 2005), suggesting that ephrin-B2 promotes venous vascular remodeling in vivo.

In addition, ephrin-B2 and ephrin-A1 can also induce an angiogenic response from subcutaneous vessels in vivo (Brantley-Sieders et al. 2004b; Maekawa et al. 2003). Moreover, hypoxia results in upregulated expression of ephrin-B2 and ephrin-A1, as well as EphB4 and EphA2, in a novel mouse skin flap hypoxia model and in cell lines subjected to hypoxic conditions (Vihanto et al. 2005). Treatment of these cells with small interfering RNAs (siRNAs) that diminished expression of the transcription factor hypoxia-inducible factor-α (HIF-1α) abrogated upregulation of Eph/ephrin expression, providing a molecular link between hypoxia and regulation of pro-angiogenic Eph family members (Vihanto et al. 2005). Ephrin-A1 and EphA2 appear to cooperate in postnatal vascular remodeling. When ephrin-A1-containing sponges were introduced into EphA2-deficient mice, the angiogenic response was greatly diminished, suggesting that vascular remodeling in response to ephrin-A1 requires EphA2 RTK (Brantley-Sieders et al. 2004b). Lung microvascular endothelial cells isolated from adult mice can also respond to ephrin-A1, which induces assembly and migration in vitro (Brantley et al. 2002; Brantley-Sieders et al. 2004b). These processes are dependent upon expression of EphA2 RTK, as endothelial cells isolated from EphA2-deficient mice display impaired angiogenic responses to ephrin-A1, and as these responses are rescued upon restoration of EphA2 expression (Brantley-Sieders et al. 2004b). Analysis of EphA2-deficient endothelial cells also provides intriguing evidence of cooperation between Eph family members in angiogenic processes. We recently reported that EphA2-deficient endothelial cells display elevated expression of EphB4 and ephrin-B2, suggesting that these Eph family members may partially compensate for the loss of EphA2 (Brantley-Sieders et al. 2005). It would be of great interest to determine whether combined loss of EphA2 and EphB4 or ephrin-B2 augments vascular remodeling defects. EphA2 may also contribute to differentiation of perivascular support cells, as suggested by studies in 10T1/2 cells, which can be induced to differentiation into pericytes/vascular smooth muscle cells upon stimulation with transforming growth factor β (TGFβ). Microarray analysis of 10T1/2 cells comparing untreated cells to cells treated and induced to differentiate in response to TGFβ revealed EphA2 as a gene that was upregulated in differentiating cells (Kale et al. 2005). This upregulation was confirmed by immunohistochemical analysis of EphA2 protein in treated cells versus unstimulated controls, suggesting that EphA2 may function in the mesenchymal component of the vasculature as well as in endothelium.

As Eph RTKs have been correlated and/or functionally implicated in pathogenesis of several angiogenesis-dependent diseases, including cancer, these studies provide a rationale for the development of Eph RTK-targeted therapies in the treatment of such diseases. In the case of tumor progression, Eph RTKs and ephrins function in both tumor cells and host vessels for several types of cancer, making these molecules attractive targets for therapeutic intervention. The function of Eph RTKs in tumor neovascularization and progression, as well as emerging strategies for targeting these molecules for clinical applications, are discussed below.

Eph Receptors and Ephrins in Tumor Angiogenesis

Acquisition of blood vessels by tumors is critical for growth, survival, and malignant progression. Solid tumors can grow to only a few millimeters in diameter without oxygen and nutrients provided by supporting blood vessels. Moreover, circulating growth factors and cytokines that facilitate tumor progression are delivered to the tumor via the tumor vasculature. Tumor vessels provide a permissive environment for growth and survival, and may actively promote malignant progression by enabling entry into circulation for dissemination of metastatic cells (Folkman 2002). Tumors may initially or exclusively "co-opt" existing host vessels for support (reviewed in: Ribatti et al. 2003), and more malignant tumor cells are able to behave like blood vessels by forming tube-like structures and activating expression of vascular adhesion molecules through the process of vascular mimicry (Hendrix et al. 2003). More recent evidence suggests that de novo differentiation of circulating endothelial cell progenitors that incorporate into tumor vessels also contributes to tumor neovascularization (reviewed in: Patan 2004; Zammaretti and Zisch 2005). However, the majority of tumors recruit new vessels through angiogenic sprouting from host vessels (reviewed in: Folkman 2002; Griffioen and Molema 2000). In addition to regulation of developmental angiogenesis, Eph RTKs and ephrins have recently emerged as critical regulators of tumor angiogenesis.

Overexpression of Eph RTKs has been observed in several types of cancer in mouse models as well as human tumor biopsies (reviewed in: Cheng et al. 2002a; Dodelet and Pasquale 2000; Sullivan and Bicknell 2003). For example, ephrin-B2 expression has been observed in tumor arterioles infiltrating transplanted Lewis lung carcinomas and B16 melanomas in mice, suggesting that this ligand may regulate tumor neovascularization (Gale et al. 2001; Shin et al. 2001). In support of this hypothesis, A375 melanomas, which overexpress endogenous EphB4, form smaller, less vascularized tumors in the presence of soluble, monomeric EphB4 in vivo (Martiny-Baron et al. 2004). Soluble EphB4 may interfere with binding of endogenous EphB4 on tumor cells with endothelium expressing ephrin-B2, thus disrupting tumor angiogenesis. This hypothesis is supported by studies in which ephrin-B2 was overexpressed in colorectal cancer cells or in which a truncated EphB4 receptor construct was overexpressed in breast cancer cells (Liu et al. 2004; Noren et al. 2004). While ephrin-B2 overexpression decreased tumor growth in xenografts, microvascular density within colon tumors was signficantly elevated (Liu et al. 2004). Overexpression of a truncated EphB4 receptor construct in which the intracellular portion was deleted while the extracellular ligand-binding domain remained intact produced increased tumor growth and vascularity in mammary tumors, probably via recruitment of ephrin-B2-expressing host endothelium through reverse signaling (Noren et al. 2004). Ephrin-B1 overexpression has been reported in hepatocellular carcinoma, and overexpression of ephrin-B1 enhances tumor neovascularization in vivo (Sawai et al. 2003). Although proliferation of ephrin-B1-overexpressing cells was not affected in culture, soluble ephrin-B1 enhanced endothelial cell proliferation and migration in vitro, suggesting that at least one function of ephrin-B1 in tumor progression involves recruitment of blood vessels through angiogenesis (Nagashima et al. 2002; Sawai et al. 2003). Taken together, these studies reveal a critical role for class B receptors and ligands in tumor progression and vascular recruitment for multiple types of human cancer.

EphA2 overexpression has been detected in melanoma, prostatic adenocarcinoma, breast adenocarcinoma, invasive ovarian and cervical carcinoma, esophageal cancer, and colorectal cancer (reviewed in: Brantley-Sieders et al. 2004a; Brantley-Sieders and Chen 2004; Ireton and Chen 2005). Ogawa et al. first noted co-expression of EphA2 RTK and its principal ligand, ephrin-A1, in both tumor cells and tumor endothelium, suggesting that this receptor–ligand pair might contribute to tumor angiogenesis (Ogawa et al. 2000). Similar expression patterns were also observed in two independent mouse models of angiogenesis-dependent cancer,

the RIP-Tag transgenic model of islet cell adenocarcinoma and the 4T1 transplantable model of mammary epithelial adenocarcinoma (Brantley et al. 2002). Expression of ephrin-A1 was predominantly detected in tumor cells, while the majority of EphA2 RTK protein localized to tumor-associated endothelium, suggesting that ephrin-A1 might serve as a pro-angiogenic signal to attract EphA2-positive endothelial cells. To test this hypothesis, tumor-bearing animals were treated with soluble EphA-Fc fusion proteins in which the ligand-binding ectodomain of EphA2 or EphA3 RTK was fused to human IgG. These reagents bind to multiple ephrinA ligands and disrupt binding and signaling through endogenous EphA RTKs (Cheng et al. 2002b). Soluble receptor (EphA2- or EphA3-Fc) treatment of 4T1 tumors transplanted into syngeneic Balb/c mice resulted in decreased tumor volume, proliferation, and survival accompanied by a decrease in microvascular density within the tumor (Brantley et al. 2002). EphA-Fc proteins also impaired endogenous tumor progression in RIP-Tag mice (Cheng et al. 2003). Soluble EphA-Fc proteins did not alter growth or survival of tumor cells in culture, suggesting that the effects observed in vivo were secondary, probably due to impaired recruitment of blood vessels supplying nutrients and oxygen necessary for growth and survival of the tumor (Brantley et al. 2002). Supporting this hypothesis, soluble EphA-Fc treatment impaired infiltration of host vessels into transplanted 4T1 and RIP-Tag tumors in cutaneous window assays (Brantley et al. 2002). Though soluble receptors do not affect proliferation or apoptosis in cultured endothelial cells, EphA-Fc proteins do impair ephrin-A1- and VEGF-induced cellular migration and sprouting (Brantley et al. 2002; Cheng et al. 2002b), suggesting that these reagents might interfere with tumor angiogenesis at the level of endothelial migration. Indeed, EphA-Fc inhibited 4T1 and RIP-Tag tumor cell-induced migration of endothelial cells in co-culture assays (Brantley et al. 2002; Cheng et al. 2003). A recent study further confirmed the anti-angiogenic activity of soluble EphA2-Fc. In this report, soluble EphA2-Fc inhibited outgrowth of new vessel sprouts from explanted aortic rings, while EphB1-Fc and EphB3-Fc induced microvessel sprouting, suggesting that EphA and EphB RTKs might have different functions in angiogenesis (Dobrzanski et al. 2004). Soluble EphA2-Fc also inhibited VEGF/FGF-induced neovascularization in Matrigel plugs, tumor angiogenesis and progression of ASPC-1 human pancreatic carcinoma xenografts, and growth and metastasis of orthotopic human pancreatic ductal adenocarcinoma (Dobrzanski et al. 2004). These data indicate that the effects of soluble EphA-Fc treatment on tumor progression involve impairment of tumor angiogenesis.

These initial studies demonstrated that class A Eph RTKs play a role in tumor angiogenesis, probably at the level of endothelial cell migration. However, since soluble receptors are global inhibitors of EphA RTK signaling, the specific class A family member target(s) in endothelium remained unidentified. Based on expression data, and the observations that endothelial cells expressing dominant negative EphA2 display defective assembly (Ogawa et al. 2000) and RIP-Tag tumor-induced migration in co-culture experiments (Cheng et al. 2003), EphA2 was the most likely candidate. To test this hypothesis, EphA2-deficient mice were analyzed. Targeted disruption of EphA2 does not affect embryonic vascular remodeling (Joseph C. Ruiz, unpublished observations; (Brantley-Sieders et al. 2004b), consistent with the lack of expression in embryonic vasculature (Ruiz and Robertson 1994). Since EphA2 is expressed in endothelial cells from mature tissues, the angiogenic potential of EphA2-deficient endothelial cells was assessed. Primary lung microvascular endothelial cells isolated from EphA2-deficient mice displayed defects in ephrin-A1 induced vascular assembly and migration in vitro relative to cells isolated from control littermates, as well as impaired assembly in vivo when transplanted into nude mice. Migration in response to ephrin-A1 requires phosphoinoside-3 kinase (PI-3 K) and Rac1 GTPase activation, the activation of which is impaired in EphA2-deficient endothelial cells (Brantley-Sieders et al. 2004b). Moreover, EphA2-deficient mice display defective angiogenic remodeling of endogenous subcutaneous vessels in response to ephrin-A1 (Brantley-Sieders et al. 2004b), suggesting that EphA2 RTK is necessary for postnatal angiogenesis

and possibly for tumor angiogenesis. Indeed, transplantation of 4T1 cells into EphA2-deficient host animals produced smaller, less vascularized tumors and impaired metastasis in vivo compared to wild-type or heterozygous controls (Brantley-Sieders et al. 2005). These defects are, at least in part, endothelial cell intrinsic, as co-transplantation of 4T1 tumor cells plus EphA2-deficient endothelial cells into wild-type hosts resulted in smaller tumors than in control endothelial cells, as well as failure of EphA2-deficient endothelial cells to survive and contribute to tumor vasculature (Brantley-Sieders et al. 2005).

While expression analysis has correlated ephrin-A1 with tumor neovascularization and progression in several mouse models of cancer and in human samples, functional evidence for ephrin-A-mediated regulation of tumor neovascularization has not been reported. Preliminary studies from our laboratory suggest that ephrin-A1 indeed regulates vascular recruitment by tumor cells in vivo. Using siRNAs to downregulate expression of ephrin-A1 in 4T1 tumor cells, we observed decreased tumor neovascularization and metastasis of ephrin-A1 "knockdown" cell lines versus controls (Brantley-Sieders et al. 2006). These data, coupled with an observed decrease in ephrin-A1 knockdown tumor cell-mediated endothelial cell migration in vitro, suggest that ephrin-A1 is a pro-angiogenic factor in tumors.

Cross-Talk Between Eph/Ephrin Family Members and Other Pro-Angiogenic Factors

Because ephrin ligands are membrane-anchored rather than soluble, the mechanism of interaction with Eph/ephrin-expressing host endothelium, which is initially distant from the tumor mass, remains unclear. One attractive explanation involves cooperation between Eph family members and other pro-angiogenic factors that are soluble. For example, substantial evidence exists for cooperation between EphA and VEGF RTK, particularly since VEGF also contributes to angiogenesis in RIP-Tag and 4T1 tumors (Bergers et al. 2000; Prewett et al. 1999). EphA-Fc treatment not only impairs ephrin-A1-induced corneal angiogenesis, it also significantly inhibits corneal neovascularization in response to VEGF (Cheng et al. 2002b, 2003). Soluble EphA receptors also inhibit VEGF-mediated endothelial cell survival, sprouting, migration, and assembly in vitro (Brantley et al. 2002; Cheng et al. 2002b). In addition, combined delivery of soluble EphA2-Fc and VEGFR2 inhibitors inhibit endothelial sprouting in aortic ring assays in a synergistic fashion, more effectively than delivery of a single inhibitor (Dobrzanski et al. 2004). More recently, it was reported that vaccination to produce an immune response targeting EphA2 inhibits VEGF-induced angiogenesis in vivo (Hatano et al. 2004). EphA2 RTK specifically cooperates with VEGF, as EphA2-deficient endothelial cells fail to undergo vascular assembly and migrate in response to VEGF as well as ephrin-A1 (Chen et al. 2005). Since VEGF, unlike ephrin ligands, is a soluble signal, VEGF signaling may initiate angiogenesis by activating host endothelial cells and inducing proliferation, a process not affected by EphA-Fc (Cheng et al. 2002b). Once host vessels infiltrate the tumor, membrane-bound ephrinA ligands may be able to bind to EphA2 RTK on adjacent endothelial cells to facilitate migration and assembly into functional tumor vessels.

Alternatively or in addition to this mechanism of activation, VEGF may also modulate ephrin-A1 expression and subsequent function within endothelial cells. Treatment of cultured endothelial cells with VEGF enhances expression of ephrin-A1 and subsequent phosphorylation of EphA2 (Cheng et al. 2002b). It is therefore possible that juxtacrine ephrin–EphA2 signaling initiated by VEGF could contribute to vascular remodeling. Studies performed in cultured cells suggest that ephrin-A1 may also mediate retraction of vascular smooth muscle cells in vascular remodeling. While ephrin-A1 stimulates chemotaxis in endothelial cells through Rac1 activation (Brantley-Sieders et al. 2004b), treatment of vascular smooth muscle cells with ephrin-A1 produces the opposite effect, with inhibition of Rac1 and cell spreading (Deroanne et al. 2003). Additional studies demonstrated that ephrin-A1 treatment activates

RhoA through EphA4 RTK-mediated activation of a novel guanine nucleotide exchange factor in vascular smooth muscle cells, Vsm-RhoGEF (Ogita et al. 2003), which could also contribute to smooth muscle contractility. Ephrin-A1 may therefore promote angiogenic remodeling not only through modulating endothelial cell migration and morphogenesis, but also, perhaps, by causing vascular smooth muscle cell retraction. This could facilitate exposure of endothelium to angiogenic stimuli and assist movement of endothelial cells by disrupting the mural cell barrier.

Cooperation and/or regulation of class B Eph RTKs and ephrins is also quite likely to contribute to tumor angiogenesis. The pro-angiogenic factors VEGF, bFGF, and HGF can upregulate expression of ephrin-B2 in cultured endothelial cells, whereas the vessel maturation factor angiopoetin-1 downregulates expression (Hayashi et al. 2005). A recently published microarray analysis revealed that mRNA levels for EphB4, ephrin-B1, ephrin-B2, and ephrin-A1 are downregulated in human microvascular endothelial cells treated with the angiostatic factor endostatin, which was confirmed by RT-PCR (Abdollahi et al. 2004). Although vasculogenesis appears to occur normally in EphB4-deficient mice (Gerety et al. 1999), a recent report suggests that EphB4 may modulate differentiation of hemangioblasts in cooperation with other pro-angiogenic factors. Wang et al. report that EphB4-deficient embryoid bodies display delayed expression of the hemangioblast marker vascular endothelial growth factor receptor-2 (VEGFR-2/Flk-1), as well as defective vascular morphogenesis in response to VEGF and basic fibroblast growth factor (bFGF) in vitro (Wang et al. 2003). These data suggest that EphB RTKs and ephrin signaling may subtly impact vasculogensis, and might explain why EphB4-deficient embryos die sooner than ephrin-B2-null mutants (Gerety et al. 1999; Wang et al. 1998). Thus, ephrins might regulate sensitivity to earlier vascular developmental cues in addition to exerting direct effects on angiogenic remodeling of embryonic vasculature. These data highlight the complex regulation of tumor angiogenesis that is facilitated by cooperation between multiple pro-angiogenic pathways, including those mediated by the Eph family.

Potential Therapeutic Strategies Targeting the Eph Family: Pros and Cons

Due to the wealth of recently published studies demonstrating a role for the Eph family in tumor angiogenesis, it is not surprising that preclinical investigation into potential therapeutic strategies targeting these family members has been initiated. One important consideration deals with the broad expression profiles of these molecules in both tumor tissue and host, including vascular endothelium. Because many of these single factors influence tumor progression in both tumor cells and host microenvironment, these molecules are attractive targets for therepeutic intervention, as the potential exists for targeting multiple aspects of tumor progression through modulation of a single factor. However, the multiple, complex functions of Eph family members in both normal tissue homeostasis and in malignant progression demand a thorough understanding of both normal functions and roles in malignancy, as well as identification of stage and of precise target tissues so as to avoid undesirable side effects or unintentional exacerbation of disease progression.

In terms of therapeutic agents, soluble receptor or soluble ligand-mediated inhibition of Eph signaling represents the best-developed strategy in preclinical models of cancer. These fusion proteins consist of the extracellular domain of receptor or ligand fused to human IgG Fc, or to some other protein component that enables relatively easy purification while eliminating the potential for dimerization. These proteins are able to bind and saturate endogenous receptors/ligands when delivered in vitro or in vivo, thus inhibiting endogenous signaling. Soluble EphA class fusion proteins, including EphA2-Fc and EphA3-Fc, have been used quite successfully to inhibit tumor angiogenesis and progression in vivo (Brantley et al. 2002; Cheng et al. 2003; Dobrzanski et al. 2004), as discussed above. Given in vitro studies in which these fusion proteins were able to abrogate endothelial cell migration, sprouting, and morphogenesis in response to ephrin-A1, it appeared that this was the major mechanism by

which these reagents impaired tumor progression. Other reports, however, demonstrate that class A Eph receptors, particularly EphA2, function within tumor cells to facilitate tumor progression. EphA2 expression has been observed in both tumor vasculature and tumor parenchyma (reviewed in: Brantley-Sieders et al. 2004a; Brantley-Sieders and Chen 2004; Ireton and Chen 2005). As overexpression of EphA2 in non-malignant cells induces oncogenic transformation in vitro and tumor formation in vivo (Zelinski et al. 2001), while siRNA-mediated inhibition of EphA2 expression impairs malignant progression (Duxbury et al. 2004a; Landen et al. 2005), it is quite possible that soluble receptors also impair tumor cell-intrinsic processes that contribute to malignancy. This is especially plausible given that host EphA2-deficiency alone produces a milder phenotype than global inhibition of EphA2 in host and tumor cells by soluble receptors in the 4T1 model (Brantley et al. 2002; Brantley-Sieders et al. 2005). Moreover, overexpression of dominant-negative mutant EphA2 receptors in 4T1 cells impair tumor progression and metastasis in vivo, providing further support for multiple functions of EphA2 in both tumor and host (Fang et al. 2005).

Soluble ligands or antibody ligand mimetics have been proposed as an effective strategy for targeting EphA2 receptor function in tumor cells. The interaction between an activating antibody (Carles-Kinch et al. 2002) or soluble ephrin-A1-Fc (Noblitt et al. 2004) and tumor cell-expressed EphA2 receptor is thought to facilitate receptor internalization and degradation, thus disrupting the malignant function of the receptor by reduced surface expression. Treatment of tumor cells or tumors expressing EphA2 receptor with soluble ephrin-A1-Fc can also reduce malignancy by modulating focal adhesion kinase (FAK) activation or expression and subsequent invasiveness (Duxbury et al. 2004b; Miao et al. 2000). These strategies present a risk, however, of enhancing malignancy in some types of cancer, based on studies showing that elevated kinase activity of EphA2 can enhance malignancy (Fang et al. 2005) and based on the role of ephrin-A1 in stimulating tumor angiogenesis in vitro and in vivo (Brantley-Sieders et al. 2004b, 2005; Cheng et al. 2003) (Brantley-Sieders et al. 2006). More information on the molecular pathways that determine positive or negative effects of targeting EphA2 through soluble ligands will be necessary before full clinical development of this strategy.

Soluble receptor strategies have also been employeed to target interactions between endogenous class B receptors and ligands. Again, as a note of caution, systemic delivery of soluble receptors presents a risk in terms of the ability of these reagents to inhibit interactions between multiple receptors and ligands that are present in both the target disease organ and in healthy tissue. In addition, activation of reverse signaling by soluble EphB receptors has been validated in several models, adding another layer of complexity to their potential use in therapeutics. For example, although monomeric soluble EphB4 inhibits tumor growth and reduces microvascular density (Martiny-Baron et al. 2004), cell surface expression of a truncated EphB4 construct in tumor cells enhances tumor malignancy and vascularity in vivo, presumably through interactions with host endothelial ephrin-B2 (Noren et al. 2004). Thus, the ability of dimeric soluble EphB4 to initiate reverse signaling through host ephrin-B ligands must be tested before clinical application. In addition, the recently reported tumor suppressor role of several EphB class receptors in colon carcinoma (Batlle et al. 2005) also demonstrates the need for caution in the application of class B inhibitors to avoid enhancing tumor malignancy through blocking EphB function.

Other strategies for targeting Eph receptors in preclinical studies include activating antibodies, small binding peptides, vaccination, and delivery of siRNAs targeting Eph receptors in vivo. As discussed above, activating antibodies that target EphA2 for degradation (Carles-Kinch et al. 2002) pose a risk for enhancing malignancy through EphA2 kinase activation in tumor cells (Fang et al. 2005) or through activation in host endothelium (Brantley-Sieders et al. 2004b; Brantley-Sieders et al. 2005). Preclinical analysis of ephrin peptide mimetics is promising, as these peptides bind with high affinity to tumor cells and endothelium expressing EphA2 and can be used to deliver phage particles to the target tissues for drug delivery (Koolpe et al. 2002). Since this peptide is capable of stimulating EphA2 phosphorylation, though,

the same concerns that exist for soluble ligands apply. The recently reported development of a vaccination strategy is also quite interesting. Peptides generated from EphA2 T-cell epitopes were used to vaccinate animals harboring tumors that were EphA2 positive or negative. The EphA2 vaccine slowed growth and metastasis of both types of tumors (Hatano et al. 2004), suggesting that host targets, such as endothelium, may also be affected by the vaccine. Finally, the first reported use of liposomes to deliver EphA2 siRNAs in vivo proved efficacious in reducing tumor growth, particularly when coupled with chemotherapy (Landen et al. 2005). Ensuring specificity of delivery to the tumor rather than non-targeted organs is crucial for further development of this strategy.

Summary

The Eph family of RTKs and their ephrin ligands have emerged as key regulators of angiogenesis during embryogenesis. In addition, they have been recently shown to regulate tumor progression and angiogenesis in several cancer models. Through cooperation with a variety of oncogenic and pro-angiogenic pathways, these factors mediate tumor progression by promoting vascular recruitment as well as through tumor cell-intrinsic modulation of oncogenic pathways. Understanding the role of these molecules in both host and tumor tissue will enhance our ability to exploit this family in the development of new anti-angiogenic and anti-cancer therapies.

Acknowledgements.

We acknowledge the investigators in the field whose work we were unable to cite due to space limitations for references. The primary references for this work may be found in the cited reviews. D. Brantley-Sieders is supported by Department of Defense postdoctoral fellowship DAMD17-03-1-0379. J. Chen is supported by National Institutes of Health grants CA95004, CA114301, CA113007, and Department of Defense grant DAMD17-02-1-0604.

References

Abdollahi A, Hahnfeldt P, Maercker C, Grone HJ, Debus J, Ansorge W, Folkman J, Hlatky L, Huber PE (2004) Endostatin's antiangiogenic signaling network. Mol Cell 13:649–663

Adams RH, Wilkinson GA, Weiss C, Diella F, Gale NW, Deutsch U, Risau W, Klein R (1999) Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. Genes Dev 13:295–306

Adams RH, Diella F, Hennig S, Helmbacher F, Deutsch U, Klein R (2001) The cytoplasmic domain of the ligand ephrinB2 is required for vascular morphogenesis but not cranial neural crest migration. Cell 104:57–69

Batlle E, Bacani J, Begthel H, Jonkheer S, Gregorieff A, van de Born M, Malats N, Sancho E, Boon E, Pawson T, et al (2005) EphB receptor activity suppresses colorectal cancer progression. Nature 435:1126–1130

Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, Tanzawa K, Thorpe P, Itohara S, Werb Z, et al (2000) Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat Cell Biol 2:737–744

Brantley DM, Cheng N, Thompson EJ, Lin Q, Brekken RA, Thorpe PE, Muraoka RS, Cerretti DP, Pozzi A, Jackson D, et al (2002) Soluble Eph A receptors inhibit tumor angiogenesis and progression in vivo. Oncogene 21:7011–7026

Brantley-Sieders DM, Chen J (2004) Eph receptor tyrosine kinases in angiogenesis: from development to disease. Angiogenesis 7:17–28

Brantley-Sieders D, Parker M, Chen J (2004a) Eph receptor tyrosine kinases in tumor and tumor microenvironment. Curr Pharm Des 10:3431–3442

Brantley-Sieders DM, Caughron J, Hicks D, Pozzi A, Ruiz JC, Chen J (2004b) EphA2 receptor tyrosine kinase regulates endothelial cell migration and vascular assembly through phosphoinositide 3-kinase-mediated Rac1 GTPase activation. J Cell Sci 117:2037–2049

Brantley-Sieders DM, Fang WB, Hicks DJ, Zhuang G, Shyr Y, Chen J (2005) Impaired tumor microenvironment in EphA2-deficient mice inhibits tumor angiogenesis and metastatic progression. FASEB J 19:1884–1886

Brantley-Sieders DM, Fang WB, Hwang Y, Hicks D, Chen J (2006) Ephrin-A1 facilitates mammary tumor metastasis through an angiogenesis-dependent mechanism by EphA2 receptor and vascular endothelial growth factor (VEGF) in mice. Cancer Res 66: 10315–10324

Carles-Kinch K, Kilpatrick KE, Stewart JC, Kinch MS (2002) Antibody targeting of the EphA2 tyrosine kinase inhibits malignant cell behavior. Cancer Res 62:2840–2847

Chen J, Hicks D, Brantley-Sieders D, Cheng N, McCollum GW, Qi-Werdich X, Penn J (2005) Inhibition of retinal neovascularization by soluble EphA2 receptor. Exp Eye Res 82:664–673

Cheng N, Brantley DM, Chen J (2002a) The ephrins and Eph receptors in angiogenesis. Cytokine Growth Factor Rev 13:75–85

Cheng N, Brantley DM, Liu H, Lin Q, Enriquez M, Gale N, Yancopoulos G, Cerretti DP, Daniel TO, Chen J (2002b) Blockade of EphA receptor tyrosine kinase activation inhibits vascular endothelial cell growth factor-induced angiogenesis. Mol Cancer Res 1:2–11

Cheng N, Brantley D, Fang WB, Liu H, Fanslow W, Cerretti DP, Bussell KN, Reith A, Jackson D, Chen J (2003) Inhibition of VEGF-dependent multistage carcinogenesis by soluble EphA receptors. Neoplasia 5:445–456

Deroanne C, Vouret-Craviari V, Wang B, Pouyssegur J (2003) EphrinA1 inactivates integrin-mediated vascular smooth muscle cell spreading via the Rac/PAK pathway. J Cell Sci 11631367–1376

Dobrzanski P, Hunter K, Jones-Bolin S, Chang H, Robinson C, Pritchard S, Zhao H, Ruggeri B (2004) Antiangiogenic and antitumor efficacy of EphA2 receptor antagonist. Cancer Res 64:910–919

Dodelet VC, Pasquale EB (2000) Eph receptors and ephrin ligands: embryogenesis to tumorigenesis. Oncogene 19:5614–5619

Duxbury MS, Ito H, Zinner MJ, Ashley SW, Whang EE (2004a) EphA2: a determinant of malignant cellular behavior and a potential therapeutic target in pancreatic adenocarcinoma. Oncogene 23:1448–1456

Duxbury MS, Ito H, Zinner MJ, Ashley SW, Whang EE (2004b) Ligation of EphA2 by Ephrin A1-Fc inhibits pancreatic adenocarcinoma cellular invasiveness. Biochem Biophys Res Commun 320:1096–1102

Fang WB, Brantley-Sieders DM, Parker MA, Reith AD, Chen J (2005) A kinase-dependent role for EphA2 receptor in promoting tumor growth and metastasis. Oncogene 24:7859––7868

Folkman J (2002) Role of angiogenesis in tumor growth and metastasis. Semin Oncol 29:15–18

Folkman J, D'Amore PA (1996) Blood vessel formation: what is its molecular basis? Cell 87:1153–115

Gale NW, Baluk P, Pan L, Kwan M, Holash J, DeChiara TM, McDonald DM, Yancopoulos GD (2001) Ephrin-B2 selectively marks arterial vessels and neovascularization sites in the adult, with expression in both endothelial and smooth-muscle cells. Dev Biol 230:151–160

Gerety SS, Anderson DJ (2002) Cardiovascular ephrinB2 function is essential for embryonic angiogenesis. Development 129:1397–1410

Gerety SS, Wang HU, Chen ZF, Anderson DJ (1999) Symmetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand ephrin-B2 in cardiovascular development. Mol Cell 4:403–414

Griffioen AW, Molema G (2000) Angiogenesis: potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation. Pharmacol Rev 52:237–268

Hatano M, Kuwashima N, Tatsumi T, Dusak JE, Nishimura F, Reilly KM, Storkus WJ, Okada H (2004) Vaccination with EphA2-derived T cell-epitopes promotes immunity against both EphA2-expressing and EphA2-negative tumors. J Transl Med 2:40

Hayashi S, Asahara T, Masuda H, Isner JM, Losordo DW (2005) Functional ephrin-B2 expression for promotive interaction between arterial and venous vessels in postnatal neovascularization. Circulation 111:2210–2218

Hendrix MJ, Seftor EA, Hess AR, Seftor RE (2003) Vasculogenic mimicry and tumour-cell plasticity: lessons from melanoma. Nat Rev Cancer 3:411–421

Ireton RC, Chen J (2005) EphA2 receptor tyrosine kinase as a promising target for cancer therapeutics. Curr Cancer Drug Targets 5:149–157

Kale S, Hanai J, Chan B, Karihaloo A, Grotendorst G, Cantley L, Sukhatme VP (2005) Microarray analysis of in vitro pericyte differentiation reveals an angiogenic program of gene expression. FASEB J 19:270–271

Koolpe M, Dail M, Pasquale EB (2002) An ephrin mimetic peptide that selectively targets the EphA2 receptor. J Biol Chem 277:46974–46979

Landen CN Jr, Chavez-Reyes A, Bucana C, Schmandt R, Deavers MT, Lopez-Berestein G, Sood AK (2005) Therapeutic EphA2 gene targeting in vivo using neutral liposomal small interfering RNA delivery. Cancer Res 65:6910–6918

Liu W, Jung YD, Ahmad SA, McCarty MF, Stoeltzing O, Reinmuth N, Fan F, Ellis LM (2004) Effects of overexpression of ephrin-B2 on tumour growth in human colorectal cancer. Br J Cancer 90:1620–1626

Maekawa H, Oike Y, Kanda S, Ito Y, Yamada Y, Kurihara H, Nagai R, Suda T (2003) Ephrin-b2 induces migration of endothelial cells through the phosphatidylinositol-3 kinase pathway and promotes angiogenesis in adult vasculature. Arterioscler Thromb Vasc Biol 23:2008–2014

Makinen T, Adams RH, Bailey J, Lu Q, Ziemiecki A, Alitalo K, Klein R, Wilkinson GA (2005) PDZ interaction site in ephrinB2 is required for the remodeling of lymphatic vasculature. Genes Dev 19:397–410

Martiny-Baron G, Korff T, Schaffner F, Esser N, Eggstein S, Marme D, Augustin HG (2004) Inhibition of tumor growth and angiogenesis by soluble EphB4. Neoplasia 6:248–257

Miao H, Burnett E, Kinch M, Simon E, Wang B (2000) Activation of EphA2 kinase suppresses integrin function and causes focal-adhesion-kinase dephosphorylation. Nat Cell Biol 2:62–69

Nagashima K, Endo A, Ogita H, Kawana A, Yamagishi A, Kitabatake A, Matsuda M, Mochizuki N (2002) Adaptor protein Crk is required for ephrin-B1-induced membrane ruffling and focal complex assembly of human aortic endothelial cells. Mol Biol Cell 13:4231–4242

Noblitt LW, Bangari DS, Shukla S, Knapp DW, Mohammed S, Kinch MS, Mittal SK (2004) Decreased tumorigenic

potential of EphA2-overexpressing breast cancer cells following treatment with adenoviral vectors that express EphrinA1. Cancer Gene Ther 11:757–766

Noren NK, Lu M, Freeman AL, Koolpe M, Pasquale EB (2004) Interplay between EphB4 on tumor cells and vascular ephrin-B2 regulates tumor growth. Proc Natl Acad Sci U S A 101:5583–5588

Ogawa K, Pasqualini R, Lindberg RA, Kain R, Freeman AL, Pasquale EB (2000) The ephrin-A1 ligand and its receptor, EphA2, are expressed during tumor neovascularization. Oncogene 19:6043–6052

Ogita H, Kunimoto S, Kamioka Y, Sawa H, Masuda M, Mochizuki N (2003) EphA4-mediated Rho activation via Vsm-RhoGEF expressed specifically in vascular smooth muscle cells. Circ Res 93:23–31

Oike Y, Ito Y, Hamada K, Zhang XQ, Miyata K, Arai F, Inada T, Araki K, Nakagata N, Takeya M, et al (2002) Regulation of vasculogenesis and angiogenesis by EphB/ephrin-B2 signaling between endothelial cells and surrounding mesenchymal cells. Blood 100:1326–1333

Pasquale EB (2005) Eph receptor signalling casts a wide net on cell behaviour. Nat Rev Mol Cell Biol 6:462–475

Patan S (2000) Vasculogenesis and angiogenesis as mechanisms of vascular network formation, growth and remodeling. J Neurooncol 50:1–15

Patan S (2004) Vasculogenesis and angiogenesis. Cancer Treat Res 117:3–32

Prewett M, Huber J, Li Y, Santiago A, O'Connor W, King K, Overholser J, Hooper A, Pytowski B, Witte L, et al (1999) Antivascular endothelial growth factor receptor (fetal liver kinase 1) monoclonal antibody inhibits tumor angiogenesis and growth of several mouse and human tumors. Cancer Res 59:5209–5218

Ribatti D, Vacca A, Dammacco F (2003) New non-angiogenesis dependent pathways for tumour growth. Eur J Cancer 39:1835–1841

Risau W, Flamme I (1995) Vasculogenesis. Annu Rev Cell Dev Biol 11:73–91

Ruiz JC, Robertson EJ (1994) The expression of the receptor-protein tyrosine kinase gene, eck, is highly restricted during early mouse development. Mech Dev 46:87–100

Sawai Y, Tamura S, Fukui K, Ito N, Imanaka K, Saeki A, Sakuda S, Kiso S, Matsuzawa Y (2003) Expression of ephrin-B1 in hepatocellular carcinoma: possible involvement in neovascularization. J Hepatol 39:991–996

Shin D, Garcia-Cardena G, Hayashi S, Gerety S, Asahara T, Stavrakis G, Isner J, Folkman J, Gimbrone MA Jr, Anderson DJ (2001) Expression of ephrinB2 identifies a stable genetic difference between arterial and venous vascular smooth muscle as well as endothelial cells, and marks subsets of microvessels at sites of adult neovascularization. Dev Biol 230:139–150

Sullivan DC, Bicknell R (2003) New molecular pathways in angiogenesis. Br J Cancer 89:228–231

Vihanto MM, Plock J, Erni D, Frey BM, Frey FJ, Huynh-Do U (2005) Hypoxia up-regulates expression of Eph receptors and ephrins in mouse skin. FASEB J 19:1689–1691

Vindis C, Cerretti DP, Daniel TO, Huynh-Do U (2003) EphB1 recruits c-Src and p52Shc to activate MAPK/ERK and promote chemotaxis. J Cell Biol 162:661–671

Wang HU, Chen ZF, Anderson DJ (1998) Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. Cell 93:741–753

Wang Z, Cohen K, Shao Y, Mole P, Dombkowski D, Scadden DT (2003) Ephrin receptor, EphB4, regulates ES cell differentiation of primitive mammalian hemangioblasts, blood, cardiomyocytes, and blood vessels. Blood 103:100–109

Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J (2000) Vascular-specific growth factors and blood vessel formation. Nature 407:242–248

Zammaretti P, Zisch AH (2005) Adult ‚endothelial progenitor cells'. Renewing vasculature. Int J Biochem Cell Biol 37:493–503

Zelinski DP, Zantek ND, Stewart JC, Irizarry AR, Kinch MS (2001) EphA2 overexpression causes tumorigenesis of mammary epithelial cells. Cancer Res 61:2301–2306

Zhang XQ, Takakura N, Oike Y, Inada T, Gale NW, Yancopoulos GD, Suda T (2001) Stromal cells expressing ephrin-B2 promote the growth and sprouting of ephrin-B2(+) endothelial cells. Blood 98:1028–1037

The Role of the Neuropilins and Their Associated Plexin Receptors in Tumor Angiogenesis and Tumor Progression

8

GERA NEUFELD and OFRA KESSLER

Abstract

The neuropilins were described as receptors for the axon guidance factors belonging to the class 3 semaphorins subfamily. They were subsequently found to be expressed in endothelial cells and to function in addition as receptors for specific splice forms of angiogenic factors belonging to the VEGF family. There is increasing evidence indicating that the neuropilins and their associated plexin and tyrosine-kinase VEGF receptors play important roles as regulators of developmental angiogenesis and in the events that initiate tumor angiogenesis. Their VEGF and class 3 semaphorin ligands were found to regulate angiogenesis as a result of their interaction with neuropilins. Furthermore, many types of cancer cells express neuropilins and there is accumulating evidence indicating that class 3 semaphorins and members of the VEGF family of growth factors can affect the behavior of cancer cells as a result of their interaction with neuropilins expressed by cancer cells. This chapter focuses on the role of the neuropilins and of their ligands in tumor angiogenesis and tumor progression.

CONTENTS

Introduction

Neuropilin-1 (npl) and neuropilin-2 (np2) were originally characterized as neuronal cell surface receptors for axon guidance factors belonging to the class 3 semaphorin subfamily. During the development of the central nervous system, class 3 semaphorins function primarily as repellants of axonal growth cones. To transduce semaphorin signals, neuropilins form complexes with members of the plexin receptor family in which the neuropilins serve as the ligand binding components and the plexins as the signal transducing components. The neuropilins were subsequently found to double as receptors for specific heparin binding splice forms of vascular endothelial growth factor (VEGF), and to be expressed in endothelial cells. This finding suggested that neuropilins as well as semaphorins and plexins may function as modulators of angiogenesis. It was indeed found that the neuropilins strongly modulate the pro-angiogenic signals of VEGF. Furthermore, several types of class 3 semaphorins, such as semaphorin-3F (s3f), function as repellents of endothelial cells, as regulators of vasculogenesis and developmental angiogenesis, and as inhibitors of tumor angiogenesis. In this chapter we cover recent developments in this rapidly evolving field of research.

The Neuropilins and the Plexins and Their Involvement in Semaphorin-induced Signal Transduction

8.2.1 The Neuropilins

The human and mouse neuropilin family consists of two genes, npl and np2. Npl was originally identified as a cell surface protein involved in neuronal recognition (Takagi et al. 1991). Subsequent research identified the neuropilins as receptors for several semaphorins belonging to the class 3 semaphorin subfamily (He and Tessier-Lavigne 1997; Kolodkin et al. 1997; Giger et al. 1998; Chen et al. 1997; Stevens and Halloran 2005). The proteins encoded by the neuropilin genes are membrane-bound receptors, although splice forms encoding soluble extracellular domains of npl and np2 have also been identified (Gagnon et al. 2000; Rossignol et al. 2000). The two neuropilins share a very similar domain structure, although the overall homology between npl and np2 is only 44% at the amino acid level (Giger et al. 1998; Chen et al. 1997). Both neuropilins contain two complement binding (CUB)-like domains (a1 and a2 domains), two coagulation factor V/VIII homology-like domains (b1 and b2 domains), and a meprin (MAM) domain thought to be important for neuropilin dimerization and possibly for the interaction of neuropilins with other membrane receptors (He and Tessier-Lavigne 1997; Giger et al. 1998) (Fig. 8.1). Neuropilins possess a very short intracellular domain that is believed to be too short to support independent signal transduction. However, this dogma may be inaccurate. The last three amino acids of npl (SEA-COOH) are highly conserved between vertebrates and bind specifically to the PSD-95/Dlg/ZO-1 domain of the NIP protein (Cai and Reed 1999) indicating that the intracellular domain is not completely inert. Additionally, there exist np2 splice forms that possess a completely different intracellular domain, once again providing indirect evidence that the intracellular domain may not be devoid of biological function (Chen et al. 1997).

8.2.2 The Semaphorins

The semaphorin family consists of more than 30 genes divided into eight classes, of which classes 1 and 2 are derived from invertebrates, classes 3–7 are the products of vertebrate semaphorin genes, and class 8 contains viral semaphorins (Fig. 8.2). The semaphorins were previously referred to by an array of confusing designations. This situation was

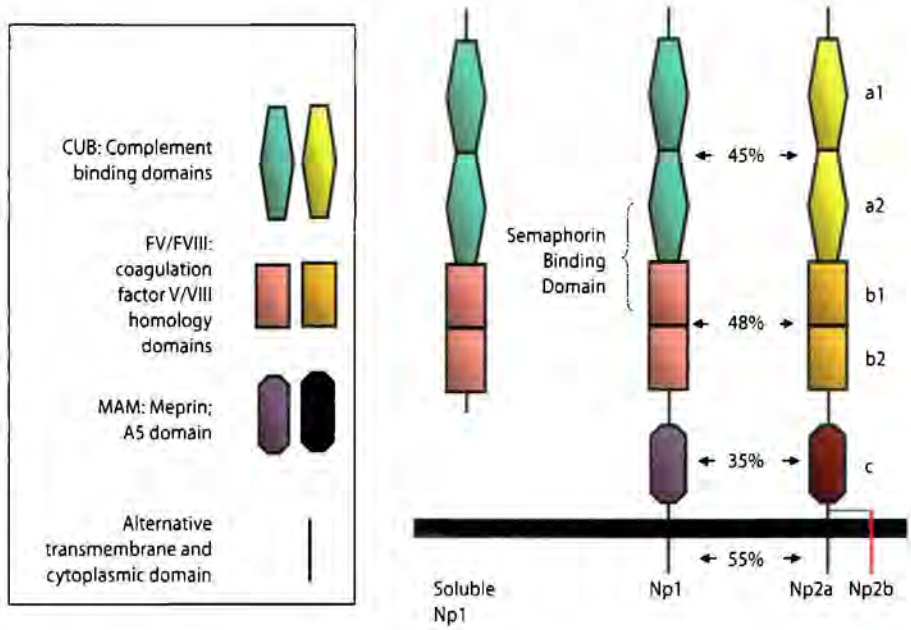


Fig. 8.1. The neuropilin receptor family. The two members of the neuropilin family are membrane-anchored receptors containing very short intracellular domains. Interestingly, there exist two np2 splice forms in which the transmembrane and intracellular domains are completely different (np2a and np2b). The s3a-binding domain of np1 is located between the a2 and b1 domains and partially overlaps the $VEGF_{165}$-binding domain. The MAM domain is required for receptor dimerization and for interaction with other receptors. For more details see the text

clarified several years ago by the adoption of a unified nomenclature for the semaphorins (Goodman et al. 1999). The semaphorins are characterized by the presence of a sema domain approximately 500 amino acids long located close to the N-terminus. The sema domain is essential for semaphorin signaling and determines the specificity of binding (Gherardi et al. 2004). The X-ray structures of the sema domains of semaphorin-3A (s3a) and semaphorin-4D (s4d) were analyzed at the atomic level revealing a conserved seven-bladed β-propeller structure (Gherardi et al. 2004).

Class 3 semaphorins are distinguished from other vertebrate semaphorins by being the only secreted semaphorins. In addition, they are distinguished by the presence of a basic domain in their C-terminus (Fig. 8.2). The class 3 semaphorins s3a and semaphorin-3D (s3d) were found to be produced as disulfide-linked homodimers, and the dimerization was found to be important for their bioactivity (Koppel and Raper 1998; Klostermann et al. 1998). It is therefore likely that the active forms of other class 3 semaphorins are also homodimeric. Various class 3 semaphorins differentiate between the two neuropilins. For example, it was found that semaphorin-3A (s3a) binds to np1 but not to np2, while semaphorin-3F (s3f) binds well to np2 but only with a much reduced affinity to np1 (He and Tessier-Lavigne 1997; Kolodkin et al. 1997; Giger et al. 1998). The binding site of s3a in np1 covers part of the second a-domain of np1 and part of the first b-domain (Fig. 8.1) (Gu et al. 2002). The class 3 semaphorins guide growth cones of elongating axons by causing a localized collapse of the cytoskeleton in the growth cone, thereby directing it in the opposite direction (Isbister and O'Connor 2000). When applied externally and non-directionally, class 3 semaphorins induce a general collapse of the cytoskeleton in responsive cells which is manifested by cell contraction (Takahashi et al. 1999). The class 3 semaphorin s3a was reported to function in addition as an inducer of apoptosis (Shirvan et al. 1999; Bagnard et al. 2004). The intracellular domain of the neuropilins is short, and is assumed not to suffice to transduce biological signals. This view is supported by experiments that have shown that although np1 is required for s3a-induced collapse of axonal growth cones, deletion of the cytoplasmic domain of np1 does not inhibit s3a activity, suggesting the existence of independent signal-transduc-

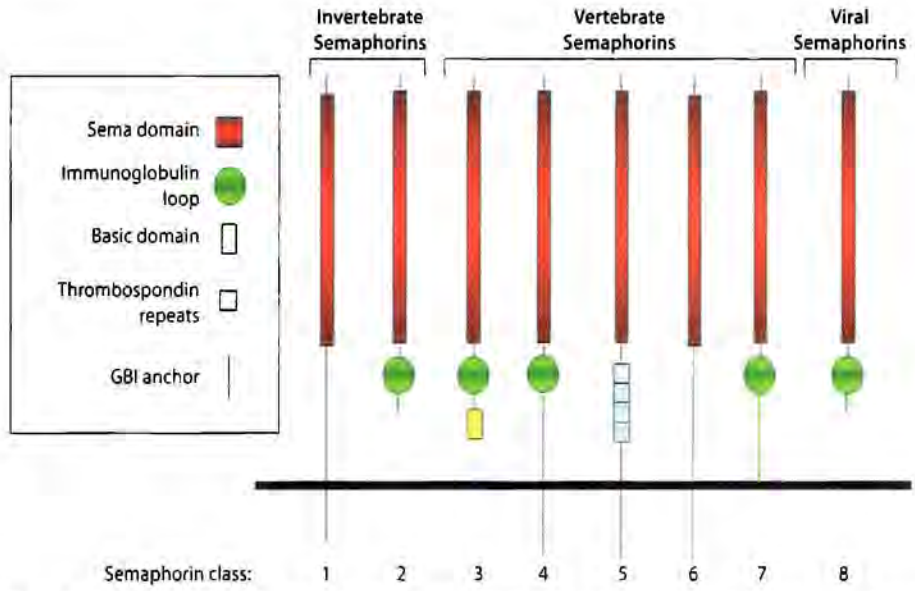


Fig. 8.2. The semaphorin family. The different semaphorin subclasses are shown. Classes 3–7 contain vertebrate semaphorins. The two main semaphorin subclasses containing members reported to function as angiogenesis regulators are class 3 and class 4. Class 3 semaphorins are the only secreted vertebrate semaphorins. The subfamily contains seven known members. They are distinguished by a small basic domain and by an Ig-like domain in addition to the sema domain which is present in all semaphorins. Class 4 semaphorins are membrane-anchored semaphorins containing an Ig loop-like domain. For more details see the text

ing moieties in semaphorin receptors (Nakamura et al. 1998). These were later identified as plexins (Takahashi et al. 1999; Tamagnone et al. 1999) (Fig. 8.3). In contrast, the other types of the vertebrate semaphorins can bind directly to plexins and do not require neuropilins for the initiation of signal transduction (Tamagnone et al. 1999).

Although the intracellular domain of the neuropilins is short, there are a few observations that suggest that this domain does have a function. The last three amino acids of npl contain a conserved SEA sequence which functions as a docking site for the postsynaptic density-95/Discs large/zona occludens-1 (PDZ) domain containing protein NIP [also known as RGS-GAIP-interacting protein (GIPC)] (Cai and Reed 1999). Therefore, although repulsion of npl-expressing growth cones does not require the presence of the intracellular domain of npl (Nakamura et al. 1998), it may yet turn out to be required for additional npl functions. This notion is also supported by the identification of a np2 splice form in which the C-terminal domain (including the transmembrane domain) is completely exchanged to yield np2b (Fig. 8.3) (Chen et al. 1997). It is unknown whether these two np2 forms have different biological functions, but their mere existence indicates that the intracellular domains of the neuropilins, although short, are likely to possess functions which have yet to be discovered.

8.2.3 The Plexins

The plexin family contains nine vertebrate members segregated into four classes. The plexins are transmembrane receptors containing a cytoplasmic sex/plexin (SP) domain that includes putative tyrosine phosphorylation sites but no known enzymatic activity. Interestingly, their extracellular domains

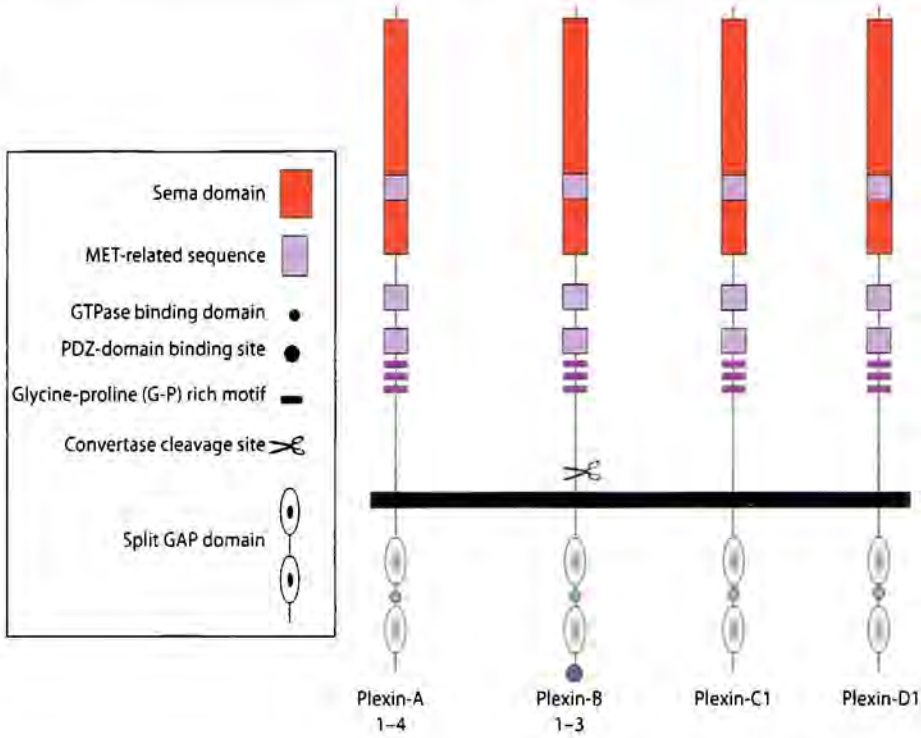


Fig. 8.3. The plexin receptor family. There are currently nine known mammalian members of this family, grouped into four subfamilies. Members of the A, B and D subfamilies have been found to function as modulators of angiogenesis. All plexins contain a sema domain and MET-related sequences. The intracellular part contains tyrosine residues that can be phosphorylated but lack tyrosine kinase activity and a split GAP domain

are distinguished by the presence of a sema domain, by the presence of a Met-related sequence (MRS) domain and by glycine-proline (G-P) rich motifs which the plexins share with the tyrosine kinase receptors belonging to the Met receptor family (Fig. 8.3) (Trusolino and Comoglio 2002). The four type A plexins were found to form complexes with neuropilins and to serve as the signal-transducing components in the resulting semaphorin holo-receptors (Tamagnone et al. 1999; Takahashi et al. 1999). Recent evidence also identified plexin-D1 as a plexin that forms complexes with neuropilins to transduce semaphorin-3C (s3c) signals (Gitler et al. 2004). Interestingly, although class 3 semaphorins such as s3a do not seem to be able to bind directly to plexins and do not activate plexin-mediated signal transduction in the absence of neuropilins, other semaphorins, including the class 3 semaphorin semaphorin-3E (s3e), do not require neuropilins and activate plexin-mediated signal transduction by direct binding to plexins (Fig. 8.4) (Gu et al. 2005; Potiron and Roche 2005). Although semaphorins such as semaphorin-6D (s6d) and s3e activate signal transduction via plexin-A1 and plexin-D1 directly, there exist other semaphorins, such as s3a and s3c, that activate the same plexins but are unable to bind directly to these plexins and require a neuropilin in order to activate signaling via these very same plexins (Fig. 8.4) (Toyofuku et al. 2004; Gitler et al. 2004; Gu et al. 2005). In only a few studies have attempts been made to identify differences in signal transduction between different plexins activated by a common semaphorin or to determine how different plexins affect the binding of semaphorins to neuropilins. These few studies indicate that the identity of the plexin affects the binding and the responses to class 3 semaphorins that bind to neuropilins (Rohm et al. 2000; Yaron et al. 2005). It is also unclear whether the direct as opposed to neuropilin-mediated activation of a plexin such as plexin-A1 or plexin-D1 leads to similar or diverse biological responses.

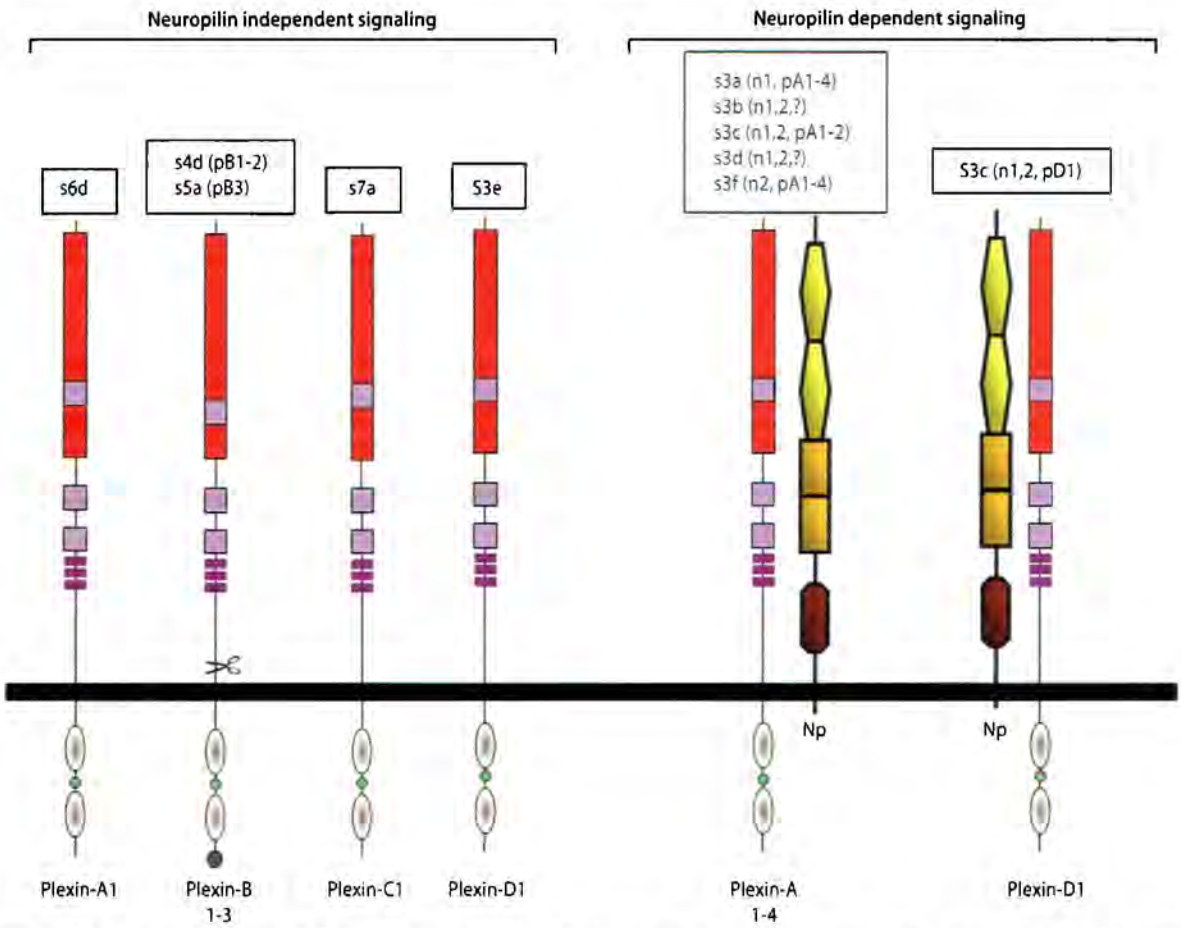


Fig. 8.4. Known interactions of different semaphorins with plexin and neuropilin receptors. The interactions of different plexins or holo-receptors containing specific neuropilin/plexin complexes are depicted. Plexins are abbreviated here with *p*; thus, plexin-B1 is designated *pB1*. neuropilins are designated *n1* or *n2*, and *n12* means that the semaphorin uses both neuropilins. For more details see the text

8.2.4 Plexin-Mediated Semaphorin Signaling

The intracellular domain of the plexins does not contain a tyrosine kinase domain. However, intracellular tyrosine kinases such as Fes/Fps bind to s3a-activated plexins such as plexin-A1 to phosphorylate tyrosine residues in the intracellular domain of plexin-A1 which serve as docking sites for downstream signal-transducing proteins (Mitsui et al. 2002). The intracellular tyrosine kinase Fyn was found to bind to the intracellular domain of the plexin-A2 receptor and phosphorylate it in response to s3a (Sasaki et al. 2002). The serine-threonine kinase Cdk-5 also associates with plexin-A2 and phosphorylates, in response to s3a, the CRMP2 protein, which serves as an important downstream target of plexins in neurons, binds to tubulin, promotes tubulin polymerization, and regulates cytoskeleton organization (Fig. 8.5) (Arimura et al. 2005; Brown et al. 2004). The intracellular domain of the Drosophila homolog of plexin-A1, plexin-A, contains in addition a binding site that enables association with

the flavoprotein oxidoreductase MICAL (molecule interacting with CasL), which was found to be essential for correct semaphorin-1a-induced axon repulsion in *Drosophila*. MICAL has several vertebrate homologs that have been found to be important for the transduction of s3a and s3f signals (Terman et al. 2002; Pasterkamp et al. 2006).

The intracellular part of several plexins such as plexin-B1 and plexin-A1 contains a domain that functions as a binding site for GTPases such as RAC1 and Rnd1. This binding site is located between the C1 and C2 domains, which function as a split GTPase-activating (GAP) domain that binds the small GTPase R-Ras and leads to its inactivation. R-Ras controls integrin function, and its inactivation leads to localized cell detachment from the extracellular matrix (Oinuma et al. 2004a). R-Ras was also found to function as an important regulator of angiogenesis (Komatsu and Ruoslahti 2005). In plexin-B1 the activation of the plexin GAP activity is associated with the binding of the small constitutively active GTPase Rnd1 disrupts the association between the two separate arms of the GAP domain of plexin-B1 enabling the inactivation of R-Ras in response to s4d (Oinuma et al. 2004b). The GAP domain is conserved quite highly throughout the plexin family, although it is unclear whether it is functional in all plexins. Additional small GTPases and their corresponding guanine-nucleotide exchange factors (GEFs) and GAPs participate in the transduction of plexin-mediated signals. It was shown that type B plexins possess a PDZ binding motif at the C-terminus through which GEFs such as PDZ-Rho-GEF and leukemia-associated Rho-GEF (LARG) bind to type B plexins (Perrot et al. 2002; Aurandt et al. 2002). The small GTPase Rho is activated following the binding of these Rho GEFs to plexin-B1, initiating a reorganization of the actin cytoskeleton in response to s4d (Fig. 8.5) (Hall 2005).

The three type B plexins do not seem to function as receptors for class 3 semaphorins. They seem to function as receptors for membrane-bound class 4 and class 5 semaphorins. The best characterized type B plexin is plexin-B1, which serves as a receptor for semaphorin-4D (s4d) (Takahashi et al. 1999). The plexin-B1 receptor can form complexes with the Met tyrosine kinase receptor, which functions as a receptor for hepatocyte growth factor/scatter factor (Bottaro et al. 1991). Interestingly, the Met receptor can be activated in response to the binding of s4d to plexin-B1 (Giordano et al. 2002).

8.2.5 Additional Neuropilin-associated Cell Surface Molecules that Modulate Neuropilin-mediated Signal Transduction

The neuropilins form complexes with additional cell surface molecules besides plexins. Npl was reported to form complexes with L1-CAM, an adhesion molecule which is primarily found in the central nervous system. Surprisingly, the association of L1-CAM with npl causes s3a to behave as an attractive molecule (Castellani et al. 2000, 2002). This conversion of a repulsive signal to an attractive signal may be associated with changes in the intracellular levels of cGMP (Polleux et al. 2000; Castellani et al. 2002). An L1-CAM derived peptide was found to inhibit endocytosis of npl- and s3a-induced cytoskeletal collapse, indicating that endocytosis of npl/L1-CAM complexes may be important for s3a signal transduction (Castellani et al. 2004). The NrCAM adhesion receptor, on the other hand, was found to form complexes with np2 and seems to be required for semaphorin-3B (s3b)-induced signaling mediated by np2 (Julien et al. 2005).

Neuropilins as VEGF Receptors

8.3.1 The VEGF Family

VEGF (also known as VEGF-A) is considered to be a major angiogenic factor that plays an essential role in embryonic vasculogenesis and angiogenesis as well as in tumor angiogenesis (Neufeld et al. 1999).

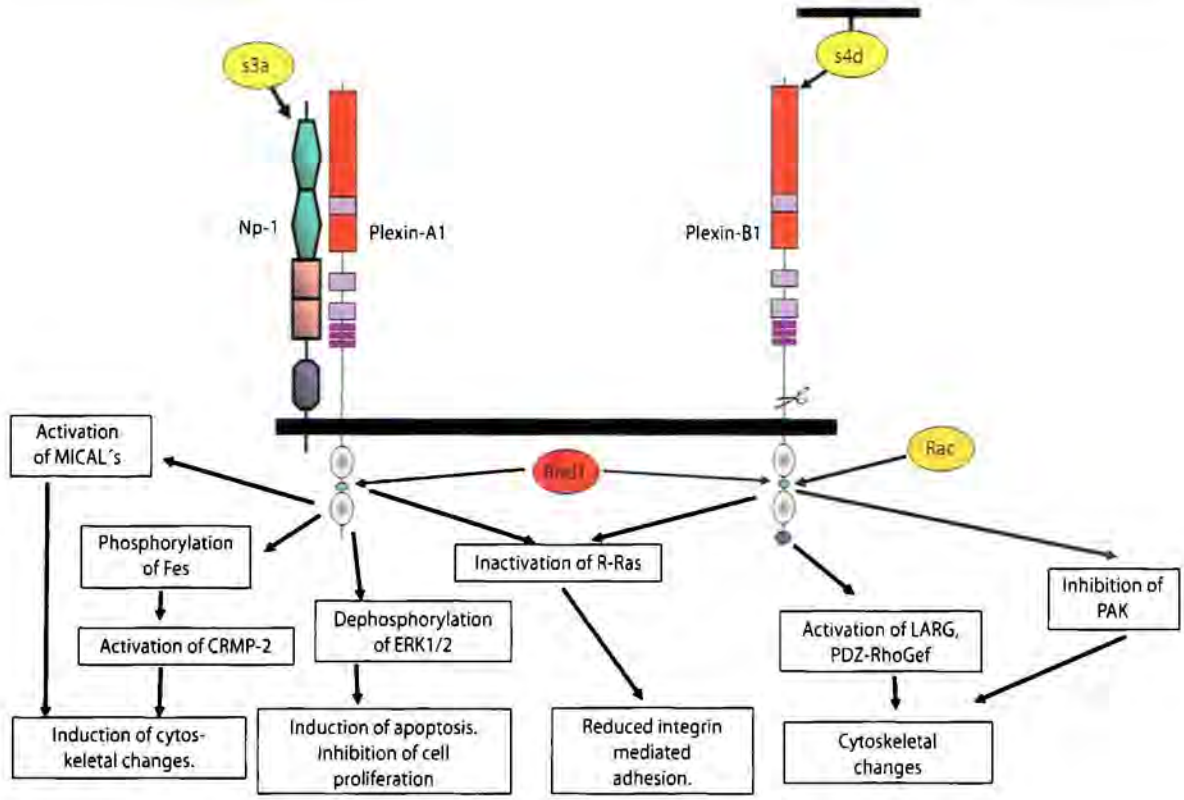


Fig. 8.5. The main signaling pathways activated by the plexin-A1 and plexin-B1 following activation by their respective s3a and s4d ligands. Following the binding of s3a to npl, which is associated with plexin-A1 in the presence or lack of s3a, plexin-A1 is activated. Rnd1 binds to the GTPase binding site leading to activation of the intrinsic GAP domain, which leads to R-Ras inactivation and inhibition of integrin function. Simultaneously, other pathways involving activation of CRMP-2 and MICALs lead to the reorganization of the actin and tubulin cytoskeleton. In the case of plexin-B1, activation occurs directly, without involvement of neuropilins. Inactivation of integrin function via R-Ras inactivation occurs similarly. Cytoskeletal changes are triggered by the activation of Rho GEFs via the PDZ-binding domain of plexin-B1. Rac is bound to plexin-B1. As a result, Rac-mediated inhibition of PAK is relieved. For more details see the text

Multiple forms of VEGF are produced as a result of alternative splicing. Three of these forms, $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$, are considered to be the major VEGF forms that are most frequently encountered. These three major VEGF forms differ in the expression of exons 6 and 7 of the VEGF gene. The 24-amino-acid peptide encoded by exon 7 is present in $VEGF_{165}$ and $VEGF_{189}$, while the 17-amino-acid peptide encoded by exon-6 is present in $VEGF_{145}$ and $VEGF_{189}$. Exons 6 and 7 encode independent heparin-binding domains, and $VEGF_{121}$, which lacks both exons, does not bind to heparan sulfates or to heparin (Neufeld et al. 1999). All the VEGF forms bind and activate the VEGFR-2 tyrosine kinase receptor, which seems to be essential for the transduction of VEGF-induced angiogenic signals (Terman et al. 1992; Shibuya 2003). The VEGFR-1 tyrosine kinase receptor, on the other hand (Devries et al. 1992), binds all the VEGF forms and is required for developmental and pathological angiogenesis (Fong et al. 1995; Luttun et al. 2002). Interestingly, knock-in experiments revealed that the tyrosine kinase domain is not required for developmental angiogenesis (Hiratsuka et al. 1998). It is, therefore, thought

that this receptor and the soluble form of VEGFR-1 function primarily as inhibitors of VEGF (Kendall and Thomas 1993; Ahmad and Ahmed 2004), although more recent evidence indicates that VEGF stimulation of VEGFR-1 plays an important role in the recruitment of macrophages and bone marrow-derived precursors of endothelial cells to tumors (Lyden et al. 2001; Luttun et al. 2002).

The VEGF family contains four additional vertebrate family members. PlGF and VEGF-B bind to VEGFR-1 but not to VEGFR-2 (Neufeld et al. 1999). The lymphangiogenesis-promoting agents VEGF-C and VEGF-D, on the other hand, bind to the VEGFR-2 receptor and induce angiogenesis, but in contrast to VEGF do not bind to VEGFR-1 (Cao et al. 1998) (Tammela et al. 2005). However, both VEGF-C and VEGF-D bind to the third member of the VEGF tyrosine kinase receptor subfamily, VEGFR-3 (Joukov et al. 1996; Achen et al. 1998), which is primarily expressed on lymphatic endothelial cells, enabling them to induce proliferation of lymphatic endothelial cells and lymphangiogenesis (Veikkola et al. 2001; Kaipainen et al. 1995; Kukk et al. 1996).

8.3.2 The Interaction of VEGF with Neuropilins and Its Effect on VEGF-induced Signaling by Tyrosine Kinase Receptors for VEGF

When the differences between the VEGF splice forms were investigated further, it was found that $VEGF_{165}$ was able to bind to a receptor that was not recognized by $VEGF_{121}$ (Gitay-Goren et al. 1996). This receptor turned out to be npl (Soker et al. 1998). It was subsequently found that np2 also distinguishes between $VEGF_{165}$ and $VEGF_{121}$ but, unlike npl, can bind the exon-6 containing $VEGF_{145}$ form of VEGF (Gluzman-Poltorak et al. 2000). The VEGF-binding domain of npl was mapped following the identification of the VEGF-binding properties of the neuropilins. It was found that the VEGF-binding site partially overlaps the s3a-binding site, so that VEGF and s3a compete for binding to npl (Gu et al. 2002). Interestingly, it was possible to introduce mutations into the ligand-binding domain of npl which resulted in the complete nullification of the VEGF-binding ability, but did not compromise the binding of s3a to npl, indicating that the binding domains of VEGF and s3a overlap but are not identical (Gu et al. 2002). In contrast, the binding of the np2 agonist s3f to np2 is not inhibited by $VEGF_{165}$, indicating that the binding sites of s3f and VEGF on np2 are independent (Gluzman-Poltorak et al. 2001).

Since both neuropilins function as splice form-specific VEGF receptors, it was not surprising that they were found to affect VEGF signaling and function in various experimental systems. Initially, the binding of $VEGF_{165}$ to npl was found to enhance $VEGF_{165}$-induced migration of endothelial cells in cells that express, in addition to npl, the VEGF receptor VEGFR-2 (Soker et al. 1998). It was subsequently observed that soluble dimers of the npl extracellular domain enhance VEGF-induced vascular development, while monomers of the soluble extracellular domain function as $VEGF_{165}$ traps and inhibit VEGF-induced vascular development (Yamada et al. 2001). The role of npl in embryonic vascular development was also studied in gene targeting experiments. Mice lacking functional npl receptors suffer from impaired neural vascularization and from defects in the development of large arteries such as brachial arch arteries. In addition, the development of the heart was strongly impaired in these mice, and heart failure was responsible for their premature death (Kawasaki et al. 1999). Knock-in mice expressing a npl variant lacking s3a binding ability but retaining VEGF binding displayed normal vascular development but abnormal neural development, indicating that the VEGF binding ability of npl is critical for proper vascular development. In contrast, the s3a binding ability is required in addition to the VEGF binding ability for proper heart development (Gu et al. 2003). These results are strengthened by experiments showing that mice lacking a functional npl gene in their endothelial cells but not in other cell types also suffer from severe vascular abnormalities (Gu et al. 2003), and by experiments which show that proper development of the vasculature in zebrafish requires npl (Lee et al. 2002), and indicate that

npl expression is critical for proper development of blood vessels during embryonic development.

The role of np2 in VEGF-induced vasculogenesis and angiogenesis is less clear. The vasculature of mice lacking a functional np2 receptor develops normally except for defects observed at birth in some lymphatic vessels (Yuan et al. 2002; Giger et al. 2000). However, these mice do not respond to $VEGF_{165}$ by retinal angiogenesis indicating that np2 is also important for angiogenesis (Shen et al. 2004). The importance of np2 to vascular development is also highlighted in experiments in which mice lacking both functional neuropilins were generated. These mice display a total lack of endothelial cells (Takashima et al. 2002), and their phenotype therefore resembles that of mice lacking functional VEGFR-2 receptors (Shalaby et al. 1995). Furthermore, mice lacking a functional np2 gene and containing only one functional npl gene also displayed vascular abnormalities that were more severe than those observed in mice that lack both npl alleles (Takashima et al. 2002).

The mechanism by which npl enhances $VEGF_{165}$-induced signal transduction via the VEGF receptor VEGFR-2 is unclear. It was suggested that npl binds $VEGF_{165}$ and presents it to the VEGFR-2 receptor, thereby increasing responsiveness to $VEGF_{165}$. Such a mechanism ought to function "in trans" too, and it was indeed found that angiogenesis is enhanced in tumors containing tumor cells expressing high levels of npl (Miao et al. 2000). It was recently suggested that npl contains a heparin-like domain that enables npl to bind a wide variety of heparin-binding growth factors such as basic fibroblast growth factor and not just $VEGF_{165}$, and that as a consequence npl is able to potentiate the activity of a wide variety of heparin-binding growth factors (West et al. 2005). On the other hand, it was also reported that npl forms complexes with VEGFR-2 directly, although there is some disagreement as to whether these complexes form as a result of stimulation by VEGF (Whitaker et al. 2001; Soker et al. 2002; Gu et al. 2002). The formation of such complexes may account, at least partially, for the npl-dependent potentiation of $VEGF_{165}$ activity.

8.3.3 The Interaction of Additional VEGF Family Members with Neuropilins

Recent evidence indicates that although PlGF does not seem to play an important role in developmental angiogenesis, it is important for pathological angiogenesis (Carmeliet et al. 2001; Luttun et al. 2002). However, these results are still controversial and under debate (Malik et al. 2005). It was observed that the heparin-binding form of PlGF, PlGF-2, binds npl. However, the functional consequences of this interaction are still unclear (Migdal et al. 1998). Likewise, it was reported that VEGF-B binds to npl, but here too the functional consequences are unclear (Makinen et al. 1999). In contrast, it was reported that the lymphangiogenic factor VEGF-C binds to neuropilin-2, which is highly expressed in lymphatics (Karkkainen et al. 2001; Yuan et al. 2002). VEGF-C activates angiogenesis via the VEGFR-2 tyrosine kinase receptor and lymphangiogenesis primarily by activating VEGFR-3 (Veikkola et al. 2001), but whether the interaction of VEGF-C with np2 modulates VEGF-C-induced lymphangiogenesis is still unclear.

8.3.4 The Role of the Neuropilins in VEGF-Induced Tumor Angiogenesis

VEGF is now thought to function as a primary inducer of tumor angiogenesis (Ferrara 2002; Neufeld et al. 1999). The importance of the neuropilins for VEGF signaling suggests that neuropilin-mediated signaling also plays an important role in the induction of tumor angiogenesis by VEGF. However, the importance of neuropilin for VEGF-induced tumor angiogenesis has not yet been assessed directly.

The Role of Class 3 Semaphorins in Normal Angiogenesis and in Tumor Angiogenesis

The class 3 semaphorins s3a, s3b, s3c, s3d and s3f bind to neuropilins, and the binding is required for initiation of signal transduction mediated by plexins (Kolodkin et al. 1997; He and Tessier-Lavigne 1997; Castro-Rivera et al. 2004; Takahashi et al. 1998; Gitler et al. 2004; Wolman et al. 2004; Chen et al. 1997). The primary neuropilin expressed in adult blood vessels is npl, and it was therefore logical to examine the effects of the npl agonist s3a on angiogenesis. S3a competes with $VEGF_{165}$ for binding to npl, inhibits $VEGF_{165}$-induced proliferation and migration of endothelial cells, and inhibits the pro-angiogenic effects of VEGF165 in angiogenesis experiments in vitro (Miao et al. 1999). Subsequent experiments indicated that s3a inhibits developmental angiogenesis (Serini et al. 2003). To date, however, no effects of s3a on tumor progression or on tumor angiogenesis have been reported.

The class 3 semaphorin S3f was originally identified as a tumor suppressor gene which is lost from small cell lung carcinoma cells (Xiang et al. 1996). Many types of tumor cells express neuropilins, and it was observed that s3f inhibits the proliferation, migration and spreading of several types of tumor cells, including lung and breast cancer-derived tumor cells, and inhibits the development of tumors from several types of small cell lung carcinoma-derived cell types (Xiang et al. 2002; Nasarre et al. 2003; Nasarre et al. 2005). Another semaphorin that was originally identified as a tumor suppressor of small cell lung carcinoma is s3b (Tomizawa et al. 2001). Interestingly, s3b was found to antagonize the anti-apoptotic effects that $VEGF_{165}$ produces in NCI-H1299 lung cancer-derived cells, probably by interfering with neuropilin-mediated VEGF signaling in these cells (Castro-Rivera et al. 2004). These findings indicate that s3b and s3f can directly affect the behavior of tumor cells that express neuropilins.

VEGF165 binds to np2 (Gluzman-Poltorak et al. 2000), but the biological consequences of this interaction have not yet been investigated in detail. The binding of the np2-specific semaphorin s3f to np2 is not inhibited by $VEGF_{165}$, suggesting that the $VEGF_{165}$ and the s3f-binding sites of np2 are independent (Gluzman-Poltorak et al. 2001). Nevertheless, s3f was found to inhibit VEGF as well as bFGF-induced proliferation of endothelial cells and to inhibit bFGF- and $VEGF_{165}$-induced angiogenesis in angiogenesis assays in vivo and in vitro. These experiments indicated that s3f may be able to inhibit tumor angiogenesis. Indeed, the development of tumors from s3f-expressing HEK293 cells implanted subcutaneously in nude mice was strongly inhibited compared to parental, empty expression vector-transfected HEK293 cells. The inhibition was probably due to inhibition of angiogenesis, because s3f did not inhibit the proliferation of HEK-293 tumor cells in vitro, and because the tumors that did develop from the s3f-expressing cells contained significantly lower concentrations of blood vessels (Kessler et al. 2004). In another study, it was observed that S3f is able to repel endothelial cells in vitro, indicating that it could also affect angiogenesis through repulsion of newly formed blood vessels. In this study it was observed that expression of recombinant s3f in highly metastatic melanoma cells which express endogenous np2 receptors inhibited their ability to form metastases. Although the expression of s3f in these cells did not prevent the formation of primary tumors, it affected tumor angiogenesis, since the primary tumors contained a much lower density of blood vessels. In this study s3f also affected the tumor cells directly, reducing their adherence to fibronectin and downregulating integrin-β1 expression (Bielenberg et al. 2004). Taken together, these studies indicate that s3f may be able to inhibit tumor progression using several mechanisms, including inhibition of angiogenesis.

Another class 3 semaphorin that activates neuropilin-mediated signaling and may affect tumor angiogenesis is s3c. S3c is a semaphorin that seems to signal through np2 or through npl/np2 complexes (Takahashi et al. 1998). Recently, it was found that both neuropilins associate with plexin-D1 to transduce s3c signals (Gitler et al. 2004). Mice lacking a functional plexin-D1 gene suffer from heart defects and vascular patterning defects, indicating

that plexin-D1 signaling plays an important role in vascular development (Feiner et al. 2001; Gitler et al. 2004; Torres-Vazquez et al. 2004). Plexin-D1 was reported to be specifically expressed in tumor-associated blood vessels and may therefore represent a target for anti-angiogenic therapy (Roodink et al. 2005). It should be noted that plexin-D1 can also be activated directly by the class 3 semaphorin s3e in the absence of neuropilins. Plexin-D1 signaling mediated by S3e was found to play an important role in the patterning of intersomitic vessels in developing embryos, indicating that s3e is an important modulator of embryonic angiogenesis (Gu et al. 2005). It was also reported that s3e undergoes proteolytic processing and that the resulting cleaved s3e is more active than the unprocessed form. The cleaved s3e promoted tumor metastasis, promoted migration of an endothelial cell-derived cell line, and activated ERK1/2 phosphorylation in these cells (Christensen et al. 2005). More work will be required to elucidate the role of neuropilin-dependent and -independent plexin-D1 signaling and the possible effects of s3c and s3e on tumor angiogenesis.

8.5 Membrane-Bound Semaphorins as Regulators of Angiogenesis

S4d is the best-characterized membrane-bound semaphorin that utilizes plexin-B1 and plexin-B2 as its receptors (Fig. 8.4). Plexin-B1 forms complexes with the MET tyrosine kinase receptor, which functions as a receptor for the angiogenic factor hepatocyte growth factor (Bussolino et al. 1992). Activation of plexin-B1 by s4d leads to the activation of the Met tyrosine kinase receptor (Giordano et al. 2002). Endothelial cells express plexin-B1 as well as Met, and it was therefore hypothesized that s4d may perhaps regulate angiogenesis. It was indeed found that s4d can function as a chemotactic factor for endothelial cells and that s4d induces angiogenesis through activation of the associated MET receptor (Conrotto et al. 2005). Interestingly, it was also reported that activation of plexin-B1 by s4d can induce angiogenesis by a Met-independent mechanism (Basile et al. 2004). A similar mechanism may also operate in the case of s6d. S6d utilizes plexin-A1 as a receptor. Plexin-A1 associates with VEGFR-2, and there is some evidence indicating that s6d can induce signaling via VEGFR-2 in this complex (Toyofuku et al. 2004).

8.6 Neuropilins as Modulators of VEGF-induced Tumor Angiogenesis

8.6.1 Effects of np1 on Tumor Angiogenesis

Many types of tumor cells, including pancreatic cancer cells, colon cancer cells, and breast cancer cells, express either np1 or np2 (Parikh et al. 2004; Fukahi et al. 2004; Cohen et al. 2002; Soker et al. 1998; Stephenson et al. 2002; Kawakami et al. 2002; Hansel et al. 2004). Membrane-bound np1, as well as soluble dimers of the extracellular domain of np1, enhances VEGF165-induced signaling mediated by the VEGFR-2 tyrosine kinase receptor, while soluble monomers of np1 extracellular domains inhibit VEGF (Yamada et al. 2001). These observations indicates that np1 may work "in trans" to enhance VEGFR-2 mediated VEGF165 signaling, indicating that np1 expressed on tumor cells may perhaps be able to potentiate VEGF-induced signaling in adjacent endothelial cells. In agreement with this hypothesis, it was found that overexpression of np1 in AT2.1 prostate cancer cells led to faster tumor development even though it did not affect the proliferation rate of the cancer cells in vitro, indicating that the effects on tumor growth are not the result of a direct effect on the cancerous cells. The enhancement in tumor growth rate was accompanied by an increased density of blood vessels in the tumors, presumably due to such a "trans" effect on VEGF signaling (Miao et al. 2000). It seems, therefore, that

the expression of neuropilins on cancer cells may enhance the pro-angiogenic effects of VEGF.

8.6.2 The Effects of Soluble Monomers of npl Extracellular Domains on Tumor Progression

Several secreted soluble splice forms of npl and np2 have been described (Rossignol et al. 2000). One of these, a VEGF-binding secreted form of npl, contains a truncated extracellular domain of npl lacking the MAM domain thought to be important for receptor dimerization (Fig. 8.2). This secreted, soluble extracellular domain of npl inhibited the activity of VEGF, presumably through trapping and sequestration of VEGF. Tumors of rat prostate carcinoma cells expressing this soluble npl form were characterized by extensive hemorrhage, damaged vessels, and by the presence of apoptotic tumor cells. It therefore seems that this monomeric soluble npl form functions as a VEGF165 antagonist and as an inhibitor of tumor angiogenesis and tumor progression (Gagnon et al. 2000).

8.7 Expression of Neuropilins in Tumor Cells and Direct Effects of Neuropilins on Tumor Cell Behavior

8.7.1 Expression of npl and np2 in Cancerous Cells

Cancer cells do not usually express tyrosine kinase VEGF receptors, but many types of cancerous cells express one or both neuropilins. Colon carcinoma cells express npl, and its expression was reported to be correlated with a better prognosis in one study (Kamiya et al. 2006). In another study npl expression was correlated with a highly malignant phenotype of gastrointestinal tumors (Hansel et al. 2004). In gliomas, overexpression of npl is correlated with poor prognosis (Osada et al. 2004). Npl overexpression is also correlated with aggressive prostate cancer (Latil et al. 2000), and androgen-independent prostate cancer-derived cells such as PC3 cells express high levels of npl (Li and Sarkar 2002). Npl was also found to be expressed in breast cancer-derived cells such as MDA-MB-231 cells (Soker et al. 1998). Myoepithelial cells of ducts and lobules in both neoplastic and non-neoplastic tissue specimens derived from neoplastic areas of breast cancer display a stronger positive reaction for npl than those in the normal breast (Stephenson et al. 2002). Both neuropilins were found to be highly expressed in tumor cells of pancreatic ductal adenocarcinoma and in cell lines derived from such tumors (Fukahi et al. 2004). Np2 is also expressed in a variety of tumors and tumor-derived cell lines. Np2 is expressed in osteosarcoma, and high expression levels correlate with poor prognosis (Handa et al. 2000). In the gastrointestinal tract, np2 is strongly expressed in neuroendocrine cells but its expression is decreased in carcinoid tumors which develop from these cells (Cohen et al. 2001). In contrast, np2 is highly expressed in endocrine pancreatic tumors.

8.7.2 The Effects of Neuropilins Expressed in Tumor Cells on Tumor Cell Behavior

The expression of neuropilins in tumor cells was in some cases found to affect the survival and migration of tumor cells directly. Thus, inhibition of npl expression or function in breast cancer-derived tumor cells resulted in the induction of apoptosis and increased the susceptibility to chemotherapy in breast cancer cells (Barr et al. 2005). Overexpression of npl in npl-deficient breast cancer cells protected them from apoptosis by an angiogenesis-independent mechanism, indicating that the presence of npl in these cells contributed to their survival (Bachelder et al. 2001). In breast cancer cells it was also found that $VEGF_{165}$ increases cell attachment and cell spreading by a npl-dependent mechanism (Nasarre et al. 2003). Furthermore, even though MCF7 breast cancer cells express npl and no np2,

it was found that s3f inhibited cell-cell adhesion, which was associated with decreased E-cadherin and beta-catenin expression. In the case of the motile C100 breast cancer cells, which do express np2, it was found that s3f had a repulsive effect (Nasarre et al. 2005).

Npl overexpression in the human pancreatic cancer cell line FG increased constitutive mitogen-activated protein kinase (MAPK) signaling, enhanced resistance to anoikis, and increased the resistance of cells to chemotherapy, while downregulation of npl expression in pancreatic cancer-derived Panc-1 cells markedly increased chemosensitivity (Wey et al. 2005). Interestingly, overexpression of a npl variant lacking a VEGF- or semaphorin-binding ability in Panc-1 cells reduced several key tumorigenic properties, including anchorage-independent cell growth and migration in vitro, and resulted in reduced tumor incidence and tumor volume in vivo. Conversely, reduction of npl expression by small interfering RNA targeting led to enhanced tumor growth (Gray et al. 2005).

Conclusions

The neuropilins have been shown to function as receptors for VEGF and for some class 3 semaphorins. As VEGF receptors they play a part in the induction of angiogenesis, but several class 3 semaphorins seem to inhibit angiogenesis as a result of their interaction with neuropilins. The neuropilins fulfill an important role in VEGF-induced developmental angiogenesis as demonstrated in gene targeting experiments, while the importance of semaphorins to developmental angiogenesis is not well established as yet and more work will be required in order to determine whether class 3 semaphorins play important roles in the regulation of developmental angiogenesis. However, there exists a growing body of evidence indicating that class 3 semaphorins such as s3f may be capable of inhibiting pathological angiogenesis such as the angiogenesis that accompanies the growth of solid tumors, and that they may, in addition, also affect tumor progression by directly affecting the behavior of tumor cells. The next few years will probably result in more information that will lead to a better understanding of the role of the neuropilins and their ligands in tumor progression. It is hoped that these new insights will lead to the development of new anti-tumorigenic drugs based upon semaphorins or on drugs targeting neuropilin-mediated VEGF-induced signal transduction.

Acknowledgements

This work was supported by grants from the Israel Science Foundation (ISF), the German-Israeli Binational Foundation (GIF), the International Union against Cancer (AICR) and by the Rappaport Family Institute for Research in the Medical Sciences of the Faculty of Medicine at the Technion, Israel Institute of Technology (to G.N.).

References

Achen MG, Jeltsch M, Kukk E, Makinen T, Vitali A, Wilks AF, Alitalo K, Stacker SA (1998) Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). Proc Natl Acad Sci U S A 95:548–553

Ahmad S, Ahmed A (2004) Elevated placental soluble vascular endothelial growth factor receptor-1 inhibits angiogenesis in preeclampsia. Circ Res 95:884–891

Arimura N, Menager C, Kawano Y, Yoshimura T, Kawabata S, Hattori A, Fukata Y, Amano M, Goshima Y, Inagaki M, Morone N, Usukura J, Kaibuchi K (2005) Phosphorylation by Rho kinase regulates CRMP-2 activity in growth cones. Mol Cell Biol 25:9973–9984

Aurandt J, Vikis HG, Gutkind JS, Ahn N, Guan KL (2002) The semaphorin receptor plexin-B1 signals through a direct interaction with the Rho-specific nucleotide exchange factor, LARG. Proc Natl Acad Sci U S A 99:12085–12090

Bachelder RE, Crago A, Chung J, Wendt MA, Shaw LM, Robinson G, Mercurio AM (2001) Vascular endothelial growth factor is an autocrine survival factor for neu-

ropilin-expressing breast carcinoma cells. Cancer Res 61:5736–5740

Bagnard D, Sainturet N, Meyronet D, Perraut M, Miehe M, Roussel G, Aunis D, Belin MF, Thomasset N (2004) Differential MAP kinases activation during semaphorin3A-induced repulsion or apoptosis of neural progenitor cells. Mol Cell Neurosci 25:722–731

Barr MP, Byrne AM, Duffy AM, Condron CM, Devocelle M, Harriott P, Bouchier-Hayes DJ, Harmey JH (2005) A peptide corresponding to the neuropilin-1-binding site on VEGF(165) induces apoptosis of neuropilin-1-expressing breast tumour cells. Br J Cancer 92:328–333

Basile JR, Barac A, Zhu T, Guan KL, Gutkind JS (2004) Class IV Semaphorins promote angiogenesis by stimulating rho-initiated pathways through plexin-B. Cancer Res 64:5212–5224

Bielenberg DR, Hida Y, Shimizu A, Kaipainen A, Kreuter M, Kim CC, Klagsbrun M (2004) Semaphorin 3F, a chemorepulsant for endothelial cells, induces a poorly vascularized, encapsulated, nonmetastatic tumor phenotype. J Clin Invest 114:1260–1271

Bottaro DP, Rubin JS, Faletto DL, Chan AM, Kmiecik TE, Vande Woude GF, Aaronson SA (1991) Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. Science 251:802–804

Brown M, Jacobs T, Eickholt B, Ferrari G, Teo M, Monfries C, Qi RZ, Leung T, Lim L, Hall C (2004) Alpha2-chimaerin, cyclin-dependent Kinase 5/p35, and its target collapsin response mediator protein-2 are essential components in semaphorin 3A-induced growth-cone collapse. J Neurosci 24:8994–9004

Bussolino F, Di Renzo MF, Ziche M, Bocchietto E, Olivero M, Naldini L, Gaudino G, Tamagnone L, Coffer A, Comoglio PM (1992) Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. J Cell Biol 119:629–641

Cai HB, Reed RR (1999) Cloning and characterization of neuropilin-1-interacting protein: a PSD-95/Dlg/ZO-1 domain-containing protein that interacts with the cytoplasmic domain of neuropilin-1. J Neurosci 19:6519–6527

Cao YH, Linden P, Farnebo J, Cao RH, Eriksson A, Kumar V, Qi JH, Claesson-Welsh L, Alitalo K (1998) Vascular endothelial growth factor C induces angiogenesis in vivo. Proc Natl Acad Sci USA 95:14389–14394

Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De Mol M, Wu Y, Bono F, Devy L, Beck H, Scholz D, Acker T, Dipalma T, Dewerchin M, Noel A, Stalmans I, Barra A, Blacher S, Vandendriessche T, Ponten A, Eriksson U, Plate KH, Foidart JM, Schaper W, Charnock-Jones DS, Hicklin DJ, Herbert JM, Collen D, Persico MG (2001) Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat Med 7:575–583

Castellani V, Chedotal A, Schachner M, Faivre-Sarrailh C, Rougon G (2000) Analysis of the L1-deficient mouse phenotype reveals cross-talk between Sema3A and L1 signaling pathways in axonal guidance. Neuron 27:237–249

Castellani V, De Angelis E, Kenwrick S, Rougon G (2002) Cis and trans interactions of L1 with neuropilin-1 control axonal responses to semaphorin 3A. EMBO J 21:6348–6357

Castellani V, Falk J, Rougon G (2004) Semaphorin3A-induced receptor endocytosis during axon guidance responses is mediated by L1 CAM. Mol Cell Neurosci 26:89–100

Castro-Rivera E, Ran S, Thorpe P, Minna JD (2004) Semaphorin 3B (SEMA3B) induces apoptosis in lung and breast cancer, whereas VEGF165 antagonizes this effect. Proc Natl Acad Sci U S A 101:11432–11437

Chen H, Chedotal A, He Z, Goodman CS, Tessier-Lavigne M (1997) Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III. Neuron 19:547–559

Christensen C, Ambartsumian N, Gilestro G, Thomsen B, Comoglio P, Tamagnone L, Guldberg P, Lukanidin E (2005) Proteolytic processing converts the repelling signal Sema3E into an inducer of invasive growth and lung metastasis. Cancer Res 65:6167–6177

Cohen T, Gluzman-Poltorak Z, Brodzky A, Meytal V, Sabo E, Misselevich I, Hassoun M, Boss JH, Resnick M, Shneyvas D, Eldar S, Neufeld G (2001) Neuroendocrine cells along the digestive tract express neuropilin-2. Biochem Biophys Res Commun 284:395–403

Cohen T, Herzog Y, Brodzky A, Greenson JK, Eldar S, Gluzman-Poltorak Z, Neufeld G, Resnick MB (2002) Neuropilin-2 is a novel marker expressed in pancreatic islet cells and endocrine pancreatic tumours. J Pathol 198:77–82

Conrotto P, Valdembri D, Corso S, Serini G, Tamagnone L, Comoglio PM, Bussolino F, Giordano S (2005) Sema4D induces angiogenesis through Met recruitment by Plexin B1. Blood 105:4321–4329

Devries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT (1992) The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science 255:989–991

Feiner L, Webber AL, Brown CB, Lu MM, Jia L, Feinstein P, Mombaerts P, Epstein JA, Raper JA (2001) Targeted disruption of semaphorin 3C leads to persistent truncus arteriosus and aortic arch interruption. Development 128:3061–3070

Ferrara N (2002) Role of vascular endothelial growth factor in physiologic and pathologic angiogenesis: therapeutic implications. Semin Oncol 29:10–14

Fong GH, Rossant J, Gertsenstein M, Breitman ML (1995) Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature 376:66–70

Fukahi K, Fukasawa M, Neufeld G, Itakura J, Korc M (2004) Aberrant expression of neuropilin-1 and -2 in human pancreatic cancer cells. Clin Cancer Res 10:581–590

Gagnon ML, Bielenberg DR, Gechtman Z, Miao HQ, Ta-

kashima S, Soker S, Klagsbrun M (2000) Identification of a natural soluble neuropilin-1 that binds vascular endothelial growth factor: in vivo expression and antitumor activity. Proc Natl Acad Sci U S A 97:2573–2578

Gherardi E, Love CA, Esnouf RM, Jones EY (2004) The sema domain. Curr Opin Struct Biol 14:669–678

Giger RJ, Cloutier JF, Sahay A, Prinjha RK, Levengood DV, Moore SE, Pickering S, Simmons D, Rastan S, Walsh FS, Kolodkin AL, Ginty DD, Geppert M (2000) Neuropilin-2 is required in vivo for selective axon guidance responses to secreted semaphorins. Neuron 25:29–41

Giger RJ, Urquhart ER, Gillespie SK, Levengood DV, Ginty DD, Kolodkin AL (1998) Neuropilin-2 is a receptor for semaphorin IV: insight into the structural basis of receptor function and specificity. Neuron 21:1079–1092

Giordano S, Corso S, Conrotto P, Artigiani S, Gilestro G, Barberis D, Tamagnone L, Comoglio PM (2002) The Semaphorin 4D receptor controls invasive growth by coupling with Met. Nat Cell Biol 4:720–724

Gitay-Goren H, Cohen T, Tessler S, Soker S, Gengrinovitch S, Rockwell P, Klagsbrun M, Levi B-Z, Neufeld G (1996) Selective binding of VEGF121 to one of the three VEGF receptors of vascular endothelial cells. J Biol Chem 271:5519–5523

Gitler AD, Lu MM, Epstein JA (2004) PlexinD1 and semaphorin signaling are required in endothelial cells for cardiovascular development. Dev Cell 7:107–116

Gluzman-Poltorak Z, Cohen T, Herzog Y, Neufeld G (2000) Neuropilin-2 and Neuropilin-1 are receptors for 165-amino acid long form of vascular endothelial growth factor (VEGF) and of placenta growth factor-2, but only neuropilin-2 functions as a receptor for the 145 amino acid form of VEGF. J Biol Chem 275:18040–18045

Gluzman-Poltorak Z, Cohen T, Shibuya M, Neufeld G (2001) Vascular endothelial growth factor receptor-1 and neuropilin-2 form complexes. J Biol Chem 276:18688–18694

Goodman CS, Kolodkin AL, Luo Y, Pueschel AW, Raper JA (1999) Unified nomenclature for the semaphorins collapsins. Cell 97:551–552

Gray MJ, Wey JS, Belcheva A, Mccarty MF, Trevino JG, Evans DB, Ellis LM, Gallick GE (2005) Neuropilin-1 suppresses tumorigenic properties in a human pancreatic adenocarcinoma cell line lacking neuropilin-1 coreceptors. Cancer Res 65:3664–3670

Gu C, Limberg BJ, Whitaker GB, Perman B, Leahy DJ, Rosenbaum JS, Ginty DD, Kolodkin AL (2002) Characterization of neuropilin-1 structural features that confer binding to semaphorin 3A and vascular endothelial growth factor 165. J Biol Chem 277:18069–18076

Gu C, Rodriguez ER, Reimert DV, Shu T, Fritzsch B, Richards LJ, Kolodkin AL, Ginty DD (2003) Neuropilin-1 conveys semaphorin and VEGF signaling during neural and cardiovascular development. Dev Cell 5:45–57

Gu C, Yoshida Y, Livet J, Reimert DV, Mann F, Merte J, Henderson CE, Jessell TM, Kolodkin AL, Ginty DD (2005) Semaphorin 3E and Plexin-D1 control vascular pattern independently of neuropilins. Science 307:265–268

Hall A (2005) Rho GTPases and the control of cell behaviour. Biochem Soc Trans 33:891–895

Handa A, Tokunaga T, Tsuchida T, Lee YH, Kijima H, Yamazaki H, Ueyama Y, Fukuda H, Nakamura M (2000) Neuropilin-2 expression affects the increased vascularization and is a prognostic factor in osteosarcoma. Int J Oncol 17:291–295

Hansel DE, Wilentz RE, Yeo CJ, Schulick RD, Montgomery E, Maitra A (2004) Expression of neuropilin-1 in high-grade dysplasia, invasive cancer, and metastases of the human gastrointestinal tract. Am J Surg Pathol 28:347–356

He Z, Tessier-Lavigne M (1997) Neuropilin is a receptor for the axonal chemorepellent Semaphorin III. Cell 90:739–751

Hiratsuka S, Minowa O, Kuno J, Noda T, Shibuya M (1998) Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. Proc Natl Acad Sci U S A 95:9349–9354

Isbister CM, O'Connor TP (2000) Mechanisms of growth cone guidance and motility in the developing grasshopper embryo. J Neurobiol 44:271–280

Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, Saksela O, Kalkkinen N, Alitalo K (1996) A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. EMBO J 15:290–298

Julien F, Bechara A, Fiore R, Nawabi H, Zhou H, Hoyo-Becerra C, Bozon M, Rougon G, Grumet M, Puschel AW, Sanes JR, Castellani V (2005) Dual functional activity of semaphorin 3B is required for positioning the anterior commissure. Neuron 48:63–75

Kaipainen A, Korhonen J, Mustonen T, Vanhinsbergh VWM, Fang GH, Dumont D, Breitman M, Alitalo K (1995) Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. Proc Natl Acad Sci USA 92:3566–3570

Kamiya T, Kawakami T, Abe Y, Nishi M, Onoda N, Miyazaki N, Oida Y, Yamazaki H, Ueyama Y, Nakamura M (2006) The preserved expression of neuropilin (NRP) 1 contributes to a better prognosis in colon cancer. Oncol Rep 15:369–373

Karkkainen MJ, Saaristo A, Jussila L, Karila KA, Lawrence EC, Pajusola K, Bueler H, Eichmann A, Kauppinen R, Kettunen MI, Yla-Herttuala S, Finegold DN, Ferrell RE, Alitalo K (2001) A model for gene therapy of human hereditary lymphedema. Proc Natl Acad Sci U S A 98:12677–12682

Kawakami T, Tokunaga T, Hatanaka H, Kijima H, Yamazaki H, Abe Y, Osamura Y, Inoue H, Ueyama Y, Nakamura M (2002) Neuropilin 1 and neuropilin 2 co-expression is significantly correlated with increased vascularity and poor prognosis in nonsmall cell lung carcinoma. Cancer 95:2196–2201

Kawasaki T, Kitsukawa T, Bekku Y, Matsuda Y, Sanbo M, Yagi T, Fujisawa H (1999) A requirement for neuropilin-1 in embryonic vessel formation. Development 126:4895–4902

Kendall RL, Thomas KA (1993) Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. Proc Natl Acad Sci USA 90:10705–10709

Kessler O, Shraga-Heled N, Lange T, Gutmann-Raviv N, Sabo E, Baruch L, Machluf M, Neufeld G (2004) Semaphorin-3F is an inhibitor of tumor angiogenesis. Cancer Res 64:1008–1015

Klostermann A, Lohrum M, Adams RH, Puschel AW (1998) The chemorepulsive activity of the axonal guidance signal semaphorin D requires dimerization. J Biol Chem 273:7326–7331

Kolodkin AL, Levengood DV, Rowe EG, Tai YT, Giger RJ, Ginty DD (1997) Neuropilin is a semaphorin III receptor. Cell 90:753–762

Komatsu M, Ruoslahti E (2005) R-Ras is a global regulator of vascular regeneration that suppresses intimal hyperplasia and tumor angiogenesis. Nat Med 11:1346–1350

Koppel AM, Raper JA (1998) Collapsin-1 covalently dimerizes, and dimerization is necessary for collapsing activity. J Biol Chem 273:15708–15713

Kukk E, Lymboussaki A, Taira S, Kaipainen A, Jeltsch M, Joukov V, Alitalo K (1996) VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development. Development 122:3829–3837

Latil A, Bieche I, Pesche S, Valeri A, Fournier G, Cussenot O, Lidereau R (2000) VEGF overexpression in clinically localized prostate tumors and neuropilin-1 overexpression in metastatic forms. Int J Cancer 89:167–171

Lee P, Goishi K, Davidson AJ, Mannix R, Zon L, Klagsbrun M (2002) Neuropilin-1 is required for vascular development and is a mediator of VEGF-dependent angiogenesis in zebrafish. Proc Natl Acad Sci U S A 99:10470–10475

Li Y, Sarkar F (2002) Down-regulation of invasion and angiogenesis-related genes identified by cDNA microarray analysis of PC3 prostate cancer cells treated with genistein. Cancer Lett 186:157–164

Luttun A, Tjwa M, Moons L, Wu Y, Angelillo-Scherrer A, Liao F, Nagy JA, Hooper A, Priller J, De Klerck B, Compernolle V, Daci E, Bohlen P, Dewerchin M, Herbert JM, Fava R, Matthys P, Carmeliet G, Collen D, Dvorak HF, Hicklin DJ, Carmeliet P (2002) Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. Nat Med 8:831–840

Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L, Chadburn A, Heissig B, Marks W, Witte L, Wu Y, Hicklin D, Zhu Z, Hackett NR, Crystal RG, Moore MA, Hajjar KA, Manova K, Benezra R, Rafii S (2001) Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat Med 7:1194–1201

Makinen T, Olofsson B, Karpanen T, Hellman U, Soker S, Klagsbrun M, Eriksson U, Alitalo K (1999) Differential binding of vascular endothelial growth factor B splice and proteolytic isoforms to neuropilin-1. J Biol Chem 274:21217–21222

Malik AK, Baldwin ME, Peale F, Fuh G, Liang WC, Lowman H, Meng G, Ferrara N, Gerber HP (2005) Redundant roles of VEGF-B and PlGF during selective VEGF-A blockade in mice. Blood 107:550–557

Miao HQ, Lee P, Lin H, Soker S, Klagsbrun M (2000) Neuropilin-1 expression by tumor cells promotes tumor angiogenesis and progression. FASEB J 14:2532–2539

Miao HQ, Soker S, Feiner L, Alonso JL, Raper JA, Klagsbrun M (1999) Neuropilin-1 mediates collapsin-1/semaphorin III inhibition of endothelial cell motility. Functional competition of collapsin-1 and vascular endothelial growth factor-165. J Cell Biol 146:233–242

Migdal M, Huppertz B, Tessler S, Comforti A, Shibuya M, Reich R, Baumann H, Neufeld G (1998) Neuropilin-1 is a placenta growth factor-2 receptor. J Biol Chem 273:22272–22278

Mitsui N, Inatome R, Takahashi S, Goshima Y, Yamamura H, Yanagi S (2002) Involvement of Fes/Fps tyrosine kinase in semaphorin3A signaling. EMBO J 21:3274–3285

Nakamura F, Tanaka M, Takahashi T, Kalb RG, Strittmatter SM (1998) Neuropilin-1 extracellular domains mediate semaphorin D/III- induced growth cone collapse. Neuron 21:1093–1100

Nasarre P, Constantin B, Rouhaud L, Harnois T, Raymond G, Drabkin HA, Bourmeyster N, Roche J (2003) Semaphorin SEMA3F and VEGF have opposing effects on cell attachment and spreading. Neoplasia 5:83–92

Nasarre P, Kusy S, Constantin B, Castellani V, Drabkin HA, Bagnard D, Roche J (2005) Semaphorin SEMA3F has a repulsing activity on breast cancer cells and inhibits E-cadherin-mediated cell adhesion. Neoplasia 7:180–189

Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z (1999) Vascular endothelial growth factor (VEGF) and its receptors. FASEB J 13:9–22

Oinuma I, Ishikawa Y, Katoh H, Negishi M (2004a) The Semaphorin 4D receptor Plexin-B1 is a GTPase activating protein for R-Ras. Science 305:862–865

Oinuma I, Katoh H, Negishi M (2004b) Molecular dissection of the semaphorin 4D receptor plexin-B1-stimulated R-Ras GTPase-activating protein activity and neurite remodeling in hippocampal neurons. J Neurosci 24:11473–11480

Osada H, Tokunaga T, Nishi M, Hatanaka H, Abe Y, Tsugu A, Kijima H, Yamazaki H, Ueyama Y, Nakamura M (2004) Overexpression of the neuropilin 1 (NRP1) gene correlated with poor prognosis in human glioma. Anticancer Res 24:547–552

Parikh AA, Fan F, Liu WB, Ahmad SA, Stoeltzing O, Rein-

muth N, Bielenberg D, Bucana CD, Klagsbrun M, Ellis LM (2004) Neuropilin-1 in human colon cancer: expression, regulation, and role in induction of angiogenesis. Am J Pathol 164:2139–2151

Pasterkamp RJ, Dai HN, Terman JR, Wahlin KJ, Kim B, Bregman BS, Popovich PG, Kolodkin AL (2006) MICAL flavoprotein monooxygenases: Expression during neural development and following spinal cord injuries in the rat. Mol Cell Neurosci 31:52–69

Perrot V, Vazquez-Prado J, Gutkind JS (2002) Plexin B regulates Rho through the guanine nucleotide exchange factors leukemia-associated RhoGEF (LARG) and PDZ-RhoGEF. J Biol Chem 278:26111–26119

Polleux F, Morrow T, Ghosh A (2000) Semaphorin 3A is a chemoattractant for cortical apical dendrites. Nature 404:567–573

Potiron V, Roche J (2005) Class 3 semaphorin signaling: the end of a dogma. Sci STKE 2005:e24

Rohm B, Ottemeyer A, Lohrum M, Puschel AW (2000) Plexin/neuropilin complexes mediate repulsion by the axonal guidance signal semaphorin 3A. Mech Dev 93:95–104

Roodink I, Raats J, Van Der ZB, Verrijp K, Kusters B, Van Bokhoven H, Linkels M, de Waal RM, Leenders WP (2005) Plexin d1 expression is induced on tumor vasculature and tumor cells: a novel target for diagnosis and therapy? Cancer Res 65:8317–8323

Rossignol M, Gagnon ML, Klagsbrun M (2000) Genomic Organization of human neuropilin-1 and neuropilin-2 genes: identification and distribution of splice variants and soluble isoforms. Genomics 70:211–222

Sasaki Y, Cheng C, Uchida Y, Nakajima O, Ohshima T, Yagi T, Taniguchi M, Nakayama T, Kishida R, Kudo Y, Ohno S, Nakamura F, Goshima Y (2002) Fyn and Cdk5 mediate semaphorin-3a signaling, which is involved in regulation of dendrite orientation in cerebral cortex. Neuron 35:907–920

Serini G, Valdembri D, Zanivan S, Morterra G, Burkhardt C, Caccavari F, Zammataro L, Primo L, Tamagnone L, Logan M, Tessier-Lavigne M, Taniguchi M, Puschel AW, Bussolino F (2003) Class 3 semaphorins control vascular morphogenesis by inhibiting integrin function. Nature 424:391–397

Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC (1995) Failure of blood-island formation and vasculogenesis in Flk-1- deficient mice. Nature 376:62–66

Shen J, Samul R, Zimmer J, Liu H, Liang X, Hackett S, Campochiaro PA (2004) Deficiency of neuropilin 2 suppresses VEGF-induced retinal neovascularization. Mol Med 10:12–18

Shibuya M (2003) Vascular endothelial growth factor receptor-2: Its unique signaling and specific ligand, VEGF-E. Cancer Sci 94:751–756

Shirvan A, Ziv I, Fleminger G, Shina R, He ZG, Brudo I, Melamed E, Barzilai A (1999) Semaphorins as mediators of neuronal apoptosis. J Neurochem 73:961–971

Soker S, Miao HQ, Nomi M, Takashima S, Klagsbrun M (2002) VEGF(165) mediates formation of complexes containing VEGFR-2 and neuropilin-1 that enhance VEGF(165)-receptor binding. J Cell Biochem 85:357–368

Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M (1998) Neuropilin-1 is expressed by endothelial and tumor cells as an isoform specific receptor for vascular endothelial growth factor. Cell 92:735–745

Stephenson JM, Banerjee S, Saxena NK, Cherian R, Banerjee SK (2002) Neuropilin-1 is differentially expressed in myoepithelial cells and vascular smooth muscle cells in preneoplastic and neoplastic human breast: a possible marker for the progression of breast cancer. Int J Cancer 101:409–414

Stevens CB, Halloran MC (2005) Developmental expression of sema3G, a novel zebrafish semaphorin. Gene Expr Patterns 5:647–653

Takagi S, Hirata T, Agata K, Mochii M, Eguchi G, Fujisawa H (1991) The A5 antigen, a candidate for the neuronal recognition molecule, has homologies to complement components and coagulation factors. Neuron 7:295–307

Takahashi T, Fournier A, Nakamura F, Wang LH, Murakami Y, Kalb RG, Fujisawa H, Strittmatter SM (1999) Plexin-neuropilin-1 complexes form functional semaphorin-3A receptors. Cell 99:59–69

Takahashi T, Nakamura F, Jin Z, Kalb RG, Strittmatter SM (1998) Semaphorins A and E act as antagonists of neuropilin-1 and agonists of neuropilin-2 receptors. Nat Neurosci 1:487–493

Takashima S, Kitakaze M, Asakura M, Asanuma H, Sanada S, Tashiro F, Niwa H, Miyazaki JJ, Hirota S, Kitamura Y, Kitsukawa T, Fujisawa H, Klagsbrun M, Hori M (2002) Targeting of both mouse neuropilin-1 and neuropilin-2 genes severely impairs developmental yolk sac and embryonic angiogenesis. Proc Natl Acad Sci U S A 99:3657–3662

Tamagnone L, Artigiani S, Chen H, He Z, Ming GI, Song H, Chedotal A, Winberg ML, Goodman CS, Poo M, Tessier-Lavigne M, Comoglio PM (1999) Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. Cell 99:71–80

Tammela T, Enholm B, Alitalo K, Paavonen K (2005) The biology of vascular endothelial growth factors. Cardiovasc Res 65:550–563

Terman BI, Dougher-Vermazen M, Carrion ME, Dimitrov D, Armellino DC, Gospodarowicz D, Bôhlen P (1992) Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. Biochem Biophys Res Commun 187:1579–1586

Terman JR, Mao T, Pasterkamp RJ, Yu HH, Kolodkin AL (2002) MICALs, a family of conserved flavoprotein oxidoreductases, function in plexin-mediated axonal repulsion. Cell 109:887–900

Tomizawa Y, Sekido Y, Kondo M, Gao B, Yokota J, Roche J, Drabkin H, Lerman MI, Gazdar AF, Minna JD (2001)

Inhibition of lung cancer cell growth and induction of apoptosis after reexpression of 3p21.3 candidate tumor suppressor gene SEMA3B. Proc Natl Acad Sci U S A 98:13954–13959

Torres-Vazquez J, Gitler AD, Fraser SD, Berk JD, Van NP, Fishman MC, Childs S, Epstein JA, Weinstein BM (2004) Semaphorin-plexin signaling guides patterning of the developing vasculature. Dev Cell 7:117–123

Toyofuku T, Zhang H, Kumanogoh A, Takegahara N, Suto F, Kamei J, Aoki K, Yabuki M, Hori M, Fujisawa H, Kikutani H (2004) Dual roles of Sema6D in cardiac morphogenesis through region-specific association of its receptor, Plexin-A1, with off-tack and vascular endothelial growth factor receptor type 2. Genes Dev 18:435–447

Trusolino L, Comoglio PM (2002) Scatter-factor and semaphorin receptors: cell signalling for invasive growth. Nat Rev Cancer 2:289–300

Veikkola T, Jussila L, Makinen T, Karpanen T, Jeltsch M, Petrova TV, Kubo H, Thurston G, McDonald DM, Achen MG, Stacker SA, Alitalo K (2001) Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. EMBO J 20:1223–1231

West DC, Chris RG, Duchesne L, Patey SJ, Terry CJ, Turnbull JE, Delehedde M, Heegaard CW, Allain F, Vanpouille C, Ron D, Fernig DG (2005) Interactions of multiple heparin-binding growth factors with neuropilin-1 and potentiation of the activity of fibroblast growth factor-2. J Biol Chem 280:13457–13464

Wey JS, Gray MJ, Fan F, Belcheva A, Mccarty MF, Stoeltzing O, Somcio R, Liu W, Evans DB, Klagsbrun M, Gallick GE, Ellis LM (2005) Overexpression of neuropilin-1 promotes constitutive MAPK signalling and chemoresistance in pancreatic cancer cells. Br J Cancer 93:233–241

Whitaker GB, Limberg BJ, Rosenbaum JS (2001) Vascular endothelial growth factor receptor-2 and neuropilin-1 form a receptor complex that is responsible for the differential signaling potency of VEGF(165) and VEGF(121). J Biol Chem 276:25520–25531

Wolman MA, Liu Y, Tawarayama H, Shoji W, Halloran MC (2004) Repulsion and attraction of axons by Semaphorin3D are mediated by different neuropilins in vivo. J Neurosci 24:8428–8435

Xiang R, Davalos AR, Hensel CH, Zhou XJ, Tse C, Naylor SL (2002) Semaphorin 3F gene from human 3p21.3 suppresses tumor formation in nude mice. Cancer Res 62:2637–2643

Xiang RH, Hensel CH, Garcia DK, Carlson HC, Kok K, Daly MC, Kerbacher K, van den BA, Veldhuis P, Buys CH, Naylor SL (1996) Isolation of the human semaphorin III/F gene (SEMA3F) at chromosome 3p21, a region deleted in lung cancer. Genomics 32:39–48

Yamada Y, Takakura N, Yasue H, Ogawa H, Fujisawa H, Suda T (2001) Exogenous clustered neuropilin 1 enhances vasculogenesis and angiogenesis. Blood 97:1671–1678

Yaron A, Huang PH, Cheng HJ, Tessier-Lavigne M (2005) Differential requirement for Plexin-A3 and -A4 in mediating responses of sensory and sympathetic neurons to distinct class 3 semaphorins. Neuron 45:513–523

Yuan L, Moyon D, Pardanaud L, Breant C, Karkkainen MJ, Alitalo K, Eichmann A (2002) Abnormal lymphatic vessel development in neuropilin 2 mutant mice. Development 129:4797–4806

Platelet-derived Growth Factor: Impact on Physiological and Tumor Angiogenesis

9

ARNE ÖSTMAN and CHRISTER BETSHOLTZ

Abstract

The PDGF growth factors make up a family of five dimeric growth factors acting through the two dimeric tyrosine kinase PDGF α- and β-receptors. This chapter summarizes the findings, predominantly from analyses of genetically modified mice, which have established important functions for PDGF receptor signaling in the recruitment and growth of vascular mural cells. The text also reviews studies which have confirmed important roles of PDGF receptor signaling in tumor angiogenesis, predominantly involving effects on the mural cells. Thereafter, pre-clinical studies demonstrating anti-angiogenic and anti-tumoral effects of PDGF inhibitors are reviewed. Finally, results from clinical studies using drugs that block PDGF-receptors are discussed.

CONTENTS

PDGF Ligands and Receptors

9.1.1 The PDGF System: Five Dimeric Ligands and Two Tyrosine Kinase Receptors

9.1.1.1 PDGF Ligands

The PDGF family of growth factors are encoded by four genes that give rise to four homodimeric proteins – PDGF-AA, -BB, -CC, -DD – and one heterodimeric form – PDGF-AB (Heldin et al. 1998). The four different PDGF genes have similar exon-intron architecture and all code for proteins with a classical amino-terminal hydrophobic signal sequence. The conserved PDGF core domain is characterized by eight conserved cysteine residues. The PDGF A-chain gene is subject to alternative splicing, which gives rise to forms with or without a carboxy-terminal negatively charged "retention sequence" (see below) (Betsholtz et al. 1986, 1990). The PDGF C- and D-genes differ from the other by encoding an amino-terminal CUB domain (see below) (Heldin et al. 2002).

Biosynthesis of the dimeric PDGF proteins involve intracellular dimerization, mediated by cross-wise disulfide formation of the second and fourth cysteine residues, which precedes processing of amino- and carboxy-terminal pro-sequences (Andersson et al. 1992; Ostman et al. 1988, 1992). The pro-sequences, which show significant variations between the PDGF isoforms, are important for determining localization and latency. PDGF-BB and dimers of the long splice form of PDGF-AA contain carboxy-terminal retention sequences mediating pericellular retention through binding to proteoglycans. PDGF-CC and -DD differ from other PDGF isoforms by being secreted as latent version, which are activated upon cleavage of the amino-terminal CUB domain. Recent studies suggest tissue plasminogen activator and urokinase plasminogen activator as the most important proteases for cleavage of the CUB domain (Fredriksson et al. 2004, 2005; Ustach and Kim 2005).

Characterization of the three-dimensional structure of PDGF-BB revealed that the chains of the dimer are arranged in an anti-parallel manner, creating an elongated bowl-shaped structure (Oefner et al. 1992). This overall structure is similar to that of vascular endothelial growth factor (VEGF), and also includes a structurally distinct pattern of disulfide bridges – designated cysteine knot – also seen in members of the TGF-beta and NGF families of growth factors (Murray-Rust et al. 1993). The receptor-binding part has been mapped, by mutational studies, to the two distal parts, which each are composed of distinct loops from the two subunits (LaRochelle et al. 1990, 1992; Ostman et al. 1991b).

9.1.1.2 PDGF Receptors

The two PDGF receptors are designated α- and β-receptors. The two receptors are encoded by distinct genes on chromosome 4 and 5, respectively, and are independently regulated (Heldin and Westermark 1999). Structural features of the receptors include an extracellular domain composed of five Ig-like domains, a single transmembrane domain and an intracellular part with a split tyrosine kinase domain surrounded by a juxtamembrane region and a carboxy-terminal tail. The two PDGF receptors are most similar in their kinase domains, which show about 80% amino acid conservation. In contrast, the extracellular parts show only 30% conservation. During biosynthesis the receptors undergo glycosylation before insertion into the plasma membrane. Turnover of receptors occurs through receptor internalization and degradation.

9.1.1.3 Ligand-Receptor Interactions

The five PDGF ligands display distinct receptor-binding specificities (Heldin and Westermark 1999). In brief, PDGF-BB can induce the formation of all three possible receptor dimers (α/α, α/β, β/β), whereas PDGF-AA and -DD signal only through α/α and β/β receptors, respectively. PDGF-AB and -CC can both induce receptor heterodimers, and

in addition signal through PDGF α-receptor homodimers.

In all cases ligand binding involves receptor dimerization through the formation of a complex composed of one bivalent dimeric PDGF ligand and two receptor molecules (Heldin et al. 1989). As of now, the structure of a PDGF ligand–receptor complex has not been solved. However, biochemical studies have indicated that ligand binding particularly involves the second and third Ig-like domains of the receptors (Heidaran et al. 1990; Miyazawa et al. 1998). Furthermore, receptor–receptor interaction, involving the fourth Ig-like domain, contributes to stabilization of ligand-induced receptor dimers (Omura et al. 1997).

9.1.2 PDGF Receptor Signaling

Characteristic cellular responses to PDGF stimulation include proliferation and chemotaxis. The molecular details underlying these responses to PDGF stimulation have been extensively studied and have revealed a complex pattern of cell-specific activation of multiple interacting pathways (Heldin et al. 1998). What follows below is a brief summary that highlights major concepts, and molecules and pathways of particular interest.

9.1.2.1 Signal Transduction Triggered by PDGF Receptor Activation

Ligand-induced receptor dimerization leads to autophosphorylation of tyrosine residues, outside the kinase domain, which induce site-specific recruitment of proteins with phospho-tyrosine-binding domains such as SH2 or PTB domains. These proteins include adaptor proteins, such as grb2/sos and shc, which both contribute to activation of ras signaling, and signaling enzymes such as tyrosine kinase c-Src, the tyrosine phosphatase SHP-2, phospho-lipase C-gamma and the lipid kinase PI-3 kinase. The activation of these enzymes include direct tyrosine phosphorylation as in the case of PLC-gamma, conformational changes as for SHP-2 or translocation to a place of substrate access, as exemplified by PI-3 kinase.

Although some initial studies indicated that strong links could be made between particular signaling enzymes and specific cellular responses, such as migration or proliferation, there is now an emerging consensus that such a notion is too simplistic. It is now rather believed that quantitative differences in signal strength and duration are more important determinants for the type of response that is elicited.

9.1.2.2 Negative Regulation of PDGF Receptor Signaling

The major mechanisms for termination of PDGF receptor signaling are ligand-induced receptor internalization and dephosphorylation by protein-tyrosine phosphatases (PTPs). Concerning PDGF-receptor-targeting PTPs, both cytosolic and receptor-like PTPs have been implicated as negative regulators, including PTP-1B, TC-PTP and DEP-1 (Haj et al. 2003; Markova et al. 2003; Persson et al. 2004). A potentially interesting concept that has emerged from these studies is that different PTPs act in a site-specific manner (Persson et al. 2004). This implies that changes in the profile of PTP activity in cells will impact on the pattern of receptor phosphorylation and thereby possibly determine the type of cellular response triggered by ligand stimulation.

9.1.2.3 Differences Between Signaling of Different PDGF Receptor Dimers

As mentioned above, the intracellular parts of the PDGF α- and β-receptors display a large degree of conservation, and most auto-phosphorylation sites are conserved between the two receptors. Some noteworthy differences include the fact that the RasGAP binding site of the β-receptor (P-Tyr-771) has no counterpart in the α-receptor, and that the Shc-binding sites of the β-receptors appear to be to unique to this isoform (Ronnstrand and Hel-

din 2001). Furthermore, detailed comparison of the phosphorylation patterns of the β-receptor in α/β- and β/β-dimers revealed reduced P-Tyr-771 phosphorylation in the heterodimer which might be biologically significant (Ronnstrand and Heldin 2001). Genetic approaches have also been employed to compare the in vivo signaling capacity of receptors (Klinghoffer et al. 2001). One intriguing finding of this study was that the α-receptor kinase domain failed to execute some of the functions of the β-receptor in vascular development.

9.2 PDGF and Physiological Angiogenesis

9.2.1 Role of PDGFs in the Development of Vascular Mural Cells

The PDGFs are structurally closely related to the VEGFs. The VEGF family of growth factors regulates angiogenesis primarily through direct effects on endothelial cells. The PDGFs play a role in angiogenesis by having direct effects on the mural cells of the vascular wall, the vascular smooth muscle cells (vSMC) and the pericytes (reviewed in: Betsholtz 2004; Hoch and Soriano 2003).

9.2.1.1 Role of PDGF α-Receptors in Mural Cell Precursors

Developing and mature pericytes express primarily PDGF β-receptors, but α-receptors are expressed as well in the vSMC of larger vessels. Knockout of the α-receptor gene, *pdgfra*, leads to defective formation of mural cells in the cardiac outflow tract and malformation of this region, contributing to the embryonic lethality of *pdgfra* null mice at around embryonic days (E) 14–16 (Soriano 1997). PDGF α-receptors play a role in the proliferation and migration of neural crest-derived mesenchyme, which may explain why mural compartments derived from neural crest, such as that of the cardiac outflow tract, are affected in *pdgfra* knockouts. The embryonic kidney is another site where mesenchymal precursors of mural cells depend on PDGF α-receptors (Ding et al. 2004). Whereas PDGF α-receptors may thus play a role in the development of certain mesenchymal precursors of mural cell precursors, it is not known whether they also play a role in the already established mural cells.

9.2.1.2 Role of PDGF β-Receptors for Mural Cell Proliferation and Migration

PDGF β-receptors appear dispensable for the induction of mural cells from immature mesenchyme; *pdgfrb* knockouts establish an early coat of mural cells around the large trunk vessels and cardiac outflow tract (Hellstrom et al. 1999). However, subsequent proliferation of mural cells and their longitudinal recruitment along angiogenic sprouts fail in *pdgfrb* knockouts. These mutants are perinatally lethal (Soriano 1994), and the cause of death appears to be widespread microvascular dysfunction caused by lack of pericytes, resulting in extensive microvascular hemorrhage and edema.

9.2.1.3 PDGF-B is the Critical PDGF β-Receptor Ligand During Development

Pdgfb knockouts (Leveen et al. 1994; Lindahl et al. 1997) and *pdgfrb* knockouts have indistinguishable phenotypes. *Pdgfd* null mice have not yet been reported, and it is therefore too early to rule out a role for PDGF-D in development. *Pdgfc* null mice share some of the characteristics of *pdgfra* null mice, and the *pdgfa*/*pdgfc* double-knockout phenocopies the *pdgfra* knockout, suggesting that PDGF-AA and PDGF-CC have cooperative functions via PDGF α-receptors during development (Ding et al. 2004). Together, these observations also suggest that PDGF-BB is the major ligand for PDGF β-receptors in the regulation of pericyte recruitment during developmental angiogenesis. However, the possibility remains that PDGF-C and -D have vascular functions

in the postnatal period. Future conditional genetic silencing of individual PDGF ligands and receptors in the postnatal period will shed additional light on these questions.

The major source of PDGF-BB for its function in pericyte recruitment appears to be the endothelial cells. The vascular endothelium expresses *pdgfb* (Lindahl et al. 1997). Particularly strong expression has been demonstrated in the endothelial tip cells of the sprouts (Gerhardt et al. 2003) and in developing arteries (Hellstrom et al. 1999), i.e. at sites where active recruitment and proliferation of mural cells take place. Endothelium-restricted ablation of PDGF-B leads to defective pericyte recruitment and a set of organ defects similar to that in the full PDGF-B knockout (Enge et al. 2003; Bjarnegard et al. 2004). Conversely, ablation of PDGF-B in hematopoietic cells through bone marrow transplantations (Kaminski et al. 2001), or neuro-ectoderm-selective ablation of PDGF-B (Enge et al. 2002), i.e. ablation in the two other major sites of PDGF-B expression during development, did not result in vascular complications.

Thus, expression patterns and genetic studies have revealed that PDGF-BB produced by endothelial cells triggers PDGF β-receptor signaling in neighboring mural cells in order to regulate their recruitment to sprouting and growing blood vessels. This paracrine mode of signaling is partially dependent on retention of secreted PDGF-B at the surface of the producing cell or in the extracellular matrix (LaRochelle et al. 1991; Ostman et al. 1991a). Deletion of the carboxy-terminal retention sequence in PDGF-B leads to aberrant detachment of pericytes from the developing microvessels and results in a microvascular phenotype similar to that of full knockout, with the difference that pericytes are present but aberrantly attached to the microvessels (Lindblom et al. 2003). The exact molecular identity of the PDGF-B retention sequence-binding epitope(s) has not yet been defined, but the abundance of basic amino acid residues within the retention sequence suggests that PDGF-B may associate with heparan sulfate proteoglycans surrounding the producer cell.

9.2.2 Consequence of Pericyte Defects for Angiogenesis

9.2.2.1 Angiogenesis in the Absence of PDGF-B or PDGFβ-Receptors

Insight into the role of pericytes for the angiogenic process has come mainly from analyses of the defective vascular development in mice carrying targeted mutations in *pdgfb* or *pdgfrb*. Studies of sprouting angiogenesis in the central nervous system (CNS) suggest that this process does not require pericytes per se. Sprouting and fusion of vessels into a primitive vascular network takes place in the absence of pericytes, and at least some degree of functionality of the vasculature is established, as organ development (including CNS development) proceeds relatively normally until E17–E19 (birth). However, vessels devoid of pericytes do not mature properly and display several abnormal features, including endothelial hypercellularity, vascular tortuosity and the formation of focal dilations (microaneurysms), altered organization of endothelial cell junctions, increased vesicular transport, increased leakage of plasma and blood cells, and aberrant formation of the luminal plasma membrane (Hellstrom et al. 2001). Many of these abnormalities are observed also in tumor vessels (Hashizume et al. 2000), which are also frequently poor in pericyte coverage. It is therefore possible that some of the aberrations typical for tumor vessels may reflect a defective pericyte coat (Abramsson et al. 2002).

It is difficult to determine the direct versus indirect effects of pericyte deficiency on endothelial cell and microvessel formation and function. As soon as the vessel functionality becomes insufficient with regard to blood and nutrient supply to the developing organs, hypoxia is likely to result, with consequent upregulation of VEGF. Embryonic development is highly sensitive to the levels of VEGF, and as little as twofold upregulation of VEGF has been shown to lead to lethal vascular aberrations (Miquerol et al. 2000). Pdgfb and pdgfrb knockouts display an approximately twofold increase in VEGF levels at late

gestation (Hellstrom et al. 2001), which might contribute to some of the above-mentioned abnormalities, in particular to the increased extravasation of plasma and blood cells. Thus, whereas the studies of pdgfb and pdgfrb mutant mice have established that pericytes have a critical role in the development of a fully functional vasculature, it is still not clear exactly how pericytes promote vessel maturation and stabilization, and what is the molecular mediator(s) of this process.

9.2.2.2 Retinal and Glomerular Capillaries Are Particularly Sensitive to Pericyte Loss

Two sites appear particularly sensitive to pericyte dysfunction (and PDGF-B/PDGF β-receptor signaling), namely the retina and the renal glomerulus. Both sites have a high abundance of pericytes (mesangial cells in the renal glomeruli), in comparison with other organs and tissues. Mutants with partial pericyte deficiency, such as the endothelial-specific *pdgfb* knockouts (Bjarnegard et al. 2004; Enge et al. 2002), the PDGF-B retention motif knockouts (Abramsson et al. 2003; Lindblom et al. 2003), the *pdgfrb* F-series (tyrosine-phenylalanine substitutions) (Tallquist et al. 2003; Tallquist et al. 2000) and PDGF β/α chimeric receptor mutants (Klinghoffer et al. 2001) all show various degrees of retinopathy and glomerulopathy, in the most severe cases leading to blindness and proteinuria. Similar effects on retinal and glomerular development have been reported following administration of neutralizing PDGF β-receptor antibodies to newborn mice (Uemura et al. 2002).

9.2.2.3 Physiological Consequences of Abnormal Extracellular Distribution of PDGF-BB

The postnatal survival of some of the *pdgfb* and *pdgfrb* mutants has permitted analysis of certain circulatory physiological parameters. It was recently reported that PDGF-B retention sequence knockouts show reduced physical performance (Nystrom et al. 2006). This correlated with two structural defects in the blood vessels. First, the aorta displayed an increased diameter and stiffness, fewer layers of vSMC and increased media collagen. No changes were observed in resistance vessels and the blood pressure was normal, suggesting a specific effect on the remodeling and function of conduit vessels. Since *pdgfb* and *pdgfrb* knockouts show aortic dilation at late gestation, it is possible that the increased stiffness and media collagen seen in adult PDGF-B retention-sequence knockouts reflect a remodeling process that has occurred secondary to the increased vessel wall tension, which is an expected consequence of the dilation (at constant blood pressure). Second, a reduced capillary density was observed in skeletal muscle. Probably, this reflects the impairment of pericyte association with the microvessels in this mutant, adding some confirmation to the notion that pericytes are needed for endothelial survival and capillary stability. The reduced capillary density provides a direct explanation for the reduced physical performance of PDGF-B retention-motif knockouts in the absence of reduced cardiac function and lower blood pressure (Nystrom et al. 2006).

PDGF and Tumor Angiogenesis

9.3.1 PDGF Receptor Expression in Human Tumors

Analyses of the physiological functions of PDGF receptors have implied that PDGF receptors are highly important for the development of mesenchymal cells, such as pericytes, fibroblasts, smooth muscle cells and mesangial cells. In most cases, this occurs through paracrine stimulation following ligand production in epithelial or endothelial cells.

In general, this pattern appears to be maintained in the common epithelial solid tumors, in which

PDGF β-receptor expression has been consistently demonstrated in pericytes and tumor fibroblasts. A recent analysis of a tumor tissue array of 25–75 samples each of different tumor types, including breast, colon, lung, prostate and ovarian cancer, has provided further support for this concept (Paulsson, Sjöblom et al., in preparation). Perivascular PDGF β-receptor staining was found in variable fractions of tumors with frequencies ranging from 56% in melanomas to 11% in prostate cancer. Tumor fibroblast PDGF β-receptor expression in tumor fibroblasts was commonly observed, with the highest frequency found in colorectal cancer (51%) and the lowest in melanoma (11). Similar analyses of PDGFα-receptor expression indicate, in general, very little staining of perivascular cells and variable expression in the fibroblasts, as exemplified by 41% positive colon cancer cases, compared to only 4% of breast cancers.

In addition to this, there are tumor types where PDGF receptor expression also occurs in the malignant cells (reviewed in (Ostman 2004). Gliomas, glioblastomas, soft tissue sarcomas and osteosarcomas are examples of tumor types where PDGF receptor signaling might be directly involved in the growth of the malignant cells.

9.3.2 Regulation of Tumor Pericytes by PDGF

The functional significance of PDGF receptor expression on tumor pericytes has been explored in various animal tumor models.

An important role for pericyte recruitment and growth of locally retained endothelial cell-derived PDGF-BB was deduced by analyzing tumor angiogenesis in which the pericellular retention of PDGF-BB produced by endothelial cells had been manipulated (Lindblom et al. 2003). Furthermore, malignant cells engineered to overproduce PDGF-BB or -DD were demonstrated to promote pericyte coverage of tumor vessels, compared to their wild-type counterparts (Furuhashi et al. 2004; Guo et al. 2003). Finally, co-injection of tumor cells and wild-type pericytes gave rise to better-organized pericyte coverage than that observed after co-injection of PDGF β-receptor-negative mesenchymal cells (Abramsson et al. 2003).

9.3.2.1 Functional Consequences of Variations in Tumor Vessel Pericyte Coverage

Some of these studies also indicated that the PDGF-dependent variations in pericyte coverage had functional consequences with regard to vessel function, tumor growth and tumor metastasis (Furuhashi et al. 2004; Xian et al. 2006). In general, higher pericyte coverage was associated with a tendency towards reduction of vessel diameter and reduced hemorrhage. One study also clearly demonstrated that the increased pericyte coverage, which in this model occurred in the absence of increase in vessel density, enhanced tumor growth (Furuhashi et al. 2004). Analyses of tumor perfusion, by functional MRI and staining of perfused vessels, indicate that the enhanced pericyte coverage is associated with enhanced perfusion, thus emphasizing the role of PDGF-dependent pericyte coverage in tumor vessel maturation and function (Robinson et al., submitted).

Deficient tumor vessel pericyte coverage was recently correlated with increased hematogenous metastasis from insulinomas in two different genetic mouse models – PDGF-B retention-sequence knockouts and mice deficient for neural cell adhesion molecule (N-CAM) (Xian et al. 2006). A negative correlation between vSMC abundance and metastasis in human tumors was also recently indicated through transcription profiling of primary solid tumors. Out of a 17-gene signature associated with increased metastatic propensity, 4 of 9 downregulated genes represent markers for vSMC (Ramaswamy et al. 2003) and may therefore reflect alterations in the tumor stroma rather than in the tumor cells themselves. A negative correlation between expression of the SMC marker h-caldesmon and metastasis in human melanoma has also been reported (Koganehira et al. 2003). Together, these observations highlight the possibility that vessels that are de-stabilized as a consequence of pericyte/vSMC deficiency may be more prone to invasion by tumor cells.

9.3.2.2 Origin of Tumor Pericytes

The origin of PDGF-dependent pericytes is still incompletely characterized. It is likely that proliferation among local pericytes is a major mechanism for expansion of this cell type during tumor angiogenesis. Also, conversion of stromal fibroblasts to pericytes has been proposed. Most recently, a role for bone-marrow-derived precursors in the formation of tumor pericytes was proposed, based on the identification of marrow-derived cells among tumor pericytes of mouse tumors (Song et al. 2005). This latter study also provided a preliminary identification, which merits further investigations, of distinct pericyte subsets with varying marker expression and PDGF dependency.

9.3.3 PDGF-dependent VEGF Production in Tumor Stromal Cells

VEGF is a key factor in tumor angiogenesis. Although the malignant cells of the tumors are in general considered as the major source of VEGF production, there is good evidence that stromal fibroblasts also represent a significant source of VEGF.

Studies of human tumor tissue have described stromal production of VEGF. Furthermore, an elegant study using mice in which GFP was expressed under the control of the VEGF promoter identified stromal fibroblasts as a major source of VEGF production activity in both subcutaneous and orthotopic experimental tumors (Fukumura et al. 1998). More recently, PDGF receptors expressed on fibroblasts were identified as key upstream regulators of stromal VEGF production. In a study using $VEGF^{-/-}$ tumor cells, tumor formation was still found to be sensitive to treatment with anti-VEGF antibodies (Dong et al. 2004). Stromal fibroblasts were identified as the source of VEGF production and, interestingly, this angiogenic activity could be blocked by interfering with the signaling of PDGF α-receptors exclusively expressed on mesenchymal stromal cells.

Together, these studies thus suggest that PDGF receptor signaling in tumor fibroblasts exerts an important indirect effect on angiogenic function by stimulating the VEGF production in these cells.

9.3.4 PDGF Receptor Expression on Lymph- and Hemangiogenic Tumor Endothelial Cells

PDGF receptor expression on tumor endothelial cells remains controversial, and the literature contains conflicting information. In general, it should be noted that many PDGF receptor antibodies, as well as phospho-PDGF receptor antibodies, that have been used in immuno-histochemical analyses have been incompletely characterized with regard to specificity. Furthermore, most standard formats for tissue analyses make it difficult to distinguish staining of pericytes and endothelial cells. Finally, phenotypic analyses of numerous PDGF-ligand and -receptor knockout mice have so far failed to provide strong evidence for functionally significant PDGF receptor signaling in endothelial cells. With these reservations in mind, some studies remain that merit discussion to encourage continued research efforts.

Analyses of a mouse prostate cancer bone metastasis model indicated that growth of bone metastases was associated with induction of PDGF β-receptors in endothelial cells (Uehara et al. 2003). Functional relevance was indicated both by demonstration of an activated state of receptors, through phospho-PDGF-receptor immunohistochemistry, and by growth-inhibiting effects of PDGF antagonists. Subsequent studies from the same group have demonstrated similar findings from models of bone metastases of breast cancer, ovarian cancer grown in the intraperitoneal cavity and orthotopically grown pancreatic carcinoma (Apte et al. 2004; Hwang et al. 2003; Kim et al. 2004; Lev et al. 2005). These findings merit continued analyses, among which detailed studies of corresponding human tumor tissue should be prioritized.

Tumor lymphangiogenesis is highly dependent on activation of VEGF-3 receptors (Alitalo et al. 2005). The first set of evidence for a functional role of PDGF

receptors in this process was recently presented, with the demonstration of enhanced tumor lymphangiogenesis in murine fibrosarcomas after overexpression of PDGF-BB in the tumor cells (Cao et al. 2004). To what extent this effect occurred through direct effects of PDGF-BB on lymphatic endothelial cells, or indirectly through induction of VEGFR-3 ligand, remained incompletely characterized. The former mechanism received some support from studies on cultured lymphatic endothelial cells. Given the significance of lymphangiogenesis for metastases, these findings obviously need further validation.

PDGF Receptors in Tumor Vessel as Cancer Drug Targets

9.4.1 PDGF Antagonists

9.4.1.1 Neutralizing Antibodies

Isoform-specific neutralizing polyclonal and monoclonal antibodies against PDGF ligands and receptors have been generated and successfully used to define PDGF-dependent processes in animal disease models (reviewed in (Ostman and Heldin 2001). Most of these studies have analyzed the importance of PDGF receptor signaling in atherosclerosis and restenosis, but significant effects have also been obtained in tumor models.

Humanized antibodies are most attractive as candidate drugs for clinical application. PDGF α-receptor-neutralizing activity of one such antibody was recently characterized (Loizos et al. 2005). Clinical cancer studies with neutralizing PDGF receptor antibodies are still scarce, and the only published example is a report where a PEG-conjugated FA fragment targeting the PDGF β-receptor was used to analyze effects on tumor perfusion (see Sect. 9.4.3) (Jayson et al. 2005).

9.4.1.2 Soluble Receptors

Recombinant soluble receptors constitute a generic class of growth factor antagonists. PDGF-antagonistic effects of such proteins have also been demonstrated. Two variants worth special attention are fusion proteins where the PDGF receptor sequence has been fused either to the conserved part of the immunoglobulin heavy chain or to GST (Heidaran et al. 1995; Leppanen et al. 2000). In both cases inhibitory effects were observed at nanomolar concentrations, which were believed to be dependent on the dimeric nature of both these receptor constructs. To date, none of these types of antagonists have been developed for clinical applications.

9.4.1.3 LMW Kinase Inhibitors

The first selective LMW inhibitor of the PDGF receptor tyrosine kinase was described in 1994 (Kovalenko et al. 1994). Since then a series of compounds have been introduced that are now in various phases of clinical development (some examples are detailed below). Two characteristics shared by all these compounds are that they inhibit both PDGF α- and β-receptors, and that they block some other tyrosine kinases at the concentration required for PDGF receptor inhibition. The manner in which the compounds differ from each other is in potency, i.e. concentration required for PDGF receptor inhibition, and selectivity profile.

Imatinib is the most established PDGF receptor inhibitor, with demonstrated clinical efficiency in PDGF-dependent malignancies such as dermatofibrosarcoma protubernas, hypereosinophilic syndrome and a subset of gastrointestinal tumors with activating PDGF α-receptor mutations (reviewed in (Capdeville et al. 2002; Ostman 2004). Two multikinase inhibitors with anti-PDGF receptor activity has also recently been approved by the US Food and Drug Administration (FDA) – sunitinib and sorafenib (www.fda.gov). Finally, a novel highly potent PDGF receptor inhibitor (CP673451) with a more specific

target profile has been introduced and is now entering clinical trials (Roberts et al. 2005).

9.4.2 Studies in Animal Models

Preclinical studies have defined three distinct concepts implying that PDGF receptors in cancer vessels are therapeutically relevant proteins. Firstly; inhibition of PDGF receptors on endothelial cells has been associated with direct anti-angiogenic effects. Secondly, PDGF-antagonist-mediated targeting of pericytes has been demonstrated to act synergistically with inhibitors of VEGF receptor. Thirdly, evidence has been presented suggesting that PDGF receptor inhibition of stromal cells, including pericytes and fibroblasts, have a beneficial effect on tumor drug uptake.

As discussed above, activated endothelial cell expression of PDGF receptors has been detected in mouse models of bone metastases of breast and prostate cancer lesions. Treatment of these with STI571 was shown to lead to reduced vessel density, apoptosis of tumor endothelial cells and concomitant reduction in growth of metastases (Uehara et al. 2003). Studies from the same group have also demonstrated anti-angiogenic effects of combination of STI571 with gemcitabine or paclitaxel in models of orthotopic growth of pancreas cancer and intraperitoneal growth of ovarian cancer, respectively (Apte et al. 2004; Hwang et al. 2003; Kim et al. 2004; Lev et al. 2005). Also, in these models, endothelial expression of activated PDGF receptors was demonstrated by immunohistochemistry.

The concept of a two-pronged attack on tumor vessels by combined targeting of PDGF-dependent pericytes and VEGF-dependent endothelial cells was first validated by Bergers and colleagues (Bergers et al. 2003). Their study compared the effects of PDGF antagonists and VEGF antagonists, and combinations thereof, in different phases of tumor growth in the RIP-Tag model of insulinomas. Whereas VEGF antagonists showed strong effects in a setting designed to prevent tumor formation, only the combination was able to reduce the size of established tumors. Convincing analyses of these tumors also demonstrated that PDGF receptor expression was restricted to the pericyte part of tumor vessels. Subsequently, similar findings were made after treatment of subcutaneously grown C6 rat gliomas (Erber et al. 2004).

Solid tumors are characterized by high interstitial fluid pressure (IFP), which has been postulated to act as a barrier to tumor drug uptake (Jain 2001). The mechanisms underlying this phenotype are incompletely understood. Tumor features which have been implicated include the absence of functional lymphatic vessels, leaky immature vasculature and the presence of activated fibroblasts (Heldin et al. 2004). In tumor models with PDGF receptor expression restricted to pericytes and fibroblasts, transient inhibition of stromal PDGF receptors has been demonstrated to reversibly reduce tumor IFP and increase tumor drug uptake (Baranowska-Kortylewicz et al. 2005; Pietras et al. 2001, 2002, 2003). Although inhibition of tumor fibroblasts has been considered as the major mediator of these interesting effects, it can still not be excluded that some of these changes also involve alterations in pericyte function.

9.4.3 Clinical Effects of Drugs with Anti-PDGF Activity Involving Anti-angiogenesis

The antitumor activity of the FDA approved multikinase inhibitors sunitinib and sorafenib are generally believed to involve anti-angiogenic effects which occur through the ability of these drugs to exert combined anti-endothelial and antipericyte actions via inhibition of both VEGF and PDGF receptors (Larkin and Eisen 2006). In renal cell cancer, phase III studies with sorafenib and sunitinib have demonstrated survival benefits of both drugs (abstracts at ASCO meetings 2005 and 2006).

These results are likely to be followed by reports of results in phase II/III studies from many other tumor types in the near future. It will be interesting to see whether these studies can be performed in a manner that can directly demonstrate that an-

tipericyte effects, involving PDGF receptor inhibition, contribute to the antitumor activity. Obvious goals for continued evaluation of anti-angiogenic strategies include the identification of markers for particularly sensitive or resistant tumors. In this context it should be remembered that tumors appear to show wide variations with regard to presence of PDGF receptor-positive pericytes. It thus seems warranted to investigate possible relationships between pericyte status and sensitivity to anti-angiogenic drugs with or without anti-PDGF receptor activity.

Alterations in vascular tumor physiology following treatment with PDGF antagonists were recently directly demonstrated in a study which applied MRI analyses of patients treated with a neutralizing PDGF receptor antibody (Jayson et al. 2005). In this study, an increase in relative blood volume was observed following PDGF receptor inhibition. This change is compatible with a reduction in IFP, mediated by tu-

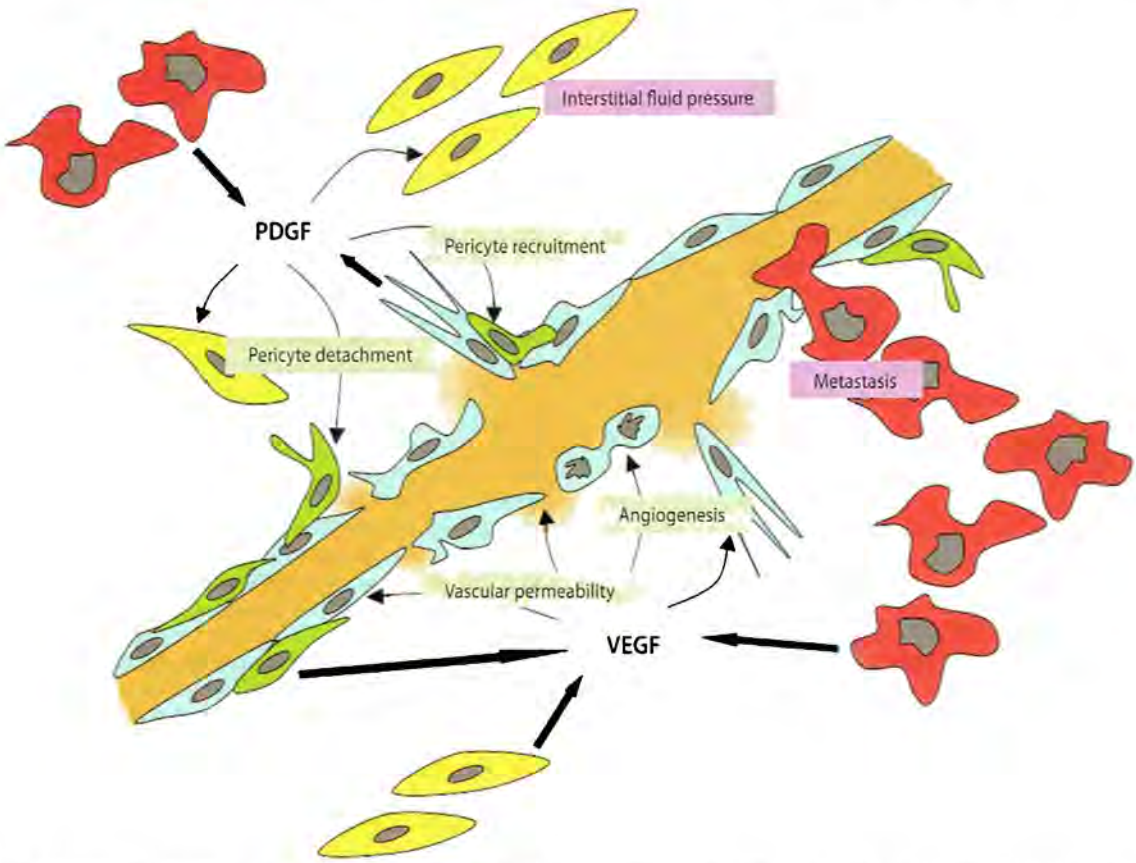


Fig. 9.1. Schematic depiction of paracrine PDGF and VEGF functions in solid tumors and their consequences for the tumor vasculature and stroma. Interplay between tumor cells (*red*), blood vessel endothelium (*blue*), pericytes (*green*) and fibroblasts (*yellow*). PDGF-BB production by sprouting endothelial cells promotes pericyte recruitment to the growing sprout. PDGF β-receptor agonists (-BB or -DD) produced by tumor cells may stimulate pericytes to detach from the vessels. PDGF α- and β-receptor agonists (-AA, -AB, -BB, -CC, -DD) produced by the tumor cells may influence fibroblast recruitment, and β-receptor agonists may increase the interstitial fluid pressure within the tumor. Pericyte deficiency or detachment from the vessels may facilitate metastatic dissemination of tumor cells. In addition to the tumor cells, both stromal fibroblasts and pericytes may contribute to the production of VEGF, which increases vascular permeability and interstitial fluid pressure, as well as triggering endothelial sprouting and proliferation as part of the tumor angiogenic response

mor fibroblasts, but might also reflect functional or structural changes of the tumor vasculature caused by perturbed pericyte functions.

Concluding Remarks

Genetic and pharmacological experiments, primarily performed in rodents, have firmly established a role of PDGF-B-containing PDGF ligands and the PDGF β-receptor in pericyte recruitment to newly formed blood vessels, both under physiological conditions and in tumors. More broadly, PDGF ligands and receptors have also been shown to have indirect roles in angiogenesis, for example by promoting the recruitment of stroma cells (e.g. "fibroblasts") as pericyte/vSMC precursors or as important sources of endothelial mitogens (such as VEGF) (Fig. 9.1). Based on data obtained from various experimental animal models, it may therefore appear attractive to attempt tumor vessel targeting, directly or indirectly, by inhibiting PDGF/PDGF receptor signaling. One possible caveat which requires further exploration is that the abnormal and dysfunctional vessels that may result from PDGF/PDGF receptor inhibition or pericyte/vSMC targeting may also be more prone to invasion by tumor cells, possibly promoting tumor dissemination. Other tumor parameters that may be influenced by PDGF/PDGF receptor targeting include IFP, reduction of which may have the beneficial effect of enhancing drug delivery to the tumor.

Altogether, there is thus now ample experimental rationale for testing PDGF or PDGF receptor antagonists clinically in human patients with the aim of inhibiting formation or maturation of tumor blood vessels. However, since many basic aspects of the PDGF, mural cell, and tumor stroma biology remain poorly understood, it is also important to continue addressing fundamental questions in various experimental model systems.

Notes added in proof: The phase III studies with sorafenib and sunitinib in renal cell cancer are now published (Escudier B et al., NEJM, 2007 and Motzer, RA et al., NEJM, 2007).

References

Abramsson A, Berlin O, Papayan H, Paulin D, Shani M, Betsholtz C (2002) Analysis of mural cell recruitment to tumor vessels. Circulation 105:112–117

Abramsson A, Lindblom P, Betsholtz C (2003) Endothelial and nonendothelial sources of PDGF-B regulate pericyte recruitment and influence vascular pattern formation in tumors. J Clin Invest 112:1142–1151

Alitalo K, Tammela T, Petrova TV (2005) Lymphangiogenesis in development and human disease. Nature 446:946–953

Andersson M, Ostman A, Backstrom G, Hellman U, George-Nascimento C, Westermark B, Heldin CH (1992) Assignment of interchain disulfide bonds in platelet-derived growth factor (PDGF) and evidence for agonist activity of monomeric PDGF. J Biol Chem 267:11260–11266

Apte SM, Fan D, Killion JJ, Fidler IJ (2004) Targeting the platelet-derived growth factor receptor in antivascular therapy for human ovarian carcinoma. Clin Cancer Res 10:897–908

Baranowska-Kortylewicz J, Abe M, Pietras K, Kortylewicz ZP, Kurizaki T, Nearman J, Paulsson J, Mosley RL, Enke CA, Ostman A (2005) Effect of platelet-derived growth factor receptor-beta inhibition with STI571 on radioimmunotherapy. Cancer Res 65:7824–7831

Bergers G, Song S, Meyer-Morse N, Bergsland E, Hanahan D (2003) Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. J Clin Invest 111:1287–1295

Betsholtz C (2004) Insight into the physiological functions of PDGF through genetic studies in mice. Cytokine Growth Factor Rev 15:215–228

Betsholtz C, Johnsson A, Heldin CH, Westermark B, Lind P, Urdea MS, Eddy R, Shows TB, Philpott K, Mellor AL, et al (1986) cDNA sequence and chromosomal localization of human platelet-derived growth factor A-chain and its expression in tumour cell lines. Nature 320:695–699

Betsholtz C, Rorsman F, Westermark B, Ostman A, Heldin CH (1990) Analogous alternative splicing. Nature 344:299

Bjarnegard M, Enge M, Norlin J, Gustafsdottir S, Fredriksson S, Abramsson A, Takemoto M, Gustafsson E, Fassler R, Betsholtz C (2004) Endothelium-specific ablation of PDGFB leads to pericyte loss and glomerular, cardiac and placental abnormalities. Development 131:1847–1857

Cao R, Bjorndahl MA, Religa P, Clasper S, Garvin S, Galter D, Meister B, Ikomi F, Tritsaris K, Dissing S, et al (2004) PDGF-BB induces intratumoral lymphangiogenesis and promotes lymphatic metastasis. Cancer Cell 6:333–345

Capdeville R, Buchdunger E, Zimmermann J, Matter A (2002) Glivec (STI571, imatinib), a rationally developed, targeted anticancer drug. Nat Rev Drug Discov 1:493–502

Ding H, Wu X, Bostrom H, Kim I, Wong N, Tsoi B, O'Rourke M, Koh GY, Soriano P, Betsholtz C, et al (2004) A specific requirement for PDGF-C in palate formation and PDGFR-alpha signaling. Nat Genet 36:1111–1116

Dong J, Grunstein J, Tejada M, Peale F, Frantz G, Liang WC, Bai W, Yu L, Kowalski J, Liang X, et al (2004) VEGF-null cells require PDGFR alpha signaling-mediated stromal fibroblast recruitment for tumorigenesis. Embo J 23:2800–2810

Enge M, Bjarnegard M, Gerhardt H, Gustafsson E, Kalen M, Asker N, Hammes HP, Shani M, Fassler R, Betsholtz C (2002) Endothelium-specific platelet-derived growth factor-B ablation mimics diabetic retinopathy. Embo J 21:4307–4316

Erber R, Thurnher A, Katsen AD, Groth G, Kerger H, Hammes HP, Menger MD, Ullrich A, Vajkoczy P (2004) Combined inhibition of VEGF and PDGF signaling enforces tumor vessel regression by interfering with pericyte-mediated endothelial cell survival mechanisms. Faseb J 18:338–340

Fredriksson L, Ehnman M, Fieber C, Eriksson U (2005) Structural requirements for activation of latent platelet-derived growth factor CC by tissue plasminogen activator. J Biol Chem 280:26856–26862

Fredriksson L, Li H, Fieber C, Li X, Eriksson U (2004) Tissue plasminogen activator is a potent activator of PDGF-CC. Embo J 23:3793–3802

Fukumura D, Xavier R, Sugiura T, Chen Y, Park EC, Lu N, Selig M, Nielsen G, Taksir T, Jain RK, Seed B (1998) Tumor induction of VEGF promoter activity in stromal cells. Cell 94 715–725

Furuhashi M, Sjoblom T, Abramsson A, Ellingsen J, Micke P, Li H, Bergsten-Folestad E, Eriksson U, Heuchel R, Betsholtz C, et al (2004) Platelet-derived growth factor production by B16 melanoma cells leads to increased pericyte abundance in tumors and an associated increase in tumor growth rate. Cancer Res 64:2725–2733

Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, Jeltsch M, Mitchell C, Alitalo K, Shima D, Betsholtz C (2003) VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. J Cell Biol 161:1163–1177

Guo P, Hu B, Gu W, Xu L, Wang D, Huang HJ, Cavenee WK, Cheng SY (2003) Platelet-derived growth factor-B enhances glioma angiogenesis by stimulating vascular endothelial growth factor expression in tumor endothelia and by promoting pericyte recruitment. Am J Pathol 162:1083–1093

Haj FG, Markova B, Klaman LD, Bohmer FD, Neel BG (2003) Regulation of receptor tyrosine kinase signaling by protein tyrosine phosphatase-1B. J Biol Chem 278:739–744

Hashizume H, Baluk P, Morikawa S, McLean JW, Thurston G, Roberge S, Jain RK, McDonald DM (2000) Openings between defective endothelial cells explain tumor vessel leakiness. Am J Pathol 156:1363–1380

Heidaran MA, Mahadevan D, Larochelle WJ (1995) Beta PDGFR-IgG chimera demonstrates that human beta PDGFR Ig-like domains 1 to 3 are sufficient for high affinity PDGF BB binding. Faseb J 9:140–145

Heidaran MA, Pierce JH, Jensen RA, Matsui T, Aaronson SA (1990) Chimeric alpha- and beta-platelet-derived growth factor (PDGF) receptors define three immunoglobulin-like domains of the alpha-PDGF receptor that determine PDGF-AA binding specificity. J Biol Chem 265:18741–18744

Heldin CH, Westermark B (1999) Mechanism of action and in vivo role of platelet-derived growth factor. Physiol Rev 79:1283–1316

Heldin CH, Ernlund A, Rorsman C, Ronnstrand L (1989) Dimerization of B-type platelet-derived growth factor receptors occurs after ligand binding and is closely associated with receptor kinase activation. J Biol Chem 264:8905–8912

Heldin CH, Ostman A, Ronnstrand L (1998) Signal transduction via platelet-derived growth factor receptors. Biochim Biophys Acta 1378:F79–113

Heldin CH, Eriksson U, Ostman A (2002) New members of the platelet-derived growth factor family of mitogens. Arch Biochem Biophys 398:284–290

Heldin CH, Rubin K, Pietras K, Ostman A (2004) High interstitial fluid pressure – an obstacle in cancer therapy. Nat Rev Cancer 4:806–813

Hellstrom M, Kalen M, Lindahl P, Abramsson A, Betsholtz C (1999) Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. Development 126:3047–3055

Hoch RV, Soriano P (2003) Roles of PDGF in animal development. Development 130:4769–4784

Hellstrom M, Gerhardt H, Kalen M, Li X, Eriksson U, Wolburg H, Betsholtz C (2001) Lack of pericytes leads to endothelial hyperplasia and abnormal vascular morphogenesis. J Cell Biol 153:543–553

Hwang RF, Yokoi K, Bucana CD, Tsan R, Killion JJ, Evans DB, Fidler IJ (2003) Inhibition of platelet-derived growth factor receptor phosphorylation by STI571 (Gleevec) reduces growth and metastasis of human pancreatic carcinoma in an orthotopic nude mouse model. Clin Cancer Res 9:6534–6544

Jain RK (2001) Delivery of molecular and cellular medicine to solid tumors. Adv Drug Deliv Rev 46:149–168

Jayson GC, Parker GJ, Mullamitha S, Valle JW, Saunders M, Broughton L, Lawrance J, Carrington B, Roberts C, Issa B, et al (2005) Blockade of platelet-derived growth factor receptor-beta by CDP860, a humanized, PEGylated di-Fab', leads to fluid accumulation and is associated with increased tumor vascularized volume. J Clin Oncol 23:973–981

Kaminski WE, Lindahl P, Lin NL, Broudy VC, Crosby JR, Hellstrom M, Swolin B, Bowen-Pope DF, Martin PJ, Ross R, et al (2001) Basis of hematopoietic defects in platelet-derived growth factor (PDGF)-B and PDGF beta-receptor null mice. Blood 97:1990–1998

Kim SJ, Uehara H, Yazici S, Langley RR, He J, Tsan R, Fan

D, Killion JJ, Fidler IJ (2004) Simultaneous blockade of platelet-derived growth factor-receptor and epidermal growth factor-receptor signaling and systemic administration of paclitaxel as therapy for human prostate cancer metastasis in bone of nude mice. Cancer Res 64:4201–4208

Klinghoffer RA, Mueting-Nelsen PF, Faerman A, Shani M, Soriano P (2001) The two PDGF receptors maintain conserved signaling in vivo despite divergent embryological functions. Mol Cell 7:343–354

Koganehira Y, Takeoka M, Ehara T, Sasaki K, Murata H, Saida T, Taniguchi S (2003) Reduced expression of actin-binding proteins, h-caldesmon and calponin h1, in the vascular smooth muscle inside melanoma lesions: an adverse prognostic factor for malignant melanoma. Br J Dermatol 148:971–980

Kovalenko M, Gazit A, Bohmer A, Rorsman C, Ronnstrand L, Heldin CH, Waltenberger J, Bohmer FD, Levitzki A (1994) Selective platelet-derived growth factor receptor kinase blockers reverse sis-transformation. Cancer Res 54:6106–6114

Larkin JM, Eisen T (2006) Kinase inhibitors in the treatment of renal cell carcinoma. Crit Rev Oncol Hematol

LaRochelle WJ, Giese N, May-Siroff M, Robbins KC, Aaronson SA (1990) Molecular localization of the transforming and secretory properties of PDGF A and PDGF B. Science 248:1541–1544

LaRochelle WJ, May-Siroff M, Robbins KC, Aaronson SA (1991) A novel mechanism regulating growth factor association with the cell surface: identification of a PDGF retention domain. Genes Dev 5:1191–1199

LaRochelle WJ, Pierce JH, May-Siroff M, Giese N, Aaronson SA (1992) Five PDGF B amino acid substitutions convert PDGF A to a PDGF B-like transforming molecule. J Biol Chem 267:17074–17077

Leppanen O, Miyazawa K, Backstrom G, Pietras K, Sjoblom T, Heldin CH, Ostman A (2000) Predimerization of recombinant platelet-derived growth factor receptor extracellular domains increases antagonistic potency. Biochemistry 39:2370–2375

Lev DC, Kim SJ, Onn A, Stone V, Nam DH, Yazici S, Fidler IJ, Price JE (2005) Inhibition of platelet-derived growth factor receptor signaling restricts the growth of human breast cancer in the bone of nude mice. Clin Cancer Res 11:306–314

Leveen P, Pekny M, Gebre-Medhin S, Swolin B, Larsson E, Betsholtz C (1994) Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. Genes Dev 8:1875–1887

Lindahl P, Johansson BR, Leveen P, Betsholtz C (1997) Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. Science 277:242–245

Lindblom P, Gerhardt H, Liebner S, Abramsson A, Enge M, Hellstrom M, Backstrom G, Fredriksson S, Landegren U, Nystrom HC, et al (2003) Endothelial PDGF-B retention is required for proper investment of pericytes in the microvessel wall. Genes Dev 17:1835–1840

Loizos N, Xu Y, Huber J, Liu M, Lu D, Finnerty B, Rolser R, Malikzay A, Persaud A, Corcoran E, et al (2005) Targeting the platelet-derived growth factor receptor alpha with a neutralizing human monoclonal antibody inhibits the growth of tumor xenografts: implications as a potential therapeutic target. Mol Cancer Ther 4:369–379

Markova B, Herrlich P, Ronnstrand L, Bohmer FD (2003) Identification of protein tyrosine phosphatases associating with the PDGF receptor. Biochemistry 42:2691–2699

Miquerol L, Langille BL, Nagy A (2000) Embryonic development is disrupted by modest increases in vascular endothelial growth factor gene expression. Development 127:3941–3946

Miyazawa K, Backstrom G, Leppanen O, Persson C, Wernstedt C, Hellman U, Heldin CH, Ostman A (1998) Role of immunoglobulin-like domains 2–4 of the platelet-derived growth factor alpha-receptor in ligand-receptor complex assembly. J Biol Chem 273:25495–25502

Murray-Rust J, McDonald NQ, Blundell TL, Hosang M, Oefner C, Winkler F, Bradshaw RA (1993) Topological similarities in TGF-beta 2, PDGF-BB and NGF define a superfamily of polypeptide growth factors. Structure 1:153–159

Nystrom HC, Lindblom P, Wickman A, Andersson I, Norlin J, Faldt J, Lindahl P, Skott O, Bjarnegard M, Fitzgerald SM, et al (2006) Platelet-derived growth factor B retention is essential for development of normal structure and function of conduit vessels and capillaries. Cardiovasc Res 71:557–565

Oefner C, D'Arcy A, Winkler FK, Eggimann B, Hosang M (1992) Crystal structure of human platelet-derived growth factor BB. Embo J 11:3921–3926

Omura T, Heldin CH, Ostman A (1997) Immunoglobulin-like domain 4-mediated receptor-receptor interactions contribute to platelet-derived growth factor-induced receptor dimerization. J Biol Chem 272:12676–12682

Ostman A (2004) PDGF receptors-mediators of autocrine tumor growth and regulators of tumor vasculature and stroma. Cytokine Growth Factor Rev 15:275–286

Ostman A, Andersson M, Betsholtz C, Westermark B, Heldin CH (1991a) Identification of a cell retention signal in the B-chain of platelet-derived growth factor and in the long splice version of the A-chain. Cell Regul 2:503–512

Ostman A, Andersson M, Hellman U, Heldin CH (1991b) Identification of three amino acids in the platelet-derived growth factor (PDGF) B-chain that are important for binding to the PDGF beta-receptor. J Biol Chem 266:10073–10077

Ostman A, Heldin CH (2001) Involvement of platelet-derived growth factor in disease: development of specific antagonists. Adv Cancer Res 80:1–38

Ostman A, Rall L, Hammacher A, Wormstead MA, Coit D, Valenzuela P, Betsholtz C, Westermark B, Heldin CH

(1988) Synthesis and assembly of a functionally active recombinant platelet-derived growth factor AB heterodimer. J Biol Chem 263:16202–16208

Ostman A, Thyberg J, Westermark B, Heldin CH (1992) PDGF-AA and PDGF-BB biosynthesis: proprotein processing in the Golgi complex and lysosomal degradation of PDGF-BB retained intracellularly. J Cell Biol 118:509–519

Persson C, Savenhed C, Bourdeau A, Tremblay ML, Markova B, Bohmer FD, Haj FG, Neel BG, Elson A, Heldin CH, et al (2004) Site-selective regulation of platelet-derived growth factor beta receptor tyrosine phosphorylation by T-cell protein tyrosine phosphatase. Mol Cell Biol 24:2190–2201

Pietras K, Ostman A, Sjoquist M, Buchdunger E, Reed RK, Heldin CH, Rubin K (2001) Inhibition of platelet-derived growth factor receptors reduces interstitial hypertension and increases transcapillary transport in tumors. Cancer Res 61:2929–2934

Pietras K, Rubin K, Sjoblom T, Buchdunger E, Sjoquist M, Heldin CH, Ostman A (2002) Inhibition of PDGF receptor signaling in tumor stroma enhances antitumor effect of chemotherapy. Cancer Res 62:5476–5484

Pietras K, Stumm M, Hubert M, Buchdunger E, Rubin K, Heldin CH, McSheehy P, Wartmann M, Ostman A (2003) STI571 enhances the therapeutic index of epothilone B by a tumor-selective increase of drug uptake. Clin Cancer Res 9:3779–3787

Ramaswamy S, Ross KN, Lander ES, Golub TR (2003) A molecular signature of metastasis in primary solid tumors. Nat Genet 33:49–54

Roberts WG, Whalen PM, Soderstrom E, Moraski G, Lyssikatos JP, Wang HF, Cooper B, Baker DA, Savage D, Dalvie D, et al (2005) Antiangiogenic and antitumor activity of a selective PDGFR tyrosine kinase inhibitor, CP-673,451. Cancer Res 65:957–966

Ronnstrand L, Heldin CH (2001) Mechanisms of platelet-derived growth factor-induced chemotaxis. Int J Cancer 91:757–762

Song S, Ewald AJ, Stallcup W, Werb Z, Bergers G (2005) PDGFRbeta+ perivascular progenitor cells in tumours regulate pericyte differentiation and vascular survival. Nat Cell Biol 7:870–879

Soriano P (1994) Abnormal kidney development and hematological disorders in PDGF beta-receptor mutant mice. Genes Dev 8:1888–1896

Soriano P (1997) The PDGF alpha receptor is required for neural crest cell development and for normal patterning of the somites. Development 124:2691–2700

Tallquist MD, Klinghoffer RA, Heuchel R, Mueting-Nelsen PF, Corrin PD, Heldin CH, Johnson RJ, Soriano P (2000) Retention of PDGFR-beta function in mice in the absence of phosphatidylinositol 3'-kinase and phospholipase Cgamma signaling pathways. Genes Dev 14:3179–3190

Tallquist MD, French WJ, Soriano P (2003) Additive effects of PDGF receptor beta signaling pathways in vascular smooth muscle cell development. PLoS Biol 1:E52

Uehara H, Kim SJ, Karashima T, Shepherd DL, Fan D, Tsan R, Killion JJ, Logothetis C, Mathew P, Fidler IJ (2003) Effects of blocking platelet-derived growth factor-receptor signaling in a mouse model of experimental prostate cancer bone metastases. J Natl Cancer Inst 95:458–470

Uemura A, Ogawa M, Hirashima M, Fujiwara T, Koyama S, Takagi H, Honda Y, Wiegand SJ, Yancopoulos GD, Nishikawa S (2002) Recombinant angiopoietin-1 restores higher-order architecture of growing blood vessels in mice in the absence of mural cells. J Clin Invest 110:1619–1628

Ustach CV, Kim HR (2005) Platelet-derived growth factor D is activated by urokinase plasminogen activator in prostate carcinoma cells. Mol Cell Biol 25:6279–6288

Xian X, Hakansson J, Stahlberg A, Lindblom P, Betsholtz C, Gerhardt H, Semb H (2006) Pericytes limit tumor cell metastasis. J Clin Invest 116:642–651

Hypoxia and Tumour Angiogenesis

10

M. Christiane Brahimi-Horn, Julie Laferrière, Nathalie Mazure, Jacques Pouyssegur

Abstract

The hypoxia-inducible factor (HIF) is a well-known transcription factor recognized to play a key role in tumour angiogenesis through a cellular response to a low tissue oxygen concentration, a feature of a rapidly expanding tumour. Malignant cells initially proliferate in a nourishing vascular environment, but as the tumour mass develops it rapidly outstrips its vascular supply of nutrients and oxygen, leading to a cellular stress response that initiates metabolic and micro-environmental adaptive changes. A low level of oxygen, or hypoxia, directly regulates these changes through the activation of HIF by oxygen-dependent modulation of its post-translational profile. Active HIF induces the transcription of a broad range of genes involved, in particular, in metabolism and angiogenesis, thereby reinforcing the proliferative capacity of tumour cells. This review will introduce the reader to environmental and tissue hypoxia, to the HIF protein and its regulation through post-translational modification, to the implication of HIF in tumour angiogenesis and, finally, to the HIF-induced metabolic changes that modulate not only the micro-environment but also the fate of tumour cells.

CONTENTS

10.1 Ambient and Tissue Oxygen Gradients

Hypoxia is a deficiency in the availability of oxygen and may be encountered at an atmospheric level, as in increasing altitude, or within mammalian tissues. Hypoxia in tissues may be a necessary part of a physiological event, in particular in embryogenesis, but may also be a characteristic of certain pathophysiological situations such as ischaemic diseases and cancer.

10.1.1 Thinning of Air with Increasing Altitude

The proportion of oxygen in the air is around 21% (160 mmHg) at sea level and decreases with increasing altitude (Fig. 10.1). High-flying (3,000–9,000 m) migratory birds that are exposed to a rarefied atmosphere show cardiopulmonary and haematological adaptation, including a high haemoglobin–oxygen affinity. While the vast majority of humans live at, or close to, sea level, some populations such as the inhabitants of the Andean mountains live at very high altitude (around 4,000 m) and have adapted to conditions of low oxygen, hypobaric hypoxia. Individuals living at high altitude are subject to tumours of the carotid body (the peripheral oxygen-sensing organ) that have been shown to result from mutations in a tumour suppressor gene, succinate dehydrogenase D. The increased penetrance and severity of these tumours in these individuals provides a link to

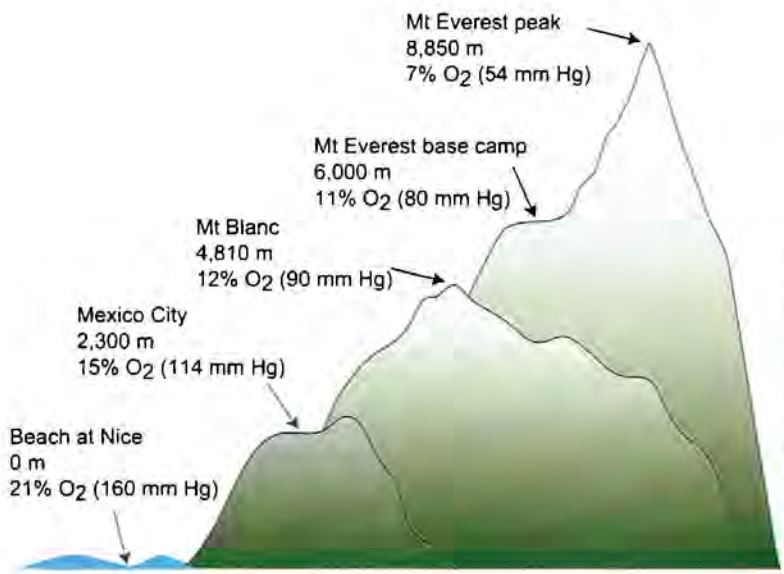


Fig. 10.1. The oxygen partial pressure (PO_2) correlated to altitude. With increasing altitude PO_2 (measured here in mmHg) drops. Individuals residing near sea level experience a physiological and biochemical response when confronted with increasing altitude, which results in an increase in respiration and cardiac output and production of molecules such as erythropoietin that increase the capacity of blood to transport oxygen (http://www.mountaineering.ie)

oxygen sensing (Astrom et al. 2003). Short-term exposure of people who live at low altitude to hypobaric hypoxia results in a physiological and biochemical response that includes, respectively, an increase in the heart and respiratory rate and an increase in the synthesis of a multitude of different proteins, including erythropoietin (Epo), that help mammals adapt to hypoxic stress. Epo, as everybody in the sporting world knows, increases the red blood cell count and thus increases the capacity of blood to transport oxygen. After much discussion the 1968 Olympic Games were held in Mexico City, but the choice of this location was controversial due to the city's high altitude of 2,300 m (Fig. 10.1) At this altitude the oxygen level is 30% less than at sea level. Athletes participating in endurance events experienced a low level of performance, while those in events requiring a rapid burst of energy were advantaged.

10.1.2 Progressive Tissue Distribution of Oxygen

At sea level the oxygen partial pressure (PO_2) of inspired air is around 160 mmHg. A substantial drop in the PO_2 is then observed in the lungs, in part due to water vapour and diffusion (Fig. 10.2). The blood flowing from the alveolar capillaries carries the oxygen, at a PO_2 of around 104 mmHg, towards organs and tissues for their oxygenation. Here a further drop in the PO_2 is observed, to a level well below that present at the peak of Mount Everest. The normal PO_2 of a given tissue depends on the type of organ; for example, rat spleen has a measured PO_2 of around 16 mmHg while for the thymus it is 10 mmHg (Braun et al. 2001). Thus, certain normal tissues can be considered as hypoxic. The normal rat retina, due to its low vascularity, is also relatively hypoxic (2–25 mmHg) (Yu and Cringle 2005). The normal tissue of the rat brain is even more hypoxic, 0.4–8.0 mmHg, depending on the location (Erecinska and Silver 2001). Oxygen has been reported to only diffuse within a distance of 100–200 μm from a capillary, and a PO_2 of almost zero has been reported at only 100 μm from blood vessels (Folkman et al. 2000; Gatenby and Gillies 2004). Thus, tissues such as the retina with a low vascularity are relatively hypoxic.

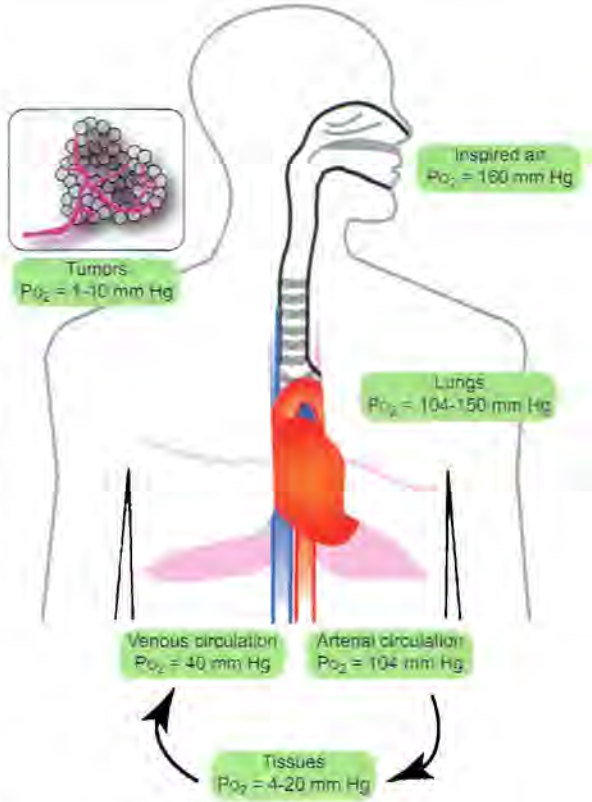


Fig. 10.2. The PO_2 gradient from inspired air to normal and tumour tissues. A significant drop in PO_2 is observed as inspired air flows from the lungs into tissues. An even greater decrease is observed in tumour tissue, in which a low level of oxygen (hypoxia) has been correlated to the expression of a transcription factor termed the hypoxia-inducible factor

10.1.3 Tumour Hypoxia

Considerable attention has been paid to investigating the instability of the human genome in cancer, but mounting evidence points more and more to epigenetic and environmental factors in tumour progression (Folkman et al. 2000). Cancerous tissues, in general, have been reported to possess extensive regions of hypoxia relative to the corresponding normal tissue (Vaupel 2004). This is due, in part, to the rapid proliferation of the tumour mass that distances the cells from the oxygen-carrying vasculature, but is also the consequence of the formation of a vasculature that is

distorted and irregular, and thus inefficient in oxygen transport. The degree of hypoxia is variable within tumours and between tumours, and is often associated with necrotic regions. Hypoxia can be measured in tissues samples either with chemical markers such as pimonidazole hydrochloride or EF5 or by using O_2 micro-electrodes or optical PO_2-measuring devices. More recently hypoxic tumours in patients were detected by positron emission tomography (PET) after injection of [^{18}F]misonidazole (Rajendran et al. 2004). The hypoxic nature of tumours is disadvantageous for clinical outcome – it has been known for some time that they are resistant to radio- and chemotherapy. In 1991 it was reported by Semenza et al. (Semenza et al. 1991) that liver cells incubated under hypoxic conditions experienced a substantial induction in the expression of the *Epo* gene, as described above for mammals exposed to acute hypobaric hypoxia. A cellular, nuclear factor appropriately termed the hypoxia-inducible factor (HIF) was subsequently identified to bind to the *Epo* gene and to induce its transcription, and thus its protein product, in hypoxia. This transcription factor was shown in vitro, in a variety of cell culture systems, to be activated at a cut-off of around 5% oxygen (40 mmHg). However, most in vitro experiments reported in the literature are performed at between 1% and 2% oxygen (8–16 mmHg), which can be considered as highly hypoxic. Under these conditions HIF is detected in the nucleus of cells, where it influences the transcription of numerous genes involved in multiple functions (Fig. 10.3).

10.2 General Introduction to the Hypoxia-inducible Factor

Signalling through HIF is a multi-step process involving first oxygen depletion, HIF α subunit stability, translocation to the nucleus, heterodimerization, binding to DNA and finally induction of transcription of a vast array of genes. HIF is often referred to as the key or master regulator in the hypoxic response of cells but in fact it is the activity of two oxygen-sensing enzymes, the prolyl hydroxylase domain (PHD) proteins and factor inhibiting HIF-1 (FIH-1) that determines its existence and activity, and thus orchestrates the hypoxic response. These proteins are dioxygenases that, as this name implies, are dependent on the presence of oxygen for activity.

10.2.1 The PHD Proteins Drive HIF-α to Destruction

The PHD proteins, of which there exists three human isoforms, are 2-oxoglutarate- and Fe^{2+}-dependent dioxygenases belonging to the largest known family of non-haem-oxidizing enzymes (EC 1.14.11.2). A number of other terms have been used to designate these proteins, including HIF-1α prolyl-4-hydroxylase (HPH) and EGL nine (EGLN). The equivalent abbreviations for the different homologues are as follows: PHD1/HPH-3/EGLN2; PHD2/HPH-2/EGLN1; PHD3/HPH-1/EGLN3. These enzymes, in the presence of oxygen, hydroxylate two prolyl residues (P402 and/or 564) of the human HIF-1α subunit in a region referred to as the oxygen-dependent degradation domain (ODDD) (Fig. 10.4). This posttranslational modification results in the rapid attraction to HIF-α of the protein von Hippel–Lindau (VHL), a component of an E3 multiprotein ubiquitin ligase complex termed VBC (VHL/elongin B/elongin C). Subsequently HIF-α becomes earmarked with multi-ubiquitin chains that drive it to destruction by the proteasomal system. HIF-α is probably a champion when it comes to half-life, determined at around 5 minutes at 21% oxygen. In general, when cells in culture are incubated under hypoxic conditions (1.5% O_2) for 2–4 h the HIF-α protein is stabilized because the PHDs, in the absence of oxygen, are inhibited. Auto-regulation in hypoxia occurs through increased expression of the genes *phd2* and *phd3*, but not *phd1*, since these genes are themselves HIF targets and thus their products are up-regulated in hypoxia. This response thereby guarantees rapid and optimal degradation of HIF-α on return of cells to normoxia. Recent reviews describing in further detail the properties and functions of the PHDs have been published by our group and by others (Berra et al. 2006; Schofield and Ratcliffe 2005).

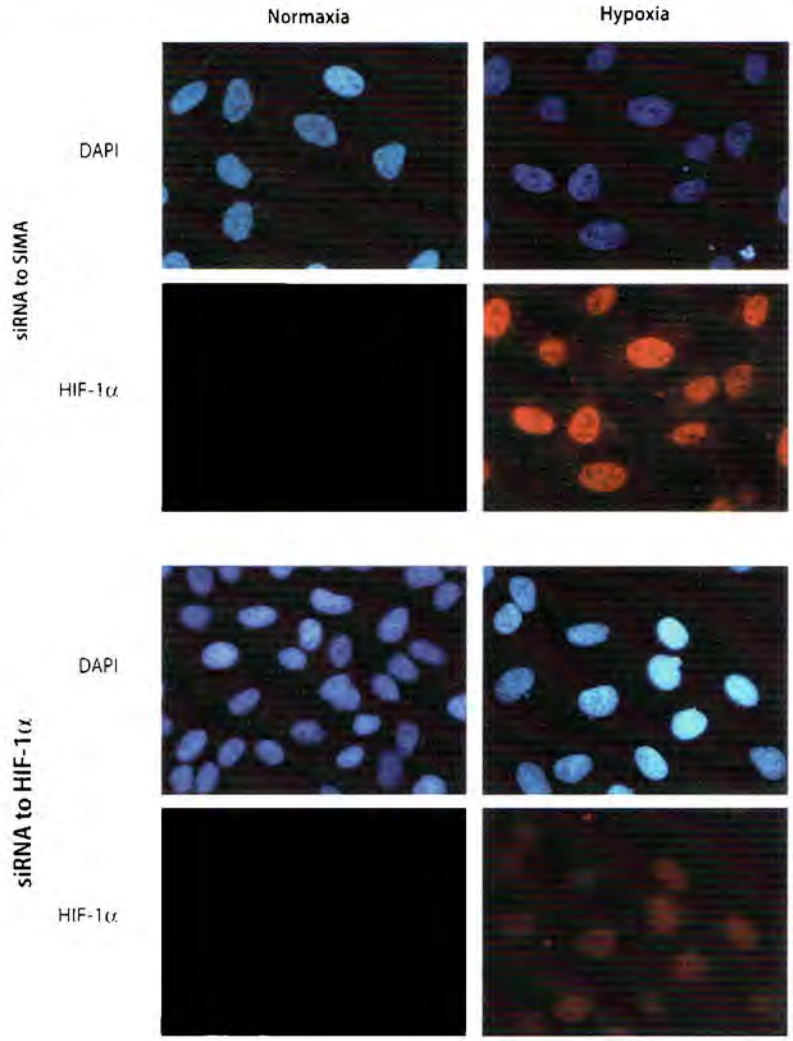


Fig. 10.3. Cellular localization of the α subunit of the hypoxia-inducible factor-1 (*HIF-1α*) in hypoxia. Under normoxic conditions HIF-1α is hardly detectable by immunofluorescence (*red staining, left panels*), while under hypoxic conditions it is detected and localized to the nucleus (*red staining, right upper panel*), where it binds to its partner HIF-1β. Cell nuclei are stained with 4',6-diamine-2-phenyl indole (*DAPI*) (*blue staining*). Transfection of cells with siRNA to HIF-1α attenuates the signal obtained for cells incubated in hypoxia (*red staining, right lower panel*), thereby validating the antibody detection of HIF-1α. Transfection of siRNA to SIMA, Drosophila HIF, was used as a control (*upper panels*)

10.2.2 The Inhibition of FIH-1 Unlocks HIF Transcriptional Activity

The HIF-α protein possesses a nuclear localisation signal sequence in its C-terminus, so once stable it is rapidly directed into the nucleus where it interacts with its partner HIF-β; the latter subunit is not affected by the level of oxygen and is constitutively present (Fig. 10.4). Hand in hand, the α and β subunits bind to so-called hypoxia response elements (HRE), of the sequence 5'-RCGTG-3', in target genes. Dimerization and DNA interaction occurs via basic helix–loop–helix (bHLH) and PER-ARNT-SIM (PAS) domains in the N-terminal part of the proteins, while the transcriptional activity is regulated at the C-terminal part via two transcriptional activation domains (TAD) termed N-TAD and C-TAD. The C-TAD activity of HIF-1α is regulated by another 2-oxoglutarate- and Fe^{2+}-dependent dioxygenase termed factor inhibiting HIF-1 (FIH-1) that hydroxylates an asparagine residue

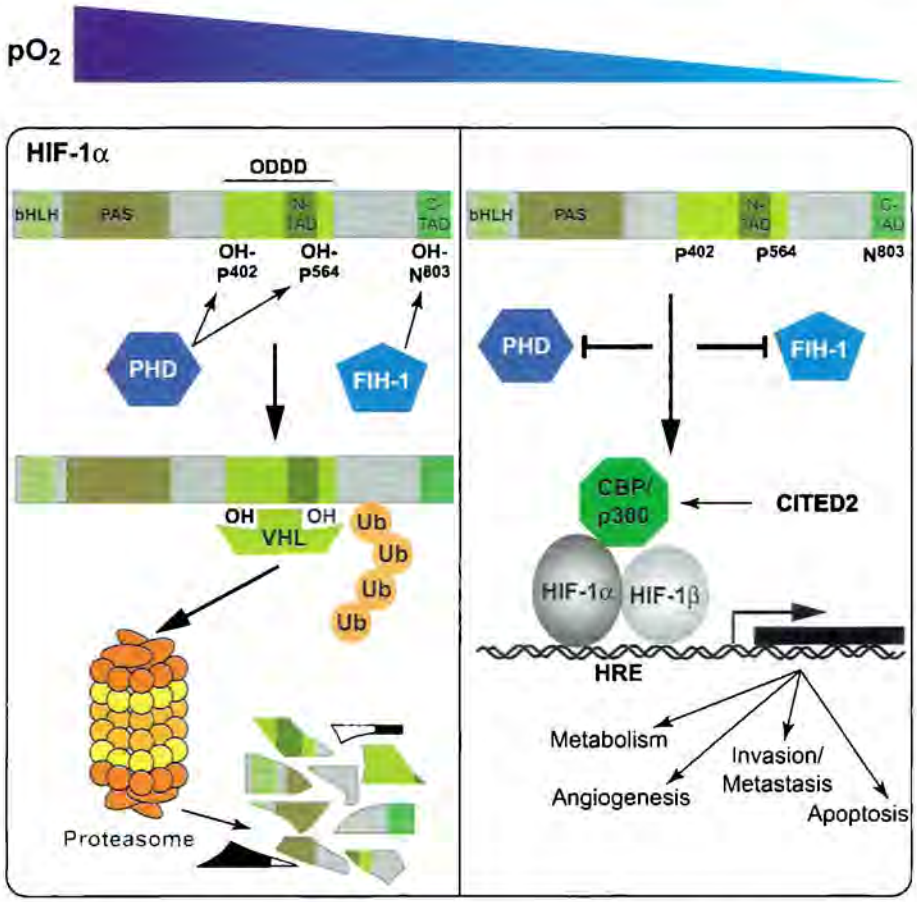


Fig. 10.4. Regulation of the stability and activity of HIF by oxygen sensors. Under normoxic condition HIF-1α is hydroxylated on two proline residues (402 and 564) in its oxygen-dependent degradation domain (*ODDD*) by prolyl hydroxylase domain (*PHD*) proteins. This hydroxylation is a signal for interaction with the von Hippel–Lindau (*VHL*) protein, a component of a E3 ubiquitin ligase, and addition to HIF-1α of multi-ubiquitin chains that address it for degradation by the proteasome. (Since the amino acids modified with ubiquitin (*Ub*) have not been identified, the indicated location is hypothetical, as is the length of the chain.) Under hypoxic conditions the PHDs, which require oxygen for activity, are inhibited and HIF-1α is no longer degraded. Another oxygen sensor, factor inhibiting HIF-1 (*FIH-1*), which hydroxylates HIF-1α in the presence of oxygen, on an asparagine (803) residue in its C-terminal transcription activation domain (*C-TAD*), is also inactive, thereby allowing interaction with co-activators such as CBP/p300. Proteins such as CITED2 can act as repressors by competing with co-activators. Together the HIF-1α and -1β subunits bind to hypoxia response elements (*HRE*) in a myriad of genes and induce or repress their transcription

(N803). This hydroxylation of HIF-α has been shown to inhibit its transcriptional activity by abrogating interaction with transcriptional co-activators such as p300 and its paralogue CBP (CREB-binding protein), which are also histone acetyltransferases (Fig. 10.4). Thus, a double lock of posttranslational hydroxylation controls HIF stability and activity.

10.2.3 Fine-tuning Through Protein Interaction and Posttranslational Modification Influences Stability and Activity

The regulation of the alpha subunits of HIF, of which there are three isoforms, involves interaction with a

number of proteins, some of which lead to its post-translational modification. The protein OS-9 been shown to interact with HIF-1α and to increase its instability by promoting interaction with PHD2 and PHD3, resulting in prolyl hydroxylation and thus VHL-dependent polyubiquitination and proteasomal degradation (Baek et al. 2005). Therefore, by favouring instability OS-9 reduces HIF transcriptional activity; however, this would require the presence of oxygen and thus would concern either residual levels of HIF-1α or HIF-1 α stabilized through non-hypoxic mechanisms such as in the presence of certain growth factors. Acetylation of HIF-1α by an acetyltransferase ARD1 has been reported to diminish stability (Jeong et al. 2002), but more recently this has been questioned (Arnesen et al. 2005b; Bilton et al. 2005; Fisher et al. 2005). The expression of ARD1 was found to be neither hypoxia nor HIF-1- or HIF-2-dependent and had no effect on the stability of HIF-1α or -2 α (Bilton et al. 2005). While acetylation of HIF-1α by ARD1 has not been convincingly confirmed, the interaction of ARD1 with HIF-1 α has (Arnesen et al. 2005b). Thus the role of ARD1 in HIF function remains to be clarified and any link to tumorigenesis through increased expression of its partner N-acetyl transferase human (NATH; also called Tubedown or Gal9) needs to be further investigated (Arnesen et al. 2005a; Brahimi-Horn et al. 2005). The α subunit of HIF undergoes other forms of posttranslational modification that result in further fine-tuning of the activity of HIF; these include phosphorylation, SUMOylation and S-nitrosylation (for review see Brahimi-Horn et al. 2005; Sects. 3.4, 3.6).

The Nitty-Gritty of the Transcriptional Activity of the Hypoxia-inducible Factor

More than 60 target genes induced by HIF have already been identified (Semenza 2003) and probably as many are repressed; however, the latter are less well studied (Manalo et al. 2005; Wang et al. 2005). A myriad of genes of varied function are HIF-dependent. The majority of the gene products involved in glycolysis are up-regulated in hypoxia through HIF induction, as are some genes for nucleotide, amino acid and iron metabolism. Other genes include those involved in such functions as cell survival, apoptosis, cell motility, cytoskeletal structure, cell adhesion, erythropoiesis, vascular tone, transcriptional regulation, epithelial homeostasis and drug resistance.

10.3.1 Differential Gene Expression Through Two Transcriptional Activation Domains

For gene regulation heterologous partners of the large family of bHLH-PAS proteins of the α and β classes are usually required. Three isoforms of the α subunit of HIF (1, 2 and 3) and three of the β subunit (1, 2 and 3; also termed ARNT) together with a number of splice variants have been identified (For a diagrammatical representation of the different isoforms and of other bHLH-PAS proteins, see Brahimi-Horn and Pouyssegur 2005.) The relative role of these isoforms, which show the same mechanisms of regulation, is still to be determined. Each possesses at least one TAD in its C-terminal part; however, HIF-1 α and -2 α have two such domains (N-TAD and C-TAD); HIF-3 α has a single N-TAD. Few transcription factors possess two TAD, and as yet their respective roles remain to be defined. Only 20% similarity exists between the human HIF-1 α N-TAD and C-TAD amino acid sequences. However, comparison of the sequence of the human HIF-1α and 2 α C-TAD shows more than 70% conservation. The human N-TAD protein sequences of HIF-2 α and -3α share, respectively, 65% and 60% identity with human HIF-1 α. Interspecies comparison between *Homo sapiens, Bos taurus, Mus musculus, Rattus norvegicus and Gallus gallus* shows that the N-TAD is highly conserved, with more than 90% similarity, while the C-TAD is even more conserved, with almost 100% identity.

RNA interference has proved to be a useful approach not only to determine the role of expressed proteins but also in validating tools such as

antibodies. In this way our laboratory previously validated antibodies to HIF-1α, PHD2 (Berra et al. 2003) and the acetyltransferase ARD1 (Bilton et al. 2005). Using specific siRNA targeting of PHD2 our laboratory was able to demonstrate that PHD2 is the key isoform controlling the stability of HIF-1 α (Berra et al. 2003). Using an RNA interference approach we are now studying the bifunctional role of the two TADs of HIF-1α. Studies on the K*m* of the two oxygen sensors, PHD and FIH-1, indicate that the PHDs require higher oxygen levels than FIH-1 for activity (Koivunen et al. 2003). Based on this study we postulate that in areas near blood vessels, where cells are maximally oxygenated, both PHD and FIH-1 will be active (Fig. 10.5). Under these conditions only minimal, basal normoxic levels of HIF-1 α will exist but will be inactive. As the cells are distanced from the blood vessels a progressive drop in oxygen pressure will occur with stabilization of HIF-1α but maintenance of the lock on C-TAD activity. However, if certain genes are driven solely by the N-TAD, i.e. they do not require the C-TAD, these genes will become activated at the intermediate oxygen level. As the oxygen pressure drops even further, the genes requiring the C-TAD with or without N-TAD activity will be induced. Our preliminary results demonstrate that this is the case, and the study of 26 different genes by real-time quantitative PCR, in a cell system where FIH-1 is either overexpressed or silenced, has allowed us to identify two groups of genes that are respectively inhibited or not by FIH-1 expression, thus C-TAD activity-independent and -dependent (Mazure et al. 2005). However, within the inhibited group we also identified a sub-class of genes that were repressed when FIH-1 was silenced and either repressed or enhanced when FIH-1 was overexpressed. Taken together, these results suggest that HIF-1α possesses a bifunctional transcriptional activity that regulates gene expression differentially.

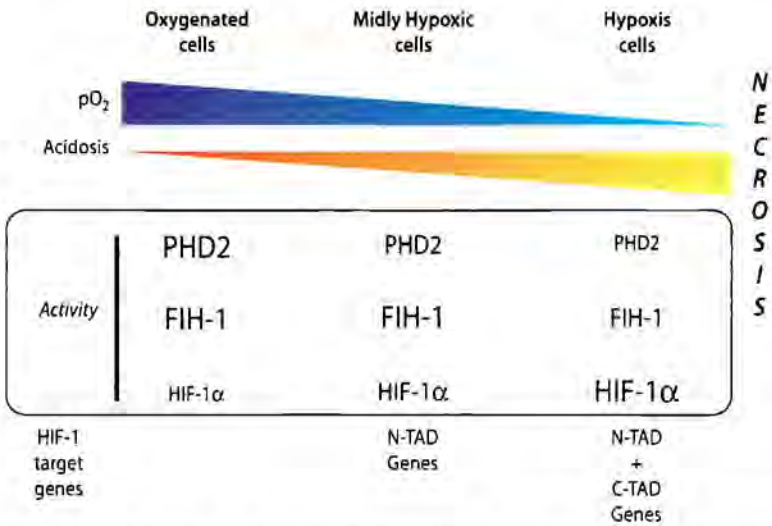


Fig. 10.5. Bifunctional role of two TAD of HIF. HIF-1α and -2α contain two TAD in the C-terminal part of the protein, termed N-TAD and C-TAD. The respective role of these two TAD has not yet been investigated and is a subject we are exploring. The two oxygen sensors, the PHD and FIH-1, control, respectively, HIF-1α stability and activity. FIH-1 targets an asparagine residue in the C-TAD and thereby blocks interaction with co-activators. A decreasing PO_2 gradient from the blood vessel to the tumour core will determine the activity of the PHDs and FIH-1. The K_m values of the PHDs and FIH-1 predict that the former is more sensitive to oxygen levels and thus is more rapidly inhibited then FIH-1. Thus at moderate oxygen concentrations HIF-1α will be stable but genes dependent on the C-TAD activity will not be induced due to maintenance of FIH-1 activity; however, genes requiring only N-TAD activity will be induced. As the PO_2 decreases further, the inhibition of C-TAD will be released and HIF-1α will attain full transcriptional activity. Using RNA interference and overexpression of FIH-1 we have demonstrated that certain genes are indeed regulated differentially by the two TAD

10.3.2 Differential Gene Expression by HIF-1 and HIF-2

Differential gene expression may also arise from the specificity of HIF-1α and -2α for different genes, i.e. there is evidence to suggest that these isoforms have preferential or exclusive activity toward a given gene (Aprelikova et al. 2004; Hu et al. 2003; Sowter et al. 2003; Wang et al. 2005). These two isoforms show substantial structural resemblance with about 48% overall amino acid sequence similarity and with even greater similarity in the bHLH-PAS domain (bHLH, 85%; PAS-A, 68%; PAS-B, 73%) (Brahimi-Horn and Pouyssegur 2005). However, they do possess differences in the sequences of their TAD, as seen above. In addition, their instability and stability, respectively, are regulated by PHDs/VHL and hypoxia and they both heterodimerize with HIF-1β to bind HRE sequences. HIF-2α was initially described in endothelial cells but later shown to be expressed in a broad range of cells of different origin. However, these isoforms do show cell-type specificity, as seen for certain VHL-deficient renal cell carcinoma (RCC) cell lines that express either both isoforms or, in the case of 786-0 cells, only HIF-2α (Maxwell et al. 1999). The latter cells have been studied with the aim of deciphering the respective roles of these isoforms in gene induction and in their association to tumorigenesis. Of particular interest, it was determined that certain glycolytic enzymes (PGK-1 and LDHA) were induced preferentially by HIF-1α (Hu et al. 2003). Although probably too simplistic, it appears that HIF-1α is preferentially implicated in breast cancer cells while HIF-2α may play a more active role in RCC (Kondo et al. 2002; Maranchie et al. 2002). The role of the third HIF-α isoform, and its six splice variants, has so far been little studied. It has a more divergent structure, though its bHLH-PAS domain shows considerable amino acid sequence similarity and it heterodimerizes with HIF-1β to bind core HRE sequences. However, it lacks a C-TAD. It may turn out that this isoform functions in competition for binding to HIF-1β, leading to repression of the hypoxic response for induction of certain genes.

Additional differential regulation may result from specific interaction with co-activators or co-repressors. Indeed, while the co-activators CBP/p300 have been shown to activate both isoforms, NEMO, the NF-κB essential modulator, has been shown to be a co-activator specific for HIF-2α (Bracken et al. 2005). Even in the context of expression of both isoforms it is conceivable that one or the other is repressed under certain conditions in certain cell types.

10.3.3 Repression of Activity Through Protein–Protein Interactions

Although FIH-1 is the major protein involved in repression of HIF, several other proteins have also been reported to repress the transcriptional activity, including CBP/p300 interacting transactivator with ED-rich tail 2 (CITED2) (Freedman et al. 2003) and PHD2 (To and Huang 2005). Repression by CITED2, as the name suggests, occurs through competition of HIF-1α for CBP/p300 and thereby results in a block in HIF-dependent transcription. However, CITED2 binds p300 with a 33-fold higher affinity than HIF-1α, but its expression is increased in hypoxia via a HRE in its promoter, providing yet another feedback loop in regulation. Yet the levels of CITED2 compared to CBP/p300 are low, so a balance towards activation may dominate. An interplay between CITED2 and FIH-1 was suggested where CITED2 may facilitate FIH-1 hydroxylation (Freedman et al. 2003). Another member of the CITED family, CITED4, also acts as a repressor of activity and its expression is inversely correlated to that of HIF-1α in breast cancer (Fox et al. 2004). Surprisingly, repression of the N-TAD activity of HIF-1 in hypoxia was also reported to occur through binding of PHD2 and was found to be independent of interaction with VHL (To and Huang 2005). The mechanism involved probably does not involve the hydroxylase activity of PHD2, since this is absent in hypoxia. PDH2 may have other functions and has been shown to also repress HIF activity in hypoxia by recruitment of a candidate tumour suppressor, inhibitor of growth family member 4 (ING4) (Ozer et al. 2005). The ING

family of proteins regulate gene expression at the level of chromatin by interacting with complexes that modulate histone acetylation. Histone acetylation by histone acetyltransferases (HATs) and histone deacetylation by histone deacetylases (HDACs) determine the level of acetylation of lysine residues of histones. This acetylation leads to modification in the charge of lysine residues, which become more acidic and thereby bind DNA less strongly, making the DNA more accessible to the transcription machinery. Thus, acetylation is associated with activation of transcription, while deacetylation is in general correlated with repression of transcription. The protein VHL has been implicated in transcriptional repression and this has been hypothesised to occur by recruitment of HDACs (Mahon et al. 2001). However, HDAC7 has been reported to interact with the inhibitory domain of HIF-1α and to activate rather that repress transcription (Kato et al. 2004). Interaction of HIF-1α with proteins that enhance its binding to VHL is another way of repressing HIF-1α activity. A protein termed VHL-associated KRAB-A domain-containing protein (VHLaK) has been shown to repress HIF-1α activity independent of VHL ubiquitin-dependent degradation (Brahimi-Horn and Pouyssegur 2005).

10.3.4 Repression Through Posttranslational Modification

Repression of the activity of a transcription factor can occur not only through protein–protein interactions but also through direct posttranslational modification of the factor itself, which may in turn solicit additional protein interactions. The posttranslational modification of a large number of proteins involved in transcription by the covalent attachment of the small ubiquitin-related modifier (SUMO) is emerging as an important mechanism in the control of transcription (Gill 2005). In addition to a variety of effects on protein function, more often than not SUMOylation of transcription factors correlates with repression of transcription. SUMO, as the name suggests, is a small polypeptide that in many ways resembles ubiquitin. However, unlike certain forms of ubiquitination that drive proteins to proteolytic degradation by the proteasome, SUMOylation does not induce proteasomal targeting and can even protect against degradation. In the case of HIF, SUMO modification of both the HIF-1β and -1α subunits has been suggested to repress its transcriptional activity without altering the stability of the latter subunit (Berta et al. 2004; Tojo et al. 2002). Repression of transcription of the key co-activators of HIF, CBP/p300, also implicates SUMOylation. This may be due to the recruitment of co-repressors, including certain HDACs that are themselves SUMO-modified proteins. It may also be the consequence of crosstalk between acetylation and SUMOylation, modifications that both occur on lysine residues, where HDACs may regulate SUMOylation. Thus, interaction with repressive molecules and posttranslational modification may represent fine-tuning feedback mechanisms that protect from an excessive hypoxic response.

10.3.5 Activation Through Protein–Protein Interactions

Rather than repressing HIF activity, interaction with a number of molecules has been shown to enhance its activity. As mentioned above, the interaction with co-activators such as CBP/p300, but also the protein steroid hormone receptor co-activator-1 (SRC-1), has a major impact on transcriptional activity. These proteins possess HAT activity, which as also mentioned above is associated with transcriptional activation. Their activity can also be potentiated by proteins such as the redox regulatory proteins redox-factor 1 (Ref-1) and thioredoxin-1. However, the acetyltransferase activity of CBP/p300 does not concern only histones, and it is now apparent that many non-histone proteins, in particular transcription factors such as p53, are substrates. As yet no evidence has been put forward to suggest that HIF-1α is acetylated by CBP/p300, and there exists considerable doubt concerning acetylation by ARD1 (Arnesen et al. 2005b;

Bilton et al. 2005; Fisher et al. 2005). The large protein p300 contains domains involved in transcriptional activation as well as acetyltransferase activity (Brahimi-Horn and Pouyssegur 2005). The first cysteine/histidine-rich (CH1) domain in the N-terminal part binds HIF-1α but also many other proteins such as p53 and CITED2. Taken that HIF activity is highly dependent on this interaction for activity, its rupture has been shown in principle to be an effective way of inhibiting HIF. Disruption of the interaction was achieved with polypeptides of either the C-TAD of HIF-1α or the CH1 minimal binding domain of p300, or with the small molecule inhibitor chetomin. Not only were these agents able to diminish HIF transcriptional activity, they also diminished tumour growth in xenograft mouse models (Kung et al. 2004). However, another study showed that deletion of the CH1 domain did not attenuate tumorigenesis significantly (Kasper et al. 2005). These studies indicated that inhibition of HIF may be of potential clinical benefit, and substantial investigation into small molecular inhibitors for therapeutic purposes is currently under way. Considerable interest is also being shown in the therapeutic use of HDAC inhibitors in cancer due to their anti-proliferative and apoptotic effects. A number of clinical trials have shown some benefit in the treatment of both haematological and solid tumours. Yet one would expect that from the point of view of HIF activation by the CBP/p300 HAT activity, such inhibitors would enhance its activity and thus tumorigenesis. However, the inhibitor trichostatin A (TSA) has been reported to be anti-angiogenic possibly through reactivation of p53 and VHL and parallel suppression of HIF-1 and VEGF (Brahimi-Horn and Pouyssegur 2005). More recently it was revealed that certain HIF-1 target genes are either CH1-dependent or -independent and that the dependent genes were sensitive to inhibition with TSA (Kasper et al. 2005). Surprisingly fewer genes were CH1-dependent than -independent. These observations reinforce the notion of differential activation of sets of genes, possibly by soliciting either N-TAD or C-TAD or both for transcriptional activation of HIF target genes.

10.3.6 Activation Through Posttranslational Modification

Posttranslational modification of a transcription factor and in particular phosphorylation is a well-known mechanism for controlling activity, and HIF-1α is no exception. When stabilized under hypoxic conditions HIF-1α is phosphorylated subsequent to activation of the Ras/ERK pathway by growth factors or oncogenes and possibly through the implication of p38 kinase and casein kinase II-like kinase (Brahimi-Horn et al. 2005). The level of phosphorylation is probably considerable, if the easily perceived shift in the mobility in the protein on SDS-PAGE is any indication. Although eight serine residues that could be consensus target sites for ERK exist in the C-TAD region of HIF-1α, the individual residues phosphorylated by these kinases have not yet been identified. Phosphorylation does not modify the stability or DNA-binding capacity of HIF-1α, but does increase the transcriptional activity, possibly by favouring heterodimerization with HIF-1β or interaction with co-activators. However, increased transcriptional activity has also been linked to the phosphorylation of the co-activator p300 by ERK. In addition, S-nitrosylation of cysteine 800 in the C-TAD of HIF-1α enhances HIF-1α activity by again increasing interaction with p300.

The Hypoxia-inducible Factor and Tumour Angiogenesis

The cellular expansion of tumours progressively distances cells from the vasculature and thus from a supply of oxygen and nutrients. In an attempt to correct this deficiency, tumour cells respond by sending out signals that initiate the formation of new blood vessels, which then penetrate the tumour mass and thus allow cells to continue to be supplied with oxygen and nutrients and maintain their prolif-

eration. This adaptive process, termed angiogenesis, is a characteristic of solid tumours and constitutes a potential therapeutic target (Carmeliet 2003; Ferrara and Kerbel 2005). However, the new vessels show structural malformations as well as fluctuations in their blood flow, and local regions of hypoxia may nonetheless prevail. Evidence that HIF plays a fundamental role in angiogenesis comes in part from the study of embryos of mice that are knockout for HIF-1α, HIF-1β or VEGF, all of which are lethal to the embryo and show defects in blood vessel development (Brahimi-Horn and Pouyssegur 2005; Pugh and Ratcliffe 2003). However, hypoxia and thus HIF influences not only the behaviour of endothelial and tumour cells but also the surrounding normal stromal cells and macrophages.

10.4.1 The Key Angiogenic Factor Vascular Endothelial Growth Factor Is HIF-mediated

Some of the major proteins involved in initiating angiogenesis are HIF-1 and -2-dependent, including vascular endothelial growth factor (VEGF), the role of which has been extensively studied (Ferrara et al. 2003). VEGF in cooperation with angiopoietin-2 (Ang-2) attracts and guides sprouting neo-vessels into oxygen-depleted regions of the tumour mass (Carmeliet 2003). While binding of HIF to the *vegf* promoter is a key determinant in its expression, other levels of control, including mRNA stability through the stress-activated kinase p38 and translation via internal ribosome entry site (IRES) sequences, also regulate expression (Pages and Pouyssegur 2005). Since under hypoxic and nutrient-depleted conditions classic cap-dependent translation is inhibited, only mRNA containing IRES will be translated. Thus translation of VEGF is assured under such conditions, as is HIF-1α which also contains IRES. We have shown in our laboratory that VEGF is up-regulated by the Ras>MEK>ERK pathway through the phosphorylation of the transcription factor Sp1 and its recruitment to the proximal region of the *vegf* promoter. Up-regulation also occurs through this pathway by HIF-1α phosphorylation (Pages and Pouyssegur 2005), and phosphorylation may improve accessibility of *vegf* to RNA polymerase II. In addition, binding of Sp1 to VHL has been shown to repress *vegf* promoter activity (Pages and Pouyssegur 2005). Since VEGF is up-regulated by HIF, a number of studies have investigated a possible correlation between HIF expression and the intratumoral microvascular density, a measure of tumour angiogenesis (Brahimi-Horn and Pouyssegur 2005). The majority of these studies show a link between the two, but the most striking and convincing association comes from studies into RCC, in which there is loss of function of VHL (Kaelin 2002). *Vhl* is a tumour-suppressor gene and its product is a component of an E3 ubiquitin ligase complex. As mentioned above, it earmarks HIF-α with ubiquitin chains that target it for degradation by the proteasome. In RCC and in VHL disease, a familial cancer syndrome, this function is lost, and as a consequence HIF-α is stable and active, and tumours are highly vascularized. VHL disease is characterized by the formation of blood vessel tumours (haemangioblastomas) of the central nervous system and retina often associated with other tumours such as RCC. These observations also support the notion that the HIF pathway plays an important role in tumorigenesis. The finding that the expression of HIF-1α is increased in a broad range of cancers (Brahimi-Horn and Pouyssegur 2005) and has been correlated to tumour grade also supports this hypothesis.

The micro-environment with respect to the degree of vascularization influences tumour progression and is HIF and VEGF dependent. This is nicely illustrated in a study in which HIF-1α-deficient astrocytes were injected into either a vessel-poor or a vessel-rich environment, respectively either subcutaneously or into the brain parenchyma (Blouw et al. 2003). In the former environment necrosis and reduced tumour growth was observed, while in the latter the tumours grew rapidly, penetrating the brain. Minimal vascularization of the tumour itself in the subcutaneous site was noted compared to the other site. In contrast, the growth of astrocytes deficient in VEGF injected at either site was reduced due to an inability to maintain co-opted vessels. Thus the differences may result from induction of VEGF in the HIF-1α-deficient cells by non-HIF-dependent mechanisms.

10.4.2
Inhibition of Angiogenesis in Relative Hypoxia

However, so-called hypoxic conditions do not necessarily lead to angiogenesis. It was noted previously that the retina is relatively hypoxic and yet in the normal situation angiogenesis is not induced, and when it is induced it leads to a pathophysiological situation. Retinal capillary quiescence is a reflection of a fine balance in pro-angiogenic and anti-angiogenic factors, in particular due to the expression of a HIF-α truncated variant exerting a dominant negative action in this hypoxic micro-environment (Makino et al. 2001). Ocular neovascularization, on the other hand, is a major feature of diabetic retinopathy and age-related macular degeneration. Retinopathy can be induced in newborn mice by alternating exposure to hyperoxia (45% oxygen) and normoxia (12.5% oxygen). The return to normoxia is in fact relative hypoxia and results in the expression of angiogenic factors and neovascularization. Knockout of HIF-1α and -2α in mice is lethal for the embryo, ruling out the investigation of the role of these proteins in mouse physiology. However, the study of HIF-2α knockdown in mice exposed to hyperoxia–normoxia in the investigation of retinopathy of prematurity leads to the conclusion that HIF-2α plays a key role in this pathology and that the angiogenic factor Epo, rather than other more frequently induced angiogenic factors, is associated with retinopathy (Morita et al. 2003).

Tumour Metabolism

It has been known for some time that tumours in general have a high rate of glucose uptake accompanied by elevated glucose consumption, i.e. glycolysis (for review see Gatenby and Gillies 2004). In fact, this characteristic of tumours has been put into clinical application through the detection by PET of [^{18}F]2-deoxy-2-fluoro-D-glucose (FDG), which accumulates in solid tumours. It is now generally accepted that the rate of glucose uptake measured by PET-FDG is an important predictor in determining the aggressivity of a broad range of types of tumours. However, detection of hypoxic regions in human tumours by PET after injection of the radioactively labelled hypoxia marker [18F]misonidazole and comparison to PET-FDG showed that some hypoxic tumours had a modest glucose uptake while other non-hypoxic tumours showed a high uptake (Rajendran et al. 2004). These results point to an overall heterogeneity of oxygenation of different tumours that, although not related to tumour size, probably reflects differences in vascularization.

10.5.1
Aerobic Versus Anaerobic Glucose Metabolism

In the 1920s Otto Warburg discovered that tumours, unlike normal cells, converted glucose to pyruvate and then to lactate, even in the presence of plentiful amounts of oxygen (the "Warburg effect") (Fig. 10.6A). This is the same metabolic pathway used by muscle tissue when oxygen is low. In normal cells, glucose is similarly converted to pyruvate (glycolysis), which is then transported to the mitochondria where it enters the tricarboxylic acid (TCA) cycle (synonyms: citric acid cycle, Krebs cycle). During subsequent oxidative phosphorylation, the NADH and $FADH_2$ that are produced transfer electrons to molecular oxygen (Fig. 10.6B). This oxygen-requiring catalytic process is highly efficient in providing energy, with the overall production of 38 ATP molecules per molecule of glucose, while direct conversion to the waste product lactate produces only two ATP molecules. It was initially thought that the choice by tumour cells to opt for or "switch" to so-called "aerobic glycolysis", an inappropriate and confusing term for a process that could be more appropriately termed "anaerobic glucose metabolism", was due to defective mitochondrial respiration, but little evidence was found, until recently, to support this. One explanation lies in chronic or transitory exposure of cells to hypoxic conditions. Stable and active HIF in these cells would induce up-regulation

a

Aerobic glucose metabolism:

$C_6H_{12}O_6 + 38\ ADP + 38\ P_i + 6\ O_2 \longrightarrow 6\ CO_2 + 44\ H_2O + 38\ ATP$

"Pasteur Effect", inhibition of glycolysis in presence of O_2

Aerobic glucose metabolism:

$C_6H_{12}O_6 + 2\ ADP + 2\ P_i \longrightarrow 2\ lactate + 2\ H^+ + 2\ H_2O + 2\ ATP$

"Warburg Effect" also termed aerobic glycolysis

b

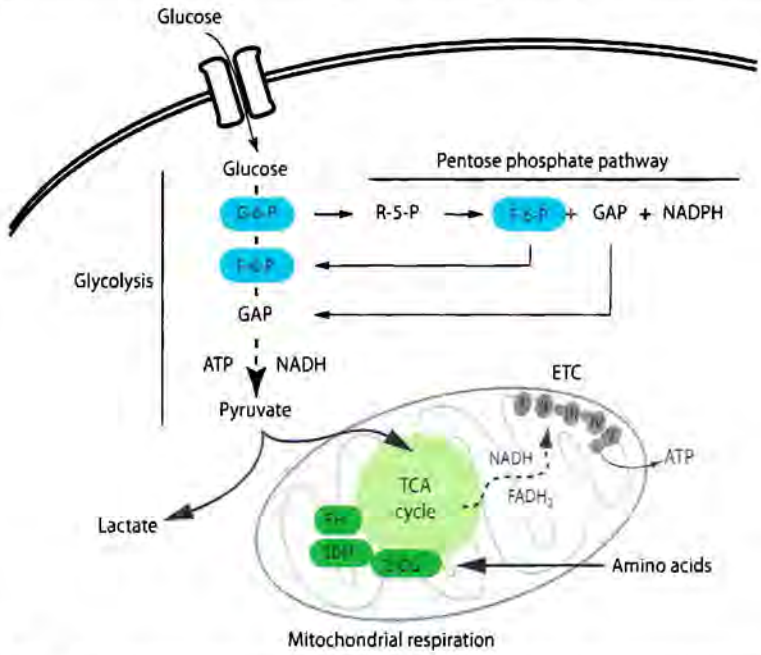


Fig. 10.6a,b. Tumour cells switch from aerobic to anaerobic glucose metabolism. Tumour cells posses a high level of uptake and consumption of glucose with the production of high amounts of lactate. a Aerobic glucose metabolism generates 38 molecules of ATP, while anaerobic glucose metabolism gives only two. The "Pasteur effect" relates to the observation that oxygen results in inhibition of aerobic glucose metabolism. The "Warburg effect" relates to tumour cells that preferentially adopt anaerobic glucose metabolism even when in the presence of oxygen, also termed "aerobic glycolysis". b Glucose oxidation may occur via glycolysis but also through the pentose phosphate pathway (PPP). In normal cells the pyruvate generated by glycolysis is metabolized through the tricarboxylic acid (*TCA*) cycle and oxidative phosphorylation, which is efficient in energy production. One of the metabolites of the TCA cycle is 2-oxoglutarate (*2-OG*) (α-ketoglutarate), which is required for PHD and FIH-1 activity. Catabolism of amino acids is also a source of 2-OG. The free energy required to generate ATP is obtained from the oxidation of NADH and $FADH_2$ by the mitochondrial electron transport chain (ETC), a series of five multi-enzyme complexes (I–V) via which electrons transfer from lower to higher reduction standard potentials. Other abbreviations: *G-6-P*, glucose-6-phosphate; *F-6-P*, fructose-6-phosphate, *R-5-P*, ribose-5-phosphate; *GAP*, glyceraldehyde-3-phosphate; *ATP*, adenosine triphosphate; *NADH*, nicotinamide adenine dinucleotide; *NADPH*, nicotinamide adenine dinucleotide phosphate; *SDH*, succinate dehydrogenase; *FH*, fumarate hydratase

of not only glucose transporters but also the enzymes involved in glycolysis. In addition, pyruvate, in inhibiting HIF-1α degradation, would enhance further the rate of glycolysis. Active HIF would also drive the reversible conversion of pyruvate to lactate by lactate dehydrogenase A (LDH-A), another HIF target that is up-regulated in transformed cells. In this way, highly proliferating cells with a high energy demand rapidly dispose of a supply of ATP. However, this picture is probably too simplistic, and the origins of transformation of cells into cancer-initiating cells, be they differentiated or stem cells in origin, requires clarification. Numerous factors in the host cell environment may trigger early stages in tumour formation. Modifications in both the c-myc oncogene and Akt kinase pathways have been reported to activate anaerobic glucose metabolism (Gatenby and Gillies 2004). c-*myc* was found to induce up-regulation of the glucose transporter GLUT1 and several glycolytic enzymes as well as lactate dehydrogenase A and thus lactate overproduction. The *Akt* oncogene also induces anaerobic glucose metabolism, and Akt-expressing cells were dependent on glucose for survival. Though conflicting evidence has been reported, HIF-1 is also activated by the PI3 K/Akt→ mTOR pathway. Nonetheless, oncogenes are more usually considered to be involved in cancer cell proliferation and survival, so their involvement in metabolism came as a surprise.

10.5.2 Modulation of HIF Signalling Through Metabolism

The concentration of glucose and of glycolytic and/or TCA cycle components in the micro-environment may influence the activity of the HIF pathway. Glucose has been shown to be required for hypoxic accumulation of HIF-1α in FaDu and HT1080 tumour cell lines, and this observation has been put forward as an explanation for the detection of only low levels of HIF-1α in FaDu xenograft tumours (Vordermark et al. 2005). 2-Oxoglutarate (2-OG) (α-ketoglutarate), a product of the TCA cycle and amino acid catabolism, is required for the activity of the oxygen sensors PHDs and FIH-1; thus, a decrease in its concentration could be expected to favour HIF-α stability and HIF activation. However, the explanation for the effect of pyruvate, mentioned above, does not seem to be due to competition for 2-OG. Otto Warburg's initial suggestion that mitochondrial function was deficient in tumour cells turns out to be, to a certain degree, correct. Mutations in the mitochondrial enzymes succinate dehydrogenase (SDH) and fumarate hydratase (FH; also termed fumarase), enzymes of the TCA cycle, promote tumorigenesis (for review see Gottlieb and Tomlinson 2005). SDH and FH are in fact tumour suppressors, and SDH provides a direct link with the respiration chain. The postulated mechanism leading to tumorigenesis includes the accumulation of succinate or fumarate, and thus inhibition of SDH or FH respectively, which represses the activity of the PHDs. This leads to stabilization of HIF-1α and induction of downstream genes such as *vegf*, with the consequence of formation of highly vascularized tumours (Gottlieb and Tomlinson 2005). In addition, the competitive inhibition of PHD by succinate was found to be reversed by an excess of 2-OG. However, succinate is also a product of oxidative decarboxylation of 2-OG by PHD. Increased production of reactive oxygen species (ROS) has also been implicated and may be linked to HIF activation by oxidation of Fe^{2+} and ascorbic acid, two co-factors required for PHD activity (Gerald et al. 2004).

10.5.3 Tumour Cells Use the Pentose PhosphatePathway

An alternative catalytic process diverging from the glycolytic pathway is the pentose phosphate pathway (PPP) or hexose monophosphate shunt that is in part responsible for nucleic acid synthesis (for a detailed schematic presentation of PPP and its interconnection with glycolysis see Coy et al. 2005). Transketolase activity controls the non-oxidative part of this pathway, and it has been proposed that the inhibition of its activity suppresses tumour growth and metastasis (Coy et al. 2005).

In addition, it has been shown that a mutated transketolase-like 1 (*tktl1*) gene is up-regulated in cancer, and thus this pathway may also be involved in lactate production and metabolic adaptation of cancer cells (Coy et al. 2005). Glycolysis and the PPP also produce respectively, NADH and NADPH, which in oxidative stress act as cofactors for antioxidant enzymes and in preserving high amounts of the cell's major antioxidant, reduced glutathione. Thus activation of these pathways through HIF may have implications for a vast number of diseases in addition to cancer, such as diabetic retinopathy, atherosclerosis and neurodegenerative diseases, where oxidative stress is suspected to play a significant role. Indeed HIF-dependent activation of glycolysis and the PPP has been reported to mediate a neuroprotective response of cells to toxic amyloid beta peptide, a peptide suspected to be a causative agent in Alzheimer's disease (Soucek et al. 2003).

The Acidic Micro-environment of Tumours

A correlation between PO_2 and pH, both of which decrease with increasing distance from blood vessels, has been reported in breast cancer (Gatenby and Gillies 2004). The high levels of lactate and CO_2 produced by tumour cells are the major contributors to acidosis. A number of transporters, exchangers and pumps, together with the enzyme carbonic anhydrase, contribute to maintaining the intracellular pH that allows cancer cells to survive acidosis.

10.6.1 Intracellular pH Regulation by Transporters and Exchangers

The lactate produced by anaerobic glucose metabolism in tumour cells is excreted from the cells via a H^+/lactate cotransporter (monocarboxylate transporter, MCT), with the consequence of a decrease in the extracellular pH (pH_e) of tumours: pH 6.2–6.8 for tumour cells compared to 7.2–7.4 for normal cells (Fig. 10.7) (Cardone et al. 2005). The pH_e may also remain acidic due to inefficient removal of this waste product by a defective micro-vasculature in tumours. In addition, active MCT even results in alkalinization of the intracellular pH (pH_i) of tumour cells beyond that of normal cells: pH 7.2–7.7 versus 6.9–7.1, respectively (Cardone et al. 2005). The amiloride-sensitive Na^+/H^+ exchanger (NHE-1), a major player in pH_i homeostasis (Counillon and Pouyssegur 2000) has even been implicated in promoting metastasis through extracellular matrix remodelling (Cardone et al. 2005). With the aim of investigating the role that the acidic extracellular environment plays in tumorigenesis, several years ago our laboratory established mutant cell lines, obtained from parental Chinese hamster lung fibroblast (CCL39) transformed with the H-*ras* oncogene, that were defective in pHi regulation through NHE-1 and/or aerobic glucose metabolism (Pouyssegur et al. 2001). Ras-transformed cells mutated for *nhe1* (*nhe1*$^-$) formed tumours when injected into nude mice, but 80% of these tumours finally regressed and disappeared, demonstrating that NHE-1 is indeed an important factor controlling tumour cell survival. We hypothesised that this regression occurred because tumour cells, producing large amounts of lactic acid, need all of the pH_i-regulating system for progression. To validate this point, we engineered a double mutant that lacked both NHE1 (*nhe1*$^-$) and the glycolytic enzyme phosphoglucose isomerase (*pgi*–) and produced 14 times less lactic acid than the parental cells. The single mutant cells (*pgi*$^-$), which rely on respiration via glucose-6-phosphate being diverted into the PPP for energy supply, formed tumours like wild-type Ras-transformed fibroblasts. Interestingly, the double mutant (*nhe1*$^-$, *pgi*$^-$) was no longer impaired in tumour formation. This finding established that NHE-1 is a key player in tumour progression in the context of an acidic micro-environment. Another cell line, mutant for oxidative phosphorylation (respiration mutant, *res*$^-$) that produced 3–4 times

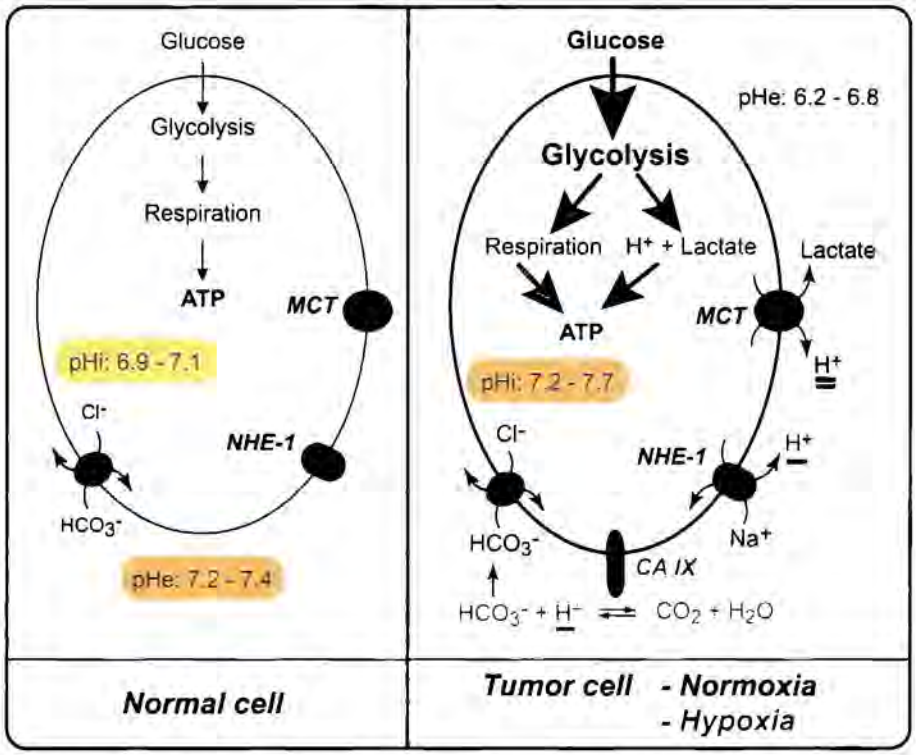


Fig. 10.7. The pH of tumours is highly acidic due to over-production of lactate and CO_2. To survive, cells must maintain a balance between the extracellular pH (pH_e) and intracellular pH (pH_i) and do so through the activity of a number of pumps, exchangers and transporters. Lactate is excreted from cells by the H^+/lactate cotransporter (monocarboxylate transporter, *MCT*), while intracellular protons are exchanged for sodium ions by the Na^+/H^+ exchanger (*NHE-1*). The extracellular activity of carbonic anhydrase IX (*CA IX*) converts CO_2 into carbonic acid, and bicarbonate ions are exchanged for chloride ions in both directions by different Na^+-dependent and -independent Cl^-/HCO_3^- exchangers. The pH_e of tumour cells is more acidic than normal cells and the pH_i is more alkaline

more lactic acid showed a 5 times lower incidence of tumour formation. These cells survived hypoxia but were very reliant on glucose for energy. Thus a low level of lactate production, due to inhibition of glycolysis, brought about a high incidence of tumour formation, while a high level of lactate, resulting from an inhibition of respiration, led to a low incidence of tumour formation. A double mutant *nhe1*$^-$ *res*$^-$ never gave rise to tumours. These results indicate that oncogenic transformation alone is not the only factor important for in vivo tumorigenesis and that pHi regulation and therefore the acidic micro-environment is also a key determinant in cancer progression.

10.6.2 Carbonic Anhydrase, a pH_i-regulating Enzyme and Marker of Tumour Hypoxia

However, lactate is not the only source of acidification of tumours. The production of CO_2 induced by anaerobic conditions also contributes to the major acid load in the tumour environment. The enzyme carbonic anhydrase (CA), which catalyzes the reversible conversion of CO_2 to carbonic acid, contributes to the increase in the pH_i of tumour cells via the uptake of HCO_3^- through Cl^-/HCO_3^- exchangers (Fig. 10.7) (Ivanov et al. 2001). CA is a family of zinc metalloenzymes (EC 4.2.1.1) of which there are

14 mammalian isoforms that vary in activity and tissue distribution. CA 9 and CA 12 are HIF-dependent genes and have been shown to be up-regulated in cells of RCC and in multiple human cancers (Brahimi-Horn and Pouyssegur 2005; Ivanov et al. 2001). The protein CA IX is localized in the plasma membrane and has an extracellular activity, which may be involved in pH sensing. A correlation between hypoxia, angiogenesis, HIF-1α and CA IX expression in primary tumours and lymph node metastases in breast cancer has been reported (Van den Eynden et al. 2005), and considerable interest is being shown in investigating immunohistochemical labelling of cancer tissue sections with antibodies to CA IX for prognostic purposes. It is of interest to note that recently, in addition to interacting with a number of membrane exchangers and cotransporters, the CA II isoform was shown to interact with MCT1 and to enhance the rate of H^+ flux mediated by MCT1 via a mechanism that did not involve its enzyme activity (Becker et al. 2005). However, the involvement of CA in tumour progression and metastasis through modulation of the pH is still to be determined.

Hypoxic Induction of Cell Survival or Cell Death

The implication of hypoxia and HIF in cell survival versus cell death is not clear-cut (for review: Greijer and van der Wall 2004). This may be due to the fact that: (1) different degrees and duration of hypoxia may promote survival or death depending on the cell type (Vaupel 2004); (2) HIF induces genes involved in both apoptosis (programmed cell death) and cell survival and proliferation, two seemingly opposite events (Semenza 2003); (3) although structurally similar, HIF-1α and HIF-2α may act in opposing ways in different cellular contexts and in response to different environmental stimuli (Acker et al. 2005); and (4) the micro-environment of hypoxic tumour cells may influence the direction cells follow (Blouw et al. 2003). It is also generally accepted that tumours that are hypoxic are more aggressive, leading to a metastatic phenotype (Vaupel 2004).

Hypoxia is undoubtedly the major stimulus regulating HIF activity; however, a number of cell-specific non-hypoxia-dependent modes of regulation also influence activity and thus cell survival/death (Semenza 2003). Some of the agents found to influence HIF activity are in fact better known for their effects on cell proliferation, others for their effects on cell death. These include a number of growth factors, such as insulin-like growth factor, epidermal growth factor, platelet growth factor and fibroblast growth factor, hormones such as the vasoactive angiotensin II and androgens, cytokines such as tumour necrosis factor-α and interleukin-1β, and finally the vasoactive molecule NO. These molecules bring into play the Ras/ERK, PI3K/Akt, NF-κB and ROS-sensitive signalling pathways.

10.7.1 Cell Survival, Growth and Metastasis

The insulin-like growth factor-2 and insulin-like growth factor binding proteins that promote cell proliferation and survival are themselves HIF-induced gene products. These growth factors bind transmembrane receptor tyrosine kinases that phosphorylate tyrosine residues of substrates that signal predominantly through the PI3K/Akt pathway. These factors cooperate with nutrients in activating mammalian target of rapamycin (mTOR), a serine/threonine protein kinase involved in regulating protein translation, for protein synthesis during cell proliferation (for review: Pouyssegur et al. 2006). When nutrients and thus energy are limited, or when cells are exposed to a hypoxic stress, mTOR is inhibited and protein synthesis shuts down.

Cells that survive acidosis are thought to have developed a selective growth advantage. In addition, the acidic extracellular environment leads to activation of proteins such as matrix metalloproteases that themselves are induced in hypoxia in a HIF-dependent manner (Petrella et al. 2005).

This contributes to disruption of cell–cell and cell–extracellular matrix (ECM) contacts that allow migration of cells through basement membranes and stromal tissue to enter blood or lymph vessels and to metastasize. The role of a number of proteins involved in cell–cell–ECM contact, such as epithelial cadherin, selectins and integrins, may be influenced directly by HIF or indirectly by its effects on the tumour environment. Thereby high lactate concentrations, even if not the cause of tumour formation, may induce adaptive processes and favour metastasis with subsequent poor survival (Walenta and Mueller-Klieser 2004). In addition, HIF is known to activate certain genes, such as c-*met* proto-oncogene and the chemokine receptor CXCR4, that are involved in invasion and metastasis (Pennacchietti et al. 2003; Staller et al. 2003). It is of interest to note that a cytokine termed autocrine motility factor (AMF) is none other than the HIF-dependent glycolytic enzyme PGI (Tsutsumi et al. 2004). The cytokine function of AMF/PGI has been attributed to a region of the protein that is distinct from the region that possesses enzyme activity. AMF/PGI is of course found to be up-regulated in a number of cancers, and the cytokine function has been implicated in a number of cancer-associated events, including transformation, proliferation, angiogenesis, apoptosis, cell migration and metastasis (Tsutsumi et al. 2004). AMF/PGI is secreted from cells and binds to a seven-transmembrane glycoprotein receptor, AMFR. We are presently examining the impact of knockdown by RNA interference of AMF/PGI on lactate production and HIF function (Laferrière et al. 2005). These studies should provide further insight into the role of glycolysis and hypoxia in tumour progression and allow distinction between acidosic and hypoxic stress.

Studies using HIF-1α deficient cells have shown conflicting results with respect to the promotion or inhibition of tumour growth, and a similar situation exists for HIF-2α (Acker et al. 2005). This may again suggest that the same genetic mutation may have different outcomes depending on the environmental conditions. Therefore further studies are required to clarify the tumour-suppressor-like versus oncogene-like functions of HIF.

A number of reports have indicated that cells may develop resistance to hypoxia-mediated cell death through successive periods of exposure to hypoxia. In these cells the translocation of the pro-apoptotic protein Bax to mitochondria was reduced and lower levels of cytochrome C released. Two anti-apoptotic proteins, the HIF-induced apoptosis inhibitor protein-2 (AIP-2) and Bcl-xL, have been implicated in this hypoxia-resistant cell survival. AIP-2 had an inhibitory effect towards the activity of caspases, proteolytic activity associated with apoptosis. Bcl-xL was found to interact with the pro-apoptotic Bax protein, thus preventing the activation of the intrinsic cascade. Protection accorded by Bcl-xL was dependent on the import of ATP, produced by glycolysis, into the mitochondria to generate an inner mitochondrial membrane potential through F_1F_0-ATP synthase, the fundamental cellular energy producer. When glucose was deficient or glycolysis was inhibited, protection from hypoxic-mediated cell death was absent – yet another example of the importance of environmental factors in determining cell fate. HIF may also indirectly favour cell survival, through angiogenic proteins or activation of signalling pathways. Thus VEGF expression enhances the expression of anti-apoptotic proteins, and activation of the PI3 K/Akt pathway can protect from apoptosis due to serum starvation (Ferrara et al. 2003).

Hypoxia can also lead to reduced cell proliferation, and there again certain HIF target genes are involved in cell-cycle arrest and proliferation, including p21 and cyclin G2 (Brahimi-Horn and Pouyssegur 2005). G1/S phase cell cycle transition implicates certain cyclin-dependent kinases (CDK)–cyclin complexes that are inhibited by $p21^{cip1}$ and $p27^{Kip1}$. A deficiency in HIF-1α in embryonic fibroblasts or splenic B lymphocytes abolished hypoxia-induced growth arrest, and hypoxia led to a HIF-1α-dependent increase in $p21^{cip1}$ and $p27^{Kip1}$. Growth arrest was also independent of p53 expression. Increased expression of $p21^{cip1}$ in hypoxia was linked to derepression by Myc through the displacement of Myc from the $p21^{cip1}$ promoter by HIF-1ô Similar findings were reported for $vhl^{-/-}$ fibrosarcoma cells (Mack et al. 2005). This result suggests that other genes regulated by Myc may also be counteracted by HIF.

10.7.2 Cell Death

Multiple mechanisms of programmed cell death, including apoptosis, necrosis and autophagy, are responsible for physiological and pathological clearing of cells (Edinger and Thompson 2004). Apoptosis is an ATP, energy-dependent mechanism that occurs either through an extrinsic (death receptor) pathway or intrinsic (mitochondrial) pathway and is dependent on the proteolytic activity of caspases. "Accidental necrosis" is caspase independent and occurs when the level of ATP is so low that cells can no longer survive. It is termed accidental because it is often observed subsequent to exposure to cytotoxic agents. Autophagic cell death involves vacuolation of cellular components for lysosomal degradation and recycling, and thereby constitutes a desperate survival strategy in response to stress, which ends in suicide. These different routes of cell death all contribute to reduce tumour progression, but the part that hypoxia and HIF play, in particular in the area of autophagy, still needs clarification. Autophagy may represent a temporary mechanism of obtaining nutrients to increase the ATP level prior to the establishment, through angiogenesis, of new blood vessels that eventually bring oxygen and nourishment to the tumour.

The protein Bcl-2/adenovirus EIB 19kDa-interacting protein 3 (BNIP3) and its homolog Nip-3-like protein X (NIX) are induced by HIF and are implicated in cell death (Greijer and van der Wall 2004). BNIP3 is overexpressed in ductal carcinoma in situ of the breast and was associated with high-grade necrotic lesions that may correlate with an invasive phenotype. However, no correlation was found for the expression of NIX, which may suggest that other factors come into play. Despite the fact that BNIP3 belongs to the family of Bcl-2 apoptotic proteins, BNIP3-mediated cell death has been suggested to resemble necrosis more than apoptosis. However, increased expression of BNIP3 on its own may not be sufficient to induce apoptosis; other additional conditions may be needed. Removal of epidermal growth factor and insulin-like growth factor in breast cancer cell line MCF7, and acidosis or glucose deprivation in cardiac myocytes (Greijer and van der Wall 2004; Webster et al. 2005), were required to lead to BNIP3-induced cell death in hypoxia. HIF also induces two other proteins that are involved in apoptosis, HGTD-P and RTP801 (Brahimi-Horn and Pouyssegur 2005). The former was found to be associated with mitochondria and promotes apoptosis by the mitochondrial pathway. RNA interference targeting HGTD-P led to protection of PC-3 prostatic cancer cells from apoptosis. RTP801 overexpression in differentiated neuronal PC12 cells and MCF7 breast carcinoma cells resulted in apoptosis through a decrease in the production of ROS.

A number of studies have investigated possible links between the HIF signalling pathway and p53, the so-called "guardian of the genome". The p53 gene is a tumour suppressor gene that is often inactivated or mutated in tumours and transformed cells. The protein product is a transcription factor that controls cell growth and DNA damage repair, and its activation leads potentially cancerous cells to autodestruct. The protein p53, in response to multiple DNA damage, induces the apoptotic proteins Bax and Bak that initiate apoptosis by regulating release of cytochrome C from mitochondria, a characteristic of the mitochondrial pathway of apoptosis. The level of p53 is normally low in cells, but it has been shown to accumulate under hypoxic stress and to interact directly with the ODDD of HIF-1α. The direct physiological consequence of this is not fully understood (Fels and Koumenis 2005). Interaction may diminish HIF activity by competing with p53 for common cofactors, such as p300/CBP, or p53 may influence HIF-1α stability or vice versa. Indeed, the ubiquitin E3 ligase murine double minute 2 (Mdm2), responsible for proteasomal targeting, and thus destruction, of p53, has been shown to interact with HIF-1α and be suppressed by it, thus leaving p53 free to act in cell-cycle arrest and apoptosis (Fels and Koumenis 2005). Cells deficient in p53 have also been shown to be less sensitive to hypoxia-induced apoptosis, giving them a survival advantage and a low responsiveness to anti-angiogenic combination therapy (Yu et al. 2002).

Conclusions

Given the substantial evidence that hypoxia- throug HIF activation of downstream genes- plays a role in tumorigenesis and in poor patient survival, it is essential that the precise mechanisms involved in this pathway be fully explored. Therapeutic strategies targeting the HIF-signalling pathway, be it through direct inhibition of HIF activity with small molecules or, better, through inhibition of HIF-dependent gene products, such as VEGF, are still in their infancy but show promise for the future. However, due to the dual effect of HIF in cell survival/death, the risk of producing tumour cells even less dependent on the vasculature and more resistant to stress-induced cell death, thus favouring metastasis, by such approaches has been raised. Only further pre-clinical and clinical evidence will assuage such concerns.

Acknowledgements

We thank members of the Pouyssegur laboratory for sharing with us their results and comments. We thank Dr Rebecca Bilton of Adelaide University, Australia, for preparing the immunofluorescence images of HIF-1α. We apologize to the many research groups whose work was cited indirectly by reference to review articles. This is due to constraints in the number of references permitted. Readers are referred to two of our recent reviews in which many of the individual studies are cited (Brahimi-Horn and Pouyssegur 2004; Brahimi-Horn and Pouyssegur 2005). Our laboratory is funded by grants from the Ligue National Contre le Cancer (Equipe labellisée), the Centre National de la Recherche Scientific (CNRS), the Ministère de l'Education, de la Recherche et de la Technologie, the Institut National de la Santé et de la Recherche Médicale (INSERM) and the National Cancer Institute of Canada. J.L. is presently a Research Fellow of The Terry Fox Foundation funded by an award from the National Cancer Institute of Canada.

References

Acker T, Diez-Juan A, Aragones J, Tjwa M, Brusselmans K, Moons L, Fukumura D, Moreno-Murciano MP, Herbert JM, Burger A, et al (2005) Genetic evidence for a tumor suppressor role of HIF-2alpha. Cancer Cell 8:131–141

Aprelikova O, Chandramouli GV, Wood M, Vasselli JR, Riss J, Maranchie JK, Linehan WM, Barrett JC (2004) Regulation of HIF prolyl hydroxylases by hypoxia-inducible factors. J Cell Biochem 92:491–501

Arnesen T, Gromyko D, Horvli O, Fluge O, Lillehaug J, Varhaug JE (2005a) Expression of N-acetyl transferase human and human arrest defective 1 proteins in thyroid neoplasms. Thyroid 15:1131–1136

Arnesen T, Kong X, Evjenth R, Gromyko D, Varhaug JE, Lin Z, Sang N, Caro J, Lillehaug JR (2005b) Interaction between HIF-1alpha (ODD) and hARD1 does not induce acetylation and destabilization of HIF-1alpha. FEBS Lett 579:6428–6432

Astrom K, Cohen JE, Willett-Brozick JE, Aston CE, Baysal BE (2003) Altitude is a phenotypic modifier in hereditary paraganglioma type 1: evidence for an oxygen-sensing defect. Hum Genet 113:228–237

Baek JH, Mahon PC, Oh J, Kelly B, Krishnamachary B, Pearson M, Chan DA, Giaccia AJ, Semenza GL (2005) OS-9 interacts with hypoxia-inducible factor 1alpha and prolyl hydroxylases to promote oxygen-dependent degradation of HIF-1alpha. Mol Cell 17:503–512

Becker HM, Hirnet D, Fecher-Trost C, Sultemeyer D, Deitmer JW (2005) Transport activity of MCT1 expressed in Xenopus oocytes is increased by interaction with carbonic anhydrase. J Biol Chem 280:39882–39889

Berra E, Benizri E, Ginouves A, Volmat V, Roux D, Pouyssegur J (2003) HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia. Embo J 22:4082–4090

Berra E, Ginouves A, Pouyssegur J (2006) The hypoxia-inducible factor hydroxylases bring fresh air into hypoxia signalling. EMBO Rep 7:41–45

Berta M, Mazure N, Hattab M, Pouysségur J, Brahimi-Horn MC (2004) Hypoxia-inducible factor-1ô is modified by covalent attachment to SUMO. Keystone symposia. Biology of hypoxia: the role of oxygen sensing in development, normal function and disease. Colorado, USA, 25–30 March 2004

Bilton R, Mazure N, Trottier E, Hattab M, Dery MA, Richard DE, Pouyssegur J, Brahimi-Horn MC (2005) Arrest-defective-1 protein, an acetyltransferase, does not alter stability of hypoxia-inducible factor (HIF)-1alpha and is not induced by hypoxia or HIF. J Biol Chem 280:31132–31140

Blouw B, Song H, Tihan T, Bosze J, Ferrara N, Gerber HP, Johnson RS, Bergers G (2003) The hypoxic response of tumors is dependent on their microenvironment. Cancer Cell 4:133–146

Bracken CP, Whitelaw ML, Peet DJ (2005) Activity of hypoxia-inducible factor 2alpha is regulated by association with the NF-kappaB essential modulator. J Biol Chem 280:14240–14251

Brahimi-Horn MC, Pouyssegur J (2005) The hypoxia-inducible factor and tumor progression along the angiogenic pathway. Int Rev Cytol 242:157–213

Brahimi-Horn C, Mazure N, Pouyssegur J (2005) Signalling via the hypoxia-inducible factor-1alpha requires multiple posttranslational modifications. Cell Signal 17:1–9

Braun RD, Lanzen JL, Snyder SA, Dewhirst MW (2001) Comparison of tumor and normal tissue oxygen tension measurements using OxyLite or microelectrodes in rodents. Am J Physiol Heart Circ Physiol 280:H2533–2544

Cardone RA, Casavola V, Reshkin SJ (2005) The role of disturbed pH dynamics and the Na+/H+ exchanger in metastasis. Nat Rev Cancer 5:786–795

Carmeliet P (2003) Angiogenesis in health and disease. Nat Med 9:653–660

Counillon L, Pouyssegur J (2000) The expanding family of eucaryotic Na(+)/H(+) exchangers. J Biol Chem 275:1–4

Coy JF, Dressler D, Wilde J, Schubert P (2005) Mutations in the transketolase-like gene TKTL1: clinical implications for neurodegenerative diseases, diabetes and cancer. Clin Lab 51:257–273

Edinger AL, Thompson CB (2004) Death by design: apoptosis, necrosis and autophagy. Curr Opin Cell Biol 16:663–669

Erecinska M, Silver IA (2001) Tissue oxygen tension and brain sensitivity to hypoxia. Respir Physiol 128:263–276

Fels DR, Koumenis C (2005) HIF-1alpha and p53: the ODD couple? Trends Biochem Sci 30:426–429

Ferrara N, Gerber HP, LeCouter J (2003) The biology of VEGF and its receptors. Nat Med 9:669–676

Ferrara N, Kerbel RS (2005) Angiogenesis as a therapeutic target. Nature 438:967–974

Fisher TS, Etages SD, Hayes L, Crimin K, Li B (2005) Analysis of ARD1 function in hypoxia response using retroviral RNA interference. J Biol Chem 280:17749–17757

Folkman J, Hahnfeldt P, Hlatky L (2000) Cancer: looking outside the genome. Nat Rev Mol Cell Biol 1:76–79

Fox SB, Braganca J, Turley H, Campo L, Han C, Gatter KC, Bhattacharya S, Harris AL (2004) CITED4 inhibits hypoxia-activated transcription in cancer cells, and its cytoplasmic location in breast cancer is associated with elevated expression of tumor cell hypoxia-inducible factor 1alpha. Cancer Res 64:6075–6081

Freedman SJ, Sun ZY, Kung AL, France DS, Wagner G, Eck MJ (2003) Structural basis for negative regulation of hypoxia-inducible factor-1alpha by CITED2. Nat Struct Biol 10:504–512

Gatenby RA, Gillies RJ (2004) Why do cancers have high aerobic glycolysis? Nat Rev Cancer 4:891–899

Gerald D, Berra E, Frapart YM, Chan DA, Giaccia AJ Mansuy D, Pouyssegur J, Yaniv M, Mechta-Grigoriou F (2004) JunD reduces tumor angiogenesis by protecting cells from oxidative stress. Cell 118:781–794

Gill G (2005) Something about SUMO inhibits transcription. Curr Opin Genet Dev 15:536–541

Gottlieb E, Tomlinson I (2005) Mitochondrial tumor suppressors: genetic and biochemical update. Nat Rev Cancer 5:857–866

Greijer AE, van der Wall E (2004) The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis. J Clin Pathol 57:1009–1014

Hu CJ, Wang LY, Chodosh LA, Keith B, Simon MC (2003) Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. Mol Cell Biol 23:9361–9374

Ivanov S, Liao SY, Ivanova A, Danilkovitch-Miagkova A, Tarasova N, Weirich G, Merrill MJ, Proescholdt MA, Oldfield EH, Lee J, et al (2001) Expression of hypoxia-inducible cell-surface transmembrane carbonic anhydrases in human cancer. Am J Pathol 158:905–919

Jeong JW, Bae MK, Ahn MY, Kim SH, Sohn TK, Bae MH, Yoo MA, Song EJ, Lee KJ, Kim KW (2002) Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation. Cell 111:709–720

Kaelin WG Jr (2002) Molecular basis of the VHL hereditary cancer syndrome. Nat Rev Cancer 2:673–682

Kasper LH, Boussouar F, Boyd K, Xu W, Biesen M, Rehg J, Baudino TA, Cleveland JL, Brindle PK (2005) Two transactivation mechanisms cooperate for the bulk of HIF-1-responsive gene expression. Embo J 24:3846–3858

Kato H, Tamamizu-Kato S, Shibasaki F (2004) Histone deacetylase 7 associates with hypoxia-inducible factor 1alpha and increases transcriptional activity. J Biol Chem 279:41966–41974

Koivunen P, Hirsila M, Gunzler V, Kivirikko KI, Myllyharju J (2003) Catalytic properties of the asparaginyl hydroxylase (FIH) in the oxygen sensing pathway are distinct from those of its prolyl 4-hydroxylases. J Biol Chem 279:9899-9904

Kondo K, Klco J, Nakamura E, Lechpammer M, Kaelin WG Jr (2002) Inhibition of HIF is necessary for tumor suppression by the von Hippel-Lindau protein. Cancer Cell 1:237–246

Kung AL, Zabludoff SD, France DS, Freedman SJ, Tanner EA, Vieira A, Cornell-Kennon S, Lee J, Wang B, Wang J, et al (2004) Small molecule blockade of transcriptional coactivation of the hypoxia-inducible factor pathway. Cancer Cell 6:33–43

Laferrière J, Mazure N, Trottier E, Pouyssegur J (2005) Role of phosphoglucose isomerase (PGI/AMF), HIF-1 and glycolysis in cancer progression. Paper presented at: 2nd Annual International Tumor Metabolism Summit, Genoa

Mack FA, Patel JH, Biju MP, Haase VH, Simon MC (2005)

Decreased growth of Vhl-/- fibrosarcomas is associated with elevated levels of cyclin kinase inhibitors p21 and p27. Mol Cell Biol 25:4565-4578

Mahon PC, Hirota K, Semenza GL (2001) FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev 15:2675-2686

Makino Y, Cao R, Svensson K, Bertilsson G, Asman M, Tanaka H, Cao Y, Berkenstam A, Poellinger L (2001) Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. Nature 414:550-554

Manalo DJ, Rowan A, Lavoie T, Natarajan L, Kelly BD, Ye SQ, Garcia JG, Semenza GL (2005) Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1. Blood 105:659-669

Maranchie JK, Vasselli JR, Riss J, Bonifacino JS, Linehan WM, Klausner RD (2002) The contribution of VHL substrate binding and HIF1-alpha to the phenotype of VHL loss in renal cell carcinoma. Cancer Cell 1:247-255

Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ (1999) The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 399:271-275.

Mazure N, Dayan F, Roux D, Berra E, Brahimi-Horn C, Pouyssegur J (2005) The oxygen-sensor factor-inhibiting HIF-1 (FIH-1) controls expression of distinct genes through the bi-functional transcription factor HIF-1ô. Paper presented at: 2nd Annual International Tumor Metabolism Summit, Genoa

Morita M, Ohneda O, Yamashita T, Takahashi S, Suzuki N, Nakajima O, Kawauchi S, Ema M, Shibahara S, Udono T, et al (2003) HLF/HIF-2alpha is a key factor in retinopathy of prematurity in association with erythropoietin. Embo J 22:1134-1146

Ozer A, Wu LC, Bruick RK (2005) The candidate tumor suppressor ING4 represses activation of the hypoxia inducible factor (HIF). Proc Natl Acad Sci U S A 102:7481-7486

Pages G, Pouyssegur J (2005) Transcriptional regulation of the vascular endothelial growth factor gene - a concert of activating factors. Cardiovasc Res 65:564-573

Pennacchietti S, Michieli P, Galluzzo M, Mazzone M, Giordano S, Comoglio PM (2003) Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. Cancer Cell 3:347-361

Petrella BL, Lohi J, Brinckerhoff CE (2005) Identification of membrane type-1 matrix metalloproteinase as a target of hypoxia-inducible factor-2 alpha in von Hippel-Lindau renal cell carcinoma. Oncogene 24:1043-1052

Pouyssegur J, Dayan F, Mazure NM (2006) Hypoxia signalling and angiogenesis and approaches to enforce tumour regression. Nature 441:437-443

Pouyssegur J, Franchi A, Pages G (2001) pHi, aerobic glycolysis and vascular endothelial growth factor in tumour growth. Paper presented at: Novartis Found Symp 240:186-196 (Wiley, Chichester)

Pugh CW, Ratcliffe PJ (2003) Regulation of angiogenesis by hypoxia: role of the HIF system. Nat Med 9:677-684

Rajendran JG, Mankoff DA, O'Sullivan F, Peterson LM, Schwartz DL, Conrad EU, Spence AM, Muzi M, Farwell DG, Krohn KA (2004) Hypoxia and glucose metabolism in malignant tumors: evaluation by [18F]fluoromisonidazole and [18F]fluorodeoxyglucose positron emission tomography imaging. Clin Cancer Res 10:2245-2252

Schofield CJ, Ratcliffe PJ (2005) Signalling hypoxia by HIF hydroxylases. Biochem Biophys Res Commun 338:617-626

Semenza GL (2003) Targeting HIF-1 for cancer therapy. Nat Rev Cancer 3:721-732

Semenza GL, Nejfelt MK, Chi SM, Antonarakis SE (1991) Hypoxia-inducible nuclear factors bind to an enhancer element located 3' to the human erythropoietin gene. Proc Natl Acad Sci U S A 88:5680-5684

Soucek T, Cumming R, Dargusch R, Maher P, Schubert D (2003) The regulation of glucose metabolism by HIF-1 mediates a neuroprotective response to amyloid beta peptide. Neuron 39:43-56

Sowter HM, Raval RR, Moore JW, Ratcliffe PJ, Harris AL (2003) Predominant role of hypoxia-inducible transcription factor (Hif)-1alpha versus Hif-2alpha in regulation of the transcriptional response to hypoxia. Cancer Res 63:6130-6134

Staller P, Sulitkova J, Lisztwan J, Moch H, Oakeley EJ, Krek W (2003) Chemokine receptor CXCR4 downregulated by von Hippel-Lindau tumour suppressor pVHL. Nature 425:307-311

To KK, Huang LE (2005) Suppression of HIF-1alpha transcriptional activity by the HIF prolyl hydroxylase EGLN1. J Biol Chem 280:38102-38107

Tojo M, Matsuzaki K, Minami T, Honda Y, Yasuda H, Chiba T, Saya H, Fujii-Kuriyama Y, Nakao M (2002) The aryl hydrocarbon receptor nuclear transporter is modulated by the SUMO-1 conjugation system. J Biol Chem 277:46576-46585.

Tsutsumi S, Yanagawa T, Shimura T, Kuwano H, Raz A (2004) Autocrine motility factor signaling enhances pancreatic cancer metastasis. Clin Cancer Res 10:7775-7784

Van den Eynden GG, Van der Auwera I, Van Laere SJ, Colpaert CG, Turley H, Harris AL, van Dam P, Dirix LY, Vermeulen PB, Van Marck EA (2005) Angiogenesis and hypoxia in lymph node metastases is predicted by the angiogenesis and hypoxia in the primary tumour in patients with breast cancer. Br J Cancer 93:1128-1136

Vaupel P (2004) Tumor microenvironmental physiology

and its implications for radiation oncology. Semin Radiat Oncol 14:198–206

Vordermark D, Kraft P, Katzer A, Bolling T, Willner J, Flentje M (2005) Glucose requirement for hypoxic accumulation of hypoxia-inducible factor-1alpha (HIF-1alpha) Cancer Lett 230:122–133

Walenta S, Mueller-Klieser WF (2004) Lactate: mirror and motor of tumor malignancy. Semin Radiat Oncol 14:267–274

Wang V, Davis DA, Haque M, Huang LE, Yarchoan R (2005) Differential gene up-regulation by hypoxia-inducible factor-1alpha and hypoxia-inducible factor-2alpha in HEK293T cells. Cancer Res 65:3299–3306

Webster KA, Graham RM, Bishopric NH (2005) BNip3 and signal-specific programmed death in the heart. J Mol Cell Cardiol 38:35–45

Yu DY, Cringle SJ (2005) Retinal degeneration and local oxygen metabolism. Exp Eye Res 80:745–751

Yu JL, Rak JW, Coomber BL, Hicklin DJ, Kerbel RS (2002) Effect of p53 status on tumor response to antiangiogenic therapy. Science 295:1526-1528

Hypoxia and Angiogenesis in Glioblastomas

TILL ACKER and KARL H. PLATE

Abstract

Malignant gliomas have retained their dismal prognosis despite an aggressive, multimodal therapeutic approach, warranting the need for novel therapeutic modalities. Highly proliferating tumors frequently outstrip their vascular supply, leading to a tumor microenvironment characterized by low PO_2, low glucose levels, and an acidic pH. Regions of low PO_2 are indeed common findings in malignant tumors, being associated with increased frequency of tumor invasion and metastasis. The ability to initiate homeostatic responses and adapt to hypoxia is a crucial aspect of solid tumor growth. The hypoxia-inducible transcription factors HIF-1α and HIF-2α act as main regulators of hypoxia-induced gene expression, determining key parameters of the tumor phenotype such as angiogenesis, energy metabolism, pH regulation, and genetic instability, as well as tumor invasion/metastasis. These adaptive responses confer an increased resistance to the hostile tumor microenvironment. Recent insights into cellular and molecular crosstalk in this microenvironment suggest a model in which hypoxia, HIF, and several HIF target genes participate in the coordinated collaboration between tumor, endothelial, inflammatory/hematopoietic, and circulating endothelial precursor cells to enhance and promote tumor growth. Interestingly, however, the HIF pathway is known to encompass tumor growth-promoting as well as -inhibiting effects, some of which may be offset by genetic alterations, suggesting a far more complex agrowth. Thus, despite its promise as a novel and potentially selective cancer treatment, therapeutic modulation of the HIF pathway may require an integrated and detailed understanding of the multifaceted nature of HIF action in tumor physiology.

CONTENTS

Introduction

Each year 20,000 new primary central nervous system (CNS) neoplasms are diagnosed In the United States. Importantly, CNS tumors affect the organ that defines the "self;" they are among the most debilitating of human malignancies, often severely compromising quality of life. A multitude of different neoplastic CNS entities are recognized by the CNS tumor classification of the World Health Organization (WHO). Among them, malignant gliomas are the most common and most studied primary malignancies. Malignant gliomas have retained their dismal prognosis despite an aggressive, multimodal therapeutic approach, warranting the need for novel therapeutic modalities.

Tumor growth and progression occurs as a result of cumulative acquisition of genetic alterations affecting oncogenes or tumor suppressor genes selecting for tumor cell clones with enhanced proliferation and survival potential. Tumor growth depends on vascular supply to sustain the metabolic needs of the tumor tissue. Indeed, the acquisition of a functional blood supply seems to be rate-limiting for the tumor's ability to grow beyond a certain size and metastasize to other sites. However, highly proliferative tumors frequently outstrip their vascular supply, leading to a tumor microenvironment characterized by low oxygen tension, low glucose levels and an acidic pH. Glioblastomas are characterized by a prominent, proliferative vascular component and necrotic areas, making them prototype tumors in the understanding of the role of hypoxia-induced mechanisms in tumor growth and progression. A series of recent cell and molecular biology studies have significantly extended our knowledge on how tumor cells exploit key regulatory mechanisms of oxygen homeostasis to adapt to changes in ambient oxygen concentrations. These studies have identified putative oxygen-sensing mechanisms, showing that reduced oxygen levels and tumor-specific genetic alterations synergistically control important physiological pathways by activating a key transcriptional system, the HIF (hypoxia-inducible factor) system, a potent inducer of gene expression in tumor cells. A mounting body of evidence suggests that hypoxia and HIF play a decisive role in tumor physiology and progression by setting and controlling a tumor-specific microenvironment essential for tumor growth. HIF and hypoxia are the major triggers for new blood vessel growth in malignant tumors, and, as recent evidence suggests, regulate a pro-invasive and -metastatic machinery crucially determining tumor aggressiveness. They induce a shift in energy metabolism from oxidative to glycolytic pathways, thus contributing to the acidic tumor microenvironment. Moreover, hypoxia induces genetic instability in tumor cells and, possibly involving HIF function, selects for apoptosis-resistant and thus malignant cell clones. Given the significance of HIF and hypoxia in tumor physiology, recent insight into the precise mechanisms of oxygen sensing and signaling may offer new promising and potentially selective targets for tumor therapy.

The Transcription Factor System HIF

Cells respond to changes in the microenvironment such as acidosis, hypoglycemia or changes in oxygen

tension by down- or upregulation of certain genes. Hypoxia, for example, upregulates the transcription of several genes. A highly developed, multi-level physiological system is devoted to oxygen homeostasis, requiring the coordinate regulation of a wide array of genes. Most of these genes are hypoxia-inducible in multiple cell types including tumor cells. The identification of the HIF transcription system in 1995 constituted a milestone in our understanding of oxygen physiology (Wang and Semenza 1995). Since then the HIF system has emerged as a key regulatory system of responses to hypoxia both on a local and on a systemic level. It is believed that approximately 1–1.5% of the genome is transcriptionally regulated by hypoxia.

The HIF transcriptional complex is widely conserved among mammalian species and invertebrate model organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans*, further stressing its importance as a key transcriptional regulator of hypoxia-induced responses throughout evolution. The HIF complex exists as a heterodimer composed of constitutively expressed HIF-β and O_2-regulated HIF-α subunits belonging to the bHLH (basic helix loop helix)-PAS (PER-ARNT-SIM) family of transcription factors. Both HIF-α and HIF-β proteins exist as isoforms [HIF-1α, HIF-2α, HIF-3α and ARNT (aryl hydrocarbon receptor nuclear translocator), ARNT2 and ARTN3, respectively] (reviewed in Wenger 2002).

11.2.1 Oxygen-Dependent HIF Regulation

HIF activity is tightly regulated throughout the range of physiological and pathological oxygen concentrations, involving multiple mechanisms of control at the level of mRNA expression, protein stability, nuclear translocation and transactivation activity. These combine to activate HIF to maximal levels under decreasing oxygen concentrations. On the molecular level this is mediated by subjecting HIF-α subunits to multiple modes of posttranslational modification, including lysine residue acetylation, phosphorylation and two different types of hydroxylation. The dominant control mechanism occurs through oxygen-dependent proteolysis of HIF-α subunits (Fig. 11.1). Cellular oxygen concentrations regulate transcriptional activity of HIF-α subunits, namely via influencing protein levels and transactivation domain functions. However, other processes, encompassing common tumor-specific genetic alterations (see below), can influence HIF function on different levels. Oxygen-dependent enzymatic hydroxylation of proline residues within HIF-α subunits constitutes the critical modification governing protein stability. Prolyl hydroxylation allows capture by the von Hippel–Lindau tumor suppressor protein (pVHL), which acts as the recognition component of an E3-ubiquitin ligase enzyme. Subsequent ubiquitination targets the complex for proteosomal degradation. As a consequence only low-level HIF-α protein expression can be detected in the presence of oxygen, increasing rapidly and exponentially with decreasing oxygen concentrations. A second oxygen-dependent switch involving hydroxylation of an asparagine residue within the transactivation domain regulates transcriptional activity, possibly by interfering with recruitment of the coactivator p300, which results in reduced transcriptional activity. Following oxygen deprivation HIF-α subunits translocate into the nucleus, where they dimerize with HIF-β subunits, allowing binding to the conserved consensus DNA-binding motif RCGTG residing in the hypoxia-responsive elements (HRE) of many oxygen-regulated genes. Transactivation is initiated by recruitment of coactivators such as CBP (CREB-binding protein)/P300, SRC (steroid receptor coactivator)-1, and TIF2 (transcriptional intermediary factor), which is promoted by the redox regulatory protein Ref (redox effector factor)-1.

11.2.2 Oxygen-Independent HIF Regulation

Though oxygen-dependent regulation seems to provide the prevailing control mechanism of HIF function, receptor-mediated phosphorylation cascades via binding of various growth factors and cytokines,

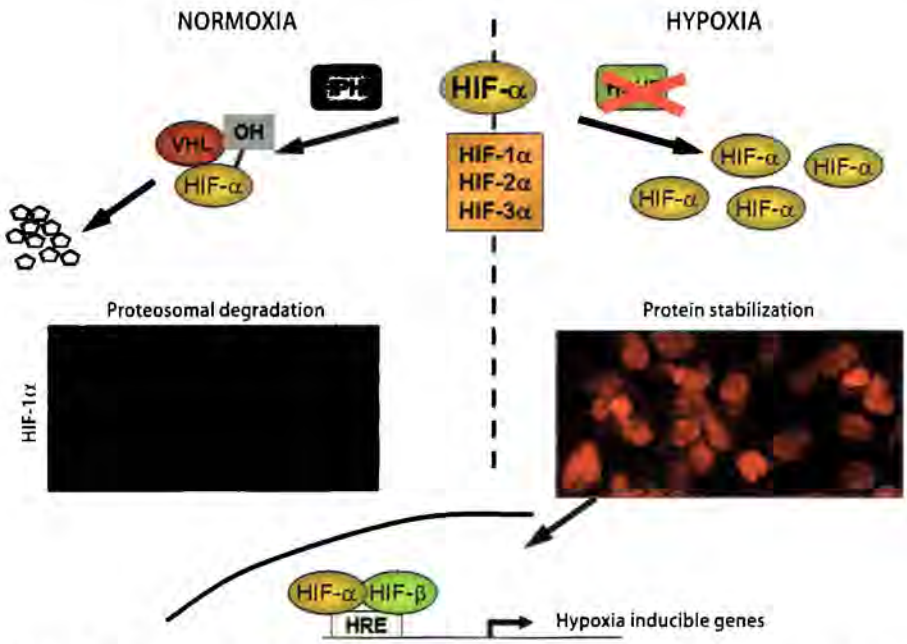


Fig. 11.1. Regulation of HIF subunits by oxygen tension: Oxygen-dependent enzymatic hydroxylation of proline residues within HIF-α subunits constitutes the critical modification governing protein stability. Under normoxia, prolyl hydroxylase (*HPHD*)-mediated hydroxylation allows capture by the von Hippel–Lindau tumor suppressor protein (*VHL*), which acts as the recognition component of an E3-ubiquitin ligase enzyme. Subsequent ubiquitination targets the complex for proteosomal degradation. As a consequence only low-level HIF-α protein expression can be detected in the presence of oxygen (immunofluorescence HIF-1 protein in *red*). Following oxygen deprivation (hypoxia), HIF-α subunit protein concentrations rapidly translocate into the nucleus (immunofluorescence HIF-1 protein in *red*), where they dimerize with HIF-β subunits, allowing binding to the conserved consensus DNA-binding motif RCGTG residing in the hypoxia-responsive elements (*HRE*) of many oxygen-regulated genes

including angiotensin II, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), tumor necrosis factor (TNF) α, insulin, and insulin-like growth factor (IGF) 1 and 2, represent an alternative way to enhance HIF activity by translational and posttranslational control under normoxia. This induction is generally less intense than that mediated by reductions in oxygen tension (reviewed in Bilton and Booker 2003). ARD1-mediated acetylation of a lysine residue (Lys 532) within the ODD (oxygen-dependent domain) represents a novel mode of posttranslational modification of HIF-α subunits under normoxia. Lysyl acetylation has been shown to modulate HIF-α protein stability by promoting VHL binding and subsequent proteosomal degradation. With decreasing oxygen tensions acetylation is gradually reduced due to decreased ARD1 mRNA levels and decreased affinity of ARD1 to HIF-α subunits (Jeong et al. 2002). A VHL-independent regulation of HIF-α stability was suggested by studies reporting that heat shock protein (Hsp) 70 and Hsp 90 protect HIF-1α from proteosomal degradation involving physical interaction and support HIF-α transcriptional activity (Isaacs et al. 2002). While these pathways do not directly mediate the response to hypoxia, interactions with HIF signaling suggest that cellular responses to hypoxia can be fine tuned by integration into the major signaling systems.

The 2-Oxoglutarate-Dependent Hydroxylases PHD and FIH

Hydroxylation provides a dual mechanism of inhibiting HIF activity, inducing proteolytic degradation and reducing transcriptional capacity. These processes are conferred by a subclass of 2-oxoglutarate-dependent hydroxylases (reviewed in Acker and Acker 2004). Interaction of VHL with HIF-α requires an oxygen- and iron-dependent hydroxylation of specific prolyl residues (Pro 402, Pro 564) within the HIF-α ODD carried out by HIF-prolyl hydroxylase (PHD) (Epstein et al. 2001). So far, four orthologs of PHD have been described (PHD I–IV). A second oxygen-dependent switch involves hydroxylation of an asparagine residue within the C-TAD of HIF-α subunits by a HIF asparaginyl hydroxylase called factor inhibiting HIF (FIH-1) (Lando et al. 2002). Asparagine hydroxylation apparently interferes with recruitment of the coactivator p300, resulting in reduced transcriptional activity. Both PHD and FIH belong to a superfamily of 2-oxoglutarate-dependent hydroxylases which employ nonheme iron in the catalytic moiety. They require oxygen in the form of dioxygen, with one oxygen atom being incorporated into the prolyl or asparagyl residue, respectively, and the other into 2-oxoglutarate, yielding succinate and CO_2. Thus, the hydroxylation reaction is inherently dependent on ambient oxygen pressure, providing a molecular basis for the oxygen-sensing function of these enzymes.

Interestingly, PHD are strikingly sensitive to graded levels of oxygen in vitro, mirroring the progressive increase in HIF-α protein stability and transactivation activity observed when cells are subjected to graded hypoxia in vitro (Epstein et al. 2001). In line with this observation, PHD have been found to have a strikingly low oxygen affinity of 178 mmHg above the concentration of dissolved oxygen in the air (Hirsilä et al. 2003). Consequently, given the regular tissue PO_2 distribution, PHD would operate under suboptimal, nonequilibrium conditions for HIF-α turnover far beyond their K_m. However, given a regular Michaelis–Menten kinetic this would allow the enzymes to operate in a highly sensitive manner, in which even small changes in oxygen concentration result in pronounced changes in enzymatic reaction velocity, and thus in HIF-α turnover. In contrast, collagen prolyl-4-hydroxylases exhibit a K_m of about 28 mmHg, one sixth of the K_m of PHD, allowing optimal hydroxyprolyl-collagen biosynthesis under the low oxygen concentrations physiologically found in the cell (Hirsilä et al. 2003). FIH was shown to have a K_m of around 64 mmHg, suggesting that also this enzyme acts as a bona fide oxygen sensor at least under conditions as found in normoxic tissues in vivo (Linke et al. 2004).

The above-mentioned characteristics of the PHD system render it highly sensitive to alterations in co-factor concentration such as ferrous iron (Knowles et al. 2003) or 2-oxoglutarate; in substrate concentration, e.g. due to changes in HIF-α synthesis; and in enzyme concentration, e.g. due to changes in mRNA expression of PHD orthologs in response to PO_2 (Epstein et al. 2001), being particularly striking for PHD3. Consistent with this hypothesis, physiological concentrations of cofactors such as ascorbate (25–50 μM) (Knowles et al. 2003) have been reported to be far below the K_m values of PHD for vitamin C (140–170 μM) (Hirsilä et al. 2003), suggesting significant alterations in PHD activity with changes in co-factor concentrations. Our understanding of the exact interplay of these factors in setting the sensitivity of the PHD/HIF system is still at the beginning, but without any doubt it is of crucial importance to understand the cell- and tissue-specific activity and response of the oxygen signaling cascade. Immunohistochemical staining of tissues for HIF-α subunits provide an indirect method to assess the activity of the PHD/HIF system in vivo. These studies have documented that HIF-α levels are generally low in rodent tissues under physiological conditions and are substantially increased in response to systemic hypoxia or tissue ischemia (Stroka et al. 2001; Wiesener et al. 2003). Interestingly, HIF-α levels remain low even in regions such as the renal medulla, which are characterized by low oxygen tensions known to enhance HIF-α protein in vitro. In addition, the extent and time course of induction as well as cell type-specific expression varies suggesting that indi-

vidual, cell-specific thresholds for activation of the response may exist.

11.4 HIF Activation in Tumors

A wide range of genes known to be involved in adaptive mechanisms to hypoxia, such as enzymes of glucose metabolism and pH regulation as well as angiogenic growth factors, have been classically associated with tumors. Many of these genes have subsequently been shown to be regulated by HIF function (see below). To date more than 60 putative HIF target genes have been identified, expression of which governs important processes such as angiogenesis and regulation of vascular tone, erythropoiesis, iron homeostasis, energy metabolism and pH regulation, as well as cell survival and proliferation (reviewed in Semenza 2003) (Fig. 11.2). A mounting body of evidence suggests that HIF activation by hypoxia and genetic alterations underlie the observed patterns of gene expression (reviewed in Acker and Plate 2002).

11.4.1 Tumor Hypoxia

Hypoxia is a common feature of solid tumor growth. Reduced PO_2 levels have been found in the majority of human tumors analyzed compared to normal tissue of the corresponding organ (Vaupel et al. 1989). Immunolabeling studies using monoclonal antibodies raised against the HIF-α subunit, which determines HIF activity, demonstrated increased HIF-1α expression in about 53% of malignant tumors, including colon, breast, gastric, lung, ovarian, pancreatic, prostate, and renal cell carcinomas (RCC), melanomas and glioblastomas, compared to the respective normal tissue (Zhong et al. 1999). Indirect experimental evidence for the induction of HIF activity by the hypoxic tumor environment came from studies showing perinecrotic expression patterns of HIF target genes or HRE-driven reporter genes (Damert et al. 1997; Plate et al. 1992). In the majority of tumors analyzed, intense HIF-α immunostaining was observed in perinecrotic tumor cells, suggesting regulation by microenvironmental tumor hypoxia. In contrast,

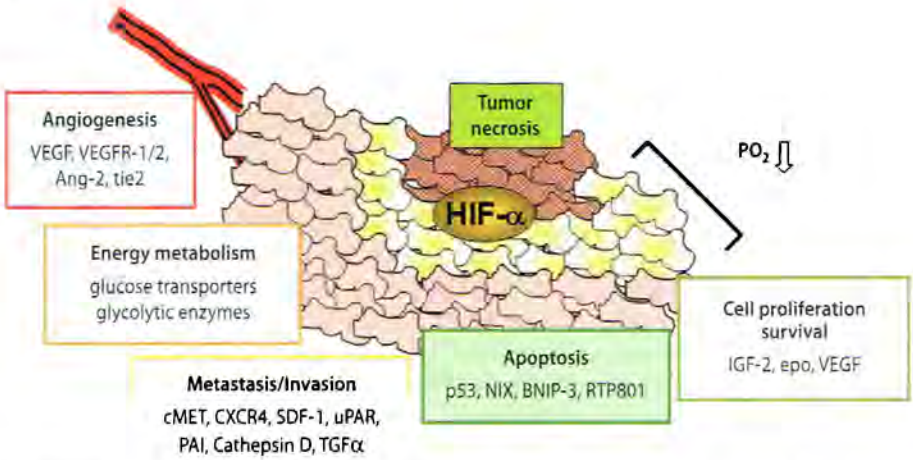


Fig. 11.2. Extended physiological responses governed by the HIF pathway. The HIF system acts as master regulator of physiological responses to hypoxia, initiating a cascade of mechanisms allowing the tumor to adapt to the hostile microenvironment. To date more than 60 putative HIF target genes have been identified. These include transactivation of genes mediating angiogenesis (VEGF), shift in energy metabolism from oxidative to glycolytic pathways (glucose transporters, glycolytic enzymes), pH regulation (CA IX) and cell survival and proliferation (IGF-2). In addition, the HIF pathway includes responses with adverse effects on cell function by inducing cell-cycle arrest-specific and pro-apoptotic proteins

HIF-α immunoreactivity in hemangioblastomas and RCC is rather homogeneous, consistent with a constitutive HIF activation being caused by VHL tumor suppressor gene inactivation (see below) (Krieg et al. 2000).

When tumors develop, they often become more malignant with time, a process termed tumor progression. HIF expression and activation seem to relate to tumor progression. In a mouse model of multi-step epidermal carcinogenesis, evolution of a more malignant phenotype was correlated with a progressive increase in HIF activation as shown by enhanced HIF-1α mRNA and HIF target gene expression (Elson et al. 2000). Overall, HIF expression is increased in more aggressive tumors, and it has been shown to correlate with tumor grade and tumor progression in a series of human brain tumors. While little or no HIF-α immunoreactivity could be detected in low-grade gliomas, glioblastomas revealed marked upregulation of HIF-1α in palisading cells adjacent to areas of necrosis (Zagzag et al. 2000). This finding correlates with previous studies showing significantly higher levels of VEGF (vascular endothelial growth factor) and increased vascularization in high-grade gliomas (Plate 1999). VEGF, as the major tumor angiogenesis and vascular permeability factor, supports glioma growth via a paracrine effect on endothelial cells (see below).

11.4.2 Genetic Alterations

Apart from microenvironmental tumor hypoxia, additional mechanisms have been identified which influence HIF function. HIF expression or activity is increased in response to genetic alterations, inactivating tumor suppressor genes or activating oncogenes, and in response to activation of various growth factor pathways.

11.4.2.1 VHL

About 100 years ago, von Hippel and Lindau were the first to describe an autosomal-dominant hereditary angiomatous syndrome, which was subsequently linked to VHL loss of function. VHL inactivation is further frequently detected in sporadic hemangioblastoma and RCC. The common hallmark of lesions associated with VHL loss of function is an angiogenic phenotype, which led to the suggestion that angiogenic factors such as VEGF are constitutively activated. Interestingly, lesions linked to the VHL syndrome occasionally overproduce erythropoietin (Epo), leading to polycythemia. Both VEGF and Epo are hypoxia-inducible genes which are regulated by HIF, connecting VHL function to HIF regulation. In support of this hypothesis, constitutive activation of the HIF system can be observed in RCC lacking a functional pVHL. Loss of VHL function leads to stabilization of HIF-α subunits already under normoxic conditions, while reintroduction of a functional VHL gene restores HIF regulation (Krieg et al. 2000; Maxwell et al. 1999). Importantly, HIF-α degradation involves a physical association between pVHL and HIF-α subunits. It was previously demonstrated that pVHL interacts with a number of proteins, namely elongin B, elongin C, Cul2 and Rbx1, forming a multimeric complex with similarities to the Skp1/Cdc53 F-Box class of ubiquitin ligases, which are classified as E3 ligases. Proteins are tagged for proteosomal-dependent destruction in cells by covalent binding of polyubiquitin chains. It was therefore suggested that pVHL acts as a substrate recognition module in an E3 ligase complex mediating HIF degradation under normoxic condition by targeting HIF for polyubiquitination. Several studies went on to show that the pVHL β-domain binds to the ODD of HIF-α subunits, which involves hydroxylation of specific prolyl residues, while the pVHL α-domain mediates interaction with elongin C. In conclusion, VHL loss of function would lead to a constitutive HIF activation by inhibiting ubiquitin-mediated proteosomal degradation, conferring a status of hypoxia mimicry to the cell.

11.4.2.2 SDH/FH

Little is known about the involvement of PHD in neoplasia. However, PHD may be a target of growth

factor signaling pathways and/or oncogenic transformation. In fact, it has been shown that certain oncogenes such as ras and src induce HIF under normoxia by inhibiting prolyl hydroxylation on Pro 564 (Chan et al. 2002). Recent findings, however, suggest a possible link between PHD function and tumorigenesis. The co-substrate 2-oxoglutarate as an intermediate of the Krebs cycle is generated in mitochondria. The two ubiquitously expressed mitochondrial enzymes, succinate dehydrogenase (SDH) and fumarate hydratase (FH), catalyze sequential steps in the Krebs cycle. Germline heterozygous mutations in the autosomally encoded enzyme and enzyme subunits are associated with hereditary predispositions to various tumors including paraganglioma, pheochromocytoma, benign smooth muscle cell tumor and RCC (Baysal et al. 2000; Tomlinson et al. 2002). Thus, both SDH and FH act as tumor suppressor genes. Interestingly, both succinate and fumarate, sequential metabolites of the mitochondrial Krebs cycle, have been shown to increase HIF-1 protein levels by PHD inhibition, indicating a direct link between PHD activity and tumorigenesis (Isaacs et al. 2005; Selak et al. 2005).

11.4.2.3 p53, PTEN

In addition, other tumor suppressor genes, including p53 and PTEN have been implicated in HIF regulation. p53 activity is induced in response to various stimuli, leading to cell cycle arrest or apoptosis. Loss of p53 function is a frequent event in tumorigenesis. Loss of p53 activity can result either from somatic mutations in the p53 gene locus or from functional inactivation, e.g. mediated by overexpression of MDM 2, a ubiquitin–protein ligase targeting p53 for proteosomal degradation. p53 has been implicated in promoting HIF degradation and decreasing transactivation of HRE-bearing genes, possibly by competing for the coactivator p300. Apparently, interaction between p53 and HIF-1α is physical, as shown by co-immunoprecipitation of both proteins. Loss of p53 activity in HCT116 human colon carcinoma cells is associated with increased HIF-1α expression and HIF-1 DNA-binding activity, as a consequence promoting tumor angiogenesis by enhancing levels of the angiogenic factor VEGF (Ravi et al. 2000). These studies suggest that apart from protecting the cell from hypoxia-mediated apoptosis, loss of p53 activity might also contribute to metabolic adaptation and angiogenesis by enhancing HIF-1 activity. PTEN is inactivated in a number of human tumors, including glioblastoma, breast cancer and prostate cancer. Induced expression of PTEN in glioblastoma or prostate cancer cells suppresses HIF accumulation under hypoxia and leads to attenuation of HIF transcriptional response, possibly by inhibiting signaling via the PI3 K(kinase)-AKT pathway (Zundel et al. 2000).

11.4.2.4 The PI3K-AKT-FRAP and the RAS-RAF-MEK-ERK Pathway

Several oncogenes have been shown to amplify the HIF pathway. V-SRC expression results in increased HIF-1α protein expression and transactivation of different HIF target genes (Jiang et al. 1997). Transformation of cells by V-SRC seems to involve two major intracellular signal transduction pathways, namely the PI3K-AKT-FRAP and the RAS-RAF-MEK-ERK pathway, the latter including p42/p44 MAP kinase. Both signaling cascades have been found to influence HIF activity, with the former inducing HIF-α protein expression and the latter HIF-α transcriptional activity. There seems to be some crosstalk between the two pathways, as H-RAS transformation appears to increase HIF-α levels under normoxia and hypoxia via the PI3 K signaling cascade (Semenza 2000).

Taken together, these studies have proven that increased HIF expression or activity within tumor cells is the result of different mechanisms such as microenvironmental hypoxia or multiple tumor growth-promoting genetic alterations. As a consequence, pathways are activated which help the tumor to adapt to the hypoxic and proliferative microenvironment, acting in concert with the selective advantage conferred by these mutations.

Tumor Angiogenesis and Hypoxia

In 1971, Folkman proposed that solid tumor growth is angiogenesis dependent. It is now widely accepted that tumors and metastases need to acquire a functional blood supply to grow beyond a volume of several cubic millimeters. Thus, absence of angiogenesis can be considered as rate-limiting for tumor growth (Carmeliet and Jain 2000). The angiogenic switch occurs when the balance between pro-angiogenic and anti-angiogenic molecules is shifted in favor of angiogenesis, permitting rapid tumor growth and subsequent development of invasive and metastatic properties, characteristics that define the lethal cancer phenotype (Hanahan and Folkman 1996). Indeed, a statistically significant correlation between vascular density as a parameter for tumor angiogenesis and patient survival has been established for a variety of tumors.

11.5.1 Molecular Regulators of Angiogenesis

Various molecular players have been identified which are involved in orchestrating specific stages and mechanisms of vascular growth in response to developmental, physiological and oncogenic stimuli. Among these, members of the VEGF and the angiopoietin (Ang) family seem to have a predominant role (Carmeliet 2000; Yancopoulos et al. 2000). Other factors that can act as inducers or modulators of angiogenesis include acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), transforming growth factor alpha and beta (TGF-α and -β), tumor necrosis factor alpha (TNF) and interleukin-8 (IL-8). In addition, factors have been reported that function as naturally occurring inhibitors of angiogenesis, like angiostatin or endostatin, derived from proteolytic fragments of larger proteins such as plasminogen or collagen type XVIII, respectively (Jansen et al. 2004).

11.5.1.1 The VEGF Family

VEGF and its tyrosine kinase receptors VEGFR-1 (flt-1) and VEGFR-2 (flk-1/KDR) are major regulators of vasculogenesis and angiogenesis. Gene targeting studies suggest that signaling via VEGFR-2 mediates vascular permeability and endothelial cell (EC) growth, while VEGFR-1 plays a negative role by either acting as a decoy receptor or suppressing signaling through VEGFR-2. However, recent studies imply a positive regulatory role of VEGFR-1 in pathological angiogenesis in vivo (see below). VEGF is an EC-specific mitogen with vascular permeability inducing properties in vivo (Keck et al. 1989). Interestingly, a fundamental link between microenvironmental tumor hypoxia and induction of angiogenesis could be established by several studies showing that VEGF expression is regulated by oxygen levels. VEGF is highly expressed in perinecrotic palisading cells but is downregulated in tumor cells adjacent to vessels, suggesting oxygen-dependent gene expression (Plate et al. 1992; Shweiki et al. 1992). VEGF expression under hypoxia is subject to multi-level regulation. The hypoxia-mediated response seems to depend on regulatory sequences in the 5' and 3' regions of the VEGF gene. It has been shown that (1) the 5' HRE binding site for HIF is necessary for the hypoxic transactivation and (2) mRNA stabilization sites in the 3' UTR of the VEGF gene restrict hypoxic gene expression to the perinecrotic palisading cells in situ. mRNA stabilization seems to involve RNA–protein complexes in the 3' UTR as formed by HuR, an RNA-binding protein, or hnRNP (heterogeneous nuclear ribonucleoprotein) L. In addition, VEGF expression is regulated on the translational level by a functional IRES (internal ribosomal entry site) in the 5' UTR which allows for efficient, cap-independent translation even under hypoxia. Further, VEGF protein export and secretion seems to be controlled by oxygen tension.

VEGF, secreted by the hypoxic tumor cell compartment, is distributed throughout the tumor by diffusion generating a gradient. The diffusion capacity differs with the different splice variants of VEGF, depending on their heparin-binding affinity, which

determines their adherence to the extracellular matrix. VEGF binding to its receptors (VEGFR) specifically expressed by EC, enhances endothelial VEGFR expression in an autocatalytic fashion. VEGFR signaling leads to a cascade of events, including EC migration and proliferation as well as induction of fenestrae and vascular permeability in tumor vessels. The importance of the VEGF family in regulating tumor angiogenesis, tumor growth and progression was verified by several reports showing that inhibition of VEGF/VEGFR-2 signaling by VEGF-neutralizing antibodies, low-molecular VEGFR-2 inhibitors or gene transfer of VEGFR-2 dominant-negative constructs led to stunted tumor growth with reduced vascularization. In an elegant approach using the well-established RIP1-Tag2 mouse model of multi-step tumorigenesis of pancreatic islet carcinoma, islet-specific VEGF deletion by means of the Cre-lox system diminished angiogenic switching and decreased tumor growth and progression (Inoue et al. 2002). The history of the identification of "VEGF and the quest for tumor angiogenesis factors" has been excellently depicted in a review by N. Ferrara (Ferrara 2002).

PlGF (placenta growth factor), another member of the VEGF family, which specifically binds to VEGFR-1, has been shown to exert important, possibly synergistic functions with VEGF in increasing tumor vascularization (Carmeliet et al. 2001). These findings have established a positive regulatory role of the VEGFR-1 signaling pathway in tumor angiogenesis. However, much of the biological function of the VEGFR-1 ligand system may lie in the regulation and recruitment of hematopoietic and inflammatory cells to the tumor site rather than acting directly on EC. Although a HIF-binding site (HRE) has not yet been identified in the PlGF gene, several reports indicate oxygen-dependent PlGF expression in certain cell types. Thus, tumor hypoxia may induce VEGFR-1 signaling synergistically by VEGF and PlGF upregulation.

11.5.1.2 The Angiopoietin/Tie Family

Angiopoietins, in particular Ang-1 and the naturally occurring antagonist Ang-2, are implicated in later stages of vascular development, i.e. during vascular remodeling and maturation (Yancopoulos et al. 2000). In adult animals, Ang-2 induction is demonstrated in EC undergoing active remodeling. Hence, it was proposed that Ang-2 induced in the vascular endothelium blocked the constitutive stabilizing influence exerted by Ang-1. This would allow the EC to revert to a more plastic and unstable state. VEGF and hypoxia have been reported to increase Ang-2 expression in EC in vitro. The observation that Ang-2 expression in tumor EC can be seen in close vicinity to VEGF-expressing tumor cells adjacent to areas of necrosis suggests that similar mechanisms take place in vivo (Stratmann et al. 2001). Moreover, tumor vessels are structurally and functionally abnormal, with excessive branching, shunts and leakiness resulting in regional heterogeneity in tumor perfusion (Carmeliet 2000). As a consequence tumor blood flow is chaotic, leading to severely hypoxic regions within the tumor, so that even EC of tumor vessels are subject to hypoxia (Helmlinger et al. 1997). Acting in concert, EC hypoxia and VEGF-mediated upregulation of Ang-2 would render EC more accessible to angiogenic inducers such as VEGF, resulting in a strong angiogenic response (Maisonpierre et al. 1997). In addition, angiopoietins have been implicated in vascular permeability. In particular Ang-1 could counter the permeability-inducing effects of VEGF. In return, its antagonist Ang-2 potentiated the VEGF-mediated increase in vascular permeability.

Taken together, these observations support the view that hypoxia and HIF are key regulators of blood vessel growth, inducing upregulation of both pivotal angiogenic ligands and their cognate receptors. Tumor hypoxia and HIF-mediated upregulation of VEGF and Ang-2 is most likely responsible for the two major obstacles to effective cancer treatment: tumor angiogenesis and edema.

11.5.2 Cellular Regulators of Angiogenesis

Apart from endothelial and perivascular cells, tumors attract a number of cell types which syner-

gistically act to augment tumor vascularization, including inflammatory/ hematopoietic cells and circulating endothelial precursor cells (CEP). Current studies indicate that tumor hypoxia not only indirectly influences these cell types by tumor cell-specific upregulation of various secreted, paracrine-acting factors but may in addition have direct cell-intrinsic effects (reviewed in Acker and Plate 2003).

11.5.2.1 Endothelial Cells

Hypoxia-mediated induction of angiogenesis is thought to be mainly conferred by transactivation of VEGF in surrounding cells, thus acting in a paracrine fashion on EC (extrinsic pathway). However, exposure of EC to intermittent and chronic hypoxic conditions has been shown to occur in vivo as a result of the structurally and functionally abnormal tumor vasculature (Helmlinger et al. 1997). Recent studies suggest that hypoxia may operate as an intrinsic regulator of EC growth and function by stimulating receptor and ligand expression. HIF-α subunits have been reported to induce VEGFR-1, VEGFR-2 and tie2. Stabilization of HIF-1α by the peptide regulator (PR) 39 in different EC lines resulted in VEGF upregulation and accelerated formation of vascular structures. Moreover, in vivo expression of PR39 targeted to the myocardium increased myocardial vascularization, though it was not clear from that study how relevant HIF pathway activation in EC was in comparison with activation in the surrounding tissue (Li et al. 2000). Interestingly, hypoxic VEGF induction in EC was also shown to promote network formation in vitro (Helmlinger et al. 2000). Thus, hypoxia-driven autocrine stimulation of EC may enhance the angiogenic pathway and participate in the formation and reorganization of the vascular network in solid tumors. This was recently confirmed by demonstrating that EC-specific loss of HIF-1 resulted in reduced tumor growth concomitantly with decreased tumor vascularization (Tang et al. 2004).

Apart from their metabolic function EC may provide inductive signals important for tumor development. A number of studies suggest that blood vessels, independent of their nourishing function, stimulate organ development and differentiation, as shown for pancreas and liver formation, and enhance tumor proliferation. Thus, signaling between tumor cells and EC might indeed be bidirectional, with tumor cells promoting blood vessel growth and EC giving tumor cell-specific differentiation and proliferation cues.

11.5.2.2 Inflammatory/Hematopoietic Cells

When in the early 1970s Folkman put forward the hypothesis that tumors induce blood vessel ingrowth by secreting diffusible angiogenic factors, his concept was much criticized as common belief stated that tumor angiogenesis was directed by the inflammatory host response. His work inspired many researchers, leading to the discovery of VEGF and its regulation by tumor hypoxia. Several decades later a mounting body of evidence suggests that tumors may indeed exploit the host-defense mechanism and attract inflammatory cells to further enhance vascularization. Interestingly, both concepts may finally be reconciled with the observation that tumor- and host cell-directed vascularization apparently makes use of similar mechanisms with analogous functions of the VEGF family and tumor hypoxia.

Induction of angiogenesis is known as a hallmark of various chronic inflammatory disorders such as rheumatoid arthritis and psoriasis. Tumors produce various cytokines and chemokines that attract inflammatory/hematopoietic cells (Rafii et al. 2002). Hematopoietic stem cells are pluripotent cells with the capacity for self-renewal and differentiation into specific lineages; for instance, when differentiating along the myeloid lineage they give rise to neutrophils and macrophages. Immune surveillance has long been viewed as keeping tumor growth at bay. However, the inflammatory cell component may have a dual role in tumors. Apart from killing neoplastic cells, leukocytes such as tumor-associated macrophages (TAM) produce an array of potent angiogenic and lymphangiogenic growth

factors, cytokines and extracellular proteases which potentiate tumor growth and progression (Carmeliet and Jain 2000). Failure to recruit TAM has been demonstrated to significantly attenuate tumor progression and metastasis (Lin et al. 2001), partly due to impaired angiogenesis. Hypoxia and the HIF transcriptional system may play an important role in these processes. It has been shown using transgenic mice expressing the green fluorescent protein under the control of the human VEGF promoter that the tumor environment is capable of inducing this HIF-responsive promoter in stromal cells of host origin (Fukumura et al. 1998). Moreover, hypoxia seems to reduce macrophage migration, potentially leading to a preferential accumulation of macrophages in hypoxic tumor regions. In line with these observations, conditional gene targeting of HIF-1α in myeloid cells blunted the inflammatory cell response by attenuating macrophage/neutrophil invasion and migration (Cramer et al. 2003). Several reports have further underlined the importance of several HIF target genes such as VEGF, PlGF and IL-8 either in the direct regulation or by promoting the release of additional factors such as MMP-9 and s-KitL in the indirect regulation of hematopoietic cell recruitment and function.

11.5.2.3 CEP

In addition to angiogenic sprouting and co-option of pre-existing vessels (Holash et al. 1999), new evidence indicates that tumor vascularization is enhanced by the mobilization and incorporation of circulating endothelial progenitor cells (CEP). CEP are bone-marrow-derived cells with a high proliferation potential. Depending on the experimental system, their contribution to tumor vessels ranges from a few to up to 90% of all vessels (Rafii et al. 2002). The VEGF family, in line with its pivotal role in tumor angiogenesis, has also been implicated in the mobilization of CEP. While signaling in hematopoietic and inflammatory cells seems to be mediated mainly via the VEGFR-1/ligand system, VEGFR-2 signaling constitutes the prevailing pathway in CEP to induce recruitment and proliferation. Interestingly, in tumors hematopoietic cells and CEP are found in close association, suggesting crosstalk between these two cell types. It remains to be determined whether hypoxia and the HIF pathway exert similar direct effects on CEP.

Tumor Physiology and Hypoxia

11.6.1 HIF and the Warburg Effect

More than 70 years ago Otto Warburg demonstrated a marked turnover of the glycolytic pathway in tumor cells even when cultured under normoxic conditions. Induction of aerobic, in contrast to anaerobic glycolysis came to be known as the Warburg effect. Metabolic adaptation of the tumor cell to intratumoral hypoxia includes switching from oxidative to glycolytic pathways. Hypoxia-responsive elements have been identified in a number of genes involved in glycolysis (Semenza 2000). In addition, expression studies in HIF-1α-deficient embryonic stem cells revealed decreased expression of 13 different genes encoding glucose transporters and glycolytic enzymes. Fructose-2,6-bisphosphate, an allosteric activator of 6-phosphofructo-1-kinase, regulating glycolytic flux, has been shown to be induced by HIF-α (Minchenko et al. 2002). Thus, HIF mediates coordinate upregulation of genes of the glycolytic pathway ranging from glucose uptake to lactate production. In line with these findings, glycolytic metabolism as measured by lactate concentration correlates with disease progression and metastasis. Though a tempting candidate, to date HIF function has not been directly linked to the Warburg effect. However, glycolytic end products such as lactate and pyruvate, which accumulate as a consequence of the Warburg effect, have been shown to further promote HIF-α stability and HIF-α-dependent gene expression (Lu et al. 2002).

Cellular proliferation inevitably increases oxygen demand. It has been proposed that the switch from oxygen-consuming to glycolytic pathways might be hardwired to the program of cellular proliferation. This would provide the cells with an alternative energy source during increased oxygen consumption and would, in addition, reduce the generation of deleterious reactive oxygen species. Indeed, HIF-1α levels are elevated in the presence of various growth factors such as angiotensin II, PDGF and thrombin in vascular smooth muscle cells and in tumor cells cultured under conditions associated with cellular proliferation. In an experimental model of epidermal wounding, HIF activation closely correlates with cellular proliferation of keratinocytes in vivo (Elson et al. 2000).

Apart from hypoxia, tumors are typically characterized by an acidic extracellular microenvironment (Helmlinger et al. 1997). Lactate production by glycolysis is thought to be the major source of protons within the tumor. Tumor pH homeostasis is tightly controlled by various proton extrusion mechanisms. Among these, the tumor-associated transmembrane carbonic anhydrases (CA) 9 and 12 provide a potential link between metabolism and pH regulation. They catalyze the reversible hydration of carbon dioxide to bicarbonate and protons. It has been proposed that this mechanism may contribute to intracellular pH homeostasis and further aggravate extracellular acidosis as bicarbonate is exchanged for intracellular chloride. Interestingly, CA9 and CA12 have been identified as a new class of HIF-α-regulated genes (Wykoff et al. 2000). In support of this finding, upregulation of CA9 and CA12 is observed in a variety of tumors by hypoxia and VHL loss of function in vitro and in vivo. Taken together, these findings suggest that HIF controls fundamental metabolic changes which are favorable for tumor growth by inducing a shift from oxidative to glycolytic pathways, while at the same time providing means to deal with the resulting increase in proton load by upregulation of CA.

11.6.2 HIF and Tumor Progression

Genetic instability leading to mutations is a hallmark of malignancy (Hanahan and Weinberg 2000). Accumulative acquisition of specific genetic alterations is implicated in tumor progression. Glioblastomas, for example, arise either de novo (primary glioblastoma) or by progression from a low-grade astrocytoma (secondary glioblastoma). Epidermal growth factor receptor overexpression and p53 mutations have been specifically associated with primary and secondary glioblastomas, respectively. Interestingly, glioma progression in vivo is associated with clonal expansion of p53 mutant cells. With these findings in mind, it is interesting to consider that genetic alterations found in tumor cells are not necessarily primary events, but may themselves be a consequence of tumor hypoxia. An elevated mutation frequency in hypoxic compared to normoxic tumor cells has been reported. The authors conclude that "the microenvironment of an incipient developing tumor might itself contribute to genomic instability and mutagenesis, leading to tumor progression and an evolution of the malignant phenotype" (Reynolds et al. 1996). A diminished DNA repair capacity reported under hypoxic conditions may underlie this phenomenon. Recently it was shown that HIF represses expression of the DNA-repair enzymes MSH2 and MSH6, providing a molecular link between tumor hypoxia and the pro-mutagenic tumor microenvironment (Koshiji et al. 2005).

Hypoxia may not only induce mutations in tumor cells, but may select for malignant cell clones with increased resistance to hypoxia-mediated apoptosis. Subjecting cells to hypoxia has been shown to induce p53 activity and apoptosis (Graeber et al. 1994). Hypoxia-induced acidosis has been implicated in this process. When p53-deficient and p53 wild-type (wt) cells were mixed in vitro, several rounds of hypoxia led to an accumulation of p53-deficient cells. In vivo, highly apoptotic regions overlap with hypoxic areas in wt p53 tumors, while only little apoptosis in hypoxic areas of p53-deficient tumors is reported (Graeber et al. 1996). A potential mechanism for HIF

involvement in hypoxia-induced p53 accumulation was proposed by a study demonstrating a physical and functional interaction of p53 with HIF-1α, with the binding of HIF-1α to p53 protecting the latter from proteosomal degradation (An et al. 1998). This apparently involves a dephosphorylated form of HIF-1α. In line with these findings, one study demonstrated that apoptosis in ES cells in response to hypoxia or hypoglycemia involved HIF-1α-mediated upregulation of p53 and downregulation of Bcl-2 (Carmeliet et al. 1998). In contrast, HIF-2α seems to be rather specifically involved in mediating hypoglycemia-induced apoptosis (Brusselmans et al. 2001). Interestingly, in mixed tumors composed of HIF-1α-deficient and HIF-1α wt ES cells, HIF-1α deficient cells preferentially accumulated at distance from existing blood vessels (Yu et al. 2001). In striking concordance, a study analyzing mixed tumors composed of p53-deficient and p53 wt HCT116 cells revealed clustering of p53-deficient cells in hypoxic regions (Yu et al. 2002). These findings suggest that tumor cell apoptosis induced in hypoxic/hypoglycemic areas, possibly mediated by HIF activation, leads to selection of cell clones which have lost p53 activity and are less vulnerable to low oxygen tension.

11.6.3 HIF and Tumor Invasion

The clinical correlation between intratumoral hypoxia and tumor aggressiveness which is in particular characterized by tumor invasion and the ability to metastasize has found a possible molecular correlate in recent studies. Thus, crucial steps in these processes, including cell mobility and migration, tissue invasion and the ability to home into specific organ sites, are governed by specific signaling pathways known to contain HIF target genes such as c-Met, the receptor for HGF (scatter factor/ hepatocyte growth factor), or the chemokine receptor CXCR4. Both receptor systems have well-established functions in tumor invasion and metastasis, as shown for example in breast cancer (Muller et al. 2001) and gliomas (reviewed in Lamszus et al. 1999). In addition, a number of factors known to determine the invasive cancer phenotype, such as cathepsin D, matrix metalloproteinase 2 and urokinase plasminogen activator receptor (uPAR), have been shown to be regulated by HIF. Indeed, inhibition of the HIF pathway by geldanamycin was shown to diminish glioma cell migration in vitro (Zagzag et al. 2003). Interestingly, though a growing body of evidence suggests that HIF-1α is the major ortholog to convey hypoxia-induced gene expression, both orthologs, HIF-1α and HIF-2α, seem to be required for hypoxia-induced cell migration, as shown by siRNA-mediated knockdown of each ortholog in breast cancer cell lines (Sowter et al. 2003). Thus, low PO_2 and activation of the HIF system cause the tumor cell to migrate away from hypoxic areas and invade further into the host tissue and organs, thus supporting tumor spread. It is interesting to note that in highly invasive glioblastoma multiforme, the most malignant and particularly hypoxic brain tumor entity, single tumor cells with high-level HIF expression have been detected at the leading tumor invasion front (Zagzag et al. 2000).

Implications for Tumor Therapy

Tumor growth and progression occurs as a result of clonal selection of cells within the tumor cell population with mutations in key tumor suppressor genes or oncogenes which confer a survival advantage within a hostile tumor environment (Hanahan and Weinberg 2000). These changes contribute to a microenvironment, characterized by low oxygen tensions, low glucose and an acidic extracellular pH, which by itself further increases genetic instability. Tumor hypoxia has several deleterious effects for patients. Hypoxia correlates with tumor malignancy, frequency of invasion and metastasis, and thus with poor patient prognosis. In addition, intratumoral hypoxia has been associated with increased resistance to radio- and chemotherapy (Jain 2001). HIF activation is commonly observed in human tumors and their metastases. As outlined, this can be partly

attributed to tumor hypoxia. In addition, mutations leading to tumor suppressor gene inactivation or oncogene activation as well as activation of various growth factor pathways lead to increased HIF activity. Thus, physiological and genetic alterations act synergistically to enhance HIF activation. As a consequence, HIF activity progressively increases with tumor progression.

The HIF system acts as master regulator of physiological responses to hypoxia, initiating a cascade of mechanisms allowing the tumor to adapt to the hostile microenvironment (Acker and Plate 2002; Semenza 2000). These include transactivation of genes mediating angiogenesis (VEGF), shift in energy metabolism from oxidative to glycolytic pathways (glucose transporters, glycolytic enzymes), pH regulation (CA IX) and cell survival and proliferation (IGF-2) (Fig. 11.2). However, clonal selection of a particular advantageous component lying downstream of HIF activation may co-select for other components that are linked through a common physiological pathway, the HIF system. These may have properties with a positive, neutral or even negative influence on the growth of the emerging cell clone. Indeed, apart from inducing pro-proliferative proteins, such as IGF (insulin-like growth factor)-2, IGF-BP (binding proteins) 1–3 and TGF (transforming growth factor) β3, the HIF pathway includes responses with adverse effects on cell function by inducing cell-cycle arrest-specific and pro-apoptotic proteins such as DEC (defective chorion)-1, BNIP (Bcl2/adenovirus E1B 19kDa-interacting protein)-3, its ortholog NIX (Nip3-like protein X) and cyclin G2. In addition, direct stabilization of the pro-apoptotic protein p53 has been suggested by studies demonstrating physical and functional interaction between HIF-1α and p53 (reviewed in Acker and Plate 2002). Thus, the HIF system transactivates an extended physiological pathway which encompasses a wide array of physiological responses to hypoxia, ranging from mechanisms which increase cell survival to those inducing cell cycle arrest or even apoptosis. Though activation of the entire pathway by widespread HIF activation in tumors seems to confer an overall growth advantage to the individual cell, single components may have opposing functions on tumor growth. However, tumors may counteract these cell death responses by selection of cells with specific genetic alterations, exploiting HIF-mediated survival mechanisms for their growth and progression. In this context, hypoxia-mediated upregulation of an antisense HIF-1α, the inhibitory PAS protein IPAS, PHD2/3 or activation of glycogen synthase kinase 3 (GSK3), resulting in reduced hypoxic HIF-α accumulation, may be viewed as a preventative and self-limiting procedure by the cell to keep HIF-α levels and function from reaching toxic concentrations (reviewed in Acker and Acker 2004).

The recent insight into the precise mechanisms of oxygen sensing and signaling may further help to develop novel strategies to specifically target the HPHD-HIF-VHL pathway in tumor cells at different levels, namely interfering with hypoxia-inducible gene function, inhibiting HIF-mediated transactivation or preventing HIF stabilization. Given the widespread HIF activation in tumors, the role of HIF in transactivating angiogenic factors and the role of angiogenic factors in tumor growth, interfering with the HIF system is particularly appealing. The rationale of this approach lies in depriving the tumor cell of oxygen and nutrients by inhibiting angiogenesis while at the same time disabling adaptive mechanisms that help the cell to survive in this microenvironment. However, as described, the HIF system may transactivate an extended physiological pathway with individual components having pro- or anti-tumorigenic effects. Based on the relative influence each component has on overall cell growth, general manipulation of the HIF system is likely to show variable outcome.

Indeed, several studies have revealed conflicting data with regard to the effect of HIF manipulation on tumor growth. Some reported reduction in tumor growth and angiogenesis by HIF inhibition using, for example, HIF-1α-deficient ES cells (Ryan et al. 1998); a peptide blocking the interaction of the HIF-1α carboxyl terminus with the transcriptional coactivator p300 (Kung et al. 2000); the histone deacetylase inhibitor trichostatin A (Kim et al. 2001); or PI3 K pathway inhibitors such as LY294002 (Blancher et al. 2001). In contrast, one group demonstrated accelerated growth of HIF-1α-defi-

cient ES cells due to a decrease in hypoxia-mediated apoptosis, though angiogenesis was also reduced (Carmeliet et al. 1998). Similarly, others described a negative influence on tumor growth by constitutive HIF-2α activation in breast cancer cell lines (Blancher et al. 2000). Interestingly, clinical studies correlating HIF-1α expression with patient prognosis have supported this variable outcome. One, for instance, positively correlated increased HIF-1α expression with a poor prognosis in patients with cervical cancer (Birner et al. 2000), while another study reported a better survival rate for patients with non-small cell lung cancer with HIF-1α-positive than -negative tumors (Volm and Koomagi 2000). A recent study (Acker et al. 2005) provided new evidence indicating that under certain conditions HIF may a have tumor-suppressive role, suggesting a dual function of HIF in tumor biology (reviewed in Acker and Acker 2004) (Fig. 11.3). Though decreasing tumor vascularization, HIF inhibition using a dominant-negative HIF transgene in gliomas or HIF-2-deficient teratomas accelerated tumor growth, partly due to a decrease in hypoxia-induced tumor apoptosis. Moreover, HIF-1 crucially determines tumor responsiveness to radiotherapy (Moeller et al. 2005). By promoting ATP metabolism, proliferation and p53 activation, HIF-1 has a radiosensitizing effect on tumors. In contrast, through stimulating endothelial cell survival, HIF-1 promotes tumor radioresistance.

Thus, in terms of both tumor physiology and tumor responsiveness to therapeutic intervention, the impact of HIF on tumor growth is complex and variable. For this reason, employing HIF inhibitors in cancer treatment requires careful consideration and characterization of pro- and anti-tumorigenic func-

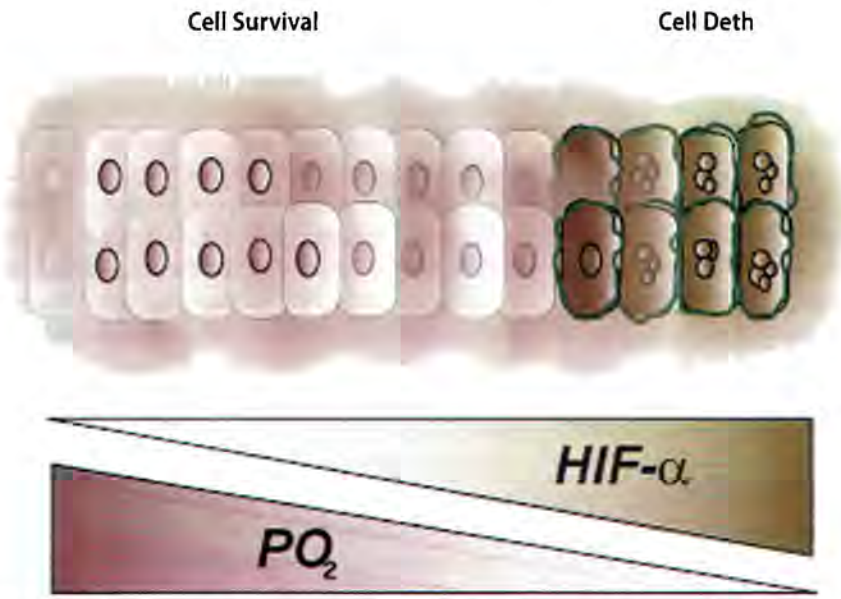


Fig. 11.3. Dual role of HIF in governing cell survival and death. The HIF system transactivates an extended physiological pathway which encompasses a wide array of physiological responses to hypoxia, ranging from mechanisms which increase cell survival to those inducing cell cycle arrest or even apoptosis. Current data suggest a model in which decreasing oxygen concentrations translate into concomitant changes in HIF-α protein levels and modifications leading to exponentially increasing HIF transactivation activity. When reaching a cell-specific threshold, HIF activity induces a qualitative switch in the cellular response from cell survival to cell death mechanisms

tions of the oxygen-sensing pathway. From a therapeutic point of view, intervention in the PHD/HIF pathway is a double-edged sword. The solution may lie in a balanced and fine-tuned manipulation of the system, as it holds true for so many matters in life.

References

Acker T, Acker H (2004) Cellular oxygen sensing need in CNS function: physiological and pathological implications. J Exp Biol 207:3171–3188

Acker T, Plate KH (2002) A role for hypoxia and hypoxia-inducible transcription factors in tumor physiology. J Mol Med 80:562–575

Acker T, Plate KH (2003) Role of hypoxia in tumor angiogenesis – molecular and cellular angiogenic crosstalk. Cell Tissue Res. 80:562–575

Acker T, Diez-Juan A, Aragones J, Tjwa M, Brusselmans K, Moons L, Fukumura D, Moreno-Murciano MP, Herbert JM, Burger A, Riedel J, Elvert G, Flamme I, Maxwell PH, Collen D, Dewerchin M, Jain RK, Plate KH, Carmeliet P (2005) Genetic evidence for a tumor suppressor role of HIF-2alpha. Cancer Cell 8:131–141

An WG, Kanekal M, Simon MC, Maltepe E, Blagosklonny MV, Neckers LM (1998) Stabilization of wild-type p53 by hypoxia-inducible factor 1alpha. Nature 392:405–408

Baysal BE, Ferrell RE, Willett-Brozick JE, Lawrence EC, Myssiorek D, Bosch A, van der MA, Taschner PE, Rubinstein WS, Myers EN, Richard CW, III, Cornelisse CJ, Devilee P, Devlin B (2000) Mutations in SDHD, a mitochondrial complex II gene, in hereditary paraganglioma. Science 287:848–851

Bilton RL, Booker GW (2003) The subtle side to hypoxia inducible factor (HIFalpha) regulation. Eur J Biochem 270:791–798

Birner P, Schindl M, Obermair A, Plank C, Breitenecker G, Oberhuber G (2000) Overexpression of hypoxia-inducible factor 1alpha is a marker for an unfavorable prognosis in early-stage invasive cervical cancer. Cancer Res 60:4693–4696

Blancher C, Moore JW, Talks KL, Houlbrook S, Harris AL (2000) Relationship of hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha expression to vascular endothelial growth factor induction and hypoxia survival in human breast cancer cell lines. Cancer Res 60:7106–7113

Blancher C, Moore JW, Robertson N, Harris AL (2001) Effects of ras and von Hippel-Lindau (VHL) gene mutations on hypoxia- inducible factor (HIF)-1alpha, HIF-2alpha, and vascular endothelial growth factor expression and their regulation by the phosphatidylinositol 3'-kinase/Akt signaling pathway. Cancer Res 61:7349–7355

Brusselmans K, Bono F, Maxwell P, Dor Y, Dewerchin M, Collen D, Herbert JM, Carmeliet P (2001) Hypoxia-inducible factor-2alpha (HIF-2alpha) is involved in the apoptotic response to hypoglycemia but not to hypoxia. J Biol Chem 276:39192–39196

Carmeliet P (2000) Mechanisms of angiogenesis and arteriogenesis. Nat Med 6:389–395

Carmeliet P, Jain RK (2000) Angiogenesis in cancer and other diseases. Nature 407:249–257

Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, Koch CJ, Ratcliffe P, Moons L, Jain RK, Collen D, Keshert E, Keshet E (1998) Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature 394:485–490

Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De Mol M, Wu Y, Bono F, Devy L, Beck H, Scholz D, Acker T, DiPalma T, Dewerchin M, Noel A, Stalmans I, Barra A, Blacher S, Vandendriessche T, Ponten A, Eriksson U, Plate KH, Foidart JM, Schaper W, Charnock-Jones DS, Hicklin DJ, Herbert JM, Collen D, Persico MG (2001) Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat Med 7:575–583

Chan DA, Sutphin PD, Denko NC, Giaccia AJ (2002) Role of prolyl hydroxylation in oncogenically stabilized hypoxia-inducible factor-1alpha. J Biol Chem 277:40112–40117

Cramer T, Yamanishi Y, Clausen BE, Forster I, Pawlinski R, Mackman N, Haase VH, Jaenisch R, Corr M, Nizet V, Firestein GS, Gerber HP, Ferrara N, Johnson RS (2003) HIF-1alpha is essential for myeloid cell-mediated inflammation. Cell 112:645–657

Damert A, Machein M, Breier G, Fujita MQ, Hanahan D, Risau W, Plate KH (1997) Up-regulation of vascular endothelial growth factor expression in a rat glioma is conferred by two distinct hypoxia-driven mechanisms. Cancer Res 57:3860–3864

Elson DA, Ryan HE, Snow JW, Johnson R, Arbeit JM (2000) Coordinate up-regulation of hypoxia inducible factor (HIF)-1alpha and HIF-1 target genes during multi-stage epidermal carcinogenesis and wound healing. Cancer Res 60:6189–6195

Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A, Tian YM, Masson N, Hamilton DL, Jaakkola P, Barstead R, Hodgkin J, Maxwell PH, Pugh CW, Schofield CJ, Ratcliffe PJ (2001) C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell 107:43–54

Ferrara N (2002) VEGF and the quest for tumour angiogenesis factors. Nat Rev Cancer 2:795–803

Fukumura D, Xavier R, Sugiura T, Chen Y, Park EC, Lu N, Selig M, Nielsen G, Taksir T, Jain RK, Seed B (1998) Tumor induction of VEGF promoter activity in stromal cells. Cell 94:715–725

Graeber TG, Peterson JF, Tsai M, Monica K, Fornace AJ Jr, Giaccia AJ (1994) Hypoxia induces accumulation of p53 protein, but activation of a G1-phase checkpoint by low-oxygen conditions is independent of p53 status. Mol Cell Biol 14:6264–6277

Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW, Giaccia AJ (1996) Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature 379:88–91

Hanahan D, Folkman J (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 86:353–364

Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100:57–70

Helmlinger G, Yuan F, Dellian M, and Jain RK (1997) Interstitial pH and pO2 gradients in solid tumors in vivo: high-resolution measurements reveal a lack of correlation. Nat Med 3:177–182

Helmlinger G, Endo M, Ferrara N, Hlatky L, Jain RK (2000) Formation of endothelial cell networks. Nature 405:139–141

Hirsilä M, Koivunen P, Gunzler V, Kivirikko KI, Myllyharju J (2003) Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor HIF. J Biol Chem 278:30772–30780

Holash J, Maisonpierre PC, Compton D, Boland P, Alexander CR, Zagzag D, Yancopoulos GD, Wiegand SJ (1999) Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. Science 284:1994–1998

Inoue M, Hager JH, Ferrara N, Gerber HP, Hanahan D (2002) VEGF-A has a critical, nonredundant role in angiogenic switching and pancreatic beta cell carcinogenesis. Cancer Cell 1:193–202

Isaacs JS, Jung YJ, Mimnaugh EG, Martinez A, Cuttitta F, Neckers LM (2002) Hsp90 regulates a von Hippel Lindau-independent hypoxia-inducible factor-1 alpha-degradative pathway. J Biol Chem 277:29936–29944

Isaacs JS, Jung YJ, Mole DR, Lee S, Torres-Cabala C, Chung YL, Merino M, Trepel J, Zbar B, Toro J, Ratcliffe PJ, Linehan WM, and Neckers L (2005) HIF overexpression correlates with biallelic loss of fumarate hydratase in renal cancer: novel role of fumarate in regulation of HIF stability. Cancer Cell 8:143–153

Jain RK (2001) Normalizing tumor vasculature with anti-angiogenic therapy: a new paradigm for combination therapy. Nat Med 7:987–989

Jansen M, Witt Hamer PC, Witmer AN, Troost D, van Noorden CJ (2004) Current perspectives on antiangiogenesis strategies in the treatment of malignant gliomas. Brain Res Brain Res Rev 45:143–163

Jeong JW, Bae MK, Ahn MY, Kim SH, Sohn TK, Bae MH, Yoo MA, Song EJ, Lee KJ, Kim KW (2002) Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation. Cell 111:709–720

Jiang BH, Agani F, Passaniti A, Semenza GL (1997) V-SRC induces expression of hypoxia-inducible factor 1 (HIF-1) and transcription of genes encoding vascular endothelial growth factor and enolase 1: involvement of HIF-1 in tumor progression. Cancer Res 57:5328–5335

Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, Connolly DT (1989) Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science 246:1309–1312

Kim MS, Kwon HJ, Lee YM, Baek JH, Jang JE, Lee SW, Moon EJ, Kim HS, Lee SK, Chung HY, Kim CW, Kim KW (2001) Histone deacetylases induce angiogenesis by negative regulation of tumor suppressor genes. Nat Med 7:437–443

Knowles HJ, Raval RR, Harris AL, Ratcliffe PJ (2003) Effect of ascorbate on the activity of hypoxia-inducible factor in cancer cells. Cancer Res 63:1764–1768

Koshiji M, To KK, Hammer S, Kumamoto K, Harris AL, Modrich P, Huang LE (2005) HIF-1alpha induces genetic instability by transcriptionally downregulating MutSalpha expression. Mol Cell 17:793–803

Krieg M, Haas R, Brauch H, Acker T, Flamme I, Plate KH (2000) Up-regulation of hypoxia-inducible factors HIF-1alpha and HIF-2alpha under normoxic conditions in renal carcinoma cells by von Hippel-Lindau tumor suppressor gene loss of function. Oncogene 19:5435–5443

Kung AL, Wang S, Klco JM, Kaelin WG, Livingston DM (2000) Suppression of tumor growth through disruption of hypoxia-inducible transcription. Nat Med 6:1335–1340

Lamszus K, Laterra J, Westphal M, Rosen EM (1999) Scatter factor/hepatocyte growth factor (SF/HGF) content and function in human gliomas. Int J Dev Neurosci 17:517–530

Lando D, Peet DJ, Gorman JJ, Whelan DA, Whitelaw ML, Bruick RK (2002) FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes Dev 16:1466–1471

Li J, Post M, Volk R, Gao Y, Li M, Metais C, Sato K, Tsai J, Aird W, Rosenberg RD, Hampton TG, Sellke F, Carmeliet P, Simons M (2000) PR39, a peptide regulator of angiogenesis. Nat Med 6:49–55

Lin EY, Nguyen AV, Russell RG, Pollard JW (2001) Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. J Exp Med 193:727–740

Linke S, Stojkoski C, Kewley RJ, Booker GW, Whitelaw ML, Peet DJ (2004) Substrate requirements of the oxygen-sensing asparaginyl hydroxylase factor inhibiting HIF. J Biol Chem 279:14391–14397

Lu H, Forbes RA, Verma A (2002) Hypoxia-inducible factor 1 activation by aerobic glycolysis implicates the Warburg effect in carcinogenesis. J. Biol. Chem

Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, Yancopoulos GD (1997) Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 277:55–60

Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ (1999) The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 399:271–275

Minchenko A, Leshchinsky I, Opentanova I, Sang N, Srinivas V, Armstead V, Caro J (2002) Hypoxia-inducible factor-1-mediated expression of the 6-phosphofructo-2- kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) gene. Its possible role in the Warburg effect. J Biol Chem 277:6183–6187

Moeller BJ, Dreher MR, Rabbani ZN, Schroeder T, Cao Y, Li CY, and Dewhirst MW (2005) Pleiotropic effects of HIF-1 blockade on tumor radiosensitivity. Cancer Cell 8:99–110

Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, Verastegui E, Zlotnik A (2001) Involvement of chemokine receptors in breast cancer metastasis. Nature 410:50–56

Plate KH (1999) Mechanisms of angiogenesis in the brain. J Neuropathol Exp Neurol 58:313–320

Plate KH, Breier G, Weich HA, Risau W (1992) Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. Nature 359:845–848

Rafii S, Lyden D, Benezra R, Hattori K, Heissig B (2002) Vascular and haematopoietic stem cells: novel targets for anti-angiogenesis therapy? Nat Rev Cancer 2:826–835

Ravi R, Mookerjee B, Bhujwalla ZM, Sutter CH, Artemov D, Zeng Q, Dillehay LE, Madan A, Semenza GL, Bedi A (2000) Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. Genes Dev 14:34–44

Reynolds TY, Rockwell S, Glazer PM (1996) Genetic instability induced by the tumor microenvironment. Cancer Res 56:5754–5757

Ryan HE, Lo J, Johnson RS (1998) HIF-1 alpha is required for solid tumor formation and embryonic vascularization. EMBO J 17:3005–3015

Selak MA, Armour SM, MacKenzie ED, Boulahbel H, Watson DG, Mansfield KD, Pan Y, Simon MC, Thompson CB, Gottlieb E (2005) Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-alpha prolyl hydroxylase. Cancer Cell 7:77–85

Semenza GL (2000) Hypoxia, clonal selection, and the role of HIF-1 in tumor progression. Crit Rev Biochem Mol Biol 35:71–103

Semenza GL (2003) Targeting HIF-1 for cancer therapy. Nat Rev Cancer 3:721–732

Shweiki D, Itin A, Soffer D, Keshet E (1992) Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 359:843–845

Sowter HM, Raval R, Moore J, Ratcliffe PJ, Harris AL (2003) Predominant role of hypoxia-inducible transcription factor (Hif)-1alpha versus Hif-2alpha in regulation of the transcriptional response to hypoxia. Cancer Res 63:6130–6134

Stratmann A, Acker T, Burger AM, Amann K, Risau W, Plate KH (2001) Differential inhibition of tumor angiogenesis by tie2 and vascular endothelial growth factor receptor-2 dominant-negative receptor mutants. Int J Cancer 91:273–282

Stroka DM, Burkhardt T, Desbaillets I, Wenger RH, Neil DA, Bauer C, Gassmann M, Candinas D (2001) HIF-1 is expressed in normoxic tissue and displays an organ-specific regulation under systemic hypoxia. FASEB J 15:2445–2453

Tang N, Wang L, Esko J, Giordano FJ, Huang Y, Gerber HP, Ferrara N, Johnson RS (2004) Loss of HIF-1alpha in endothelial cells disrupts a hypoxia-driven VEGF autocrine loop necessary for tumorigenesis. Cancer Cell 6:485–495

Tomlinson IP, Alam NA, Rowan AJ, Barclay E, Jaeger EE, Kelsell D, Leigh I, Gorman P, Lamlum H, Rahman S, Roylance RR, Olpin S, Bevan S, Barker K, Hearle N, Houlston RS, Kiuru M, Lehtonen R, Karhu A, Vilkki S, Laiho P, Eklund C, Vierimaa O, Aittomaki K, Hietala M, Sistonen P, Paetau A, Salovaara R, Herva R, Launonen V, Aaltonen LA (2002) Germline mutations in FH predispose to dominantly inherited uterine fibroids, skin leiomyomata and papillary renal cell cancer. Nat Genet 30:406–410

Vaupel P, Kallinowski F, Okunieff P (1989) Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. Cancer Res 49:6449–6465

Volm M, Koomagi R (2000) Hypoxia-inducible factor (HIF-1) and its relationship to apoptosis and proliferation in lung cancer. Anticancer Res 20:1527–1533

Wang GL, Semenza GL (1995) Purification and characterization of hypoxia-inducible factor 1. J Biol Chem 270:1230–1237

Wenger RH (2002) Cellular adaptation to hypoxia: O2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O2-regulated gene expression. FASEB J 16:1151–1162

Wiesener MS, Jurgensen JS, Rosenberger C, Scholze CK, Horstrup JH, Warnecke C, Mandriota S, Bechmann I, Frei UA, Pugh CW, Ratcliffe PJ, Bachmann S, Maxwell PH, Eckardt KU (2003) Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs. FASEB J 17:271–273

Wykoff CC, Beasley NJ, Watson PH, Turner KJ, Pastorek J, Sibtain A, Wilson GD, Turley H, Talks KL, Maxwell PH, Pugh CW, Ratcliffe PJ, Harris AL (2000) Hypoxia-inducible expression of tumor-associated carbonic anhydrases. Cancer Res 60:7075–7083

Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J (2000) Vascular-specific growth factors and blood vessel formation. Nature 407:242–248

Yu JL, Rak JW, Carmeliet P, Nagy A, Kerbel RS, Coomber BL (2001) Heterogeneous vascular dependence of tumor cell populations. Am J Pathol 158:1325–1334

Yu JL, Rak JW, Coomber BL, Hicklin DJ, Kerbel RS (2002) Effect of p53 status on tumor response to antiangiogenic therapy. Science 295:1526–1528

Zagzag D, Zhong H, Scalzitti JM, Laughner E, Simons JW, Semenza GL (2000) Expression of hypoxia-inducible factor 1alpha in brain tumors: association with angiogenesis, invasion, and progression. Cancer 88:2606–2618

Zagzag D, Nomura M, Friedlander DR, Blanco CY, Gagner JP, Nomura N, Newcomb EW (2003) Geldanamycin inhibits migration of glioma cells in vitro: a potential role for hypoxia-inducible factor (HIF-1alpha) in glioma cell invasion. J Cell Physiol 196:394–402

Zhong H, de Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL, Simons JW (1999) Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res 59:5830–5835

Zundel W, Schindler C, Haas-Kogan D, Koong A, Kaper F, Chen E, Gottschalk AR, Ryan HE, Johnson RS, Jefferson AB, Stokoe D, Giaccia AJ (2000) Loss of PTEN facilitates HIF-1-mediated gene expression. Genes Dev 14:391–396

Endogenous Inhibitors of Angiogenesis

12

MALIN SUND and RAGHU KALLURI

Abstract

During angiogenesis new blood vessels are formed from pre-existing blood vessels. Tumor angiogenesis enables a small colony of malignantly transformed cells to grow and develop into an invasive cancer. This process is believed to be regulated by a change in the balance between endogenous pro-angiogenic and anti-angiogenic factors, and thus tumor growth requires a shift towards pro-angiogenesis. There are now extensive genetic data available from animal models that illustrate that the ceiling growth rate of a cancer is strongly influenced by the angiogenic potential of the host. This concept will be discussed in this chapter. We will also introduce the reader to the known matrix-derived and other endogenous inhibitors on angiogenesis, and their mechanism of action.

CONTENTS

12.1 Introduction

Endogenous inhibitors of angiogenesis are defined as proteins or fragments of proteins that are formed in the body, which subsequently can inhibit the formation of blood vessels by disrupting the angiogenic process (Kalluri 2003; Nyberg et al. 2005). These substances can be found both in the circulation and

sequestered in the extracellular matrix (ECM) surrounding cells. It was previously thought that the ECM merely functions as a structural scaffold for cells. However, the highly active role of the ECM in modulating most aspects of cell behavior in the multicellular organism is becoming increasingly appreciated (Folkman and Kalluri 2003; Kalluri 2003; Nyberg et al. 2005). In the context of angiogenesis this becomes evident by the many endogenous inhibitors of angiogenesis that are fragments of larger ECM molecules. These fragments become released upon proteolysis of the ECM and the vascular basement membrane (VBM) by enzymes of the tumor microenvironment, such as matrix metalloproteinases (MMPs), cathepsins and elastases (Kalluri 2003; Sund et al. 2004). Therefore, many endogenous inhibitors of angiogenesis are bio-active fragments from the ECM and VBM degradome (Kalluri 2003; Sund et al. 2004). Besides the matrix-derived molecules, the quite heterogeneous group of other endogenous inhibitors of angiogenesis contains molecules that are fragments or metabolites of hormones, clotting factors or proteins of the immune system (Folkman 2004; Nyberg et al. 2005). Thus through the concept of the protein degradome, a 'second wave' of defense against pathologic neovasculature, fought by fragments of proteins that are released by enzymes of the tumor microenvironment, becomes evident.

The ECM is central to a functioning vasculature in many ways. A specialized ECM structure, the VBM, is found beneath the endothelial cell (EC) layer of all blood vessels and serves as an scaffold for the ECs (Fig. 12.1) (Kalluri 2003; Sund et al. 2004). On the outside the VBM also serves as the anchoring dock for the pericytes, cells which provide the vessels with additional structural support. The ECM surrounding blood vessels also sequesters growth factors and angiogenic factors which influence the vasculature in multiple ways (Fig. 12.1) (Kalluri 2003; Sund et al. 2004). In angiogenesis, new blood vessels are formed by sprouting from pre-established vasculature. During this process the VBM is degraded, which allows the ECs to detach and migrate into the extracellular space. In the extracellular space the ECs become surrounded by a provisional ECM, form a new lumen, a new VBM and become enveloped by pericytes to form a mature blood vessel (Fig. 12.2) (Kalluri 2003; Sund et al. 2004).

What regulates the formation of new blood vessels in a tumor? One key factor is the physiological balance between pro-angiogenic factors and the anti-angiogenic factors in the body and their subsequent effect on the ECs (Folkman and Kalluri 2003). A new blood vessel will be formed when the balance is switched towards pro-angiogenesis, caused by either an excess of angiogenic factors or a lack of anti-angiogenic factors. The angiogenic process can thus potentially be manipulated by decreasing the former or increasing the level of the latter. All tissues, including tumors, depend on oxygen delivery for survival (Folkman and Kalluri 2003; Hanahan and Folkman 1996; Jain 1988), and therefore a shift of the angiogenic balance towards anti-angiogenesis offers a new way of influencing tumor growth and an additional method of cancer treatment.

We will in this chapter discuss both matrix-derived and other endogenous inhibitors of angiogenesis. We will also discuss the genetic evidence available from animal studies that clearly indicates that shifting the angiogenic balance towards increased anti-angiogenesis prevents the formation and survival of tumors.

Matrix-Derived Endogenous Inhibitors of Angiogenesis

12.2.1 Fragments of Type IV Collagen – Tumstatin, Arresten and Canstatin

The structural network formed by type IV collagen is essential for the stability and assembly of all basement membranes (Kuhn et al. 1981; Timpl et al. 1981). There are six different type IV collagen α-chains in mammals. The α1- and α2-chains are found in most basement membranes, whereas the α3- to α6-chains are found in specialized

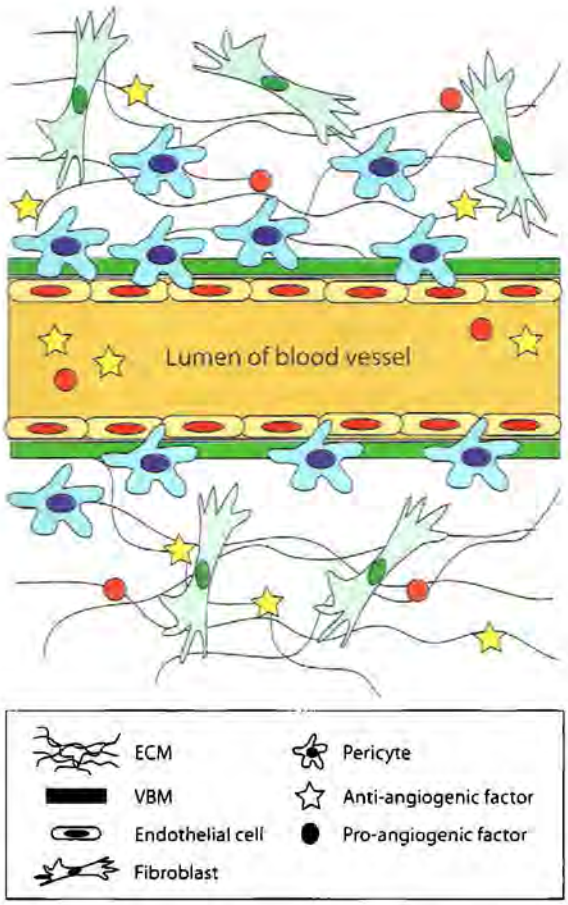


Fig. 12.1. The extracellular matrix (*ECM*) and blood vessel. The blood vessel wall consists of an endothelial cell lining, a vascular basement membrane (*VBM*) and a supporting outer pericyte layer. The VBM is a highly specialized form of ECM. Outside of the pericyte layer the vessel is surrounded by the ECM, which functions as a supportive structure but also sequesters both pro- and anti-angiogenic molecules

basement membranes, and thus display a more restricted expression pattern (Kalluri 2003). Mice deficient of the α1- and α2-chains die during early development, whereas α3-chain-deficient mice initially survive but later succumb due to renal failure (Cosgrove et al. 1996; Poschl et al. 2004). All type IV collagen α-chains share the same structure, with an N-terminal 7S domain, a middle triple-helicalcollagenous domain, and a C-terminal globular noncollagenous (NC) domain (Kalluri 2003). Our laboratory has discovered that the degradation of VBM preparations with tumor-associated enzymes such as MMPs, elastases and cathepsins results in the release of bio-active type IV collagen fragments named tumstatin, arresten and canstatin (Table 12.1) (Kalluri 2003).

Tumstatin is the 28-kDa NC1 domain of the α3-chain of type IV collagen (Table 12.1). Tumstatin was recombinantly produced and found to specifically induce apoptosis of proliferating ECs (Maeshima et al. 2000a), and to have potent anti-angiogenic activity in several in vitro angiogenesis assays and in vivo mouse tumor models (Maeshima et al. 2000a,b, 2001a,b, 2002). Tumstatin can also be found in the blood of mice at physiological levels of approximately 300–350 ng/ml (Hamano et al. 2003), most likely due to the regular basement membrane (BM) turnover process by MMPs (Hamano et al. 2003). The EC receptor β3-integrin was shown to be the functional receptor for tumstatin (Maeshima et al. 2000a,b, 2001a,b, 2002), and the binding to this integrin leads to an EC-specific inhibition of CAP-dependent protein translation, and thus decreased EC proliferation (Maeshima et al. 2000a,b, 2001a,b, 2002; Sudhakar et al. 2003). The importance of β3-integrin is further underlined by the finding that murine lung ECs (MLECs) isolated from mice deficient of β3-integrin do not display decreased proliferation when treated with recombinant tumstatin, and that vascular endothelial growth factor (VEGF)-induced neovascularization of Matrigel plugs in β3-integrin-deficient mice could not be inhibited by tumstatin treatment (Hamano et al. 2003).

Arresten is the 26-kDa NC1 domain of the α1-chain of type IV collagen (Table 12.1) (Colorado et al. 2000). Arresten inhibits EC proliferation, migration, tube formation, neovascularization of Matrigel plugs and the growth of primary tumors and metastases in mouse xenograft tumor models (Colorado et al. 2000; Sudhakar et al. 2005). The receptor for arresten on ECs is α1-integrin, and arresten competes with type IV collagen for the binding to this receptor. Recently, we have shown that arresten inhibits the focal adhesion kinase (FAK)/c-Raf/MEK/ERK1/2/p38 MAPK activation in ECs, without affecting the phosphatidylinositol 3-kinase (PI3 K)/Akt pathway (Sudhakar et al.

2005). Furthermore, arresten was also shown to inhibit the expression of hypoxia-inducible factor-1α (HIF-1α) and VEGF during hypoxia by inhibiting ERK1/2 and p38 activation (Sudhakar et al. 2005). In line with the hypothesis of the essential role of α1-integrin for the function of arresten, no inhibition of this pathway could be observed on ECs derived from α1-integrin-deficient mice (Sudhakar et al. 2005). When tumors were implanted on α1-integrin-deficient mice, no effect on tumor angiogenesis and growth could be obtained with arresten treatment, providing further evidence for the importance of α1-integrin for the anti-angiogenic effect of arresten (Sudhakar et al. 2005).

Canstatin is the 26-kDa NC1 domain of the α2-chain of type IV collagen (Table 12.1). This molecule has been shown to have anti-angiogenic activity in vitro by significantly inhibiting EC proliferation, migration, tube formation, and leading to an induction of apoptosis of these cells (Kamphaus et al. 2000; Petitclerc et al. 2000). In vivo canstatin treatment led to reduced growth of xenograft tumors in mice (Kamphaus et al. 2000). Recently, it was shown by using a canstatin–human serum albumin fusion protein that the strong induction of apoptosis by canstatin is mediated by the αvβ3- and αvβ5-integrins (Magnon et al. 2005). However, this effect was not EC specific, and could also be observed on tumor cells. Thus canstatin, besides its anti-angiogenic effect, also has potent anti-tumor activity (Magnon et al. 2005).

Interestingly, the NC1 domain of the α6-chain of type IV collagen has also been described to possess anti-angiogenic activity (Petitclerc et al. 2000).

12.2.2 The Endostatin Fragment of Type XVIII and XV Collagen

Most vascular and other basement membranes contain type XVIII and XV collagen (Hagg et al.

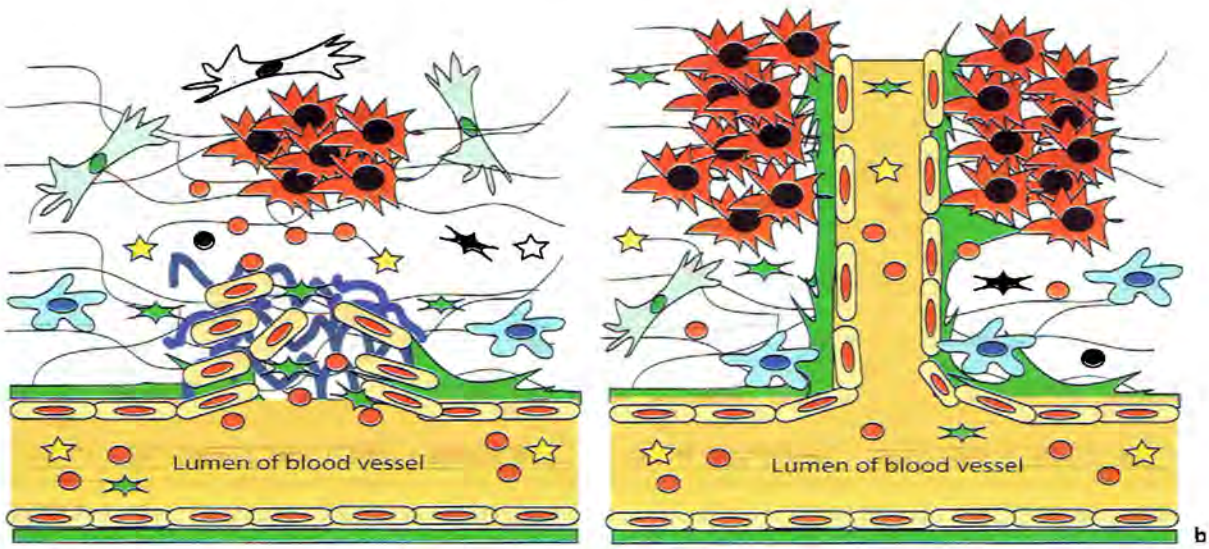


Fig. 12.2a,b. The angiogenic process. a In the initiation of the angiogenic process pericytes detach from the vessel wall and the VBM is degraded by tumor microenvironment-associated enzymes. This is influenced by a general increase of pro-angiogenic molecules from the tumor, the circulation and the ECM. Endothelial cells (ECs) are released, proliferate and migrate under the influence of angiogenic and anti-angiogenic stimulators. During this process the ECs are surrounded by a provisional ECM. b After the new blood vessel is formed, proliferation and migration is downregulated and a new VBM assembled. Blood vessels reach maturity when the newly synthesized vessel obtains appropriate pericyte coverage. However, note that the pericyte coverage is reduced compared to normal vessels. Also the VBM displays irregularities and the EC lining contains gaps

1997; Pihlajaniemi and Rehn 1995; Saarela et al. 1998). The former is a heparan sulfate proteoglycan (HSPG) (Rehn and Pihlajaniemi 1994) and the latter a chondroitin sulfate proteoglycan (CSPG) (Li et al. 2000), both of which are important for the structural integrity of the BM, as a deficiency of these molecules leads to various BM defects (Eklund et al. 2001; Fukai et al. 2002). These collagens share a similar structure with large non-collagenous domains at the N- and C-termini of the molecules, and a middle collagenous domain with multiple interruptions (Pihlajaniemi and Rehn 1995). The highest homology between type XVIII and XV collagen is found in the C-terminus of the molecules (Sasaki et al. 2000).

Endostatin is a 20- to 22-kDa C-terminal fragment of type XVIII collagen (O'Reilly et al. 1997) with very potent anti-angiogenic activity (Table 12.1). This fragment can be cleaved from the parent molecule by at least cathepsin-L, elastin and matrilysin (Felbor et al. 2000; Lin et al. 2001; Wen et al. 1999). Type XV collagen also has an endostatin-like domain with anti-angiogenic activity (Table 12.1) (Ramchandran et al. 1999; Sasaki et al. 2000), although this fragment has been much less studied than the endostatin of type XVIII collagen. Endostatin derived from type XVIII collagen can be found circulating in the blood at physiological levels of 20–35 ng/ml (Rehn and Pihlajaniemi 1994).

Endostatin has multiple ways of negatively affecting tumor growth. This molecule inhibits EC proliferation and migration, induces apoptosis and causes a G1 arrest of ECs (Dhanabal et al. 1999a,b; Yamaguchi et al. 1999). Several EC receptors have in vitro been described to bind endostatin, including α5β1-, αvβ3- and αvβ5-integrins as well as glypicans (Karumanchi et al. 2001; Rehn et al. 2001; Sudhakar et al. 2003). We observed that the

Table 12.1. Matrix derived endogenous inhibitors of angiogenesis

Inhibitor	Parent molecule	EC receptor	Tumor growth	EC proliferation	EC migration	EC apoptosis
Tumstatin	α3(IV) collagen	αvβ3 integrin	↓	↓	-	↑
Arresten	α1(IV) collagen	α1β1 integrin	↓	↓	↓	↑
Canstatin	α2(IV) collagen	αvβ3 and αvβ5 integrin	↓	↓	↓	↑
α6(IV) NC1	α6(IV) collagen	NA	NA	↓	NA	NA
Endostatin	α1(XVIII) collagen	α5β1-integrin, tropomyosin	↓	-*	↓	↑
Endostatin (COL XV)	α1(XV) collagen	NA	↓	↓	↓	NA
Endorepellin	Perlecan	α2β1-integrin	NA	NA	↓	NA
Anastellin	Fibronectin	NA	↓	NA	NA	NA
TSP-1	-	CD36	↓	↓	↓	↑
TSP-2	-	NA	↓	↓	↓	↑
Fibulin-5	-	NA	NA	↓	NA	NA

*Mouse endostatin has also been shown to inhibit EC proliferation. Abbreviations: EC, endothelial cell; NC, non-collagenous domain; NA, not

interaction of endostatin with α5-integrin leads to the inhibition of the FAK/c-Raf/MEK1/2/p38/ERK1 mitogen-activated pathway in vitro, without an effect on PI3 K/Akt/mTOR/4E-BP1 and CAP-dependent translation (Sudhakar et al. 2003). The binding of endostatin to α5-integrin has also been shown to cause a disruption of focal adhesions and actin stress fibers by downregulating RhoA (Wickstrom et al. 2002, 2004).

The indirect effects on angiogenesis by endostatin include inhibition of MMP-2 activity, blocking the binding of VEGF isoforms $VEGF_{165}$ and $VEGF_{121}$ to VEGFR2, and the stabilization of cell–cell and cell–matrix adhesions, which prevents the loosening of these junctions required during vascular sprout formation (Dixelius et al. 2002; Kim et al. 2000, 2002). Endostatin has been extensively studied and is currently in phase II clinical trials (Folkman 2004).

12.2.3 Endorepellin – a Fragment of Perlecan

Perlecan is a large HSPG of the BM, which is important for BM structural stability (Costell et al. 1999). Endorepellin is an 81-kDa C-terminal fragment of perlecan with anti-angiogenic activity (Table 12.1). This molecule inhibits EC migration, tube formation and vessel growth in angiogenesis assays both in vitro and in vivo (Mongiat et al. 2003). EC α2β1-integrin has been shown to be the functional receptor for endorepellin (Bix et al. 2004). The binding to this receptor leads to an increase in intracellular cAMP and the activation protein kinase A/FAK/p38MAPK and HSP27, followed by a downregulation of p38MAPK and HSP27 (Bix et al. 2004). This results in the disassembly of actin stress fibers and focal adhesions, with subsequent inhibition of EC migration and angiogenesis (Bix et al. 2004). A terminal laminin-like globular (LG3) domain of endorepellin has been shown to contain the active site of endorepellin (Gonzalez et al. 2005). The proteolytic processing of endorepellin by the BMP-1/Tolloid family of metalloproteases most likely occurs physiologically, as the LG3 fragment has been found in the urine of patients with end-stage renal disease and in the amniotic fluid of women with premature rupture of fetal membranes during pregnancy (Gonzalez et al. 2005). However, whether endorepellin is found in the circulation is currently unknown.

12.2.4 Thrombospondin-1 and -2

The secreted glycoproteins thrombospondin (TSP)-1 and -2 can be found in the ECM and pericellular matrix (Table 12.1) (Armstrong and Bornstein 2003; Lawler 2002). TSP-1 is a multi-functional protein that directly interacts with many ECM proteins and influences levels of extracellular proteases, as well as affecting the ECM by activating transforming growth factor β (TGF-β) (Lawler 2002). TSP-1 is a highly potent anti-angiogenic molecule and the active site responsible for this effect has been shown to be located in the N-terminal heparin-binding domain of the protein (Ferrari do Outeiro-Bernstein et al. 2002). Although many cell surface receptors bind TSP-1 (Lawler 2002), it is currently believed that CD36 on the ECs is the major receptor for the anti-angiogenic effect (Armstrong and Bornstein 2003; Lawler 2002). The whole TSP-1 protein is too large to be used as a therapeutic agent, which has led to the development of several mimetics to the anti-angiogenic region. These are currently being tested for anti-tumor activity in phase II clinical trials.

Thrombospondin-2 (TSP-2) also possesses anti-angiogenic activity. TSP-2 inhibits EC migration and tube formation, as well as increasing EC specific apoptosis through an 80-kDa fragment in the N-terminal region of the molecule (Noh et al. 2003).

12.2.5 Anastellin – a Fragment of Fibronectin

Fibronectin is a highly abundant ECM protein produced by most cell types that forms an

adhesive fibrillar meshwork. Fibronectin is a multi-domain protein, and it has been shown that the first type III repeat is important for fibronectin self-assembly (Yi and Ruoslahti 2001). Anastellin is an anti-angiogenic, 76-amino-acid fibronectin fragment, the III1-C peptide from the first type III repeat (Table 12.1) (Yi and Ruoslahti 2001). Anastellin has been found to form an active complex with soluble fibronectin and fibrinogen with potent anti-angiogenic effect (Yi et al. 2003). One hypothesis is that this active complex interacts with EC integrins, leading to the observed anti-angiogenic effect (Yi et al. 2003). The anti-angiogenic activity of anastellin is dependent on soluble fibronectin, as it is lost when injecting anastellin into mice deficient of soluble fibronectin (Yi et al. 2003). Interestingly, endostatin and antithrombin were also found to be dependent on soluble fibronectin, which adds active complex formation with fibronectin to the growing list of indirect angiogenesis effects caused by these molecules (Akerman et al. 2005; Yi et al. 2003). It has also been shown recently that anastellin inhibits EC signaling pathways and prevents cell proliferation by modulating ERK signaling pathways and downregulating expression of cell cycle-regulatory genes (Ambesi et al. 2005).

12.2.6 Fibulin-5

Fibulin-5 is a member of the fibulin family of secreted ECM glycoproteins. This molecule is strongly expressed in large arteries. It has been shown that fibulin-5 mediates EC binding to the ECM and scaffolds cells to elastic fibers. Fibulin-5 binds to $\alpha v\beta 3$-, $\alpha v\beta 5$- and $\alpha 9\beta 1$-integrins on the cell surface (Timpl et al. 2003). In vitro studies with overexpression of fibulin-5 in ECs have indicated anti-angiogenic activity with inhibition of EC proliferation, disturbed angiogenic sprouting and reduced invasion through Matrigel matrices (Table 12.1) (Albig and Schiemann 2004). Furthermore, fibulin-5 enhances TSP-1 expression from ECs, leading to an even stronger anti-angiogenic effect (Albig and Schiemann 2004).

Other Endogenous Inhibitors of Angiogenesis

12.3.1 Fragments of Blood Coagulation Factors

12.3.1.1 Angiostatin

Angiostatin is a fragment of plasminogen with potent anti-angiogenic activity (Table 12.2). Several MMPs have been shown to cleave plasminogen and to release anti-angiogenic peptides containing various members of the five plasminogen kringle domains, depending on the site of proteolysis (the 45-kDa kringle-1 to -4 or 38-kDa kringle-1 to -3). These anti-angiogenic peptides are collectively called angiostatin (Cornelius et al. 1998; Geiger and Cnudde 2004). The plasminogen kringle-5 domain by itself is also a potent inhibitor of angiogenesis (Zhang et al. 2004). Angiostatin inhibits EC proliferation, migration and tube formation and induces EC apoptosis (Claesson-Welsh et al. 1998; O'Reilly et al. 1994).

Angiostatin has been extensively studied and it has been shown to affect angiogenesis by several means (Geiger and Cnudde 2004). EC apoptosis is triggered by binding to the ATP synthase (Moser et al. 1999). Both angiostatin and plasmin specifically bind to the $\alpha v\beta 3$-integrin, and angiostatin counteracts and interferes with the plasmin-induced cell migration of ECs (Tarui et al. 2001). It has also been shown that treatment with angiostatin leads to an induction of FAK activity. FAK activity can also be induced by the internalization of angiostatin after binding to the receptor angiomotin (Troyanovsky et al. 2001). Treatment of ECs with angiostatin in the absence of growth factors results in increased apoptosis, whereas proliferation does not change (Claesson-Welsh et al. 1998). Recombinant human angiostatin is currently being tested in phase II clinical trials.

Table 12.2. Other endogenous inhibitors of angiogenesis

Inhibitor	Parent molecule	EC receptor	Tumor growth	EC proliferation	EC migration	EC apoptosis	Indirect mechanisms of action
Angiostatin	Plasminogen	ATP-synthase, annexin II, αvβ3-integrin, angiomotin	↓	↓	↓	↑	-
aaATIII	Antithrombin III	NA	↓	↓	NA	NA	-
PF-4	-	Interacts with heparin-linke GAGs on EC	↓	↓	NA	NA	Prevents bFGF dimerization, inhibits bFGF, VEGF and EGF action
INF-α and -β	-	NA	↓	NA	NA	↑	Reduces IL-8, modifies protease activity, reduces bFGF expression
IL-1β, -4, -12 and -18	-	NA	↓	↓	NA	NA	Multiple anti-angiogenic actions
2-ME	Estradiol	NA	↓	↓	↓	↑	Reduces VEGF levels through HIF-1α
Tetrahydro-cortisol	Cortisone	NA	↓	NA	NA	NA	Affects BM turnover
16 K PRL	Prolactin	NA	↓	↓	NA	↑	Inhibits VEGF action, modifies protease activity
PEDF	-	NA	↓	NA	NA	↑	-
TIMP-2	-	α3β1 integrin	NA	↓	↓	NA	Affects BM turnover
HRGP fragment	HRGP	NA	↓	↓	↓	↑	-

Abbreviations: EC, endothelial cell; NA, not assessed; BM, basement membrane; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor, GAG, glycosaminoglycan

12.3.1.2 Antithrombin III

A certain cleaved form of antithrombin III and the prothrombin kringle-2 domain have been shown to possess anti-angiogenic properties (Table 12.2) (Larsson et al. 2000; Lee et al. 1998; O'Reilly et al. 1999). This form has been named anti-angiogenic antithrombin III (aaATIII) and affects EC proliferation (O'Reilly et al. 1999). In addition, the latent form of intact antithrombin, which is similar in conformation to the cleaved molecule, also inhibits angiogenesis and tumor growth (O'Reilly et al. 1999). aaATIII has potent anti-angiogenic and anti-tumor activity in several mouse models (Larsson et al. 2000; Lee et al. 1998; O'Reilly et al. 1999).

12.3.1.3 Platelet Factor-4

Platelet factor-4 (PF-4) is a protein that can be released from blood platelet a-granules during blood aggregation and which has been shown to have anti-angiogenic properties both in vitro and in vivo through its C-terminal heparin-binding domain (Table 12.2) (Bikfalvi 2004; Maione et al. 1990).

PF-4 has been shown to inhibit the dimerization of the angiogenesis stimulator fibroblast growth factor (FGF)-2 and to block its binding to ECs. PF-4 also binds proteoglycans and potentially through this mechanism growth factor activities. There are also reports that PF-4 activates cell surface receptors on ECs and induces inhibitory signals (Bikfalvi 2004). Modified PF-4 peptides have been generated and represent a new class of anti-angiogenic agents with a strong in vivo activity (Hagedorn et al. 2002).

12.3.2 Molecules of the Immune System with Anti-angiogenic Activity

12.3.2.1 Interferons

The first described endogenous inhibitors of angiogenesis were the interferons (IFNs), cytokines that are involved in the regulation of anti-viral, anti-tumor, apoptotic and cellular immune responses. The effect on angiogenesis is the related to the presence or absence of the ELR domain, a structural/functional motif on the interferon molecule. Both IFN-α and IFN-β have been shown to inhibit angiogenesis in mouse models in vivo by modulating the pro-angiogenic signals generated by tumor cells (Table 12.2) (Mitsuyasu 1991). In vitro the treatment of tumor cells with IFN-α leads to a drop in their secretion of interleukin-8 (IL-8), the major angiogenic factor produced by tumors (Lingen et al. 1998). This interferon also modifies the activity and expression of several proteases, including MMP-9 (Ma et al. 2001), as well as the activity of urokinase-type plasminogen activator and plasminogen activator inhibitor-1 (Pepper et al. 1994). A further effect on angiogenesis is the downregulation of bFGF expression (Dinney et al. 1998). Thus IFN-α is currently an established treatment for pulmonary, brain and other hemangiomas, angioblastomas and giant cell tumors – all of which produce high levels of bFGF (Folkman 2004).

12.3.2.2 Interleukins

Interleukins (ILs) are proteins produced by leukocytes that are involved in many cellular events, including angiogenesis. Many of the ILs have pro-angiogenic activity, such as IL-8, whereas others, e.g. IL-1β, IL-4, IL-12 and IL-18, have anti-angiogenic activity (Table 12.2) (Nyberg et al. 2005). IL-1β inhibits FGF-stimulated angiogenesis by an autocrine pathway (Cozzolino et al. 1990). IL-4 inhibits bFGF-induced angiogenesis (Volpert et al. 1998). IL-12 and IL-18 are IFN-γ-inducing cytokines with anti-angiogenic activity. Both treatment with IL-12 of mice with tumors and the increased IL-12 delivery through gene transfer resulted in decreased tumor growth (Morini et al. 2004; Yao et al. 2000). The anti-angiogenic activity of IL-12 is believed to be mediated through downstream chemokines (Yao et al. 2000). IL-18 can prevent FGF-stimulated EC proliferation in vitro and in the corneal neovascularization assay in mice (Cao et al. 1999).

12.3.3 Hormones, Their Fragments and Metabolites

12.3.3.1 2-Methoxyestradiol

2-Methoxyestradiol (2-ME) is an estradiol metabolite which inhibits the growth of many tumor cell lines and also has anti-angiogenic activity in vitro. In vivo, 2-ME has been shown to be a very effective inhibitor of tumor growth and angiogenesis in numerous models (LaVallee et al. 2002). 2-ME can bind to the colchicine-binding site of tubulin and inhibit superoxide dismutase enzymatic activity (Cushman et al. 1995; D'Amato et al. 1994). 2-ME has also been shown to destabilize microtubules, resulting in a block in nuclear accumulation and the activity of HIF-1α, thus reducing VEGF levels (Table 12.2) (Mabjeesh et al. 2003).

12.3.3.2 Tetrahydrocortisol

Tetrahydrocortisol is the most potent of the naturally occurring anti-angiogenic steroids (Table 12.2) (Folkman and Ingber 1987). Tetrahydrocortisol is a metabolite of cortisone and can be found in the circulation and urine. The anti-angiogenic mechanism of action of tetrahydrocortisol is believed to be due to its ability to alter the BM turnover of the proliferating blood vessels (Folkman and Ingber 1987).

12.3.3.3 Prolactin Fragment

The hormone prolactin (PRL) is cleaved in many tissues to generate a 16-kDa (16 K) and a 8-kDa fragment (Ferrara et al. 1991). The generation of the 16 K PRL fragment has anti-angiogenic activity, although the full-length PRL is pro-angiogenic (Table 12.2) (Corbacho et al. 2002; Struman et al. 1999). The 16 K PRL inhibits VEGF-induced activation of Ras in ECs as well as interacting with the MAPK signaling pathway (D'Angelo et al. 1995, 1999). The inhibition of the p38 MAPK/Stat1/IRF-1 pathway by 16 K PRL attenuates the iNOS/NO production in ECs (Lee et al. 2005).

12.3.4 Growth Factors and Other Miscellaneous Endogenous Inhibitors

12.3.4.1 PEDF

Pigment epithelium-derived factor (PEDF) is a member of the serpin superfamily. PEDF is responsible for the avascularity of ocular compartments and it is one of the most potent endogenous inhibitors of angiogenesis (Table 12.2) (Bouck 2002; Volpert et al. 2002). It has been shown that under physiologic conditions PEDF counteracts the activities of VEGF, and thus a balance between these two molecules exist. This balance is central to the prevention of choroidal neovascularization (Duh et al. 2004). The anti-angiogenic activity of PEDF only targets proliferating vessels and its action is reversible.

Overexpression of PEDF was found to significantly inhibit melanoma growth and vessel formation in mice (Abe et al. 2004). PEDF also induces FasL-dependent apoptosis on both tumor cells and ECs. The anti-angiogenic activity of PEDF, both in vitro and in vivo, has been shown to be dependent on the induction of apoptosis through the induction of Fas and FasL (Volpert et al. 2002).

12.3.4.2 TIMP-2

Tissue inhibitors of MMPs (TIMP) suppress the activity of MMPs and thus regulate the ECM turnover. Independent of their regulation of MMP activity, these proteins also directly affect cell growth, apoptosis and differentiation (Baker et al. 2002; Jiang et al. 2002). TIMP-2 has been shown to inhibit EC proliferation in vitro and angiogenesis in vivo by binding to the $\alpha 3\beta 1$-integrin on ECs (Table 12.2) (Nisato et al. 2005).

12.3.4.3 Fragment of HRGP

The histidine-rich glycoprotein (HRGP) is a protein found in the circulation in relatively high concentrations (1.5 μM), but with unknown function. Recombinant HRGP has been shown to have potent anti-angiogenic properties in murine tumor models in vivo (Table 12.2) (Olsson et al. 2004). Treatment with HRGP resulted in increased apoptosis and reduced proliferation of tumors implanted on mice. HRGP causes a rearrangement of focal adhesions and decreased attachment of ECs to vitronectin, leading to a decrease in EC migration. A fragment of HRPG, the 150-amino-acid His/Pro-rich domain, which can be released by spontaneous proteolysis, has been shown to mediate the anti-migratory effect of HRPG (Olsson et al. 2004). The actual receptor of the HRPG fragment on the ECs is unknown. However, on the surface of FGF-2-activated ECs at least two binding sites for HPRG, tropomyosin and

HSPGs have been identified. As the anti-angiogenic activity of HPRG in the Matrigel plug assay has been shown to be partially inhibited by soluble tropomyosin, this molecule at least partially mediates the anti-angiogenic effects of HPRG (Olsson et al. 2004).

Genetic Evidence for the Therapeutic Effect of Modulating the Angiogenic Balance in Cancer

The concept of the angiogenic balance states that there is a natural balance of pro-angiogenic and anti-angiogenic molecules in the body. This balance dictates whether a new blood vessel will be formed (Folkman and Kalluri 2003; Kalluri 2003). In most circumstances the physiological balance is believed to be tilted towards anti-angiogenesis. However, in many pathological situations there is a shift towards pro-angiogenesis, which leads to the formation of pathological neovasculature. It is known from human autopsy materials that most of us have multiple areas with malignant cells in the body (Folkman and Kalluri 2004). However, only very few of these actually develop into a cancer. This phenomenon has been named 'cancer without disease' (Folkman and Kalluri 2004). Is the development of a cancer from a group of malignant cells due to a local and/or systemic shift from anti-angiogenesis to pro-angiogenesis? Do tumors grow quicker when the pro-angiogenic exceed the anti-angiogenic molecules? There are now several findings from genetically modified mouse strains that support this hypothesis.

The most conclusive data to support this theory are found for the endogenous angiogenesis inhibitor tumstatin. Mice with an inactivation of the gene for the α3-chain of type IV collagen, and thus also lacking tumstatin, display accelerated tumor growth associated with enhanced pathological angiogenesis compared to wild-type mice (Hamanoet al. 2003). However, when such tumstatin-deficient mice were given recombinant tumstatin so that they achieved normal physiological concentrations, the rate of tumor growth slowed down to normal (Hamano et al. 2003). The tumor-associated proteinase MMP-9 most efficiently cleaves tumstatin from the type IV collagen α3-chain (Hamano et al. 2003). Interestingly, mice deficient of MMP-9 subsequently display decreased circulating levels of tumstatin and thus also accelerated tumor growth compared to wild-type mice (Hamano et al. 2003). It has also been shown that the lack of the tumstatin receptor β3-integrin leads to increased pathological angiogenesis and tumor growth (Reynolds et al. 2002). Although the lack of β3-integrin naturally influences the potential function of many other endogenous inhibitors of angiogenesis besides tumstatin, these results nevertheless provide evidence that a lack of tumstatin, the enzyme that activates it (MMP-9) or its functional receptor on ECs (β3-integrin) all lead to a shift in the angiogenic balance towards favoring increased tumor growth (Fig. 12.3) (Hamano et al. 2003; Reynolds et al. 2002).

Further evidence for the notion that cancer is not growing at ceiling rate but is influenced by endogenous angiogenesis inhibitors is provided by the results from mice deficient of type XVIII collagen and thus also of endostatin (Sund et al. 2005). When tumors were implanted on these mice, increased growth and higher tumor vascularity was observed than for tumors implanted on wild-type litter mates (Sund et al. 2005). Interestingly, when tumors were implanted on mice that were genetically altered to overexpress endostatin, reduced tumor growth and decreased vascularity of tumors were observed (Sund et al. 2005). These results show that the circulating levels of endostatin directly influences the growth rate of the same tumor.

Similar findings have also been shown for the thrombospondins. Tumors implanted on mice genetically deficient of TSP-1 grew faster and displayed a higher vascular density than tumors on wild-type mice (Lawler et al. 2001). Increasing the levels of TSP-1 resulted in the opposite finding of reduced tumor angiogenesis (Streit et al. 1999). Mice deficient of the tumor suppressor gene p53 are susceptible to spontaneous cancer develop-

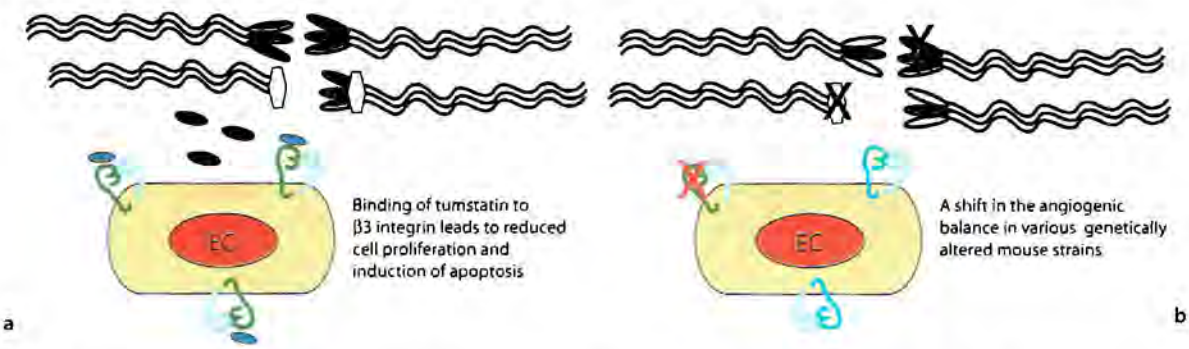


Fig. 12.3a,b. Genetic evidence for the importance of endogenous inhibitors of angiogenesis. a Tumstatin is the NC1 domain of the α3-chain of type IV collagen. This molecule can be cleaved from the type IV collagen trimer by the protease MMP-9 and it exerts its anti-angiogenic effect via binding to β3-integrin on the EC surface. This binding leads to decreased EC proliferation and induction of apoptosis. b This anti-angiogenic effect was lost in mice genetically deficient of tumstatin, MMP-9 or β3-integrin. This indicates that there is an angiogenic balance that the endogenous inhibitors of angiogenesis maintain (Hamano et al. 2003)

ment. Most interestingly, when the TSP1-deficient mice were crossed to the p53-deficient mice, decreased survival due to an even greater increase in tumor growth was observed. This is probably due to a shift in the angiogenic balance towards pro-angiogenesis (Lawler et al. 2001). Mice deficient of TSP-2 also display significantly increased tumor vascularization (Hawighorst et al. 2001). However, increasing the circulating level of TSP-2 by injecting fibroblasts which overexpress this molecule resulted in an inhibition of tumor growth and angiogenesis in mice (Streit et al. 2002).

Our laboratory has also shown that the angiogenic balance can be manipulated further by simultaneously altering the levels of many different inhibitors (Sund et al. 2005) (Fig. 12.4). Thus tumors on endostatin-, tumstatin- or TSP-1-deficient mice grow 2–3 times faster than on wild-type mice. However, if the host is deficient of both tumstatin and TSP-1, the tumors grow 4–6 times faster than those implanted on wild-type mice (Sund et al. 2005). Thus there is evidence from genetic studies that combining multiple anti-angiogenic approaches will have a cumulative effect on tumor growth.

12.5 Concluding Remarks

Endogenous stimulators and matrix-derived inhibitors of angiogenesis are molecules that are naturally produced and circulate in the body. These molecules are important in maintaining the angiogenic balance that influences the rate of new blood vessel formation in both the physiological and the pathological context. The development of a few malignant cells into invasive carcinomas is likely to be partly driven by a shift towards increased angiogenetic potential. This shift can be due to both an increase in angiogenic factors, such as VEGF, or decrease in the levels of endogenous inhibitors of angiogenesis, such as tumstatin and endostatin. Most likely this is a process influenced by oncogenes and tumor suppressor genes. Many of the matrix-derived endogenous angiogenesis inhibitors are products from the ECM degradome released by tumor-associated proteolytic enzymes or are molecules sequestered pericellularly in the ECM. The availability of these inhibitors is dictated by the activity of the releasing enzymes and

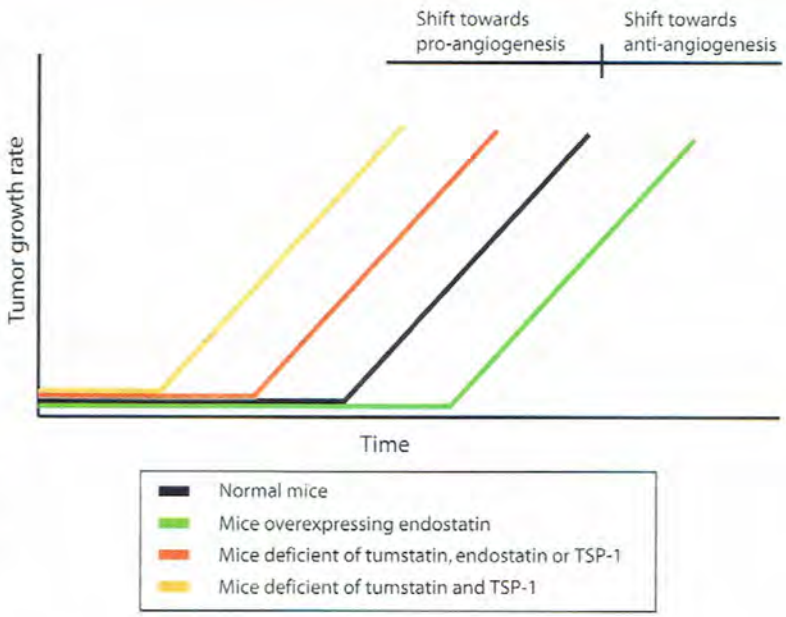


Fig. 12.4. Manipulation of the angiogenic balance alters the growth rate of tumors. B16F10 mouse melanoma cells were implanted on normal wild-type mice (*black*), mice overexpressing endostatin (*green*), mice missing either endostatin, tumstatin or TSP-1 (*red*) as well as mice deficient of both tumstatin and TSP-1 (*orange*). In mice with overexpression of endostatin reduced tumor growth rates could be observed, indicating that the angiogenic balance had shifted towards anti-angiogenesis compared to the wild-type mice. In the mice deficient of the various anti-angiogenic molecules the opposite could we observed, tumors grew faster when compared to wild-type mice due to a shift towards pro-angiogenesis (Sund et al. 2005)

thus this activity also indirectly is of importance in maintaining the angiogenic balance. This also is the case for many of the non-matrix-derived endogenous inhibitors of angiogenesis that need to be modified or released by proteolysis before activity. There is now considerable genetic evidence for the importance of the angiogenic balance in the context of tumor growth and vascularization. Many of the endogenous inhibitors are currently in the process of clinical trials for consideration as future cancer therapeutics.

Acknowledgements

This work was partially supported by NIH grants DK62987, DK55001 and research funds from the Center for Matrix Biology at the Beth Israel Deaconess Medical Center (BIDMC), the Sigrid Juselius Foundation (MS) and the Finnish Medical Society Duodecim (MS).

References

Abe R, Shimizu T, Yamagishi S, Shibaki A, Amano S, Inagaki Y, Watanabe H, Sugawara H, Nakamura H, Takeuchi M et al (2004) Overexpression of pigment epithelium-derived factor decreases angiogenesis and inhibits the growth of human malignant melanoma cells in vivo. Am J Pathol 164:1225–1232

Akerman ME, Pilch J, Peters D, Ruoslahti E(2005) Angiostatic peptides use plasma fibronectin to home to angiogenic vasculature. Proc Natl Acad Sci USA 102:2040–2045

Albig AR, Schiemann WP (2004) Fibulin-5 antagonizes vascular endothelial growth factor (VEGF) signaling and angiogenic sprouting by endothelial cells. DNA Cell Biol 23:367–379

Ambesi A, Klein RM, Pumiglia KM, McKeown-Longo PJ (2005) Anastellin, a fragment of the first type III repeat of fibronectin, inhibits extracellular signal-regulated kinase and causes G(1) arrest in human microvessel endothelial cells. Cancer Res 65:148–156

Armstrong LC, Bornstein P (2003) Thrombospondins 1 and 2 function as inhibitors of angiogenesis. Matrix Biol 22:63–71

Baker EA, Stephenson TJ, Reed MW, Brown NJ (2002) Expression of proteinases and inhibitors in human breast cancer progression and survival. Mol Pathol 55:300–304

Bikfalvi A(2004) Platelet factor 4: an inhibitor of angiogenesis. Semin Thromb Hemost 30:379–385

Bix G, Fu J, Gonzalez EM, Macro L, Barker A, Campbell S, Zutter MM, Santoro SA, Kim JK, Hook M et al (2004) Endorepellin causes endothelial cell disassembly of actin cytoskeleton and focal adhesions through alpha2beta1 integrin. J Cell Biol 166:97–109

Bouck N (2002) PEDF: anti-angiogenic guardian of ocular function. Trends Mol Med 8:330–334

Cao R, Farnebo J, Kurimoto M, Cao Y (1999) Interleukin-18 acts as an angiogenesis and tumor suppressor. Faseb J 13:2195–2202

Claesson-Welsh L, Welsh M, Ito N, Anand-Apte B, Soker S, Zetter B, O'Reilly M, Folkman J (1998) Angiostatin induces endothelial cell apoptosis and activation of focal adhesion kinase independently of the integrin-binding motif RGD. Proc Natl Acad Sci USA 95:5579–5583

Colorado PC, Torre A, Kamphaus G, Maeshima Y, Hopfer H, Takahashi K, Volk R, Zamborsky ED, Herman S, Sarkar PK et al (2000) Anti-angiogenic cues from vascular basement membrane collagen. Cancer Res 60:2520–2526

Corbacho AM, Martinez De La Escalera G, Clapp C (2002) Roles of prolactin and related members of the prolactin/growth hormone/placental lactogen family in angiogenesis. J Endocrinol 173:219–238

Cornelius LA, Nehring LC, Harding E, Bolanowski M, Welgus HG, Kobayashi DK, Pierce RA, Shapiro SD (1998) Matrix metalloproteinases generate angiostatin: effects on neovascularization. J Immunol 161:6845–6852

Cosgrove D, Meehan DT, Grunkemeyer JA, Kornak JM, Sayers R, Hunter WJ, Samuelson GC (1996) Collagen COL4A3 knockout: a mouse model for autosomal Alport syndrome. Genes Dev 10:2981–2992

Costell M, Gustafsson E, Aszodi A, Morgelin M, Bloch W, Hunziker E, Addicks K, Timpl R, Fassler R (1999) Perlecan maintains the integrity of cartilage and some basement membranes. J Cell Biol 147:1109–1122

Cozzolino F, Torcia M, Aldinucci D, Ziche M, Almerigogna F, Bani D, Stern DM (1990) Interleukin 1 is an autocrine regulator of human endothelial cell growth. Proc Natl Acad Sci USA 87:6487–6491

Cushman M, He HM, Katzenellenbogen JA, Lin CM, Hamel E (1995) Synthesis, antitubulin and antimitotic activity, and cytotoxicity of analogs of 2-methoxyestradiol, an endogenous mammalian metabolite of estradiol that inhibits tubulin polymerization by binding to the colchicine binding site. J Med Chem 38:2041–2049

D'Amato RJ, Lin CM, Flynn E, Folkman J, Hamel E (1994) 2-Methoxyestradiol, an endogenous mammalian metabolite, inhibits tubulin polymerization by interacting at the colchicine site. Proc Natl Acad Sci USA 91:3964–3968

D'Angelo G, Struman I, Martial J, Weiner RI (1995) Activation of mitogen-activated protein kinases by vascular endothelial growth factor and basic fibroblast growth factor in capillary endothelial cells is inhibited by the antiangiogenic factor 16-kDa N-terminal fragment of prolactin. Proc Natl Acad Sci USA 92:6374–6378

D'Angelo G, Martini JF, Iiri T, Fantl WJ, Martial J, Weiner RI (1999) 16 K human prolactin inhibits vascular endothelial growth factor-induced activation of Ras in capillary endothelial cells. Mol Endocrinol 13:692–704

Dhanabal M, Ramchandran R, Waterman MJ, Lu H, Knebelmann B, Segal M, Sukhatme VP (1999a) Endostatin induces endothelial cell apoptosis. J Biol Chem 274:11721–11726

Dhanabal M, Ramchandran R, Volk R, Stillman IE, Lombardo M, Iruela-Arispe ML, Simons M, Sukhatme VP (1999b) Endostatin: yeast production, mutants, and antitumor effect in renal cell carcinoma. Cancer Res 59:189–197

Dinney CP, Bielenberg DR, Perrotte P, Reich R, Eve BY, Bucana CD, Fidler IJ (1998) Inhibition of basic fibroblast growth factor expression, angiogenesis, and growth of human bladder carcinoma in mice by systemic interferon-alpha administration. Cancer Res 58:808–814

Dixelius J, Cross M, Matsumoto T, Sasaki T, Timpl R, Claesson-Welsh L (2002) Endostatin regulates endothelial cell adhesion and cytoskeletal organization. Cancer Res 62:1944–1947

Duh EJ, Yang HS, Haller JA, De Juan E, Humayun MS, Gehlbach P, Melia M, Pieramici D, Harlan JB, Campochiaro PA, Zack DJ (2004) Vitreous levels of pigment epithelium-derived factor and vascular endothelial growth factor: implications for ocular angiogenesis. Am J Ophthalmol 137:668–674

Eklund L, Piuhola J, Komulainen J, Sormunen R, Ongvarrasopone C, Fassler R, Muona A, Ilves M, Ruskoaho H, Takala TE, Pihlajaniemi T (2001) Lack of type XV collagen causes a skeletal myopathy and cardiovascular defects in mice. Proc Natl Acad Sci USA 98:1194–1199

Felbor U, Dreier L, Bryant RA, Ploegh HL, Olsen BR, Mothes W (2000) Secreted cathepsin L generates endostatin from collagen XVIII. Embo J 19:1187–1194

Ferrara N, Clapp C, Weiner R (1991) The 16 K fragment of prolactin specifically inhibits basal or fibroblast growth factor stimulated growth of capillary endothelial cells. Endocrinology 129:896–900

Ferrari do Outeiro-Bernstein MA, Nunes SS, Andrade AC, Alves TR, Legrand C, Morandi V (2002) A recombinant NH(2)-terminal heparin-binding domain of the adhesive glycoprotein, thrombospondin-1, promotes endothelial tube formation and cell survival: a possible role for syndecan-4 proteoglycan. Matrix Biol 21:311–324

Folkman J (2004) Endogenous angiogenesis inhibitors. Apmis 112:496–507

Folkman J, Ingber DE (1987) Angiostatic steroids. Method of discovery and mechanism of action. Ann Surg 206:374–383

Folkman J, Kalluri R (2003) Tumor angiogenesis. In: Kufe DW, Pollock RE, Weichselbaum RR, Bast RC Jr, Gansler TS, Holland JF, Frei E III (eds) Cancer medicine 6. Delger, Hamilton, Canada, pp 161–194

Folkman J, Kalluri R (2004) Cancer without disease. Nature 427:787

Fukai N, Eklund L, Marneros AG, Oh SP, Keene DR, Tamarkin L, Niemela M, Ilves M, Li E, Pihlajaniemi T, Olsen BR (2002) Lack of collagen XVIII/endostatin results in eye abnormalities. Embo J 21:1535–1544

Geiger JH, Cnudde SE (2004) What the structure of angiostatin may tell us about its mechanism of action. J Thromb Haemost 2:23–34

Gonzalez EM, Reed CC, Bix G, Fu J, Zhang Y, Gopalakrishnan B, Greenspan DS, Iozzo RV (2005) BMP-1/tolloid-like metalloproteases process endorepellin, the angiostatic C-terminal fragment of perlecan. J Biol Chem 280:7080–7087

Hagedorn M, Zilberberg L, Wilting J, Canron X, Carrabba G, Giussani C, Pluderi M, Bello L, Bikfalvi A (2002) Domain swapping in a COOH-terminal fragment of platelet factor 4 generates potent angiogenesis inhibitors. Cancer Res 62:6884–6890

Hagg PM, Hagg PO, Peltonen S, Autio-Harmainen H, Pihlajaniemi T (1997) Location of type XV collagen in human tissues and its accumulation in the interstitial matrix of the fibrotic kidney. Am J Pathol 150:2075–2086

Hamano Y, Zeisberg M, Sugimoto H, Lively JC, Maeshima Y, Yang C, Hynes RO, Werb Z, Sudhakar A, Kalluri R (2003) Physiological levels of tumstatin, a fragment of collagen IV alpha3 chain, are generated by MMP-9 proteolysis and suppress angiogenesis via alphaV beta3 integrin. Cancer Cell 3:589–601

Hanahan D, Folkman J (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 86:353–364

Hawighorst T, Velasco P, Streit M, Hong YK, Kyriakides TR, Brown LF, Bornstein P, Detmar M (2001) Thrombospondin-2 plays a protective role in multistep carcinogenesis: a novel host anti-tumor defense mechanism. Embo J 20:2631–2640

Jain RK (1988) Determinants of tumor blood flow: a review. Cancer Res 48:2641–2658

Jiang Y, Goldberg ID, Shi YE (2002) Complex roles of tissue inhibitors of metalloproteinases in cancer. Oncogene 21:2245–2252

Kalluri R (2003) Basement membranes: structure, assembly and role in tumour angiogenesis. Nat Rev Cancer 3:422–433

Kamphaus GD, Colorado PC, Panka DJ, Hopfer H, Ramchandran R, Torre A, Maeshima Y, Mier JW, Sukhatme VP, Kalluri R (2000) Canstatin, a novel matrix-derived inhibitor of angiogenesis and tumor growth. J Biol Chem 275:1209–1215

Karumanchi SA, Jha V, Ramchandran R, Karihaloo A, Tsiokas L, Chan B, Dhanabal M, Hanai JI, Venkataraman G, Shriver Z et al (2001) Cell surface glypicans are low-affinity endostatin receptors. Mol Cell 7:811–822

Kim YM, Jang JW, Lee OH, Yeon J, Choi EY, Kim KW, Lee ST, Kwon YG (2000) Endostatin inhibits endothelial and tumor cellular invasion by blocking the activation and catalytic activity of matrix metalloproteinase. Cancer Res 60:5410–5413

Kim YM, Hwang S, Pyun BJ, Kim TY, Lee ST, Gho YS, Kwon YG (2002) Endostatin blocks vascular endothelial growth factor-mediated signaling via direct interaction with KDR/Flk-1. J Biol Chem 277:27872–27879

Kuhn K, Wiedemann H, Timpl R, Risteli J, Dieringer H, Voss T, Glanville RW (1981) Macromolecular structure of basement membrane collagens. FEBS Lett 125:123–128

Larsson H, Sjoblom T, Dixelius J, Ostman A, Ylinenjarvi K, Bjork I, Claesson-Welsh L (2000) Antiangiogenic effects of latent antithrombin through perturbed cell–matrix interactions and apoptosis of endothelial cells. Cancer Res 60:6723–6729

LaVallee TM, Zhan XH, Herbstritt CJ, Kough EC, Green SJ, Pribluda VS (2002) 2-Methoxyestradiol inhibits proliferation and induces apoptosis independently of estrogen receptors alpha and beta. Cancer Res 62:3691–3697

Lawler J (2002) Thrombospondin-1 as an endogenous inhibitor of angiogenesis and tumor growth. J Cell Mol Med 6:1–12

Lawler J, Miao WM, Duquette M, Bouck N, Bronson RT, Hynes RO (2001) Thrombospondin-1 gene expression affects survival and tumor spectrum of p53-deficient mice. Am J Pathol 159:1949–1956

Lee SH, Nishino M, Mazumdar T, Garcia GE, Galfione M, Lee FL, Lee CL, Liang A, Kim J, Feng L et al (2005) 16-kDa prolactin downregulates inducible nitric oxide synthase expression through inhibition of the signal transducer and activator of transcription 1/IFN regulatory factor-1 pathway. Cancer Res 65:7984–7992

Lee TH, Rhim T, Kim SS (1998) Prothrombin kringle-2 domain has a growth inhibitory activity against basic fibroblast growth factor-stimulated capillary endothelial cells. J Biol Chem 273:28805–28812

Li D, Clark CC, Myers JC (2000) Basement membrane zone type XV collagen is a disulfide-bonded chondroitin sulfate proteoglycan in human tissues and cultured cells. J Biol Chem 275:22339–22347

Lin HC, Chang JH, Jain S, Gabison EE, Kure T, Kato T, Fukai N, Azar DT (2001) Matrilysin cleavage of corneal collagen type XVIII NC1 domain and generation of a 28-kDa fragment. Invest Ophthalmol Vis Sci 42:2517–2524

Lingen MW, Polverini PJ, Bouck NP (1998) Retinoic acid and interferon alpha act synergistically as antiangiogenic and antitumor agents against human head and neck squamous cell carcinoma. Cancer Res 58:5551–5558

Ma Z, Qin H, Benveniste EN (2001) Transcriptional suppression of matrix metalloproteinase-9 gene expression by IFN-gamma and IFN-beta: critical role of STAT-1alpha. J Immunol 167:5150–5159

Mabjeesh NJ, Escuin D, LaVallee TM, Pribluda VS, Swartz GM, Johnson MS, Willard MT, Zhong H, Simons JW, Gi-

annakakou P (2003) 2ME2 inhibits tumor growth and angiogenesis by disrupting microtubules and dysregulating HIF. Cancer Cell 3:363–375

Maeshima Y, Colorado PC, Torre A, Holthaus KA, Grunkemeyer JA, Ericksen MB, Hopfer H, Xiao Y, Stillman IE, Kalluri R (2000a) Distinct antitumor properties of a type IV collagen domain derived from basement membrane. J Biol Chem 275:21340–21348

Maeshima Y, Colorado PC, Kalluri R (2000b) Two RGD-independent alpha vbeta 3 integrin binding sites on tumstatin regulate distinct anti-tumor properties. J Biol Chem 275:23745–23750

Maeshima Y, Manfredi M, Reimer C, Holthaus KA, Hopfer H, Chandamuri BR, Kharbanda S, Kalluri R (2001a) Identification of the anti-angiogenic site within vascular basement membrane-derived tumstatin. J Biol Chem 276:15240–15248

Maeshima Y, Yerramalla UL, Dhanabal M, Holthaus KA, Barbashov S, Kharbanda S, Reimer C, Manfredi M, Dickerson WM, Kalluri R (2001b) Extracellular matrix-derived peptide binds to alpha(v)beta(3) integrin and inhibits angiogenesis. J Biol Chem 276:31959–31968

Maeshima Y, Sudhakar A, Lively JC, Ueki K, Kharbanda S, Kahn CR, Sonenberg N, Hynes RO, Kalluri R (2002) Tumstatin, an endothelial cell-specific inhibitor of protein synthesis. Science 295:140–143

Magnon C, Galaup A, Mullan B, Rouffiac V, Bouquet C, Bidart JM, Griscelli F, Opolon P, Perricaudet M (2005) Canstatin acts on endothelial and tumor cells via mitochondrial damage initiated through interaction with alphavbeta3 and alphavbeta5 integrins. Cancer Res 65:4353–4361

Maione TE, Gray GS, Petro J, Hunt AJ, Donner AL, Bauer SI, Carson HF, Sharpe RJ (1990) Inhibition of angiogenesis by recombinant human platelet factor-4 and related peptides. Science 247:77–79

Mitsuyasu RT (1991) Interferon alpha in the treatment of AIDS-related Kaposi's sarcoma. Br J Haematol 79 [Suppl 1]:69–73

Mongiat M, Sweeney SM, San Antonio JD, Fu J, Iozzo RV (2003) Endorepellin, a novel inhibitor of angiogenesis derived from the C terminus of perlecan. J Biol Chem 278:4238–4249

Morini M, Albini A, Lorusso G, Moelling K, Lu B, Cilli M, Ferrini S, Noonan DM (2004) Prevention of angiogenesis by naked DNA IL-12 gene transfer: angioprevention by immunogene therapy. Gene Ther 11:284–291

Moser TL, Stack MS, Asplin I, Enghild JJ, Hojrup P, Everitt L, Hubchak S, Schnaper HW, Pizzo SV (1999) Angiostatin binds ATP synthase on the surface of human endothelial cells. Proc Natl Acad Sci USA 96:2811–2816

Nisato RE, Hosseini G, Sirrenberg C, Butler GS, Crabbe T, Docherty AJ, Wiesner M, Murphy G, Overall CM, Goodman SL, Pepper MS (2005) Dissecting the role of matrix metalloproteinases (MMP) and integrin alpha(v)beta3 in angiogenesis in vitro: absence of hemopexin C domain bioactivity, but membrane-Type 1-MMP and alpha(v)beta3 are critical. Cancer Res 65:9377–9387

Noh YH, Matsuda K, Hong YK, Kunstfeld R, Riccardi L, Koch M, Oura H, Dadras SS, Streit M, Detmar M (2003) An N-terminal 80 kDa recombinant fragment of human thrombospondin-2 inhibits vascular endothelial growth factor induced endothelial cell migration in vitro and tumor growth and angiogenesis in vivo. J Invest Dermatol 121:1536–1543

Nyberg P, Xie L, Kalluri R (2005) Endogenous inhibitors of angiogenesis. Cancer Res 65:3967–3979

O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, Lane WS, Cao Y, Sage EH, Folkman J (1994) Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell 79:315–328

O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR, Folkman J (1997) Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell 88:277–285

O'Reilly MS, Pirie-Shepherd S, Lane WS, Folkman J (1999) Antiangiogenic activity of the cleaved conformation of the serpin antithrombin. Science 285:1926–1928

Olsson AK, Larsson H, Dixelius J, Johansson I, Lee C, Oellig C, Bjork I, Claesson-Welsh L (2004) A fragment of histidine-rich glycoprotein is a potent inhibitor of tumor vascularization. Cancer Res 64:599–605

Pepper MS, Vassalli JD, Wilks JW, Schweigerer L, Orci L, Montesano R(1994) Modulation of bovine microvascular endothelial cell proteolytic properties by inhibitors of angiogenesis. J Cell Biochem 55:419–434

Petitclerc E, Boutaud A, Prestayko A, Xu J, Sado Y, Ninomiya Y, Sarras MP Jr, Hudson BG, Brooks PC (2000) New functions for non-collagenous domains of human collagen type IV. Novel integrin ligands inhibiting angiogenesis and tumor growth in vivo. J Biol Chem 275:8051–8061

Pihlajaniemi T, Rehn M (1995) Two new collagen subgroups: membrane-associated collagens and types XV and XVII. Prog Nucleic Acid Res Mol Biol 50:225–262

Poschl E, Schlotzer-Schrehardt U, Brachvogel B, Saito K, Ninomiya Y, Mayer U (2004) Collagen IV is essential for basement membrane stability but dispensable for initiation of its assembly during early development. Development 131:1619–1628

Ramchandran R, Dhanabal M, Volk R, Waterman MJ, Segal M, Lu H, Knebelmann B, Sukhatme VP (1999) Antiangiogenic activity of restin, NC10 domain of human collagen XV: comparison to endostatin. Biochem Biophys Res Commun 255:735–739

Rehn M, Pihlajaniemi T (1994) Alpha 1(XVIII), a collagen chain with frequent interruptions in the collagenous sequence, a distinct tissue distribution, and homology with type XV collagen. Proc Natl Acad Sci USA 91:4234–4238

Rehn M, Veikkola T, Kukk-Valdre E, Nakamura H, Ilmonen M, Lombardo C, Pihlajaniemi T, Alitalo K, Vuori K (2001)

Interaction of endostatin with integrins implicated in angiogenesis. Proc Natl Acad Sci USA 98:1024–1029

Reynolds LE, Wyder L, Lively JC, Taverna D, Robinson SD, Huang X, Sheppard D, Hynes RO, Hodivala-Dilke KM (2002) Enhanced pathological angiogenesis in mice lacking beta3 integrin or beta3 and beta5 integrins. Nat Med 8:27–34

Saarela J, Rehn M, Oikarinen A, Autio-Harmainen H, Pihlajaniemi T (1998) The short and long forms of type XVIII collagen show clear tissue specificities in their expression and location in basement membrane zones in humans. Am J Pathol 153:611–626

Sasaki T, Larsson H, Tisi D, Claesson-Welsh L, Hohenester E, Timpl R (2000) Endostatins derived from collagens XV and XVIII differ in structural and binding properties, tissue distribution and anti-angiogenic activity. J Mol Biol 301:1179–1190

Streit M, Velasco P, Brown LF, Skobe M, Richard L, Riccardi L, Lawler J, Detmar M (1999) Overexpression of thrombospondin-1 decreases angiogenesis and inhibits the growth of human cutaneous squamous cell carcinomas. Am J Pathol 155:441–452

Streit M, Stephen AE, Hawighorst T, Matsuda K, Lange-Asschenfeldt B, Brown LF, Vacanti JP, Detmar M (2002) Systemic inhibition of tumor growth and angiogenesis by thrombospondin-2 using cell-based antiangiogenic gene therapy. Cancer Res 62:2004–2012

Struman I, Bentzien F, Lee H, Mainfroid V, D'Angelo G, Goffin V, Weiner RI, Martial JA (1999) Opposing actions of intact and N-terminal fragments of the human prolactin/growth hormone family members on angiogenesis: an efficient mechanism for the regulation of angiogenesis. Proc Natl Acad Sci USA 96:1246–1251

Sudhakar A, Sugimoto H, Yang C, Lively J, Zeisberg M, Kalluri R (2003) Human tumstatin and human endostatin exhibit distinct antiangiogenic activities mediated by alpha v beta 3 and alpha 5 beta 1 integrins. Proc Natl Acad Sci USA 100:4766–4771

Sudhakar A, Nyberg P, Keshamouni VG, Mannam AP, Li J, Sugimoto H, Cosgrove D, Kalluri R (2005) Human alpha1 type IV collagen NC1 domain exhibits distinct antiangiogenic activity mediated by alpha1beta1 integrin. J Clin Invest 115:2801–2810

Sund M, Xie L, Kalluri R (2004) The contribution of vascular basement membranes and extracellular matrix to the mechanics of tumor angiogenesis. Apmis 112:450–462

Sund M, Hamano Y, Sugimoto H, Sudhakar A, Soubasakos M, Yerramalla U, Benjamin LE, Lawler J, Kieran M, Shah A, Kalluri R (2005) Function of endogenous inhibitors of angiogenesis as endothelium-specific tumor suppressors. Proc Natl Acad Sci USA 102:2934–2939

Tarui T, Miles LA, Takada Y (2001) Specific interaction of angiostatin with integrin alpha(v)beta(3) in endothelial cells. J Biol Chem 276:39562–39568

Timpl R, Wiedemann H, van Delden V, Furthmayr H, Kuhn K (1981) A network model for the organization of type IV collagen molecules in basement membranes. Eur J Biochem 120:203–211

Timpl R, Sasaki T, Kostka G, Chu ML (2003) Fibulins: a versatile family of extracellular matrix proteins. Nat Rev Mol Cell Biol 4:479–489

Troyanovsky B, Levchenko T, Mansson G, Matvijenko O, Holmgren L (2001) Angiomotin: an angiostatin binding protein that regulates endothelial cell migration and tube formation. J Cell Biol 152:1247–1254

Wen W, Moses MA, Wiederschain D, Arbiser JL, Folkman J (1999) The generation of endostatin is mediated by elastase. Cancer Res 59:6052–6056

Wickstrom SA, Alitalo K, Keski-Oja J (2002) Endostatin associates with integrin alpha5beta1 and caveolin-1, and activates Src via a tyrosyl phosphatase-dependent pathway in human endothelial cells. Cancer Res 62:5580–5589

Wickstrom SA, Alitalo K, Keski-Oja J (2004) An endostatin-derived peptide interacts with integrins and regulates actin cytoskeleton and migration of endothelial cells. J Biol Chem 279:20178–20185

Volpert OV, Fong T, Koch AE, Peterson JD, Waltenbaugh C, Tepper RI, Bouck NP (1998) Inhibition of angiogenesis by interleukin 4. J Exp Med 188:1039–1046

Volpert OV, Zaichuk T, Zhou W, Reiher F, Ferguson TA, Stuart PM, Amin M, Bouck NP (2002) Inducer-stimulated Fas targets activated endothelium for destruction by anti-angiogenic thrombospondin-1 and pigment epithelium-derived factor. Nat Med 8:349–357

Yamaguchi N, Anand-Apte B, Lee M, Sasaki T, Fukai N, Shapiro R, Que I, Lowik C, Timpl R, Olsen BR (1999) Endostatin inhibits VEGF-induced endothelial cell migration and tumor growth independently of zinc binding. EMBO J 18:4414–4423

Yao L, Pike SE, Setsuda J, Parekh J, Gupta G, Raffeld M, Jaffe ES, Tosato G (2000) Effective targeting of tumor vasculature by the angiogenesis inhibitors vasostatin and interleukin-12. Blood 96:1900–1905

Yi M, Ruoslahti E (2001) A fibronectin fragment inhibits tumor growth, angiogenesis, and metastasis. Proc Natl Acad Sci USA 98:620–624

Yi M, Sakai T, Fassler R, Ruoslahti E (2003) Antiangiogenic proteins require plasma fibronectin or vitronectin for in vivo activity. Proc Natl Acad Sci USA 100:11435–11438

Zhang SX, Sima J, Shao C, Fant J, Chen Y, Rohrer B, Gao G, Ma JX (2004) Plasminogen kringle 5 reduces vascular leakage in the retina in rat models of oxygen-induced retinopathy and diabetes. Diabetologia 47:124–131

Thrombospondins and Angiogenesis

13

NATHAN V. LEE and M. LUISA IRUELA-ARISPE

Abstract

Thrombospondins (TSPs) include a multimeric family of secreted glycoproteins broadly expressed by several cells and tissues. They have been shown to support cell attachment through interactions with multiple cell-adhesion receptors, bind to other extracellular matrix proteins, and regulate cell shape, adhesion and migration. At the tissue level, TSPs have been implicated in the regulation of several complex processes, including angiogenesis and wound healing. More recently, some TSPs have been associated with genetic predisposition for myocardial infarction and vascular disease through genome-wide scans and SNP studies. In this chapter, we summarize the current information on the biology of TSPs, focusing more directly on the angioregulatory roles of TSP1 and -2. We will further discuss the most recent genetic and biochemical advances and their therapeutic exploration.

CONTENTS

13.1 Thrombospondins: Structure, Synthesis and Degradation

The first and prototypical member of the thrombospondin (TSP) family, TSP1, was discovered using biochemical techniques and reported as a high-molecular-weight glycoprotein present in platelets (Lawler et al. 1978). The other four members of the family were identified because of the quick advances in cloning and were subsequently reported in the early to middle 1990s. The link between the five members of the TSP family relies more specifically on their structure than on their overall amino acid similarities. The multidomain organization of the TSP1 and -2 -includes five basic domains that comprise, from amino- to carboxyl-terminal: (a) an amino-terminal heparin-binding domain,

(b) the procollagen or von Willebrand's factor region, (c) three type I or properdin repeats, (d) three type II or EGF-like repeats, (e) seven type III or Ca^{2+}-binding domains and (f) a carboxyl-terminal cell-binding domain. The other three TSPs (3–5) lack many of the amino-terminal domains, specifically the heparin-binding region, the procollagen region and the properdin repeats (Fig. 13.1). Due to these differences, the five TSP proteins have been further classified into two main subgroups. Subgroup A includes the long TSPs 1 and 2 that are organized as homotrimers, while TSPs 3–5 comprise subgroup B and are organized as pentamers (Adams and Lawler 2004). Several studies have stressed the relevance of oligomerization for specific properties displayed by the different TSP proteins. For example, the trimeric structure of TSP1 and -2 is essential for cell spreading and fascin spike organization (Anilkumar et al. 2002). Overall, the structure of all TSPs resembles a bouquet of flowers in which the coiled-coil domain located in the amino-terminal portion provides the link between the three arms of subgroup A and the five arms of each protein in subgroup B. This structure most likely offers the opportunity to create multivalent interactions of the carboxyl-terminal domain with cell-surface receptors, while other areas of the protein can interact with matrix proteins.

Although the structural differences for each of the TSP proteins impact their overall functional properties, i.e., the anti-angiogenic features are specific to TSP1 and -2, all of the TSPs share some fundamental cellular themes. For example, all five TSPs have been shown to support cell attachment in a calcium-dependent manner and interact with many other extracellular matrix glycoproteins and proteoglycans.

The regulation of TSP synthesis has been most studied for TSP1 and -2. One of the first features identified in the TSP1 promoter was the presence of a serum-responsive element; thus, most cells in culture express high levels of TSP1. The biological relevance of this expression, however, should be interpreted with caution, as in vivo, expression of TSP1 is relatively low in most uninjured tissues, except for the ovary and bone marrow. In contrast, during pathological/repair settings, TSP1 is rapidly and robustly induced. This has been demonstrated in a variety of cancers, wound healing, arthritis, and endometrial repair following menstruation (Alvarez et al. 2001; Arbeille et al. 1991; Bertin et al. 1997; Iruela-Arispe et al. 1996). Thus, it is not surprising to note that TSP1 is upregulated after exposure to sev-

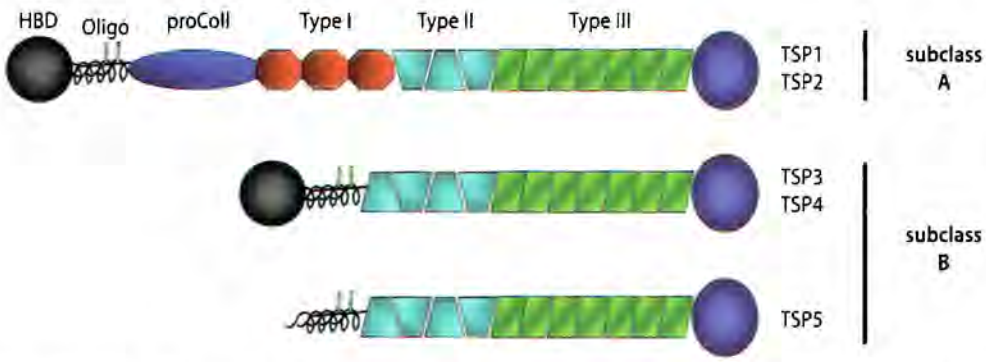


Fig. 13.1. The thrombospondin family. The five members of the TSP family are subdivided into subclass A, which comprises TSP1 and -2, and subclass B, which includes TSP3, -4, and -5. The structural domain organization of TSP1 and -2 is identical and comprises a heparin binding domain (*HBD*), an oligomerization region (*Oligo*), a procollagen domain (*proColl*), three type I (properdin or TSR) domains, three type II (or EGF) domains, seven type III (or calcium-binding) repeats and a carboxyl-terminal domain (*CBD*). The other TSPs lack amino-terminal domains, such as proColl and TSR repeats

eral growth factors in vitro, including PDGF, FGF-2, TGF-beta, KGF and TNF-alpha, heat shock and hypoxia (Adams and Tucker 2000).

Like many other matrix proteins, TSP1 is modified in the extracellular environment by proteolysis. In fact, intact TSP1 can be detected in vivo only when isolated from platelet alpha-granules. Purification of TSP1 from tissue has indicated that extracellular processing events are multiple and, in some cases, tissue-specific. This information underscores the relevance of understanding extracellular proteolysis to the functional properties of this protein. Thus far, TSP1 has been shown to be cleaved by thrombin, factor XIIIa, cathepsin, and elastase (Adams and Tucker 2000; Hogg 1994). However, there is very little information as to the biological significance of these fragments. While the tendency is to assume that cleavage equals degradation, a large body of evidence is now demonstrating that when it comes to extracellular matrix proteins, processing can generate a pool of polypeptides with functions that either enhance, nullify, or modify the contribution of these proteins to specific biological processes (Lopez-Otin and Overall 2002; Overall and Lopez-Otin 2002).

13.2 Functional Properties

TSP1 and -2 act at the cell–matrix interface to provide contextual cues important in matrix assembly and cell function. Thus, they appear to coordinate cell–matrix cross-talk. The regulation of matrix structure occurs via its ability to bind directly to fibronectin, fibrin and fibrillar collagens and by modulating MMP and plasmin activities. As this coordination of extracellular proteins takes place, TSPs also convey migratory, proliferative, apoptotic and/or adhesive signals to cells. This sophistication of cellular regulation, as well as matrix coordination has prompted the classification of TSPs as matricellular proteins (Bornstein 2000), a term that encompasses the ability of these proteins to serve as molecular bridges between the extracellular matrix and the cell surface. This has been most clearly demonstrated during wound healing, where TSPs act temporally and spatially to transmit signals that trigger specific cellular responses in the wound. A major function of TSP in this regard has been its ability to regulate cell adhesion. Nonetheless, TSP1 also participates in platelet aggregation, inflammatory response and regulation of angiogenesis during both wound healing and tumor growth. In many cases, some of these functions are shared with its closest relative, TSP2. This is not surprising given the similarities between their structures.

In addition to their well-known roles during development, TSPs also fulfill important functions in the homeostasis of several tissues, and loss of these proteins results in deleterious effects to a number of organs, including lung, skin, blood vessels and bone. The specific functions attributed to TSP1 and -2 are discussed below.

13.2.1 The Link Between Function and Expression

Although it is easy to think of TSP1 and TSP2 together due to their structural resemblance, it is critical to keep in mind that these proteins display almost exclusive patterns of expression during development and in the adult. TSP1 expression is frequently associated with epithelial/endothelial tissues, in contrast to the more stromal/mesenchymal nature of TSP2 (Iruela-Arispe et al. 1993, 1996; Kyriakides et al. 1998). The regulation of both promoters is also notably different. TSP1 is rapidly induced by injury and inflammatory mediators (Bornstein 1992, 2000, 2001; Chen et al. 2000). In contrast, expression of TSP2 does not seem to be affected by the same cohort of inflammatory cytokines (Bornstein et al. 2000; Chen et al. 2000). Interestingly, both proteins have been shown to inhibit angiogenesis in vivo and in vitro (Armstrong and Bornstein 2003; Lawler and Detmar 2004).

13.2.2 The Prototypical Endogenous Angiogenesis Inhibitor

The anti-angiogenic properties of TSP1 were first described by Bouck and colleagues in a search for proteins upregulated by tumor suppressor genes (Good et al. 1990; Polverini 1996). Since then, the angiostatic/angiotoxic effects of TSP1 have been well documented using a large spectrum of assays and models (Canfield and Schor 1995; Doll et al. 2001; Iruela-Arispe et al. 1991, 1999; Sheibani et al. 1997; Taraboletti et al. 1997; Tolsma et al. 1993). In addition, the sum of genetic manipulations that result in TSP1 overexpression using tissue-specific promoters strongly supports the participation of this protein in the regulation of vascular growth and vessel diameter. Specifically, two transgenic overexpression studies using the K14 promoter to drive TSP1 in the skin (Hawighorst et al. 2002; Streit et al. 2000) and the MMTV promoter to target TSP1 expression to the mammary epithelium (Rodriguez-Manzaneque et al. 2001) support a role for this protein in the regulation of vascular morphogenesis in vivo. Combined, these studies argue for the contribution of TSP1 in the regulation of vascular growth in whole-animal settings during normal and pathological conditions. The studies also support the concept that TSP1 is a pleiotropic regulator of vascular morphogenesis, with roles far more elaborate than restricting the number of available endothelial cells through regulation of apoptosis. Particularly, both studies showed that TSP1 modulates the size of vascular channels. These functions are also shared by TSP2 (Lawler and Detmar 2004).

Loss-of-function studies also support a direct role for TSP1 and -2 in the regulation of angiogenesis during tumor expansion. Tumor progression is greatly accelerated in mice that are null for TSP1 and also have one of the following genetic profiles: $p53^{-/-}$, $APCMin/^{+}$ or amplification of neu/erbB2 oncogene (Gutierrez et al. 2003; Lawler et al. 2001; Rodriguez-Manzaneque et al. 2001). Furthermore, the survival of p53-null and p53-heterozygous mice is reduced in the absence of TSP1 (Lawler et al. 2001).

In addition to their effects on genetically induced models of cancer, TSP1 and -2 have also been shown to accelerate tumor progression in chemically induced models. For example, frequency of tumor lesions, degree of malignancy, and lymph node metastasis was greater in TSP2-deficient mice (Hawighorst et al. 2001).

Of direct importance to angiogenesis, TSP1 is down-regulated by a significant and impressive pool of oncogenes, including c-fos, c-jun, v-src, Ras and myc (Mettouchi et al. 1994; Watnick et al. 2003). This explains the marked reduction in TSP1 transcript levels noted in all carcinomas examined. The reduction in TSP facilitates angiogenic progression and tumor expansion. Interestingly, tumors are frequently contained by the high TSP1 and -2 response mounted by the host stroma at the interface between the tumor and the normal tissue. This response is not present in TSP1- and -2-deficient mice, providing an explanation of the rampant growth observed in all the experimental tumors examined.

The mechanism developed by tumors to suppress TSP1 expression has been more clearly documented for Ras (Watnick et al. 2003). In these tumors, high levels of oncogenic Ras lead to hyperactivation of PI3 kinase/Rho that, when combined with myc mutation, results in a drastic reduction of TSP1 mRNA. The mechanisms behind myc-mediated suppression of TSP1 are complex and appear to rely more on the increase of mRNA turnover than in the suppression of transcription (Janz et al. 2000). In addition to these events, epigenic suppression of TSP1 has also been described in human neuroblastoma (Yang et al. 2003). Here, loss of TSP1 has been associated with methylation of CpG islands located in the 5' region of the TSP1 gene.

A large number of preclinical studies have been performed with TSP1 protein, protein fragments or peptide mimetics. The efficacy of TSP1 has been scrutinized in a large array of tumor types, either as xenografts or as endogenous transgenic tumors. For example, systemic exposure of tumor-bearing mice to TSP1 protein or peptides has been shown to inhibit tumor growth (Guo et al. 1997a). Combination therapy with TSP1 has revealed impressive outcomes. For example, a combination of radiotherapy

and exogenous TSP1 prevents the growth of dormant micrometastases from grafted human melanomas (Rofstad et al. 2003). Other groups have documented similar findings with short peptides based on the sequence located in the TSR of TSP1 (Vailhe and Feige 2003). More recently, clinical trials have been initiated with similar peptides, and their outcome will shortly be revealed.

13.2.2.1 Structure–Function Studies

The anti-angiogenic domain in TSP1 and TSP2 has been mapped to the type I (TSR or properdin) repeats present in both TSP1 and -2, but absent from TSP3–5 (Guo et al. 1997b; Iruela-Arispe et al. 1999; Jimenez et al. 2000; Shafiee et al. 2000; Tolsma et al. 1993, 1997). This structural feature has explained the functional differences, i.e., anti-angiogenic properties, between subgroups A and B in the TSP family.

Interestingly, presence of the TSR domain is not unique to TSP1 or -2. In fact, TSR domains have been found in a large number of proteins and this has been used to link these proteins into another group called the thrombospondin repeat (TSR) superfamily. Most of the proteins in this family have been included based on sequence analysis; a few have been also evaluated functionally to determine their potential anti-angiogenic effects (Iruela-Arispe et al. 2004) and other actions. Interestingly, some of these proteins have been shown to play important roles in cell–cell and cell–matrix interactions (TRAP, properdin and BAI), neuronal guidance (F-spondin, SCO-spondin, and semaphorins) and extracellular proteolysis (ADAMTS family) (for a review on this subject see Tucker 2004). A blast search shows that there are 187 TSRs in the human genome, distributed in 41 different human proteins (Tan et al. 2002a,b). Interestingly, TSRs are also found in *Drosophila* (a total of 45 in 14 proteins) and in *Caenorhabditis elegans* (total 90 in 27 proteins).

Structurally, the type I repeats display a rather unique antiparallel three-stranded structure. The first strand (the A strand) is irregular, but the other two strands (B and C) have a beta structure. The loop located between the B strand and the C strand has two prominent "handles" that appear to be stabilized by hydrogen bonds between the glutamic acid residue and the first tryptophan in the A strand, but the other polar residues of all of the conserved tryptophans are exposed to ligands. The analysis of TSR structure has contributed to our understanding of some of the experimental observations initiated by empirical means. For instance, two of the peptides known to block angiogenesis in vivo and in vitro have been mapped to the exposed sequences on the A and B strands, while some nonreproducible data from a peptide reported to block CD36 correspond to the AV loop that was found to be O-fucosylated and involved in disulfide bonding. The information from these structural studies have helped guide the development of a new generation of therapeutic reagents aiming at suppressing vascular growth. This information is yet to become available to the scientific community at large, but it has engendered a lot of excitement in meetings related to this field of study.

Functionally, the TSR repeats in TSP1 and -2 have been shown to bind to CD36 and to support attachment of multiple cell types. However as it relates to suppression of angiogenesis, TSR functions in a multifaceted mode that so far is known to include: (1) induction of endothelial cell apoptosis; (2) inhibition of endothelial cell migration; (3) direct interaction with growth factors; and (4) regulation of extracellular enzymatic activity. The effects of TSP1 on endothelial cell apoptosis are reportedly mediated by CD36, a cell surface receptor located on endothelial cells, monocytes, platelets and endothelial cells. A series of studies using mice null for CD36, c-jun, and fyn have shown that all these are required for the induction of endothelial cell apoptosis triggered by TSP1 (Jimenez et al. 2000; Armstrong and Bornstein 2003). The downstream effectors include caspase 3 and p38 (Jimenez et al. 2000). Furthermore, TSP1 downregulates several survival pathways (Armstrong and Bornstein 2003). The effect of TSP1 on migration has not been explored in great detail, but may relate to its ability to suppress adhesion and modify the cytoskeleton. Binding of TSP1 to growth factors has been a typical property of this protein. TSP1 has been shown to bind and

activate TGF-beta (Murphy-Ullrich and Poczatek 2000; Young and Murphy-Ullrich 2004), and to bind and to suppress the functional properties of VEGF and FGF-2 (Gupta et al. 1999). The regulation of extracellular enzymatic activity by TSP1 has been known for more than a decade, but its biological significance was only recently appreciated. TSP1 is an inhibitor of plasmin, uPA, neutrophil elastase, matrix metalloproteinase (MMP)2 and MMP9 (Anonick et al. 1993). The anticipated biological implications associated with these effects are multiple but, again, contextual. Experimentally, we have shown that TSP1 can inhibit activation of pro-MMP9 by MMP3 and therefore restrict the availability of active MMP9. In turn, lack of active MMP9 prevents the release of VEGF from the extracellular matrix influencing angiogenesis and tumor progression (Rodriguez-Manzaneque et al. 2001).

Clearly, the vast body of experimental support for an anti-angiogenic role of TSP1 has propelled interest in the development of therapeutic strategies aimed at exploiting this function in cancer. In all fairness, however, a few studies have also proposed a pro-angiogenic role for TSP1 under some experimental conditions (de Fraipont et al. 2001). The pro-angiogenic effect has been attributed to either the presentation of the protein (bound versus soluble) and/or to the ability of this protein to mediate the recruitment and migration of smooth muscle and inflammatory cells that release pro-angiogenic factors. While this controversy is yet to be resolved, the overwhelming evidence for a vasculosuppressive role has been the major force behind the pursuit of clinical trials, which have now advanced to phase III, supporting the success of a TSP therapy. Returning to the issue of bound versus soluble, it has been well recognized that the amino-terminal region of TSP1, also known as the heparin-binding domain, conveys opposite signals to cells depending on its soluble or bound status. Thus, when soluble this region is de-adhesive, while when it is immobilized it can support cellular adhesion to a variable extent (Ferrari do Outeiro-Bernstein et al. 2002). The valency of this domain also contributes to its effects on cells. When presented as a monomer this region inhibits basal and FGF2-induced chemotaxis, but as a trimer it stimulates chemotaxis (Calzada et al. 2003). One explanation is that depending on its form, this region might be able to ligate multiple and/or a different array of receptors; convey altered affinity, and/or alter uptake and receptor turnover. While the mechanistic details behind these effects are yet to be understood, it is important to stress that presentation and monomeric/trimeric status can translate into different cellular effects.

13.2.3 TSP Function in Wound Healing

Tissue damage is almost always associated with bleeding and the formation of a hemostatic plug, which provides a means to prevent excessive blood loss and enables the formation of provisional matrix in which cell repair will be initiated (Fig. 13.2). Degranulation of platelets mediates the release of growth factors and adhesive proteins that facilitate an inflammatory response and stimulate the proliferation of fibroblasts and keratinocytes. Along with growth factors, TSP1 also becomes incorporated in the hemostatic plug and in the provisional matrix that expands into the dermis. The presence of TSP1 facilitates the formation of a multiprotein complex through its ability to bind to fibronectin and fibrin. This fibrin–fibronectin–TSP1 provisional matrix acts as a framework that facilitates and stimulates cell adhesion and migration. Furthermore, TSP1 is quickly upregulated and secreted by the fibroblasts associated with the wound. The kinetics of this process have been carefully evaluated and shown to be dynamic, with high levels of TSP1 during the first day post-wound and decreasing thereafter to almost negligible transcript levels by day 4 post-wound (Reed et al. 1995). TSP2 is also secreted by the dermal fibroblasts, but with slower kinetics (Reed et al. 1993). The biological relevance of this upregulation is still under study; however, considering the properties of TSP1 and -2 in matrix assembly; the central speculation is that these proteins directly participate in the reorganization of the wounded matrix. Furthermore, the presence of TSP delays a rampant angiogenic response and enables the

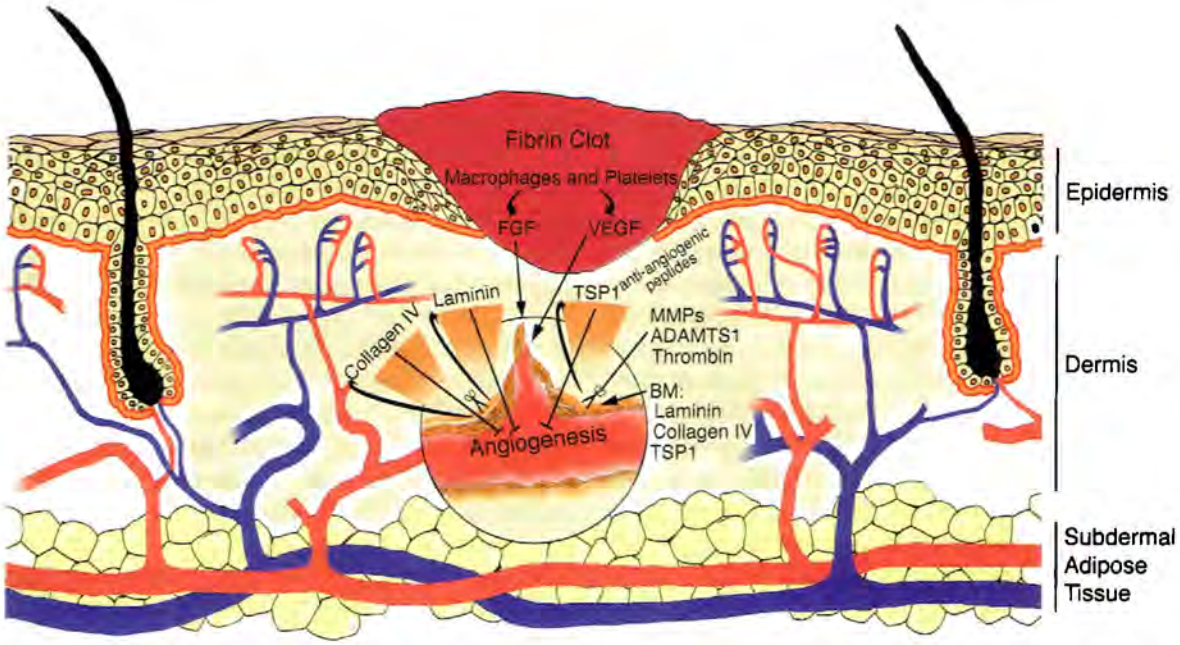


Fig. 13.2. Schematic representation of the molecular processes associated with dermal wound healing. Dermal repair requires growth of blood vessels in a manner similar to that stimulated by a tumor. The fibrin clot, located in the epidermis and dermis, contains growth factors released from the activated platelets during coagulation. The gradient of FGF and VEGF stimulates growth of blood vessels from pre-existent vessels. The process, however, is regulated by the presence of TSP1 and TSP2 in the extracellular environment

organization of capillary growth in a more orderly fashion, less permeable and with thinner diameters. The experimental support for these conclusions comes from the evaluation of wound-healing events in TSP1- and TSP2-null mice, as well as in K14-TSP1-/TSP2-overexpressing mice.

Of paramount relevance during wound healing is the contribution of TSP1 to the activation of TGF-beta. The effect has been mapped to a tripeptide (RFK) that is located in the type I repeats of TSP1 but not present in TSP2 (Schultz-Cherry et al. 1995).

Furthermore, a clear understanding of TSP1 biology during wound healing, has to take into consideration processing and proteolysis. Particularly in this biological scenario, it is extremely likely that TSP1 undergoes multiple, time- and space-restricted proteolytic events with impact on its biological properties. While much has been done to understand the kinetics of TSP1 expression and its spatial distribution in relation to tissue-healing progression, less is known about the spectrum of polypeptides released upon TSP1 exposure to extracellular proteases and its potential biological functions. Some of the enzymes that cleave TSP1 have been already discussed; nonetheless, to date, no in vivo experimentation has addressed this question directly.

The role of TSP2 in matrix assembly and wound healing was more clearly elucidated by studies in the TSP2-null mouse (Kyriakides et al. 1998). Lack of TSP2 results in altered collagen fibrillogenesis with consequences for matrix organization, vascularization and tissue repair. A more in-depth analysis of this phenotype revealed that TSP2 binds MMP2 and suppresses its catalytic properties (Yang et al. 2000). Fibroblasts that lack TSP2 showed adhesion and spreading defects in vitro, a

phenotype that is explained by the excess of MMP2 in those cultures (Yang et al. 2000). These findings were further confirmed in the TSP2-null mouse and explain the wound-healing defects, as well as the hemorrhages in the dermis of these mice (Yang et al. 2001).

Combined, the studies of TSP1 and -2 during wound healing have revealed their potential for acceleration of wound repair and minimization of scar tissue (Bornstein et al. 2004). At the moment, there are no clinical applications for TSP-derived therapies aimed at wound healing. The reasons are the multiple contextual functions displayed by these proteins and the difficulty in determining precisely when and where would they be beneficial. As the molecular nuances of TSP1 biology begin to unravel, its exploitation in therapies aiming at tissue repair will most likely flourish.

13.2.4 Additional Functions Revealed in Genetically Modified Mice and SNP Studies

Similarly to many other proteins, the inactivation of TSP1 and -2 in mice has revealed some unpredicted physiological activities performed by these proteins (Kyriakides et al. 1999; Lawler et al. 1998). TSP1-null mice develop and breed normally; however, they show some degree of embryonic lethality (25–35%) for reasons that are yet to be understood. They also have a mild defect in wound healing, which is exacerbated in $TSP1^{-/-}$ $TSP2^{-/-}$ mice (Agah et al. 2002). In addition, TSP1-null mice display mild skeletal defects, most noticeable in the spinal cord, and they have increased susceptibility to pneumonia and inflammation (Lawler et al. 1998). The pulmonary findings were unexpected and revealed a previously unknown role for TSP1 in lung homeostasis. It is likely that the mechanism behind this effect relates to the role of TSP1 in TGF-beta activation (Crawford et al. 1998a,b; Schultz-Cherry and Murphy-Ullrich 1993). In fact, some of the effects can be significantly diminished by delivery of TGF-beta-activating TSP1 peptide (Crawford et al. 1998b). Another unexpected finding in the TSP1-null mouse was hyperplasia of pancreatic islands, indicating that TSP1 is a pleiotropic growth regulator of epithelial expansion (Lawler et al. 1998). Again, reconstitution of the TGF-beta activation activity using TSP1 peptides reversed this pathology (Crawford et al. 1998b). However, the skeletal defects and the wound-healing problems were not reduced by the inclusion of this peptide. Together, the findings revealed that some, but not all, of the phenotypes displayed by TSP1 were linked to TGF-beta (Crawford et al. 1998b).

Added to its contribution as an anti-angiogenic modulator, TSP2 has complex and important roles in the assembly of connective tissue and extracellular matrix that were only uncovered through studies in the TSP2-null mouse (for review see Lawler 2000).

In addition to the functions discussed above, TSP1, -2 and -4 have been identified as novel risk factors for familial premature myocardial infarction by analysis of a series of single-nucleotide polymorphisms (SNPs) (Topol et al. 2001). The initial findings have been corroborated by two subsequent studies that yielded similar outcomes. Because of the important implications of these studies, we will discuss them more in depth. The study conducted by Topol and colleagues was quite rigorous: genetic association was considered only in high-frequency (>5%) SNPs. Criteria for the patient population included at least one affected sibling with history of premature (males, <45 years old; females, <50 years old) coronary artery disease, which was defined as myocardial infarction (54% of patients), percutaneous coronary intervention, or stenosis (70%) of a major epicardial artery by angiography. The control group consisted of 418 unselected Caucasian Americans; more importantly, it did not exclude individuals with coronary artery disease. Considering this stringency, the study probably underestimated the disease association of the test SNPs. Interestingly, the three SNPs showing the highest and most significant association with the disease were all the members of the TSP family (1, 2 and 4). Following this study, two additional large-scale SNP analysis have been reported with slightly different experimental approaches. The first was conducted

in 909 unrelated myocardial infarction patients and then expanded to 2,819 myocardial infarction subjects and 2,244 controls evaluating 112 SNPs in 71 candidate genes (Yamada et al. 2002). In that study too, one of the TSP4 SNPs was highly correlated with myocardial infarction in males. A following study of 3,085 unrelated subjects with coronary artery disease and 2,832 controls was analyzed, and there too the TSP4 polymorphism was found (Kato et al. 2003).

Although substantial additional evidence is needed to fully understand the findings regarding the SNPs and the mechanisms of action, the results presented to date indicate marked effects of these SNPs on the structure and function of TSPs. Worth mentioning is the induction of TSP in atherosclerotic lesions (Riessen et al. 1998). Further supporting these predictions, no coronary abnormality has been reported in the TSP1-null mouse (Crawford et al. 1998b; Lawler et al. 1998), which would support a gain of function induced by the TSP SNPs, as proposed. Naturally, experiments to test this hypothesis would include the development of TSP1 overexpressors and TSP4 mutants that mimic the SNPs shown by the human studies.

Compared to TSP1 and -2, less is known about the functional properties displayed by the TSPs 3, 4 and 5. TSP5, also known as COMP (cartilage oligomeric matrix protein), is mostly expressed in cartilage and has roles related to chondrocyte growth, survival and differentiation (reviewed in Unger and Hecht 2001). In fact, mutations in the coding region of TSP5 result in pseudoachondroplastic dysplasia, a hereditary autosomal dominant disorder that result in a premature arrest of bone growth and laxity of joints (Merritt et al. 2006).

13.2.5 Effects of TSPs at the Cellular Level

As alluded to previously, all TSPs have been shown to support cell attachment and participate in the assembly of the extracellular matrix via their selective, but multiple interactions with other matrix proteins. Depending on the cell type and on the specific family member, TSPs have been also implicated in cell migration and regulation of the cytoskeleton; cell growth and apoptosis; modulation of growth factors and protease function. Due to the myriad of effects, the specific role of TSPs in a particular biological process have been difficult to delineate with precision, as the specific context of matrix and growth factors can alter the outcome of TSP functions.

Furthermore, the array of responses mediated by TSPs relies on the specific cohort of receptors present at the cell surface. The list of receptors identified for TSP1 is extensive and cell-specific. They include: CD36 on monocytes, endothelial cells, epithelial cells and platelets (Vischer and Buddecke 1991); several integrins (Lawler and Hynes 1989; Lawler et al. 1988; Vischer 1990; Yabkowitz et al. 1993); integrin-associated protein (IAP or CD47) (Gao and Frazier 1994); and proteoglycans (Adams 2001). Integrins that recognize TSP1 include alphaIIb beta3 on platelets (Lawler and Hynes 1989), alpha4 beta1 in lymphocytes (Li et al. 2002), and alphaV beta3 (Lawler et al. 1988), alpha3 beta1 (Krutzsch et al. 1999), and alpha6 beta1 (Calzada et al. 2003) on vascular cells. The beta3 integrins recognize TSP1 in an RGD-dependent manner, while the other integrins appear to interact with TSP1 through other regions. The different receptors are responsible for mediating distinct and even opposite cellular responses to TSP1. For example, TSP1 mediates chemotaxis of smooth muscle cells through CD47 (Wang and Frazier 1998) and alphaV beta3 (Patel et al. 1997). It promotes proliferation of smooth muscle cells through the same integrin. (Stouffer et al. 1998). In contrast; TSP1 induces apoptosis of fibroblasts and T cells via CD47 (Graf et al. 2002; Manna and Frazier 2003) and apoptosis of endothelial cells via CD36 (Jimenez et al. 2000). As can be concluded from the example above, the engagement of multiple receptors results in complex responses that, in several cases, are cell-specific. Thus, extrapolation of results from one cell type to the next is not an option when it comes to TSPs.

Also pertinent to their cellular function, levels of TSP1 and TSP2 are regulated by uptake through endocytosis followed by degradation in the lysosomal compartment. Endocytosis is mediated by the low-density lipoprotein receptor in a complex with calreticulin, two additional TSP receptors (Godyna et al. 1995). Interestingly, from all of the receptors identified for TSP thus far, none is TSP1 or -2 specific. These are all shared by a cohort of matrix proteins; consequently, the context, i.e., abundance of other matrix proteins, as well as relative affinities, also play an important role in the specific response conveyed by TSP1.

Finally, an important function attributed to TSP1 has been its ability to bind and sequester growth factors. For example, the 140-kDa carboxyl-terminal domain of TSP1 binds FGF-2 and VEGF, blocking their respective effects on endothelial cells (Kanda et al. 1999).

Emergent Themes for Future Research

Our understanding of the biological functions of the TSP family of proteins has increased in a remarkable manner during the past 10 years. However, the quest for the biological and molecular function of TSPs continues to grow. While the contribution made by the knockouts has been extremely valuable, they have not answered all the questions. In particular, a detailed mechanistic understanding of TSP1 signaling remains elusive; while many receptors have been identified, the sum of their pathways towards a particular functional outcome is still unclear. Much work remains to be done to bring us closer to a more comprehensive knowledge of the cellular responses to TSP and enable future therapeutic exploration.

Acknowledgements

This work was supported by grants from the National Institutes of Health to MLIA (CA77420 and CA65624). Nathan V. Lee was supported by the Vascular Biology Training Grant at UCLA (T32HL69766).

References

Adams JC (2001) Thrombospondins: multifunctional regulators of cell interactions. Annu Rev Cell Dev Biol 17:25–51

Adams JC, Lawler J (2004) The thrombospondins. Int J Biochem Cell Biol 36:961–968

Adams JC, Tucker RP (2000) The thrombospondin type 1 repeat (TSR) superfamily: diverse proteins with related roles in neuronal development. Dev Dyn 218:280–299

Agah A, Kyriakides TR, Lawler J, Bornstein P (2002) The lack of thrombospondin-1 (TSP1) dictates the course of wound healing in double-TSP1/TSP2-null mice. Am J Pathol 161:831–839

Alvarez AA, Axelrod JR, Whitaker RS, Isner PD, Bentley RC, Dodge RK, Rodriguez GC (2001) Thrombospondin-1 expression in epithelial ovarian carcinoma: association with p53 status, tumor angiogenesis, and survival in platinum-treated patients. Gynecol Oncol 82:273–278

Anilkumar N, Annis DS, Mosher DF, Adams JC (2002) Trimeric assembly of the C-terminal region of thrombospondin-1 or thrombospondin-2 is necessary for cell spreading and fascin spike organisation. J Cell Sci 115:2357–2366

Anonick PK, Yoo JK, Webb DJ, Gonias SL (1993) Characterization of the antiplasmin activity of human thrombospondin-1 in solution. Biochem J 289:903–909

Arbeille BB, Fauvel-Lafeve FM, Lemesle MB, Tenza D, Legrand YJ (1991) Thrombospondin: a component of microfibrils in various tissues. J Histochem Cytochem 39:1367–1375

Armstrong LC, Bornstein P (2003) Thrombospondins 1 and 2 function as inhibitors of angiogenesis. Matrix Biol 22:63–71

Bertin N, Clezardin P, Kubiak R, Frappart L (1997) Thrombospondin-1 and -2 messenger RNA expression in normal, benign, and neoplastic human breast tissues: correlation with prognostic factors, tumor angiogenesis, and fibroblastic desmoplasia. Cancer Res 57:396–399

Bornstein P (1992) Thrombospondins: structure and regulation of expression. FASEB J 6:3290–3299

Bornstein P (2000) Matricellular proteins: an overview. Matrix Biol 19:555–556

Bornstein P (2001) Thrombospondins as matricellular modulators of cell function. J Clin Invest 107:929–934

Bornstein P, Armstrong LC, Hankenson KD, Kyriakides TR, Yang Z (2000) Thrombospondin 2, a matricellular protein with diverse functions. Matrix Biol 19:557–568

Bornstein P, Agah A, Kyriakides TR (2004) The role of thrombospondins 1 and 2 in the regulation of cell-matrix

interactions, collagen fibril formation, and the response to injury. Int J Biochem Cell Biol 36:1115–1125

Calzada MJ, Sipes JM, Krutzsch HC, Yurchenco PD, Annis DS, Mosher DF, Roberts DD (2003) Recognition of the N-terminal modules of thrombospondin-1 and thrombospondin-2 by alpha6beta1 integrin. J Biol Chem 278:40679–4087

Canfield AE, Schor AM (1995) Evidence that tenascin and thrombospondin-1 modulate sprouting of endothelial cells. J Cell Sci 108:797–809

Chen H, Herndon ME, Lawler J (2000) The cell biology of thrombospondin-1. Matrix Biol 19:597–614

Crawford SE, Flores-Stadler E.M, Huang L, Tan XD, Ranalli M, Mu Y, Gonzalez-Crussi F (1998a) Rapid growth of cutaneous metastases after surgical resection of thrombospondin-secreting small blue round cell tumor of childhood. Hum Pathol 29:1039–1044

Crawford SE, Stellmach V, Murphy-Ullrich JE, Ribeiro SM, Lawler J, Hynes RO, Boivin GP, Bouck N (1998b) Thrombospondin-1 is a major activator of TGF-beta1 in vivo. Cell 93:1159–1170

De Fraipont F, Nicholson AC, Feige JJ, van Meir EG (2001) Thrombospondins and tumor angiogenesis. Trends Mol Med 7:401–407

Doll JA, Reiher FK, Crawford SE, Pins MR, Campbell SC, Bouck NP (2001) Thrombospondin-1, vascular endothelial growth factor and fibroblast growth factor-2 are key functional regulators of angiogenesis in the prostate. Prostate 49:293–305

Ferrari do Outeiro-Bernstein MA, Nunes SS, Andrade AC, Alves TR, Legrand C, Morandi V (2002) A recombinant NH(2)-terminal heparin-binding domain of the adhesive glycoprotein, thrombospondin-1, promotes endothelial tube formation and cell survival: a possible role for syndecan-4 proteoglycan. Matrix Biol 21:311–324

Gao AG, Frazier WA (1994) Identification of a receptor candidate for the carboxyl-terminal cell binding domain of thrombospondins. J Biol Chem 269:29650–29657

Godyna S, Liau G, Popa I, Stefansson S, Argraves WS (1995) Identification of the low density lipoprotein receptor-related protein (LRP) as an endocytic receptor for thrombospondin-1. J Cell Biol 129:1403–1410

Good DJ, Polverini PJ, Rastinejad F, Le Beau MM, Lemons RS, Frazier WA, Bouck NP (1990) A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. Proc Natl Acad Sci USA 87:6624–6628

Graf R, Freyberg M, Kaiser D, Friedl P (2002) Mechanosensitive induction of apoptosis in fibroblasts is regulated by thrombospondin-1 and integrin associated protein (CD47). Apoptosis 7:493–498

Guo N, Krutzsch HC, Inman JK, Roberts DD (1997a) Thrombospondin 1 and type I repeat peptides of thrombospondin 1 specifically induce apoptosis of endothelial cells. Cancer Res 57:1735–1742

Guo NH, Krutzsch HC, Inman JK, Shannon CS, Roberts DD (1997b) Antiproliferative and antitumor activities of D-reverse peptides derived from the second type-1 repeat of thrombospondin-1. J Pept Res 50:210–221

Gupta K, Gupta P, Wild R, Ramakrishnan S, Hebbel RP (1999) Binding and displacement of vascular endothelial growth factor (VEGF) by thrombospondin: effect on human microvascular endothelial cell proliferation and angiogenesis. Angiogenesis. 3:147–158

Gutierrez LS, Suckow M, Lawler J, Ploplis VA, Castellino FJ (2003) Thrombospondin 1 – a regulator of adenoma growth and carcinoma progression in the APC(Min/+) mouse model. Carcinogenesis. 24:199–207

Hawighorst T, Velasco P, Streit M, Hong YK, Kyriakides TR, Brown LF, Bornstein P, Detmar M (2001) Thrombospondin-2 plays a protective role in multistep carcinogenesis: a novel host anti-tumor defense mechanism. EMBO J 20:2631–2640

Hawighorst T, Oura H, Streit M, Janes L, Nguyen L, Brown LF, Oliver G, Jackson DG, Detmar M (2002) Thrombospondin-1 selectively inhibits early-stage carcinogenesis and angiogenesis but not tumor lymphangiogenesis and lymphatic metastasis in transgenic mice. Oncogene 21:7945–7956

Hogg PJ (1994) Thrombospondin 1 as an enzyme inhibitor. Thromb Haemost 72:787–792

Iruela-Arispe ML, Bornstein P, Sage H (1991) Thrombospondin exerts an antiangiogenic effect on cord formation by endothelial cells in vitro. Proc Natl Acad Sci USA 88:5026–5030

Iruela-Arispe ML, Liska DJ, Sage EH, Bornstein P (1993) Differential expression of thrombospondin 1, 2, and 3 during murine development. Dev Dyn 197:40–56

Iruela-Arispe ML, Porter P, Bornstein P, Sage EH (1996) Thrombospondin-1, an inhibitor of angiogenesis, is regulated by progesterone in the human endometrium. J Clin Invest 97:403–412

Iruela-Arispe ML, Lombardo M, Krutzsch HC, Lawler J, Roberts DD (1999) Inhibition of angiogenesis by thrombospondin-1 is mediated by 2 independent regions within the type 1 repeats. Circulation 100:1423–1431

Iruela-Arispe ML, Luque A, Lee N (2004) Thrombospondin modules and angiogenesis. Int J Biochem Cell Biol 36:1070–1078

Janz A, Sevignani C, Kenyon K, Ngo CV, Thomas-Tikhonenko A (2000) Activation of the myc oncoprotein leads to increased turnover of thrombospondin-1 mRNA. Nucleic Acids Res 28:2268–2275

Jimenez B, Volpert OV, Crawford SE, Febbraio M, Silverstein RL, Bouck N (2000) Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. Nat Med 6:41–48

Kanda S, Shono T, Tomasini-Johansson B, Klint P, Saito Y (1999) Role of thrombospondin-1-derived peptide, 4N1 K, in FGF-2-induced angiogenesis. Exp Cell Res 252:262–272

Kato T, Noda A, Izawa H, Nishizawa T, Somura F, Yamada

A, Nagata K, Iwase M, Nakao A, Yokota M (2003) Myocardial velocity gradient as a noninvasively determined index of left ventricular diastolic dysfunction in patients with hypertrophic cardiomyopathy. J Am Coll Cardiol 42:278–285

Krutzsch HC, Choe BJ, Sipes JM, Guo N, Roberts DD (1999) Identification of an alpha(3)beta(1) integrin recognition sequence in thrombospondin-1. J Biol Chem 274:24080–24086

Kyriakides TR, Zhu YH, Yang Z, Bornstein P (1998) The distribution of the matricellular protein thrombospondin 2 in tissues of embryonic and adult mice. J Histochem Cytochem 46:1007–1015

Kyriakides TR, Leach KJ, Hoffman AS, Ratner BD, Bornstein P (1999) Mice that lack the angiogenesis inhibitor, thrombospondin 2, mount an altered foreign body reaction characterized by increased vascularity. Proc Natl Acad Sci USA 96:4449–4454

Lawler J (2000) The functions of thrombospondin-1 and-2. Curr Opin Cell Biol 12:634–640

Lawler J, Detmar M (2004) Tumor progression: the effects of thrombospondin-1 and -2. Int J Biochem Cell Biol 36:1038–1045

Lawler J, Hynes RO (1989) An integrin receptor on normal and thrombasthenic platelets that binds thrombospondin. Blood 74:2022–2027

Lawler JW, Slayter HS, Coligan JE (1978) Isolation and characterization of a high molecular weight glycoprotein from human blood platelets. J Biol Chem 253:8609–8616

Lawler J, Weinstein R, Hynes RO (1988) Cell attachment to thrombospondin: the role of ARG-GLY-ASP, calcium, and integrin receptors. J Cell Biol 107:2351–2361

Lawler J, Sunday M, Thibert V, Duquette M, George EL, Rayburn H, Hynes RO (1998) Thrombospondin-1 is required for normal murine pulmonary homeostasis and its absence causes pneumonia. J Clin Invest 101:982–992

Lawler J, Miao WM, Duquette M, Bouck N, Bronson RT, Hynes RO (2001) Thrombospondin-1 gene expression affects survival and tumor spectrum of p53-deficient mice. Am J Pathol 159:1949–1956

Li SS, Ivanoff A, Bergstrom SE, Sandstrom A, Christensson B, van Nerven J, Holgersson J, Hauzenberger D, Arencibia I, Sundqvist KG (2002) T lymphocyte expression of thrombospondin-1 and adhesion to extracellular matrix components. Eur J Immunol 32:1069–1079

Lopez-Otin C, Overall CM (2002) Protease degradomics: a new challenge for proteomics. Nat Rev Mol Cell Biol 3:509–519

Manna PP, Frazier WA (2003) The mechanism of CD47-dependent killing of T cells: heterotrimeric Gi-dependent inhibition of protein kinase A. J Immunol 170:3544–3553

Merritt TM, Alcorn JL, Haynes R, Hecht JT (2006) Expression of mutant cartilage oligomeric matrix protein in human chondrocytes induces the pseudoachondroplasia phenotype. J Orthop Res 24:700–707

Mettouchi A, Cabon F, Montreau N, Vernier P, Mercier G, Blangy D, Tricoire H, Vigier P, Binetruy B (1994) SPARC and thrombospondin genes are repressed by the c-jun oncogene in rat embryo fibroblasts. EMBO J 13:5668–5678

Murphy-Ullrich JE, Poczatek M (2000) Activation of latent TGF-beta by thrombospondin-1: mechanisms and physiology. Cytokine Growth Factor Rev 11:59–69

Overall CM, Lopez-Otin C (2002) Strategies for MMP inhibition in cancer: innovations for the post-trial era. Nat Rev Cancer 2:657–672

Patel MK, Lymn JS, Clunn GF, Hughes AD (1997) Thrombospondin-1 is a potent mitogen and chemoattractant for human vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 17:2107–2114

Polverini PJ (1996) How the extracellular matrix and macrophages contribute to angiogenesis-dependent diseases. Eur J Cancer 32A:2430–2437

Reed MJ, Puolakkainen P, Lane TF, Dickerson D, Bornstein P, Sage EH (1993) Differential expression of SPARC and thrombospondin 1 in wound repair: immunolocalization and in situ hybridization. J Histochem Cytochem 41:1467–1477

Reed MJ, Iruela-Arispe L, O'Brien E.R, Truong T, LaBell T, Bornstein P, Sage EH (1995) Expression of thrombospondins by endothelial cells. Injury is correlated with TSP-1. Am J Pathol 147:1068–1080

Riessen R, Kearney M, Lawler J, Isner JM (1998) Immunolocalization of thrombospondin-1 in human atherosclerotic and restenotic arteries. Am Heart J 135:357–364

Rodriguez-Manzaneque JC, Lane TF, Ortega MA, Hynes RO, Lawler J, Iruela-Arispe ML (2001) Thrombospondin-1 suppresses spontaneous tumor growth and inhibits activation of matrix metalloproteinase-9 and mobilization of vascular endothelial growth factor. Proc Natl Acad Sci USA 98:12485–12490

Rofstad EK, Henriksen K, Galappathi K, Mathiesen B (2003) Antiangiogenic treatment with thrombospondin-1 enhances primary tumor radiation response and prevents growth of dormant pulmonary micrometastases after curative radiation therapy in human melanoma xenografts. Cancer Res 63:4055–4061

Schultz-Cherry S, Murphy-Ullrich JE (1993) Thrombospondin causes activation of latent transforming growth factor-beta secreted by endothelial cells by a novel mechanism. J Cell Biol 122:923–932

Schultz-Cherry S, Chen H, Mosher DF, Misenheimer TM, Krutzsch HC, Roberts DD, Murphy-Ullrich JE (1995) Regulation of transforming growth factor-beta activation by discrete sequences of thrombospondin 1. J Biol Chem 270:7304–7310

Shafiee A, Penn JS, Krutzsch HC, Inman JK, Roberts DD, Blake DA (2000) Inhibition of retinal angiogenesis by peptides derived from thrombospondin-1. Invest Ophthalmol Vis Sci 41:2378–2388

Sheibani N, Newman PJ, Frazier WA (1997) Thrombospondin-1, a natural inhibitor of angiogenesis, regulates

platelet-endothelial cell adhesion molecule-1 expression and endothelial cell morphogenesis. Mol Biol Cell 8:1329–1341

Stouffer GA, Hu Z, Sajid M, Li H, Jin G, Nakada MT, Hanson SR, Runge MS (1998) Beta3 integrins are upregulated after vascular injury and modulate thrombospondin- and thrombin-induced proliferation of cultured smooth muscle cells. Circulation 97:907–915

Streit M, Velasco P, Riccardi L, Spencer L, Brown LF, Janes L, Lange-Asschenfeldt B, Yano K, Hawighorst T, Iruela-Arispe L, Detmar M (2000) Thrombospondin-1 suppresses wound healing and granulation tissue formation in the skin of transgenic mice. EMBO J 19:3272–3282

Tan K, Duquette M, Liu JH, Dong Y, Zhang R, Joachimiak A, Lawler J, Wang JH (2002a) Crystal structure of the TSP-1 type 1 repeats: a novel layered fold and its biological implication. J Cell Biol 159:373–382

Tan XJ, Lang JH, Liu DY, Shen K, Leng JH, Zhu L (2002b) Expression of vascular endothelial growth factor and thrombospondin-1 mRNA in patients with endometriosis. Fertil Steril 78:148–153

Taraboletti G, Belotti D, Borsotti P, Vergani V, Rusnati M, Presta M, Giavazzi R (1997) The 140-kilodalton antiangiogenic fragment of thrombospondin-1 binds to basic fibroblast growth factor. Cell Growth Differ 8:471–479

Tolsma SS, Volpert OV, Good DJ, Frazier WA, Polverini PJ, Bouck N (1993) Peptides derived from two separate domains of the matrix protein thrombospondin-1 have anti-angiogenic activity. J Cell Biol 122:497–511

Tolsma SS, Stack MS, Bouck N (1997) Lumen formation and other angiogenic activities of cultured capillary endothelial cells are inhibited by thrombospondin-1. Microvasc Res 54:13–26

Topol EJ, McCarthy J, Gabriel S, Moliterno DJ, Rogers WJ, Newby LK, Freedman M, Metivier J, Cannata R, O'Donnell CJ, Kottke-Marchant K, Murugesan G, Plow EF, Stenina O, Daley GQ (2001) Single nucleotide polymorphisms in multiple novel thrombospondin genes may be associated with familial premature myocardial infarction. Circulation 104:2641–2644

Tucker RP (2004) The thrombospondin type 1 repeat superfamily. Int J Biochem Cell Biol 36:969–974

Unger S, Hecht JT (2001) Pseudoachondroplasia and multiple epiphyseal dysplasia: New etiologic developments. Semin Med Genet 106:244–250

Vailhe B, Feige JJ (2003) Thrombospondins as anti-angiogenic therapeutic agents. Curr Pharm Des 9:583–588

Vischer P (1990) Modulation of extracellular matrix proteins and the influence of fucoidan on cell proliferation of smooth muscle cells. Biochem Soc Trans 18:962–963

Vischer P, Buddecke E (1991) Different action of heparin and fucoidan on arterial smooth muscle cell proliferation and thrombospondin and fibronectin metabolism. Eur J Cell Biol 56:407–414

Wang XQ, Frazier WA (1998) The thrombospondin receptor CD47 (IAP) modulates and associates with alpha2 beta1 integrin in vascular smooth muscle cells. Mol Biol Cell 9:865–874

Watnick RS, Cheng YN, Rangarajan A, Ince TA, Weinberg RA (2003) Ras modulates Myc activity to repress thrombospondin-1 expression and increase tumor angiogenesis. Cancer Cell 3:219–231

Yabkowitz R, Dixit VM, Guo N, Roberts DD, Shimizu Y (1993) Activated T-cell adhesion to thrombospondin is mediated by the alpha 4 beta 1 (VLA-4) and alpha 5 beta 1 (VLA-5) integrins. J Immunol 151:149–158

Yamada Y, Izawa H, Ichihara S, Takatsu F, Ishihara H, Hirayama H, Sone T, Tanaka M, and Yokota M (2002. Prediction of the risk of myocardial infarction from polymorphisms in candidate genes. N Engl J Med. 347:1916–23

Yang QW, Liu S, Tian Y, Salwen HR, Chlenski A, Weinstein J, Cohn SL (2003) Methylation-associated silencing of the thrombospondin-1 gene in human neuroblastoma. Cancer Res 63:6299–6310

Yang Z, Kyriakides TR, Bornstein P (2000) Matricellular proteins as modulators of cell-matrix interactions: adhesive defect in thrombospondin 2-null fibroblasts is a consequence of increased levels of matrix metalloproteinase-2. Mol Biol Cell 11:3353–3364

Yang Z, Strickland DK, Bornstein P (2001) Extracellular matrix metalloproteinase 2 levels are regulated by the low density lipoprotein-related scavenger receptor and thrombospondin 2. J Biol Chem 276:8403–8408

Young GD, Murphy-Ullrich JE (2004) Molecular interactions that confer latency to transforming growth factor-beta (TGF-beta). J Biol Chem 279(36):38032–38039

Molecular and Cellular Aspects of Heparanase

14

Neta Ilan, Liat Fux, Michael Elkin, and Israel Vlodavsky

Abstract

Heparanase is an endoglycosidase involved in cleavage of heparan sulfate and hence in degradation and remodeling of the basement membrane and extracellular matrix (ECM). Heparanase activity facilitates cell invasion associated with cancer metastasis, angiogenesis, autoimmunity and inflammation. The enzyme is preferentially expressed in human tumors and its over-expression in tumor cells confers an invasive phenotype. Heparanase also releases angiogenic factors from the ECM and thereby induces an angiogenic response in *vivo*. Heparanase upregulation correlates with increased tumor vascularity and poor postoperative survival of cancer patients. Moreover, heparanase levels in the urine and plasma of cancer patients often correlate with the severity of the disease and response to anti-cancer treatments. These observations, the anti-cancerous effect of heparanase gene silencing and of heparanase-inhibiting molecules, as well as the unexpected identification of a single functional heparanase, suggest that the enzyme is a valid target for anti-cancer drug development and a promising tumor marker. Heparanase also exhibits non-enzymatic activities, stimulating, among other effects, cell adhesion, Akt signaling and PI3K-dependent endothelial cell migration and invasion. It also promotes VEGF expression via the Src pathway and hence may activate endothelial cells and elicit angiogenic and survival responses. Studies with heparanase over-expressing transgenic mice revealed that the enzyme functions in normal processes (i.e., wound healing) involving cell mobilization, HS turnover, tissue vascularization and remodeling. Inhibitors directed against domains critical for heparanase secretion and signaling, combined with inhibitors of heparanase enzymatic activity (i.e., non-anticoagulant glycol-split heparin) are being developed to halt tumor growth, angiogenesis and metastasis. In this review, we summarize the current status of heparanase' research, emphasizing molecular and cellular aspects of the enzyme, including its mode of processing and activation, control of heparanase gene expression, cytoplasmic vs. nuclear localization, enzymatic and non-enzymatic functions, causal involvement in cancer metastasis and angiogenesis, and strategies for the development of heparanase inhibitors.

CONTENTS

Introduction

In a given organ, the cells normally occupy only a certain portion of the volume. A substantial part is filled with a network of macromolecules defined as extracellular matrix (ECM). The ECM is composed of a variety of proteins and polysaccharides secreted by the cells. Protein components are adhesive molecules such as laminin, fibronectin and vitronectin, and structural molecules such as collagen and elastin. Heparan sulfate proteoglycans (HSPGs) are members of the glycosaminoglycan (GAG) family, a class of molecules that consists of unbranched, repeated disaccharide units attached to a core protein. Heparan sulfate glycosaminoglycans (HS) are abundant components of the ECM. By binding several major ECM constituents (i.e., laminin, fibronectin, collagen type IV), HS are thought to contribute significantly to ECM self-assembly and integrity. HS also tether a multitude of growth factors, chemokines, cytokines and enzymes to the ECM and cell surface, providing a low-affinity storage depot (Bernfield et al. 1999; Folkman et al. 1988; Vlodavsky et al. 1987). Cleavage of HS side chains is expected not only to alter the integrity of the ECM, but also to release HS-bound biological mediators. In addition, HS fragments are also capable of modulating the activity of growth factors such as bFGF and enzymes such as thrombin. The ECM provides an essential physical barrier between cells and tissues, as well as a scaffold for cell growth, migration, differentiation and survival, and undergoes continuous remodeling during development and in certain pathological conditions such as wound healing and cancer (Fata et al. 2004). ECM-remodeling enzymes are thus expected to profoundly affect cell and tissue function. While intensive research focused on enzymes capable of degrading and remodeling protein components in the ECM (Vu and Werb 2000; Werb 1997), less attention was paid to enzymes cleaving GAG side chains. Heparanase is an endo-β-D-glucuronidase capable of cleaving HS side chains at a limited number of sites, yielding HS fragments of still appreciable size (~5–7 kDa) (Freeman and Parish 1998; Pikas et al. 1998; Vlodavsky and Goldshmidt 2001). Heparanase activity has long been detected in a number of cell types and tissues. Importantly, heparanase activity correlated with the metastatic potential of tumor-derived cells; this was attributed to enhanced cell dissemination as a consequence of HS cleavage and remodeling of the ECM barrier (Parish et al. 2001; Vlodavsky and Friedmann 2001). Similarly, heparanase activity was implicated in neovascularization, inflammation and autoimmunity, involving migration of vascular endothelial cells and activated cells of the immune system (Dempsey et al. 2000; Parish et al. 2001; Vlodavsky and Friedmann 2001). In spite of the attractive clinical relevance of the pro-metastatic, pro-inflammatory and pro-angiogenic activities of heparanase, progress in the field was slow, largely due to the lack of a simple bioassay to quantitative heparanase activity and the low abundance of the enzyme. Heparanase activity was attributed to proteins with molecular weights ranging from 8 to 130 kDa, raising the possible existence of several

HS-degrading endoglycosidic enzymes (Dempsey et al. 2000; Parish et al. 2001; Vlodavsky and Friedmann 2001). This confusion was solved when the cloning of a single human heparanase cDNA sequence was independently reported by several groups (Hulett et al. 1999; Kussie et al. 1999; Toyoshima and Nakajima 1999; Vlodavsky et al. 1999). With the availability of appropriate reagents (i.e., molecular probes, antibodies, recombinant enzymes) heparanase research entered a new era. Recent studies have shown that heparanase is upregulated in an increasing number of primary human tumors (see below). Heparanase upregulation was correlated with increased lymph node and distant metastasis, greater micro-vessel density, and reduced postoperative survival of cancer patients (see below), providing a strong clinical support for the pro-metastatic and pro-angiogenic features of the enzyme. Here, we summarize recent progress in molecular and cellular aspects of heparanase, emphasizing its causal involvement in cancer metastasis and angiogenesis, and discuss novel strategies for the development of heparanase inhibitors. Notably, heparanase activity liberates short HS fragments that may exert multiple effects of their own. This aspect has been reviewed by others (Sanderson et al. 2004, 2005; Sasisekharan et al. 2002) and will not be discussed here.

Molecular Aspects

14.2.1 Heparanase Structure

Only one gene (*HPSE*) has been shown to encode for a protein with heparanase activity (Hulett et al. 1999; Kussie et al. 1999; Toyoshima and Nakajima 1999; Vlodavsky et al. 1999). Sequence analysis revealed that heparanase is ubiquitous in mammals, with similar sequences found in human, rat, mouse, chicken and cow (Goldshmidt et al. 2001; Parish et al. 2001). Human heparanase cDNA contains an open reading frame that encodes a polypeptide of 543 amino acids with a molecular weight of 61.2 kDa. The active heparanase purified from placenta, platelets, and various cell lines was found to lack its N-terminal 156 amino acids, suggesting post-translational proteolysis of the heparanase polypeptide (Parish et al. 1999; Toyoshima and Nakajima 1999; Vlodavsky et al. 1999). In fact, active heparanase was subsequently reported to be a heterodimer consisting of a 50-kDa subunit (Lys^{158}–Ile^{543}) associated non-covalently with an 8-kDa peptide (Gln^{36}–Glu^{109}) (Fairbanks et al. 1999; Levy-Adam et al. 2003; McKenzie et al. 2003). Based on the predicted amino acid sequence, the 50-kDa subunit of human heparanase contains six putative *N*-glycosylation sites. Although glycosylation is not required for enzyme activity, secretion of heparanase is regulated by glycosylation (Simizu et al. 2004b). The sequence also contains a 35-amino-acid N-terminal signal sequence (Met^{1}–Ala^{35}) and a C-terminal hydrophobic domain (Pro^{515}–Ile^{534}). While the 3D structure of heparanase has not been resolved, heparanase has been shown to be related to members of the clan A glycosyl hydrolyses (GH-A) (Hulett et al. 2000). Protein sequence alignment approaches in combination with secondary structure predictions indicated that heparanase contains sequences that are homologous to families 10, 39, and 51 of the GH-A, especially in terms of the active-site regions (Hulett et al. 2000). This clan of enzymes uses a general acid catalysis mechanism for the hydrolysis of glycosidic bonds. The mechanism requires two critical residues, a proton donor and a nucleophile, both of which appear to be conserved in heparanase, at Glu^{225} and Glu^{343}, respectively (Hulett et al. 2000). Site-directed mutagenesis of these residues completely abolished heparanase activity (Goldshmidt et al. 2003; Hulett et al. 2000), suggesting that heparanase uses a catalytic mechanism characteristic of GH-A glycosyl hydrolyses (Hulett et al. 2000).

14.2.2 Heparanase Gene Regulation

Induced expression of heparanase in human cancer (see below), inflammation (Edovitsky et al. 2006;

Parish et al. 2001; Vlodavsky et al. 1992), diabetic nephropathy (Katz et al. 2002; Levidiotis et al. 2001, 2004a, 2004b, 2005), and other pathological conditions suggests a transcriptional regulation. In addition to multiple genetic alterations that govern cell transformation, epigenetic processes, marked by hypermethylation of the promoter region, contribute significantly to gene transcription and cancer progression, for example by downregulation of tumor suppressor and DNA repair genes. Several studies have convincingly shown that promoter methylation status plays an important role in heparanase gene transcription. By examining a series of tumor-derived cell lines, Shteper et al. found that cells which exhibit heparanase activity also harbor at least one unmethylated allele (Shteper et al. 2003). In contrast, cell lines which exhibit no heparanase expression or activity, such as C6 rat glioma and JAR human choriocarcinoma, were found to harbor fully methylated alleles. Treating these cells with demethylating agents such as 5-azacytidine restored heparanase activity and was accompanied by augmented metastatic capacity in vivo (Shteper et al. 2003). While treatment with demethylating agents is likely to affect the expression of many genes, lung colonization was suppressed in mice treated with laminaran sulfate (Shteper et al. 2003), a compound that inhibits heparanase activity and experimental metastasis (Miao et al. 1999), supporting the intimate involvement of heparanase activity in metastatic dissemination (see below). Subsequent studies revealed a similar correlation with prostate and bladder cancer-derived cell lines, and, moreover, with prostate and bladder tissues. Thus, significantly higher promoter methylation was found in benign prostatic hyperplasia (BPH) and in normal bladder than in carcinomas, inversely correlating with heparanase expression (Ogishima et al. 2005a, b). Interestingly, Ogishima et al. have noted a correlation between heparanase expression by bladder and prostate carcinomas and the expression levels of early growth response 1 (EGR1), a transcription factor implicated in heparanase gene transcription (de Mestre et al. 2003, 2005). In contrast with other transcription factors such as SP1 and Ets, associated with basal heparanase transcription levels (Jiang et al. 2002), EGR1 appears to be related to inducible transcription of the heparanase gene (de Mestre et al. 2003, 2005). EGR1 is rapidly induced in response to a variety of signals such as growth factors, cytokines and injury, is upregulated in human tumors (Abdulkadir et al. 2001), and is strongly implicated in tumor angiogenesis (Khachigian 2004). The latter function may be related, at least in part, to the strong pro-angiogenic capacity of heparanase (see below).

14.2.2.1 Transcriptional Regulation of Heparanase Gene in Breast Cancer by Estrogen

While gene methylation plays a critical role in heparanase transcriptional regulation, additional regulatory mechanisms may operate, locally or systemically. An example of a systemic regulator is the hormone estrogen, one of the main driving forces in breast tumorigenesis. Elkin et al. have identified four putative estrogen response elements in the heparanase promoter and demonstrated their functionality by a luciferase reporter gene driven by the heparanase promoter (Elkin et al. 2003). Physical association between estrogen receptor (ER) and the heparanase promoter was confirmed by chromatin immunoprecipitation (ChIP) analysis. Luciferase activity and heparanase mRNA levels were significantly increased in ER-positive MCF7 cells, but not in ER-negative human breast carcinoma cells upon treatment with estrogen, induction that could be prevented by the estrogen inhibitor ICI (Elkin et al. 2003). Similarly, estrogen facilitated heparanase expression by MCF7 cells embedded in Matrigel and implanted subcutaneously, resulting in plugs that are more vascularized, again supporting the pro-angiogenic properties of the enzyme. A correlation between heparanase and ER levels was confirmed by tissue array analysis of breast carcinoma specimens (our unpublished results), indicating the in vivo relevance of this regulatory mechanism. These results indicate that heparanase expression induced in breast epithelium by estrogen contributes, among other factors, to primary breast tumor growth and neovascularization. This effect could be particularly important at the initial stages of breast tumorigenesis, when more than 70% of all tumors are reportedly

ER positive. Notably, the partial antagonist tamoxifen (TAM), the most widely used drug in endocrine therapy of breast cancer, was found to exert an estrogen-like promoting effect on heparanase gene expression. ChIP assay confirmed TAM-ER complex binding to the heparanase promoter, suggesting that it may be held responsible, at least in part, for tumor resistance or even progression observed in many TAM-treated breast cancer patients (our unpublished results). While these observations are relevant to breast cancer, mechanisms that promote heparanase expression in tissues other than breast are currently poorly understood.

14.2.2.2 Tumor Suppressor p53 Regulates Heparanase Gene Expression

Little is known about physiologically relevant repressors of heparanase gene transcription. Applying ChIP analysis, we recently demonstrated that wild-type p53 inhibits transcription of the heparanase gene by direct binding to its promoter. Moreover, this inhibition involved recruitment of histone deacetylases (HDACs). On the other hand, mutated, tumor-derived variants of p53 lose this inhibitory ability and in some cases even upregulate heparanase gene expression (Baraz et al. 2006). These results indicate that under normal conditions the heparanase gene is constitutively inhibited by wild-type p53. Mutational inactivation of p53 during cancer development leads to transcriptional activation of heparanase expression, providing a possible molecular mechanism for the frequent increase in heparanase levels observed in the course of tumorigenesis (see below).

14.2.2.3 Heparanase Gene Regulation by Inflammatory Mediators

Polyanionic compounds which inhibit heparanase enzymatic activity (e.g., heparin) also inhibit inflammatory responses (Bartlett et al. 1995; Lider et al. 1989; Parish et al. 1998). This effect may be attributed to inhibition of heparanase produced by activated T lymphocytes, regarded as the primary cellular source of the enzyme in inflammation (Naparstek et al. 1984; Vlodavsky et al. 1992). We have recently examined the role of heparanase in a delayed-type hypersensitivity (DTH) mouse model. Surprisingly, it was noted by immunohistochemistry that endothelial cells are the primary source of the enzyme under this experimental setting (Edovitsky et al. 2006). Furthermore, TNFα and IFN-γ, key mediators of DTH inflammation, upregulated heparanase gene expression and enzymatic activity in cultured endothelial cells (Edovitsky et al. 2006) and T lymphocytes (Sotnikov et al. 2004), consistent with a recent report demonstrating induced heparanase expression and secretion by endothelial cells treated with TNFα (Chen et al. 2004). Computerized analysis of the heparanase gene 1.9-kb promoter sequence revealed two consensus interferon-stimulated response elements (ISREs) that specifically bind transcription factors activated by interferon (Edovitsky et al. 2006). These results point to the vascular endothelium as an important cellular source of heparanase enzymatic activity that, in turn, allows for remodeling of the vascular basement membrane, increased vessel permeability, and extravasation of tumor cells, leukocytes and plasma proteins. In vivo administration of anti-heparanase siRNA, or an inhibitor of heparanase enzymatic activity (non-anticoagulant glycol split heparin, see below), effectively halted the DTH inflammatory response. Altogether, our results (Edovitsky et al. 2006) highlight the decisive role of endothelial heparanase in DTH inflammation and its potential as a promising target for anti-inflammatory drug development.

14.2.3 Heparanase Processing and Cellular Localization

As already discussed, given the multitude of polypeptides associated with HS on the cell surface and ECM and their ability to profoundly affect cell and tissue function, heparanase activity and bioavailability should be kept tightly regulated. Regulation

at the transcriptional level represents one type of control mechanism. Regulation factors at the post-translational level, namely heparanase processing, cellular localization and secretion, are now recognized as additional key regulatory mechanisms. A major 50-kDa protein is detected in cell lysates following transfection and overexpression of the heparanase cDNA, correlating with high levels of enzymatic activity (Vlodavsky et al. 1999). In contrast, a 65-kDa protein was found in the cell-conditioned medium, raising the possibility that the protein is first synthesized as a latent proenzyme, which is then activated by proteolytic processing. Purifying human heparanase to homogeneity allowed Fairbanks et al. to determine the N-terminus sequence of the 50-kDa protein (Fairbanks et al. 1999). Interestingly, the purified heparanase preparation was noted to include an 8-kDa protein, and further analysis revealed that this protein is derived from the N-terminus region (Gln^{36}–Glu^{109}) of heparanase, immediately next to the signal sequence (Fairbanks et al. 1999). This finding led the authors to suggest that active heparanase is a hetrodimer composed of the 8-kDa and 50-kDa subunits. Indeed, attempts to express the truncated 50-kDa (Lys^{158}–Ile^{543}) protein alone yielded no enzymatic activity (Hulett et al. 2000), indicating that an N-terminus sequence is required. This hypothesis was confirmed by co-transfection and immunoprecipitation approaches, convincingly demonstrating that the two subunits are associated with each other, and that enzymatic activity is only obtained by coexpression of both the 8- and 50-kDa subunits (Levy-Adam et al. 2003; McKenzie et al. 2003). Multiple sequence alignment and secondary structure prediction suggest that heparanase adopts a TIM barrel fold, similar to other glycosyl hydrolases (Hulett et al. 2000). This fold motif usually consists of eight alternating α-helices and β-strands. Within the heparanase 50-kDa subunit, clear homology was noted for only six α/β units, leading Nardella et al. to suggest that the two other units are contributed by the 8-kDa subunit. Indeed, structural prediction revealed the presence of a β/α/β element in the 8-kDa subunit, which may thus contribute the missing TIM barrel units (Nardella et al. 2004). These and other studies (McKenzie et al. 2003) indicated that the linker domain (Ser^{110}–Gln^{157}) inhibits heparanase activity and needs to be fully removed. Adopting site-directed mutagenesis approach to identify amino acids essential for cleavage at Glu^{109}–Ser^{110} (site 1) and Gln^{157}–Lys^{158} (site 2), Abboud-Jarrous et al. reported that none of the mutations generated at site 1 and its flanking regions had an effect on heparanase processing and activity (Abboud-Jarrous et al. 2005). In contrast, substitution of Tyr^{156} (site 2) by alanine or glutamine rendered heparanase inactive and improperly processed (Abboud-Jarrous et al. 2005). Subsequent studies revealed that a bulky hydrophobic amino acid (i.e., Tyr^{156}) at position 2 (P2) of the cleavage site (Gln^{157}–Lys^{158}) is absolutely required for heparanase processing and activation, resembling the cleavage specificity of cathepsin L (Abboud-Jarrous et al. 2005). Indeed, incubation of purified latent 65-kDa heparanase with cathepsin L yielded properly processed and active heparanase enzyme composed of the 50- and 8-kDa subunits. Processing and activation of the pro-enzyme by intact cells and in a cell-free system was inhibited in the presence of a specific, cell-permeable inhibitor of cathepsin L (Abboud-Jarrous et al. 2005). Applying a structural model, these authors demonstrated that the linker segment, or even a small 1-kDa portion at its C-terminus, renders the active site inaccessible to the HS substrate. Moreover, heparanase activity was inhibited in the presence of a 1-kDa peptide corresponding to the C-terminus of the linker segment. While predicted model structures do provide important information (Fig. 14.1), it is limited by the relatively low sequence homology with other glycosyl hydrolases, and awaits further confirmation by crystallization and X-ray analysis.

More recent findings applying site-directed mutagenesis of aromatic residues along the linker segment support a mechanism by which cathepsin L is not only responsible for cleavage at site 2, but is actually capable of a stepwise cleavage and removal of the entire linker region, suggesting that processing and activation of pro-heparanase can be brought about solely by cathepsin L and/or cathepsin L-like proteases (our unpublished results). It is likely that cathepsins other than cathepsin L

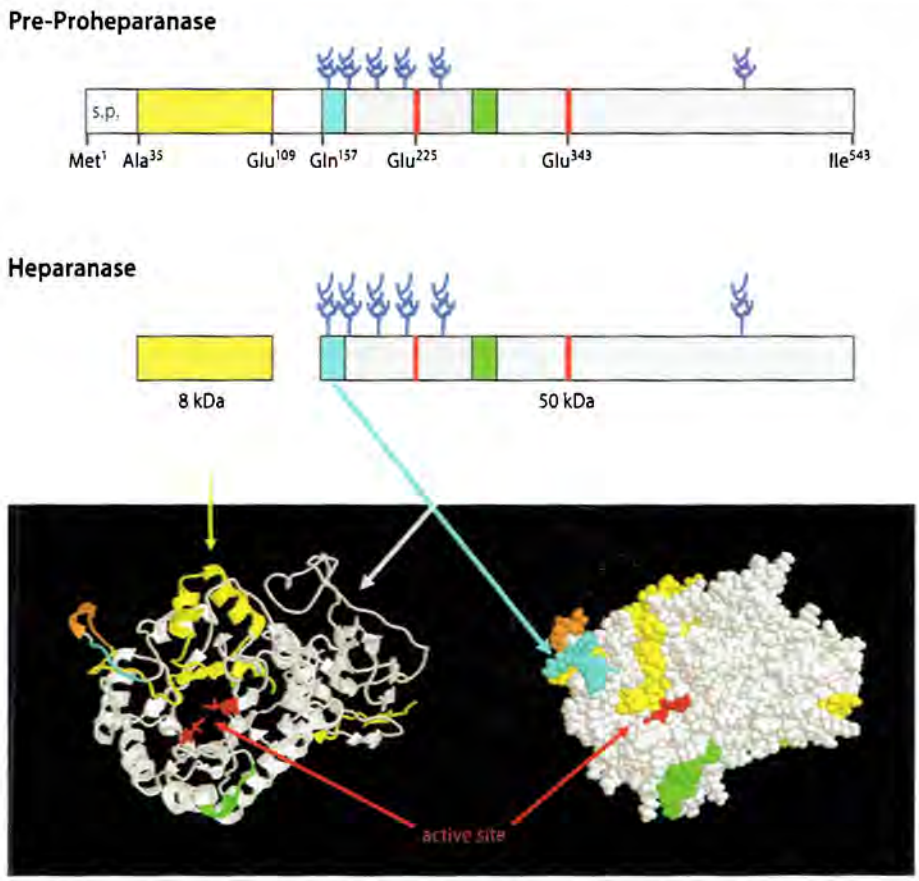


Fig. 14.1. Primary structure, critical amino acids and predicted three-dimensional structure of the heparanase heterodimer. Pre-pro-heparanase harbors a signal peptide (s.p., Met^{1}-Ala^{35}) which is removed upon entering the ER. The protein is then subjected to glycosylation at six N-glycosylation sites (*blue "trees"*) and secreted as a latent ~65 kDa protein (*top panel*). Proteolytic processing removes the linker domain (Ser^{110}-Gln^{157}), resulting in 8-kDa (Gln^{36}-Glu^{109}) and 50-kDa (Lys^{158}-Ile^{543}) subunits (*middle panel*) that heterodimerize to yield an active enzyme. A predicted three-dimensional structure of the heparanase heterodimer (*bottom panel*) was generated based on homology with β-D-xylosidase. Shown (*left*) are the 8-kDa (*blue*) and 50-kDa subunits (*yellow*), and glutamic acid residues 225 and 343, which comprise the enzymatically active site (*red*). Close proximity of the heparin-binding domains (*blue and green*) to the enzymatically active site (*red*) is shown on the right

may activate pro-heparanase, possibly in a cell- and tissue- dependent manner.

Of note, impaired hair follicle morphogenesis and cycling, and abnormal bone development, were noted in cathepsin L-deficient and cathepsin L knockout mice (Benavides et al. 2002; Potts et al. 2004). Intriguingly, heparanase overexpression in the skin and bone of transgenic mice resulted in the opposite phenomena: enhanced hair follicle cycling and hair growth (Zcharia et al. 2004, 2005a, 2005b), and increased trabecular connectivity and bone mass (Kram et al. 2006), suggesting that alterations associated with cathepsin L deficiency are due, in part, to the possible lack of heparanase activity.

Additional studies are required to elucidate the effect of cathepsin deficiency on heparanase function.

An important issue is the nature of the processing organelle. Exogenously added pro-heparanase rapidly interacts with cells in culture, followed by internalization and conversion into a highly active enzyme, raising the possibility that processing occurs at the cell surface (Nadav et al. 2002; Vlodavsky and Friedmann 2001). Recent findings indicated, however, that heparanase processing occurs intracellularly (Gingis-Velitski et al. 2004b; Vreys et al. 2005; Zetser et al. 2004), pointing to acidic vesicles, most likely lysosomes, as the processing organelle. Following exogenous addition, heparanase was noted to reside within perinuclear endocytic vesicles identified as endosomes (Nadav et al. 2002) and lysosomes (Goldshmidt et al. 2002). Applying anti-heparanase antibodies that distinguish between the latent and processed heparanase forms, Zetser et al. demonstrated that not only the processed, but also the 65-kDa latent heparanase was localized to endocytic vesicles, indicating that processing does not take place at the cell surface (Zetser et al. 2004). Complete inhibition of heparanase processing by chloroquine and bafilomycin A1, reagents that raise the pH of acidic vesicles and thus inhibit the enzymatic activity of resident enzymes, further points to acidic vesicles as the heparanase processing site (Zetser et al. 2004). Taking this notion a step further, Cohen et al. utilized a cell fractionation approach and demonstrated that lysosomal/endosomal, but not cytoplasmic, preparation is capable of heparanase processing, yielding an active enzyme (Cohen et al. 2005). Moreover, processing by the lysosomal/endosomal preparation was most efficient in acidic pH conditions (pH 4–5) (Cohen et al. 2005), typical of the lysosomal compartment. These results and the ability of cathepsin L, a characteristic lysosomal enzyme, to accurately process and activate pro-heparanase, strongly support the lysosomal compartment as the site of processing. It should be noted, nonetheless, that most studies were performed with transfected cells engineered to overexpress heparanase, or in response to exogenous addition of the latent enzyme, situations that may not fully reflect the physiological conditions.

14.2.4 Heparanase Secretion and Extracellular Retention

In spite of its localization to a highly active protein degradation environment such as the lysosome, heparanase exhibits a half-life of about 30 h (Gingis-Velitski et al. 2004b), relatively long compared with a $t_{1/2}$ of 2–6 h, and 25 min of transmembrane and GPI-anchored HSPGs, respectively. Residence and accumulation of heparanase in late endosomes and lysosomes may indicate that the enzyme functions in physiological turnover of cellular HSPGs (Fuller et al. 2006). Being not readily accessible to its extracellular substrate points to the existence of regulatory mechanism(s) by which intracellular, lysosomal heparanase is secreted in response to local or systemic cues. Recent observations may support the occurrence of such a scenario. For example, treatment of human microvascular endothelial cells (EC) with the pro-inflammatory cytokines TNFα and IL-1β resulted in a marked increase in heparanase secretion (Chen et al. 2004), although this observation was not supported by measurements of heparanase enzymatic activity. Secretion of heparanase in response to TNFα was also noted in human peripheral T-cells (Sotnikov et al. 2004). Interestingly, TNFα and IL-1β had no effect on heparanase secretion from tumor-derived cells (our unpublished results), suggesting that effective stimuli may vary among cell types and biological settings. Instead, nucleotides, such as ATP, ADP and adenosine, were most effective in stimulating secretion of active heparanase by tumor cells (Shafat et al., in preparation). Regarded as a universal source of metabolic energy, extracellular ATP and other nucleotides are capable of initiating signaling cascades through two classes of P2 receptors: P2X, which has an intrinsic activity of the ion channel; and P2Y, a G-protein coupled receptor (Communi et al. 2000). P2Y receptor activation is coupled to phospholipase C and adenylate cyclase, leading to PKC and PKA activation (Abbracchio and Burnstock 1998; Communi et al. 2000; van der Weyden et

al. 2000). Remarkably, each and every cell line examined responded to nucleotides (ATP, ADP, adenosine) by a stimulated secretion of active heparanase (Shafat et al., in preparation). Importantly, ATP exerted its maximal effect at a physiological concentration (1 μM) (Gordon 1986), emphasizing the biological relevance of this mediator, and heparanase secretion was inhibited by PKC inhibitors and P2Y receptor antagonists. Several lines of evidence suggest that the secreted heparanase originated from intracellular pools, most likely endosomes and lysosomes. The kinetics of heparanase secretion elicited by ATP resembled that of the lysosomal enzyme cathepsin D, supporting the notion that both enzymes were secreted from intracellular vesicles. Moreover, immunofluorescence staining revealed a clear transition in the localization of heparanase-positive vesicles towards the cell periphery, in response to stimulation with ATP. Thus, although not considered as typical secretory vesicles, lysosomes may secrete their content under certain conditions and in response to a proper stimuli, in agreement with the elevated levels of secreted cathepsins found in several human malignancies (Turk et al. 2000, 2004).

Apart from storage in the endosomal/lysosomal compartment, efficient uptake of exogenous heparanase by primary fibroblasts and EC, as well as by tumor-derived cells (Gingis-Velitski et al. 2004a, 2004b; Nadav et al. 2002; Zetser et al. 2004) provides an additional mechanism that limits the retention of the enzyme extracellularly. Several lines of evidence indicate that heparanase uptake is mediated by cell surface HS. We have demonstrated that addition of heparin or xylosides results in accumulation of heparanase in the culture medium of heparanase transfected cells. Heparanase uptake was attenuated in HS-deficient cells and in cells that were treated with bacterial heparinase, but not with chondroitinase ABC. In addition, transfection and overexpression of heparanase in HS-deficient cells resulted in accumulation of the latent pro-enzyme in the culture medium, concomitant with decreased levels of the intracellular processed enzyme (Gingis-Velitski et al. 2004b). This result suggests that intracellular accumulation of processed heparanase occurs following uptake of the secreted latent protein (Gingis-Velitski et al. 2004b) (Fig. 14.2). Sequence alignment of heparin-binding domains in the heparanase molecule revealed the existence of two domains that match consensus sequences for heparin binding. These were mapped at Lys^{158}–Asp^{162} at the N-terminus of the 50-kDa heparanase subunit and at Pro^{271}–Met^{278} (Levy-Adam et al. 2005). A peptide containing the Lys^{158}–Asp^{162} sequence (KKDC) exhibited firm binding to heparin and HS and inhibited both heparanase uptake and enzymatic activity, most likely due to competition with the HS substrate (Levy-Adam et al. 2005). Furthermore, heparanase deletion mutants lacking each of the heparin-binding domains exhibited no enzymatic activity. Deletion of the KKDC sequence (65Δ15) resulted in intracellular accumulation of the 65-kDa pro-enzyme that failed to get secreted. Deletion of the Pro^{271}–Met^{278} sequence (65Δ10) led to accumulation of the pro-enzyme in the cell-conditioned medium (Levy-Adam et al. 2005), further supporting a critical role for HS in heparanase uptake and processing. Notably, the KKDC sequence, and specifically $lysine^{158}$ and $lysine^{159}$, involved in interaction of the enzyme with heparin/HS (Levy-Adam et al. 2005), appeared to reside in close proximity to Glu^{225} and Glu^{343}, comprising the enzyme's active site in a micro-pocket domain (Hulett et al. 2000) (Fig. 14.1). The predicted 3D model further emphasizes the micro-pocket region, and in particular the KKDC sequence (Fig. 14.1), as a valid target for the development of heparanase-inhibiting molecules (see below). More recently, Verys et al. have identified two additional cell surface receptors that mediate heparanase uptake, namely the low-density lipoprotein receptor-related protein (LRP) and the mannose 6-phosphate receptor (Vreys et al. 2005). Binding affinities to each component have not been determined, and the precise contribution of each receptor species to heparanase uptake is yet to be demonstrated. Collectively, the studies described above clearly emphasize the complexity and tight regulation of heparanase expression, processing and secretion (Fig. 14.2), supporting its potency and significance in normal and pathological conditions.

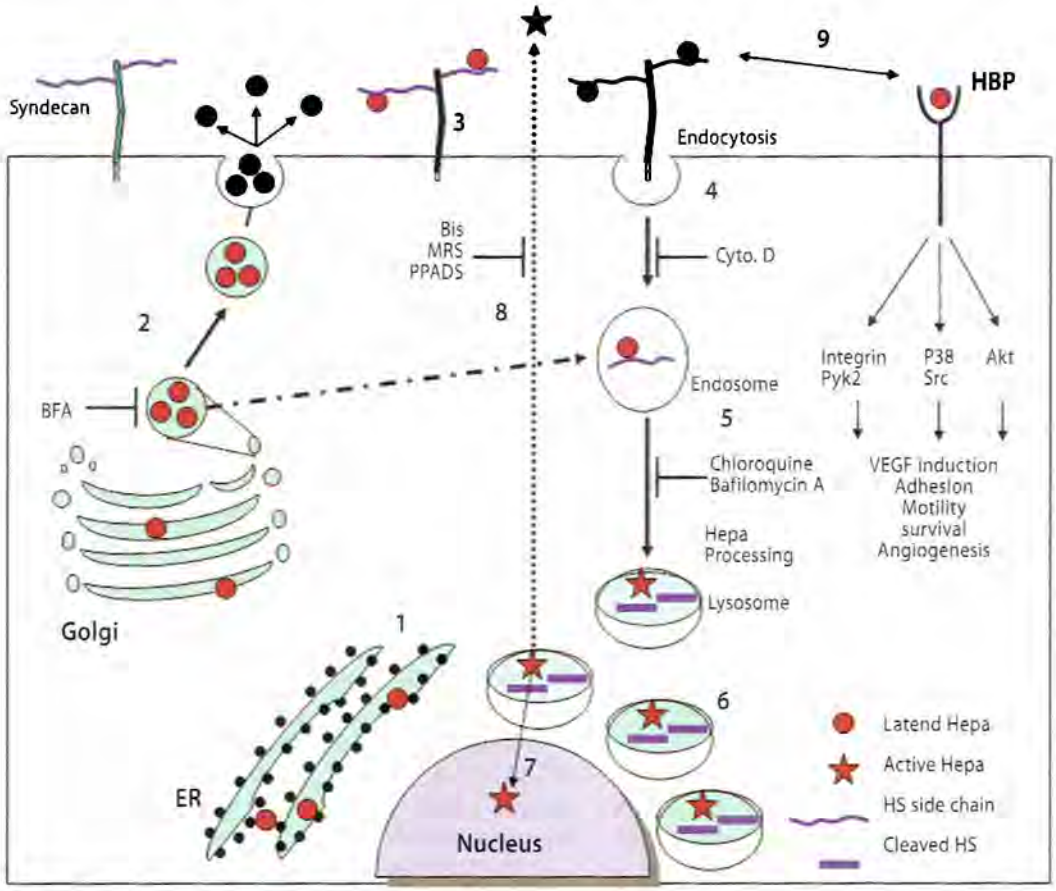


Fig. 14.2. Schematic presentation of heparanase biosynthesis, trafficking and pro-angiogenic activity. Pre-pro-heparanase is first targeted to the ER lumen via its signal peptide (Met^{1}-Ala^{35}) (*1*). The 65-kDa pro-heparanase (*red circles*) is then shuttled to the Golgi apparatus and is subsequently secreted via vesicles that bud from the Golgi (*2*), a step that is inhibited by brefeldin A (*BFA*) (Nadav et al. 2002). Once secreted, heparanase rapidly interacts with cell membrane HSPGs (i.e., syndecan family members) (*3*) (Gingis-Velitski et al. 2004b), mannose-6-phosphate receptor and LRP (Vreys et al. 2005), followed by a rapid endocytosis of the heparanase–HSPG complex (*4*) that appears to accumulate in endosomes (Nadav et al. 2002). This step is inhibited by cytochalasin D (*Cyto. D*) (Nadav et al. 2002), heparinase, or heparin (Gingis-Velitski et al. 2004b). Conversion of endosomes to lysosomes results in heparanase processing and activation (*red stars*) (*5*) that, in turn, participates in the turnover of HS side chains in the lysosome. Heparanase processing and activation is inhibited by chloroquine or bafilomycin A, inhibitors of lysosomal proteinases. Typically, heparanase appears at perinuclear lysosomal vesicles (*6*). Such a trafficking route may be bypassed in several potential ways, such as direct conversion of exocytosed vesicles to endosomes (*dash-dotted arrow*). Lysosomal heparanase may translocate to the nucleus, where it affects gene transcription, thus contributing to a more differentiated state of carcinoma cells (Ohkawa et al. 2004; Takaoka et al. 2003) (*7*), or can get secreted in response to local or systemic cues. Secretion of active heparanase heterodimer (*dotted arrow*) is inhibited by PKC inhibitors (Bis), and P2Y receptor antagonists (*MRS, PPADS*) (*8*). Both the latent and active secreted heparanase are also capable of interacting with cell surface heparanase-binding protein (*HBP*) and thereby activate signaling components such as Akt, p38, Src, Pyk2 and integrins, leading to enhanced cell adhesion, migration, VEGF expression and angiogenesis (Gingis-Velitski et al. 2004a; Goldshmidt et al. 2003; Sotnikov et al. 2004; Zetser et al. 2003, 2006) (*9*)

Cellular Aspects

14.3.1 Heparanase and Cancer Metastasis: Experimental Evidence

Heparanase activity in tumor cells was initially investigated in B16 melanoma (Nakajima et al. 1983, 1984) and T-lymphoma cells (Vlodavsky et al. 1983), resulting in HS fragments 5–6 times smaller than intact HS side chains. Heparanase activity was further characterized as an endoglucuronidase and found to be inhibited by species of heparin (Bar-Ner et al. 1985; Nakajima et al. 1984). This latter finding was confirmed by other laboratories in multitude experimental settings, providing the basis for the development of heparanase-inhibiting non-anticoagulant species of heparin (see below).

Heparanase activity correlated with the metastatic potential of mouse B16 melanoma and Eb lymphoma cells. Thus, sublines with higher potential for metastasis and organ colonization exhibited a higher enzymatic activity than low- or non-metastatic cells. These early observations gained substantial support when specific molecular probes became available shortly after cloning the heparanase gene. Hulett et al. employed Northern blot analysis to study heparanase expression in cells and tissues (Hulett et al. 1999), while Vlodavsky et al. utilized a transfection approach (Vlodavsky et al. 1999). In these studies, highly metastatic rat mammary adenocarcinoma cell lines (13762 MAT, DMBA-8A) were noted to express high levels of heparanase mRNA transcripts compared with their non-metastatic counterpart cells (Hulett et al. 1999). Subcutaneous inoculation of non-metastatic Eb lymphoma cells engineered to overexpress heparanase (*hpa*-Eb) resulted in a significant decrease in survival time of the mice due to a massive liver infiltration (Vlodavsky et al. 1999), further supporting the correlation between heparanase expression and the metastatic capacity of cancer cells. In a myeloma cell model, Yang et al. demonstrated that enhanced heparanase expression markedly induced spontaneous metastasis into various organs, depending on the site of primary tumor inoculation. Subcutaneously injected myeloma cells metastasized to the spleen, liver, lung and bone, while cells injected into the bone selectively disseminated to other bones (Yang et al. 2005). Moreover, vigorous bone resorption was noted in SCID mice following inoculation of heparanase-transfected MDA-231 human breast carcinoma cells into the mammary fat pad, although bone metastases were not detected (Kelly et al. 2005). It appears that heparanase overexpression in tumor cells can exert a systemic effect, resulting in elevation of soluble factors that stimulate osteolysis and, perhaps, progression of bone-homing tumors (Kelly et al. 2005; Sanderson et al. 2005). In other studies, siRNA and ribozyme technologies were employed to reduce heparanase expression levels in a specific manner. Transfection and stable expression of anti-heparanase ribozyme construct in human MDA-MB-435 breast carcinoma cells, known to express high levels of heparanase activity, or in Eb mouse lymphoma cells engineered to overexpress the human heparanase gene (*hpa*-Eb), resulted in a marked decrease in heparanase levels evaluated by reverse-transcription polymerase chain reaction (RT-PCR) and heparanase enzymatic activity. This decrease correlated with a 55–65% reduction in cellular invasion through a reconstituted basement membrane (Matrigel) (Edovitsky et al. 2004). Moreover, mice inoculated (s.c) with *hpa*-Eb lymphoma cells transfected with anti-heparanase ribozyme exhibited a marked decrease in liver metastasis and survived significantly longer than mice inoculated with cells transfected with control ribozyme. Similarly, lung colonization of B16-BL6 melanoma cells was markedly (>90%) reduced when applying cells transfected with anti-heparanase siRNA due to a marked inhibition of both heparanase gene expression and enzymatic activity, naturally expressed by these cells (Edovitsky et al. 2004). Subcutaneous primary tumors produced by *hpa*-Eb cells expressing the anti-heparanase ribozyme were less vascularized, supporting the pro-angiogenic function of the enzyme (see below). Altogether, both overexpression and silencing of the heparanase gene clearly indicate that heparanase not only enhances

cell dissemination, but also promotes the establishment of a vascular network that accelerates primary tumor growth and provides a gateway for invading metastatic cells.

14.3.1.1 Heparanase and Cancer Metastasis: Clinical Relevance

While the studies described above provide a proof-of-concept for the pro-metastatic and pro-angiogenic capacity of heparanase, the clinical significance of the enzyme in tumor progression emerges from a systematic evaluation of heparanase expression in primary human tumors. Immunohistochemistry, in situ hybridization, RT-PCR and real-time PCR analyses revealed that heparanase is upregulated in essentially all human tumors examined. These include carcinomas of the colon (Friedmann et al. 2000; Sato et al. 2004), thyroid (Xu et al. 2003), liver (El-Assal et al. 2001), pancreas (Kim et al. 2002; Koliopanos et al. 2001; Rohloff et al. 2002), bladder (Gohji et al. 2001a,b), cervix (Shinyo et al. 2003), breast (Maxhimer et al. 2002), gastric (Takaoka et al. 2003; Tang et al. 2002), prostate (Ogishima et al. 2005a), head and neck (Beckhove et al. 2005; Mikami et al. 2001; Simizu et al. 2003) and oral cavities, as well as multiple myeloma (Kelly et al. 2003), leukemia and lymphoma (Bitan et al. 2002). In most cases, elevated levels of heparanase were detected in about 50% of the tumor specimens, with a higher incidence in pancreatic (78%) and gastric (80%) carcinomas and in multiple myeloma (86%). In all cases, the normal-looking tissue adjacent to the malignant lesion expressed low or no detectable levels of heparanase, suggesting that epithelial cells do not normally express the enzyme. This is in agreement with the notion that under normal conditions heparanase expression is restricted primarily to the placenta, keratinocytes, lymphocytes, neutrophils, macrophages and platelets (Parish et al. 2001; Vlodavsky and Friedmann 2001). In several carcinomas, the most intense heparanase staining was localized to the invasive front of the tumor (Beckhove et al. 2005; Gohji et al. 2001b; Ohkawa et al. 2004; Tang et al. 2002), supporting a role for heparanase in cell invasion. Furthermore, patients diagnosed as heparanase-positive exhibited a significantly higher rate of local and distant metastasis as well as reduced postoperative survival compared with patients diagnosed as heparanase-negative (Gohji et al. 2001b; Kelly et al. 2003; Rohloff et al. 2002; Sato et al. 2004; Takaoka et al. 2003; Tang et al. 2002; Bar-Sela et al. 2006; Ben-Izhak et al. 2006; Doweck et al. 2006) (Fig. 14.3). Collectively, these studies provide a strong clinical support for the pro-metastatic function of heparanase. Interestingly, patient's survival was noted to correlate not only with heparanase levels, but also with its cellular localization. In addition to localization in the cytoplasm, heparanase was also noted to assume nuclear localization, demonstrated by cell fractionation (Schubert et al. 2004), and by immunostaining of cultured cells (Schubert et al. 2004) and tumor biopsies (Ohkawa et al. 2004; Takaoka et al. 2003). Interestingly, nuclear localization was correlated with maintained cellular differentiation and favorable outcome (Ohkawa et al. 2004; Takaoka et al. 2003; Doweck et al. 2006) (Fig. 14.4), suggesting that heparanase is intimately involved in gene regulation. Whether gene transcription and maintained cellular differentiation is due to direct interaction of heparanase with the DNA, or is a consequence of heparanase-mediated nuclear-HS degradation, is yet to be demonstrated.

In addition, heparanase upregulation in primary human tumors correlated in some cases with greater size of tumors (El-Assal et al. 2001; Maxhimer et al. 2002; Tang et al. 2002) and with enhanced microvessel density (El-Assal et al. 2001; Gohji et al. 2001a; Kelly et al. 2003; Sato et al. 2004; Shinyo et al. 2003; Watanabe et al. 2003), providing clinical support for the pro-angiogenic function of the enzyme.

14.3.2 Heparanase Pro-angiogenic Properties: Combining Molecular and Cellular Functions

Angiogenesis is a process of blood vessel formation in which new capillaries sprout from a pre-existing vascular network. The process requires that endothelial cells (EC) detach from the underlying base-

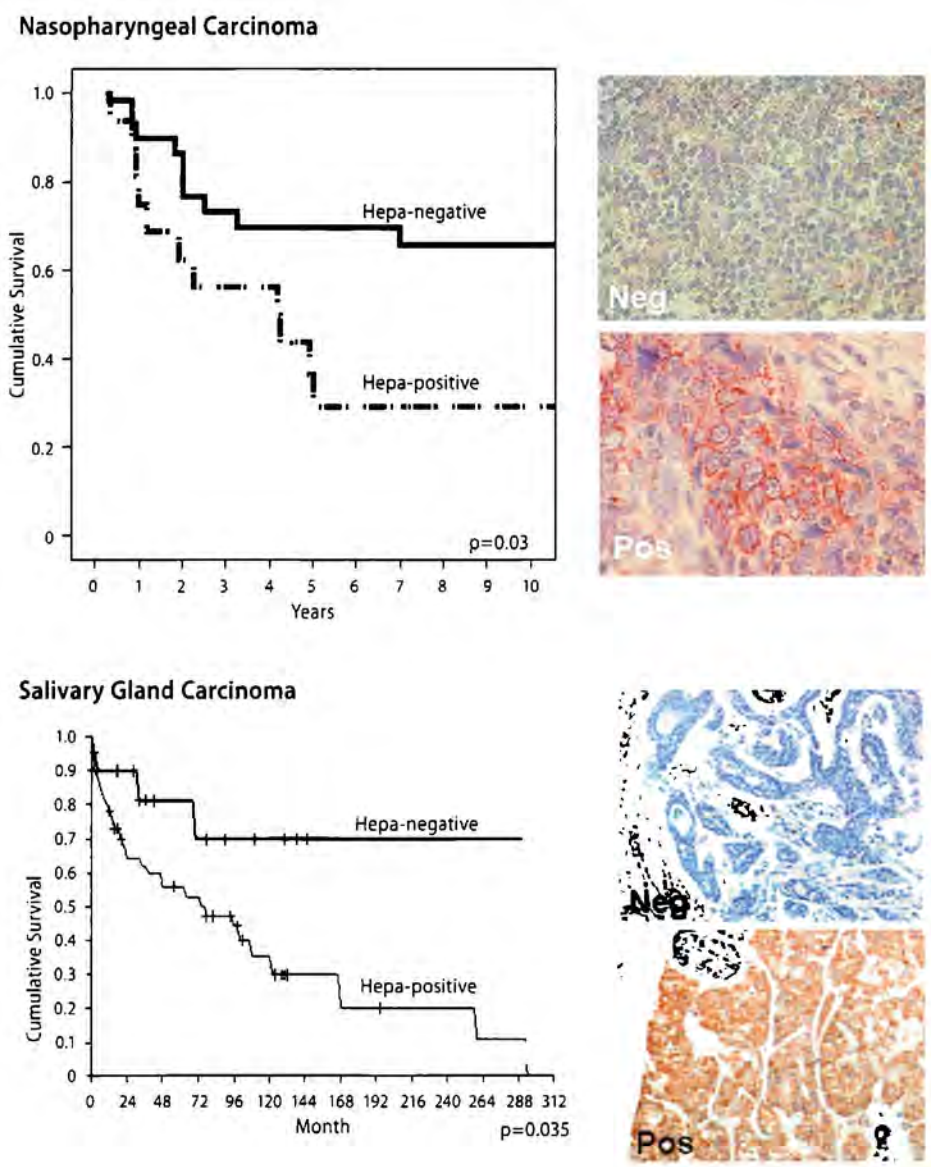


Fig. 14.3. Heparanase expression inversely correlates with patient survival. Kaplan–Meier analysis of overall survival of patients with heparanase-positive and -negative nasopharyngeal (*upper panel*, $p=0.03$) and salivary gland (*lower panel*, $p=0.035$) carcinoma

ment membrane (BM), migrate, proliferate, and reestablish new cell–cell contacts to form functional capillary tubes (Folkman 2003). Subsequent deposition of ECM components and recruitment of pericytes are required for vessel maturation (Folkman 2003). HSPGs are prominent components of blood vessels, and HSPG-degrading enzymes have long been implicated in a number of angiogenesis-related cellular processes. A critical early event in the angiogenic process is degradation of the subendothelial BM, followed by EC migration toward the angiogenic stimulus. Similar to its involvement in tumor cell dissemination, it is conceivable that by degrading HS in the BM, heparanase may directly facilitate EC invasion and sprouting. Indeed, heparanase expression

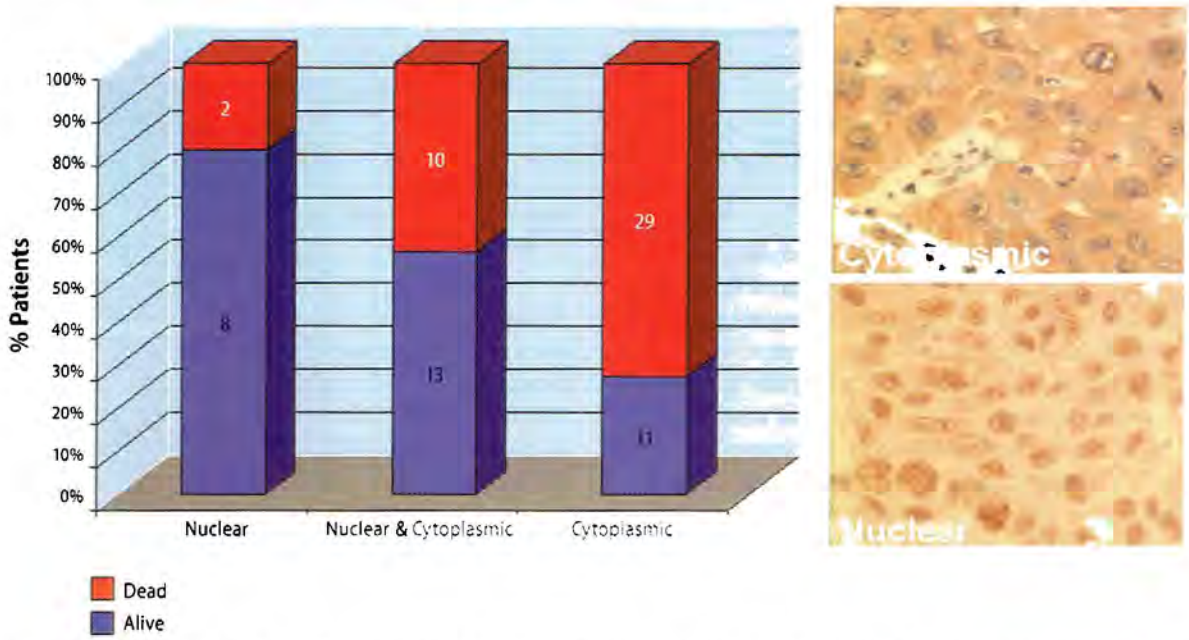


Fig. 14.4. Nuclear localization of heparanase predicts favorable outcome. Head and neck carcinoma specimens were stained with anti-heparanase antibodies and staining was categorized as nuclear (*right panel, bottom*), cytoplasmic (*right panel, top*) or both. Patient's status was analyzed according to heparanase cellular localization (*left panel*). Note the greater 5-year survival of patients exhibiting nuclear localization of heparanase

by bFGF-stimulated, bone marrow-derived EC was demonstrated by RT-PCR (Elkin et al. 2001). Immunohistochemistry of tumor specimens revealed heparanase staining of EC in capillaries, but not mature blood vessels (Elkin et al. 2001; Friedmann et al. 2000). Moreover, by releasing HS-bound angiogenic growth factors (i.e., bFGF, VEGF) from the ECM (Folkman et al. 1988), heparanase may indirectly facilitate EC migration and proliferation (Vlodavsky et al. 1996). In fact, given the multitude of biological mediators that are sequestered by HS in the ECM (Vlodavsky et al. 1991), heparanase activity liberates a number of active molecules that may act cooperatively or synergistically to promote neovascularization.

14.3.2.1 Wound Angiogenesis

Wound healing orchestrates multiple cell types (i.e., neutrophils, macrophages, fibroblasts, keratinocytes, EC), as well as soluble (i.e., growth factors, cytokines, chemokines) and insoluble (ECM components) mediators, in a complex sequence of events. Orchestration and regulation of the rapidly developing new tissue observed in wound healing depend not only on cells and bioactive polypeptides, but also on the ECM microenvironment, and require new blood vessel formation to nourish the newly formed granulation tissue. Elevated heparanase expression was observed in the wound granulation tissue and blood vessels (Zcharia et al. 2005b). Heparanase contribution to wound healing and wound angiogenesis has been demonstrated in several experimental settings. Increased amounts of heparanase were found in the wound fluid of

heparanase transgenic (*hpa*-tg) versus control mice (Zcharia et al. 2005b), in agreement with heparanase expression in healing wounds. Moreover, elevated heparanase levels in the wound fluid correlated with a comparable elevation of bFGF (Zcharia et al. 2005b), providing in vivo support for the ability of heparanase to release heparin-binding pro-angiogenic factors. Enhanced wound angiogenesis was further demonstrated in the *hpa*-tg mice by applying MRI (Zcharia et al. 2005b). Control mice exhibited an increased apparent vessel density (AVD) in the wound area that peaked 3 days after wounding, followed by a gradual decrease. In contrast, a marked fourfold increase in AVD was evident in the *hpa*-tg mice already 1 day after wounding and persisted until the end of the experiment on day 7. This effect was inhibited by non-anticoagulant glycol-split heparin (ST1514), shown to inhibit heparanase enzymatic activity at nM concentrations (Naggi et al. 2005) (see below), thus clearly supporting a role for heparanase in wound angiogenesis (Zcharia et al. 2005b). In a rat/flap punch model, topical application of highly active recombinant heparanase improved wound healing by 40% (Zcharia et al. 2005b) and enhanced wound angiogenesis (Elkin et al. 2001). Measurements taken in the area of flap incisions revealed a significant increase in epithelium thickness (Zcharia et al. 2005b), suggesting that heparanase promotes keratinocyte proliferation due to an improved bioavailability of factors such as KGF and HB-EGF. In addition, immunostaining of wound sections with anti-smooth muscle actin (SMA) antibody revealed a sevenfold increase in SMA-positive blood vessels in response to heparanase treatment (Zcharia et al. 2005b) (Fig. 14.5). Similarly, enhanced recruitment of SMA-positive cells was noted in a rat C6 glioma xenograft produced by heparanase transfected cells (our unpublished results, Fig. 14.5). Thus, heparanase accelerates wound healing by enhanced migration and proliferation of keratinocytes and stimulation of wound blood vessel formation and maturation. The coordinate, simultaneous release of a combination of HS-bound growth factors (i.e., bFGF, VEGF, HB-EGF, KGF) is unique to heparanase and may account for its efficient neovascularization and wound healing-promoting effect. It should be noted that wound healing is only one example of the involvement of heparanase in tissue remodeling, regeneration and neovascularization. For example, heparanase upregulation was observed during liver regeneration following hepatectomy (Goldshmidt et al. 2004). Furthermore, *hpa*-tg exhibited excess branching and widening of mammary gland ducts and an accelerated rate of hair growth, both associated with enhanced vascularization (Zcharia et al. 2004, 2005a), implying that heparanase contributes to neovascularization under normal and pathological conditions.

14.3.2.2 Tumor Models and Akt Activation

Heparanase overexpression in human U87 glioma (Zetser et al. 2003), HT 29 colon carcinoma (Doviner et al. 2006), CAG myeloma (Yang et al. 2005), and MCF7 (Cohen et al. 2006), MDA-MB-231 (Kelly et al. 2005), and MDA-MB-435 (Zetser et al. 2006) breast carcinoma cells correlated with enhanced xenograft tumor growth and vascularization. Using the RIP-Tag2 tumor model, Joyce et al. have recently demonstrated elevated levels of heparanase mRNA and protein upon the transition from normal to angiogenic islets, which further increased when solid tumors were detected (Joyce et al. 2005). These studies support the notion that heparanase not only facilitates tumor metastasis, but also contributes to the angiogenic switch and subsequent growth of the primary tumor. In these studies, enhanced tumor progression correlated with elevation in blood vessel density, revealed by staining with anti-PECAM-1 antibodies, as well as by MRI analysis (Cohen et al. 2006). At the molecular level, heparanase overexpression was noted to facilitate adhesion and migration of tumor cells, primary EC and T lymphocytes, mediated, at least in part, by β1-integrin and Rac activation (Goldshmidt et al. 2003; Sotnikov et al. 2004; Zetser et al. 2003). Heparanase overexpression in U87 glioma, as well as in several other tumor-derived cells, correlated with enhanced Akt/PKB phosphorylation levels (Zetser et al. 2003). Moreover, exogenous addition of heparanase to primary EC markedly stimulated Akt phosphorylation (Gin-

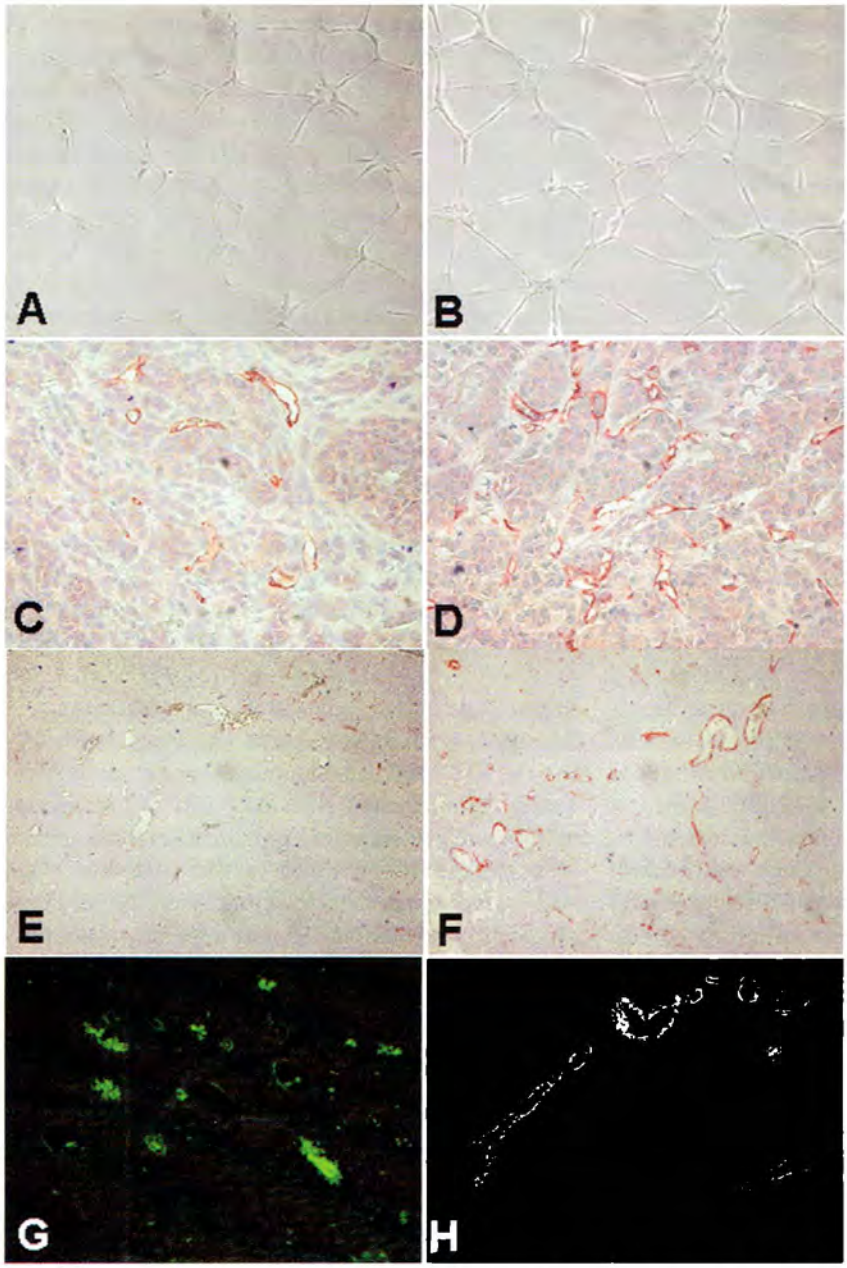


Fig. 14.5A–H. Angiogenic properties of heparanase. a, b In vitro angiogenesis. Human umbilical vein endothelial cells (HUVEC) were plated onto Matrigel-coated dishes in the absence (A) or presence (B) of latent 65-kDa heparanase (1 μg/ml) and organization into tube-like structures was examined after 24 h. C–F Tumor xenografts. Human breast carcinoma MDA-MB-435 (C, D) and rat C6 glioma (E, F) cells were stable transfected with control empty vector (C, E) or with heparanase cDNA (D, F), inoculated into mice, and tumor xenografts were stained with anti-PECAM-1 (C, D), or anti-smooth muscle actin (E, F) antibodies. Note enhanced tumor vascularity and blood vessels maturation upon heparanase overexpression. G, H Wound angiogenesis. Sections from control (*Con.*; G) or heparanase-treated (*Hepa*; H) full-thickness incision wound were stained with anti-smooth muscle actin antibody. Note a marked increase of blood vessel maturation upon heparanase treatment

gis-Velitski et al. 2004a). This effect occurred both in the presence and the absence of HS on the cell surface, was independent of heparanase enzymatic activity, and was augmented by heparin (Gingis-Velitski et al. 2004a). At the cellular level, heparanase addition stimulated PI 3-kinase-dependent EC migration and invasion and significantly improved EC rearrangement into lumen-containing tube-like structures (Gingis-Velitski et al. 2004a) (Fig. 14.5). These observations imply that heparanase is capable of eliciting angiogenic responses by a direct effect on EC. The ability of heparanase to stimulate Akt phosphorylation suggests that heparanase may protect tumor cells from apoptosis (Cohen et al. 2006), although the survival-promoting mechanism has not been sufficiently elucidated. The ability of exogenously added heparanase to activate signal transduction cascades, and its augmentation by heparin may indicate the existence of a cell surface heparanase receptor (Fig. 14.2, HBP), possibly LRP (Vreys et al. 2005), yet this aspect awaits further research and proper confirmation.

14.3.2.3 VEGF Induction and Src Activation

In addition to the above-described pro-angiogenic effects attributed to heparanase enzymatic and non-enzymatic activities, heparanase is also closely involved in VEGF gene regulation. Transfection and overexpression of heparanase in rat C6 glioma, MDA-MB-435 human breast carcinoma and human embryonic kidney HEK293 cells were accompanied by a three- to sixfold increase in VEGF mRNA and protein levels, correlated with enhanced Matrigel and tumor xenograft vascularization (Zetser et al. 2006). Moreover, transfection of the highly metastatic mouse B16-BL6 melanoma cells with heparanase-specific siRNA resulted in a 75% decrease in both heparanase mRNA and VEGF levels. This implies that endogenous heparanase is intimately involved in VEGF gene regulation. Interestingly, VEGF elevation by heparanase correlated with increased p38 phosphorylation levels, a signaling pathway implicated in VEGF induction (Zetser et al. 2006). Nonetheless, p38 inhibitors had no effect on heparanase-mediated VEGF upregulation, suggesting the operation of another signaling pathway(s) elicited by heparanase. Screening of several additional inhibitors led to the identification of Src, a kinase that was shown to modulate VEGF transcription (Ellis et al. 1998; Jiang et al. 1997; Mukhopadhyay et al. 1995), as a mediator of VEGF upregulation by heparanase. Moreover, Src inhibitors prevented VEGF induction by heparanase and significantly attenuated cell migration enhanced by heparanase, positioning Src as a critical downstream component that mediates heparanase functions (Zetser et al. 2006). VEGF induction and Src activation require heparanase secretion that was recapitulated by exogenous addition of the enzyme. Importantly, Src activation was noted also upon exogenous addition of point-mutated (Glu^{225}, Glu^{343}) heparanase that lacks enzymatic activity. Thus, heparanase exerts enzymatic activity-dependent (i.e., release of bFGF) and -independent (i.e., VEGF induction) pro-angiogenic effects. Given the large number of Src substrates, molecules other than VEGF are expected to be affected in response to Src activation. Indeed, phosphorylation of the catenin family member p120cat, a protein that was originally identified as a Src substrate, was markedly increased upon heparanase overexpression, in a Src-dependent manner (Zetser et al. 2006), likely representing only one example of Src substrates activated in response to heparanase overexpression. Overexpression of heparanase was also noted to upregulate cyclooxygenase-2 (COX-2) mRNA and protein in esophageal cancer cells, suggesting an involvement in COX-2-mediated tumor angiogenesis (Okawa et al. 2005). It therefore appears that in addition to its pro-metastatic function, heparanase affects several key components in tumor progression, resulting in increased blood vessel density and maturation, enhanced tumor cell motility, and activation of signaling mediators that govern tumor cell proliferation (i.e., Src) and survival (Akt). Collectively, these effects position heparanase as an attractive target for the development of anti-cancer drugs.

Inhibitory Molecules and Clinical Considerations

Attempts to inhibit heparanase enzymatic activity were initiated in the early days of heparanase research, in parallel with the emerging clinical relevance of this activity. More recently, with the availability of recombinant heparanase and the establishment of high-throughput screening methods, a variety of inhibitory molecules have been developed, including neutralizing antibodies, peptides, small molecules and modified non-anticoagulant species of heparin, as well as several other polyanionic molecules, such as laminaran sulfate, suramin and PI-88 (Ferro et al. 2004; Simizu et al. 2004a). PI-88 (phosphomannopentaose sulfate) exhibits anti-angiogenic, anti-metastatic, and anti-restenotic activities (Parish et al. 1999) and is undergoing phase II clinical trials for treatment of melanoma, myeloma and lung carcinoma (see Khachigian and Parish 2004 for recent review). This drug, which is composed primarily of sulfated phosphomannopentaose and phosphomannotetraose oligosaccharide units, was comparable with heparin in terms of its heparanase-inhibiting activity (Simizu et al. 2004a). PI-88, however, interferes with the action of HS-binding growth factors such as FGF-1, FGF-2, and VEGF, making interpretation regarding specificity and mode of action questionable, similar to other polyanionic compounds (Miao et al. 1999).

Suramin is a polysulfonated naphthyl urea, mimicking HS and having antineoplastic effects attributed to its ability to inhibit cell proliferation and block angiogenesis (Stein 1993). Suramin and chemically modified species of suramin exhibit a remarkable inhibitory activity toward melanoma cell invasion, attributed to its potent heparanase-inhibiting function (Marchetti et al. 2003; Nakajima et al. 1991). Suramin, however, has not been widely used due to its significant toxic effects in humans, including neurotoxicity, renal toxicity, adrenal insufficiency, and anticoagulant-mediated blood dyscrasias (Stein 1993).

14.4.1 Modulation of the Heparanase-Inhibiting Activity of Heparin Through Selective Desulfation, Graded N-Acetylation, and Glycol Splitting

Heparin is widely used as an anticoagulant for cancer patients at risk for venous thrombo-embolism. Recent clinical trials with low-molecular-weight heparin (LMWH) and meta-analyses of earlier clinical trials with unfractionated heparin indicate that heparin also exerts an anti-metastatic effect (Castelli et al. 2004; Kragh and Loechel 2005; Thodiyil and Kakkar 2002). Animal studies using non-anticoagulant species of heparin indicate that it is possible to separate the anti-metastatic and anticoagulant activities of heparin. The use of native heparin as an anti-metastatic agent is limited due to its potent anticoagulant activity. Non-anticoagulant heparins are of clinical potential because they could be administered at higher doses, thereby fully exploiting the anti-metastatic component of heparin, and because they could be applied to cancer patients with bleeding complications, where the use of heparin is precluded. The mechanism by which non-anticoagulant heparin inhibits metastasis is not fully understood. One possibility is that heparin inhibits metastasis by blocking platelet--tumor cell interactions, thereby inhibiting aggregates of tumor cells from lodging in the microvasculature (Borsig et al. 2001). Non-anticoagulant heparin also inhibits selectin-mediated cell–cell interactions (Borsig 2004), thus preventing, for example, extravasation of blood-borne cells. It stimulates tissue factor pathway inhibitor (TFPI) release and inhibits inflammatory responses (Thodiyil and Kakkar 2002). These effects, the inhibition of heparanase enzymatic activity and the encouraging animal studies and clinical trials clearly warrant further investigation of non-anticoagulant heparins as a promising therapeutic strategy for the inhibition of cancer metastasis.

As an analog of its natural HS substrate, heparin is commonly considered as a potent inhibitor of heparanase (Bar-Ner et al. 1987). This activity is attributed, in part, to its high-affinity interaction

with the enzyme and limited degradation, serving as an alternative substrate (Nasser et al. 2006). Early reports showed that heparin and some chemically modified species of heparin inhibit experimental metastasis in animal models, while other related compounds (e.g., chondroitin sulfate, carrageenan-κ, hyaluronic acid) that lack heparanase-inhibiting activity fail to exert an anti-metastatic effect (Nakajima et al. 1988; Parish et al. 1987; Vlodavsky et al. 1994). Screening of heparin derivatives permitted identification of some of its structural features associated with inhibition of the enzyme. We have analyzed the heparanase-inhibiting effect of heparin derivatives differing in degrees of 2-O- and 6-O-sulfation, N-acetylation, and glycol splitting of non-sulfated uronic acid residues (Naggi et al. 2005). The contemporaneous presence of sulfate groups at O-2 of IdoA and O-6 of GlcN was not essential for effective inhibition of heparanase activity, provided that one of the two positions retained a high degree of sulfation. N-desulfation/N-acetylation led to a marked decrease in inhibitory activity, suggesting that at least one NSO3 group per a disaccharide unit is involved in interaction with the enzyme. On the other hand, glycol splitting of preexisting or of both preexisting and chemically generated non-sulfated uronic acids dramatically increased the heparanase-inhibiting activity of N-acetylated heparin, irrespective of the degree of N-acetylation (Naggi et al. 2005). In fact, N-acetylated heparins in their glycol-split forms inhibit heparanase as effectively as the corresponding N-sulfated derivatives. While heparin and N-acetyl heparins containing unmodified GlcA residues inhibit heparanase by acting, at least in part, as substrates, their glycol-split derivatives are no longer susceptible to cleavage by heparanase (Naggi et al. 2005). It appears that formation of glycol-split residues generates three additional degrees of rotational freedom per each split residue, thus facilitating docking of heparin sequences to sites essential for heparanase activity. Notably, glycol-split N-acetyl heparins exhibit a marked decrease in the ability to release FGF-2 from ECM and to stimulate its mitogenic activity. Moreover, glycol-splitting involves substantial loss of the anticoagulant activity of heparin due to a complete loss of the heparin affinity for antithrombin (Casu et al. 2002). Collectively, the combination of high inhibition of heparanase, lack of anticoagulant activity and low release/potentiation of ECM-bound FGF-2 points to N-acetylated, glycol-split heparins as potential antiangiogenic and antimetastatic agents, more effective and specific than their counterparts with unmodified backbones (Naggi et al. 2005).

14.4.2 Other Inhibitory Strategies

An attractive approach for the inhibition of heparanase is the development and use of neutralizing monoclonal antibodies to the protein. Recently, a monoclonal antibody has been reported which effectively abolishes the activity of recombinant heparanase. Three companies entered into an alliance to develop monoclonal antibodies to heparanase, and the lead antibody (OGS-MDX-001) has already entered pre-clinical safety assessment (Al-Sarraf et al. 1998). Random, high-throughput screening of chemical libraries and microbial metabolites and rational design of compounds that block the active site or ligand-binding domain of heparanase are among the other approaches applied to develop effective heparanase inhibitors (Ferro et al. 2004; Pan et al. 2006; Simizu et al. 2004a). Natural endogenous heparanase inhibitors may also be identified. It would seem plausible that further defining the heparanase substrate specificity, catalytic and non-catalytic activities and the enzyme X-ray crystal structure would be invaluable for pursuing a more 'rational' approach to develop effective and highly specific heparanase-inhibiting molecules.

Conclusions and Perspectives

Although significant progress has been made during the past several years in understanding heparanase biology, there is much to be learned. Ac-

cumulation of compelling evidence implies that the enzyme is upregulated in primary human tumors and inversely correlates with survival rate of cancer patients post operation. While angiogenesis is the primary 'suspect' that governs heparanase-mediated tumor progression, this mode of action and the related clinical applications await further confirmation and require new molecular tools such as small inhibitory molecules, neutralizing antibodies and more stable, flexible and specific heparanase-inhibiting species of heparin and other saccharides. Although a few mechanisms that promote heparanase induction under pathological conditions have been reported, heparanase regulation at the transcriptional level requires further investigation. The ability of heparanase to function in a manner apparently independent of enzymatic activity, noted in several experimental settings (Gingis-Velitski et al. 2004a; Goldshmidt et al. 2003; Sotnikov et al. 2004; Zetser et al. 2003, 2006), is intriguing and affects the way the protein is envisioned. Thus, while attention was mainly focused on compounds that inhibit heparanase enzymatic activity, no information is available on protein domains responsible for the non-enzymatic functions of the heparanase molecule. In this respect, identification of a putative heparanase receptor is a major future challenge. Another important objective is the establishment of a reliable diagnostic assay to monitor heparanase levels and activity in plasma and urine of cancer patients by mean of a sensitive, high-throughput activity assay and ELISA method. The secreted nature of the enzyme and its induction in primary human tumors predict that under certain conditions the protein is present in body fluids. It is conceivable that elevated levels of heparanase are found in the plasma and/or urine of cancer patients, as well as in other pathological disorders such as diabetes (Katz et al. 2002; Levidiotis et al. 2004b; Shafat et al. 2006). Induction of heparanase already at early phases of cancer progression supports an important diagnostic and, possibly, prognostic value of such assays.

Recent publications clearly imply that heparanase may be involved in pathological conditions other than cancer. An intriguing example is the observation that heparanase-overexpressing transgenic mice escaped amyloid deposition in experimental models of inflammatory-associated amyloidosis (Li Jin-Ping et al. 2004) and prion disease (our unpublished observations). The possible involvement of heparanase in degenerative diseases such as Alzheimer's disease is only just starting to emerge and should be perused. Likewise, the causal involvement of heparanase in diabetic nephropathy (Levidiotis et al. 2005), autoimmunity (Lider et al. 1989) and inflammatory disorders (Edovitsky et al. 2006) should be investigated.

Functional domains other than the basic heterodimer structure (Fairbanks et al. 1999; Levy-Adam et al. 2003; McKenzie et al. 2003) and amino acids (Glu^{225}, Glu^{343}) critical for the enzyme' catalytic activity (Hulett et al. 2000), have not yet been identified in the heparanase protein, making screens for inhibitory molecules random in nature. The identification of heparin-binding domains (Levy-Adam et al. 2005) and the ability of the corresponding KKDC peptide, especially when applied as a dimer, to inhibit heparanase enzymatic activity (Levy-Adam et al. 2005; Zetser et al. 2004), clearly emphasize the need for crystallization and accurate understanding of the 3D structure of the enzyme toward an efficient drug development program.

While it is now well accepted that a single active heparanase enzyme is expressed by mammals, heparanase splice variants have recently been characterized in the Mole rat (Nasser et al. 2005). Although expected, due to the appearance of at least two mRNA transcripts in Northern blot analysis (Hulett et al. 1999), the existence of the same or other splice variants in mammals has not been confirmed, and their role has not been established. The heparanase system may be envisaged to include heparanase 1 and its splice variants, the three splice variants of heparanase 2 (McKenzie et al. 2000), the heparanase-processing protease and, possibly, the heparanase cell surface receptor. More information will enable the development of new inhibitory strategies directed against the enzyme's enzymatic and non-enzymatic functions, altogether offering better therapeutic opportunities.

Acknowledgements

This work was supported by grants from the Israel Science Foundation (grant 532/02); National Cancer Institute, NIH (grant RO1-CA106456); the Israel Cancer Research Fund; and the Rappaport Family Institute Fund.

References

Abboud-Jarrous G, Rangini-Guetta Z, Aingorn H, Atzmon R, Elgavish S, Peretz T, Vlodavsky I (2005) Site-directed mutagenesis, proteolytic cleavage, and activation of human proheparanase. J Biol Chem. 280:13568–13575

Abbracchio MP, Burnstock G (1998) Purinergic signalling: pathophysiological roles. Jpn J Pharmacol 78:113–145

Abdulkadir SA, Qu Z, Garabedian E, Song SK, Peters TJ, Svaren J, Carbone JM, Naughton CK, Catalona WJ, Ackerman JJ, Gordon JI, Humphrey PA, and Milbrandt J (2001) Impaired prostate tumorigenesis in Egr1-deficient mice. Nat Med 7:101–107

Al-Sarraf M, LeBlanc M, Giri PG, Fu KK, Cooper J, Vuong T, Forastiere AA, Adams G, Sakr WA, Schuller DE, Ensley JF (1998) Chemoradiotherapy versus radiotherapy in patients with advanced nasopharyngeal cancer: phase III randomized Intergroup study 0099. J Clin Oncol 16:1310–1317

Baraz L, Haupt Y, Elkin M, Peretz T, Vlodavsky I (2006) Tumor suppressor p53 regulates heparanase gene expression. Oncogene 25:3939–3947

Bar-Ner M, Kramer MD, Schirrmacher V, Ishai-Michaeli R, Fuks Z, Vlodavsky I (1985) Sequential degradation of heparan sulfate in the subendothelial extracellular matrix by highly metastatic lymphoma cells. Int J Cancer 35:483–491

Bar-Ner M, Eldor A, Wasserman L, Matzner Y, Cohen IR, Fuks Z, Vlodavsky I (1987) Inhibition of heparanase-mediated degradation of extracellular matrix heparan sulfate by non-anticoagulant heparin species. Blood 70:551–557

Bar-Sela G, Kaplan-Cohen V, Ilan N, Vlodavsky I, Ben-Izhak O (2006) Heparanase expression in nasopharyngeal carcinoma inversely correlates with patients' survival. Histopathology 49:188–193

Bartlett MR, Underwood PA, Parish CR (1995) Comparative analysis of the ability of leucocytes, endothelial cells and platelets to degrade the subendothelial basement membrane: evidence for cytokine dependence and detection of a novel sulfatase. Immunol Cell Biol 73:113–124

Beckhove P, Helmke BM, Ziouta Y, Bucur M, Dorner W, Mogler C, Dyckhoff G, Herold-Mende C (2005) Heparanase expression at the invasion front of human head and neck cancers and correlation with poor prognosis. Clin Cancer Res 11:2899–2906

Benavides F, Starost MF, Flores M, Gimenez-Conti IB, Guenet, Conti CJ (2002) Impaired hair follicle morphogenesis and cycling with J.-L. abnormal epidermal differentiation in nackt mice, a cathepsin L-deficient mutation. Am J Pathol 161:693–703

Ben-Izhak O, Kaplan-Cohen V, Ilan N, Gan S, Vlodavsky I, Nagler R (2006) Heparanase expression in malignant salivary gland tumors inversely correlates with long-term survival. Neoplasia 8:879–884

Bernfield M, Gotte M, Park PW, Reizes O, Fitzgerald ML, Lincecum J, Zako M (1999) Functions of cell surface heparan sulfate proteoglycans. Annu Rev Biochem 68:729–777

Bitan M, Polliack A, Zecchina G, Nagler A, Friedmann Y, Nadav L, Deutsch V, Pecker I, Eldor A, Vlodavsky I, Katz BZ (2002) Heparanase expression in human leukemias is restricted to acute myeloid leukemias. Exp Hematol 30:34–41

Borsig L (2004) Selectins facilitate carcinoma metastasis and heparin can prevent them. News Physiol Sci 19:16–21

Borsig L, Wong R, Feramisco J, Nadeau DR, Varki NM, Varki A (2001) Heparin and cancer revisited: mechanistic connections involving platelets, P-selectin, carcinoma mucins, and tumor metastasis. Proc Natl Acad Sci USA 98:3352–3357

Castelli R, Porro F, Tarsia P (2004) The heparins and cancer: review of clinical trials and biological properties. Vasc Med 9:205–213

Casu B, Guerrini M, Naggi A, Perez M, Torri G, Ribatti D, Carminati P, Giannini G, Penco S, Pisano C, Belleri M, Rusnati M, Presta M (2002) Short heparin sequences spaced by glycol-split uronate residues are antagonists of fibroblast growth factor 2 and angiogenesis inhibitors. Biochemistry 41:10519–10528

Chen G, Wang D, Vikramadithyan R, Yagyu H, Saxena U, Pillarisetti S, Goldberg IJ (2004) Inflammatory cytokines and fatty acids regulate endothelial cell heparanase expression. Biochemistry 43:4971–4977

Cohen E, Atzmon R, Vlodavsky I, Ilan N (2005) Heparanase processing by lysosomal/endosomal protein preparation. FEBS Lett 579:2334–2338

Cohen I, Pappo O, Elkin M, San T, Bar-Shavit R, Hazan R, Peretz T, Vlodavsky I, Abramovitch R (2006) Heparanase promotes growth, angiogenesis and survival of primary breast tumors. Int J Cancer 118:1609–1617

Communi D, Janssens R, Suarez-Huerta N, Robaye B, Boeynaems JM (2000) Advances in signalling by extracellular nucleotides. the role and transduction mechanisms of P2Y receptors. Cell Signal 12:351–360

De Mestre AM, Khachigian LM, Santiago FS, Staykova MA, Hulett MD (2003) Regulation of inducible heparanase gene transcription in activated T cells by early growth response 1. J Biol Chem 278:50377–50385

De Mestre AM, Rao S, Hornby JR, Soe-Htwe T, Khachigian LM, Hulett MD (2005) Early growth response gene 1 (EGR1) regulates heparanase gene transcription in tumor cells. J Biol Chem 280:35136–35147

Dempsey LA, Brunn GJ, Platt JL (2000) Heparanase, a potential regulator of cell-matrix interactions. Trends Biochem Sci 25:349–351

Doviner V, Mally B, Kaplan V, Gingis-Velitski S, Ilan N, Vlodavsky I, Sherman Y (2006) Spatial and temporal heparanase expression in colon mucosa throughout the adenoma-carcinoma sequence. Mod Pathol 19:878–888

Doweck I, Kaplan-Cohen V, Naroditsky I, Ilan N, Vlodavsky I (2006) Heparanase localization and expression by head and neck cancer: correlation with tumor progression and patient survival. Neoplasia 8:1055–1061

Edovitsky E, Elkin M, Zcharia E, Peretz T, Vlodavsky I (2004) Heparanase gene silencing, tumor invasiveness, angiogenesis, and metastasis. J Natl Cancer Inst 96:1219–1230

Edovitsky E, Lerner I, Zcharia E, Peretz T, Vlodavsky I, Elkin M (2006) Role of endothelial heparanase in delayed-type hypersensitivity. Blood 3609–3616

El-Assal ON, Yamanoi A, Ono T, Kohno H, Nagasue N (2001) The clinicopathological significance of heparanase and basic fibroblast growth factor expressions in hepatocellular carcinoma. Clin Cancer Res 7:1299–1305

Elkin M, Ilan N, Ishai-Michaeli R, Friedmann Y, Papo O, Pecker I, Vlodavsky I (2001) Heparanase as mediator of angiogenesis: mode of action. FASEB J 15:1661–1663

Elkin M, Cohen I, Zcharia E, Orgel A, Guatta-Rangini Z, Peretz T, Vlodavsky I, Kleinman HK (2003) Regulation of heparanase gene expression by estrogen in breast cancer. Cancer Res 63:8821–8826

Ellis LM, Staley CA, Liu W, Fleming RY, Parikh NU, Bucana CD, Gallick GE (1998) Down-regulation of vascular endothelial growth factor in a human colon carcinoma cell line transfected with an antisense expression vector specific for c-src. J Biol Chem 273:1052–1057

Fairbanks MB, Mildner AM, Leone JW, Cavey GS, Mathews WR, Drong RF, Slightom JL, Bienkowski MJ, Smith CW, Bannow CA, Heinrikson RL (1999) Processing of the human heparanase precursor and evidence that the active enzyme is a heterodimer. J Biol Chem 274:29587–29590

Fata JE, Werb Z, Bissell MJ (2004) Regulation of mammary gland branching morphogenesis by the extracellular matrix and its remodeling enzymes. Breast Cancer Res 6:1–11

Ferro V, Hammond E, Fairweather JK (2004) The development of inhibitors of heparanase, a key enzyme involved in tumour metastasis, angiogenesis and inflammation. Mini Rev Med Chem 4:693–702

Folkman J (2003) Fundamental concepts of the angiogenic process. Curr Mol Med 3:643–651

Folkman J, Klagsbrun M, Sasse J, Wadzinski M, Ingber D, Vlodavsky I (1988) A heparin-binding angiogenic protein – basic fibroblast growth factor – is stored within basement membrane. Am J Pathol 130:393–400

Freeman C, Parish CR (1998) Human platelet heparanase: purification, characterization and catalytic activity. Biochem J 330:1341–1350

Friedmann Y, Vlodavsky I, Aingorn H, Aviv A, Peretz T, Pecker I, Pappo O (2000) Expression of heparanase in normal, dysplastic, and neoplastic human colonic mucosa and stroma. Evidence for its role in colonic tumorigenesis. Am J Pathol 157:1167–1175

Fuller M, Chau A, Nowak RC, Hopwood JJ, Meikle PJ (2006) A defect in exo-degradative pathways provides insight into endo-degradation of heparan and dermatan sulfates. Glycobiology 16:318–325

Gingis-Velitski S, Zetser A, Flugelman MY, Vlodavsky I, Ilan N (2004a) Heparanase induces endothelial cell migration via protein kinase B/Akt activation. J Biol Chem 279:23536–23541

Gingis-Velitski S, Zetser A, Kaplan V, Ben-Zaken O, Cohen E, Levy-Adam F, Bashenko Y, Flugelman MY, Vlodavsky I, Ilan N (2004b) Heparanase uptake is mediated by cell membrane heparan sulfate proteoglycans. J Biol Chem 279:44084–44092

Gohji K, Hirano H, Okamoto M, Kitazawa S, Toyoshima M, Dong J, Katsuoka Y, Nakajima M (2001a) Expression of three extracellular matrix degradative enzymes in bladder cancer. Int J Cancer 95:295–301

Gohji K, Okamoto M, Kitazawa S, Toyoshima M, Dong J, Katsuoka Y, Nakajima M (2001b) Heparanase protein and gene expression in bladder cancer. J Urol 166:1286–1290

Goldshmidt O, Zcharia E, Aingorn H, Guatta-Rangini Z, Atzmon R, Michal I, Pecker I, Mitrani E, Vlodavsky I (2001) Expression pattern and secretion of human and chicken heparanase are determined by their signal peptide sequence. J Biol Chem 276:29178–29187

Goldshmidt O, Nadav L, Aingorn H, Irit C, Feinstein N, Ilan N, Zamir E, Geiger B, Vlodavsky I, Katz BZ (2002) Human heparanase is localized within lysosomes in a stable form. Exp Cell Res 281:50–62

Goldshmidt O, Zcharia E, Cohen M, Aingorn H, Cohen I, Nadav L, Katz B-Z, Geiger B, Vlodavsky I (2003) Heparanase mediates cell adhesion independent of its enzymatic activity. FASEB J 17:1015–1025

Goldshmidt O, Yeikilis R, Mawasi N, Paizi M, Gan N, Ilan N, Pappo O, Vlodavsky I, Spira G (2004) Heparanase expression during normal liver development and following partial hepatectomy. J Pathol 203:594–602

Gordon JL (1986) Extracellular ATP: effects, sources and fate. Biochem J 233:309–319

Hulett MD, Freeman C, Hamdorf BJ, Baker RT, Harris MJ, Parish CR (1999) Cloning of mammalian heparanase, an important enzyme in tumor invasion and metastasis. Nat Med 5:803–809

Hulett MD, Hornby JR, Ohms SJ, Zuegg J, Freeman C, Gready JE, Parish CR (2000) Identification of active-site residues

of the pro-metastatic endoglycosidase heparanase. Biochemistry 39:15659–15667

Jiang BH, Agani F, Passaniti A, Semenza GL (1997) V-SRC induces expression of hypoxia-inducible factor 1 (HIF-1) and transcription of genes encoding vascular endothelial growth factor and enolase 1: involvement of HIF-1 in tumor progression. Cancer Res 57:5328–5335

Jiang P, Kumar A, Parrillo JE, Dempsey LA, Platt JL, Prinz RA, Xu X (2002) Cloning and characterization of the human heparanase-1 (HPR1) gene promoter: role of GA-binding protein and Sp1 in regulating HPR1 basal promoter activity. J Biol Chem 277:8989–8998

Joyce JA, Freeman C, Meyer-Morse N, Parish CR, Hanahan D (2005) A functional heparan sulfate mimetic implicates both heparanase and heparan sulfate in tumor angiogenesis and invasion in a mouse model of multistage cancer. Oncogene 24:4037–4051

Katz A, Van-Dijk DJ, Aingorn H, Erman A, Davies M, Darmon D, Hurvitz H, Vlodavsky I (2002) Involvement of human heparanase in the pathogenesis of diabetic nephropathy. Isr Med Assoc J 4:996–1002

Kelly T, Miao H.-Q, Yang Y, Navarro E, Kussie P, Huang Y, MacLeod V, Casciano J, Joseph L, Zhan F, Zangari M, Barlogie B, Shaughnessy J, Sanderson RD (2003) High heparanase activity in multiple myeloma is associated with elevated microvessel density. Cancer Res 63:8749–8756

Kelly T, Suva LJ, Huang Y, Macleod V, Miao HQ, Walker RC, Sanderson RD (2005) Expression of heparanase by primary breast tumors promotes bone resorption in the absence of detectable bone metastases. Cancer Res 65:5778–5784

Khachigian LM (2004) Early growth response-1: blocking angiogenesis by shooting the messenger. Cell Cycle 3:10–11

Khachigian LM, Parish CR (2004) Phosphomannopentaose sulfate (PI-88): heparan sulfate mimetic with clinical potential in multiple vascular pathologies. Cardiovasc Drug Rev 22:1–6

Kim AW, Xu X, Hollinger EF, Gattuso P, Godellas CV, Prinz RA (2002) Human heparanase-1 gene expression in pancreatic adenocarcinoma. J Gastrointest Surg 6:167–172

Koliopanos A, Friess H, Kleeff J, Shi X, Liao Q, Pecker I, Vlodavsky I, Zimmermann A, Buchler MW (2001) Heparanase expression in primary and metastatic pancreatic cancer. Cancer Res 61:4655–4659

Kragh M, Loechel F (2005) Non-anti-coagulant heparins: a promising approach for prevention of tumor metastasis (review). Int J Oncol 27:1159–1167

Kram V, Zcharia E, Yacoby-Zeevi O, Metzger S, Chajek-Shaul T, Gabet Y, Muller R, Vlodavsky I, Bab I (2006) Heparanase is expressed in osteoblastic cells and stimulates bone formation and bone mass. J Cell Physiol 207:784–792

Kussie PH, Hulmes JD, Ludwig DL, Patel S, Navarro EC, Seddon AP, Giorgio NA, Bohlen P (1999) Cloning and functional expression of a human heparanase gene. Biochem Biophys Res Commun 261:183–187

Levidiotis V, Kanellis J, Ierino FL, Power DA (2001) Increased expression of heparanase in puromycin aminonucleoside nephrosis. Kidney Int 60:1287–1296

Levidiotis V, Freeman C, Punler M, Martinello P, Creese B, Ferro V, van der Vlag J, Berden JH, Parish CR, Power DA (2004a) A synthetic heparanase inhibitor reduces proteinuria in passive Heymann nephritis. J Am Soc Nephrol 15:2882–2892

Levidiotis V, Freeman C, Tikellis C, Cooper ME, Power DA (2004b) Heparanase is involved in the pathogenesis of proteinuria as a result of glomerulonephritis. J Am Soc Nephrol 15:68–78

Levidiotis V, Freeman C, Tikellis C, Cooper ME, Power DA (2005) Heparanase inhibition reduces proteinuria in a model of accelerated anti-glomerular basement membrane antibody disease. Nephrology (Carlton) 10:167–173

Levy-Adam F, Miao HQ, Heinrikson RL, Vlodavsky I, Ilan N (2003) Heterodimer formation is essential for heparanase enzymatic activity. Biochem Biophys Res Commun 308:885–891

Levy-Adam F, Abboud-Jarrous G, Guerrini M, Beccoti D, Vlodavsky I, Ilan N (2005) Identification and characterization of heparin/heparan sulfate binding domains of the endoglycosidase heparanase. J Biol Chem 280:20457–20466

Li Jin-Ping MLEG, Zhang X, Zcharia E, Metzger S, Vlodavsky I, Kisilevsky R, Lindahl U (2004) In vivo fragmentation of heparan sulfate by heparanase overexpression renders mice resitant to amyloid protein A amyloidosis. PNAS 102:6473–6477

Lider O, Baharav E, Mekori YA, Miller T, Naparstek Y, Vlodavsky I, Cohen IR (1989) Suppression of experimental autoimmune diseases and prolongation of allograft survival by treatment of animals with low doses of heparins. J Clin Invest 83:752–756

Marchetti D, Reiland J, Erwin B, Roy M (2003) Inhibition of heparanase activity and heparanase-induced angiogenesis by suramin analogues. Int J Cancer 104:167–174

Maxhimer JB, Quiros RM, Stewart R, Dowlatshahi K, Gattuso P, Fan M, Prinz RA, Xu X (2002) Heparanase-1 expression is associated with the metastatic potential of breast cancer. Surgery 132:326–333

McKenzie E, Tyson K, Stamps A, Smith P, Turner P, Barry R, Hircock M, Patel S, Barry E, Stubberfield C, Terrett J, Page M (2000) Cloning and expression profiling of Hpa2, a novel mammalian heparanase family member. Biochem Biophys Res Commun 276:1170–1177

McKenzie E, Young K, Hircock M, Bennett J, Bhaman M, Felix R, Turner P, Stamps A, McMillan D, Saville G, Ng S, Mason S, Snell D, Schofield D, Gong H, Townsend R, Gallagher J, Page M, Parekh R, Stubberfield C (2003) Biochemical characterisation of the active heterodimer form of human heparanase (Hpa1) protein expressed in insect cells. Biochem J 373:423–435

Miao HQ, Elkin M, Aingorn E, Ishai-Michaeli R, Stein CA, Vlodavsky I (1999) Inhibition of heparanase activity and tumor metastasis by laminarin sulfate and synthetic phosphorothioate oligodeoxynucleotides. Int J Cancer 83:424–431

Mikami S, Ohashi K, Usui Y, Nemoto T, Katsube K, Yanagishita M, Nakajima M, Nakamura K, Koike M (2001) Loss of syndecan-1 and increased expression of heparanase in invasive esophageal carcinomas. Jpn J Cancer Res 92:1062–1073

Mukhopadhyay D, Tsiokas L, Zhou XM, Foster D, Brugge JS, Sukhatme VP (1995) Hypoxic induction of human vascular endothelial growth factor expression through c-Src activation. Nature 375:577–581

Nadav L, Eldor A, Yacoby-Zeevi O, Zamir E, Pecker I, Ilan N, Geiger B, Vlodavsky I, Katz BZ (2002) Activation, processing and trafficking of extracellular heparanase by primary human fibroblasts. J Cell Sci 115:2179–2187

Naggi A, Casu B, Perez M, Torri G, Cassinelli G, Penco S, Pisano C, Giannini G, Ishai-Michaeli R, Vlodavsky I (2005) Modulation of the heparanase-inhibiting activity of heparin through selective desulfation, graded N-acetylation, and glycol splitting. J Biol Chem 280:12103–12113

Nakajima M, Irimura T, DiFerrante D, DiFerrante N, Nicolson GL (1983) Heparan sulfate degradation: relation to tumor invasion and metastatic properties of Mouse B 16 Melanoma sublines. Science 220:611–613

Nakajima M, Irimura T, di Ferrante N, Nicolson GL (1984) Metastatic melanoma cell heparanase. Characterization of heparan sulfate degradation fragments produced by B16 melanoma endoglucuronidase. J Biol Chem 259:2283–2290

Nakajima M, Irimura T, Nicolson GL (1988) Heparanases and tumor metastasis. J Cell Biochem 36:157–167

Nakajima M, DeChavigny A, Johnson CE, Hamada J, Stein CA, Nicolson GL (1991) Suramin. A potent inhibitor of melanoma heparanase and invasion. J Biol Chem 266:9661–9666

Naparstek Y, Cohen IR, Fuks Z, Vlodavsky I (1984) Activated T lymphocytes produce a matrix-degrading heparan sulphate endoglycosidase. Nature 310:241–244

Nardella C, Lahm A, Pallaoro M, Brunetti M, Vannini A, Steinkuhler C (2004) Mechanism of activation of human heparanase investigated by protein engineering. Biochemistry 43:1862–1873

Nasser NJ, Nevo E, Shafat I, Ilan N, Vlodavsky I, Avivi A (2005) Adaptive evolution of heparanase in hypoxia-tolerant Spalax: gene cloning and identification of a unique splice variant. PNAS 102:15161–15166

Nasser NJ, Sarig G, Brenner B, Nevo E, Goldshmidt O, Zcharia E, Li JP, Vlodavsky I (2006) Heparanase neutralizes the anticoagulation properties of heparin and low-molecular-weight heparin. J Thromb Haemost 4:560–565

Ogishima T, Shiina H, Breault JE, Tabatabai L, Bassett WW, Enokida H, Li LC, Kawakami T, Urakami S, Ribeiro-Filho LA, Terashima M, Fujime M, Igawa M, Dahiya R (2005a) Increased heparanase expression is caused by promoter hypomethylation and up-regulation of transcriptional factor early growth response-1 in human prostate cancer. Clin Cancer Res 11:1028–1036

Ogishima T, Shiina H, Breault JE, Terashima M, Honda S, Enokida H, Urakami S, Tokizane T, Kawakami T, Ribeiro-Filho LA, Fujime M, Kane CJ, Carroll PR, Igawa M, Dahiya R (2005b) Promoter CpG hypomethylation and transcription factor EGR1 hyperactivate heparanase expression in bladder cancer. Oncogene 24:6765–6772

Ohkawa T, Naomoto Y, Takaoka M, Nobuhisa T, Noma K, Motoki T, Murata T, Uetsuka H, Kobayashi M, Shirakawa Y, Yamatsuji T, Matsubara N, Matsuoka J, Haisa M, Gunduz M, Tsujigiwa H, Nagatsuka H, Hosokawa M, Nakajima M, Tanaka N (2004. Localization of heparanase in esophageal cancer cells: respective roles in prognosis and differentiation. Lab Invest. 84:1289–304

Okawa T, Naomoto Y, Nobuhisa T, Takaoka M, Motoki T, Shirakawa Y, Yamatsuji T, Inoue H, Ouchida M, Gunduz M, Nakajima M, Tanaka N (2005) Heparanase is involved in angiogenesis in esophageal cancer through induction of cyclooxygenase-2. Clin Cancer Res 11:7995–8005

Pan W, Miao HQ, Xu YJ, Navarro EC, Tonra JR, Corcoran E, Lahiji A, Kussie P, Kiselyov AS, Wong WC, Liu H (2006) 1-[4-(1H-Benzoimidazol-2-yl)-phenyl]-3-[4-(1H-benzoimidazol-2-yl)-phenyl]- urea derivatives as small molecule heparanase inhibitors. Bioorg Med Chem Lett 16:409–412

Parish CR, Coombe DR, Jakobsen KB, Bennett FA, Underwood PA (1987) Evidence that sulphated polysaccharides inhibit tumour metastasis by blocking tumour-cell-derived heparanases. Int J Cancer 40:511–518

Parish CR, Hindmarsh EJ, Bartlett MR, Staykova MA, Cowden WB, Willenborg DO (1998) Treatment of central nervous system inflammation with inhibitors of basement membrane degradation. Immunol Cell Biol 76:104–113

Parish CR, Freeman C, Brown KJ, Francis DJ, Cowden WB (1999) Identification of sulfated oligosaccharide-based inhibitors of tumor growth and metastasis using novel in vitro assays for angiogenesis and heparanase activity. Cancer Res 59:3433–3441

Parish CR, Freeman C, Hulett MD (2001) Heparanase: a key enzyme involved in cell invasion. Biochim Biophys Acta 1471:M99–M108

Pikas DS, Li J-P, Vlodavsky I, Lindahl U (1998) Substrate specificity of heparanases from human hepatoma and platelets. J Biol Chem 273:18770–18777

Potts W, Bowyer J, Jones H, Tucker D, Freemont AJ, A. Millest, Martin C, Vernon W, Neerunjun D, Slynn G, Harper F, Maciewicz R (2004) Cathepsin L-deficient mice exhibit abnormal skin and bone development and show increased resistance to osteoporosis following ovariectomy. Int J Exp Pathol 85:85–96

Rohloff J, Zinke J, Schoppmeyer K, Tannapfel A, Witzig-

mann H, Mossner J, Wittekind C, Caca K (2002) Heparanase expression is a prognostic indicator for postoperative survival in pancreatic adenocarcinoma. Br J Cancer 86:1270–1275

Sanderson RD, Yang Y, Suva LJ, Kelly T (2004) Heparan sulfate proteoglycans and heparanase – partners in osteolytic tumor growth and metastasis. Matrix Biol 23:341–352

Sanderson RD, Yang Y, Kelly T, Macleod V, Dai Y, Theus A (2005) Enzymatic remodeling of heparan sulfate proteoglycans within the tumor microenvironment: growth regulation and the prospect of new cancer therapies. J Cell Biochem 96:897–905

Sasisekharan R, Shriver Z, Venkataraman G, Narayanasami U (2002) Roles of heparan-sulphate glycosaminoglycans in cancer. Nat Rev Cancer 2:521–528

Sato T, Yamaguchi A, Goi T, Hirono Y, Takeuchi K, Katayama K, Matsukawa S (2004) Heparanase expression in human colorectal cancer and its relationship to tumor angiogenesis, hematogenous metastasis, and prognosis. J Surg Oncol 87:174–181

Schubert SY, Ilan N, Shushy M, Ben-Izhak O, Vlodavsky I, Goldshmidt O (2004) Human heparanase nuclear localization and enzymatic activity. Lab Invest 84:535–544

Shafat I, Zcharia E, Nisman B, Nadir Y, Nakhoul F, Vlodavsky I, Ilan N (2006) An ELISA method for the detection and quantification of human heparanase. Biochem Biophys Res Commun 341:958–963

Shinyo Y, Kodama J, Hongo A, Yoshinouchi M, Hiramatsu Y (2003) Heparanase expression is an independent prognostic factor in patients with invasive cervical cancer. Ann Oncol 14:1505–1510

Shteper PJ, Zcharia E, Ashhab Y, Peretz T, Vlodavsky I, Ben-Yehuda D (2003) Role of promoter methylation in regulation of the mammalian heparanase gene. Oncogene 22:7737–7749

Simizu S, Ishida K, Wierzba MK, Sato TA, Osada H (2003) Expression of heparanase in human tumor cell lines and human head and neck tumors. Cancer Lett 193:83–89

Simizu S, Ishida K, Osada H (2004a) Heparanase as a molecular target of cancer chemotherapy. Cancer Sci 95:553–558

Simizu S, Ishida K, Wierzba MK, Osada H (2004b) Secretion of heparanase protein is regulated by glycosylation in human tumor cell lines. J Biol Chem 279:2697–2703

Sotnikov I, Hershkoviz R, Grabovsky V, Ilan N, Cahalon L, Vlodavsky I, Alon R, Lider O (2004) Enzymatically quiescent heparanase augments T cell interactions with VCAM-1 and extracellular matrix components under versatile dynamic contexts. J Immunol 172:5185–5193

Stein CA (1993) Suramin: a novel antineoplastic agent with multiple potential mechanisms of action. Cancer Res 53:2239–2248

Takaoka M, Naomoto Y, Ohkawa T, Uetsuka H, Shirakawa Y, Uno F, Fujiwara T, Gunduz M, Nagatsuka H, Nakajima M, Tanaka N, Haisa M (2003) Heparanase expression correlates with invasion and poor prognosis in gastric cancers. Lab Invest 83:613–622

Tang W, Nakamura Y, Tsujimoto M, Sato M, Wang X, Kurozumi K, Nakahara M, Nakao K, Nakamura M, Mori I, Kakudo K (2002) Heparanase: a key enzyme in invasion and metastasis of gastric carcinoma. Mod Pathol 15:593–598

Thodiyil P, Kakkar AK (2002) Can low-molecular-weight heparins improve outcome in patients with cancer? Cancer Treat Rev 28:151–155

Toyoshima M, Nakajima M (1999) Human heparanase. Purification, characterization, cloning, and expression. J Biol Chem 274:24153–24160

Turk B, Turk D, Turk V (2000) Lysosomal cysteine proteases: more than scavengers. Biochim Biophys Acta 1477:98–111

Turk V, Kos J, Turk B (2004) Cysteine cathepsins (proteases) – on the main stage of cancer? Cancer Cell 5:409–410

Van der Weyden L, Conigrave AD, Morris MB (2000) Signal transduction and white cell maturation via extracellular ATP and the P2Y11 receptor. Immunol Cell Biol 78:369–374

Vlodavsky I, Friedmann Y (2001) Molecular properties and involvement of heparanase in cancer metastasis and angiogenesis. J Clin Invest 108:341–347

Vlodavsky I, Goldshmidt O (2001) Properties and function of heparanase in cancer metastasis and angiogenesis. Haemostasis 31:60–63

Vlodavsky I, Fuks Z, Bar-Ner M, Ariav Y, Schirrmacher V (1983) Lymphoma cells mediated degradation of sulfated proteoglycans in the subendothelial extracellular matrix: relation to tumor cell metastasis. Cancer Res 43:2704–2711

Vlodavsky I, Folkman J, Sullivan R, Fridman R, Ishai-Michaeli R, Sasse J, Klagsbrun M (1987) Endothelial cell-derived basic fibroblast growth factor: synthesis and deposition into subendothelial extracellular matrix. Proc Natl Acad Sci USA 84:2292–2296

Vlodavsky I, Bar-Shavit R, Ishai-Michaeli R, Bashkin P, Fuks Z (1991) Extracellular sequestration and release of fibroblast growth factor: a regulatory mechanism? Trends Biochem Sci 16:268–271

Vlodavsky I, Eldor A, Haimovitz-Friedman A, Matzner Y, Ishai-Michaeli R, Lider O, Naparstek Y, Cohen IR, Fuks Z (1992) Expression of heparanase by platelets and circulating cells of the immune system: possible involvement in diapedesis and extravasation. Invasion Metastasis 12:112–127

Vlodavsky I, Mohsen M, Lider O, Svahn CM, Ekre HP, Vigoda M, Ishai-Michaeli R, Peretz T (1994) Inhibition of tumor metastasis by heparanase inhibiting species of heparin. Invasion Metastasis 14:290–302

Vlodavsky I, Miao HQ, Medalion B, Danagher P, Ron D (1996) Involvement of heparan sulfate and related molecules in sequestration and growth promoting activ-

ity of fibroblast growth factor. Cancer Metastasis Rev 15:177–186

Vlodavsky I, Friedmann Y, Elkin M, Aingorn H, Atzmon R, Ishai-Michaeli R, Bitan M, Pappo O, Peretz T, Michal I, Spector L, Pecker I (1999) Mammalian heparanase: gene cloning, expression and function in tumor progression and metastasis. Nat Med 5:793–802

Vreys V, Delande N, Zhang Z, Coomans C, Roebroek A, Durr J, David G (2005) Cellular uptake of mammalian heparanase precursor involves low density lipoprotein receptor-related proteins, mannose 6-phosphate receptors, and heparan sulfate proteoglycans. J Biol Chem 280:33141–33148

Vu TH, Werb Z (2000) Matrix metalloproteinases: effectors of development and normal physiology. Genes Dev 14:2123–21233

Watanabe M, Aoki Y, Kase H, Tanaka K (2003) Heparanase expression and angiogenesis in endometrial cancer. Gynecol Obstet Invest 56:77–82

Werb Z (1997) ECM and cell surface proteolysis: regulating cellular ecology. Cell 91:439–442

Xu X, Quiros RM, Maxhimer JB, Jiang P, Marcinek R, Ain KB, Platt JL, Shen J, Gattuso P, Prinz RA (2003) Inverse correlation between heparan sulfate composition and heparanase-1 gene expression in thyroid papillary carcinomas: a potential role in tumor metastasis. Clin Cancer Res 9:5968–5979

Yang Y, Macleod V, Bendre M, Huang Y, Theus AM, Miao HQ, Kussie P, Yaccoby S, Epstein J, Suva LJ, Kelly T, Sanderson RD (2005) Heparanase promotes the spontaneous metastasis of myeloma cells to bone. Blood 105:1303–1309

Zcharia E, Metzger S, Chajek-Shaul T, Aingorn H, Elikn M, Friedmann Y, Weinstein T, Jin-Ping L, Lindahl U, Vlodavsky I (2004) Transgenic expression of mammalian heparanase uncovers physiological functions of heparan sulfate in tissue morphogenesis, vascularization, and feeding behavior. FASEB J 18:252–263

Zcharia E, Philp D, Edovitsky E, Aingorn H, Metzger S, Kleinman HK, Vlodavsky I, Elkin M (2005a) Heparanase regulates murine hair growth. Am J Pathol 166:999–1008

Zcharia E, Zilka R, Yaar A, Yacoby-Zeevi AO, Zetser, Metzger S, Sarid R, Naggi A, Casu B, Ilan N, Vlodavsky I, Abramovitch R (2005b) Heparanase accelerates wound angiogenesis and wound healing in mouse and rat models. FASEB J 19:211–221

Zetser A, Bashenko Y, Miao H.-Q, Vlodavsky I, Ilan N (2003) Heparanase affects adhesive and tumorigenic potential of human glioma cells. Cancer Res 63:7733–7741

Zetser A, Bashenko Y, Edovitsky E, Levy-Adam F, Vlodavsky I, Ilan N (2006) Heparanase induces vascular endothelial growth factor expression: correlation with p38 phosphorylation levels and Src activation 10.1158/0008–5472.CAN-05–1811. Cancer Res 66:1455–1463

Zetser A, Levy-Adam F, Kaplan V, Gingis-Velitski S, Bashenko Y, Schubert S, Flugelman MY, Vlodavsky I, Ilan N (2004) Processing and activation of latent heparanase occurs in lysosomes. J Cell Sci 117:2249–2258

Vessel Maturation and Perivascular Cells

15

ANNE M. GOODWIN and PATRICIA A. D'AMORE

Abstract

Maturation of new vessels involves interaction of vascular endothelial cells with surrounding mural cells, namely smooth muscle cells or pericytes. In this chapter we will focus on the role of pericytes, as well as the various factors secreted by pericytes, in promoting microvascular growth, differentiation and function. We will discuss perivascular cell contributions to blood–neural barrier function and fenestrations, specialized phenotypes of microvascular endothelial cells. Finally, we will address clinical implications of pericyte function and vessel maturation in cancer, diabetic retinopathy and therapeutic angiogenesis.

CONTENTS

15.1 Introduction

15.1.1 Vascular Development

Blood vessels have at least two major structural components: the vessel intima, composed of a single layer of endothelial cells surrounding the vessel lumen, and the vessel wall, primarily composed of mural cells apposing the abluminal surface of the endothelium. Vessel formation, therefore, must involve both formation of an endothelial cell tube and association of mural cells with the endothelium. In larger vessels – arteries,

large arterioles and veins – the mural cells are smooth muscle; in the microvessels – precapillary arterioles, capillaries and postcapillary venules – the mural cells are pericytes. The interactions between mural cells and endothelial cells play critical roles in promoting maturation, stability and normal function of blood vessels.

There are two processes by which new blood vessels form: vasculogenesis and angiogenesis. Vasculogenesis is the de novo formation of blood vessels from endothelial precursor cells. This process forms the initial vascular network during embryonic development and contributes to subsequent vessel formation in some tissues. Angiogenesis, on the other hand, describes the sprouting of new vessels from a pre-existing vascular network. Angiogenesis involves degradation of the basement membrane surrounding the original vessel, proliferation and migration of endothelial cells to form a new vessel sprout, and recruitment of mural cells. A mature microvessel is characterized histologically by its association with mural cells and surrounding basement membrane. Further sprouting or regression of vessels may follow formation of an initial vessel network, as determined by tissue needs, and surviving vessels acquire hierarchical characteristics of arterioles, capillaries or venules. Enlargement of vessels to accommodate increased blood flow, a process called arteriogenesis, can also occur subsequent to formation of the initial vascular network (reviewed in Carmeliet 2000).

15.1.2 Mural Cells

Pericytes and smooth muscle cells play critical support roles for their respective vessels. While certain distinctions can be made between the phenotypes of these two mural cell types, in fact they have similar origins and locations relative to the vascular endothelium.

15.1.2.1 Mural Cell Origin

Mural cells that associate with a new vessel either differentiate from surrounding precursor cells or migrate out from a pre-existing vessel. Mural cell precursors are most commonly found in the mesenchyme surrounding the new vessel, though neural crest and epicardial progenitors may contribute to the mural cells associated with vessels of the forebrain and coronary vessels, respectively (reviewed in Gerhardt and Betsholtz 2003). Differentiation of mesenchymal cells into pericytes or smooth muscle cells involves contact with the endothelial cells and subsequent activity of transforming growth factor β-1 (TGFβ1; reviewed in Lebrin et al. 2005). Pericytes may also originate from bone marrow-derived precursor cells or transdifferentiate from endothelial cells (reviewed in Armulik et al. 2005), though the exact mechanisms that regulate these differentiation processes are poorly understood. In addition to these pericyte sources, mural cells of pre-existing vessels can migrate onto the new vessel during angiogenic vessel sprouting, a process most likely mediated by platelet-derived growth factor-B (PDGF-B; reviewed in Darland and D'Amore 2001).

15.1.2.2 Mural Cell Location

Although endothelial cell morphology is similar in vessels of varying size, mural cell structure and density change depending on the vessel type. Mural cells form a continuum from arterial smooth muscle to pericytes to venous smooth muscle along the vascular tree. Arteries and veins have vessel walls largely composed of smooth muscle cells, which form a continuous layer around the endothelium. Pericytes, on the other hand, are discontinuous, and extend processes both around and along the microvessels, with process patterns differing between arterioles and venules (Fig. 15.1a). Pericyte processes often associate with several endothelial cells along a vessel, and may even extend between vessels (reviewed in Allt and Lawrenson 2001).

In contrast to smooth muscle cells, which completely encase the vascular endothelium, the degree of pericyte coverage of vessels depends on the tissue location and vessel type. In rats, pericyte coverage of the abluminal endothelial surface in capillaries ranges from 11% in cardiac muscle to 48% in retinal capillaries, and postcapillary venules typically have more pericytes than do capillaries (reviewed in Sims 1991).

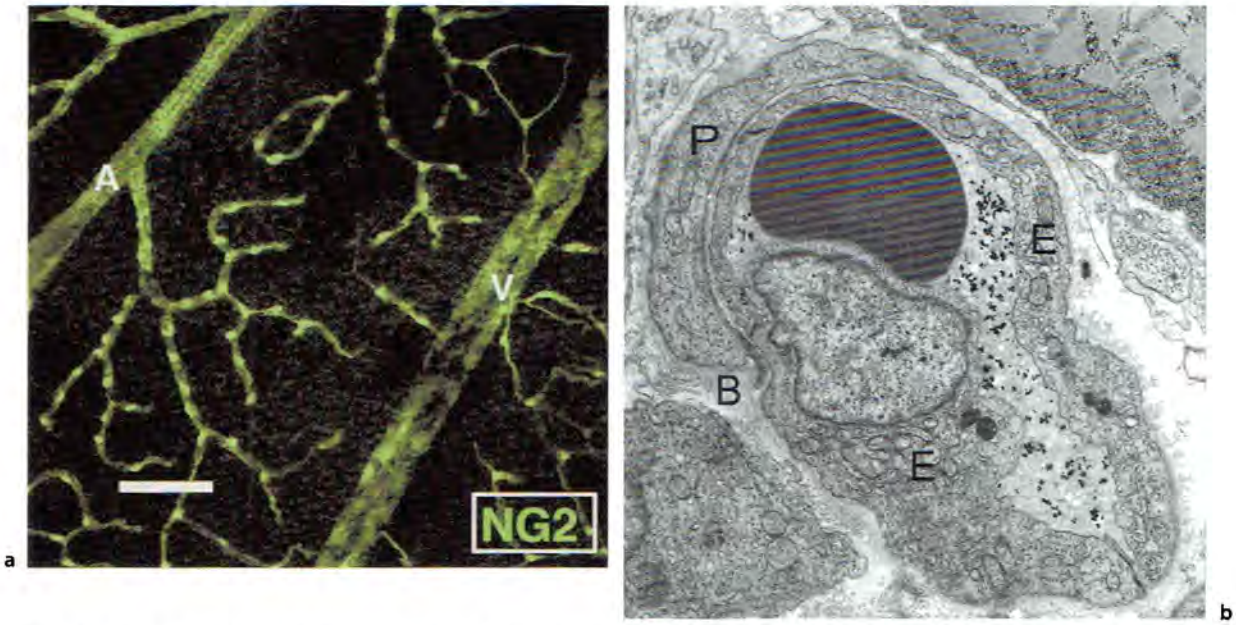


Fig. 15.1a,b. Pericytes in the microvasculature. (a) Pericytes of the the rat retinal vessels at postnatal day 15 are labeled with NG2 proteoglycan (*green fluorescence*). *A*, arteriole; *V*, venule; *scale bar* 25 μm. Copyright 2005, *Microcirculation* (Chan-Ling and Hughes 2005). (b) Photograph of a skin capillary which shows a red blood cell and previously injected colloidal carbon particles in the lumen. Two endothelial cells are connected by interendothelial cell junctions. A pericyte encloses a large portion of the capillary. *E*, endothelial cell; *P*, pericyte; *B*, basement membrane. Photo courtesy of Ann Dvorak, Beth Israel Deaconess Medical Center, Boston, MA.

In rat skeletal muscle, for example, 81% of the venule surface is covered by pericytes, compared to 21% for capillaries (reviewed in Sims 1991). Vessel pressure might also influence pericyte coverage, as venules in the more inferior regions of the torso and legs, which experience greater venous pressure due to gravity, have increased coverage by pericytes compared to venules in other regions of the body (reviewed in Sims 2000). These differences in pericyte density may contribute to the maturation of microvessels into arterioles, capillaries and postcapillary venules.

15.1.3 Pericytes

As most vessel growth and maturation involves microvessels, we will focus on the roles of pericytes in vascular development. It should be noted again, however, that smooth muscle cells and pericytes exist in a continuum along the vasculature. These cells originate from the same precursor cells, express similar proteins, and can even transition from smooth muscle to pericyte and from pericyte to smooth muscle (reviewed in Gerhardt and Betsholtz 2003).

15.1.3.1 Pericyte–Endothelial Cell Communication

Pericytes are in particularly close contact with the endothelium, and this interaction is critical for vessel maturation. Pericytes reside within the endothelial basement membrane (Fig. 15.1b) and contribute to this matrix. Pericytes and endothelial cells also make direct contact through openings in the basement membrane, forming peg-and-socket contacts that contain gap junctions and adherens junctions

(reviewed in Gerhardt and Betsholtz 2003). Interestingly, these sites of direct contact primarily occur at junctions between endothelial cells (reviewed in Allt and Lawrenson 2001). Certain aspects of vessel maturation require this direct contact between endothelial cells and pericytes, while other aspects depend on factors secreted by vascular cells. Vessel growth and function therefore depends not only on pericyte proximity, but also on the timing, amount and localization of growth factors secreted by endothelial cells and pericytes.

Table 15.1. Markers of mural cells

Marker protein	Smooth muscle cells	Pericytes
α-Smooth muscle actin	+	+/-
Aminopeptidase A	+	+
Caldesmon	+	-
Calponin	+	-
Desmin	+	+
NG2 proteoglycan	+	+
PDGF receptor β	+	+
RGS5	+	+

15.1.3.2 Pericyte Identification

There are two commonly used techniques for identifying pericytes: electron microscopy and immunohistochemistry. Pericytes are morphologically unique in that they are encased within the capillary basement membrane (Fig. 15.1b). Electron microscopy is not practical for examining large regions of the vasculature, however, and the basement membrane has not yet completely formed in vessels at early stages of development.

Pericytes may also be identified by immunolocalization of one or more marker proteins. No single protein is expressed solely by pericytes, and overlap is seen in protein expression between pericytes and smooth muscle cells. Protein expression combined with proximity to microvessels does, however, adequately identify pericytes in most vessel beds. Marker proteins expressed by pericytes include aminopeptidase A, desmin, NG2 proteoglycan, PDGFRβ, regulator of G-protein signaling-5 (RGS5) and, sometimes, αSMA (Table 15.1) (Hughes and Chan-Ling 2004; reviewed in Darland and D'Amore 2001; Gerhardt and Betsholtz 2003). In many cases, these proteins are also expressed by smooth muscle cells, though perhaps at different levels. NG2 proteoglycan, for example, is expressed at much lower levels in smooth muscle cells than in pericytes (Hughes and Chan-Ling 2004). Marker proteins that are expressed by smooth muscle cells, but not by pericytes, include caldesmon and calponin (Hughes and Chan-Ling 2004).

Variability of marker protein expression can occur within pericyte populations, depending on the maturity of the vessel and the location of the vessel bed. For example, many studies of pericyte function have used αSMA as a pericyte marker, but in fact this protein is expressed by only a small proportion of pericytes in the adult retina (Hughes and Chan-Ling 2004), and is not expressed at all by pericytes associated with early retinal angiogenesis (Witmer et al. 2004) or with brain capillaries of the smallest diameter (Hellström et al. 1999). Aminopeptidase A is strongly expressed by pericytes in angiogenic vessels, but expression is weak or absent in pericytes of normal, stable vessels (Marchio et al. 2004), and while NG2 proteoglycan is expressed at all stages of retinal pericyte development, expression levels are decreased in the adult retina compared to the developing retina (Hughes and Chan-Ling 2004). Identification of all pericytes in a tissue may therefore require utilization of a panel of pericyte markers.

15.1.3.3 Pericyte Functions

Smooth muscle cells clearly have contractile roles, but the functions of pericytes in the microvasculature are more complex. Various studies have shown that pericytes can contract in vitro, though there is little evidence indicating that this occurs in vivo (reviewed in Allt and Lawrenson 2001). Pericytes can function as phagocytes, particularly in the central nervous system, and may also serve as a smooth muscle stem cell population to be utilized for arteriogenesis

(reviewed in Gerhardt and Betsholtz 2003). Perhaps most importantly, pericytes are involved with regulation of vessel formation, stability and function. Pericyte processes extend to multiple endothelial cells and sometimes even to multiple capillaries, and pericyte-derived growth factors affect vessel maturation and function.

Pericytes in Angiogenesis and Vessel Maturation

15.2.1 Pericytes in Vessel Formation

It has long been believed that pericytes are recruited to capillaries after formation of endothelial sprouts, either by differentiation from surrounding mesenchymal precursors or by migration from the mural wall of the adjacent vessel (reviewed in Gerhardt and Betsholtz 2003). Formation of the endothelial cell tube prior to pericyte recruitment may always occur for vasculogenesis, but evidence is accumulating that pericytes may sometimes precede endothelial cells in angiogenesis. Individual pericytes can be found at the tips of angiogenic sprouts in the corpus luteum (reviewed in Gerhardt and Betsholtz 2003), and pericyte sleeves occasionally extend beyond the endothelial sprout tip in tumors and in the developing retina (Morikawa et al. 2002; Ozerdem and Stallcup 2003). In addition, pericyte processes connect adjacent vessels prior to formation of anastamoses (Nehls et al. 1992). A leading role for pericytes in angiogenesis would not be surprising, given that vascular endothelial growth factor A (VEGF), a pro-angiogenic factor secreted by pericytes exposed to hypoxia (Yamagishi et al. 1999), stimulates proliferation and migration of endothelial cells. The exact timing of pericyte arrival at a vessel sprout may therefore vary, but subsequent association of pericytes with the endothelium is undeniably a critical component of vessel maturation.

15.2.2 Pericyte Investment of Vessels

The endothelium plays an active role in recruitment of pericytes through secretion of various factors (Fig. 15.2). The best-understood means of recruiting pericytes involves activity of PDGF-B, a factor produced by sprouting endothelial cells whose receptor, PDGFRβ, is expressed on mural cells and mural cell precursors (reviewed in Betsholtz 2004). Mice deficient for PDGF-B or PDGFRβ die during embryonic development with widespread microvascular defects. Pericyte association with vessels is drastically reduced in most tissues of these animals, a finding that is not surprising, given that PDGF-B promotes pericyte precursor cell proliferation and migration. While PDGF-B can be produced by several cell types, targeted disruption of PDGF-B in endothelial cells demonstrated that endothelial production of this factor is required for pericyte recruitment in most tissues (reviewed in Betsholtz 2004).

PDGF-B is clearly not the only factor responsible for pericyte recruitment, as mice deficient for PDGF-B or PDGFRβ have normal pericyte coverage in the liver perisinusoidal capillaries (Hellström et al. 1999). About 50% of the normal number of pericytes are recruited to capillaries of the adrenal gland and the placenta in these mice, and even in tissues where more than 90% of the normal number of vessel-associated pericytes are absent, a small number of pericytes continues to be recruited (Hellström et al. 1999). Other factors that may contribute to this process, either directly or indirectly, include TGFβ1, VEGF, and nitric oxide (NO).

PDGF-B is produced by endothelial cells. In vitro, this factor stimulates migration of smooth muscle cells, pericytes, and mural cell precursors (reviewed in Darland and D'Amore 2001). Once mesenchymal cells come into contact with endothelial cells, activation of latent TGFβ1 contributes to differentiation of precursor cells into pericytes or smooth muscle cells (reviewed in Darland and D'Amore 2001). As further indication of the im-

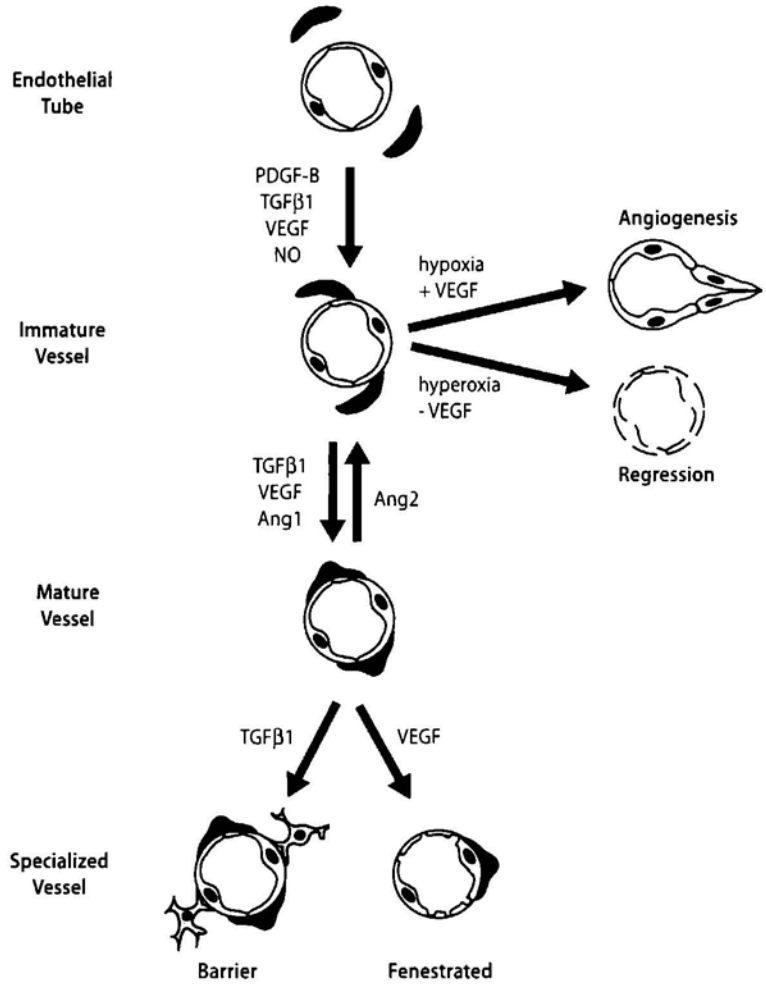


Fig. 15.2. Model of endothelial cell–pericyte interactions in microvascular maturation. Abbreviations are explained in the text

portance of TGFβ1 for mural cell differentiation, embryonic vessels of mice deficient for endoglin, a TGFβ1 co-receptor, display reduced association with smooth muscle cells and pericytes (Li et al. 1999).

VEGF, too, can affect pericyte recruitment and maintenance. Although this factor is not secreted by endothelial cells in vivo, it is expressed by other cell types exposed to hypoxia. VEGF directly induces proliferation and migration of pericytes (Yamagishi et al. 1999), and intraocular injection of VEGF increases the rate of αSMA-positive pericyte recruitment to the retinal vessels of rats (Benjamin et al. 1998). VEGF might also indirectly stimulate pericyte recruitment via endothelial cell production of NO. VEGF stimulates activity of endothelial NO synthase, and the resulting NO production mediates numerous VEGF effects, including vessel growth and increased vascular permeability (reviewed in Duda et al. 2004). NO also promotes mural precursor cell migration in vitro and pericyte recruitment to tumor vessels in vivo (Kashiwagi et al. 2005). It is possible that VEGF and/or NO contribute to pericyte recruitment in tissues that are less-affected by PDGF-B deficiency, such as the liver, placenta and adrenal gland.

15.2.3 Vessel Stabilization

Pericyte recruitment is a critical step in vascular maturation (Fig. 15.2); vessels lacking pericytes are dilated and irregularly shaped, and form microaneurysms. Pericyte-free vessels lose distinction between arterioles, capillaries and venules, and leak fluid and blood into the surrounding tissue (reviewed in Betsholtz 2004). Aspects of vessel stabilization mediated by pericytes include inhibition of endothelial cell proliferation, survival of endothelial cells, establishment of hierarchical organization of the vasculature, and maintenance of a normal degree of permeability for the capillary bed.

15.2.3.1 Inhibition of Endothelial Proliferation

Pericytes play a critical role in converting endothelial cells from a proliferating, angiogenic state to a quiescent, stable state. Indeed, vessels with the slowest rate of endothelial cell proliferation have the greatest association with pericytes and, conversely, pathological decreases in pericyte coverage are associated with excessive endothelial cell proliferation (reviewed in Allt and Lawrenson 2001). Loss of more than 50% of the pericytes leads to endothelial cell proliferation and microaneurysm formation in mice, and similar vascular alterations occur with pericyte loss in the retinas of diabetic humans (reviewed in Betsholtz 2004). Furthermore, in mice that have chimeric PDGF-B deficiency, only the vessels lacking pericytes display abnormal increases in vessel diameter, whereas contiguous vessel sections associated with pericytes have normal diameter (reviewed in Betsholtz 2004). Pericytes are therefore essential for inducing quiescence of vascular endothelial cells, a process that likely involves activity of TGFβ1.

TGFβ1, a factor secreted by both endothelial cells and pericytes, modulates several aspects of vessel stability. TGFβ1 can inhibit endothelial cell proliferation and migration (reviewed in Lebrin et al. 2005). Microvessels of mice deficient for TGFβ1 signaling components are dilated and irregular in shape, as are vessels associated with the human disorder hereditary hemorrhagic telangiectasia, caused by mutations in TGFβ1 signaling components (reviewed in Lebrin et al. 2005). In vitro, pericyte-mediated inhibition of endothelial cell proliferation requires direct contact between the two cell types, and abrogation of TGFβ1 signaling completely blocks this inhibitory effect (reviewed in Allt and Lawrenson 2001). Latent TGFβ1 is localized to the sites of direct endothelial cell-pericyte contact, as are proteolytic enzymes that convert TGFβ1 to its active form (reviewed in Allt and Lawrenson 2001). Close interaction between pericytes and endothelial cells, in conjunction with TGFβ1 activation, is therefore critical for stabilization of lumen diameter.

15.2.3.2 Survival of Endothelial Cells

Endothelial cell survival in mature vessels is maintained by at least two factors: VEGF and angiopoietin-1 (Ang1). Pericytes that contact endothelial cells produce VEGF both in vitro and in vivo, and disruption of VEGF signaling results in increased apoptosis of both cultured endothelial cells (Darland et al. 2003) and tumor endothelial cells in vivo (Benjamin et al. 1999). Interestingly, tumor vessels lacking αSMA-positive pericytes are more sensitive to VEGF withdrawal, as evidenced by increased endothelial apoptosis and vessel regression, compared to vessels associated with αSMA-positive pericytes (Benjamin et al. 1999). Although αSMA negativity does not necessarily mean that pericytes are absent (Hughes and Chan-Ling 2004; Witmer et al. 2004), this study does point to a role for pericyte-derived VEGF in promoting endothelial cell survival. Furthermore, VEGF is expressed and the VEGF receptor-1 is constitutively phosphorylated in mature vessels of adult animals (Maharaj et al. 2006), indicating that VEGF signaling occurs even in the absence of angiogenesis. Indeed, treatment of adult mice with an inhibitor of VEGF signaling results in reduction of the number of tracheal capillaries (Baffert et al. 2006). Given that VEGF inhibitors are being developed as treatments for diseases ranging from age-related macular degeneration to cancer, it

will be important to better define the role of VEGF in maintenance of the normal vasculature.

Angl, too, is a pericyte-derived factor that facilitates endothelial cell survival. Angl protects endothelial cells from a variety of apoptotic stimuli (reviewed in Jones et al. 2001). Furthermore, the receptor for Angl, Tie2, is both expressed and activated in endothelial cells of adult tissues, and Tie2 inactivation results in endothelial cell apoptosis (reviewed in Peters et al. 2004). Mice deficient for either Angl or Tie2 die during embryonic development with vascular defects similar to those observed for PDGF-B-deficient mice, namely enlarged, leaky and hemorrhagic microvessels (reviewed in Jones et al. 2001). Taken together, these results support the idea that Angl signaling is required to maintain a mature, stable vasculature.

Unlike VEGF, which can stimulate endothelial proliferation, Angl is not mitogenic for endothelial cells (reviewed in Jones et al. 2001). This characteristic, combined with the Angl effects on vascular hierarchy and endothelial permeability that will be described below, makes Angl a particularly promising vessel maturation factor for pro-angiogenic therapeutic use. In a mouse model of diabetic retinopathy, in which high blood glucose levels ultimately result in death of retinal endothelial cells, treatment with Angl reduces endothelial apoptosis without causing abnormal neovascularization (Joussen et al. 2002). Angl might also be used to stabilize vessels in other pathologic states.

15.2.3.3
Establishment of Hierarchical Vascular Organization

A mature vascular bed has distinct hierarchical organization, with microvessels in the form of arterioles, capillaries and venules. Pericytes play an important role in establishing this hierarchy. Some aspects of vessel organization are established even in the absence of pericytes, such as increased expression of the protein PECAM in arteries compared to veins (Uemura et al. 2002), but overall distinctions between microvessel types are lost in pericyte-free vessels (reviewed in Betsholtz 2004). Tumor vessels, too, lack microvascular hierarchy, perhaps because pericytes are only loosely attached to tumor vessels (reviewed in Baluk et al. 2005).

One pericyte-derived factor that has been implicated in vessel organization is Angl. Treatment with an inhibitor of Angl signaling during retinal development results in a retinal microvasculature lacking hierarchical organization (Uemura et al. 2002), and deficiency for Tie2 prevents vessels from organizing properly into arterioles, capillaries and veins (reviewed in Peters et al. 2004). When normal vessel hierarchy is lost due to absence of pericytes, as when retinal pericyte recruitment is impaired via blockade of PDGF-B signaling, treatment with Angl partially restores hierarchical morphology of vessels lacking mural cells (Uemura et al. 2002). Interestingly, in mice that overexpress Angl, vessels that should be capillaries are the size of venules and express venule marker proteins (Thurston et al. 1999). Furthermore, the mural cells associated with these vessels have the morphological characteristics of venule pericytes rather than capillary pericytes (Thurston et al. 1999). Angl clearly plays a role in regulating hierarchical organization of the vasculature; it remains to be determined which other factors might also participate in this process.

15.2.3.4
Reduction of Vascular Permeability

Capillaries in most tissues are selectively permeable, a characteristic promoted by pericytes. Indeed, one of the hallmark defects of vessels lacking pericytes, both in mice and in humans, is increased vascular leakiness (reviewed in Betsholtz 2004). Perhaps the most potent anti-permeability factor in the microvasculature is Angl. Treatment with Angl restores normal permeability of vessels lacking pericytes (Uemura et al. 2002), and Angl greatly reduces leakiness of vessels exposed to inflammatory factors such as mustard oil, platelet-activating factor and serotonin (Thurston et al. 1999). An alternate name for VEGF is vascular permeability factor; Angl also counteracts VEGF-induced endothelial leakiness (Thurston et al. 1999). Constitutive, pericyte-mediated Angl signaling in mature vessels may therefore help to maintain the vessel in a non-leaky state, in addition to promoting endothelial cell survival.

15.2.4 Vessel Bed Remodeling: Angiogenesis and Regression

Vessel bed remodeling occurs in response to environmental conditions, particularly oxygen excess or insufficiency. Interestingly, to complete formation of a functional vessel bed, it may be necessary to first make existing vessels less mature via alterations in pericyte-endothelial cell communication (Fig. 15.2). Induction of pericyte dissociation accelerates vascular regression during retinal exposure to hyperoxia (Benjamin et al. 1998). Although it is possible for pericyte-invested capillaries to regress (Hughes and Chan-Ling 2004), tumor vessels associated with ôSMA-positive pericytes are less susceptible to regression than are vessels lacking these pericytes (Benjamin et al. 1999). Pericyte dissociation also promotes angiogenic vessel growth. Retinal vessels that are not associated with pericytes display a greater angiogenic response to hyperoxia than do pericyte-associated vessels (Wilkinson-Berka et al. 2004), and angiogenesis is increased in retinal vessels with reduced pericyte coverage in a rodent model of diabetic retinopathy (Enge et al. 2002). This is not surprising, given that pericyte association with endothelial cells inhibits endothelial proliferation.

Pericyte dissociation from vessels may be physiologically mediated by angiopoietin-2 (Ang2), an endothelial cell-derived inhibitor of Angl signaling. Ang2, like Angl, binds to the receptor Tie2, but in most cases does not cause Tie2 autophosphorylation (reviewed in Peters et al. 2004). Ang2 can act as a competitive inhibitor of Angl signaling, abrogating Angl-mediated Tie2 phosphorylation and endothelial cell migration. Further evidence for the antagonistic role of Ang2 in Angl signaling is the observation that Ang2-overexpressing mice display a phenotype similar to that of Tie2-deficient animals (reviewed in Peters et al. 2004).

Ang2 expression levels are highest in tissues undergoing angiogenesis and regression (reviewed in Peters et al. 2004). During angiogenesis, Ang2 is expressed by endothelial cells located at the leading edge of proliferating vessels. Angl, on the other hand, is expressed behind the leading edge of angiogenic vessels, a position consistent with vessel maturation (reviewed in Peters et al. 2004). VEGF increases production of Ang2, and Ang2 overexpression by endothelial cells results in dissociation of pericytes from vessels (Zhang et al. 2003). Pericyte dissociation of diabetic retinopathy, too, can be mimicked by treatment with Ang2 (reviewed in Hammes 2005).

Ang2 activity is associated not only with pericyte dissociation, but also with vessel growth and regression. Proliferating vessels of retinal development or a mouse model of retinopathy of prematurity increase angiogenesis when treated with Ang2 (Oshima et al. 2005), and treatment with another antagonist of Angl results in increased vascular proliferation during relative hypoxia in retinopathy of prematurity (Hoffmann et al. 2005). Vessel regression, too, can be enhanced by Ang2, as was observed when Ang2 was overexpressed in a model of ischemic retinopathy (Oshima et al. 2005). Interestingly, mature, stable vessels are largely unaffected by Ang2. The effects of Ang2 may therefore depend on the microenvironment; for example, Ang2 expression in the presence of high VEGF levels or hypoxia promotes angiogenesis, whereas Ang2 in the presence of low VEGF levels promotes vessel regression (Fig. 15.2).

Physiological Alterations in Vessel Permeability

The architecture of the microvasculature is specialized to meet the metabolic and functional needs of the tissues that it supplies (Fig. 15.2). At opposite ends of the spectrum are the barrier functions of the central nervous system microvasculature and the fenestrated capillaries of a number of filtering and secretory organs, including the kidney, pancreas and choroid plexus.

15.3.1 Blood–Brain Barrier

The blood–brain barrier functions to maintain a constant ionic milieu and to protect the nervous system from a variety of injurious agents, including toxins, pathogens and immune cells. The barrier function of the central nervous system microvasculature is mediated by extensive inter-endothelial tight junctions, a low rate of endothelial pinocytosis and transcytosis, and the expression of specialized cell surface transporters and receptors on the capillary endothelial cells. In addition, the astrocytes, which extend end feet onto the abluminal capillary surface (Fig. 15.3a), express cell surface receptors and enzymes that contribute to maintaining ionic homeostasis (reviewed in Benarroch 2005). Loss of barrier function is associated with a number of pathologies such as stroke, Alzheimer's disease and multiple sclerosis. In spite of its critical role in promoting the health and function of the nervous system, very little is known about the molecular regulation of barrier function development and maintenance.

A significant body of experimental data points to a role for astrocytes in the induction of barrier function (reviewed in Haseloff et al. 2005). Early tissue culture studies have demonstrated that medium conditioned by astrocytes can induce tight junction formation in capillary endothelial cells (Arthur et al. 1987). Subsequent characterization of astrocyte–endothelial interactions have identified a number of factors that can modulate the expression of tight junctions and/or transendothelial permeability, including TGFβ1. TGFβ1 has been shown to induce the expression of occludin and gamma-glutamyl transferase by endothelial cells (Garcia et al. 2004). In addition, Src-suppressed C-kinase substrate (SSeCKS) has been reported to induce astrocyte expression of Ang1 and increase occludin expression by endothelial cells (Lee et al. 2003). Not surprisingly, the effects between endothelial cells and astrocytes are reciprocal, with interactions between the two cell types leading to alterations in astrocyte shape and growth (Garcia et al. 2004).

Pericytes and neurons are also observed in the vicinity of the capillary and may influence barrier function. While the astrocytes have been most extensively been studied in this regard, it is worth noting that the number of pericytes associated with blood–neural barrier capillaries is higher than with non-barrier capillaries (reviewed in Sims 1991). Pericytes, too, may play a role in the induction and/or maintenance of barrier function. A greater understanding of the mechanisms that underlie the formation and maintenance of the blood neural barrier will be relevant to a variety of pathologies characterized by loss of barrier function, including stroke, Alzheimer's disease, brain tumors and inflammatory processes such as multiple sclerosis.

15.3.2 Fenestrated Microvessels

There are three types of capillaries: continuous, discontinuous and fenestrated. Fenestrae are small openings of about 80–100 nm in diameter that are covered by a small, non-membranous diaphragm. The basement membrane of endothelial cells is continuous over the fenestrae. Fenestrae allow greater permeability and the rapid passage of macromolecules smaller than plasma proteins. Fenestrated capillaries are found in the intestine and endocrine glands, and a special type of fenestrated capillary with no diaphragm is found in the renal glomerulus. As is the case for the blood–neural barrier, there has been a long-standing interest in understanding the mechanism by which fenestrated capillaries acquire their specializations.

Early evidence for the influence of the microenvironment came from studies in which culture of adrenal cortex capillary endothelial cells on basement membrane derived from kidney epithelial cells was shown to induce the formation of fenestrations (Milici et al. 1985). Many years later, additional insight into the possible mechanism of basement membrane-induced fenestrations was provided by the presence of VEGF in the basement membrane (Esser et al. 1998). In these studies, fenestrae were induced in epithelial cells transfected

with VEGF, but not by untransfected cells. Fenestrae also formed in response to epithelial-produced basement membranes, but not collagen alone. These observations strongly supported a role for VEGF in induction of fenestrae, but did not address the question of whether maintenance of fenestrae also required VEGF.

VEGF-producing epithelial cells can be observed in close proximity to fenestrated microvessel beds in the adult (Maharaj et al. 2006), indicating that VEGF might also be required for stabilization of the fenestrated phenotype. In support of this concept, pre-eclampsia, a pathology characterized by glomerular endothelial dysfunction, is associated with excess levels of soluble VEGF receptor 1 (Maynard et al. 2003). Interestingly, the hallmarks of pre-eclampsia, hypertension and proteinuria, are also the most common side effects of systemic anti-VEGF therapies in humans. More recently, the effects of VEGF neutralization on fenestrations and capillary stability have been investigated. Administration of any one of a number of VEGF-blocking agents was shown to lead to the regression not only of tumor blood vessels, but also of normal, fenestrated microvessels in a number of tissues, including pancreatic islets, thyroid, adipose, intestinal villi, adrenal

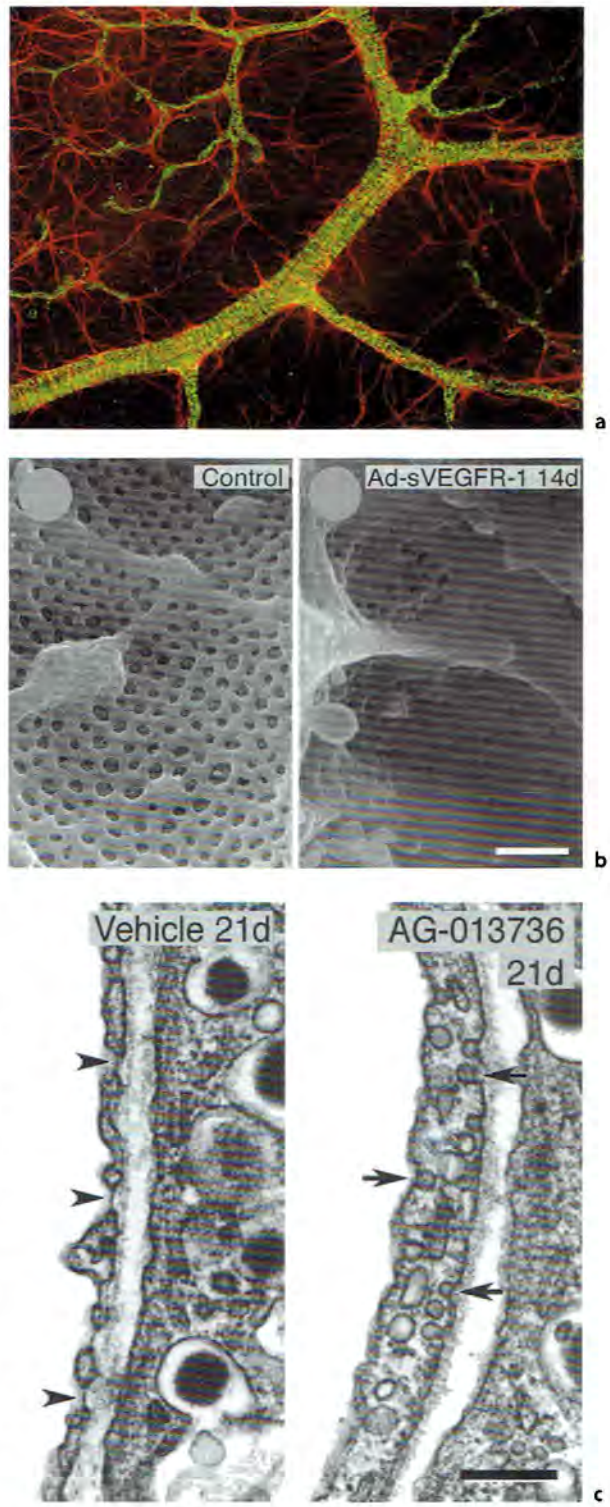


Fig. 15.3a–c. Microvascular specializations: blood–neural barrier and fenestrae. (a) Astrocytes (GFAP staining, *red fluorescence*) extend processes to blood vessels (BS1 isolectin, *green fluorescence*) in the adult mouse retina. Photo courtesy of Scott Plotkin, Schepens Eye Research Institute and Massachusetts General Hospital, Boston, MA. (b) These scanning electron micrographs demonstrate that endothelial cells of the glomerular capillaries display fenestrae (*left panel*), and that fenestrations is reduced following treatment with Ad-sVEGFR-1, an inhibitor of VEGF signaling (*right panel*). Scale bar 0.5 μm. (c) These transmission electron micrographs show thin endothelium with fenestrations (*arrowheads, left panel*) in islet capillaries, and demonstrate endothelial thickening, numerous caveolae and loss of fenestrations result from treatment with AG-013736, an inhibitor of VEGF signaling (*arrows, right panel*). Scale bar 0.3 μm. Photographs in b and c are reproduced with permission from the *American Journal of Physiolology: Heart and Circulatory Physiology* (Kamba et al. 2006)

cortex, pituitary and choroid plexus (Fig. 15.3b, c; Kamba et al. 2006). Furthermore, ultrastructural analysis revealed that only 7 days of treatment with VEGF-neutralizing agents could lead to the loss of fenestrations in a number of tissue beds. The extent of capillary loss varied among the tissues, with the greatest capillary loss (more than 65%) measured in the thyroid (Baffert et al. 2006). Interestingly, a majority of the capillaries regrew within 2 weeks of withdrawal of the VEGF inhibitor. Analysis of the mechanism of capillary loss revealed a sequence of events that began with loss of blood flow followed by the appearance of apoptotic capillary endothelial cells, leaving "empty sleeves of basement membrane" (Baffert et al. 2006). Because some of the inhibitors used in these studies (VEGF receptor tyrosine kinase inhibitor and sFlt-1) are not specific for VEGF and can also neutralize the effects of placental growth factor, another VEGF family member, the specific factor that mediates vessel stability and specialization is not clear. In spite of this caveat, these findings, along with clinical observations of the side effects of VEGF inhibition and the etiology of pre-eclampsia, strongly point to a role for VEGF in the maintenance of the adult microvasculature.

Vessel Maturation in Pathology

15.4.1 Pericytes and Disease

Abnormal pericyte morphology and function have been associated with the pathogenesis of a number of diseases involving the vasculature. Vessel abnormalities associated with pericyte loss, including microaneurysms and increased vessel leakiness, are also associated with numerous pathological states (reviewed in Sims 1991). Two diseases in which pericyte dysfunction is particularly well characterized are cancer and diabetic retinopathy.

15.4.1.1 Cancer

Tumor vessels differ from normal vessels in many ways. Structurally, tumor vessels are irregularly shaped, lack a normal hierarchy of vessel types, contain endothelial cell protrusions into the vessel lumen, and have altered interactions between pericytes and endothelial cells (Fig. 15.4a; reviewed in Baluk et al. 2005). Tumor vessels also display functional defects, including increases in permeability, vessel regression and need for growth factors to maintain vascular survival (reviewed in Baluk et al. 2005).

A subset of tumor vessels are clearly immature. These vessels have abnormal or decreased pericyte coverage, contain proliferating endothelial cells, and are prone to regression (Gee et al. 2003). Whereas in normal vessels the pericytes and smooth muscle cells tightly appose the endothelial tube, in tumor vessels the mural cells are only loosely associated with the vessels, and are in some locations completely dissociated from them (Fig. 15.4a; reviewed in Baluk et al. 2005). When tumors are treated with anti-angiogenic agents, it is primarily the immature, pericyte-deficient vessels that regress (Gee et al. 2003). Prevention of pericyte recruitment or destabilization of pericyte-invested vessels might therefore improve outcomes of anti-angiogenic therapy.

In fact, anti-cancer therapy targeting both PDGF activity, which primarily affects pericyte recruitment, and VEGF activity, which primarily controls endothelial proliferation and survival, is more effective than targeting either VEGF or PDGF alone (reviewed in Baluk et al. 2005). Treatment with kinase inhibitors that target PDGF signaling results in dissociation of pericytes from the endothelial cells in tumors (Bergers et al. 2003). The pericyte-deficient capillaries are distinctly abnormal, with dilated lumina and distorted morphology. Most notably, this treatment did not affect capillaries in adjacent normal tissues (Bergers et al. 2003). This is not surprising, given that mature vessels in the retina do not undergo pericyte dissociation following treatment with Ang2 (Oshima et al. 2005) or

excessive PDGF-B (Benjamin et al. 1998). Most current anti-angiogenic therapies target endothelial proliferation, particularly via inhibition of VEGF signaling; these agents will likely be even more effective when combined with pericyte-destabilizing factors.

15.4.1.2 Diabetic Retinopathy

Diabetic retinopathy is a leading cause of blindness and involves dysfunction of the retinal vasculature. Vascular abnormalities include excessive vessel permeability, resulting in edema; accumulation of acellular capillaries, resulting in vascular occlusion; and formation of microaneurysms, often accompanied by hemorrhage (reviewed in Hammes 2005). In diabetic retinopathy, chronic exposure to high blood glucose levels ultimately results in capillary occlusion due to endothelial cell loss, decreased pericyte number in the retina, and formation of acellular capillaries (Fig. 15.4b; reviewed in Sims 1991). The resulting retinal ischemia then stimulates growth of new vessels, which are often torturous, leaky and dilated (reviewed in Hammes 2005). Deficient pericyte coverage has been associated with the abnormal neovascularization of diabetic retinopathy. In mice, proliferative retinopathy similar to that observed for diabetic retinopathy was observed when pericyte coverage was less than half of that seen with normal mice (Enge et al. 2002). These alterations in pericyte coverage are probably mediated by Ang2.

Ang2 is expressed at higher levels in the eyes of diabetic rats than in control rats prior to the onset of pericyte loss, and intraocular injection of Ang2 results in pericyte deficiency in retinal vessels (reviewed in Hammes 2005). Even heterozygous loss of Ang2 expression eliminates the pericyte deficiency normally associated with diabetes (reviewed in Hammes 2005), supporting a direct role for this molecule in pericyte dissociation from retinal vessels during diabetic retinopathy. The best-understood function for Ang2 is that of acting as a competitive inhibitor of Ang1. Indeed, Ang1 treatment of mice with impaired PDGF-B signaling reduces edema and hemorrhage even in capillaries lacking pericytes (Uemura et al. 2002). Ang1 treatment also reduces both vessel leakiness and endothelial cell damage in the retinas of diabetic rats (Joussen

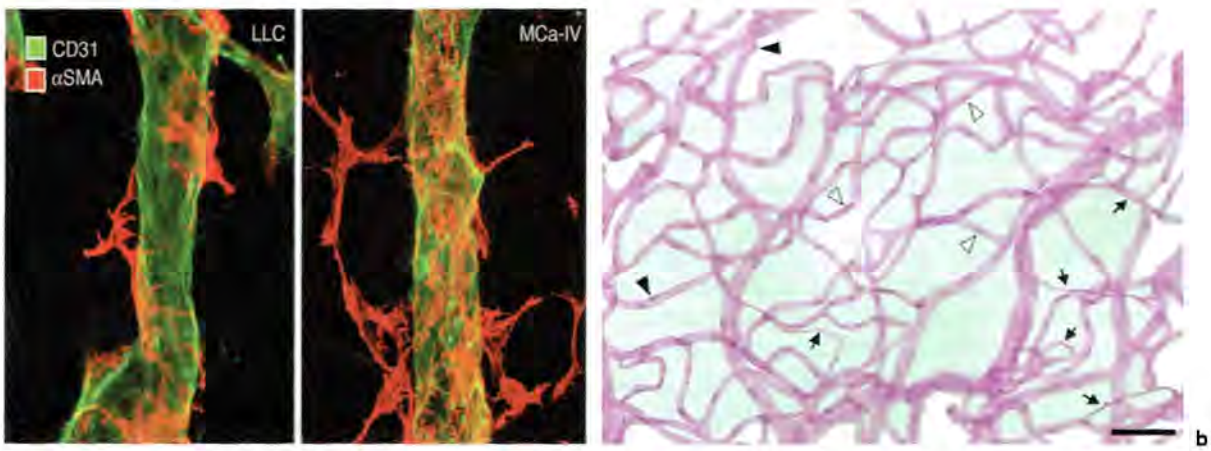


Fig. 15.4a,b. Microvascular pathologies: cancer and diabetic retinopathy. (a) Vessels from Lewis lung carcinoma (*left panel*) and Mca-IV carcinoma (*right panel*) demonstrate loose association between pericytes (αSMA staining, red fluorescence) and endothelial cells (CD31 staining, *green fluorescence*). Photos are reproduced with permission from the American Journal of Pathology (Morikawa et al. 2002). (b) Retinal trypsin digest showing acellular capillaries in a rat with streptozotocin-induced diabetes for the previous 9 months. *Arrows*, acellular capillaries; *filled arrowheads*, pericyte nuclei; *empty arrowheads*, endothelia cell nuclei; *scale bar* 50 μm. Photo courtesy of Mara Lorenzi, Schepens Eye Research Institute, Boston, MA.

et al. 2002). Agents that promote vessel stabilization may therefore provide novel treatment strategies for diabetic retinopathy.

15.4.2 Pericytes in Therapeutic Angiogenesis

Induction of angiogenesis is desirable in the treatment of myocardial infarction and peripheral vascular disease, and is critical for formation and regeneration of tissues and organs. It is important that new vessels have characteristics of stable vessels, however; they must have permeability appropriate to the vessel bed, and should be maintained without exogenous growth factors.

Traditionally, pro-angiogenic therapies have focused on stimulation of endothelial cell proliferation and migration with factors such as VEGF. VEGF potently induces angiogenesis, but also has the unwanted side effect of making vessels very leaky, often resulting in edema. It is important that the permeability of new vessels be reduced, either by investing the vessels with pericytes or by treating the vessels with a permeability-reducing factor. Indeed, when VEGF is expressed in the presence of Angl, the degree of vessel leakiness is similar to that observed for control mice (Thurston et al. 1999).

For a vessel to function normally and be maintained long-term, it must be invested with mural cells. Large vessels have been engineered with both endothelial and smooth muscle layers (reviewed in Stegemann et al. 2005), but stable microvessels are particularly important for sustaining new tissues. In recognition of this need, combinations of growth factors are being tested to ensure that the vasculature in a new tissue contains both endothelial cells and pericytes. For example, implantation of a polymer scaffold to provide sustained, localized delivery of both VEGF and PDGF-B resulted in formation and maintenance of a mature vascular network in the tissue (Richardson et al. 2001). Better understanding of the cell–cell interactions in vessel maturation will likely lead to further improvements in pro-angiogenic therapies.

Conclusions

Great strides have been made in our understanding of the regulation of blood vessel assembly. The next phase of investigation will clearly focus on analysis of the later phases of vessel formation, including arteriogenesis, remodeling and specialization. These process display tissue-specific differences and will require an examination of complex intercellular interactions such as those that occur among the endothelium, pericytes, astrocytes and neural elements in the central nervous system. More detailed information about vessel remodeling and specialization will be central to translational aspects of microvascular research, including pro- and anti-angiogenic therapies and tissue engineering.

Acknowledgements

The authors would like to thank Diane Darland for critically reading the manuscript, as well as Ann Dvorak, Mara Lorenzi and Scott Plotkin for providing images. P.A.D. is supported by EY05318, EY15435 and CA45548. P.A.D. is a Research to Prevent Blindness Senior Scientist.

References

Allt G, Lawrenson JG (2001) Pericytes: cell biology and pathology. Cells Tissues Organs 169:1–11

Armulik A, Abramsson A, Betsholtz C (2005) Endothelial/pericyte interactions. Circ Res 97:512–523

Arthur FE, Shivers RR, Bowman PD (1987) Astrocyte-mediated induction of tight junctions in brain capillary endothelium: an efficient in vitro model. Dev Brain Res 36:155–159

Baffert F, Le T, Sennino B, Thurston G, Kuo CJ, Hu-Lowe D, McDonald DM (2006) Cellular changes in normal blood capillaries undergoing regression after inhibition of VEGF signaling. Am J Physiol Heart Circ Physiol 290: H547–559

Baluk P, Hashizume H, McDonald DM (2005) Cellular abnormalities of blood vessels as targets in cancer. Curr Opin Genet Dev 15:102–111

Benarroch EE (2005) Neuron-astrocyte interactions: part-

nership for normal function and disease in the central nervous system. Mayo Clin Proc 80:1326–1338

Benjamin LE, Golijanin D, Itin A, Pode D, Keshet E (1999) Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. J Clin Invest 103:159–165

Benjamin LE, Hemo I, Keshet E (1998) A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. Development 125:1591–1598

Bergers G, Song S, Meyer-Morse N, Bergsland E, Hanahan D (2003) Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. J Clin Invest 111:1287–1295

Betsholtz C (2004) Insight into the physiological functions of PDGF through genetic studies in mice. Cytokine Growth Factor Rev 15:215–228

Carmeliet P (2000) Mechanisms of angiogenesis and arteriogenesis. Nat Med 6:389–395

Darland DC, D'Amore PA (2001) Cell–cell interactions in vascular development. Curr Top Dev Biol 52:107–149

Chan-Ling T, Hughes S (2005) NG2 can be used to identify arteries versus veins enabling the characterization of the different functional roles of arterioles and venules during microvascular network growth and remodeling. Microcirculation 12:539–540

Darland DC, Massingham LJ, Smith SR, Piek E, Saint-Geniez M, D'Amore PA (2003) Pericyte production of cell-associated VEGF is differentiation-dependent and is associated with endothelial survival. Dev Biol 264:275–288

Duda DG, Fukumura D, Jain RK (2004) Role of eNOS in neovascularization: NO for endothelial progenitor cells. Trends Mol Med 10:143–145

Enge M, Bjarnegard M, Gerhardt H, Gustafsson E, Kalen M, Asker N, Hammes HP, Shani M, Fassler R, Betsholtz C (2002) Endothelium-specific platelet-derived growth factor-B ablation mimics diabetic retinopathy. EMBO J 21:4307–4316

Esser S, Wolburg K, Wolburg H, Breier G, Kurzchalia T, Risau W (1998) Vascular endothelial growth factor induces endothelial fenestrations in vitro. J Cell Biol 140:947–959

Garcia CM, Darland DC, Massingham LJ, D'Amore PA (2004) Endothelial cell–astrocyte interactions and TGF beta are required for induction of blood–neural barrier properties. Brain Res Dev Brain Res 152:25–38

Gee MS, Procopio WN, Makonne JS, Feldman MD, Yeilding NM, Lee WM (2003) Tumor vessel development and maturation impose limits on the effectiveness of anti-vascular therapy. Am J Pathol 162:183–193

Gerhardt H, Betsholtz C (2003) Endothelial-pericyte interactions in angiogenesis. Cell Tissue Res 314:15–23

Hammes HP (2005) Pericytes and the pathogenesis of diabetic retinopathy. Horm Metab Res 37 [Suppl 1]:39–43

Haseloff, RF, Blasig EE, Bauer HC, Bauer H (2005) In search of the astrocytic factor(s) modulating blood–brain barrier functions in brain capillary endothelial cells in vitro. Cell Mol Neurobiol 25:25–39

Hellström M, Kalén M, Lindahl P, Abramsson A, Betsholtz C (1999) Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. Development 126:3047–3055

Hoffmann J, Feng Y, Vom Hagen F, Hillenbrand A, Lin J, Erber R, Vajkoczy P, Gourzoulidou E, Waldmann H, Giannis A, et al (2005) Endothelial survival factors and spatial completion, but not pericyte coverage of retinal capillaries, determine vessel plasticity. FASEB J, DOI 10.1096/fj.04–2109fje

Hughes S, Chan-Ling T (2004) Characterization of smooth muscle cell and pericyte differentiation in the rat retina in vivo. Invest Ophthalmol Vis Sci 45:2795–2806

Jones N, Iljin K, Dumont DJ, Alitalo K (2001) Tie receptors: new modulators of angiogenic and lymphangiogenic responses. Nat Rev Mol Cell Biol 2:257–267

Joussen AM, Poulaki V, Tsujikawa A, Qin W, Qaum T, Xu Q, Moromizato Y, Bursell SE, Wiegand SJ, Rudge J, et al (2002) Suppression of diabetic retinopathy with angiopoietin-1. Am J Pathol 160:1683–1693

Kamba T, Tam BY, Hashizume H, Haskell A, Sennino B, Mancuso MR, Norberg SM, O'Brien SM, Davis RB, Gowen LC, et al (2006) VEGF-dependent plasticity of fenestrated capillaries in the normal adult microvasculature. Am J Physiol Heart Circ Physiol 290:H560–576

Kashiwagi S, Izumi Y, Gohongi T, Demou ZN, Xu L, Huang PL, Buerk DG, Munn LL, Jain RK, Fukumura D (2005) NO mediates mural cell recruitment and vessel morphogenesis in murine melanomas and tissue-engineered blood vessels. J Clin Invest 115:1816–1827

Lebrin F, Deckers M, Bertolino P, Ten Dijke P (2005) TGF-beta receptor function in the endothelium. Cardiovasc Res 65:599–608

Lee SW, Kim WJ, Choi YK, Song HS, Son MJ, Gelman IH, Kim YJ, Kim KW (2003) SSeCKS regulates angiogenesis and tight junction formation in blood–brain barrier. Nat Med 9:900–906

Li DY, Sorensen LK, Brooke BS, Urness LD, Davis EC, Taylor DG, Boak BB, Wendel DP (1999) Defective angiogenesis in mice lacking endoglin. Science 284:1534–1537

Maharaj ASR, Saint-Geniez M, Maldonado AE, D'Amore PA (2006) Vascular endothelial growth factor localization in the adult. Am J Pathol 168:639–648

Marchio S, Lahdenranta J, Schlingemann RO, Valdembri D, Wesseling P, Arap MA, Hajitou A, Ozawa MG, Trepel M, Giordano RJ, et al (2004) Aminopeptidase A is a functional target in angiogenic blood vessels. Cancer Cell 5:151–162

Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, Libermann TA, Morgan JP, Sellke FW, Stillman IE, et al (2003) Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hy-

pertension, and proteinuria in preeclampsia. J Clin Invest 111:649–658

Milici AJ, Furie MB, Carley WW (1985) The formation of fenestrations and channels by capillary endothelium in vitro. Proc Natl Acad Sci U S A 82:6181–6185

Morikawa S, Baluk P, Kaidoh T, Haskell A, Jain RK, McDonald DM (2002) Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. Am J Pathol 160:985–1000

Nehls V, Denzer K, Drenckhahn D (1992) Pericyte involvement in capillary sprouting during angiogenesis in situ. Cell Tissue Res 270:469–474

Oshima Y, Oshima S, Nambu H, Kachi S, Takahashi K, Umeda N, Shen J, Dong A, Apte RS, Duh E, et al (2005) Different effects of angiopoietin-2 in different vascular beds: new vessels are most sensitive. FASEB J 19: 963–965

Ozerdem U, Stallcup WB (2003) Early contribution of pericytes to angiogenic sprouting and tube formation. Angiogenesis 6:241–249

Peters KG, Kontos CD, Lin PC, Wong AL, Rao P, Huang L, Dewhirst MW, Sankar S (2004) Functional significance of Tie2 signaling in the adult vasculature. Recent Prog Horm Res 59:51–71

Richardson TP, Peters MC, Ennett AB, Mooney DJ (2001) Polymeric system for dual growth factor delivery. Nat Biotechnol 19:1029–1034

Sims DE (1991) Recent advances in pericyte biology – implications for health and disease. Can J Cardiol 7:431–443

Sims DE (2000) Diversity within pericytes. Clin Exp Pharmacol Physiol 27:842–846

Stegemann JP, Hong H, Nerem RM (2005) Mechanical, biochemical, and extracellular matrix effects on vascular smooth muscle cell phenotype. J Appl Physiol 98:2321–2327

Thurston G, Suri C, Smith K, McClain J, Sato TN, Yancopoulos GD, McDonald DM (1999) Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. Science 286:2511–2514

Uemura A, Ogawa M, Hirashima M, Fujiwara T, Koyama S, Takagi H, Honda Y, Wiegand SJ, Yancopoulos GD, Nishikawa S (2002) Recombinant angiopoietin-1 restores higher-order architecture of growing blood vessels in mice in the absence of mural cells. J Clin Invest 110:1619–1628

Wilkinson-Berka JL, Babic S, de Gooyer T, Stitt AW, Jaworski K, Ong LG, Kelly DJ, Gilbert RE (2004) Inhibition of platelet-derived growth factor promotes pericyte loss and angiogenesis in ischemic retinopathy. Am J Pathol 164:1263–1273

Witmer AN, van Blijswijk BC, van Noorden CJ, Vrensen GF, Schlingemann RO (2004) In vivo angiogenic phenotype of endothelial cells and pericytes induced by vascular endothelial growth factor-A. J Histochem Cytochem 52:39–52

Yamagishi S, Yonekura H, Yamamoto Y, Fujimori H, Sakurai S, Tanaka N, Yamamoto H (1999) Vascular endothelial growth factor acts as a pericyte mitogen under hypoxic conditions. Lab Invest 79:501–509

Zhang L, Yang N, Park JW, Katsaros D, Fracchioli S, Cao G, O'Brien-Jenkins A, Randall TC, Rubin SC, Coukos G (2003) Tumor-derived vascular endothelial growth factor up-regulates angiopoietin-2 in host endothelium and destabilizes host vasculature, supporting angiogenesis in ovarian cancer. Cancer Res 63:3403–3412

Adhesion Molecules in the Vascular Cell Cross-Talk

16

Ugo Cavallaro

Abstract

In the context of the vascular system, cell–cell adhesion is of paramount importance, as demonstrated by the fact that the inhibition of this process during embryogenesis is usually incompatible with normal development and often leads to death. Indeed, interendothelial contacts not only contribute to preserving the structure of the whole vascular tree, they also play a key role in regulating the exchanges of fluids, solutes and cells between the circulation and the peripheral tissues. This implies that the vascular cell–cell junctions are by no means static structures that simply link adjacent cells to each other; instead, they are highly dynamic in order to ensure a rapid response of the vascular system to microenvironmental stimuli. This chapter will provide an overview of the structural organization and of the molecules involved in the assembly and function of the cell–cell junctions in the vascular network. Based on the essential role of intercellular adhesion in various steps of the angiogenic process, it is conceivable that similar mechanisms are involved in tumor-associated neovascularization. Nevertheless, a clear definition of the molecular mechanisms that link interendothelial junctions to the formation of tumor vessels is still elusive. Progress in this field will have a profound impact on the design of innovative therapeutic strategies aimed at interfering with tumor angiogenesis and metastasis.

CONTENTS

Introduction

The communication between cells and their microenvironment is crucial both during development and for the maintenance of tissue architecture and organization. Indeed, cells need to perceive the signals coming from their neighborhood in order to "keep an eye" on the surrounding environment and react promptly to changes that may occur. Although various molecular mechanisms account for the ability of cells to "sense" the microenvironment, they can be essentially grouped into two major classes: (1) the transmission of signals in the form of soluble molecules which interact with cellular receptors, such as growth factors, cytokines, hormones, etc., and (2) the interaction of cells with "structural" components of their environment, namely other cells and the extracellular matrix (ECM).

The latter process requires specialized cellular structures that anchor the cells to each other (cell–cell adhesion) or to the ECM substrate (cell–matrix adhesion). These structures are formed by various proteins that are recruited to discrete areas of the cell periphery, where they assemble into adhesion complexes. In the case of cell–matrix adhesion (also known as focal adhesion), specific transmembrane proteins called integrins act as "hooks" through the direct binding of their extracellular portion to ECM components. On the intracellular side, integrins interact with proteins that connect the adhesion complexes to the cytoskeleton, thus stabilizing the cell–matrix contacts. Although different integrins bind to different ECM proteins and the components of the focal adhesion complexes vary depending on the cellular context and on other factors, the general organization of these adhesive structures is rather conserved. This is not the case for cell–cell adhesion, which involves various types of complexes that differ not only in their biochemical composition and structural properties, but also because they serve diverse functions. Consequently, along the intercellular cleft one can identify different classes of adhesive structures, such as adherens, tight, and gap junctions, which are spatially and functionally distinct. Nevertheless, their formation and activity needs to be regulated in a coordinated manner, and extensive interplay occurs between different junctions. Indeed, several research groups focus their studies on this cross-talk, trying to dissect it at the molecular level and to explore its implications for various pathophysiological processes.

This chapter will focus on the main features of the adhesive complexes that are present at vascular cell–cell boundaries, with a particular emphasis on novel aspects of their functions, such as the role in intracellular signaling and in the cross-talk of endothelial cells with other cell types.

Vascular Cell–Cell Junctions

Cell adhesion and its regulation are particularly important in the context of the vascular system, in that all the processes that govern the ontogenesis, maintenance and proper functioning of the vessel network depend on the ability of the endothelial cells to interact with each other and with the surrounding tissue. Notably, the adhesive properties of endothelial cells exert a regulatory function on, and are themselves controlled by, other cellular events such as proliferation, survival, migration, and morphogenesis. Endothelial cell adhesion plays an essential role also in the vascular response to pathological conditions, such as inflammation, ischemia, wound healing and, in particular, cancer. Indeed, as described in other chapters of this book, tumor-associated angiogenesis is key to cancer progression and metastasis, and vascular adhesion molecules are undoubtedly major players in this context.

In most epithelial tissues, the location of the different types of junctions along the intercellular cleft follows a general order, with tight junctions towards the apical portion of the cleft and adherens junctions and desmosomes in the basolateral region (Perez-Moreno et al. 2003). This spatial distribution contributes to determine the apical-basal polarity of epithelial cells. In the case of endothelial cells,

however, the junctional organization is not as rigid, resulting in tight junctions being often intermingled with adherens junctions along the intercellular boundaries. In addition, endothelial cells do not contain desmosomes, although some desmosomal components are found in the complex adherens, a junctional structure specific to certain specialized vascular districts, such as a subset of lymphatic vessels and of veins (Hammerling et al. 2006).

Within the vessel wall, the intercellular junctions undergo a process of maturation and stabilization during the polarization of the endothelial barrier. The important implications of these structures in endothelial cell physiology are underscored, for example, by the dramatically different behavior that endothelial cells show when cultured as a sparse cell population vs. a dense monolayer (Dejana 2004). It is well known that confluent endothelial cells are contact inhibited (i.e., they stop proliferating and enter a quiescent state), become resistant to pro-apoptotic stimuli, and show a reduced response to growth factors. All these effects are reversible, as shown by shifting the same cells to low-density conditions. Besides this, the confluence state has obvious effects on another crucial function of the endothelium, namely the permeability to solutes as well as to cells (e.g., of the immune system). Finally, there is increasing evidence that cell density influences the signal transduction emanating from adhesion molecules and, even more important, the gene expression profile of endothelial cells (Dejana 2004). Taken together, these observations indicate that the formation and maturation of endothelial cell junctions have a profound impact on the vascular physiology.

Based on this, an increasing number of groups in the last years have focused on the structural and functional characterization of cell–cell junctions within the vascular network. Although these efforts are still in progress and our current knowledge is less advanced than that accumulated, for example, in epithelial cells, it has become clear that vascular intercellular adhesion exhibits cell type-specific features that account for the specialized roles of the adhesive junctions in the endothelium. The definition of the molecular mechanisms that control the formation and the activity of endothelial adhesion complexes is likely to impact on novel therapeutic approaches for the treatment of various vascular disorders, including cancer-associated angiogenesis.

Vascular Adherens Junctions

Adherens junctions (AJs) are the adhesive structures that have been most extensively characterized in endothelial cells. The main function of AJs is the formation and stabilization of physical contacts between adjacent cells, with obvious implications for tissue architecture and homeostasis. AJs have been mostly studied in epithelial cells, where an impressive amount of information has been obtained since the identification of E-cadherin. The latter is a calcium-dependent adhesion molecule that binds homophilically with E-cadherin molecules on the surface of adjacent cells in a zipper-like fashion. This interaction triggers the recruitment of intracellular proteins, termed catenins, which anchor the adhesion complex to the actin cytoskeleton, resulting in the stabilization of the intercellular contacts (Bazzoni and Dejana 2004). As outlined below, catenins have also emerged as important signaling proteins, an activity that is tightly controlled by their subcellular localization. Thus, AJ-mediated cell–cell adhesion not only has crucial structural implications, but also modulates signal transduction inside the cell.

16.3.1 VE-Cadherin

Although the general organization of vascular AJs is similar to that found in epithelial cells, certain biochemical and functional properties are restricted to the vessel network. The main difference between epithelial and endothelial AJs is that the latter do not contain E-cadherin but an endothelial-specific

cadherin, called vascular endothelial (VE) cadherin. The expression of VE-cadherin is essentially restricted to cells of the endothelial lineage and starts very early during the differentiation of endothelial cell precursors. In this context, the VE-cadherin gene promoter has been successfully used to induce endothelial cell-specific expression of transgenes in the mouse (Gory et al. 1999), and the recently reported expression of Cre recombinase under the control of the VE-cadherin gene promoter is a promising tool for the inactivation of specific genes in the embryonic and adult vasculature (Alva et al. 2006). Although VE-cadherin is found in all endothelial cell types, its levels vary in different vascular districts and during angiogenesis, including tumor vascularization. Indeed, the expression of VE-cadherin is enhanced in activated, cancer-associated vessels, suggesting a causal involvement in tumor angiogenesis (Prandini et al. 2005).

VE-cadherin exhibits the typical molecular structure of the so-called "classical" cadherins, with five extracellular cadherin-type repeats, a single transmembrane domain, and a cytoplasmic tail containing the docking sites for the catenins. By analogy to E-cadherin in epithelial cells, the homophilic and calcium-dependent interaction between VE-cadherin molecules on adjacent endothelial cells is thought to be the first step in the formation of vascular AJs. This initial binding induces lateral clusterization of VE-cadherin and the recruitment of beta-catenin and plakoglobin (also known as gamma-catenin). The latter then binds to alpha-catenin, which, in turn, is linked to the actin cytoskeleton, either by direct binding or via other actin-binding proteins such as vinculin and alpha-actinin (Perez-Moreno et al. 2003). Figure 16.1 shows a schematic view of the cadherin/catenin complex at the endothelial AJs.

The functional contribution of VE-cadherin to the development of the vascular system was unequivocally demonstrated by the ablation of the gene in mice. VE-cadherin-null mouse embryos, indeed, died at mid-gestation because of severe vascular defects (Carmeliet et al. 1999; Gory-Faure et al. 1999). Interestingly, the phenotypic analysis of the mutant embryos revealed that not only cell adhesion, but also survival and intracellular signaling were affected in VE-cadherin-deficient endothelial cells. Moreover, the overall morphogenetic program that normally underlies vessel development was disrupted by the loss of VE-cadherin, confirming that the function of this protein extends beyond the mere pro-adhesive activity.

In the past few years, the characterization of the molecular mechanisms underlying such a broad spectrum of VE-cadherin properties have become the focus of intense research, resulting in the discovery of novel and intriguing aspects of cadherin biology. Experimental evidence in vivo as well as in endothelial cell cultures pointed to an interplay between VE-cadherin-mediated adhesion and endothelial cell survival (i.e., resistance to programmed cell death or apoptosis). The molecular basis of this cross-talk probably lies in the ability of VE-cadherin to activate the phosphatidyl inositol-3 kinase (PI3K) pathway, an enzymatic cascade that ultimately leads to the inhibition of apoptosis (Carmeliet et al. 1999). The VE-cadherin/PI3K interaction requires an intact binding of VE-cadherin to beta-catenin, although it remains elusive whether the latter acts as an adaptor molecule or is actively involved in the recruitment of PI3K to the AJs.

16.3.1.1 The VE-Cadherin/VEGFR-2 Complex

An important function of cadherins (but also of other adhesion molecules such as integrins) is the modulation of the signaling downstream of growth factor receptors (or receptor tyrosine kinases, RTKs). Although this interplay has long been proposed, only recently have researchers begun to unravel its molecular terms (reviewed by Cavallaro and Christofori 2004). For example, both epidermal growth factor receptor (EGFR) and hepatocyte growth factor receptor (HGFR or c-Met) have been detected in the AJs of epithelial cells, where E-cadherin was shown to influence their response to the specific growth factors. Similarly, as schematically depicted in Fig. 16.1, VE-cadherin is able to associate with one of the vascular endothelial growth factor receptors, namely VEGFR-2 (Carmeliet et al. 1999).

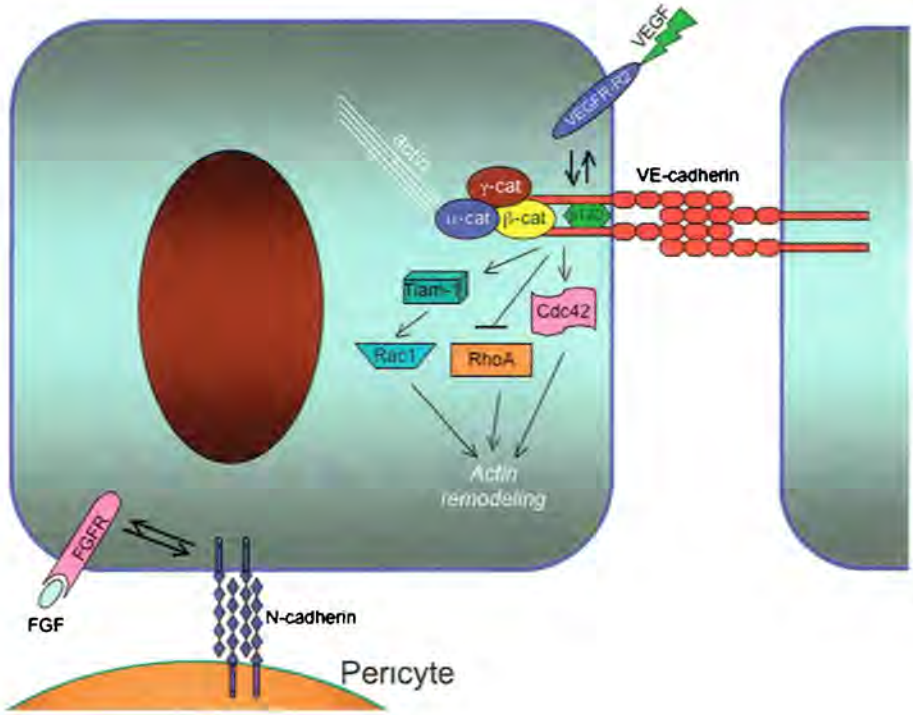


Fig. 16.1. Vascular adherens junctions. The molecular organization of the adherens junctions between endothelial cells is illustrated in a schematic manner, together with the main signaling pathways emanating from these structures. In addition, N-cadherin-mediated adhesion between endothelial cells and pericytes is shown, along with the cross-talk between N-cadherin and FGFR. See the text for more detail. Abbreviations: *cat*, catenin; *p120*, p120-catenin

This complex is induced by the stimulation of endothelial cells with VEGF and requires the presence of beta-catenin, suggesting that the binding involves the intracellular portion of the two transmembrane proteins. The formation of the VE-cadherin/VEGFR-2 complex promotes a widely known feature of endothelial cells, the contact inhibition of cell growth. This consists of stopping the cell proliferation that follows the maturation of intercellular junctions, aimed at preventing uncontrolled cell growth in normal tissues. When VEGFR-2 associates to VE-cadherin in confluent cells, its auto-phosphorylation (i.e., activation) in response to VEGF is dramatically attenuated. This effect has been attributed to the VE-cadherin-induced recruitment of VEGFR-2 close to the junctional phosphatase DEP-1/CD148, which interferes with the receptor activation (Lampugnani et al. 2003). Hence, endothelial cells no longer enter the mitotic program upon VEGF stimulation, in sharp contrast to sparse cells, which exhibit a strong proliferative response to VEGF. This recapitulates to some extent the situation in quiescent vs. angiogenic vessels, where endothelial cells are in close or loose contact with each other, respectively. In the latter case, VEGF induces cell proliferation and vessel growth, while non-angiogenic vessels do not exhibit this response. This does not mean that confluent endothelial cells (or mature vessels) do not respond to VEGF. Indeed, the association with clustered VE-cadherin induces as yet unidentified molecular changes in VEGFR-2, so that confluent endothelial cells react to VEGF by increasing their resistance to pro-apoptotic stimuli, a characteristic that is not present in low-density cells. While the proliferative response is mediated by VEGF-induced activation of mitogen-activated protein kinase (MAPK), cell survival requires the induction of the PI3K pathway (Carmeliet et al. 1999; Lampugnani et al. 2003). Therefore, as schematically depicted in Fig. 16.2, VE-cadherin acts a molecular switch for VEGFR-2, driving the response of endothelial cells to divergent pathways depending on the cellular and microenvironmental context.

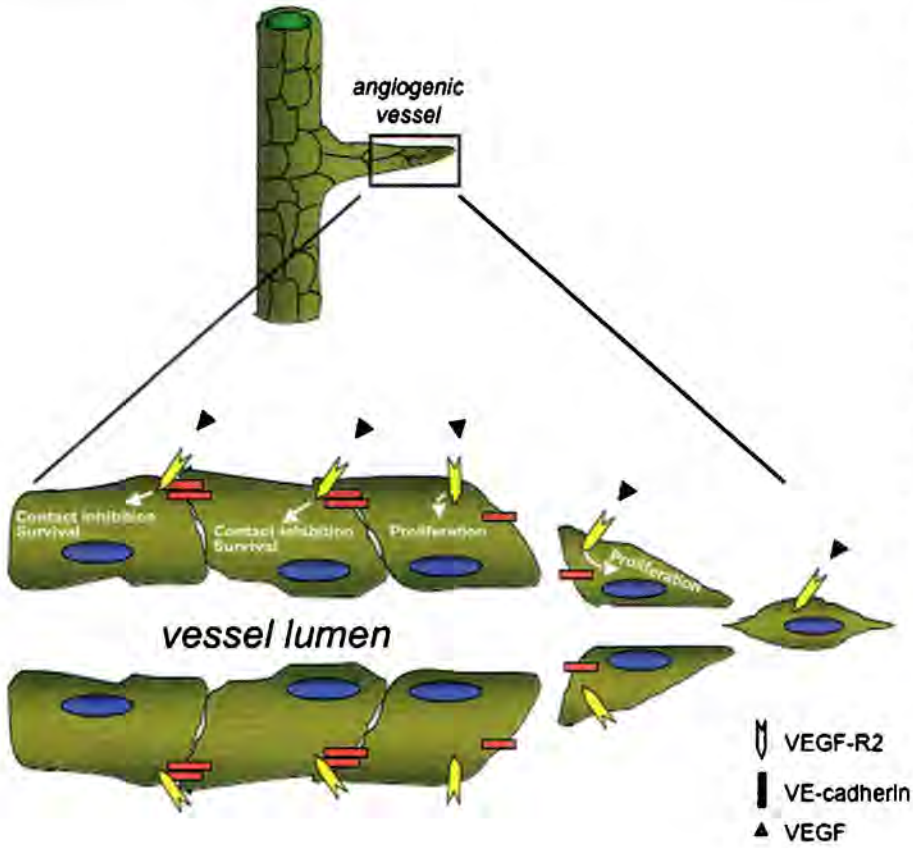


Fig. 16.2. VE-cadherin regulation of VEGF-R2 signaling. In mature endothelial AJs, VE-cadherin forms a complex with VEGF-R2, while the association is disrupted in loosely adherent or migrating endothelial cells, e.g., in the sprouts of angiogenic vessels. The association with VE-cadherin switches VEGF-R2 signaling towards cell survival, while the stimulation of "free" VEGF-R2 induces endothelial cell proliferation, a hallmark of angiogenesis. See the text for more detail

16.3.1.2 VE-Cadherin and the Endothelial Cytoskeleton

Recent evidence has implicated VE-cadherin in the regulation of endothelial cell cytoskeleton, an activity that has relevant implications for a broad spectrum of vascular functions, ranging from cell morphology and migration to differentiation and remodeling. The effects of VE-cadherin on the cytoskeleton are largely mediated by members of the Rho family of small GTPases (Fig. 16.1). Indeed, the clustering of VE-cadherin at endothelial AJs induces the recruitment of Tiam-1, a guanosine-exchange factor that specifically activates the small GTPase Rac1. The latter is known to control actin dynamics and, hence, cell shape and motility. In addition to Rac1 activation, VE-cadherin downregulates the activity of RhoA, another small GTPase involved in cytoskeletal remodeling (Lampugnani et al. 2002). Finally, also the third prototypic member of the family, Cdc42, has been shown to act downstream of VE-cadherin in the formation of cell membrane protrusions, and the VE-cadherin/Cdc42 cross-talk appeared to be endothelial-specific (Kouklis et al. 2003). Notably, this interplay between VE-cadherin-mediated adhesion and Rho-family GTPases is bidirectional. Indeed, Rac1 exerts a regulatory function during the early phase of vascular AJ formation, by controlling the spatial organization of the cadherin–catenin complexes (Cascone et al. 2003). Rac1 was also shown to induce the redistribution of VE-cadherin and the disruption of endothelial cell–cell adhesion, an effect that required Rac-induced production of reactive oxygen species (ROS) (van Wetering et al. 2002). The emerging picture was made even more complicated by the observation that

the inhibition of VE-cadherin function induced a rapid activation of Rac1 and the generation of ROS, which preceded the actual loss of cell–cell adhesion (van Buul et al. 2005). Thus, Rac1 would mediate the disruption of the endothelial barrier function induced by the dysfunction of VE-cadherin. These events acquire a significant relevance in inflammation, where Rac1-triggered production of ROS might contribute to the well-described endothelial damage.

Recent observations implicated Rac also in the cross-talk between VEGF signaling and VE-cadherin. Indeed, VEGF regulates the endothelial barrier function by inducing the phosphorylation of VE-cadherin complexes, and this effect specifically requires Rac activity (Seebach et al. 2005). Finally, all the prototypic members of the Rho family have been shown to influence vascular permeability, a function that depends on the regulation of VE-cadherin-dependent formation and stabilization of endothelial AJ (Wojciak-Stothard and Ridley 2002; Broman et al. 2006).

Thus, VE-cadherin represents an integration point between interendothelial adhesion and cytoskeletal regulation, and futures studies should define the molecular details of these concerted actions.

16.3.2 The Cytoplasmic Partners of VE-Cadherin

As mentioned earlier, catenins are the main interacting partners of the cytoplasmic tail of VE-cadherin. While beta-catenin, plakoglobin and p120-catenin (p120*ctn*) bind directly to VE-cadherin, alpha-catenin acts as a bridge between the cadherin–catenin complex and the actin cytoskeleton. These interactions stabilize the junctional complexes and preserve the integrity of the endothelial layer within the vessel wall. Indeed, interfering with the binding of catenins to VE-cadherin (e.g., by deleting the catenin-binding sites from the cytoplasmic domain of VE-cadherin) results in the disruption of interendothelial adhesion, with dramatic alterations in vessel morphogenesis and function (Carmeliet et al. 1999). This essential function of catenins has highlighted their structural implications in the vascular junctions, somehow promoting the idea that they mainly act as scaffolds. However, this view has dramatically changed in the past few years, due to the increasing evidence that catenins play also a crucial role in very dynamic intracellular events, such as signaling and the modulation of gene expression, as discussed below.

16.3.2.1 Beta-Catenin

Unlike the epithelial cells, our knowledge on the functions of beta-catenin in the vascular network is still very limited and, although certain similarities with the epithelium appear evident, preliminary observations point to endothelial-specific features. A significant step forward in the definition of beta-catenin's activities in vascular cells has been provided by the endothelial-specific inactivation of the gene in the mouse. This approach highlighted an essential role of endothelial beta-catenin during embryonic development, as demonstrated by the death of the mutant embryos at embryonic day 13.5 (Cattelino et al. 2003). Interestingly, beta-catenin did not appear to play a major role in the early phases of vasculogenesis and angiogenesis. In contrast, the loss of beta-catenin caused dramatic alterations in vascular patterning in various districts of the embryo proper and of the yolk sac. At the cellular level, beta-catenin-null endothelial cells showed a clear deficiency in the formation and maintenance of intercellular contacts, and the junctional complexes exhibited an altered composition and organization. A detailed characterization of the cell–cell adhesion complexes revealed that beta-catenin is required for the recruitment of alpha-catenin and, hence, for the anchorage of the junctions to the actin cytoskeleton. The loss of beta-catenin led not only to the decrease in alpha-catenin, but also to its replacement by desmoplakin, which preferentially binds to vimentin filaments, thus inducing a major shift in the cytoskeletal connections of the endothelial junctions. These alterations resulted in morphological changes, with cells assuming an elongated shape instead of the classical

cobblestone-like appearance, and in fenestration of the endothelial wall. In turn, these structural abnormalities in beta-catenin knockout cells had a remarkable functional outcome, exemplified by the reduced strength of interendothelial adhesion and the paracellular permeability of the endothelium (Cattelino et al. 2003).

These defects were obviously related to the loss of the junctional properties of beta-catenin, i.e., its participation in the adhesion complexes, confirming the importance of this protein in the organization and stabilization of the interendothelial junctions, just like in epithelial cells. However, beta-catenin plays crucial roles also when it is not part of the junctions, in particular in the context of the Wnt signaling pathway, a major regulator of developmental and pathophysiological processes (reviewed by Gregorieff and Clevers 2005). Upon disruption of cell–cell contacts, beta-catenin becomes soluble in the cytosol, and is normally targeted to the beta-catenin destruction complex (BDC), composed of the scaffolding proteins adenomatous polyposis coli (APC) and axin and the enzymes glycogen synthase kinase 3β (GSK3β) and casein kinase-1. The two kinases phosphorylate specific residues of beta-catenin, thus earmarking it for proteasome-mediated degradation. This default pathway of beta-catenin destruction is interrupted upon the activation of the Wnt signaling pathway (Fig. 16.3). Indeed, the binding of the extracellular factors of the Wnt family to their surface receptors, known as Frizzled, leads to the inhibition of GSK3β and, hence, to the stabilization and accumulation of beta-catenin. The latter eventually translocates to the nucleus, where it associates to transcription factors of the lymphoid enhancer factor-1 (Lef-1)/T-cell factor (TCF) family. The beta-catenin/TCF complex directly regulates the expression of a number of genes, for example cyclin D1 and c-Myc, which then impact on cell proliferation. As a consequence, an excessive transcriptional activity of beta-catenin in epithelial cells, such as that resulting from mutations in its gene or from the loss of function of APC in the colon, has been directly implicated in cell transformation and tumorigenesis (Gregorieff and Clevers 2005).

The Wnt/beta-catenin/TCF axis has been shown to operate also in endothelial cells, where, however, only few studies have been performed with controversial results. Indeed, some reports have shown that, by analogy to epithelial cell types, Wnt signaling promotes cell proliferation and requires beta-catenin-dependent gene expression (Wright et al. 1999; Masckauchan et al. 2005). On the other hand, the Wnt/beta-catenin/TCF pathway was also reported to repress endothelial cell proliferation and to induce intercellular adhesion (Cheng et al. 2003). This discrepancy might be attributed to the heterogeneity of endothelial cells or to different culture conditions; however, it may also imply that the vascular response to Wnt signaling is context-dependent, a hypothesis that deserves further investigation. The effect of the Wnt/beta-catenin/TCF cascade in endothelial cells are by no means restricted to the mere control of cell proliferation. Indeed, it turned out that among the transcriptional targets of beta-catenin in endothelial cells are members of the vascular endothelial growth factor (VEGF) family, namely VEGF-A and -C. Thus, the stabilization of beta-catenin leads to the autocrine activation of VEGF receptor signaling, as demonstrated by the induction of VEGF-R2 phosphorylation, which, in turn, activates the PI3K/Akt pathway. These molecular events underlie endothelial cell differentiation and remodeling in vitro and a strong angiogenic response in vivo (Skurk et al. 2005).

As discussed above, VEGF can have different effects on VE-cadherin-based junctions, depending on the density of endothelial cells and on whether VEGF-R2 is associated with VE-cadherin. Notably, VEGF induces the phosphorylation of specific tyrosine residues in the cytoplasmic tail of VE-cadherin, leading to the uncoupling of beta-catenin and $p120^{ctn}$ (Potter et al. 2005). The strong angiogenic effect of VEGF depends also on its ability to induce a mesenchymal conversion in endothelial cells. This phenotypic change is essential to endow the cells with migratory and remodeling potential and, hence, to support vessel invasion and angiogenesis. The detachment of beta-catenin and p120ctn from the junctions has been implicated in the maintenance of VEGF-induced mesenchymal state in

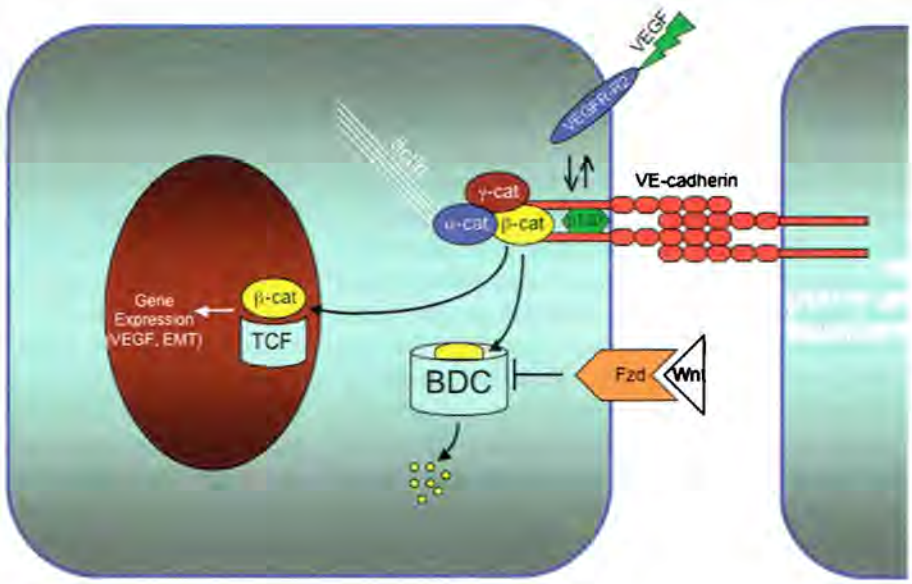


Fig. 16.3. The dual role of beta-catenin. The figure depicts in a schematic manner the two main functions of beta-catenin: as an integral component of the adherens junctions, and as an effector in Wnt signaling. The latter represses the degradation of beta-catenin, thus allowing its translocation to the nucleus and inducing its transcriptional activity. See the text for more detail. Abbreviations: *cat*, catenin; *p120*, p120-catenin; *BDC*, beta-catenin destruction complex; *Fzd*, frizzled; *EMT*, endothelial-to-mesenchymal transition

endothelial cells (Potter et al. 2005). Therefore, it is conceivable that the stabilization of cytosolic beta-catenin and its participation in Wnt signaling activate a VEGF-mediated feedback loop which leads to a sustained endothelial-to-mesenchymal transition (EMT), thus promoting an angiogenic response. The role of beta-catenin in the EMT of vascular cells has been also recently documented in vivo, both in mouse (Liebner et al. 2004) and in zebrafish (Hurlstone et al. 2003). In particular, Wnt signaling was shown to trigger EMT in the endocardial cells that underlies the formation of the heart cushion, which then gives rise to the atrio-ventricular valves. This EMT process is repressed in mice lacking endothelial beta-catenin, and has been shown to depend on the transcriptional activity of the beta-catenin/TCF complex (Liebner et al. 2004). The genes and pathways that act under the control of endothelial beta-catenin remain elusive and are the focus of intense research in various laboratories. Nevertheless, these findings highlight a key role for the Wnt/beta-catenin/TCF axis in vascular pathophysiology and raise the hypothesis that a dynamic balance between junctional and soluble beta-catenin determines the behavior (i.e., quiescence vs angiogenesis) of several endothelial cell types in vivo.

16.3.2.2 p120-Catenin

p120-Catenin certainly represents one of the most intriguing cytosolic partners of cadherins. This protein was initially identified as a prominent substrate of the non-receptor tyrosine kinase Src, and indeed cadherin-bound $p120^{ctn}$ is normally tyrosine-phosphorylated. Nevertheless, the impact of phosphorylation on $p120^{ctn}$ function has remained elusive.

By analogy to beta-catenin, $p120^{ctn}$ can be found either as an integral component of AJs or as a soluble protein in the cytosol, and the two pools appear to serve different functions. In endothelial cells, junctional $p120^{ctn}$ binds directly to the juxta-membrane region of VE-cadherin (Fig. 16.1); however, it exhibits neither direct nor indirect links to the cytoskeleton, in sharp contrast with the other catenins. As this suggests that $p120^{ctn}$ is not involved in the structural organization of the junctional complex, researchers have focused on the hypothesis that it exerts a regulatory function. This hypothesis was supported by the observation that the cellular level of $p120^{ctn}$ determines the amount of VE-cadherin, in particular by controlling its membrane trafficking (Xiao et al. 2003). More recently, Xiao and colleagues

have provided further insight into this regulatory activity, showing that p120^{ctn} stabilizes interendothelial junctions by preventing the internalization and lysosomal degradation of VE-cadherin (Xiao et al. 2005). This interplay between p120ctn and VE-cadherin has important functional consequences as, for example, it is required for the barrier function of the endothelium. Notably, either a decrease or an excess in p120^{ctn} levels results in the disruption of its barrier activity (Iyer et al. 2004), highlighting the importance of a tight control of p120^{ctn} expression for the pathophysiology of the endothelium. The alterations induced by excess p120^{ctn}, i.e. by the pool that is not bound to VE-cadherin, also indicate that non-junctional p120^{ctn} has important functions. Intriguingly, soluble p120^{ctn} was found to transduce signals to the cytoskeleton via the Rho-family small GTPases, with an inhibitory effect on RhoA and the positive regulation of Rac1 and Cdc42 (Anastasiadis et al. 2000; Noren et al. 2000; Grosheva et al. 2001). It remains to be investigated whether and how this interplay between p120^{ctn} and Rho GTPases modulates the permeability of the endothelial barrier, and also whether cytosolic p120^{ctn} has additional functions. Nevertheless, taken together, these observations put p120^{ctn} in a central position to integrate adhesive and cytoskeletal properties of vascular cells, most likely due to its ability to shuttle between a VE-cadherin-bound state and a cytosolic pool.

Finally, it should be mentioned that, by analogy to beta-catenin, also p120^{ctn} can translocate to the nucleus, where it has been shown to bind to and inactivate the transcriptional repressor Kaiso (Daniel and Reynolds 1999; Daniel et al. 2002). Furthermore, the expression of Wnt target genes appears to be coordinately regulated by the p120^{ctn}/Kaiso and beta-catenin/TCF complexes. Although the transcriptional effects of p120^{ctn} in endothelial cells have not yet been addressed, these results raise the fascinating possibility that, besides their crucial function within the adhesive structures, vascular catenins regulate important processes such as angiogenesis also by an integrated control on gene expression.

Although all the findings discussed above support the notion that p120^{ctn} is implicated in a variety of cellular processes, they refer to studies on cultured cells. The relative contribution of these p120^{ctn}-mediated functions in mammals has not yet been investigated. The ablation of the p120^{ctn} gene in the mouse results in embryonic lethality (Davis and Reynolds 2006), but to date a phenotypic characterization of the mutant embryos has not been reported. These studies, and in particular the analysis of the p120ctn-deficient vascular system will help to define the function of this protein in the endothelium.

In summary, the experimental evidence has conclusively supported a dual role for the catenin partners of VE-cadherin, as structural components of the interendothelial junctions and as important cytoplasmic effectors of different signaling pathways. Therefore, VE-cadherin, given its role in determining the subcellular localization of endothelial catenins, emerges as a key regulator of a complex network of signals that coordinates a broad spectrum of events in the vascular system.

16.3.3 N-Cadherin

From the data discussed so far, the role of VE-cadherin in the differentiation and function of the endothelium is quite clear, which also accounts for the attention that this protein has received since its discovery. However, VE-cadherin is not the only member of the cadherin family that is found in endothelial cells. Actually, the latter express levels of neural (N)-cadherin which are usually comparable to those of VE-cadherin. Besides endothelial cells, N-cadherin is expressed in neural tissue, myocytes, and fibroblasts. Notably, de novo expression of N-cadherin has been reported in various cell types concomitant with the acquisition of an invasive phenotype. Although this phenomenon is prominent during cancer progression, it also occurs in different phases of embryonic development (reviewed by Cavallaro and Christofori 2004). Such an induction of N-cadherin has important functional implications since, unlike E- or VE-cadherin, N-cadherin has been shown to enhance cell motility and invasiveness (Hazan et al. 2004). The cellular and molecular determinants that control the balance

between the adhesive and pro-migratory activities of N-cadherin remain to be elucidated.

In the context of the vascular system, the role of N-cadherin has long been overshadowed by that of VE-cadherin. Nevertheless, morphological studies soon ruled out a role of N-cadherin in endothelial cell–cell adhesion (Salomon et al. 1992). In addition, some reports have indicated a cadherin "hierarchy" in cultured cells, in that VE-cadherin was shown to displace N-cadherin from the junctions, thus dictating its subcellular localization. Indeed, in the presence of VE-cadherin, N-cadherin shows diffuse expression over the endothelial cell surface (Salomon et al. 1992; Navarro et al. 1998; Jaggi et al. 2002), although this has been recently challenged by the detection of N-cadherin at cell–cell junctions even in VE-cadherin-expressing endothelial cells (Luo and Radice 2005). All these studies, however, were conducted on cultured endothelial cells, thus not taking into account the higher complexity of the vessel wall in vivo. Indeed, in normal vessels the endothelium is surrounded by mural cells, namely smooth muscle cells in large vessels and pericytes in capillaries. The endothelial coverage by mural cells is critical for the correct formation and function of the vascular network, and this role appears to extend beyond a mere structural, scaffolding action. Indeed, it has become evident that perivascular cells promote vessel maturation and stabilization through a complex, yet poorly defined, array of intercellular signals (reviewed by von Tell et al. 2006).

Recent data have provided insightful information that assign very important functions to vascular N-cadherin in the endothelial-pericyte interplay. First of all, N-cadherin has been reported to cluster at the contact sites between endothelial and mural cells in the vessels of various tissues (Gerhardt et al. 2000; Liebner et al. 2000; Paik et al. 2004). This N-cadherin-mediated heterotypic adhesion is causally involved in vascular assembly, as the neutralization of N-cadherin in the developing brain led to an aberrant vessel network and to hemorrhages (Gerhardt et al. 2000). These observations were recently confirmed and extended by Tillet and colleagues, who showed that N-cadherin-null embryonic stem cells retain their ability to differentiate into endothelial cells and to undergo sprouting angiogenesis. However, the loss of N-cadherin resulted in the lack of pericyte recruitment to newly formed vessels (Tillet et al. 2005). Hence, N-cadherin would not be required for the early steps of vasculogenesis and angiogenesis, but rather for the subsequent, pericyte-mediated maturation of developing vessels. With regard to the molecular mechanisms that control the N-cadherin-mediated cross-talk between endothelial and mural cells, recent data have implicated the platelet-derived lipid mediator sphingosine 1-phosphate (S1P) and its receptor. Perturbation of S1P signaling led to the re-distribution of N-cadherin at endothelial cell–cell contacts, impairment in pericyte coverage of the endothelium, and lack of vessel maturation (Liu et al. 2000; Paik et al. 2004).

The heterotypic interactions mediated by endothelial N-cadherin are not limited to mural cells. For example, N-cadherin has been shown to enhance the adhesion of melanoma cells to the endothelium of tumor-associated vessels. Following the initial intercellular contact, N-cadherin is phosphorylated, causing the release of beta-catenin from the junctions and its translocation to the nucleus. The coordinated action of N-cadherin and beta-catenin results in the transendothelial migration of melanoma cells, a key step in the metastatic cascade (Qi et al. 2005). Based on the wide range of epithelial tumors which exhibit a strong induction of N-cadherin concomitant with cancer progression (Cavallaro 2004), it is likely that the role of this cadherin in tumor cell intravasation is not limited to melanoma.

The abrogation of N-cadherin expression in mice resulted in dramatic developmental defects and embryonic lethality at day 10 of gestation. Along with striking alterations in the neural tube, somites and myocardium, Radice et al. reported aberrant formation of the yolk sac vasculature, which could account for the developmental arrest of the mutant embryos (Radice et al. 1997). This initial indication of an important role for N-cadherin in vasculogenesis and early angiogenesis was confirmed by the endothelial-specific inactivation of the gene in the mouse. Indeed, conditional knockout embryos died at mid-gestation due to an impaired development of both the intra- and extraembryonic vasculature (Luo and

Radice 2005). The anatomical and functional alterations in developing vessels occurred earlier than the endothelial coverage by mural cells, indicating that the role of vascular N-cadherin is not restricted to the interaction between endothelial and perivascular cells. One possibility, supported by the detection of N-cadherin at interendothelial junctions in mouse embryos (Luo and Radice 2005), is that N-cadherin is also involved in homotypic cell–cell adhesion in the endothelium of developing vessels. Another intriguing hypothesis was raised by the detailed phenotypic analysis of the embryos carrying the endothelial-specific deletion of N-cadherin. The alterations exhibited by these mutant embryos were strikingly similar to those described in VE-cadherin-knockout mice (Carmeliet et al. 1999; Gory-Faure et al. 1999), thus highlighting a possible cross-talk between these two cadherins. Indeed, Luo and Radice found that the loss of N-cadherin correlated with a dramatic down-regulation of VE-cadherin in endothelial cells, both in vivo and in vitro (Luo and Radice 2005). Hence, N-cadherin might regulate vascular development by controlling the level of VE-cadherin, which, as discussed above, plays a pivotal role in vasculogenesis and angiogenesis. Interestingly, N-cadherin-mediated modulation of VE-cadherin did not occur at the mRNA level, suggestive of a post-transcriptional mechanism. Since the loss of N-cadherin also induced a marked reduction in $p120^{ctn}$, which stabilizes junctional VE-cadherin (see above), it is conceivable that $p120^{ctn}$ serves as an effector in N-cadherin-dependent pathways that control VE-cadherin turnover.

Additional findings on N-cadherin-deficient endothelial cells implicated this molecule also in the positive modulation of cell proliferation, which might contribute to impaired angiogenesis in mutant embryos, and in the repression of endothelial cell motility. The latter observation is in sharp contrast with the pro-migratory activity of N-cadherin reported in other cell types (as discussed above).

An interesting property of N-cadherin is its ability to associate with, and modulate the function of, the fibroblast growth factor receptor (FGFR), a receptor tyrosine kinase known to activate various signaling pathways. While FGFR has been implicated in N-cadherin-induced cell motility in breast cancer cells (Suyama et al. 2002), in endothelial cells it acts downstream of N-cadherin to prevent apoptosis (Erez et al. 2004). Taken together, these observations imply that N-cadherin acts in a cell context-dependent manner, and future research should aim at defining the cellular and microenvironmental factors that modulate the function of this cadherin in the vascular system.

Moreover, many lines of evidence described above point to a bidirectional interplay between VE and N-cadherin in endothelial cells, which is much more complex than the mutual competition that was initially hypothesized. This is the first example of cross-talk between classical cadherins, and the characterization of the underlying molecular mechanisms would have a major impact on our understanding of physiological and pathological neovascularization.

Vascular Tight Junctions

In contrast to AJs, the molecular architecture and the function of tight junctions (TJs) within the vascular system have been only poorly elucidated, and most of the current knowledge derives from the TJ characterization in epithelial cells (reviewed by Bazzoni and Dejana 2004). Indeed, both endothelial and epithelial TJs play a key role in controlling paracellular permeability to fluids and solutes. Moreover, the overall organization of the TJs is largely conserved between the two cell types. For some aspects, however, this extrapolation appears inappropriate, since remarkable structural and functional differences between endothelial and epithelial TJs have emerged. For example, TJs represent themost apical junctions in the epithelial intercellular cleft, while they are frequently intermingled with AJs in endothelial cells. Furthermore, the discovery of claudin-5 as an endothelial-specific member of the TJs (Morita et al. 1999) suggests that also the biochemical composition of vascular TJs is distinct from that found in the epithelium.

Endothelial TJs shows remarkable differences in different vascular districts. In particular, they appear well organized into arteries and arterioles, while a loose structure is often observed in veins and postcapillary venules. In the microcirculation, the presence of less developed TJs normally correlates with the need to ensure transendothelial trafficking of plasma components and blood cells. In contrast, well-developed TJs are found in those districts where the exchanges between intra- and extravascular spaces have to be tightly controlled. This is best exemplified by the brain circulation, where endothelial TJs contribute to establish the blood–brain barrier, which accounts for the extremely low and selective rate of trafficking across the vascular endothelium (Wolburg and Lippoldt 2002).

The organization and function of epithelial TJs have been thoroughly described in excellent recent reviews (see, for example, Kohler and Zahraoui 2005; Matter et al. 2005; Miyoshi and Takai 2005) and are out of the scope of this chapter. Moreover, as mentioned above, it is not yet clear what part of this information is transferable to the endothelium. Therefore, I will focus on endothelial-related features of TJ structure and activity.

16.4.1 Junctional Adhesion Molecules

Junctional adhesion molecules (JAMs) form a group of transmembrane proteins belonging to the immunoglobulin (Ig) superfamily, due to the presence of two Ig domains in their extracellular portion. After the identification of the first JAM (Martin-Padura et al. 1998), named JAM-A, other four members of the family were discovered, namely JAM-B, JAM-C, JAM-4, and JAM-L. This nomenclature follow the one recently proposed (Muller 2003) to overcome the confusion generated by the different names initially given to mouse and human orthologs. Another protein called ESAM (endothelial cell-selective adhesion molecule) was reported to be highly related to JAMs (Hirata et al. 2001).

In spite of their prominent role in TJ formation and function in both endothelial and epithelial cells, JAMs appear to be associated with TJs rather than being integral components. In fact, the localization of JAMs is not restricted to TJ complexes and they are also found along the intercellular clefts. Many lines of evidence indicate that JAMs have multiple functions in endothelial cells. As suggested by their name, a prominent feature of JAMs is their ability to promote intercellular adhesion via homophilic binding (Bazzoni et al. 2000a). However, it is not clear to what extent the pro-adhesive function of JAMs is relevant in vivo. The inactivation of the JAM-A gene in mice, either in the whole organism or in the vasculature only, did not lead to significant alterations in interendothelial contacts (Cera et al. 2004), arguing against an essential role of JAM-A in cell–cell adhesion. However, based on the co-expression of other members of the JAM family in vascular cells, it is possible that in knockout cells the function of JAM-A is replaced by other JAMs.

The JAM family appears to play an important role in the recruitment of various proteins to the TJs. Indeed, JAM-A associates with zonula occludens-1 (ZO-1), cingulin, and occludin, inducing their localization at TJs (Fig. 16.4; Bazzoni et al. 2000b). In addition to these structural components, JAM-A also interacts with a broad spectrum of scaffolding and signaling proteins, including AF6/afadin, the PAR-3/aPKC-PAR-6 complex, and MUPP1 (reviewed by Ebnet et al. 2004). While some of these interactions appear to mediate the anchorage of TJs to the cytoskeleton, others (such as those with MUPP1 and the PAR-3/aPKC-PAR-6 complex) have been implicated in another important function of JAMs, namely the induction and maintenance of cell polarity. Although most of these observations have been made in non-endothelial cell types, the regulatory role of JAMs in cell polarization can be extended to the vascular system. Indeed, JAM-A-deficient endothelial cells exhibit an abnormal polarized motility, which leads to increased random migration (Bazzoni et al. 2005).

The cellular functions that have been assigned to JAMs so far, such as regulation of TJs and cell polarity, have important implications for the angiogenic cascade. Hence, vascular JAM proteins

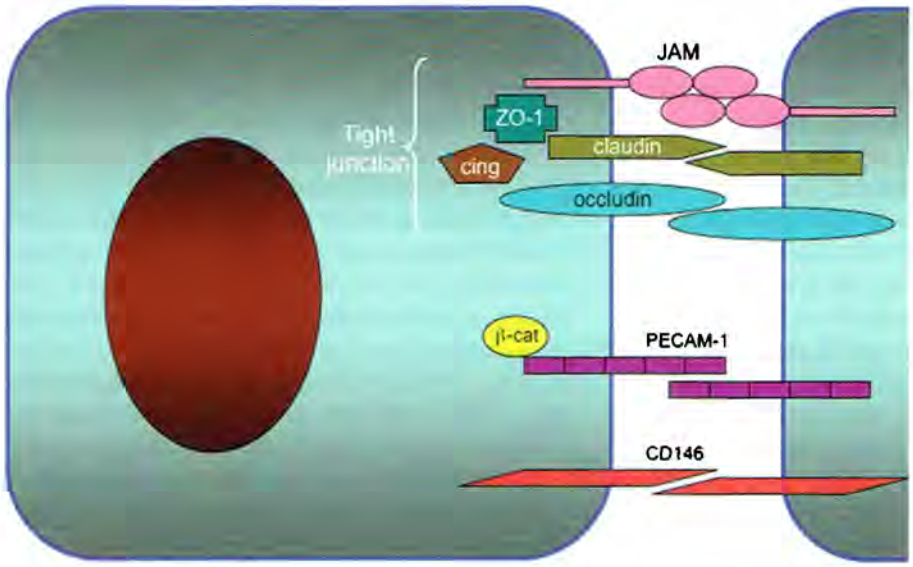


Fig. 16.4. Vascular tight junctions. The molecular organization of the tight junctions between endothelial cells is illustrated in a schematic manner, together with the non-junctional adhesion provided by PECAM-1 and CD146. See the text for more detail. Abbreviations: *cat*, catenin; *cing*, cingulin

could play a direct role in the vascularization process, and indeed recent data have confirmed this hypothesis. In particular, JAM-A was implicated as an important mediator downstream of a strong angiogenic factor, the basic fibroblast growth factor (bFGF). It is noteworthy that these studies revealed a novel cross-talk between JAM-A and $\alpha v\beta 3$, an integrin involved in capillary morphogenesis. Furthermore, JAM-A appears to modulate intracellular signaling during angiogenesis, as bFGF failed to induce the usual signaling cascade in JAM-A-depleted endothelial cells (Naik et al. 2003a, 2003b). Finally, recent observations have raised the possibility that JAMs are involved in tumor angiogenesis. Indeed, an antibody against JAM-C was reported to interfere with cancer growth by preventing neovascularization (Lamagna et al. 2005). Future studies should address this novel role of JAMs in a systematic manner, aimed at verifying whether this family of proteins could be exploited as novel therapeutic targets to block tumor angiogenesis.

A major function of JAM proteins is their ability to regulate the trafficking of leukocytes and dendritic cells across the endothelium, a process that has crucial implications for the inflammatory response. Interfering with JAM function in vivo, e.g., by using neutralizing antibodies, blocks the transendothelial migration of monocytes and neutrophils in experimental models of inflammation. Vascular JAMs facilitate leukocyte–endothelium interactions by heterophilic binding to blood cell integrins (reviewed by Ebnet et al. 2004). In particular, leukocyte function-associated antigen-1 (LFA-1; integrin $\alpha L\beta 2$) binds to JAM-A, very late antigen-4 (VLA-4; integrin $\alpha 4\beta 1$) binds to JAM-B, and Mac-1 (integrin $\alpha M\beta 2$) binds to JAM-C. Moreover, JAMs are also found in certain inflammatory cell types, where they mediate transendothelial migration. For example, JAM-A$^{-/-}$ polymorphonuclear leukocytes exhibit a reduced ability to cross the vessel wall and to infiltrate the perivascular tissue during inflammation (Corada et al. 2005). Notably, dendritic cells from JAM-A-deficient mice cross the lymphatic endothelium more efficiently than their wild-type counterpart (Cera et al. 2004). Thus, JAM-A can have different effects on transendothelial migration, depending on microenvironmental factors and on the inflammatory cell type.

16.4.2 Other Tight Junction Components

Besides JAMs, the transmembrane TJ components that have been identified in endothelial cells include occludin and the claudins 1, 3, 5 (which is restricted to the endothelium), and 12 (Nitta et al. 2003; Bazzoni and Dejana 2004). The functional contribution of these proteins to the regulation of vascular permeability can be already deduced from their distribution along the vascular tree. Occludin, for example, is abundantly expressed in the blood-brain barrier but its level drops in endothelial cells from other vascular districts, where it also exhibits a discontinuous distribution (Hirase et al. 1997). Both occludin and the endothelial claudins are able to induce the formation of TJ-like structures when ectopically expressed in fibroblasts (Furuse et al. 1998), which implies that they are not only constituents of the TJs but also regulate their organization. Moreover, the regulatory roles of occludin and claudins in the endothelium appear to extend beyond the mere sealing of the endothelium, and to entail the selectivity properties of the barrier (Miyoshi and Takai 2005). Other non-junctional activities of TJ proteins include the ability of claudin-5 to promote angiogenesis-related events (Miyamori et al. 2001) and that of claudin-1 to translocate to the nucleus and regulate gene expression (Dhawan et al. 2005). Claudin-1 shares this transcriptional activity with intracellular components of TJs, such as ZO-1. The latter, indeed, has been shown to induce the expression of specific genes, some of which may have important implications in angiogenesis (Polette et al. 2005).

Taken together, the findings described above point to a finely tuned interplay between junctional and transcriptional activities of TJ proteins, which is probably involved in important aspects of endothelial cell pathophysiology.

While functional TJs are normally conserved in cultured epithelial cells, they rapidly disorganize when primary endothelial cells are kept in culture, indicating that in the vascular system TJs are regulated by non-cell-autonomous mechanisms. Indeed, co-culture systems in which brain endothelial cells are in contact with astrocytes, or treatment of endothelial cells with astrocyte-derived conditioned medium, restore their TJ-dependent barrier function (Wolburg and Lippoldt 2002). The microenvironmental factors that contribute to the formation and maturation of TJs in the endothelium remain elusive. However, based on the increasing attention that this field has received in the last decade, significant progress in this regard can be expected in the near future. The knowledge derived from these studies would open novel perspectives, for example because of the possibility to modulate the permeability of the blood–brain barrier and, hence, the therapeutic access to the brain. In addition, based on the role of vascular TJs in the transendothelial trafficking of inflammatory cells (see above), the characterization of extrinsic regulatory mechanisms might have important implications also for novel anti-inflammatory therapies.

Non-Junctional Adhesion Molecules

Some adhesion molecules expressed in endothelial cells do not show a specific association to junctional complexes, but appear distributed along the intercellular cleft or over the whole cell surface. Platelet-endothelial cell adhesion molecule-1 (PECAM-1, also known as CD31) mediates interendothelial adhesion through homophilic binding. In addition, PECAM-1 has been implicated in a broad spectrum of vascular processes, including endothelial cell migration, survival, remodeling and angiogenesis (reviewed by Jackson 2003). In spite of these findings and of the early expression of PECAM-1 during the embryonic development of the vasculature, PECAM-1-knockout mice are viable and fertile, and do not exhibit vascular abnormalities (Duncan et al. 1999). To date, the role of PECAM-1 in embryonic vasculogenesis and

angiogenesis has remained elusive, and compensatory mechanisms during development have not been identified. Instead, a critical role for vascular PECAM-1 during the inflammatory response has been established. By analogy to JAMs, PECAM-1 modulates vascular permeability and the transendothelial migration of leukocytes through the endothelial cell junctions, mainly via homophilic binding between PECAM-1 molecules present on both cell types (Muller 2003). Moreover, PECAM-1 directly associates with beta-catenin (Fig. 16.4) and, in addition, induces its accumulation and nuclear translocation (Biswas et al. 2003), suggesting that PECAM-1 regulates the transcriptional activity of beta-catenin. Finally, recent data have also implicated PECAM-1 as an effector in various intracellular signaling pathways. A detailed description of these PECAM-1 properties is out of the scope of this chapter, but has been provided in excellent reviews (Ilan and Madri 2003; Newman and Newman 2003).

The adhesion molecule CD146 is widely expressed in endothelial cells and appears to be an early marker of the endothelial lineage. Antibodies against CD146 have become a widespread tool to identify vessels in tissue sections and to isolate circulating endothelial cells from the blood of patients affected by vascular disease. In spite of intriguing data that implicate CD146 in interendothelial adhesion and in signal transduction (Anfosso et al. 2001; Bardin et al. 2001), a clear definition of its role in the vascular system has remained elusive.

Endothelial Adhesion Molecules As Therapeutic Targets for Tumor Angiogenesis

Many of the adhesion molecules described above have been shown to be required for vascular remodeling and for the angiogenic cascade. Thus, it is conceivable that strategies aimed at interfering with their function could prove useful as novel avenues to repress tumor vascularization. A limited number of proof-of-concept experiments in this regard have been performed already. For example, neutralizing antibodies against VE-cadherin were shown to inhibit angiogenesis in mouse tumor models (Liao et al. 2000). Furthermore, subsequent studies have led to the generation of antibodies that specifically interfere with tumor vascularization, avoiding the disruption of VE-cadherin-based adhesion in normal vessels and, hence, the toxic effects due to diffused vascular permeability (Corada et al. 2002; Liao et al. 2002).

Members of the JAM family have also been implicated in tumor angiogenesis. The inhibition of JAM-C function prevented cancer vascularization and growth (Lamagna et al. 2005), confirming that JAMs could serve as targets in anti-angiogenic therapies. Another property of JAMs that should be considered in this context is their role in modulating the transendothelial migration of leukocytes. The inflammatory response that is often induced by solid tumors contributes to tumor progression also because the infiltrating cells secrete various angiogenic growth factors, thus promoting tumor neovascularization. Moreover, the intra- and extravasation processes that are utilized by infiltrating leukocytes are remarkably similar to the transendothelial migration of tumor cells during the metastatic dissemination. This implies that endothelial molecules, such as JAMs, PECAM-1 and CD146, that are involved in inflammatory infiltration could also facilitate the trafficking of tumor cells across the vascular wall. Hence, the therapeutic inhibition of adhesion molecules promoting transendothelial migration of inflammatory cells could prove useful also as a strategy to repress the metastatic dissemination of tumor cells.

Several studies under way in various laboratories focus on the role of vascular adhesion molecules in tumor angiogenesis and progression. This research will certainly have a significant impact on the possibility to develop novel anti-tumor therapies that could be used in combination with the classical chemotherapeutic approaches.

Acknowledgements

I am grateful to Elisabetta Dejana for her helpful comments on this chapter. I apologize to all those colleagues whose important work could not be cited due to space limitations.

References

Alva JA, Zovein AC, Monvoisin A, Murphy T, Salazar A, Harvey NL, Carmeliet P, Iruela-Arispe ML (2006) VE-Cadherin-Cre-recombinase transgenic mouse: a tool for lineage analysis and gene deletion in endothelial cells. Dev Dyn 235:759–767

Anastasiadis PZ, Moon SY, Thoreson MA, Mariner DJ, Crawford HC, Zheng Y, Reynolds AB (2000) Inhibition of RhoA by p120 catenin. Nat Cell Biol 2:637–644

Anfosso F, Bardin N, Vivier E, Sabatier F, Sampol J, Dignat-George F (2001) Outside-in signaling pathway linked to CD146 engagement in human endothelial cells. J Biol Chem 276:1564–1569

Bardin N, Anfosso F, Masse J-M, Cramer E, Sabatier F, Bivic AL, Sampol J, Dignat-George F (2001) Identification of CD146 as a component of the endothelial junction involved in the control of cell–cell cohesion. Blood 98:3677–3684

Bazzoni G, Dejana E (2004) Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. Physiol Rev 84:869–901

Bazzoni G, Martinez-Estrada OM, Mueller F, Nelboeck P, Schmid G, Bartfai T, Dejana E, Brockhaus M (2000a) Homophilic interaction of junctional adhesion molecule. J Biol Chem 275:30970–30976

Bazzoni G, Martinez-Estrada OM, Orsenigo F, Cordenonsi M, Citi S, Dejana E (2000b) Interaction of junctional adhesion molecule with the tight junction components ZO-1, cingulin, and occludin. J Biol Chem 275:20520–20526

Bazzoni G, Tonetti P, Manzi L, Cera MR, Balconi G, Dejana E (2005) Expression of junctional adhesion molecule-A prevents spontaneous and random motility. J Cell Sci 118:623–632

Biswas P, Canosa S, Schoenfeld J, Schoenfeld D, Tucker A, Madri JA (2003) PECAM-1 promotes beta-catenin accumulation and stimulates endothelial cell proliferation. Biochem Biophys Res Commun 303:212–218

Broman MT, Kouklis P, Gao X, Ramchandran R, Neamu RF, Minshall RD, Malik AB (2006) Cdc42 regulates adherens junction stability and endothelial permeability by inducing alpha-catenin interaction with the vascular endothelial cadherin complex. Circ Res 98:73–80

Carmeliet P, Lampugnani MG, Moons L, Breviario F, Compernolle V, Bono F, Balconi G, Spagnuolo R, Oostuyse B, Dewerchin M, Zanetti A, Angellilo A, Mattot V, Nuyens D, Lutgens E, Clotman F, de Ruiter MC, Gittenberger-de Groot A, Poelmann R, Lupu F, Herbert JM, Collen D, Dejana E (1999) Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. Cell 98:147–157

Cascone I, Giraudo E, Caccavari F, Napione L, Bertotti E, Collard JG, Serini G, Bussolino F (2003) Temporal and spatial modulation of Rho GTPases during in vitro formation of capillary vascular network. Adherens junctions and myosin light chain as targets of Rac1 and RhoA. J Biol Chem 278:50702–50713

Cattelino A, Liebner S, Gallini R, Zanetti A, Balconi G, Corsi A, Bianco P, Wolburg H, Moore R, Oreda B, Kemler R, Dejana E (2003) The conditional inactivation of the beta-catenin gene in endothelial cells causes a defective vascular pattern and increased vascular fragility. J Cell Biol 162:1111–1122

Cavallaro U (2004) N-cadherin as an invasion promoter: a novel target for antitumor therapy? Curr Opin Investig Drugs 5:1274–1278

Cavallaro U, Christofori G (2004) Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. Nat Rev Cancer 4:118–132

Cera MR, Del Prete A, Vecchi A, Corada M, Martin-Padura I, Motoike T, Tonetti P, Bazzoni G, Vermi W, Gentili F, Bernasconi S, Sato TN, Mantovani A, Dejana E (2004) Increased DC trafficking to lymph nodes and contact hypersensitivity in junctional adhesion molecule-A-deficient mice. J Clin Invest 114:729–738

Cheng CW, Smith SK, Charnock-Jones DS (2003) Wnt-1 signaling inhibits human umbilical vein endothelial cell proliferation and alters cell morphology. Exp Cell Res 291:415–425

Corada M, Chimenti S, Cera MR, Vinci M, Salio M, Fiordaliso F, De Angelis N, Villa A, Bossi M, Staszewsky LI, Vecchi A, Parazzoli D, Motoike T, Latini R, Dejana E (2005) Junctional adhesion molecule-A-deficient polymorphonuclear cells show reduced diapedesis in peritonitis and heart ischemia-reperfusion injury. Proc Natl Acad Sci U S A 102:10634–10639

Corada M, Zanetta L, Orsenigo F, Breviario F, Lampugnani MG, Bernasconi S, Liao F, Hicklin DJ, Bohlen P, Dejana E (2002) A monoclonal antibody to vascular endothelial-cadherin inhibits tumor angiogenesis without side effects on endothelial permeability. Blood 100:905–911

Daniel JM, Reynolds AB (1999) The Catenin p120ctn interacts with Kaiso, a novel BTB/POZ domain zinc finger transcription factor. Mol Cell Biol 19:3614–3623

Daniel JM, Spring CM, Crawford HC, Reynolds AB, Baig A (2002) The p120ctn-binding partner Kaiso is a bi-modal DNA-binding protein that recognizes both a sequence-specific consensus and methylated CpG dinucleotides. Nucl Acids Res 30:2911–2919

Davis MA, Reynolds AB (2006) Blocked acinar development, E-Cadherin reduction, and intraepithelial neoplasia upon ablation of p120-catenin in the mouse salivary gland. Developmental Cell 10:21–31

Dejana E (2004) Endothelial cell–cell junctions: happy together. Nat Rev Mol Cell Biol 5:261–270

Dhawan P, Singh AB, Deane NG, No Y, Shiou SR, Schmidt C, Neff J, Washington MK, Beauchamp RD (2005) Claudin-1 regulates cellular transformation and metastatic behavior in colon cancer. J Clin Invest 115:1765–1776

Duncan GS, Andrew DP, Takimoto H, Kaufman SA, Yoshida H, Spellberg J, Luis de la Pompa J, Elia A, Wakeham A, Karan-Tamir B, Muller WA, Senaldi G, Zukowski MM, Mak TW (1999) Genetic evidence for functional redundancy of platelet/endothelial cell adhesion molecule-1 (PECAM-1): CD31-deficient mice reveal PECAM-1-dependent and PECAM-1-independent functions. J Immunol 162:3022–3030

Ebnet K, Suzuki A, Ohno S, Vestweber D (2004) Junctional adhesion molecules (JAMs): more molecules with dual functions? J Cell Sci 117:19–29

Erez N, Zamir E, Gour BJ, Blaschuk OW, Geiger B (2004) Induction of apoptosis in cultured endothelial cells by a cadherin antagonist peptide: involvement of fibroblast growth factor receptor-mediated signalling. Exp Cell Res 294:366–378

Furuse M, Sasaki H, Fujimoto K, Tsukita S (1998) A single gene product, claudin-1 or -2, reconstitutes tight junction strands and recruits occludin in fibroblasts. J Cell Biol 143:391–401

Gerhardt H, Wolburg H, Redies C (2000) N-cadherin mediates pericytic–endothelial interaction during brain angiogenesis in the chicken. Dev Dyn 218:472–479

Gory S, Vernet M, Laurent M, Dejana E, Dalmon J, Huber P (1999) The vascular endothelial-cadherin promoter directs endothelial-specific expression in transgenic mice. Blood 93:184–192

Gory-Faure S, Prandini MH, Pointu H, Roullot V, Pignot-Paintrand I, Vernet M, Huber P (1999) Role of vascular endothelial-cadherin in vascular morphogenesis. Development 126:2093–2102

Gregorieff A, Clevers H (2005) Wnt signaling in the intestinal epithelium: from endoderm to cancer. Genes Dev 19:877–890

Grosheva I, Shtutman M, Elbaum M, Bershadsky AD (2001) p120 catenin affects cell motility via modulation of activity of Rho-family GTPases: a link between cell–cell contact formation and regulation of cell locomotion. J Cell Sci 114:695–707

Hammerling B, Grund C, Boda-Heggemann J, Moll R, Franke W (2006) The complexus adhaerens of mammalian lymphatic endothelia revisited: a junction even more complex than hitherto thought. Cell Tissue Res 324:55–67

Hazan RB, Qiao R, Keren R, Badano I, Suyama K (2004) Cadherin switch in tumor progression. Ann N Y Acad Sci 1014:155–163

Hirase T, Staddon JM, Saitou M, Ando-Akatsuka Y, Itoh M, Furuse M, Fujimoto K, Tsukita S, Rubin LL (1997) Occludin as a possible determinant of tight junction permeability in endothelial cells. J Cell Sci 110:1603–1613

Hirata K-i, Ishida T, Penta K, Rezaee M, Yang E, Wohlgemuth J, Quertermous T (2001) Cloning of an immunoglobulin family adhesion molecule selectively expressed by endothelial cells. J Biol Chem 276:16223–16231

Hurlstone AF, Haramis AP, Wienholds E, Begthel H, Korving J, Van Eeden F, Cuppen E, Zivkovic D, Plasterk RH, Clevers H (2003) The Wnt/beta-catenin pathway regulates cardiac valve formation. Nature 425:633–637

Ilan N, Madri JA (2003) PECAM-1: old friend, new partners. Curr Opin Cell Biol 15:515–524

Iyer S, Ferreri DM, DeCocco NC, Minnear FL, Vincent PA (2004) VE-cadherin-p120 interaction is required for maintenance of endothelial barrier function. Am J Physiol Lung Cell Mol Physiol 286:L1143–1153

Jackson DE (2003) The unfolding tale of PECAM-1. FEBS Letters 540:7–14

Jaggi M, Wheelock MJ, Johnson KR (2002) Differential displacement of classical cadherins by VE-cadherin. Cell Commun Adhes 9:103–115

Kohler K, Zahraoui A (2005) Tight junction: a co-ordinator of cell signalling and membrane trafficking. Biol Cell 97:659–665

Kouklis P, Konstantoulaki M, Malik AB (2003) VE-cadherin-induced Cdc42 signaling regulates formation of membrane protrusions in endothelial cells. J Biol Chem 278:16230–16236

Lamagna C, Hodivala-Dilke KM, Imhof BA, Aurrand-Lions M (2005) Antibody against junctional adhesion molecule-C inhibits angiogenesis and tumor growth. Cancer Res 65:5703–5710

Lampugnani MG, Zanetti A, Breviario F, Balconi G, Orsenigo F, Corada M, Spagnuolo R, Betson M, Braga V, Dejana E (2002) VE-cadherin regulates endothelial actin activating Rac and increasing membrane association of Tiam. Mol Biol Cell 13:1175–1189

Lampugnani MG, Zanetti A, Corada M, Takahashi T, Balconi G, Breviario F, Orsenigo F, Cattelino A, Kemler R, Daniel TO, Dejana E (2003) Contact inhibition of VEGF-induced proliferation requires vascular endothelial cadherin, beta-catenin, and the phosphatase DEP-1/CD148. J Cell Biol 161:793–804

Liao F, Doody JF, Overholser J, Finnerty B, Bassi R, Wu Y, Dejana E, Kussie P, Bohlen P, Hicklin DJ (2002) Selective targeting of angiogenic tumor vasculature by vascular endothelial-cadherin antibody inhibits tumor growth without affecting vascular permeability. Cancer Res 62:2567–2575

Liao F, Li Y, O'Connor W, Zanetta L, Bassi R, Santiago A, Overholser J, Hooper A, Mignatti P, Dejana E, Hicklin DJ, Bohlen P (2000) Monoclonal antibody to vascular endothelial-cadherin is a potent inhibitor of angiogenesis, tumor growth, and metastasis. Cancer Res 60:6805–6810

Liebner S, Cattelino A, Gallini R, Rudini N, Iurlaro M, Piccolo S, Dejana E (2004) Beta-catenin is required for endothelial-mesenchymal transformation during heart cushion development in the mouse. J Cell Biol 166:359–367

Liebner S, Gerhardt H, Wolburg H (2000) Differential expression of endothelial beta-catenin and plakoglobin during development and maturation of the blood–brain and blood–retina barrier in the chicken. Dev Dyn 217:86–98

Liu Y, Wada R, Yamashita T, Mi Y, Deng CX, Hobson JP, Rosenfeldt HM, Nava VE, Chae SS, Lee MJ, Liu CH, Hla T, Spiegel S, Proia RL (2000) Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. J Clin Invest 106:951–961

Luo Y, Radice GL (2005) N-cadherin acts upstream of VE-cadherin in controlling vascular morphogenesis. J Cell Biol 169:29–34

Martin-Padura I, Lostaglio S, Schneemann M, Williams L, Romano M, Fruscella P, Panzeri C, Stoppacciaro A, Ruco L, Villa A, Simmons D, Dejana E (1998) Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. J Cell Biol 142:117–127

Masckauchan TN, Shawber CJ, Funahashi Y, Li CM, Kitajewski J (2005) Wnt/beta-catenin signaling induces proliferation, survival and interleukin-8 in human endothelial cells. Angiogenesis 8:43–51

Matter K, Aijaz S, Tsapara A, Balda MS (2005) Mammalian tight junctions in the regulation of epithelial differentiation and proliferation. Curr Opin Cell Biol 17:453–458

Miyamori H, Takino T, Kobayashi Y, Tokai H, Itoh Y, Seiki M, Sato H (2001) Claudin promotes activation of pro-matrix metalloproteinase-2 mediated by membrane-type matrix metalloproteinases. J Biol Chem 276:28204–28211

Miyoshi J, Takai Y (2005) Molecular perspective on tight-junction assembly and epithelial polarity. Adv Drug Delivery Rev 57:815–855

Morita K, Sasaki H, Furuse M, Tsukita S (1999) Endothelial claudin: Claudin-5/TMVCF constitutes tight junction strands in endothelial cells. J Cell Biol 147:185–194

Muller WA (2003) Leukocyte-endothelial-cell interactions in leukocyte transmigration and the inflammatory response. Trends Immunol 24:327–334

Naik MU, Mousa SA, Parkos CA, Naik UP (2003a) Signaling through JAM-1 and ôvô3ś required for the angiogenic action of bFGF: dissociation of the JAM-1 and ôvô3 œmplex. Blood 102:2108–2114

Naik MU, Vuppalanchi D, Naik UP (2003b) Essential role of junctional adhesion molecule-1 in basic fibroblast growth factor-induced endothelial cell migration. Arterioscler Thromb Vasc Biol 23:2165–2171

Navarro P, Ruco L, Dejana E (1998) Differential localization of VE- and N-cadherins in human endothelial cells: VE-cadherin competes with N-cadherin for junctional localization. J Cell Biol 140:1475–1484

Newman PJ, Newman DK (2003) Signal transduction pathways mediated by PECAM-1: new roles for an old molecule in platelet and vascular cell biology. Arterioscler Thromb Vasc Biol 23:953–964

Nitta T, Hata M, Gotoh S, Seo Y, Sasaki H, Hashimoto N, Furuse M, Tsukita S (2003) Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. J Cell Biol 161:653–660

Noren NK, Liu BP, Burridge K, Kreft B (2000) p120 catenin regulates the actin cytoskeleton via Rho family GTPases. J Cell Biol 150:567–580

Paik JH, Skoura A, Chae SS, Cowan AE, Han DK, Proia RL, Hla T (2004) Sphingosine 1-phosphate receptor regulation of N-cadherin mediates vascular stabilization. Genes Dev 18:2392–2403

Perez-Moreno M, Jamora C, Fuchs E (2003) Sticky business: orchestrating cellular signals at adherens junctions. Cell 112:535–548

Polette M, Gilles C, Nawrocki-Raby B, Lohi J, Hunziker W, Foidart J-M, Birembaut P (2005) Membrane-type 1 matrix metalloproteinase expression is regulated by zonula occludens-1 in human breast cancer cells. Cancer Res 65:7691–7698

Potter MD, Barbero S, Cheresh DA (2005) Tyrosine phosphorylation of VE-cadherin prevents binding of p120- and beta-catenins and maintains the cellular mesenchymal state. J Biol Chem 280:31906–31912

Prandini MH, Dreher I, Bouillot S, Benkerri S, Moll T, Huber P (2005) The human VE-cadherin promoter is subjected to organ-specific regulation and is activated in tumour angiogenesis. Oncogene 24:2992–3001

Qi J, Chen N, Wang J, Siu CH (2005) Transendothelial migration of melanoma cells Involves N-cadherin-mediated adhesion and activation of the {beta}-catenin signaling pathway. Mol Biol Cell 16:4386–4397

Radice GL, Rayburn H, Matsunami H, Knudsen KA, Takeichi M, Hynes RO (1997) Developmental defects in mouse embryos lacking N-cadherin. Dev Biol 181:64–78

Salomon D, Ayalon O, Patel-King R, Hynes RO, Geiger B (1992) Extrajunctional distribution of N-cadherin in cultured human endothelial cells. J Cell Sci 102:7–17

Seebach J, Madler HJ, Wojciak-Stothard B, Schnittler HJ (2005) Tyrosine phosphorylation and the small GTPase rac cross-talk in regulation of endothelial barrier function. Thromb Haemost 94:620–629

Skurk C, Maatz H, Rocnik E, Bialik A, Force T, Walsh K (2005) Glycogen-synthase kinase 3beta/beta-catenin axis promotes angiogenesis through activation of vascular endothelial growth factor signaling in endothelial cells. Circ Res 96:308–318

Suyama K, Shapiro I, Guttman M, Hazan RB (2002) A signaling pathway leading to metastasis is controlled by N-cadherin and the FGF receptor. Cancer Cell 2:301–314

Tillet E, Vittet D, Feraud O, Moore R, Kemler R, Huber P (2005) N-cadherin deficiency impairs pericyte recruitment, and not endothelial differentiation or sprouting,

in embryonic stem cell-derived angiogenesis. Exp Cell Res 310:392–400

van Buul JD, Anthony EC, Fernandez-Borja M, Burridge K, Hordijk PL (2005) Proline-rich tyrosine kinase 2 (Pyk2) mediates vascular endothelial-cadherin-based cell–cell adhesion by regulating beta-catenin tyrosine phosphorylation. J Biol Chem 280:21129–21136

van Wetering S, van Buul JD, Quik S, Mul FP, Anthony EC, ten Klooster JP, Collard JG, Hordijk PL (2002) Reactive oxygen species mediate Rac-induced loss of cell–cell adhesion in primary human endothelial cells. J Cell Sci 115:1837–1846

von Tell D, Armulik A, Betsholtz C (2006) Pericytes and vascular stability. Exp Cell Res 312:623–629

Wojciak-Stothard B, Ridley AJ (2002) Rho GTPases and the regulation of endothelial permeability. Vascul Pharmacol 39:187–199

Wolburg H, Lippoldt A (2002) Tight junctions of the blood-brain barrier: development, composition and regulation. Vascul Pharmacol 38:323–337

Wright M, Aikawa M, Szeto W, Papkoff J (1999) Identification of a Wnt-responsive signal transduction pathway in primary endothelial cells. Biochem Biophys Res Commun 263:384–388

Xiao K, Allison DF, Buckley KM, Kottke MD, Vincent PA, Faundez V, Kowalczyk AP (2003) Cellular levels of p120 catenin function as a set point for cadherin expression levels in microvascular endothelial cells. J Cell Biol 163:535–545

Xiao K, Garner J, Buckley KM, Vincent PA, Chiasson CM, Dejana E, Faundez V, Kowalczyk AP (2005) p120-Catenin regulates clathrin-dependent endocytosis of VE-cadherin. Mol Biol Cell

Homing and Differentiation of Endothelial Progenitor Cells

17

CARMEN URBICH, EMMANOUIL CHAVAKIS, STEFANIE DIMMELER

Abstract

Endothelial progenitor cells (EPC), which presumable originate from bone marrow-derived progenitor cells, circulate with the blood on mobilization from the bone marrow, and home to sites of active vessel growth, a process termed "adult vasculogenesis". Infusion of EPC improves neovascularization in mice and in clinical trials. Several studies demonstrated that EPC contribute to tumor angiogenesis. However, the extent of endothelial incorporation and the precise mechanisms by which EPC contribute to neovessel formation are still under debate. Defining the events in progenitor cell homing and differentiation may enable novel therapeutic strategies to improve or block vascular regeneration. Homing is a multi-step cascade including the initial adhesion to activated endothelium or exposed matrix, transmigration through the endothelium, and, finally, migration and invasion into the target tissue. This review will summarize the current understanding of the molecular mechanisms mediating EPC homing.

CONTENTS

17.1 Introduction

In 1997, Asahara and colleagues reported the isolation of putative endothelial progenitor cells (EPC) from human peripheral blood. These cells were positive for the hematopoietic stem cell marker CD34 and endothelial markers such as VEGF-R2 (KDR). They differentiate to endothelial cells in vitro and incorporate into newly formed vessels during angiogenesis in vivo (Asahara et al. 1997). Meanwhile, several studies have shown that endothelial cells can be ex vivo differentiated from various cell populations including peripheral blood-derived mononuclear cells, hematopoietic and mesenchymal stem cells,

and tissue-residing stem cells (for review see Urbich and Dimmeler 2004). Subsequently, functional studies demonstrated that EPC are preferentially recruited to sites of ischemia, incorporate into vascular structures and functionally improve neovascularization after critical ischemia (Asahara et al. 1997; Shi et al. 1998). Furthermore, EPC attach to denuded arteries after balloon injury, and contribute to endothelial regeneration (Walter et al. 2002; Werner et al. 2002).

Several studies also clearly suggest the contribution of bone marrow-derived EPC to tumor angiogenesis (Garcia-Barros et al. 2003; Lyden et al. 2001) although the extent of contribution as well as the precise mechanism are still under debate (for review see Carmeliet 2005; Ferrara and Kerbel 2005). The incorporation rate of EPC into tumor endothelium ranges from 0–100% depending on the experimental set-up, e.g. tumor type, host and stage of tumorigenesis (de Palma et al. 2003a,b; Garcia-Barros et al. 2003; Li et al. 2004; Lyden et al. 2001; Spring et al. 2005). Using the tumor-resident Id-mutant mice, EPC give rise to a large proportion of endothelial cells within xenografted tumors (Lyden et al. 2001). In addition, bone marrow-derived endothelial precursors contribute functionally to neovasculature of some but not all spontaneous $Pten^{+/-}$ tumors in Id-deficient mice, although to a lesser extent (Ruzinova et al. 2003). In a recent study from Peters et al. the importance of bone marrow-derived EPC in human tumor neovascularization after bone marrow transplantation was assessed (Peters et al. 2005). Using FISH with X- and Y-chromosome-specific probes, the proportion of bone marrow-derived endothelial cells incorporated into the tumor vasculature ranged from 1% to 12% and averaged 4.9% (Peters et al. 2005). However, de Palma and collaborators proposed that the percentage of incorporated EPC into vessels is very low and that the majority of bone marrow-derived cells are accessory perivascular mononuclear cells which release angiogenic factors to stimulate angiogenesis of tumor-resident endothelial cells (de Palma et al. 2003b). In accordance, a subset of myeloid bone marrow mononuclear cells stimulates neovascularization by releasing pro-angiogenic factors such as MMP-9 (Grunewald et al. 2006). These bone marrow-derived circulating cells are recruited and retained by VEGF and SDF-1, respectively, and are positive for VEGF-R1, CD45, CX3CR1, and the myeloid marker CD11b. Two other studies also described the recruitment of $VEGF\text{-}R1^{+}$ hematopoietic progenitors that augmented revascularization and metastasis (Jin et al. 2006a; Kaplan et al. 2005). Moreover, immune cells such as monocytes, macrophages, dendritic cells, and B lymphocytes have been found to be recruited to areas of neo-angiogenesis, suggesting that the co-mobilization of different subtypes of bone marrow mononuclear cells may contribute to new vessel formation either via the release of paracrine factors ($VEGF\text{-}R1^{+}$ cells) or via physical incorporation ($VEGF\text{-}R2^{+}$ cells). Similar to ischemia, the hypoxic core of growing tumors involving the release of growth factors may influence progenitor-mediated tumor vasculogenesis (Annabi et al. 2004). Recently, it has been shown that growth factor-enriched conditioned medium isolated from several tumor cell lines induces bone marrow stromal cell proliferation, migration and tubulogenesis. Interestingly, a specific VEGF-blocking antibody, bevacizumab, decreased the number of viable circulating progenitor cells associated with a reduction in tumor perfusion and tumor growth (Willett et al. 2004). Since different tumors release a specific mixture of growth factors, it is conceivable that distinct tumors may attract different progenitor cell subpopulations with variable vasculogenic capacity.

Taking all of these findings together, bone marrow-derived EPC clearly contribute to tumor neovascularization, although the extent of their contribution as wells as the precise mechanism (incorporation vs paracrine effects) remains to be elucidated.

Mechanism of EPC Homing and Differentiation

Although the contribution of EPC to tumor angiogenesis and ischemia-induced neovascularization is under intensive investigation, little is known about the mechanism of homing and differentiation of EPC. Using intravital fluorescence videomicroscopy, the analysis of in vivo homing of embryonic EPC (eEPC) elucidated the multistep process of eEPC homing and incorporation. This cascade involves the arrest of circulating cells within tumor microvessels, extravasation into the interstitium, and incorporation into new vessels, suggesting that adhesion and transmigration are involved in the recruitment of EPC to sites of tumor angiogenesis (Vajkoczy et al. 2003). In analogy, ex vivo expanded adult EPC and hematopoietic stem/progenitor cells may use similar pathways for recruitment and incorporation in growing vessels during ischemia and tumor growth. Thus, the recruitment and incorporation of EPC requires a coordinated sequence of adhesion, transmigration, invasion, and, finally, the differentiation to endothelial cells (Fig. 17.1).

17.2.1 Homing of EPC

EPC are preferentially recruited to sites of ischemia and can improve neovascularization by direct incorporation into vascular structures and differentiation to the endothelial cell phenotype or by paracrine effects (Aicher et al. 2003; Asahara et al. 1997, 1999; Kalka et al. 2000b; Takahashi et al. 1999; Urbich and Dimmeler 2004). Both the paracrine effects and the differentiation of the EPC to the endothelial cell phenotype are dependent on the homing of EPC to sites of active neovascularization. While the homing of leukocytes to sites of inflammation is well studied (Imhof and Aurrand-Lions 2004; Kinashi 2005; Luster et al. 2005; Muller 2002), the mechanisms by which EPC home to sites of ischemia or to sites of active tumor neovascularization are poorly understood. During inflammation, the recruitment of inflammatory cells requires a coordinated sequence of multistep adhesive and signaling events including selectin-mediated rolling, leukocyte activation by chemokines leading to activation of integrins, integrin-mediated firm adhesion on endothelial cell monolayers, diapedesis through the endothelial cell monolayers and finally migration/invasion into the extracellular matrix involving integrin-dependent processes and matrix-degrading proteases (Carlos and Harlan 1994; Imhof and Aurrand-Lions 2004; Kinashi 2005; Luster et al. 2005; Muller 2002; Schenkel et al. 2004; Springer 1994). Evidence from recent studies suggests that the homing mechanisms of EPC to sites of tumor neovascularization and to sites of ischemia have at least some features in common with the homing of leukocytes to sites of inflammation. For the initialization of both the EPC homing to sites of ischemia/active neovascularization and the homing of leukocytes to sites of inflammation, the action of chemokines is mandatory (Abbott et al. 2004; Walter et al. 2005). In a study assessing the in vivo homing of murine embryonic EPC to sites of tumor angiogenesis by intravital microscopy, the EPC arrested within tumor microvessels, extravasated into the interstitium and incorporated into new vessels, suggesting that adhesion and transendothelial migration are involved in the recruitment of EPC to sites of tumor angiogenesis (Vajkoczy et al. 2003). Furthermore, recent studies support the idea that EPC and other progenitor cells engage adhesion molecules for homing to sites of neovascularization, similar to the engagement of adhesion molecules by leukocytes for recruitment to sites of inflammation (Chavakis et al 2003; Chavakis et al. 2005; Jin et al. 2006b; Yamaguchi et al. 2003). In the following sections we will focus on the role of chemokines, adhesion molecules and proteases for the homing of EPC to ischemic tissues and to sites of active neovascularization.

17.2.1.1 Role of Chemo-/Cytokines in EPC Homing

Activation by chemokines is an important step during EPC homing to recruit a reasonable number of progenitor cells to the ischemic tissues or to sites of

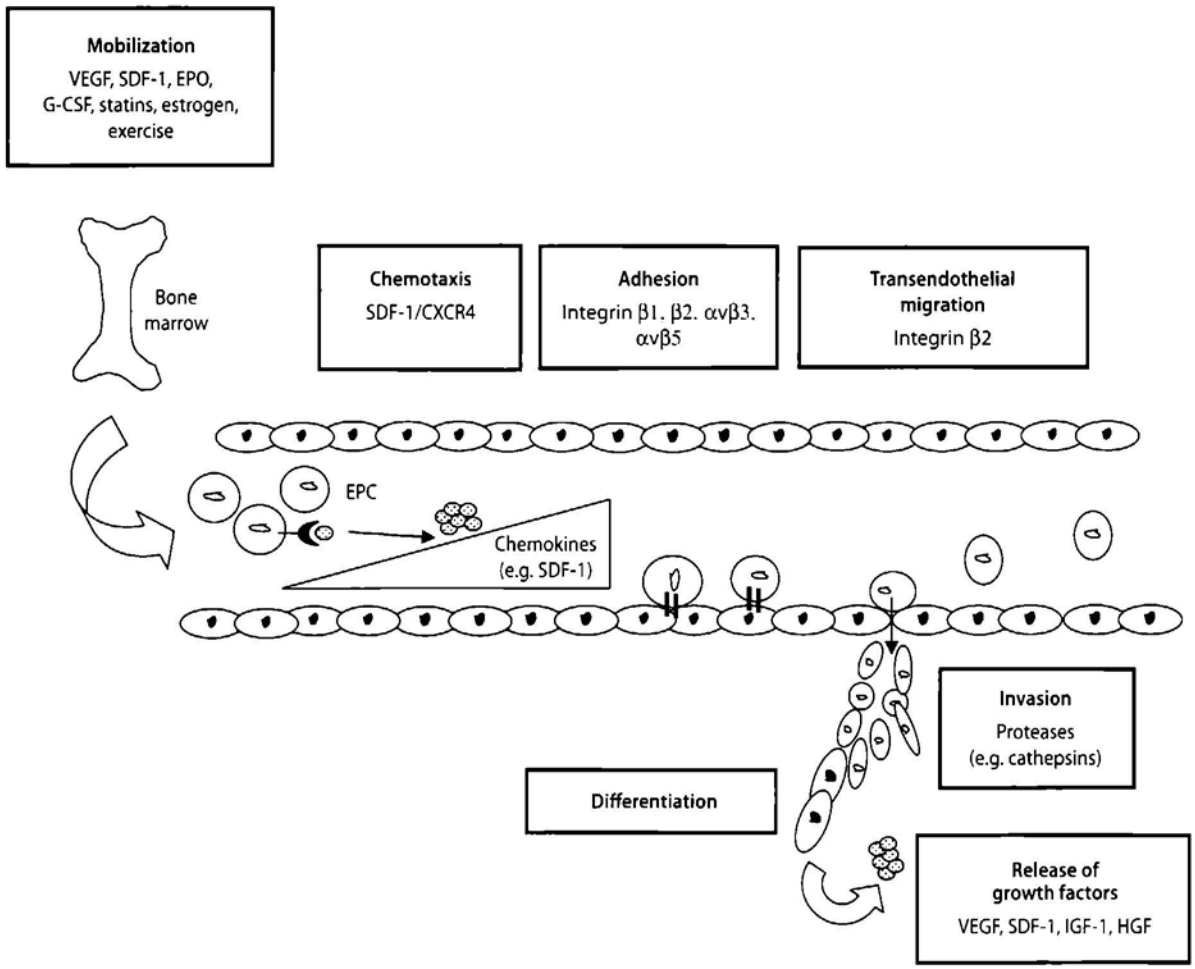


Fig. 17.1. Multistep process of EPC homing. Recruitment and incorporation of EPC into ischemic tissue requires a coordinated multistep process including mobilization, adhesion, transmigration, migration/chemotaxis, tissue invasion and in situ differentiation. The factors which are proposed to regulate the distinct steps are indicated

active tumor angiogenesis. The factors that attract circulating EPC to the ischemic and to tumor tissues may be similar to those regulating hematopoietic stem cell engraftment to the bone marrow. Indeed, SDF-1 has been shown to stimulate not only stem cell engraftment but also the recruitment of progenitor cells to the ischemic tissue (Lapidot 2001; Wright et al. 2002; Yamaguchi et al. 2003). The expression of SDF-1 is upregulated during ischemia (Abbott et al. 2004; Ceradini et al. 2004; de Falco et al. 2004). Moreover, Abbott and colleagues could demonstrate that inhibition of the SDF-1/CXCR4 axis partially blocks the homing of progenitor/stem cells to the ischemic myocardium (Abbott et al. 2004). In line with these data, inhibition of CXCR4 by neutralizing anti-CXCR4 antibodies significantly reduced SDF-1-induced adhesion of EPC to mature endothelial cell monolayers, the migration of EPC in vitro (Ceradini et al. 2004), and the in vivo homing of EPC to the ischemic limb in the model of hind limb

ischemia (Walter et al. 2005). Moreover, overexpression of SDF-1 enhanced stem cell homing and incorporation into ischemic tissues (Askari et al. 2003; Yamaguchi et al. 2003).

Recent evidence additionally suggests that other chemokines may also be involved in the homing of EPC to sites of ischemia and active angiogenesis. CXC-chemokines IL-8/Gro-α and their cellular receptors CXCR2 and CXCR1 seem to be involved in the homing of intravenous infused $CD34^+$ progenitor cells to the ischemic myocardium (Kocher et al. 2006). IL-8 is an inflammatory chemokine which is able to stimulate angiogenesis (Muller 2002; Strieter et al. 2005). Myocardial infarction leads to an increase of the expression of IL-8/Gro-α mRNA in the heart and of the serum concentration of IL-8/Gro-α (Kocher et al. 2006). $CD34^+/CD117^{bright}$ progenitor cells demonstrated a chemotactic response to IL-8 (ligand for CXCR1 and CXCR2) in vitro (Kocher et al. 2006). Moreover, local injection of IL-8 in the non-ischemic myocardium was able to increase the recruitment of $CD34^+$ cells (Kocher et al. 2006). Neutralizing anti-IL-8/Gro-α antibodies or neutralizing antibodies against CXCR1 or CXCR2 (receptors for IL-8) partially blocked the recruitment of EPC to the ischemic myocardium and the EPC-induced neovascularization, establishing a role for CXC chemokines (IL-8/Gro-α) in the homing and neovascularization capacity of EPC. Furthermore, ischemia-induced VEGF acts as a chemoattractant to EPC (Kalka et al. 2000a; Lee et al. 2000; Shintani et al. 2001). Interestingly, VEGF is sufficient to induce the organ recruitment of bone marrow-derived circulating myeloid cells and their perivascular localization via induction of SDF-1 expression by perivascular myofibroblasts, suggesting that different cytokines may cooperate during homing of bone marrow cells (Grunewald et al. 2006). In addition, immune competent cells that have invaded the ischemic tissue may release further chemokines, such as MCP-1 or interleukins, that can attract circulating progenitor cells (Fujiyama et al. 2003). Beside stimulating migration, MCP-1 and VEGF are capable of inducing the transendothelial migration of human EPC derived from peripheral blood in a β2-integrin-dependent manner in vitro (Chavakis et al. 2005). In accordance with these data, Spring and colleagues demonstrated recently the expression of the chemokine receptors CCR2 and CCR5 in EPC and the expression of C-C chemokines in tumor vessels (Spring et al. 2005). In the same study, the inhibition of chemokine receptor signaling by PTX reduced significantly the homing of EPC and the incorporation of EPC into tumor vessels (Spring et al. 2005), supporting the involvement of G-protein coupled chemokine receptors in the homing of EPC to sites of tumor angiogenesis. In conclusion, these data suggest that chemokines – IL-8, SDF-1 and probably others – are involved in the trafficking of EPC from the bloodstream to ischemic tissues and to sites of tumor neovascularization. Beside classical chemokines, other factors that could be present in the ischemic myocardium may also influence the recruitment of EPC. For instance, high-mobility group box-1 (HMGB-1) is a nuclear protein which is released extracellularly upon activation of cells by inflammatory cytokines and during cell necrosis and acts as a chemoattractant for inflammatory cells and stem cells in vitro and in vivo (Chavakis et al. 2003; Yamaguchi et al. 2003; Lotze and Tracey 2005; Palumbo et al. 2004). Since necrosis and inflammation are hallmarks of ischemic and tumor tissues, it is conceivable that HMGB-1 may be involved in the homing of EPC. However, while the role of chemokines as chemoattractants during migration of EPC is well established, less is known about the role of chemokines/chemokine receptors for other steps of EPC homing such as adhesion and transendothelial migration.

17.2.1.2 Role of Adhesion Molecules in EPC Homing

Role of Selectins in Rolling

In the paradigm of leukocyte homing to sites of inflammation the initial step involves the rolling of the leukocytes on the endothelium (Muller 2002). Circulating leukocytes must come into brief low-affinity contacts (rolling) with the endothelial cell monolayer in order to get activated by cytokines and to arrest in the subsequent step on endothelial cells. In circulating leukocytes, rolling on the

endothelial cell monolayer is predominantly mediated by selectins and selectin ligands (Luster et al. 2005; McEver 2002; Muller 2002). The selectin family of adhesion molecules consists of three related molecules. L-selectin is constitutively expressed in most leukocytes (McEver 2002). The expression of E-selectin is restricted to endothelial cells activated by inflammatory cytokines, while P-selectin expression is restricted to both endothelial cells and platelets. P-selectin expression on the surface of platelets and endothelial cells can rapidly be induced by exocytosis. Moreover, E-selectin expression in endothelial cells can be induced by inflammatory cytokines. Selectins bind sialyl-Lewis-X-like carbohydrate ligands presented by sialomucin-like surface molecules such as P-selectin-glycoprotein ligand-1 (PSGL-1) (Luster et al. 2005). The selectin-mediated binding to the selectin counterligands is transient, allowing the rolling of the leukocytes. Beside selectins, α4β1-integrin also was reported to mediate rolling on VCAM-1 (Berlin et al. 1995; Henderson et al. 2001).

Interestingly, using intravital microscopy in a model of tumor angiogenesis no rolling of embryonic EPC was observed (Vajkoczy et al. 2003). In the same study, however, in vivo blocking experiments provided evidence that E- and P-selectin and PSGL-1 are involved in the initial cell arrest of embryonic EPC (Vajkoczy et al. 2003). Additionally, in another study also using intravital microscopy, it was reported that murine bone marrow-derived progenitor cells (Sca-1$^+$/Lin$^-$cells) display rolling on the endothelium of the tumor vasculature before firm adhesion (Jin et al. 2006b). Moreover, human hematopoietic progenitor CD34+ cells show efficient rolling on P-selectin, E-selectin, and the CD44 ligand hyaluronic acid under physiological shear flow in vitro (Peled et al. 1999). In addition, L-selectin is expressed by EPC, and L-selectin interactions with the respective ligands seem to support homing of EPC to endothelioma tumors in SCID mice (Biancone et al. 2004).

Role of Integrins in Adhesion, Transendothelial Migration and Migration of EPC

The process of rolling is reversible. Many leukocytes that roll in vivo will not stop, but dissociate from the vessel surface and reenter the bloodstream. To stop rolling, the low-affinity rolling interactions must be replaced by high-affinity adhesion. Thus, the subsequent step of leukocyte homing consists of integrin activation by chemokines and the integrin-dependent arrest/firm adhesion of the leukocytes on the endothelium. Integrins are glycosylated heterodimeric proteins expressed on the cell surface that mediate the adhesion of cells to extracellular matrix proteins (cell–matrix adhesion) and to other cells (cell–cell adhesion) (Hynes 2002). Integrins consist of non-covalent bound α- and β-subunits (Hynes 2002). For the homing of leukocytes to sites of inflammation, cell–cell interactions of the leukocytes with the endothelium of the vessels are mandatory. Leukocytes possess a unique family of integrins, the β2-integrins (CD11a/CD18, CD11b/CD18, CD11c/CD18). In addition, many leukocytes express the β1-integrin α4β1 (very late antigen-4, VLA-4, CD49d/CD29) and the integrin α4β7. The β2-integrins, α4β1-integrin and α4β7-integrin are all able to mediate cell–cell interactions to counterligands expressed on the surface of endothelial cells. CD11a/CD18 (LFA-1, αLβ2) is able to bind to ICAM-1, -2 and -3 and JAM-A expressed by endothelial cells. In contrast, CD11b/CD18 (Mac-1, αMβ2) is a multiligand integrin receptor which binds to ICAM-1, RAGE and JAM-C on endothelial cells and to extracellular proteins such as fibrinogen, coagulation factor X and iC3b (Carlos and Harlan 1994; Chavakis et al. 2003; Plow et al. 2000; Santoso et al. 2002; Springer 1994). The α4β1-integrin (VLA-4, CD49d/CD29) binds to VCAM-1 expressed by endothelial cells activated with cytokines (Carlos and Harlan 1994; Plow et al. 2000; Springer 1994). In order to extravasate from the bloodstream at sites of infection, inflammation, tissue damage and at lymphoid organs, various rolling leukocytes captured from the blood flow must rapidly establish adhesive interactions with the endothelial cells, which are resistant to detachment by disruptive shear forces. Leukocytes arrest on endo-

thelial integrin ligands after variable periods of selectin-mediated rolling, only after proper activation of the integrins (Kinashi 2005; Laudanna and Alon 2006). There are two mechanisms regulating the adhesive activity of integrins. One pathway involves the regulation of integrin affinity for the respective ligands mediated by conformational changes of the integrin subunits (Carman and Springer 2003; Dustin et al. 2004; Kinashi 2005). Interestingly, integrins seem to exist in low-, intermediate- and high-affinity conformations (Shimaoka et al. 2003). The other pathway involves the regulation of integrin valency mediated by changes of integrin distribution on the cell surface (lateral motility) (Bazzoni and Hemler 1998; Kinashi 2005). Both mechanisms seem to be involved concomitantly in the regulation of leukocyte arrest (adhesion) on the endothelium (Kinashi 2005; Laudanna and Alon 2006). Recent evidence suggests that rapid triggering of integrin-dependent adhesion of leukocytes is preferentially transduced by G-protein-coupled receptors occupied by immobilized, endothelial-presented chemokines, rather than by their soluble blood-circulating counterparts (Grabovsky et al. 2000; Peled et al. 1999; Shamri et al. 2005). Chemokines are presented by the activated endothelial cells to the rolling leukocytes and lead by intracellular signaling (inside–outside signaling) to an increase of integrin activity of leukocytes (Kinashi 2005). When activated, the leukocyte integrins bind to their respective ligands expressed by the endothelium and induce the arrest of the rolling leukocytes (Kinashi 2005; Laudanna and Alon 2006). The chemokine-induced signaling leading to integrin activation and subsequent arrest of rolling leukocytes seems to happen in an immediate rather than in a stepwise successive manner (Shamri et al. 2005). Regarding hematopoietic progenitor $CD34^+$ cells it was demonstrated that SDF-1 expressed on vascular endothelium is crucial for translation of rolling adhesion of $CD34^+$ progenitor cells into firm adhesion by increasing the adhesivity of the integrins $\alpha4\beta1$ and LFA-1 to their respective endothelial ligands, VCAM-1 and ICAM-1 (Peled et al. 1999).

Recent evidence supports the involvement of integrins in the homing of EPC and progenitor cells to sites of active neovascularization. Human adult peripheral blood-derived EPC, murine adult bone marrow-derived EPC ($VEGFR2^+/Lin^-$ cells) and bone marrow-derived hematopoietic progenitor/stem cells ($Sca\text{-}1^+/Lin^-$ cells) express the leukocyte $\beta2$-integrins (Chavakis et al. 2005). The expression of $\beta2$-integrins in bone marrow-derived hematopoietic progenitor/stem cells ($Sca\text{-}1^+/Lin^-$ cells) was also reported by other groups (Becker et al. 1999; Jin et al. 2006b; Orschell-Traycoff et al. 2000). In vitro adhesion studies revealed that $\beta2$-integrins mediate the adhesion of human EPC (derived from peripheral blood) to mature endothelial cell monolayers, while the $\alpha4\beta1$-integrin was not involved in this process (Chavakis et al. 2005). Interestingly, murine bone marrow Lin^-progenitor cells adhere to mature endothelial cell monolayers via both the $\beta2$-integrins and $\alpha4\beta1$-integrin (unpublished data), suggesting that progenitor cells of different origins engage different mechanisms for adhesion to mature endothelial cells. In addition, $\beta2$-integrins play an essential role for the homing of murine bone marrow $Sca\text{-}1^+/Lin^-$ hematopoietic progenitor cells and murine $VEGF\text{-}R2^+/Lin^-$ bone marrow EPC to ischemic tissues and for the neovascularization capacity of these cells in vivo (Chavakis et al. 2003; Yamaguchi et al. 2003; Chavakis et al. 2005). Since the $\beta2$-integrins led only to a partial inhibition of the homing to sites of ischemia and of the neovasculatory capacity of progenitor cells, it is conceivable that other integrins may also be involved in these processes. Interestingly, in a recent study the blockade of $\alpha4\beta1$-integrin did not inhibit homing of bone marrow-derived EPC to ischemic tissues but increased mobilization of progenitor cells from the bone marrow and enhanced the progenitor cell-mediated neovascularization in the context of ischemia (Qin et al. 2006). Nevertheless, in a tumor model the inhibition of $\alpha4\beta1$-integrin significantly blocked adhesion and homing of bone marrow progenitor cells to sites of active tumor neovascularization as assessed by intravital microscopy (Jin et al. 2006b). A conceivable explanation for this discrepancy is that different integrins may play distinct context-specific roles (ischemic vs tumor neovascularization) for homing of progenitor cells.

After establishing adhesive contracts to the endothelium the leukocytes move in a β2-integrin-dependent manner from the site of firm adhesion to the nearest junctions (Schenkel et al. 2004). Then leukocytes pass across them in a process called transendothelial migration or diapedesis. During diapedesis the leukocytes squeeze (in ameboid fashion) between the tightly apposed endothelial cells. Two routes of leukocyte diapedesis have been found so far: a paracellular route that dominates most extravasation and a transcellular route (Carman and Springer 2004; Muller 2002, 2003). Interaction of integrins with their respective ligands expressed by endothelial cells like ICAM-1 and VCAM-1 seems to be involved in the process of leukocyte transmigration (Carman and Springer 2004; Oppenheimer-Marks et al. 1991). Moreover, junctional adhesion molecules (JAM) localized at the cell junctions are counter-receptors for integrins. Indeed, it has been demonstrated that JAM-A is a ligand of CD11a/CD18, JAM-B a ligand of α4β1-integrin and JAM-C a ligand of CD11b/CD18 (Cunningham et al. 2002; Ostermann et al. 2002; Santoso et al. 2002). There is evidence that the interaction of integrins with the respective junctional ligands is involved in diapedesis (Chavakis et al. 2004; Keiper et al. 2005; Ostermann et al. 2002). Moreover, homophilic interactions of leukocyte PECAM-1 with endothelial PECAM-1 and of leukocyte CD99 with endothelial CD99 play an essential role in the paracellular diapedesis of leukocytes (Muller 2003; Schenkel et al. 2002).

Less is known about the transendothelial migration of EPC. In vivo studies demonstrated that embryonic EPC and murine bone marrow progenitor cells are able to extravasate (Jin et al. 2006b; Vajkoczy et al. 2003). However, it is not clear whether EPC can follow a paracellular and a transcellular route during diapedesis as has been reported for leukocytes. In vitro studies revealed that the β2-integrins play an essential role for the chemokine-induced transendothelial migration of human adult peripheral blood-derived EPC, while α4β1-integrin is not involved in this process (Chavakis et al. 2005). However, the role of PECAM-1 and CD99 in the diapedesis of EPC has not been elucidated so far.

After transmigration the subsequent step of leukocyte homing is the migration through the basal lamina and through the interstitial extracellular matrix. These processes require cell–matrix interactions. It was shown that the laminin-binding integrin α6β1 is important for the migration of leukocytes through the basal lamina (Dangerfield et al. 2002, 2005). Moreover, it was demonstrated that both β1- and β2-integrins mediate the in vivo migration of leukocytes (Werr et al. 1998). Specifically, the β2-integrin CD11b/CD18 binds to fibrinogen, which is a component of extracellular matrix in the context of inflammation and mediates migration (Forsyth et al. 2001). The role of integrins in the in vivo migration of EPC has not been established so far. However, β1-integrins appear to mediate the chemokine-induced migration of adult human peripheral blood-derived EPC on the matrix protein fibronectin, while the β2-integrins mediate the EPC migration to fibrinogen (unpublished data).

17.2.1.3 Role of Proteases in EPC Homing and Differentiation

Proteases are well established to be involved in angiogenesis, in particular in migration of endothelial cells. Growing evidence suggests that pericellular proteases also play an important role in vasculogenesis (Fig. 17.2). Thus, the mobilization, recruitment and invasion of stem and progenitor cells during vasculogenesis also involves proteolytic activity. Proteases comprise a large family of matrix metalloproteinases (MMPs), the related "a disintegrin and metalloprotease domain" proteins (ADAMs) and the catalytic classes of serine, aspartic and cysteine proteases exhibiting endo- or exopeptidase activities or a combination of both. Extracellular proteases may affect neovascularization by degradation of the extracellular matrix and cell surface receptors and activation, liberation and modification of angiogenic growth factors. Intracellularly, proteases mediate protein maturation and processing (van Hinsbergh et al. 2006). One important intracellular target is the angiogenesis-regulating transcription factor HIF-1α (Salceda and Caro 1997).

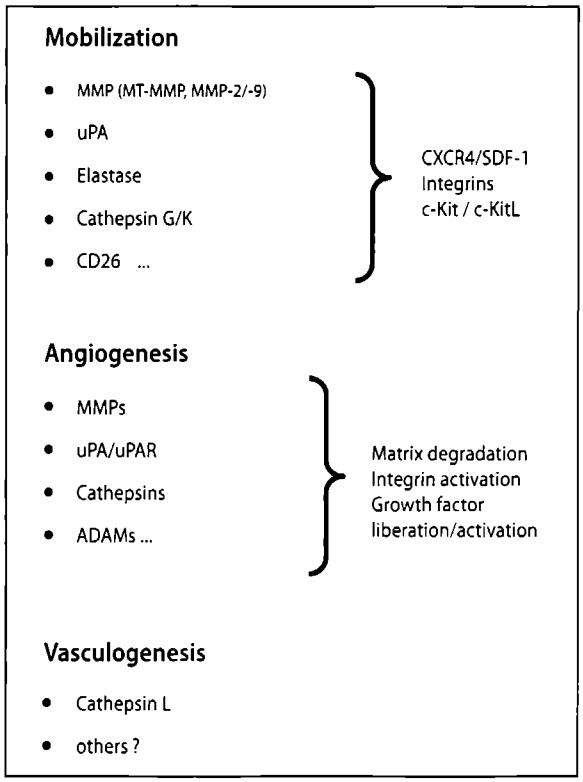


Fig. 17.2. Summary of proteases involved in angiogenesis and vasculogenesis

In the following section, we will introduce the protease families MMPs, ADAMs, cysteine and serine proteases and describe their contribution to EPC homing and differentiation.

Metalloproteinases

MMPs are zinc-dependent endopeptidases that are either soluble or membrane-type (MT) MMPs. Soluble MMPs are expressed as inactive pro-enzymes that become activated within the extracellular environment. MMPs cleave extracellular matrix components, other MMPs and growth factors. Based on their substrate specificity they are involved in cell migration and invasion and in remodeling processes such as angiogenesis, arterial remodeling and wound healing. The endogenous inhibitors of MMPs are tissue inhibitors of metalloproteinases (TIMPs). The soluble MMPs comprise collagenases (MMP-1, MMP-8, and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10, and MMP-11), matrilysins (MMP-7 and MMP-25), and others (MMP-12 and MMP-26). The MT-MMPs consist of six family members (MT1- to MT6-MMP) that are activated intracellularly by furin-like enzymes. The MT-MMP substrates include extracellular matrix proteins, such as collagen, laminin, fibronectin, vitronectin and fibrin, and growth factors and cytokines.

ADAMs (a disintegrin and metalloproteinase domain) and ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs) also belong to the family of metalloproteinases. ADAMs are membrane-spanning proteins and ADAMTSs are secreted.

MMPs as well as ADAMs are well established to contribute to angiogenesis (for review see van Hinsbergh et al. 2006); however, for vasculogenesis only a few studies have assessed the role of MMPs. As shown by Rafii's group, the mobilization of stem- and progenitor cells requires MMP-9 mediated release of kit-ligand (Heissig et al. 2002). Thus, bone marrow (BM) ablation induces the upregulation of MMP-9 within the BM microenvironment. MMP-9 releases soluble Kit ligand (sKitL), which augments mobility of EPC and hematopoietic stem cells (HSC) and mobilization to the peripheral circulation (Heissig et al. 2002). Moreover, circulating VEGF induced MMP-9 expression in the BM, leading to the release of sKitL. In accordance, the reduction of MMP-9 activity may underlie the defective mobilization described in $eNOS^{-/-}$ mice (Aicher et al. 2003), suggesting a specific role of MMP-9 in mobilization.

MMP-2 and other MMPs can also cleave and inactivate SDF-1 and its receptor CXCR4 (McQuibban et al. 2001), a chemokine which potently recruits stem and progenitor cells. Moreover, VEGF is a target of MMP-3 and MMP-9 (Lee et al. 2005), suggesting that protease activity can interfere with the bioavailability of cytokines and chemokines involved in vasculogenesis. Using positron emission tomography for in vivo real-time investigation of trafficking of EPC revealed that recruitment of EPC to the tumor tis-

sue was only slightly reduced by treatment with the MMP inhibitor MMI270 (Tamura et al. 2004). Moreover, MMP inhibitors did not affect EPC invasion in vitro (Urbich et al. 2005b), leading to the speculation that MMPs may be specifically involved in EPC mobilization rather than homing to the target tissue. This is supported by the finding that homing of MMP-9-deficient bone marrow mononuclear cells into ischemic hind limbs is not impaired (Urbich et al. 2005b). However, it is also conceivable that different subsets of progenitor cells may use different sets of proteases for either mobilization and/or homing. Thus, recruited myeloid VEGF-R1$^+$ cells release MMP-9, which may in turn facilitate the homing of other progenitor cells (Grunewald et al. 2006). However, further studies are required to analyze role of proteases in the complex scenario of EPC homing.

Cathepsins

In 1948 cathepsin C, also known as dipeptidyl peptidase I or DPPI, was found as the first pure enzyme of the lysosomal cysteine protease family. Around 30 years later cathepsins B, H, and L were identified. Meanwhile, more than 10 human cathepsins are known. Lysosomal cysteine proteases are active in slightly acidic conditions. Most of the cathepsins are endopeptidases, composed of disulfide-connected heavy and light chains. Similar to MMPs, most of the cathepsins are expressed as inactive pro-enzymes, which are activated by removal of amino acids from the N-terminus. The processing is mediated either by activation through other proteases (e.g., pepsin or cathepsin D), or by autocatalytic activation at acidic pH. The activity of cathepsins is further regulated by endogenous protein inhibitors, cystatins.

Several in vitro studies as well as knockout studies revealed an important role of cathepsins not only in protein degradation as expected, but also in specialized cellular processes such as invasion and proliferation. Cathepsin K is crucial for normal bone remodeling, whereas cathepsins S and L are involved in MHC class II processing. In addition, cathepsin S knockout mice showed impaired microvessel growth (Shi et al. 2003). Interestingly, cathepsins B, K, and L are also localized and active extracellularly (Graf et al. 1981; Mason et al. 1987; Tepel et al. 2000). Yet, there is growing evidence for specific and individual intra- and extracellular functions for these lysosomal enzymes, especially leading to tumor invasion and metastasis. In detail, cathepsin D promotes tumor progression by modulating proliferation and angiogenesis (Berchem et al. 2002), and cathepsin L anti-sense oligonucleotides inhibit invasion of osteosarcoma cells (Krueger et al. 2001). For their extracellular actions, lysosomal peptidases are secreted in considerable amounts. For example, early investigations on the cysteine peptidase cathepsin L revealed that this protease is identical to the "major excreted protein" of malignantly transformed mouse fibroblasts (Mason et al. 1987). Specifically, tumor-released cathepsin L has been shown to produce the angiogenesis inhibitor endostatin from collagen XVIII that is the core protein of heparan sulfate proteoglycans in vascular and epithelial basement membranes (Felbor et al. 2000). Moreover, cathepsin L exerts specific physiological functions, including the regulation of epidermal homeostasis, hair follicle cycling, cardiac function, and MHC class II-mediated antigen presentation (Honey et al. 2002; Nakagawa et al. 1998; Roth et al. 2000; Stypmann et al. 2002; Tobin et al. 2002). With respect to vasculogenesis, we have recently shown that cathepsin L is specifically required for EPC-induced neovascularization. Thus, EPC showed a high expression and activity of cathepsin L. The improvement of neovascularization after hind limb ischemia was significantly impaired in cathepsin L$^{-/-}$ mice, and infused cathepsin L$^{-/-}$ progenitor cells failed to home to sites of ischemia and to augment neovascularization. This seems to be specific for cathepsin L, since cathepsin D$^{-/-}$ or MMP-9$^{-/-}$ progenitor cells did not show an impaired phenotype. Mechanistically, cathepsin L was necessary for EPC invasion and proteolytic matrix-degrading activity of EPC (Urbich et al. 2005b). Moreover, our study revealed that ischemia-induced cathepsin L activity depends on irradiation-sensitive cells (most probably bone marrow-derived cells), whereas ischemia-induced MMP-9 activity was independent on bone marrow-derived cells (most probably tissue-residing cells) (Urbich et al. 2005b).

Serine Proteases and Others

Serine proteases are endopeptidases that are mainly synthesized as zymogens and are activated by N-terminal cleavage. The serine protease urokinase-type plasminogen activator (u-PA) proteolytically activates plasminogen into plasmin. Tissue-type plasminogen activator (t-PA) cleaves plasminogen in the presence of fibrin during fibrinolysis of the blood. Both u-PA and t-PA are inhibited by plasminogen activator inhibitors (PAIs). u-PA and its receptor u-PAR are well established to be involved in cell migration and invasion (for review see Andreasen et al. 1997; Pepper 2001). A recent study have shown that EPC-derived endothelial cells (EPDC) displayed high levels of u-PA and u-PAR (Basire et al. 2006). Moreover, inhibition of u-PA by blocking antibodies reduced proliferation, migration and tube-forming activity of EPDC, suggesting that the u-PA-/u-PAR-dependent proteolysis contributes not only to angiogenesis but also to vasculogenesis.

Beside the u-PA system, other proteases may be involved in vasculogenesis. Thus, the chemoattractant SDF-1 is processed by the aminopeptidase DPPIV/CD26 and carboxypeptidase N (Davis et al. 2005; de la Luz Sierra et al. 2004), thus regulating its biological activity.

Taken together, proteases (especially MMPs, cysteine and serine proteases) have attracted increasing interest during the past few years with regard to vasculogenesis. They can modulate EPC homing in many different ways. In this context it will also be important to identify specific targets of proteases.

17.2.2 Differentiation and Release of Growth Factors

Although the role of EPC in the improvement of neovascularization is under intensive investigation, the mechanisms of action are still not clear. Moreover, the physical contribution of EPC to tumor vessels is variable. Two main mechanisms may contribute to the functional activity of EPC, namely incorporation and differentiation to endothelial cells and the release of paracrine factors. Several studies have clearly demonstrated that EPC incorporate and differentiate to endothelial cells in various animal models of ischemia, injury or tumor growth. In addition, EPC also release a variety of growth factors such VEGF, SDF-1, IGF-1, and HGF, which in turn may stimulate angiogenesis and the recruitment and survival of other progenitor cells, such as cardiac-resident progenitor cells (Urbich et al. 2005a). However, the relative contribution of incorporation versus paracrine effects is not known and is difficult to assess in vivo.

The genetic cascades regulating maturation of EPC to functional endothelial cells in the adult system are largely unknown. During embryonic development, VEGF and its receptors play a crucial role for stimulating endothelial differentiation of the hemangioblasts (Ferrara et al. 1996; Fong et al. 1995; Shalaby et al. 1995). Likewise, VEGF induces ex vivo endothelial differentiation of a variety of adult progenitor populations such as $CD34^+$, $CD133^+$, and peripheral blood mononuclear cells (Asahara et al. 1997; Dimmeler et al. 2001; Gehling et al. 2000; Kalka et al. 2000b). During embryonic development, the orphan homeobox gene Hex (also termed Prh) is required for differentiation of the hemangioblast into the definitive hematopoietic progenitors and also for endothelial differentiation (Guo et al. 2003). Beside embryonic development, homeobox transcription factors seem to be of importance for the adult organism. Thus, the homeobox factor HoxA9 acts as a master switch to regulate expression of prototypical endothelial-committed genes such as eNOS, VEGF-R2, and VE-cadherin and mediates shear stress-induced maturation of endothelial cells. Consistently, HoxA9-deficient mice exhibit lower numbers of EPC and showed an impaired postnatal neovascularization capacity after induction of ischemia (Rossig et al. 2005). Additionally, the serine/threonine kinase Pim-1 was recently discovered as a VEGF-responsive gene that contributes to endothelial differentiation from embryonic stem cells (Zippo et al. 2004). However, further studies are necessary to elucidate the coordinated interplay of transcription factors and other signaling molecules during EPC differentiation.

Conclusion/Open Questions

In the past few years the insights gained into the role of EPC activation by chemokines, the role of integrins in EPC adhesion, transendothelial migration and matrix migration, and the role of proteases in matrix migration/invasion have raised new questions in the field of EPC trafficking to sites of active neovascularization: Are the mechanisms of homing tissue-specific (e.g., ischemic heart vs ischemic limb) and/or context-specific (ischemia- vs tumor-induced neovascularization)? Moreover, since distinct subpopulations of progenitor cells are able to support neovascularization, a relevant question is whether these subpopulations are engaging the same molecular mechanisms for homing to sites of ischemia and tumor neovascularization. Finally, less is known about the intracellular signaling mediating the chemokine-induced activation of effector molecules in EPC (such as integrins, proteases), which are essential for the homing. Transgenic animal models, which enable us to perform either gain- or loss-of-function studies in tissue-specific fashion or in a conditional manner, will help to clarify the signaling cascades that are essential for the recruitment of EPC.

In conclusion, the recent evidence suggests that the EPC homing to sites of active neovascularization is a complex process dependent on a timely and spatially orchestrated interplay between chemokines, chemokine receptors, intracellular signaling, adhesion molecules (selectins and integrins) and proteases. The elucidation of the molecular mechanisms of homing of the different progenitor cell subpopulations to sites of neovascularization is essential for the development of new specific therapeutic strategies, in order to improve the efficacy of cell-based therapies in patients with ischemic disorders and to inhibit EPC-mediated neovascularization in patients with tumors.

References

Abbott JD, Huang Y, Liu D, Hickey R, Krause DS, Giordano FJ (2004) Stromal cell-derived factor-1alpha plays a critical role in stem cell recruitment to the heart after myocardial infarction but is not sufficient to induce homing in the absence of injury. Circulation 110:3300–3305

Aicher A, Heeschen C, Mildner-Rihm C, Urbich C, Ihling C, Technau-Ihling K, Zeiher AM, Dimmeler S (2003) Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. Nat Med 9:1370–1376

Andreasen PA, Kjoller L, Christensen L, Duffy MJ (1997) The urokinase-type plasminogen activator system in cancer metastasis: a review. Int J Cancer 72:1–22

Annabi B, Naud E, Lee YT, Eliopoulos N, Galipeau J (2004) Vascular progenitors derived from murine bone marrow stromal cells are regulated by fibroblast growth factor and are avidly recruited by vascularizing tumors. J Cell Biochem 91:1146–1158

Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM (1997) Isolation of putative progenitor endothelial cells for angiogenesis. Science 275:964–967

Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, Kearne M, Magner M, Isner JM (1999) Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 85:221–228

Askari AT, Unzek S, Popovic ZB, Goldman CK, Forudi F, Kiedrowski M, Rovner A, Ellis SG, Thomas JD, DiCorleto PE, Topol EJ, Penn MS (2003) Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. Lancet 362:697–703

Basire A, Sabatier F, Ravet S, Lamy E, Mialhe A, Zabouo G, Paul P, Gurewich V, Sampol J, Dignat-George F (2006) High urokinase expression contributes to the angiogenic properties of endothelial cells derived from circulating progenitors. Thromb Haemost 95:678–688

Bazzoni G, Hemler ME (1998) Are changes in integrin affinity and conformation overemphasized? Trends Biochem Sci 23:30–34

Becker PS, Nilsson SK, Li Z, Berrios VM, Dooner MS, Cooper CL, Hsieh CC, Quesenberry PJ (1999) Adhesion receptor expression by hematopoietic cell lines and murine progenitors: modulation by cytokines and cell cycle status. Exp Hematol 27:533–541

Berchem G, Glondu M, Gleizes M, Brouillet JP, Vignon F, Garcia M, Liaudet-Coopman E (2002) Cathepsin-D affects multiple tumor progression steps in vivo: proliferation, angiogenesis and apoptosis. Oncogene 21:5951–5955

Berlin C, Bargatze RF, Campbell JJ, von Andrian UH, Szabo MC, Hasslen SR, Nelson RD, Berg EL, Erlandsen SL, Butcher EC (1995) alpha 4 integrins mediate lymphocyte attachment and rolling under physiologic flow. Cell 80:413–422

Biancone L, Cantaluppi V, Duo D, Deregibus MC, Torre C, Camussi G (2004) Role of L-selectin in the vascular homing of peripheral blood-derived endothelial progenitor cells. J Immunol 173:5268–5274

Carlos TM, Harlan JM (1994) Leukocyte-endothelial adhesion molecules. Blood 84:2068–2101

Carman CV, Springer TA (2003) Integrin avidity regulation: are changes in affinity and conformation underemphasized? Curr Opin Cell Biol 15:547–556

Carman CV, Springer TA (2004) A transmigratory cup in leukocyte diapedesis both through individual vascular endothelial cells and between them. J Cell Biol 167:377–388

Carmeliet P (2005) Angiogenesis in life, disease and medicine. Nature 438:932–936

Ceradini DJ, Kulkarni AR, Callaghan MJ, Tepper OM, Bastidas N, Kleinman ME, Capla JM, Galiano RD, Levine JP, Gurtner GC (2004) Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. Nat Med 10:858–864. Epub Jul 2004

Chavakis E, Aicher A, Heeschen C, Sasaki K, Kaiser R, El Makhfi N, Urbich C, Peters T, Scharffetter-Kochanek K, Zeiher AM, Chavakis T, Dimmeler S (2005) Role of beta2-integrins for homing and neovascularization capacity of endothelial progenitor cells. J Exp Med 201:63–72

Chavakis T, Bierhaus A, Al-Fakhri N, Schneider D, Witte S, Linn T, Nagashima M, Morser J, Arnold B, Preissner KT, Nawroth PP (2003) The pattern recognition receptor (RAGE) is a counterreceptor for leukocyte integrins: a novel pathway for inflammatory cell recruitment. J Exp Med 198:1507–1515

Chavakis T, Keiper T, Matz-Westphal R, Hersemeyer K, Sachs UJ, Nawroth PP, Preissner KT, Santoso S (2004) The junctional adhesion molecule-C promotes neutrophil transendothelial migration in vitro and in vivo. J Biol Chem 279:55602–55608

Cunningham SA, Rodriguez JM, Arrate MP, Tran TM, Brock TA (2002) JAM2 interacts with alpha4beta1. Facilitation by JAM3. J Biol Chem 277:27589–27592

Dangerfield J, Larbi KY, Huang MT, Dewar A, Nourshargh S (2002) PECAM-1 (CD31) homophilic interaction up-regulates alpha6beta1 on transmigrated neutrophils in vivo and plays a functional role in the ability of alpha6 integrins to mediate leukocyte migration through the perivascular basement membrane. J Exp Med 196:1201–1211

Dangerfield JP, Wang S, Nourshargh S (2005) Blockade of alpha6 integrin inhibits IL-1beta- but not TNF-alpha-induced neutrophil transmigration in vivo. J Leukoc Biol 77:159–165

Davis DA, Singer KE, De La Luz Sierra M, Narazaki M, Yang F, Fales HM, Yarchoan R, Tosato G (2005) Identification of carboxypeptidase N as an enzyme responsible for C-terminal cleavage of stromal cell-derived factor-1alpha in the circulation. Blood 105:4561–4568

De Falco E, Porcelli D, Torella AR, Straino S, Iachininoto MG, Orlandi A, Truffa S, Biglioli P, Napolitano M, Capogrossi MC, Pesce M (2004) SDF-1 involvement in endothelial phenotype and ischemia-induced recruitment of bone marrow progenitor cells. Blood 104:3472–3482

De la Luz Sierra M, Yang F, Narazaki M, Salvucci O, Davis D, Yarchoan R, Zhang HH, Fales H, Tosato G (2004) Differential processing of stromal-derived factor-1alpha and stromal-derived factor-1beta explains functional diversity. Blood 103:2452–2459

De Palma M, Venneri MA, Naldini L (2003a) In vivo targeting of tumor endothelial cells by systemic delivery of lentiviral vectors. Hum Gene Ther 14:1193–1206

De Palma M, Venneri MA, Roca C, Naldini L (2003b) Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells. Nat Med 9:789–795

Dimmeler S, Aicher A, Vasa M, Mildner-Rihm C, Adler K, Tiemann M, Rutten H, Fichtlscherer S, Martin H, Zeiher AM (2001) HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. J Clin Invest 108:391–397

Dustin ML, Bivona TG, Philips MR (2004) Membranes as messengers in T cell adhesion signaling. Nat Immunol 5:363–372

Felbor U, Dreier L, Bryant RA, Ploegh HL, Olsen BR, Mothes W (2000) Secreted cathepsin L generates endostatin from collagen XVIII. EMBO J 19:1187–1194

Ferrara N, Kerbel RS (2005) Angiogenesis as a therapeutic target. Nature 438:967–974

Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW (1996) Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 380:439–442

Fong GH, Rossant J, Gertsenstein M, Breitman ML (1995) Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature 376:66–70

Forsyth CB, Solovjov DA, Ugarova TP, Plow EF (2001) Integrin alpha(M)beta(2)-mediated cell migration to fibrinogen and its recognition peptides. J Exp Med 193:1123–1133

Fujiyama S, Amano K, Uehira K, Yoshida M, Nishiwaki Y, Nozawa Y, Jin D, Takai S, Miyazaki M, Egashira K, Imada T, Iwasaka T, Matsubara H (2003) Bone marrow monocyte lineage cells adhere on injured endothelium in a monocyte chemoattractant protein-1-dependent manner and accelerate reendothelialization as endothelial progenitor cells. Circ Res 93:980–989. Epub 2003 Oct 2002

Garcia-Barros M, Paris F, Cordon-Cardo C, Lyden D, Rafii S, Haimovitz-Friedman A, Fuks Z, Kolesnick R (2003) Tumor response to radiotherapy regulated by endothelial cell apoptosis. Science 300:1155–1159

Gehling UM, Ergun S, Schumacher U, Wagener C, Pantel K, Otte M, Schuch G, Schafhausen P, Mende T, Kilic N, Kluge K, Schafer B, Hossfeld DK, Fiedler W (2000) In vitro differentiation of endothelial cells from AC133-positive progenitor cells. Blood 95:3106–3112

Grabovsky V, Feigelson S, Chen C, Bleijs DA, Peled A, Cinamon G, Baleux F, Arenzana-Seisdedos F, Lapidot T, van Kooyk Y, Lobb RR, Alon R (2000) Subsecond induction

of alpha4 integrin clustering by immobilized chemokines stimulates leukocyte tethering and rolling on endothelial vascular cell adhesion molecule 1 under flow conditions. J Exp Med 192:495–506

Graf M, Baici A, Strauli P (1981) Histochemical localization of cathepsin B at the invasion front of the rabbit V2 carcinoma. Lab Invest 45:587–596

Grunewald M, Avraham I, Dor Y, Bachar-Lustig E, Itin A, Yung S, Chimenti S, Landsman L, Abramovitch R, Keshet E (2006) VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. Cell 124:175–189

Guo Y, Chan R, Ramsey H, Li W, Xie X, Shelley WC, Martinez-Barbera JP, Bort B, Zaret K, Yoder M, Hromas R (2003) The homeoprotein Hex is required for hemangioblast differentiation. Blood 102:2428–2435. Epub 2003 Jun 2425

Heissig B, Hattori K, Dias S, Friedrich M, Ferris B, Hackett NR, Crystal RG, Besmer P, Lyden D, Moore MA, Werb Z, Rafii S (2002) Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. Cell 109:625–637

Henderson RB, Lim LH, Tessier PA, Gavins FN, Mathies M, Perretti M, Hogg N (2001) The use of lymphocyte function-associated antigen (LFA)-1-deficient mice to determine the role of LFA-1, Mac-1, and alpha4 integrin in the inflammatory response of neutrophils. J Exp Med 194:219–226

Honey K, Nakagawa T, Peters C, Rudensky A (2002) Cathepsin L regulates CD4+ T cell selection independently of its effect on invariant chain: a role in the generation of positively selecting peptide ligands. J Exp Med 195:1349–1358

Hynes RO (2002) Integrins: bidirectional, allosteric signaling machines. Cell 110:673–687

Imhof BA, Aurrand-Lions M (2004) Adhesion mechanisms regulating the migration of monocytes. Nat Rev Immunol 4:432–444

Jin DK, Shido K, Kopp HG, Petit I, Shmelkov SV, Young LM, Hooper AT, Amano H, Avecilla ST, Heissig B, Hattori K, Zhang F, Hicklin DJ, Wu Y, Zhu Z, Dunn A, Salari H, Werb Z, Hackett NR, Crystal RG, Lyden D, Rafii S (2006a) Cytokine-mediated deployment of SDF-1 induces revascularization through recruitment of CXCR4(+) hemangiocytes. Nat Med 12:557–567

Jin H, Aiyer A, Su J, Borgstrom P, Stupack D, Friedlander M, Varner J (2006b) A homing mechanism for bone marrow-derived progenitor cell recruitment to the neovasculature. J Clin Invest 116:652–662

Kalka C, Masuda H, Takahashi T, Gordon R, Tepper O, Gravereaux E, Pieczek A, Iwaguro H, Hayashi SI, Isner JM, Asahara T (2000a) Vascular endothelial growth factor(165) gene transfer augments circulating endothelial progenitor cells in human subjects. Circ Res 86:1198–1202

Kalka C, Masuda H, Takahashi T, Kalka-Moll WM, Silver M, Kearney M, Li T, Isner JM, Asahara T (2000b) Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proc Natl Acad Sci USA 97:3422–3427

Kaplan RN, Riba RD, Zacharoulis S, Bramley AH, Vincent L, Costa C, MacDonald DD, Jin DK, Shido K, Kerns SA, Zhu Z, Hicklin D, Wu Y, Port JL, Altorki N, Port ER, Ruggero D, Shmelkov SV, Jensen KK, Rafii S, Lyden D (2005) VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. Nature 438:820–827

Keiper T, Al-Fakhri N, Chavakis E, Athanasopoulos AN, Isermann B, Herzog S, Saffrich R, Hersemeyer K, Bohle RM, Haendeler J, Preissner KT, Santoso S, Chavakis T (2005) The role of junctional adhesion molecule-C (JAM-C) in oxidized LDL-mediated leukocyte recruitment. FASEB J 19:2078–2080

Kinashi T (2005) Intracellular signalling controlling integrin activation in lymphocytes. Nat Rev Immunol 5:546–559

Kocher AA, Schuster MD, Bonaros N, Lietz K, Xiang G, Martens TP, Kurlanski P, Witkowski P, Boyle A, Homma S, Wang SF, Itescu S (2006) Myocardial homing and neovascularization by human bone marrow angioblasts is regulated by IL-8/ Gro CXC chemokines. J Mol Cell Cardiol 40:455–464

Krueger S, Kellner U, Buehling F and Roessner A (2001) Cathepsin L antisense oligonucleotides in a human osteosarcoma cell line: effects on the invasive phenotype. Cancer Gene Ther 8:522–528

Lapidot T (2001) Mechanism of human stem cell migration and repopulation of NOD/SCID and B2mnull NOD/SCID mice. The role of SDF-1/CXCR4 interactions. Ann NY Acad Sci 938:83–95

Laudanna C, Alon R (2006) Right on the spot. Chemokine triggering of integrin-mediated arrest of rolling leukocytes. Thromb Haemost 95:5–11

Lee S, Jilani SM, Nikolova GV, Carpizo D, Iruela-Arispe ML (2005) Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. J Cell Biol 169:681–691

Lee SH, Wolf PL, Escudero R, Deutsch R, Jamieson SW, Thistlethwaite PA (2000) Early expression of angiogenesis factors in acute myocardial ischemia and infarction. N Engl J Med 342:626–633

Li H, Gerald WL, Benezra R (2004) Utilization of bone marrow-derived endothelial cell precursors in spontaneous prostate tumors varies with tumor grade. Cancer Res 64:6137–6143

Lotze MT, Tracey KJ (2005) High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. Nat Rev Immunol 5:331–342

Luster AD, Alon R, von Andrian UH (2005) Immune cell migration in inflammation: present and future therapeutic targets. Nat Immunol 6:1182–1190

Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L, Chadburn A, Heissig B, Marks W, Witte L, Wu Y, Hicklin

D, Zhu Z, Hackett NR, Crystal RG, Moore MA, Hajjar KA, Manova K, Benezra R, Rafii S (2001) Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat Med 7:1194–1201

Mason RW, Gal S, Gottesman MM (1987) The identification of the major excreted protein (MEP) from a transformed mouse fibroblast cell line as a catalytically active precursor form of cathepsin L. Biochem J 248:449–454

McEver RP (2002) Selectins: lectins that initiate cell adhesion under flow. Curr Opin Cell Biol 14:581–586

McQuibban GA, Butler GS, Gong JH, Bendall L, Power C, Clark-Lewis I, Overall CM (2001) Matrix metalloproteinase activity inactivates the CXC chemokine stromal cell-derived factor-1. J Biol Chem 276:43503–43508

Muller WA (2002) Leukocyte-endothelial cell interactions in the inflammatory response. Lab Invest 82:521–533

Muller WA (2003) Leukocyte-endothelial-cell interactions in leukocyte transmigration and the inflammatory response. Trends Immunol 24:327–334

Nakagawa T, Roth W, Wong P, Nelson A, Farr A, Deussing J, Villadangos JA, Ploegh H, Peters C, Rudensky AY (1998) Cathepsin L: critical role in Ii degradation and CD4 T cell selection in the thymus. Science 280:450–453

Oppenheimer-Marks N, Davis LS, Bogue DT, Ramberg J, Lipsky PE (1991) Differential utilization of ICAM-1 and VCAM-1 during the adhesion and transendothelial migration of human T lymphocytes. J Immunol 147:2913–2921

Orschell-Traycoff CM, Hiatt K, Dagher RN, Rice S, Yoder MC, Srour EF (2000) Homing and engraftment potential of Sca-1(+)lin(-) cells fractionated on the basis of adhesion molecule expression and position in cell cycle. Blood 96:1380–1387

Ostermann G, Weber KS, Zernecke A, Schroder A, Weber C (2002) JAM-1 is a ligand of the beta(2) integrin LFA-1 involved in transendothelial migration of leukocytes. Nat Immunol 3:151–158

Palumbo R, Sampaolesi M, de Marchis F, Tonlorenzi R, Colombetti S, Mondino A, Cossu G, Bianchi ME (2004) Extracellular HMGB1, a signal of tissue damage, induces mesoangioblast migration and proliferation. J Cell Biol 164:441–449

Peled A, Grabovsky V, Habler L, Sandbank J, Arenzana-Seisdedos F, Petit I, Ben-Hur H, Lapidot T, Alon R (1999) The chemokine SDF-1 stimulates integrin-mediated arrest of CD34(+) cells on vascular endothelium under shear flow. J Clin Invest 104:1199–1211

Pepper MS (2001) Role of the matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis. Arterioscler Thromb Vasc Biol 21:1104–1117

Peters BA, Diaz LA, Polyak K, Meszler L, Romans K, Guinan EC, Antin JH, Myerson D, Hamilton SR, Vogelstein B, Kinzler KW, Lengauer C (2005) Contribution of bone marrow-derived endothelial cells to human tumor vasculature. Nat Med 11:261–262

Plow EF, Haas TA, Zhang L, Loftus J, Smith JW (2000) Ligand Binding to Integrins. J Biol Chem 275:21785–21788

Qin G, Ii M, Silver M, Wecker A, Bord E, Ma H, Gavin M, Goukassian DA, Yoon YS, Papayannopoulou T, Asahara T, Kearney M, Thorne T, Curry C, Eaton L, Heyd L, Dinesh D, Kishore R, Zhu Y, Losordo DW (2006) Functional disruption of alpha4 integrin mobilizes bone marrow-derived endothelial progenitors and augments ischemic neovascularization. J Exp Med 203:153–163

Rossig L, Urbich C, Bruhl T, Dernbach E, Heeschen C, Chavakis E, Sasaki K, Aicher D, Diehl F, Seeger F, Potente M, Aicher A, Zanetta L, Dejana E, Zeiher AM, Dimmeler S (2005) Histone deacetylase activity is essential for the expression of HoxA9 and for endothelial commitment of progenitor cells. J Exp Med 201:1825–1835

Roth W, Deussing J, Botchkarev VA, Pauly-Evers M, Saftig P, Hafner A, Schmidt P, Schmahl W, Scherer J, Anton-Lamprecht I, von Figura K, Paus R, Peters C (2000) Cathepsin L deficiency as molecular defect of furless: hyperproliferation of keratinocytes and pertubation of hair follicle cycling. FASEB J 14:2075–2086

Ruzinova MB, Schoer RA, Gerald W, Egan JE, Pandolfi PP, Rafii S, Manova K, Mittal V, Benezra R (2003) Effect of angiogenesis inhibition by Id loss and the contribution of bone-marrow-derived endothelial cells in spontaneous murine tumors. Cancer Cell 4:277–289

Salceda S, Caro J (1997) Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. J Biol Chem 272:22642–22647

Santoso S, Sachs UJ, Kroll H, Linder M, Ruf A, Preissner KT, Chavakis T (2002) The junctional adhesion molecule 3 (JAM-3) on human platelets is a counterreceptor for the leukocyte integrin Mac-1. J Exp Med 196:679–691

Schenkel AR, Mamdouh Z, Chen X, Liebman RM, Muller WA (2002) CD99 plays a major role in the migration of monocytes through endothelial junctions. Nat Immunol 3:143–150

Schenkel AR, Mamdouh Z, Muller WA (2004) Locomotion of monocytes on endothelium is a critical step during extravasation. Nat Immunol 5:393–400

Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 376:62–66

Shamri R, Grabovsky V, Gauguet JM, Feigelson S, Manevich E, Kolanus W, Robinson MK, Staunton DE, von Andrian UH, Alon R (2005) Lymphocyte arrest requires instantaneous induction of an extended LFA-1 conformation mediated by endothelium-bound chemokines. Nat Immunol 6:497–506

Shi GP, Sukhova GK, Kuzuya M, Ye Q, Du J, Zhang Y, Pan JH, Lu ML, Cheng XW, Iguchi A, Perrey S, Lee AM, Chapman HA, Libby P (2003) Deficiency of the cysteine protease cathepsin S impairs microvessel growth. Circ Res 92:493–500

Shi Q, Rafii S, Wu MH, Wijelath ES, Yu C, Ishida A, Fujita Y, Kothari S, Mohle R, Sauvage LR, Moore MA, Storb RF, Hammond WP (1998) Evidence for circulating bone marrow-derived endothelial cells. Blood 92:362–367

Shimaoka M, Xiao T, Liu JH, Yang Y, Dong Y, Jun CD, McCormack A, Zhang R, Joachimiak A, Takagi J, Wang JH, Springer TA (2003) Structures of the alpha L I domain and its complex with ICAM-1 reveal a shape-shifting pathway for integrin regulation. Cell 112:99–111

Shintani S, Murohara T, Ikeda H, Ueno T, Honma T, Katoh A, Sasaki K, Shimada T, Oike Y, Imaizumi T (2001) Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. Circulation 103:2776–2779

Spring H, Schuler T, Arnold B, Hammerling GJ, Ganss R (2005) Chemokines direct endothelial progenitors into tumor neovessels. Proc Natl Acad Sci USA 102:18111–18116

Springer TA (1994) Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 76:301–314

Strieter RM, Burdick MD, Gomperts BN, Belperio JA, Keane MP (2005) CXC chemokines in angiogenesis. Cytokine Growth Factor Rev 16:593–609

Stypmann J, Glaser K, Roth W, Tobin DJ, Petermann I, Matthias R, Monnig G, Haverkamp W, Breithardt G, Schmahl W, Peters C, Reinheckel T (2002) Dilated cardiomyopathy in mice deficient for the lysosomal cysteine peptidase cathepsin L. Proc Natl Acad Sci USA 99:6234–6239

Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, Magner M, Isner JM, Asahara T (1999) Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med 5:434–438

Tamura M, Unno K, Yonezawa S, Hattori K, Nakashima E, Tsukada H, Nakajima M, Oku N (2004) In vivo trafficking of endothelial progenitor cells their possible involvement in the tumor neovascularization. Life Sci 75:575–584

Tepel C, Bromme D, Herzog V, Brix K (2000) Cathepsin K in thyroid epithelial cells: sequence, localization and possible function in extracellular proteolysis of thyroglobulin. J Cell Sci 113:4487–4498

Tobin DJ, Foitzik K, Reinheckel T, Mecklenburg L, Botchkarev VA, Peters C, Paus R (2002) The lysosomal protease cathepsin L is an important regulator of keratinocyte and melanocyte differentiation during hair follicle morphogenesis and cycling. Am J Pathol 160:1807–1821

Urbich C, Dimmeler S (2004) Endothelial progenitor cells: characterization and role in vascular biology. Circ Res 95:343–353

Urbich C, Aicher A, Heeschen C, Dernbach E, Hofmann WK, Zeiher AM, Dimmeler S (2005a) Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac resident progenitor cells. J Mol Cell Cardiol 39:733–742

Urbich C, Heeschen C, Aicher A, Sasaki K, Bruhl T, Farhadi MR, Vajkoczy P, Hofmann WK, Peters C, Pennacchio LA, Abolmaali ND, Chavakis E, Reinheckel T, Zeiher AM, Dimmeler S (2005b) Cathepsin L is required for endothelial progenitor cell-induced neovascularization. Nat Med 11:206–213

Vajkoczy P, Blum S, Lamparter M, Mailhammer R, Erber R, Engelhardt B, Vestweber D, Hatzopoulos AK (2003) Multistep nature of microvascular recruitment of ex vivo-expanded embryonic endothelial progenitor cells during tumor angiogenesis. J Exp Med 197:1755–1765

Van Hinsbergh VW, Engelse MA, Quax PH (2006) Pericellular proteases in angiogenesis and vasculogenesis. Arterioscler Thromb Vasc Biol 26:716–728

Walter DH, Rittig K, Bahlmann FH, Kirchmair R, Silver M, Murayama T, Nishimura H, Losordo DW, Asahara T, Isner JM (2002) Statin therapy accelerates reendothelialization: a novel effect involving mobilization and incorporation of bone marrow-derived endothelial progenitor cells. Circulation 105:3017–3024

Walter DH, Haendeler J, Reinhold J, Rochwalsky U, Seeger F, Honold J, Hoffmann J, Urbich C, Lehmann R, Arenzana-Seisdesdos F, Aicher A, Heeschen C, Fichtlscherer S, Zeiher AM, Dimmeler S (2005) Impaired CXCR4 signaling contributes to the reduced neovascularization capacity of endothelial progenitor cells from patients with coronary artery disease. Circ Res 97:1142–1151

Werner N, Priller J, Laufs U, Endres M, Bohm M, Dirnagl U, Nickenig G (2002) Bone marrow-derived progenitor cells modulate vascular reendothelialization and neointimal formation: effect of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibition. Arterioscler Thromb Vasc Biol 22:1567–1572

Werr J, Xie X, Hedqvist P, Ruoslahti E, Lindbom L (1998) beta1 integrins are critically involved in neutrophil locomotion in extravascular tissue In vivo. J Exp Med 187:2091–2096

Willett CG, Boucher Y, di Tomaso E, Duda DG, Munn LL, Tong RT, Chung DC, Sahani DV, Kalva SP, Kozin SV, Mino M, Cohen KS, Scadden DT, Hartford AC, Fischman AJ, Clark JW, Ryan DP, Zhu AX, Blaszkowsky LS, Chen HX, Shellito PC, Lauwers GY, Jain RK (2004) Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. Nat Med 10:145–147. Epub 2004 Jan 2025

Wright DE, Bowman EP, Wagers AJ, Butcher EC, Weissman IL (2002) Hematopoietic stem cells are uniquely selective in their migratory response to chemokines. J Exp Med 195:1145–1154

Yamaguchi J, Kusano KF, Masuo O, Kawamoto A, Silver M, Murasawa S, Bosch-Marce M, Masuda H, Losordo DW, Isner JM, Asahara T (2003) Stromal cell-derived factor-1 effects on ex vivo expanded endothelial progenitor cell recruitment for ischemic neovascularization. Circulation 107:1322–1328

Zippo A, De Robertis A, Bardelli M, Galvagni F, Oliviero S (2004) Identification of Flk-1-target genes in vasculogenesis: Pim-1 is required for endothelial and mural cell differentiation in vitro. Blood 24:24

Molecular Players in Lymphangiogenesis

18

MARJA LOHELA and KARI ALITALO

Abstract

Lymph vessels are essential for the maintenance of tissue fluid homeostasis and immune surveillance and are involved in the pathogenesis of several human diseases, such as lymphoedema, inflammation and tumour metastasis. The identification of lymphangiogenic growth factors and their receptor, and of transcription factors that control lymphangiogenesis, have provided significant insight into the biology of lymphatic vasculature. Another important advance has been the recent development of mouse, frog and fish models that allow the study of lymphangiogenesis and diseases affecting lymph vessels. In this review we discuss some of the major molecular players involved in regulation of lymphangiogenesis and lymph vessel remodelling, and the role of lymph vessels and their regulators in human disease.

CONTENTS

The main function of the lymphatic system is to transport interstitial fluid, extravasated plasma proteins and cells back into the blood circulation. Lymph vessels also form a part of the immune system together with the lymphoid organs, and are involved in absorption and transport of digested fat from the intestine. Blind-ended lymphatic capillaries in peripheral tissues collect fluid that is passed on to pre-collecting and collecting vessels, going through lymph nodes on its way back to the venous circulation via the final collecting vessel, the thoracic duct. Lymphatic capillaries consist of a single layer of endothelial cells with overlapping junctions, have a discontinuous basement membrane or none at all, and have no surrounding pericytes or smooth muscle cells. These morphological features make lymphatic capillaries highly per-

meable to large macromolecules and easily accessible to extravasated white blood cells. Attached to capillary lymphatic endothelial cells are thin anchoring filaments that link the capillaries to extracellular matrix, preventing their collapse at high interstitial pressure. Collecting lymph vessels have sparse smooth muscle cell coverage, a basement membrane, and irregularly spaced valves. Lymph is propelled forward by contractions of the smooth muscle cells and surrounding skeletal muscle, valves preventing backflow. The structure, function and development of the lymphatic system has been described in more detail in several review articles (Alitalo et al. 2005; Hong et al. 2004b; Jeltsch et al. 2003; Karpanen and Makinen 2006; Oliver and Alitalo 2005; Tammela et al. 2005a; Witte et al. 2001). Lymphoedema, the accumulation of protein-rich fluid in interstitial tissues, occurs in humans either as a hereditary disease or due to lymph vessel damage or removal. Lymph vessels also serve as a major metastatic route for tumour cells, and metastasis to the regional lymph nodes is one of the most important indicators of tumour aggressiveness (reviewed by Achen et al. 2005; Karpanen and Alitalo 2001; Pepper 2001).

Lymph vessels arise from embryonic veins through a process termed lymphangiogenesis. More than a century ago, Florence Sabin proposed that the primary lymph sacs, first observed in humans in 6- to 7-week embryos, arise by endothelial cell budding from pre-existing veins (Sabin 1902). In mice, lymph sacs are seen around embryonic day 10 (E10), and current evidence from mouse experiments supports Sabin's hypothesis. A primitive plexus of capillary-like lymph vessels is first formed, followed by remodelling into hierarchy of a superficial capillary plexus and a system of collecting lymph vessels (Fig. 18.1). However, at least in avian models (Papoutsi et al. 2001; Wilting et al. 2000) and *Xenopus* tadpoles (Ny et al. 2005), mesoderm-derived precursors termed lymphangioblasts may contribute to lymphangiogenesis, and there is some evidence of a small contribution of circulating endothelial progenitors to lymph vessels also in mouse embryos (Sebzda et al. 2006). In zebrafish, which were recently discovered to possess a functional lymphatic circulation, elegant lineage-tracing experiments have shown that lymphatic endothelial cells first forming the thoracic duct have a venous origin, and no mesenchymal precursor cells were found to be involved (Yaniv et al. 2006).

Knowledge about different molecules involved in lymphangiogenic processes in health and disease has been accumulating rapidly during the last few years, and complexity is added to the picture all the time. Vascular endothelial growth factors (VEGFs) and their tyrosine kinase receptors (VEGFRs) have long been known to be central to the processes of vasculogenesis and angiogenesis. The discovery of VEGFR-3 as the first lymphatic endothelial marker (Kaipainen et al. 1995a), and its ligand VEGF-C as the first factor capable of stimulating lymphangiogenesis (Jeltsch et al. 1997; Joukov et al. 1996), awakened interest in lymphatic research after a long period of inactivity since the use of electron microscopy in the 1960s had allowed characterisation of the fine structure of lymph vessels (reviewed by Leak 1970).

VEGFR-3 and its ligands VEGF-C and VEGF-D have now been firmly established as the major players in lymphangiogenesis, although the strongly angiogenic VEGFR-2 and VEGF-A may also have lymphangiogenic roles. Members of other signalling systems known to be important for angiogenesis, such as Tie/angiopoietin, Eph/Ephrin and PDGFR/PDGF families, neuropilins and some integrins, have also emerged as lymphangiogenic regulators. Among the transcription factors known to be important for lymphangiogenesis, Prox1 is the major fate-determining factor for lymphatic differentiation (Wigle et al. 2002; Wigle and Oliver 1999), whereas FoxC2 is involved in controlling the maturation of lymph vessels (Petrova et al. 2004). These important molecules, along with some about which less is known, are discussed in more detail below.

18.1 Lymphatic Differentiation: Prox1

Prox1 is a homeobox transcription factor related to the *Drosophila* Prospero, and its expression is first observed in a subpopulation of LYVE-1-expressing venous cells in mouse embryos at E9.5 (Wigle and Oliver 1999). After Prox1 expression starts, LYVE-1 expression becomes restricted to, and VEGFR-3 upregulated in, the Prox1-positive cells, which then migrate and proliferate to form the primary lymph sacs (Fig. 18.1; Wigle et al. 2002; Wigle and Oliver 1999). No lymph sacs or lymph vessels are formed in *Prox1*-null mouse embryos, and the subpopulation of cells budding off from veins fail to express lymphatic markers but continue to express blood vascular genes, indicating that Prox1 is instrumental in endothelial cell commitment to the lymphatic phenotype (Wigle et al. 2002). Accordingly, in zebrafish an antisense morpholino against Prox1 abrogated the formation of thoracic duct and lymph vasculature (Yaniv et al. 2006), and *Prox1* knockdown in *Xenopus* tadpoles caused lymphatic defects and lymphoedema by impairing lymphatic commitment (Ny et al. 2005). The observation that overexpression of Prox1 in cultured blood vascular endothelial cells can shift their gene expression towards a lymphatic endothelial cell profile further highlights this function (Hong et al. 2002; Petrova et al. 2002). It is currently unknown what induces Prox1 expression in the defined subpopulation of venous endothelial cells that are to acquire a lymphatic identity. Interleukin-3 (IL-3) and IL-7 have been suggested to induce Prox1 in vitro, but it is not known whether they regulate Prox1 and participate in lymphatic differentiation in vivo (Al-Rawi et al. 2005; Groger et al. 2004).

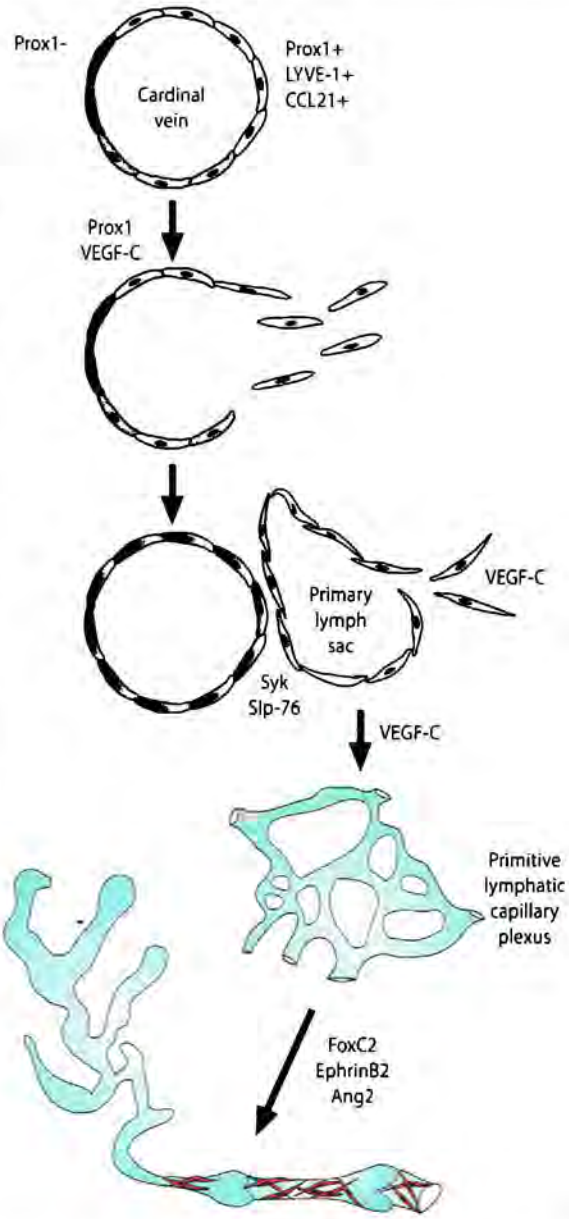


Fig. 18.1. Development of the lymphatic system. At E9–E9.5, a subpopulation of endothelial cells of the cardinal vein begin expressing Prox1, which drives their commitment to lymphatic fate. Prox1 and VEGF-C are required for budding, migration and proliferation of these cells to form the primary lymph sacs at E10–E11. From the lymph sacs, lymphatic endothelial cells then sprout to form the primary plexus of capillary-like lymph vessels, established between E11.5–E14.5. Signalling molecules Syk and Slp-76 are required to keep the developing lymphatic system separate from blood vasculature. FoxC2, EphrinB2 and Ang2 are involved in maturation of the lymph vasculature, which continues from E14.5 to the first postnatal week. The maturation process leads to the formation of a hierarchy of vessels starting from lymphatic capillaries and extending via collecting lymph vessels to the thoracic duct. Mature collecting vessels have acquired a coating of tightly associated smooth muscle cells, and have irregularly spaced valves to prevent backflow of lymph

Lymphatic Sprouting and Growth: VEGFR-3 and VEGF-C/D

During murine embryogenesis, VEGFR-3 is initially widely expressed in endothelial cells, but later during development its expression becomes largely restricted to the lymphatic endothelium (Aprelikova et al. 1992; Galland et al. 1993; Kaipainen et al. 1995b; Pajusola et al. 1992). In adult human tissues VEGFR-3 is restricted to lymphatic endothelium, with the exception of certain fenestrated and discontinuous blood vessel endothelia, found in tissues with highly active transport/exchange of molecules across the vessel wall (Partanen et al. 2000). VEGFR-3 expression is high in lymphatic capillaries, but downregulated in collecting lymph vessels, which in turn express higher levels of VEGFR-2 than lymphatic capillaries (Saaristo et al. 2002b). It appears that VEGFR-3 has a role in mouse embryonic angiogenesis, since genetic disruption of *Vegfr3* results in defective remodelling of the primary vascular plexus, disturbed haematopoiesis, cardiovascular failure and embryonic death by E9.5 (Dumont et al. 1998). In zebrafish, VEGFR-3 and its ligand VEGF-C are involved in lymphatic development: the formation of a thoracic duct can be blocked by the injection of morpholinos against *Vegfc* or of mRNA producing a soluble zebrafish VEGFR-3 ortholog (also called zebrafish Flt4), (Kuchler et al. 2006; Yaniv et al. 2006). However, the zebrafish VEGF-C also appears to be required for vasculogenesis and angiogenesis (Ober et al. 2004), morpholinos against *Vegfr3/Flt4* cause variable defects in segmental artery formation, and genetic interaction is seen between *Vegfr3/Flt4* and the functional zebrafish ortholog of *Vegfr2 (kdra)* in artery development (Covassin et al. 2006). In *Xenopus* tadpoles, knockdown of *Vegfc* caused lymphatic defects, but also impaired sprouting, branching and remodelling of blood vessels (Ny et al. 2005). Ligand-stimulated VEGFR-3 can heterodimerise with VEGFR-2 in human primary endothelial cells, and signal transduction downstream of the heterodimers and homodimers may differ (Dixelius et al. 2003), but it is currently unknown whether this phenomenon is involved in the angiogenic properties of VEGFR-3 in mammals.

Ablation of *Vegfc* leads to a complete absence of lymph vessels in mouse embryos, due to the failure of the first lymphatic endothelial cells to migrate and proliferate to form the lymph sacs, but the blood vasculature appears to develop normally (Karkkainen et al. 2004). Mice heterozygous for *Vegfc* deletion display delayed development of lymph vasculature and severe lymphatic hypoplasia in the skin, and a similar phenotype is seen in mice harbouring a kinase-inactivating mutation in one *Vegfr3* allele (Karkkainen et al. 2001, 2004). These mice serve as a model for the rare autosomal dominant human lymphoedema syndrome called Milroy disease, which is also caused by heterozygous missense point mutations that inactivate the VEGFR-3 tyrosine kinase (Irrthum et al. 2000; Karkkainen et al. 2000, 2001). Despite the crucial role of VEGFR-3 signalling for lymphangiogenesis, VEGF-D seems to be dispensable for the development of the lymphatic system, and its physiological role remains unclear (Baldwin et al. 2005). In vitro stimulation of VEGFR-3 protects lymphatic endothelial cells from apoptosis and stimulates their proliferation and migration (Joukov et al. 1998; Makinen et al. 2001b). VEGFR-3 phosphorylation has been shown to lead to PI3K-dependent Akt activation and protein kinase C-dependent activation of the p42/p44 MAPK pathway (Makinen et al. 2001b).

Both VEGF-C and VEGF-D, and also an engineered VEGFR-3-specific ligand called VEGF-C156S, induce robust sprouting lymphangiogenesis when expressed ectopically as transgenes or via viral vectors, and in various tumour models (Fig. 18.2) (Byzova et al. 2002; Enholm et al. 2001; Jeltsch et al. 1997; Karpanen et al. 2001; Mandriota et al. 2001; Saaristo et al. 2002a,b; Stacker et al. 2001; Veikkola et al. 2001). Importantly, these molecules have also successfully been used therapeutically to grow new lymph vessels in mouse models of primary and secondary lymphoedema (Karkkainen et al. 2001, 2004; Saaristo et al. 2002b, 2004). Pro-

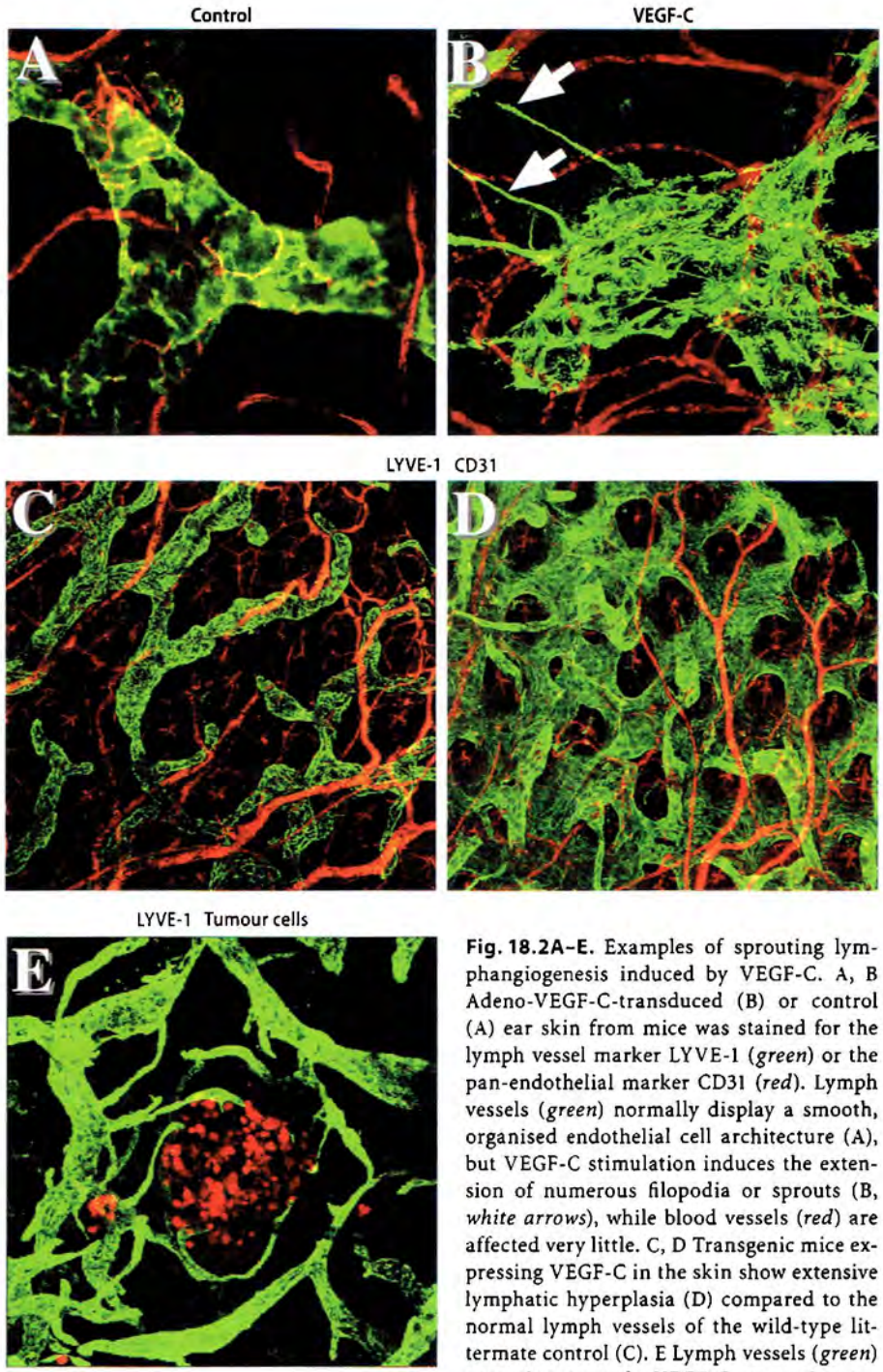


Fig. 18.2A–E. Examples of sprouting lymphangiogenesis induced by VEGF-C. A, B Adeno-VEGF-C-transduced (B) or control (A) ear skin from mice was stained for the lymph vessel marker LYVE-1 (*green*) or the pan-endothelial marker CD31 (*red*). Lymph vessels (*green*) normally display a smooth, organised endothelial cell architecture (A), but VEGF-C stimulation induces the extension of numerous filopodia or sprouts (B, *white arrows*), while blood vessels (*red*) are affected very little. C, D Transgenic mice expressing VEGF-C in the skin show extensive lymphatic hyperplasia (D) compared to the normal lymph vessels of the wild-type littermate control (C). E Lymph vessels (*green*) sprouting towards VEGF-C-expressing tumour cells (*red*). Panel b, picture by Tuomas Tammela; panel E from He et al. (2005)

teolytic processing of propeptides of VEGF-C and VEGF-D increases their affinity for VEGFR-3, and the mature forms also bind VEGFR-2, with the exception of mouse VEGF-D, which is specific for VEGFR-3 (Achen et al. 1998; Joukov et al. 1997). The fully processed, mature forms of VEGF-C and VEGF-D are also capable of inducing angiogenesis in some settings (Byzova et al. 2002; Saaristo et al. 2002a; Stacker et al. 2001). Conversely, expression of VEGFR-3 extracellular domain fused to the Fc domain of human IgG, a functional VEGF-C/D trap, leads to regression of newly forming lymph vessels, excluding large collecting vessels, via endothelial cell apoptosis during embryogenesis and the first few postnatal weeks (Karpanen et al. 2006b; Makinen et al. 2001a). VEGF-C/D trap does not have any effect on adults, and inhibition of VEGFR-3 signalling by a blocking antibody only blocks the formation of new lymph vessels without effects on pre-existing ones, indicating that mature lymph vessels are independent of VEGFR-3 signalling for survival (Karpanen et al. 2006b; Pytowski et al. 2005). Curiously, some lymph vessels in young mice with saturating concentrations of VEGF-C/D trap in the blood could re-grow, indicating that in some circumstances, other mechanisms can substitute for the VEGF-C/D pathway in lymphangiogenesis (Karpanen et al. 2006b; Makinen et al. 2001a).

Overexpressed VEGF-A has been shown to induce lymphatic hyperplasia when delivered via an adenovirus into the skin, and also in models of wound healing and skin tumours (Hirakawa et al. 2005; Hong et al. 2004a; Nagy et al. 2002), but at least some of this effect is probably due to the recruitment of inflammatory cells producing VEGF-C and VEGF-D (Baluk et al. 2005; Cursiefen et al. 2004; Schoppmann et al. 2002). Other growth factors that can induce lymphangiogenesis include basic fibroblast growth factor (FGF-2) and hepatocyte growth factor (HGF), shown to function indirectly via the VEGF-C/D/VEGFR-3 pathway (Cao et al. 2006; Chang et al. 2004; Kubo et al. 2002), although there is some evidence that HGF might also act on lymphatic endothelial cells directly (Kajiya et al. 2005; Tammela and Alitalo 2006).

18.2.1 VEGF-C, VEGF-D and VEGFR-3 in Cancer

VEGF-C/D expression in both experimental and human tumours correlates with vascular invasion, lymphatic vessel and lymph node involvement, distant metastasis, and, in some instances, poor clinical outcomes (reviewed in Achen et al. 2005; Stacker et al. 2002). The first evidence of lymphangiogenesis in cancer and its association with metastasis emerged over 7 years ago (see Alitalo 2000). Studies of various xenotransplantation or transgenic tumour models have shown that VEGF-C and VEGF-D overexpression can enhance lymphatic metastasis, while VEGF-C/D trap inhibited lymphatic metastasis (He et al. 2002; Hirakawa et al. 2007; Karpanen et al. 2001; Mandriota et al. 2001; Skobe et al. 2001b; Stacker et al. 2001). VEGF-C/D trap was found to block lymphatic, but not lung metastases in some models, whereas in others both routes of metastasis were inhibited by VEGF-C/D trap or blocking anti-VEGFR-3 antibodies (He et al. 2002; Krishnan et al. 2003; Roberts et al. 2006). Interestingly, blocking antibodies against VEGFR-3 can significantly inhibit the growth of several human tumour xenografts in mice (Kubo et al. 2000; Laakkonen et al. 2007). Microhaemorrhages and disruption of the endothelial integrity of postcapillary venules were found in tumours treated with the monoclonal antibody AFL4 against mouse VEGFR-3, but it is not clear how this antibody works (Kubo et al. 2000). Another monoclonal anti-VEGFR-3 antibody, mF4-31C1, blocked tumour lymphangiogenesis and also reduced tumour blood capillary density, increasing hypoxia and necrosis (Laakkonen et al. 2007). The analysis of the mechanisms behind these effects is in progress. In a recent study, tumours that expressed VEGF-C were more likely to metastasise to distant organs as well as to sentinel lymph nodes, and in tumour-bearing mice VEGF-C induced the expansion of lymphatic networks within the lymph node even before the onset of metastasis, possibly thus facilitating the spread of tumour cells further (Hirakawa et al. 2007). Lymph vessel proliferation seen in tumour models overexpressing the

lymphangiogenic factors may not occur to a significant extent in several human cancers, and is probably not even necessary for enhanced metastasis in most solid tumours. While intratumoral lymphatic vessels have been detected in some solid tumours, such as melanomas and head and neck carcinomas (Dadras et al. 2003; Maula et al. 2003), at least in experimental tumours their collapse due to high intratumoral pressure may render them dysfunctional (Padera et al. 2004). Instead, the pressure gradient and lymph vessels situated at the tumour margin may be more important in spreading tumour cells through the process of vessel sprouting stimulated by tumour-secreted VEGF-C or VEGF-D (He et al. 2005; Padera et al. 2002). In this process the endothelial cells send long filopodia towards the growth factor-producing tumour cells and then form tumour-directed vessel sprouts, where the vessel lumen opens up and may allow facilitated access of tumour cells to the lumen (Figs. 18.3, 18.2E) (He et al. 2005). Furthermore, the collecting lymph vessels draining fluid from the tumour area dilate via the process of endothelial proliferation in response to tumour-produced VEGF-C, conceivably allowing easier transit for clumps of metastatic tumour cells with the lymph (He et al. 2005). The VEGF-C/D trap inhibited sprouting and vessel dilation, and seemed to restore the integrity of the vessel wall (He et al. 2005). As well as ligand trap molecules and antibodies against VEGFR-3, blocking monoclonal antibodies that target VEGF-C, VEGF-D or VEGFR-2 and small molecules that inhibit the tyrosine kinase catalytic domain of these receptors could be used for the inhibition of tumour metastasis.

Separation of Vascular Domains: Syk and Slp-76

Tyrosine kinase Syk and adaptor protein Slp-76 are haematopoietic intracellular signalling proteins that are necessary for the development of a functional lymphatic system (Abtahian et al. 2003). Loss of either protein in mice leads to haemorrhage and perinatal death, and closer examination of Syk or Slp-76 deficient embryos has shown that abnormal connections exist between blood and lymph circulations, leading to blood-filled lymph vessels (Abtahian et al. 2003). Interestingly, neither Syk nor Slp-76 are expressed in mature endothelial cells, and overexpression of Slp-76 in a subset of haematopoietic cells under the GATA1 promoter is sufficient to rescue the lymphatic defects in Slp76-null mice (Sebzda et al. 2006). Studies with chimeric embryos indicate that Syk and Slp-76 act cell-autonomously, and are required in haematopoietic cells that may contribute directly to vascular development as endothelial precursors (Sebzda et al. 2006). The mechanisms by which Syk and Slp-76 exert their effects on separation of the blood- and lymph vasculature remain unknown, although Sebzda and co-workers (2006) speculate that they could be required for the efficient migration of endothelial progenitor cells, allowing entry and exit through vessel walls.

Adhesion and Migration: Podoplanin, Neuropilin-2 and Integrin $\alpha 9\beta 1$

Podoplanin is a membrane glycoprotein that is highly expressed in lymphatic endothelial cells, although its expression is seen also in some non-endothelial cell types such as podocytes and lung alveolar cells (Breiteneder-Geleff et al. 1997, 1999). Podoplanin$^{-/-}$ mice die shortly after birth due to respiratory failure, displaying swollen limbs and dysfunctional lymph drainage (Schacht et al. 2003). Loss of podoplanin also leads to dilated and disorganised lymphatic capillary networks, and a similar but much milder phenotype was seen in adult podoplanin$^{+/-}$ mice (Schacht et al. 2003). In vitro experiments have suggested that podoplanin in endothelial cells might stimulate their migration and adhesion (Schacht et al. 2003).

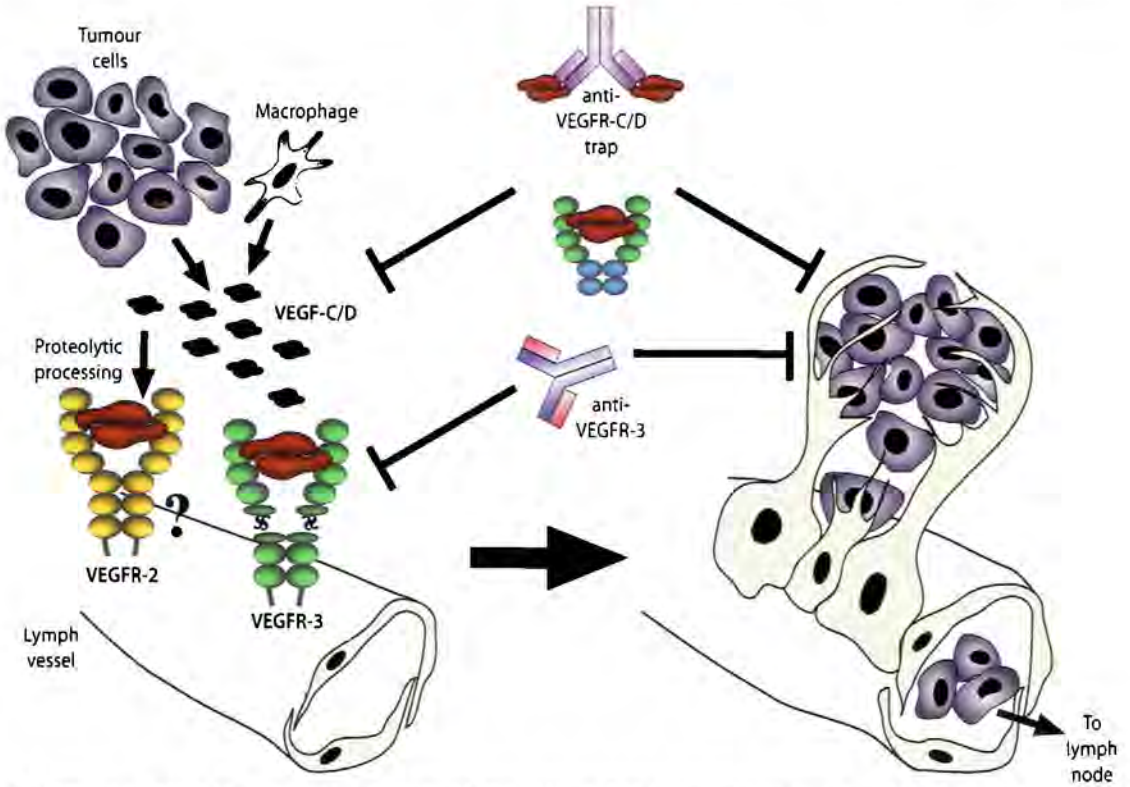


Fig. 18.3. Model of VEGF-C/D involvement in initial steps of lymphatic metastasis. Tumour cells and tumour-associated macrophages secrete VEGF-C and VEGF-D, which stimulate VEGFR-3 on endothelial cells of the lymph vessels within or close to the tumour. VEGFR-3 signalling causes sprouting of the lymphatic endothelium, along with lymphatic endothelial proliferation and widening of the lymph vessels. Endothelial sprouting towards the source of VEGF-C opens up the vessels, which together with vessel dilation allows easy access and transport for metastasising tumour cells (see also Fig. 2E). Lymphatic endothelial cells may also actively attract tumour cells, possibly by mechanisms normally used for interactions with inflammatory cells. Treatment of experimental tumours with VEGF-C/D trap or blocking antibodies to VEGFR-3 has been shown to inhibit metastasis, and antibodies to VEGF-C and VEGF-D could also be used this way. Blocking antibodies to VEGFR-3 also reduce tumour angiogenesis in several xenotransplant models. For references, see text

The semaphorin receptor neuropilin-2 (Nrp2) is involved in axon guidance, but also interacts with VEGFR-3 as a co-receptor for VEGF-C and VEGF-D (Karkkainen et al. 2001; Karpanen et al. 2006a). In *Nrp2*-null mice, transient loss of lymphatic capillaries and small lymph vessels during development was observed, followed by postnatal regeneration; adult animals had slightly abnormally patterned but seemingly functional lymphatic vasculature (Yuan et al. 2002). Collecting lymph vessels did not appear to be affected by loss of Nrp2 (Yuan et al. 2002).

Targeted deletion of *integrinα9*, which together with integrin β1 subunit forms a receptor for the extracellular matrix proteins osteopontin and tenascin C as well as vascular cell adhesion molecule-1 (VCAM-1), leads to fatal bilateral chylothorax at 6–12 days of age (Huang et al. 2000). Integrin α9 is highly expressed in lymphatic endothelial cells (Petrova et al. 2002), and in vitro data suggest that VEGF-C and VEGF-D can bind to integrin α9β1 (Vlahakis et al. 2005). When bound to matrix components such as collagen or fibronectin, integrin β1 can interact with VEGFR-3 and weakly activate its tyrosine phosphor-

ylation (Wang et al. 2001). Both of the above studies on cultured cells show evidence that integrin α9β1 is involved in VEGF-C/D/VEGFR-3-mediated migration of endothelial cells (Vlahakis et al. 2005; Wang et al. 2001), and together with neuropilin-2, and probably other effectors, it may be involved in differentially regulating the VEGFR-3 pathway in the functionally diverse lymphatic vessels of different sizes in different tissues.

Remodelling and Maturation of Lymph Vessels

Recently, a lot of progress has been made in identifying the mechanisms and molecules involved in the maturation of the primary lymphatic capillary plexus into a hierarchical network of capillaries and collecting vessels of different sizes. Especially the importance of controlled recruitment of smooth muscle cells/pericytes onto collecting vessels has been highlighted, establishing another parallel between angiogenesis and lymphangiogenesis. The molecules that have been found to be most important for remodelling and maturation so far are discussed below (see also Fig. 18.1).

18.5.1 Tie Receptors and Angiopoietins

The three members of the angiopoietin (Ang) family, namely Angl, Ang2 and Ang3/4, are ligands for the Tie2 receptor, and it has recently been shown that Angl can also activate Tiel (Davis et al. 1997; Kim et al. 1999; Lee et al. 2004; Maisonpierre et al. 1997; Saharinen et al. 2005; Valenzuela et al. 1999). Tiel and Tie2 are expressed in both blood vascular and lymphatic endothelial cells, and targeted deletions of both lead to embryonic death due to severe blood vascular remodelling defects and defects in vascular pericyte/smooth muscle cell coverage (Dumont et al. 1994; Iljin et al. 2002; Patan 1998; Puri et al. 1995; Sato et al. 1995). Interestingly, *Ang2*-null mice display chylous ascites, subcutaneous oedema and disorganised lymphatic vasculature with loss of tight smooth muscle cell association in collecting vessels after birth, and this phenotype can be rescued by insertion of *Angl* into the *Ang2* locus (Gale et al. 2002). In blood vessels, Ang2 is thought to act as an antagonist of Tie2, destabilising the endothelium at sites of vascular remodelling (Maisonpierre et al. 1997). Accordingly, blood vascular remodelling defects seen in Ang2-deficient mice cannot be rescued by Angl (Gale et al. 2002). Furthermore, overexpression of Angl can induce enlargement of lymph vessels, and proliferation and sprouting of lymphatic endothelium in a VEGFR-3-dependent manner, suggesting that crosstalk between the VEGF and angiopoietin systems, also thought to occur in angiogenesis, plays a role in lymphangiogenesis as well (Morisada et al. 2005; Tammela et al. 2005b).

18.5.2 FoxC2, PDGF-B and EphrinB2

Heterozygous loss of function mutations of forkhead transcription factor *FoxC2* have been identified as the cause of the lymphoedema-distichiasis (LD) syndrome, characterised by late-onset lymphoedema due to lymph backflow, a double row of eyelashes, and increased risk for early-onset varicose veins (Brice et al. 2002; Fang et al. 2000). FoxC2 is highly expressed in developing lymph vessels, and high levels of expression persist in adult lymphatic valves (Dagenais et al. 2004; Petrova et al. 2004). In both $FoxC2^{-/-}$ mice and affected tissue in LD patients, lymphatic capillaries are abnormally covered by smooth muscle cells, patterning of lymph vessels is abnormal, and the collecting lymph vessels lack valves (Petrova et al. 2004). $FoxC2^{-/-}$ lymphatic capillaries in mouse embryos upregulated PDGF-B and endoglin, both involved in pericyte/smooth muscle cell recruitment, and also the basement membrane protein collagen IV was upregulated (Petrova et al. 2004). Since loss of FoxC2 results in collecting vessel- or blood vessel-like characteristics in lymphatic capillaries, it appears to have an important

role in the establishment of capillary phenotype in lymphatic development (Petrova et al. 2004). FoxC2 is also essential for the development of lymphatic valves, and it is likely to play a role in their maintenance, perhaps partly by keeping them free of smooth muscle cells by repression of PDGF-B (Petrova et al. 2004). Interaction between FoxC2 and PDGF-B might also have broader significance, since in xenotransplanted tumours PDGF-B was reported to be lymphangiogenic and cause an increase of lymphatic metastasis (Cao et al. 2004), but further research is needed to establish the possible role of PDGF-B in normal lymph vessels. FoxC2 also seems to co-operate with the VEGFR-3 pathway in lymphatic development, as embryos heterozygous for both *Foxc2* and *Vegfr3* had a phenotype closely resembling that caused by complete loss of FoxC2 (Petrova et al. 2004). Targets of FoxC2 in lymphatic endothelial cells are currently unknown, but it was shown recently that FoxC2 can bind and activate the promoter of *Delta-like 4 ligand (Dll4)*, suggesting that FoxC2 might interact with the Notch signalling pathway (Seo et al. 2006).

EphrinB2 is a transmembrane ligand that, together with its receptor EphB4, is essential for remodelling of the primary blood capillary plexus into functional vessels of venous and arterial identity (Adams et al. 1999; Wang et al. 1998). EphrinB2 is also expressed in the primary lymphatic capillary plexus during development, and collecting lymph vessels in adults, while EphB4 is expressed in all lymph vessels, at least in the skin (Makinen et al. 2005). Surprisingly, mutant mice that lack the PDZ domain binding motif of EphrinB2 survived the requirement for EphrinB2 in blood vascular remodelling, but had lymphatic defects such as chylothorax, retrograde lymph flow, collecting vessel hyperplasia and valve agenesis, and abnormal smooth muscle cell coverage of lymphatic capillaries (Makinen et al. 2005). The similar phenotypes resulting from loss of FoxC2 and the *EphrinB2* PDZ mutation establish these molecules as important players in remodelling and maturation of lymph vasculature, and central for the acquisition of a distinct molecular identity for collecting lymph vessels versus lymphatic capillaries.

Lymph Vessels and Inflammation

Lymph vessels are needed for the transport of inflammatory cells from peripheral tissues into the secondary lymphoid organs. The chemokine CCL21/SLC expressed by lymphatic endothelial cells is a ligand for the chemokine receptor CCR7 expressed on dermal dendritic cells (DCs), and CCR7 is required for entry of DCs into the dermal lymph vessels in order to be transported to lymph nodes (Ohl et al. 2004). Other molecules known to be involved in interactions of inflammatory cells with lymphatic endothelium include mannose receptor, which is expressed in lymph node sinuses and bound by L-selectin on lymphocytes (Irjala et al. 2001), and common lymphatic endothelial and vascular endothelial receptor-1 (CLEVER-1), which can mediate leukocyte transmigration in culture through both blood vascular and lymphatic endothelia (Salmi et al. 2004). It has long been known that lymph vessels proliferate during inflammation (Pullinger and Florey 1937), and inflammatory lymphangiogenesis has now been documented in several settings. Massive lymphangiogenesis is associated with inflammatory infiltrates in rejected human kidney transplants (Kerjaschki et al. 2004), and lymphatic hyperplasia is also seen in psoriatic lesions (Kunstfeld et al. 2004). Tumour-associated macrophages can express VEGF-C and VEGF-D (Schoppmann et al. 2002; Skobe et al. 2001a), and VEGF was shown to recruit VEGF-C/D-expressing macrophages in rabbit cornea model of inflammatory lymph-/angiogenesis, where blockage of VEGF or depletion of macrophages also lead to inhibition of lymphangiogenesis (Cursiefen et al. 2004). DCs expressing both VEGF-C and VEGFR-3 were found in inflamed mouse corneas (Hamrah et al. 2003), while in mouse corneal transplants, trafficking of DCs to lymph nodes, induction of delayed-type hypersensitivity and graft rejection were blocked by ocular administration of VEGF-C/D trap (Chen et al. 2004). VEGF-C/D trap also blocked the robust inflammatory cell-driven lymphangiogenesis caused by *Mycoplasma pulmonis* infection of mouse airway epithelium,

exacerbating mucosal oedema and lessening the reactive lymphadenitis (Baluk et al. 2005). Interestingly, in mice immunised with complete Freund's adjuvant, lymph node sinus expansion was shown to enhance DC migration into the lymph nodes, an effect that was found to be dependent on lymph node B-cells, and could be partially blocked by antibodies to VEGFR-2 or VEGFR-3 (Angeli et al. 2006). Together with the finding that sentinel lymph node sinusoidal endothelium can expand even before the arrival of first metastases (Hirakawa et al. 2007), this suggests a more extensive role for the sinus network in lymph nodes than previously thought. The results presented above illustrate a two-way communication, where inflammatory cells can both induce lymphangiogenesis and respond to lymphangiogenic signals in general, and signals specifically from lymphatic endothelial cells.

Concluding Remarks

Beginning with the discovery of VEGFR-3 and VEGF-C, a decade of intensive research has seen major advances in understanding the mechanisms of lymphangiogenesis and the molecular players involved. The molecules described above have all been found to be important for lymphatic development and growth, but downstream signalling and interactions between the known pathways still need to be characterised to get a more complete picture of the mechanisms of lymphangiogenesis. Among the unresolved questions is how it all begins: What is the signal that makes a defined subpopulation of venous endothelial cells upregulate Prox1 expression? Another mystery is encountered later in the development, when the absolute requirement for VEGF-C seems to be lost; some lymph vessels grow back and become functional despite saturating concentrations of VEGF-C/D trap in the serum (Makinen et al. 2001a). It is not known what mechanisms allow this to happen, and why some vessels grow back and some never recover – there are obviously significant differences between lymphatic endothelial cells in different vascular beds, as would of course be expected given the variety of functions that the vessels have to perform, but very little is known about this heterogeneity. It cannot be forgotten that vessels residing in different environments will differ in the communication between endothelial cells, supporting cells and the extracellular matrix. The first pieces of information about these interactions have started to trickle in with the smooth muscle cell/pericyte and basal lamina abnormalities seen in *FoxC2*-null and *EphrinB2*-mutant mice (Makinen et al. 2005; Petrova et al. 2004), which also shed some light on how collecting lymph vessels might acquire their phenotypic characteristics distinct from lymphatic capillaries. Ang2 also seems to be involved in the maturation process (Gale et al. 2002), but it is totally unknown how it exerts its effects, and what the exact roles of Tie receptors and Angl might be. Despite the obvious importance of lymph vessels in immune reactions, research into inflammatory lymphangiogenesis and the active role of lymphatic endothelium in transport of inflammatory cells both in the periphery and in lymphoid organs has only recently begun to emerge, and a lot remains to be discovered about the mechanisms and functional significance of these processes. The active participation of lymphatic endothelium in the entry and transport of metastasising tumour cells, and the emerging role of lymphatic sinuses of the lymph nodes in the metastatic process, are important issues to clarify in order to achieve successful therapeutic intervention in the future.

References

Abtahian F, Guerriero A, Sebzda E, Lu MM, Zhou R, Mocsai A, Myers EE, Huang B, Jackson DG, Ferrari VA et al (2003) Regulation of blood and lymphatic vascular separation by signaling proteins SLP-76 and Syk. Science 299:247–251

Achen MG, Jeltsch M, Kukk E, Makinen T, Vitali A, Wilks AF, Alitalo K, Stacker SA (1998) Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). Proc Natl Acad Sci USA 95:548–553

Achen MG, McColl BK, Stacker SA (2005) Focus on lymphangiogenesis in tumor metastasis. Cancer Cell 7:121–127

Adams RH, Wilkinson GA, Weiss C, Diella F, Gale NW, Deutsch U, Risau W, Klein R (1999) Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. Genes Dev 13:295–306

Al-Rawi MA, Watkins G, Mansel RE, Jiang WG (2005) The effects of interleukin-7 on the lymphangiogenic properties of human endothelial cells. Int J Oncol 27:721–730

Alitalo K (2000) VEGF Family and Lymphangiogenesis. In: Witte MH (ed) Conquering lymphatic disease: setting the research agenda. University of Arizona Press, Tucson

Alitalo K, Tammela T, Petrova TV (2005) Lymphangiogenesis in development and human disease. Nature 438:946–953

Angeli V, Ginhoux F, Llodra J, Quemeneur L, Frenette PS, Skobe M, Jessberger R, Merad M, Randolph GJ (2006) B cell-driven lymphangiogenesis in inflamed lymph nodes enhances dendritic cell mobilization. Immunity 24:203–215

Aprelikova O, Pajusola K, Partanen J, Armstrong E, Alitalo R, Bailey SK, McMahon J, Wasmuth J, Huebner K, Alitalo K (1992) FLT4, a novel class III receptor tyrosine kinase in chromosome 5q33-qter. Cancer Res 52:746–748

Baldwin ME, Halford MM, Roufail S, Williams RA, Hibbs ML, Grail D, Kubo H, Stacker SA, Achen MG (2005) Vascular endothelial growth factor D is dispensable for development of the lymphatic system. Mol Cell Biol 25:2441–2449

Baluk P, Tammela T, Ator E, Lyubynska N, Achen MG, Hicklin DJ, Jeltsch M, Petrova TV, Pytowski B, Stacker SA et al (2005) Pathogenesis of persistent lymphatic vessel hyperplasia in chronic airway inflammation. J Clin Invest 115:247–257

Breiteneder-Geleff S, Matsui K, Soleiman A, Meraner P, Poczewski H, Kalt R, Schaffner G, Kerjaschki D (1997) Podoplanin, novel 43-kd membrane protein of glomerular epithelial cells, is down-regulated in puromycin nephrosis. Am J Pathol 151:1141–1152

Breiteneder-Geleff S, Soleiman A, Kowalski H, Horvat R, Amann G, Kriehuber E, Diem K, Weninger W, Tschachler E, Alitalo K, Kerjaschki D (1999) Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries: podoplanin as a specific marker for lymphatic endothelium. Am J Pathol 154:385–394

Brice G, Mansour S, Bell R, Collin JR, Child AH, Brady AF, Sarfarazi M, Burnand KG, Jeffery S, Mortimer P, Murday VA (2002) Analysis of the phenotypic abnormalities in lymphoedema-distichiasis syndrome in 74 patients with FOXC2 mutations or linkage to 16q24. J Med Genet 39:478–483

Byzova TV, Goldman CK, Jankau J, Chen J, Cabrera G, Achen MG, Stacker SA, Carnevale KA, Siemionow M, Deitcher SR, DiCorleto PE (2002) Adenovirus encoding vascular endothelial growth factor-D induces tissue-specific vascular patterns in vivo. Blood 99:4434–4442

Cao R, Bjorndahl MA, Religa P, Clasper S, Garvin S, Galter D, Meister B, Ikomi F, Tritsaris K, Dissing S et al (2004) PDGF-BB induces intratumoral lymphangiogenesis and promotes lymphatic metastasis. Cancer Cell 6:333–345

Cao R, Bjorndahl MA, Gallego MI, Chen S, Religa P, Hansen AJ, Cao Y (2006) Hepatocyte growth factor is a lymphangiogenic factor with an indirect mechanism of action. Blood 107:3531–3536

Chang LK, Garcia-Cardena G, Farnebo F, Fannon M, Chen EJ, Butterfield C, Moses MA, Mulligan RC, Folkman J, Kaipainen A (2004) Dose-dependent response of FGF-2 for lymphangiogenesis. Proc Natl Acad Sci USA 101:11658–11663

Chen L, Hamrah P, Cursiefen C, Zhang Q, Pytowski B, Streilein JW, Dana MR (2004) Vascular endothelial growth factor receptor-3 mediates induction of corneal alloimmunity. Nat Med 10:813–815

Covassin LD, Villefranc JA, Kacergis MC, Weinstein BM, Lawson ND (2006) Distinct genetic interactions between multiple Vegf receptors are required for development of different blood vessel types in zebrafish. Proc Natl Acad Sci USA 103:6554–6559

Cursiefen C, Chen L, Borges LP, Jackson D, Cao J, Radziejewski C, D'Amore PA, Dana MR, Wiegand SJ, Streilein JW (2004) VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment. J Clin Invest 113:1040–1050

Dadras SS, Paul T, Bertoncini J, Brown LF, Muzikansky A, Jackson DG, Ellwanger U, Garbe C, Mihm MC, Detmar M (2003) Tumor lymphangiogenesis: a novel prognostic indicator for cutaneous melanoma metastasis and survival. Am J Pathol 162:1951–1960

Dagenais SL, Hartsough RL, Erickson RP, Witte MH, Butler MG, Glover TW (2004) Foxc2 is expressed in developing lymphatic vessels and other tissues associated with lymphedema-distichiasis syndrome. Gene Exp Patterns 4:611–619

Davis S, Aldrich TH, Jones PF, Acheson A, Compton DL, Jain V, Ryan TE, Bruno J, Raziejewski C, Maisonpierre PC, Yancopoulos GD (1997) Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. Cell 87:1161–1169

Dixelius J, Makinen T, Wirzenius M, Karkkainen MJ, Wernstedt C, Alitalo K, Claesson-Welsh L (2003) Ligand-induced vascular endothelial growth factor receptor-3 (VEGFR-3) heterodimerization with VEGFR-2 in primary lymphatic endothelial cells regulates tyrosine phosphorylation sites. J Biol Chem 278:40973–40979

Dumont DJ, Gradwohl G, Fong GH, Puri MC, Gertsenstein M, Auerbach A, Breitman ML (1994) Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. Genes Dev 8:1897–1909

Dumont DJ, Jussila L, Taipale J, Lymboussaki A, Mustonen T, Pajusola K, Breitman M, Alitalo K (1998) Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. Science 282:946–949

Enholm B, Karpanen T, Jeltsch M, Kubo H, Stenback F, Prevo R, Jackson DG, Yla-Herttuala S, Alitalo K (2001) Adenoviral expression of vascular endothelial growth factor-C induces lymphangiogenesis in the skin. Circ Res 88:623–629

Fang J, Dagenais SL, Erickson RP, Arlt MF, Glynn MW, Gorski JL, Seaver LH, Glover TW (2000) Mutations in FOXC2 (MFH-1), a forkhead family transcription factor, are responsible for the hereditary lymphedema-distichiasis syndrome. Am J Hum Genet 67:1382–1388

Gale NW, Thurston G, Hackett SF, Renard R, Wang Q, McClain J, Martin C, Witte C, Witte MH, Jackson D et al (2002) Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by Angiopoietin-1. Dev Cell 3:411–423

Galland F, Karamysheva A, Pebusque M-J, Borg J-P, Rottapel R, Dubreuil P, Rosnet O, Birnbaum D (1993) The FLT4 gene encodes a transmembrane tyrosine kinase related to the vascular endothelial growth factor receptor. Oncogene 8:1233–1240

Groger M, Loewe R, Holnthoner W, Embacher R, Pillinger M, Herron GS, Wolff K, Petzelbauer P (2004) IL-3 induces expression of lymphatic markers Prox-1 and podoplanin in human endothelial cells. J Immunol 173:7161–7169

Hamrah P, Chen L, Zhang Q, Dana MR (2003) Novel expression of vascular endothelial growth factor receptor (VEGFR)-3 and VEGF-C on corneal dendritic cells. Am J Pathol 163:57–68

He Y, Kozaki K, Karpanen T, Koshikawa K, Yla-Herttuala S, Takahashi T, Alitalo K (2002) Suppression of tumor lymphangiogenesis and lymph node metastasis by blocking vascular endothelial growth factor receptor 3 signaling. J Natl Cancer Inst 94:819–825

He Y, Rajantie I, Pajusola K, Jeltsch M, Holopainen T, Yla-Herttuala S, Harding T, Jooss K, Takahashi T, Alitalo K (2005) Vascular endothelial cell growth factor receptor 3-mediated activation of lymphatic endothelium is crucial for tumor cell entry and spread via lymphatic vessels. Cancer Res 65:4739–4746

Hirakawa S, Kodama S, Kunstfeld R, Kajiya K, Brown LF, Detmar M (2005) VEGF-A induces tumor and sentinel lymph node lymphangiogenesis and promotes lymphatic metastasis. J Exp Med 201:1089–1099

Hirakawa S, Brown LF, Kodama S, Paavonen K, Alitalo K, Detmar M (2007) VEGF-C-induced lymphangiogenesis in sentinel lymph nodes promotes tumor metastasis to distant sites. Blood 109(3):1010–1017

Hong YK, Harvey N, Noh YH, Schacht V, Hirakawa S, Detmar M, Oliver G (2002) Prox1 is a master control gene in the program specifying lymphatic endothelial cell fate. Dev Dyn 225:351–357

Hong YK, Lange-Asschenfeldt B, Velasco P, Hirakawa S, Kunstfeld R, Brown LF, Bohlen P, Senger DR, Detmar M (2004a) VEGF-A promotes tissue repair-associated lymphatic vessel formation via VEGFR-2 and the alpha1beta1 and alpha2beta1 integrins. FASEB J 18:1111–1113

Hong YK, Shin JW, Detmar M (2004b) Development of the lymphatic vascular system: a mystery unravels. Dev Dyn 231:462–473

Huang XZ, Wu JF, Ferrando R, Lee JH, Wang YL, Farese RV Jr, Sheppard D (2000) Fatal bilateral chylothorax in mice lacking the integrin alpha9beta1. Mol Cell Biol 20:5208–5215

Iljin K, Petrova TV, Veikkola T, Kumar V, Poutanen M, Alitalo K (2002) A fluorescent Tie1 reporter allows monitoring of vascular development and endothelial cell isolation from transgenic mouse embryos. FASEB J 16:1764–1774

Irjala H, Johansson EL, Grenman R, Alanen K, Salmi M, Jalkanen S (2001) Mannose receptor is a novel ligand for L-selectin and mediates lymphocyte binding to lymphatic endothelium. J Exp Med 194:1033–1042

Irrthum A, Karkkainen MJ, Devriendt K, Alitalo K, Vikkula M (2000) Congenital hereditary lymphedema caused by a mutation that inactivates VEGFR3 tyrosine kinase. Am J Hum Genet 67:295–301

Jeltsch M, Kaipainen A, Joukov V, Meng X, Lakso M, Rauvala H, Swartz M, Fukumura D, Jain RK, Alitalo K (1997) Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. Science 276:1423–1425

Jeltsch M, Tammela T, Alitalo K, Wilting J (2003) Genesis and pathogenesis of lymphatic vessels. Cell Tissue Res 314:69–84

Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, Saksela O, Kalkkinen N, Alitalo K (1996) A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. EMBO J 15:290–298

Joukov V, Sorsa T, Kumar V, Jeltsch M, Claesson-Welsh L, Cao Y, Saksela O, Kalkkinen N, Alitalo K (1997) Proteolytic processing regulates receptor specificity and activity of VEGF-C. EMBO J 16:3898–3911

Joukov V, Kumar V, Sorsa T, Arighi E, Weich H, Saksela O, Alitalo K (1998) A recombinant mutant vascular endothelial growth factor-C that has lost vascular endothelial growth factor receptor-2 binding, activation, and vascular permeability activities. J Biol Chem 273:6599–6602

Kaipainen A, Korhonen J, Mustonen T, van Hinsbergh VW, Fang GH, Dumont D, Breitman M, Alitalo K (1995a) Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. Proc Natl Acad Sci USA 92:3566–3570

Kaipainen A, Korhonen J, Mustonen T, van Hinsbergh VW. M, Fang G-H, Dumont D, Breitman M, Alitalo K (1995b) Expression of the fms-like tyrosine kinase FLT4 gene becomes restricted to lymphatic endothelium during development. Proc Natl Acad Sci USA 92:3566–3570

Kajiya K, Hirakawa S, Ma B, Drinnenberg I, Detmar M (2005) Hepatocyte growth factor promotes lymphatic vessel formation and function. EMBO J 24:2885–2895

Karkkainen MJ, Ferrell RE, Lawrence EC, Kimak MA, Levinson KL, McTigue MA, Alitalo K, Finegold DN (2000) Missense mutations interfere with VEGFR-3 signalling in primary lymphoedema. Nat Genet 25:153–159

Karkkainen MJ, Saaristo A, Jussila L, Karila KA, Lawrence EC, Pajusola K, Bueler H, Eichmann A, Kauppinen R, Kettunen MI et al (2001) A model for gene therapy of human hereditary lymphedema. Proc Natl Acad Sci USA 98:12677–12682

Karkkainen MJ, Haiko P, Sainio K, Partanen J, Taipale J, Petrova TV, Jeltsch M, Jackson DG, Talikka M, Rauvala H et al (2004) Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. Nat Immunol 5:74–80

Karpanen T, Alitalo K (2001) Lymphatic vessels as targets of tumor therapy? J Exp Med 194:757–782

Karpanen T, Makinen T (2006) Regulation of lymphangiogenesis-From cell fate determination to vessel remodeling. Exp Cell Res 312:575–583

Karpanen T, Egeblad M, Karkkainen MJ, Kubo H, Jackson DG, Ylä-Herttuala S, Jäättelä M, Alitalo K (2001) Vascular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. Cancer Res 61:1786–1790

Karpanen T, Heckman CA, Keskitalo S, Jeltsch M, Ollila H, Neufeld G, Tamagnone L, Alitalo K (2006a) Functional interaction of VEGF-C and VEGF-D with neuropilin receptors. FASEB J 20:1462–1472

Karpanen T, Wirzenius M, Makinen T, Veikkola T, Haisma HJ, Achen MG, Stacker SA, Pytowski B, Yla-Herttuala S, Alitalo K (2006b) Lymphangiogenic growth factor responsiveness is modulated by postnatal lymphatic vessel maturation. Am J Pathol 169:708–718

Kerjaschki D, Regele HM, Moosberger I, Nagy-Bojarski K, Watschinger B, Soleiman A, Birner P, Krieger S, Hovorka A, Silberhumer G et al (2004) Lymphatic neoangiogenesis in human kidney transplants is associated with immunologically active lymphocytic infiltrates. J Am Soc Nephrol 15:603–612

Kim I, Kwak HJ, Ahn JE, So JN, Liu M, Koh KN, Koh GY (1999) Molecular cloning and characterization of a novel angiopoietin family protein, angiopoietin-3. FEBS Lett 443:353–356

Krishnan J, Kirkin V, Steffen A, Hegen M, Weih D, Tomarev S, Wilting J, Sleeman JP (2003) Differential in vivo and in vitro expression of vascular endothelial growth factor (VEGF)-C and VEGF-D in tumors and its relationship to lymphatic metastasis in immunocompetent rats. Cancer Res 63:713–722

Kubo H, Fujiwara T, Jussila L, Hashi H, Ogawa M, Shimizu K, Awane M, Sakai Y, Takabayashi A, Alitalo K et al (2000) Involvement of vascular endothelial growth factor receptor-3 in maintenance of integrity of endothelial cell lining during tumor angiogenesis. Blood 96:546–553

Kubo H, Cao R, Brakenhielm E, Makinen T, Cao Y, Alitalo K (2002) Blockade of vascular endothelial growth factor receptor-3 signaling inhibits fibroblast growth factor-2-induced lymphangiogenesis in mouse cornea. Proc Natl Acad Sci USA 99:8868–8873

Kuchler AM, Gjini E, Peterson-Maduro J, Cancilla B, Wolburg H, Schulte-Merker S (2006) Development of the zebrafish lymphatic system requires VEGFC signaling. Curr Biol 16:1244–1248

Kunstfeld R, Hirakawa S, Hong YK, Schacht V, Lange-Asschenfeldt B, Velasco P, Lin C, Fiebiger E, Wei X, Wu Y et al (2004) Induction of cutaneous delayed-type hypersensitivity reactions in VEGF-A transgenic mice results in chronic skin inflammation associated with persistent lymphatic hyperplasia. Blood 104:1048–1057

Laakkonen P, Waltari M, Holopainen T, Takahashi T, Pytowski B, Steiner P, Hicklin D, Persaud K, Tonra JR, Witte L, Alitalo K (2007) Vascular endothelial growth factor receptor 3 is involved in tumor angiogenesis and growth. Cancer Res 67:593–599

Leak LV (1970) Electron microscopic observations on lymphatic capillaries and the structural components of the connective tissue-lymph interface. Microvisc Res 2:361–391

Lee HJ, Cho CH, Hwang SJ, Choi HH, Kim KT, Ahn SY, Kim JH, Oh JL, Lee GM, Koh GY (2004) Biological characterization of angiopoietin-3 and angiopoietin-4. FASEB J 18:1200–1208

Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N et al (1997) Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 277:55–60

Makinen T, Jussila L, Veikkola T, Karpanen T, Kettunen MI, Pulkkanen KJ, Kauppinen R, Jackson DG, Kubo H, Nishikawa S et al (2001a) Inhibition of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble VEGF receptor-3. Nat Med 7:199–205

Makinen T, Veikkola T, Mustjoki S, Karpanen T, Catimel B, Nice EC, Wise L, Mercer A, Kowalski H, Kerjaschki D et al (2001b) Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3. EMBO J 20:4762–4773

Makinen T, Adams RH, Bailey J, Lu Q, Ziemiecki A, Alitalo K, Klein R, Wilkinson GA (2005) PDZ interaction site in ephrinB2 is required for the remodeling of lymphatic vasculature. Genes Dev 19:397–410

Mandriota SJ, Jussila L, Jeltsch M, Compagni A, Baetens D, Prevo R, Banerji S, Huarte J, Montesano R, Jackson DG et al (2001) Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. EMBO J 20:672–682

Maula SM, Luukkaa M, Grenman R, Jackson D, Jalkanen S,

Ristamaki R (2003) Intratumoral lymphatics are essential for the metastatic spread and prognosis in squamous cell carcinomas of the head and neck region. Cancer Res 63:1920–1926

Morisada T, Oike Y, Yamada Y, Urano T, Akao M, Kubota Y, Maekawa H, Kimura Y, Ohmura M, Miyamoto T et al (2005) Angiopoietin-1 promotes LYVE-1-positive lymphatic vessel formation. Blood 105:4649–4656

Nagy JA, Vasile E, Feng D, Sundberg C, Brown LF, Detmar MJ, Lawitts JA, Benjamin L, Tan X, Manseau EJ et al (2002) Vascular permeability factor/vascular endothelial growth factor induces lymphangiogenesis as well as angiogenesis. J Exp Med 196:1497–1506

Ny A, Koch M, Schneider M, Neven E, Tong RT, Maity S, Fischer C, Plaisance S, Lambrechts D, Heligon C et al (2005) A genetic Xenopus laevis tadpole model to study lymphangiogenesis. Nat Med 11:998–1004

Ober EA, Olofsson B, Makinen T, Jin SW, Shoji W, Koh GY, Alitalo K, Stainier DY (2004) Vegfc is required for vascular development and endoderm morphogenesis in zebrafish. EMBO Rep 5:78–84

Ohl L, Mohaupt M, Czeloth N, Hintzen G, Kiafard Z, Zwirner J, Blankenstein T, Henning G, Forster R (2004) CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. Immunity 21:279–288

Oliver G, Alitalo K (2005) The lymphatic vasculature: recent progress and paradigms. Annu Rev Cell Dev Biol 21:457–483

Padera TP, Kadambi A, di Tomaso E, Carreira CM, Brown EB, Boucher Y, Choi NC, Mathisen D, Wain J, Mark EJ et al (2002) Lymphatic metastasis in the absence of functional intratumor lymphatics. Science 296:1883–1886

Padera TP, Stoll BR, Tooredman JB, Capen D, di Tomaso E, Jain RK (2004) Pathology: cancer cells compress intratumour vessels. Nature 427:695

Pajusola K, Aprelikova O, Korhonen J, Kaipainen A, Pertovaara L, Alitalo R, Alitalo K (1992) FLT4 receptor tyrosine kinase contains seven immunoglobulin-like loops and is expressed in multiple human tissues and cell lines. Cancer Res 52:5738–5743

Papoutsi M, Tomarev SI, Eichmann A, Prols F, Christ B, Wilting J (2001) Endogenous origin of the lymphatics in the avian chorioallantoic membrane. Dev Dyn 222:238–251

Partanen TA, Arola J, Saaristo A, Jussila L, Ora A, Miettinen M, Stacker SA, Achen MG, Alitalo K (2000) VEGF-C and VEGF-D expression in neuroendocrine cells and their receptor, VEGFR-3, in fenestrated blood vessels in human tissues. FASEB J 14:2087–2096

Patan S (1998) TIE1 and TIE2 receptor tyrosine kinases inversely regulate embryonic angiogenesis by the mechanism of intussusceptive microvascular growth. Microvasc Res 56:1–21

Pepper MS (2001) Lymphangiogenesis and tumor metastasis: myth or reality? Clin Cancer Res 7:462–468

Petrova TV, Makinen T, Makela TP, Saarela J, Virtanen I, Ferrell RE, Finegold DN, Kerjaschki D, Yla-Herttuala S, Alitalo K (2002) Lymphatic endothelial reprogramming of vascular endothelial cells by the Prox-1 homeobox transcription factor. EMBO J 21:4593–4599

Petrova TV, Karpanen T, Norrmen C, Mellor R, Tamakoshi T, Finegold D, Ferrell R, Kerjaschki D, Mortimer P, Yla-Herttuala S et al (2004) Defective valves and abnormal mural cell recruitment underlie lymphatic vascular failure in lymphedema distichiasis. Nat Med 10:974–981

Pullinger BD, Florey HW (1937) Proliferation of lymphatics in inflammation. J Pathol Bacteriol 45:157–170

Puri MC, Rossant J, Alitalo K, Bernstein A, Partanen J (1995) The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells. EMBO J 14:5884–5891

Pytowski B, Goldman J, Persaud K, Wu Y, Witte L, Hicklin DJ, Skobe M, Boardman KC, Swartz MA (2005) Complete and specific inhibition of adult lymphatic regeneration by a novel VEGFR-3 neutralizing antibody. J Natl Cancer Inst 97:14–21

Roberts N, Kloos B, Cassella M, Podgrabinska S, Persaud K, Wu Y, Pytowski B, Skobe M (2006) Inhibition of VEGFR-3 activation with the antagonistic antibody more potently suppresses lymph node and distant metastases than inactivation of VEGFR-2. Cancer Res 66:2650–2657

Saaristo A, Veikkola T, Enholm B, Hytonen M, Arola J, Pajusola K, Turunen P, Jeltsch M, Karkkainen MJ, Kerjaschki D et al (2002a) Adenoviral VEGF-C overexpression induces blood vessel enlargement, tortuosity, and leakiness but no sprouting angiogenesis in the skin or mucous membranes. FASEB J 16:1041–1049

Saaristo A, Veikkola T, Tammela T, Enholm B, Karkkainen MJ, Pajusola K, Bueler H, Yla-Herttuala S, Alitalo K (2002b) Lymphangiogenic gene therapy with minimal blood vascular side effects. J Exp Med 196:719–730

Saaristo A, Tammela T, Timonen J, Yla-Herttuala S, Tukiainen E, Asko-Seljavaara S, Alitalo K (2004) Vascular endothelial growth factor-C gene therapy restores lymphatic flow across incision wounds. FASEB J 18:1707–1709

Sabin FR (1902) On the origin of the lymphatic system from the veins and the development of the lymph hearts and thoracic duct in the pig. Am J Anat 1:367–391

Saharinen P, Kerkela K, Ekman N, Marron M, Brindle N, Lee GM, Augustin H, Koh GY, Alitalo K (2005) Multiple angiopoietin recombinant proteins activate the Tie1 receptor tyrosine kinase and promote its interaction with Tie2. J Cell Biol 169:239–243

Salmi M, Koskinen K, Henttinen T, Elima K, Jalkanen S (2004) CLEVER-1 mediates lymphocyte transmigration through vascular and lymphatic endothelium. Blood 104:3849–3857

Sato TN, Tozawa Y, Deutsch U, Wolburg-Buchholz K, Fujiwara Y, Gendron-Maguire M, Gridley T, Wolburg H, Risau W, Qin Y (1995) Distinct roles of the receptor tyrosine

kinases Tie-1 and Tie-2 in blood vessel formation. Nature 376:70–74

Schacht V, Ramirez MI, Hong YK, Hirakawa S, Feng D, Harvey N, Williams M, Dvorak AM, Dvorak HF, Oliver G, Detmar M (2003) T1alpha/podoplanin deficiency disrupts normal lymphatic vasculature formation and causes lymphedema. EMBO J 22:3546–3556

Schoppmann SF, Birner P, Stockl J, Kalt R, Ullrich R, Caucig C, Kriehuber E, Nagy K, Alitalo K, Kerjaschki D (2002) Tumor-associated macrophages express lymphatic endothelial growth factors and are related to peritumoral lymphangiogenesis. Am J Pathol 161:947–956

Sebzda E, Hibbard C, Sweeney S, Abtahian F, Bezman N, Clemens G, Maltzman JS, Cheng L, Liu F, Turner M et al (2006) Syk and Slp-76 mutant mice reveal a cell-autonomous hematopoietic cell contribution to vascular development. Dev Cell 11:349–361

Seo S, Fujita H, Nakano A, Kang M, Duarte A, Kume T (2006) The forkhead transcription factors, Foxc1 and Foxc2, are required for arterial specification and lymphatic sprouting during vascular development. Dev Biol 294:458–470

Skobe M, Hamberg LM, Hawighorst T, Schirner M, Wolf GL, Alitalo K, Detmar M (2001a) Concurrent induction of lymphangiogenesis, angiogenesis, and macrophage recruitment by vascular endothelial growth factor-C in melanoma. Am J Pathol 159:893–903

Skobe M, Hawighorst T, Jackson DG, Prevo R, Janes L, Velasco P, Riccardi L, Alitalo K, Claffey K, Detmar M (2001b) Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. Nat Med 7:192–198

Stacker SA, Caesar C, Baldwin ME, Thornton GE, Williams RA, Prevo R, Jackson DG, Nishikawa S, Kubo H, Achen MG (2001) VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. Nat Med 7:186–191

Stacker SA, Achen MG, Jussila L, Baldwin ME, Alitalo K (2002) Lymphangiogenesis and cancer metastasis. Nat Rev Cancer 2:573–583

Tammela T, Alitalo K (2006) Yet another function for hepatocyte growth factor. Blood 107:3424–3425

Tammela T, Petrova TV, Alitalo K (2005a) Molecular lymphangiogenesis: new players. Trends Cell Biol 15:434–441

Tammela T, Saaristo A, Lohela M, Morisada T, Tornberg J, Norrmen C, Oike Y, Pajusola K, Thurston G, Suda T et al (2005b) Angiopoietin-1 promotes lymphatic sprouting and hyperplasia. Blood 105:4642–4648

Valenzuela DM, Griffiths JA, Rojas J, Aldrich TH, Jones PF, Zhou H, McClain J, Copeland NG, Gilbert DJ, Jenkins NA et al (1999) Angiopoietins 3 and 4: diverging gene counterparts in mice and humans. Proc Natl Acad Sci USA 96:1904–1909

Veikkola T, Jussila L, Makinen T, Karpanen T, Jeltsch M, Petrova TV, Kubo H, Thurston G, McDonald DM, Achen MG et al (2001) Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. EMBO J 20:1223–1231

Vlahakis NE, Young BA, Atakilit A, Sheppard D (2005) The lymphangiogenic vascular endothelial growth factors VEGF-C and -D are ligands for the integrin alpha9beta1. J Biol Chem 280:4544–4552

Wang HU, Chen ZF, Anderson DJ (1998) Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. Cell 93:741–753

Wang JF, Zhang X-F, Groopman JE (2001) Stimulation of b1 integrin induces tyrosine phosphorylation of VEGF receptor-3 and modulates cell migration. J Biol Chem 276:41950–41957

Wigle JT, Oliver G (1999) Prox1 function is required for the development of the murine lymphatic system. Cell 98:769–778

Wigle JT, Harvey N, Detmar M, Lagutina I, Grosveld G, Gunn MD, Jackson DG, Oliver G (2002) An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. EMBO J 21:1505–1513

Wilting J, Papoutsi M, Schneider M, Christ B (2000) The lymphatic endothelium of the avian wing is of semitic origin. Dev Dyn 217:271–278

Witte MH, Bernas MJ, Martin CP, Witte CL (2001) Lymphangiogenesis and lymphangiodysplasia: from molecular to clinical lymphology. Microsc Res Tech 55:122–145

Yaniv K, Isogai S, Castranova D, Dye L, Hitomi J, Weinstein BM (2006) Live imaging of lymphatic development in the zebrafish. Nat Med 12:711–716

Yuan L, Moyon D, Pardanaud L, Breant C, Karkkainen MJ, Alitalo K, Eichmann A (2002) Abnormal lymphatic vessel development in neuropilin 2 mutant mice. Development 129:4797–4806

The Relationship Between Tumors and the Lymphatics: Consequences for Metastasis

19

JONATHAN P. SLEEMAN

Abstract

Metastasis of tumor cells via the lymphatic vasculature is of major clinical relevance, and lymph node metastases are highly prognostically significant. Factors such as the structure of the lymphatic capillaries, tumor-induced lymphangiogenesis and the gene expression profiles expressed by the tumor cells all contribute to this process. Despite rapid progress in recent years, there is still much to be learned about the relationship between tumors and the lymphatics, particularly with regard to its significance in regulating metastatic spread to organs other than lymph nodes.

CONTENTS

19.1 Introduction

It is widely recognized that metastasis is the major cause of death for cancer patients. Aside from metastasis by direct extension, which plays a significant role in only a few types of cancers, solid tumors disseminate by entering and being transported in vessels of the circulatory system (Fig. 19.1). Invasive tumor cells can either enter directly into the blood vasculature and thereby be transported to other organs, or they can enter the lymphatics and be transported to regional lymph nodes (reviewed in Sleeman 2000). Within the lymph nodes, these tumor cells either die, remain dormant, proliferate or pass through the node. Proliferation results in the formation of lymph node metastases, either within the subcapsular lumen of the lymph node or within the parenchyma of the lymph node after tumor cells invade into it. These metastases can

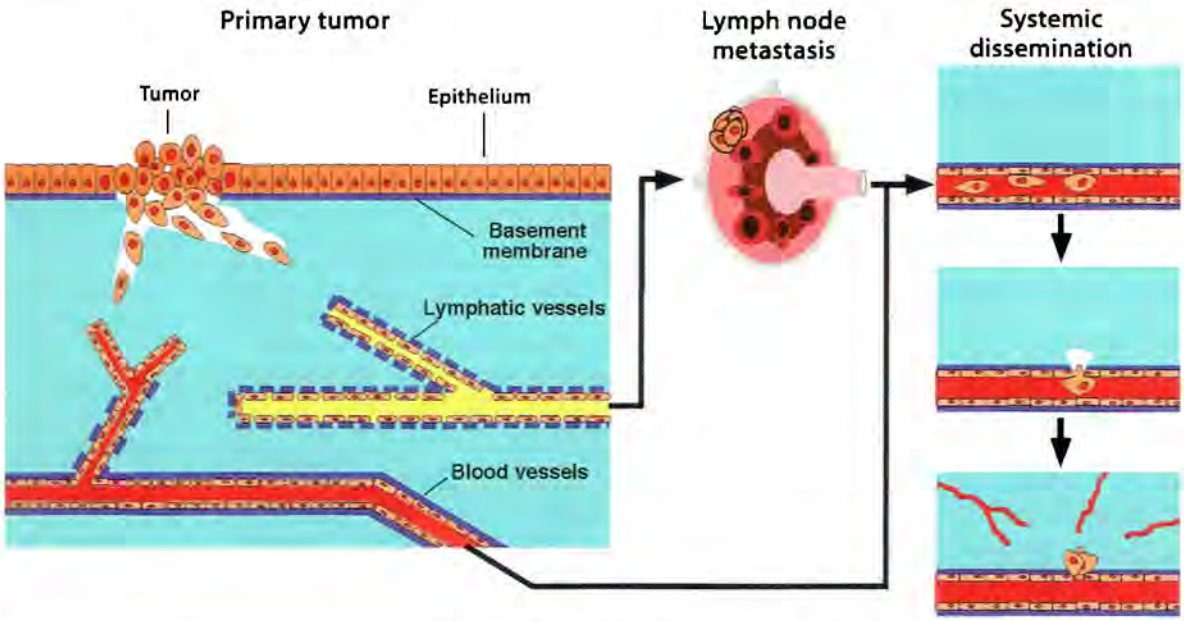


Fig. 19.1. Major routes of dissemination for metastasizing tumors. Invasive tumor cells may enter the blood vasculature and thereby be transported to distant organs, where they extravasate and go on to form angiogenic tumors. Alternatively they can enter the lymphatic system. Within the lymphatic system they may form metastases in regional lymph nodes that then shed further tumor cells into the efferent lymphatics. Tumor cells within the lymphatics ultimately empty into the blood stream via anastomoses, generally the thoracic duct

then seed further metastatic tumor cells into the efferent lymphatics that in turn form metastases in lymph nodes higher up the lymphatic drainage, or ultimately enter the blood stream via anastomoses, in the main via the thoracic duct. Thus there are two routes by which tumors cells can ultimately reach vital organs such as the lungs and liver (Fig. 19.1): directly via the blood circulatory system or indirectly via the lymphatics before entering the blood circulatory system via anastomoses.

A number of observations suggest that the indirect route to vital organs via the lymph nodes plays a central role in the metastatic process. For carcinomas, regional lymph nodes are frequently colonized by metastatic cells and are often the first sites of metastatic spread (Leong et al. 2006). Frequent and early lymph node involvement is also a typical feature of melanomas (Leiter et al. 2004). Early lymph node involvement is the foundation of tumor staging schemes and assessment of prognosis for many tumor types (Beahrs and Myers 1983), and further gives rise to the concept of the sentinel lymph node, which is the basis of sentinel lymphonodectomy (Cabanas 1977). The sentinel node is the first lymph node in a lymphatic basin that receives lymph flow from a primary tumor, and should therefore be the first to receive metastatic cells from the tumor. Analysis of the sentinel node for the presence of metastases should therefore indicate whether more distant metastases are likely. Follow-up analysis of sentinel lymphonodectomies suggests that only 20% of systemic metastases are derived from tumor cells that bypass the lymphatic route (Leong et al. 2006).

Lymph node metastases are often the first indication that tumor progression has occurred, despite the fact that tumor cells can be observed in the blood early after tumorigenesis. Furthermore, the prognostic significance of circulating tumor cells in the blood remains open (Pantel et al. 2003; Pierga et al. 2004). Moreover, in animal models it has been demonstrated that well-differentiated, non-metastasizing tumors shed more than a million

tumor cells per gram of tumor into the blood system every day without giving rise to systemic metastases (Butler and Gullino 1975). The analysis of metastases in patients with inoperable cancer who had peritovenous shunts to relieve the symptoms of ascites also illustrates the same principle, namely that the presence of tumor cells in the blood does not necessarily equate with metastasis (Tarin et al. 1984). In this study, despite the fact that the patients received direct infusion of tumor cells into the bloodstream, not all patients developed metastases despite long survival times, although quiescent tumor cells were detectable in tissues of some patients. In those patients in which metastases did form, they were small and asymptomatic.

After consideration of these observations, the question therefore arises as to why the lymphatic route is preferentially used during tumor dissemination, and why lymph node metastases are of such important prognostic significance. These questions are the focus of this chapter.

The Structure and Function of Lymphatic Capillaries Facilitates Metastasis via the Lymphatics

Several biological and physical attributes make the lymphatic system a relatively easy entry site into the circulatory system for tumor cells, and also favor metastasis formation (reviewed in Sleeman et al. 2001). In comparison to blood capillaries, the lymphatic endothelium has loose junctions which readily permit the passage of large biological macromolecules, pathogens and migrating cells, including tumor cells. Furthermore, the lymphatic capillaries have no or at best only an incomplete basement membrane, in contrast to the basement membrane of blood vessels, which tumor cells need to penetrate into order to enter and exit the circulatory system. These features mean that the lymphatic system is relatively easy for tumor cells to access and disseminate in. Furthermore, the high interstitial pressure within tumors means that interstitial fluid flows away from the tumors into the draining lymphatics (Baxter and Jain 1989; Jain 1989), and therefore tumor cells which have detached from the primary tumor mass will be channeled into local lymphatic vessels by the flow of lymphatic fluid. Moreover, the flow of lymphatic fluid is passive, at least in the peripheral lymphatic vessels. Tumor cells in lymphatic capillaries are therefore not subject to the same shear forces experienced by tumor cells in the blood system, substantially reducing shear stress-mediated attrition of circulating tumor cells within the lymphatic capillaries in comparison to tumor cells within the blood vasculature.

Within the lymph nodes the tumor cells are channeled into a confined space, the subcapsular sinuses, and thus the filter function of the lymph node increases local tumor cell concentration. This is in sharp contrast to cells in the blood system, which are distributed over a large capillary bed in the next organ they reach after entering the blood stream, for example the lung or liver. This aggregation of tumor cells in the subcapsular sinuses of lymph nodes may be a critical factor in understanding the propensity of metastasizing tumor cells to initially colonize the regional lymph nodes, as it is well established that emboli or clusters of tumor cells form metastases much more efficiently than single tumor cells (Nicolson 1988). Furthermore, metastasis formation can occur directly within the subcapsular space, which obviates the need for metastasizing cells to possess properties necessary for extravasation, as would be required for tumor cells within the blood circulatory system, further increasing the efficiency of metastasis formation.

The relative ease with which tumor cells can enter the lymphatic system and form metastases within regional lymph nodes means that the selective pressure put to bear on the metastasizing tumor cells is less than that experienced by tumor cells within the blood. This could conceivably have several consequences (reviewed in Sleeman 2000). Firstly, the formation of metastases via the lymphatics is likely to be more efficient, as cells with metastatic potential survive better and metastasize more easily. Secondly, tumor cell subpopulations that have acquired

metastatic potential and that have successfully metastasized to regional lymph nodes are amplified as a result of the growth of the lymph node metastasis. Subsequent shedding from these metastases of tumor cells that go on to enter the blood stream means that the number of circulating metastatically competent tumor cells in the blood increases, thereby increasing the efficiency of systemic metastasis formation.

Tumor-induced Lymphangiogenesis Promotes Lymph Node Metastasis Formation

The metastatically advantageous structure and function of the lymphatics is not the only reason why the lymphatic route plays a decisive role in metastatic dissemination. In recent years it has become clear that an inductive relationship that promotes metastasis to regional lymph nodes exists between tumors and the lymphatics, namely tumor-induced lymphangiogenesis (Alitalo et al. 2005). Lymphangiogenesis describes the process of new lymphatic vessel formation. The paradigm that has emerged is that during tumor progression tumors begin to produce factors that promote lymphangiogenesis (Fig. 19.2). This increases the density of lymphatic vessels in the vicinity of the tumor, either by increasing the number of vessels through the promotion of sprouting lymphangiogenesis, or by increasing lymphatic vessel diameter. Thereby the probability that invasive tumor cells are able to enter the lymphatics and subsequently to be transported to regional lymph nodes is increased. Thus metastasis to regional lymph nodes is promoted.

Evidence supporting the concept that tumor-induced lymphangiogenesis promotes metastasis to regional lymph nodes comes from both experimental animal studies and human tumor data. Most work in this area has focused on VEGFR-3, a transmembrane receptor tyrosine kinase that is located in the plasma membrane of lymphatic endothelial cells (Tammela et al. 2005). It is activated in response to its two known ligands, VEGF-C and VEGF-D, and thereby orchestrates lymphangiogenesis. In the past couple of years it has, however, become apparent in other contexts that a variety of other receptor–ligand pairs are able to regulate lymphangiogenesis, including VEGF-A, hepatocyte growth factor and members of the fibroblast growth factor, angiopoietin, platelet-derived growth factor and insulin-like growth factor families (reviewed in Achen and Stacker 2006). Many of these factors are also expressed in the context of tumors, and it remains to be seen to what extent they play a role in regulating tumor-induced lymphangiogenesis in comparison to VEGFR-3 and its ligands.

The notion that tumor-induced lymphangiogenesis driven by VEGFR-3 activation is able to promote metastasis to regional lymph nodes has been substantiated in a number of animal models in which VEGFR-3-dependent lymphangiogenesis has been modulated in the context of experimental tumors. We and others have shown that experimental overexpression of VEGF-C or VEGF-D stimulates lymphangiogenesis in the vicinity of tumors and is sufficient to promote metastasis to regional lymph nodes (Mandriota et al. 2001; Karpanen et al. 2001; Skobe et al. 2001; Stacker et al. 2001; Yanai et al. 2001; He et al. 2002; Mattila et al. 2002; Krishnan et al. 2003). Conversely, blockade of the activity of VEGF-C and VEGF-D in tumors using soluble VEGFR-3 receptor proteins or VEGFR-3-blocking antibody inhibits tumor-induced lymphangiogenesis and suppresses the metastasis of tumors to regional lymph nodes (He et al. 2002, 2005; Krishnan et al. 2003; Shimizu et al. 2004; Lin et al. 2005). The onset of lymphangiogenesis is later than angiogenesis and functionally correlates with lymph node metastasis formation (He et al. 2005). These data support the notion that enhanced numbers or dilation of lymphatic vessels in the vicinity of tumors promotes metastasis to regional lymph nodes by providing a greater number of entry sites into the lymphatic system for invading tumor cells.

Additional evidence supporting the notion that VEGFR-3-regulated tumor-induced lymphangiogenesis is important in the regulation of lymph node

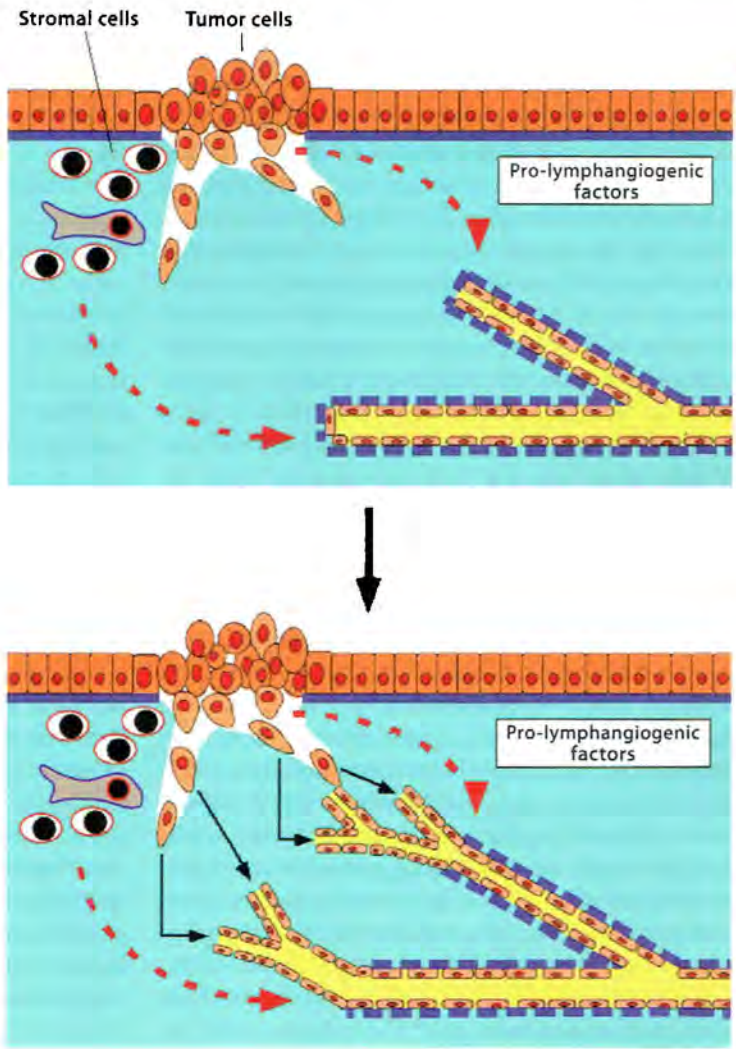


Fig. 19.2. Tumor-induced lymphangiogenesis promotes metastasis via the lymphatics. The production of pro-lymphangiogenic factors by tumor cells or tumor-associated stromal cells such as macrophages and fibroblasts stimulates expansion of the lymphatic vasculature in the vicinity of the tumor. Invasive tumor cells therefore have a much higher chance of penetrating and entering the lymphatic system. In turn, this potentiates the formation of lymph node metastases

metastasis formation comes from the analysis of human tumor material. A large body of literature now exists in which VEGF-C or VEGF-D expression or tumor-associated lymphatic vessel density has been compared with lymph node metastasis formation and prognosis in human tumors (reviewed in Thiele and Sleeman 2006). In many different types of cancer, a statistically significant correlation or at least a tendency has been demonstrated between VEGF-C or VEGF-D expression or lymphatic vessel density, and lymph node metastasis formation and prognosis.

19.4 The Inductive Relationship Between Tumors and the Lymphatics: More than Lymphangiogenesis?

Not all studies find a statistically significant correlation between VEGF-C and VEGF-D expression and lymphatic density, regional lymph node metastasis formation or poor prognosis, and the situation regarding tumor-induced lymphangiogenesis appears more complex than first thought (Thiele and Sleeman

2006). Reasons for this include the possible multifactorial regulation of tumor-induced lymphangiogenesis by mechanisms other than the regulation of VEGFR-3, for example by the pro-lymphangiogenic factors listed above. Furthermore, if the primary tumor is located in a tissue that has a relatively high lymphatic vascular density, entry of invasive tumor cells into the lymphatics may occur efficiently in the absence of neo-lymphangiogenesis. Moreover, several studies provide evidence that tumors can coopt pre-existing lymphatic vessels, again probably affording efficient entry of invasive tumor cells into the lymphatics (e.g. Agarwal et al. 2005).

Several studies report a higher peritumoral lymphatic vessel density at the invasive border of tumors in the absence of intratumoral lymphatics. However, there is also now compelling evidence that lymphangiogenesis can also occur within the tumor, although it is important to bear in mind that these studies often rely on Lyve-1 as a single marker of lymphatic vessels (reviewed in Thiele and Sleeman 2006). The extent to which the observed intratumoral lymphatics are really lymphatic capillaries that have been coopted by tumors, or whether their existence is due to lymphangiogenesis is still unclear. The proliferative activity of intratumoral lymphatics observed in head and neck cancer argues against the hypothesis of cooption of lymphatics by the tumor in these cases (Beasley et al. 2002; Kyzas et al. 2005). Furthermore, tumor emboli have been reported within proliferating intratumoral lymphatics (Kyzas et al. 2005), although other studies suggest that intratumoral lymphatics may not be functional (Padera et al. 2002). What regulates whether lymphangiogenesis occurs peritumorally, intratumorally or both is not clear. We also have no clear idea of the relative importance of the peritumoral lymphatics compared to intratumoral lymphatics in terms of entry of tumor cells into the lymphatics, their dissemination therein, and subsequent lymph node metastasis formation. The literature contains contradictory findings (e.g. Maula et al. 2003; Franchi et al. 2004).

It has recently emerged that intratumoral lymphatic vessels may be formed at least in part by vascular mimicry or transdifferentiation. Several studies have shown that circulating CD11b-positive, LYVE1-positive macrophages are able to integrate into lymphatic vessels and to form lumen-containing capillaries (Maruyama et al. 2005; Kerjaschki et al. 2006). This has also been reported for tumor-associated lymphatics (Schledzewski et al. 2006). The relative contribution of these macrophage-derived cells to the lymphatic vasculature in the context of tumors, the regulation of their recruitment into the lymphatic capillaries and their importance for metastasis via the lymphatics remains to be demonstrated. Initial observations with animal models suggest that lymphangiogenesis from pre-existing lymphatic vessels accounts for most of the tumor-associated lymphatics (He et al. 2005), while studies with human melanomas suggest that a significant proportion of intratumoral lymphatics in melanomas may be derived from macrophage precursors (Schledzewski et al. 2006).

A recent study suggests that tumors may induce lymphangiogenesis not only in their immediate vicinity, but also distally. Hirakawa et al. (2007) have reported that VEGF-C-expressing tumors also induce lymphangiogenesis in sentinel lymph nodes in addition to the local tumor environment. Sentinel lymph node lymphangiogenesis occurs before metastatic cells enter the lymph nodes and is further augmented once metastases form. The authors also suggest that sentinel lymph node lymphangiogenesis may be connected with metastasis to other organs, although this remains to be functionally demonstrated.

Tumor-intrinsic Changes in Gene Expression That Promote Metastasis to Regional Lymph Nodes

The observation that tumor cells often acquire the ability to express lymphangiogenesis-inducing factors during tumor progression touches on the broader point of tumor-intrinsic changes in gene expression that promote metastasis via the lymphatics. A long-standing notion is that metastatic tumor

cells represent subpopulations of tumor cells that have acquired gene expression patterns prerequisite for successful dissemination through the effects of genomic instability and subsequent selection (Fidler and Kripke 1977; Fidler 2002). However, recent advances in gene expression profiling techniques have led to an alternative concept, namely that the original oncogenic insults that lead to transformation lay down the basic gene expression profile framework that determines the metastatic propensity of the tumor (reviewed in Weigelt et al. 2005). In addition, regional changes in gene expression within tumors, for example at the invasive front, may be decisive (e.g. Gavert et al. 2005). Whatever mechanism lies behind tumor-intrinsic metastasis-associated gene expression patterns, clearly gene expression patterns within tumor cells must contribute to the process of metastasis via the lymphatic system. These gene expression patterns may act generally to promote tumor cell motility and invasiveness, endowing the tumor cells with the potential to escape from the primary tumor and enter both the bloodstream and the lymphatics. However, there are indications that specific changes in gene expression may promote homing of disseminating tumor cells to the lymphatics. For example, certain chemokines and their receptors have been associated with metastasis formation in lymph nodes (Muller et al. 2001; Kaifi et al. 2005).

Clearly there is still a lot to be understood about the tumor-intrinsic genetic determinants that contribute to lymphatic metastasis. An additional complicating factor is the realization that cancer stem cells (otherwise called tumor-initiating cells) exist not only in leukemias but also in solid tumors. Within a population of tumor cells it is thought that the majority of the cancer cells have a limited ability to divide, but that a small subpopulation of cancer stem cells has the exclusive ability to extensively proliferate and form new tumors (Al Hajj and Clarke 2004). These cells drive the growth of tumors and metastases. The role of cancer stem cells in the process of metastasis is currently the subject of speculation (Brabletz et al. 2005), and so far no studies have addressed their relationship to metastasis via the lymphatics.

The Broader Significance of Lymph Node Metastases in the Course of Tumor Progression: Indicators or Players?

The predilection of carcinomas to metastasize via the lymphatics, together with the observations that tumor-induced lymphangiogenesis is able to promote metastasis to regional lymph nodes, has given credence to the idea that it might be possible to block or retard the process of metastasis by controlling the entry of tumor cells into the lymphatics. To this end there is currently great interest in identifying ways of inhibiting tumor-induced lymphangiogenesis (McColl et al. 2005). However, for such approaches to be effective, lymph node metastases need to play a significant role in the systemic dissemination of tumors, as they are themselves not usually life-threatening and can in the main be successfully treated surgically. Thus a major outstanding issue that needs to be resolved is the extent to which regional lymph node metastases are only indicators that metastatic progression has occurred in the primary tumor and do not contribute themselves to systemic dissemination to any great extent, compared to the possibility that lymph node metastases are important players in systemic dissemination by seeding metastatic tumor cells into the blood that go on to colonize vital organs.

Clinical studies suggest that for a variety of tumor types there is no survival difference between cancer patients whose regional lymph nodes are removed and those who receive only partial or no dissection of these nodes (Gervasoni et al. 2000; Thiele and Sleeman 2006). These data seem to speak for an indicator function for lymph node metastases, and at first sight do not support the notion that lymph node metastases play a major role in systemic dissemination. There are a number of important caveats, however, not least that surgical intervention may have disrupted the normal lymphatic connections in the tissues concerned, and thus metastasis via the lymphatics might not necessarily be able to proceed in the normal way.

While there are difficulties in directly addressing the role of lymph node metastases in the further dissemination of human cancers, experimental tumors in animal models are perhaps more informative. In some studies it was observed that upon stimulating tumor-induced lymphangiogenesis, not only was metastasis to regional lymph nodes promoted, but also metastasis to the lungs (Skobe et al. 2001; Krishnan et al. 2003; Hirakawa et al. 2007). Importantly, suppression of tumor-induced lymphangiogenesis inhibited the formation of metastases not only in regional lymph nodes, but also in the lung (Krishnan et al. 2003). These observations provide evidence that tumor-induced lymphangiogenesis can promote metastasis formation not only in the regional lymph nodes, but also in other organs. However, this has not been observed in all studies. Padera et al. (2002) reported that VEGF-C overexpression in B16 melanomas and T241 fibrosarcomas stimulated metastasis formation in regional lymph nodes, but had no influence on metastasis to the lung.

Conclusions

Recent advances in understanding the complex relationship between tumor cells and the lymphatics and the molecular understanding of how lymphangiogenesis is controlled have opened up new avenues for possible therapeutic intervention in metastatic progression. However, there are still a large number of open questions regarding the nature, regulation and function of tumor-associated lymphatics. Most importantly, understanding the role and significance of regional lymph node metastases in the systemic dissemination of tumors will be pivotal for determining whether or not manipulation of the relationship between tumor cells and the lymphatics can make a meaningful contribution to the control of neoplastic disease.

Acknowledgements

This work was supported by a grant from the Deutsche Forschungsgemeinschaft under the auspices of SPP 1190 "The tumor-vessel interface".

References

Achen MG, Stacker SA (2006) Tumor lymphangiogenesis and metastatic spread - new players begin to emerge. Int J Cancer 119:1755–1760

Agarwal B, Saxena R, Morimiya A, Mehrotra S, Badve S (2005) Lymphangiogenesis does not occur in breast cancer. Am J Surg Pathol 29:1449–1455

Al-Hajj M, Clarke MF (2004) Self-renewal and solid tumor stem cells. Oncogene 23:7274–7282

Alitalo K, Tammela T, Petrova TV (2005) Lymphangiogenesis in development and human disease. Nature 438:946–953

Baxter LT, Jain RK (1989) Transport of fluid and macromolecules in tumors. I. Role of interstitial pressure and convection. Microvasc Res 37:77–104

Beahrs O, Myers M (1983) Purposes and principles of staging. In: Beahrs O, Myers M (eds) Manual for staging of cancer. Lippincott, Philadelphia, pp 3–5

Beasley NJ, Prevo R, Banerji S, Leek RD, Moore J, van Trappen P, Cox G, Harris AL, Jackson DG (2002) Intratumoral lymphangiogenesis and lymph node metastasis in head and neck cancer. Cancer Res 62:1315–1320

Brabletz T, Jung A, Spaderna S, Hlubek F, Kirchner T (2005) Opinion: migrating cancer stem cells - an integrated concept of malignant tumour progression. Nat Rev Cancer 5:744–749

Butler TP, Gullino PM (1975) Quantitation of cell shedding into efferent blood of mammary adenocarcinoma. Cancer Res 35:512–516

Cabanas RM (1977) An approach for the treatment of penile carcinoma. Cancer 39:456–466

Fidler IJ (2002) Critical determinants of metastasis. Semin Cancer Biol 12:89–96

Fidler IJ, Kripke ML (1977) Metastasis results from preexisting variant cells within a malignant tumor. Science 197:893–895

Franchi A, Gallo O, Massi D, Baroni G, Santucci M (2004) Tumor lymphangiogenesis in head and neck squamous cell carcinoma: a morphometric study with clinical correlations. Cancer 101:973–978

Gavert N, Conacci-Sorrell M, Gast D, Schneider A, Altevogt P, Brabletz T, Ben-Ze'ev A (2005) L1, a novel target of beta-catenin signaling, transforms cells and is expressed at the invasive front of colon cancers. J Cell Biol 168:633–642

Gervasoni JE Jr, Taneja C, Chung MA, Cady B (2000) Axillary dissection in the context of the biology of lymph node metastases. Am J Surg 180:278–283

He Y, Kozaki K, Karpanen T, Koshikawa K, Yla-Herttuala S, Takahashi T, Alitalo K (2002) Suppression of tumor lymphangiogenesis and lymph node metastasis by blocking vascular endothelial growth factor receptor 3 signaling. J Natl Cancer Inst 94:819–825

He Y, Rajantie I, Pajusola K, Jeltsch M, Holopainen T, Yla-Herttuala S, Harding T, Jooss K, Takahashi T, Alitalo K (2005) Vascular endothelial cell growth factor receptor 3-mediated activation of lymphatic endothelium is crucial for tumor cell entry and spread via lymphatic vessels. Cancer Res 65:4739–4746

Hirakawa S, Brown LF, Kodama S, Paavonen K, Alitalo K, Detmar M (2007) VEGF-C-induced lymphangiogenesis in sentinel lymph nodes promotes tumor metastasis to distant sites. Blood 109:1010–1017

Jain RK (1989) Delivery of novel therapeutic agents in tumors: physiological barriers and strategies. J Natl Cancer Inst 81:570–576

Kaifi JT, Yekebas EF, Schurr P, Obonyo D, Wachowiak R, Busch P, Heinecke A, Pantel K, Izbicki JR (2005) Tumor-cell homing to lymph nodes and bone marrow and CXCR4 expression in esophageal cancer. J Natl Cancer Inst 97:1840–1847

Karpanen T, Egeblad M, Karkkainen MJ, Kubo H, Yla-Herttuala S, Jaattela M, Alitalo K (2001) Vascular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. Cancer Res 61:1786–1790

Kerjaschki D, Huttary N, Raab I, Regele H, Bojarski-Nagy K, Bartel G, Krober SM, Greinix H, Rosenmaier A, Karlhofer F, Wick N, Mazal PR (2006) Lymphatic endothelial progenitor cells contribute to de novo lymphangiogenesis in human renal transplants. Nat Med 12:230–234

Krishnan J, Kirkin V, Steffen A, Hegen M, Weih D, Tomarev S, Wilting J, Sleeman JP (2003) Differential in vivo and in vitro expression of vascular endothelial growth factor (VEGF)-C and VEGF-D in tumors and its relationship to lymphatic metastasis in immunocompetent rats. Cancer Res 63:713–722

Kyzas PA, Geleff S, Batistatou A, Agnantis NJ, Stefanou D (2005) Evidence for lymphangiogenesis and its prognostic implications in head and neck squamous cell carcinoma. J Pathol 206:170–177

Leiter U, Meier F, Schittek B, Garbe C (2004) The natural course of cutaneous melanoma. J Surg Oncol 86:172–178

Leong SP, Cady B, Jablons DM, Garcia-Aguilar J, Reintgen D, Jakub J, Pendas S, Duhaime L, Cassell R, Gardner M, Giuliano R, Archie V, Calvin D, Mensha L, Shivers S, Cox C, Werner JA, Kitagawa Y, Kitajima M (2006) Clinical patterns of metastasis. Cancer Metastasis Rev 25:221–232

Lin J, Lalani AS, Harding TC, Gonzalez M, Wu WW, Luan B, Tu GH, Koprivnikar K, VanRoey MJ, He Y, Alitalo K, Jooss K (2005) Inhibition of lymphogenous metastasis using adeno-associated virus-mediated gene transfer of a soluble VEGFR-3 decoy receptor. Cancer Res 65:6901–6909

Mandriota SJ, Jussila L, Jeltsch M, Compagni A, Baetens D, Prevo R, Banerji S, Huarte J, Montesano R, Jackson DG, Orci L, Alitalo K, Christofori G, Pepper MS (2001) Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. EMBO J 20:672–682

Maruyama K, Ii M, Cursiefen C, Jackson DG, Keino H, Tomita M, Van Rooijen N, Takenaka H, D'Amore PA, Stein-Streilein J, Losordo DW, Streilein JW (2005) Inflammation-induced lymphangiogenesis in the cornea arises from CD11b-positive macrophages. J Clin Invest 115:2363–2372

Mattila MM, Ruohola JK, Karpanen T, Jackson DG, Alitalo K, Harkonen PL (2002) VEGF-C induced lymphangiogenesis is associated with lymph node metastasis in orthotopic MCF-7 tumors. Int J Cancer 98:946–951

Maula SM, Luukkaa M, Grenman R, Jackson D, Jalkanen S, Ristamaki R (2003) Intratumoral lymphatics are essential for the metastatic spread and prognosis in squamous cell carcinomas of the head and neck region. Cancer Res 63:1920–1926

McColl BK, Loughran SJ, Davydova N, Stacker SA, Achen MG (2005) Mechanisms of lymphangiogenesis: targets for blocking the metastatic spread of cancer. Curr Cancer Drug Targets 5:561–571

Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, Verastegui E, Zlotnik A (2001) Involvement of chemokine receptors in breast cancer metastasis. Nature 410:50–56

Nicolson GL (1988) Organ specificity of tumor metastasis: role of preferential adhesion, invasion and growth of malignant cells at specific secondary sites. Cancer Metastasis Rev 7:143–188

Padera TP, Kadambi A, di Tomaso E, Carreira CM, Brown EB, Boucher Y, Choi NC, Mathisen D, Wain J, Mark EJ, Munn LL, Jain RK (2002) Lymphatic metastasis in the absence of functional intratumor lymphatics. Science 296:1883–1886

Pantel K, Muller V, Auer M, Nusser N, Harbeck N, Braun S (2003) Detection and clinical implications of early systemic tumor cell dissemination in breast cancer. Clin Cancer Res 9:6326–6334

Pierga JY, Bonneton C, Vincent-Salomon A, de Cremoux P, Nos C, Blin N, Pouillart P, Thiery JP, Magdelenat H (2004) Clinical significance of immunocytochemical detection of tumor cells using digital microscopy in peripheral blood and bone marrow of breast cancer patients. Clin Cancer Res 10:1392–1400

Schledzewski K, Falkowski M, Moldenhauer G, Metharom P, Kzhyshkowska J, Ganss R, Demory A, Falkowska-Hansen B, Kurzen H, Ugurel S, Geginat G, Arnold B, Goerdt S (2006) Lymphatic endothelium-specific hyaluronan receptor LYVE-1 is expressed by stabilin-1+, F4/80+, CD11b+ macrophages in malignant tumours and wound

healing tissue in vivo and in bone marrow cultures in vitro: implications for the assessment of lymphangiogenesis. J Pathol 209:67–77

Shimizu K, Kubo H, Yamaguchi K, Kawashima K, Ueda Y, Matsuo K, Awane M, Shimahara Y, Takabayashi A, Yamaoka Y, Satoh S (2004) Suppression of VEGFR-3 signaling inhibits lymph node metastasis in gastric cancer. Cancer Sci 95:328–333

Skobe M, Hamberg LM, Hawighorst T, Schirner M, Wolf GL, Alitalo K, Detmar M (2001) Concurrent induction of lymphangiogenesis, angiogenesis, and macrophage recruitment by vascular endothelial growth factor-C in melanoma. Am J Pathol 159:893–903

Sleeman JP (2000) The lymph node as a bridgehead in the metastatic dissemination of tumors. Rec Res Cancer Res 157:55–81

Sleeman JP, Krishnan J, Kirkin V, Baumann P (2001) Markers for the lymphatic endothelium: in search of the holy grail? Microsc Res Tech 55:61–69

Stacker SA, Caesar C, Baldwin ME, Thornton GE, Williams RA, Prevo R, Jackson DG, Nishikawa S, Kubo H, Achen MG (2001) VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. Nat Med 7:186–191

Tammela T, Enholm B, Alitalo K, Paavonen K (2005) The biology of vascular endothelial growth factors. Cardiovasc Res 65:550–563

Tarin D, Price JE, Kettlewell MG, Souter RG, Vass AC, Crossley B (1984) Mechanisms of human tumor metastasis studied in patients with peritoneovenous shunts. Cancer Res 44:3584–3592

Thiele W, Sleeman JP (2006) Tumor-induced lymphangiogenesis: a target for cancer therapy? J Biotechnol 124:224–241

Weigelt B, Peterse JL, van 't Veer LJ (2005) Breast cancer metastasis: markers and models. Nat Rev Cancer 5:591–602

Yanai Y, Furuhata T, Kimura Y, Yamaguchi K, Yasoshima T, Mitaka T, Mochizuki Y, Hirata K (2001) Vascular endothelial growth factor C promotes human gastric carcinoma lymph node metastasis in mice. J Exp Clin Cancer Res 20:419–428

Inflammation and Angiogenesis: Innate Immune Cells as Modulators of Tumor Vascularization

20

MARGARETA M. MUELLER

Abstract

Inflammation is an essential process for survival and for the physiological defense against pathogens. After early reports by Virchow suggesting a functional connection between inflammation and cancer, the pathological role of inflammation in promoting tumor growth and invasion has recently again entered the focus of attention. There is now clear evidence that one essential contribution of the inflammatory infiltrate to tumor growth is the stimulation of angiogenesis. Different cell types of the innate immune system, particularly macrophages, mast cells, and neurophils, play an active role in enhancing tumor angiogenesis – either directly, via the release of vesicle-stored growth factors, cytokines and proteolytic enzymes, or indirectly, via paracrine signalling cascades. This concept of an indirect inflammation-dependent induction of angiogenesis places inflammation as a target for tumor therapy and, even better, for the prevention of tumor angiogenesis by anti-inflammatory agents.

CONTENTS

20.1 Introduction

Inflammation is an age-old process that has proved essential for survival. As a crucial function of the innate immune system it protects against pathogens by destroying infectious agents, gathers intelligence for the immune system by initiating specific and long-term immunity, and repairs damaged tissue. Acute inflammation is a rapid self-limiting process that is closely interlaced with the process of repair and reconstruction. However, it does not always resolve in due time, but may be maintained for a prolonged time and/or become chronic. There is increasing evidence that chronic, often subclinical

inflammation lies at the basis of many of the diseases of advanced age, such as arthritis, heart attack, Alzheimer's disease or cancer. The association between inflammation and cancer was discovered as early as 1863 by Virchow, who first described the presence of a leukocytic infiltrate in tumor tissues and concluded that there ought to be a functional connection between inflammation and cancer (Virchow 1863). Indeed, population-based studies show that the susceptibility to cancer increases when tissues are chronically inflamed and that long-term use of non-steroidal anti-inflammatory drugs reduces the risk of several cancers (Gupta and Dubois 2001; Sugar 2006). Thus, in the past decade the concept of Virchow has re-emerged, and inflammation is now considered a key factor in many cancers (Balkwill et al. 2005; Mueller 2006). The tumor cell, having lost its normal growth control program, alters the microenvironment and recapitulates a range of effects typical of an injury, such as the inflammatory response and the formation of new blood vessels – the angiogenesis process. While most of the studies on angiogenesis in tumor growth and development have concentrated on the endothelial cells and their activation by tumor cell-derived factors, recent data suggest that inflammatory cells infiltrating the tumor can contribute to angiogenesis by activating and recruiting endothelial cells. Indeed, even physiological inflammation involves, after an initial leukocyte intervention, the activation of stromal and endothelial cell as well as angiogenesis. Ultimately this results in newly formed blood vessels providing nutrients to the repairing tissue and allowing the trafficking of immune cells (Albini et al. 2005). This review will concentrate on the indirect effect of the inflammatory infiltrate on tumor angiogenesis and highlight the experimental evidence for a functional connection between the two processes and the role played in this connection by different cell types of the innate immune system (Fig. 20.1).

Angiogenesis and the Inflammatory Infiltrate

Under physiological conditions, angiogenesis is dependent on the balance between positive and negative angiogenic modulators within the vascular microenvironment and requires the activities of a number of factors, including angiogenic growth factors, extracellular matrix (ECM) proteins, adhesion receptors and proteolytic enzymes (Hanahan and Folkman 1996). Accordingly, an angiogenic endothelial cell is characterized by the expression of a specific set of proteolytic enzymes and adhesion molecules (Korff and Augustin 1999). There is a tight interplay between the activated endothelial cells and the innate immune system. In response to the expression of specific growth factors, activated endothelial cells mediate leukocyte recruitment into tissues by the expression of the appropriate adhesion molecules. In this context tumor necrosis factor alpha (TNF alpha) seems to play a central role. TNF alpha and also interleukin-1 (IL-1) augment the expression of adhesion molecules such as E-selectin and vascular adhesion molecule-1 (VCAM-1) on endothelial cells, thereby promoting leukocyte adhesion and homing to sites of inflammation (Balkwill 2002). Accordingly, continuous endothelial cell activation by transfection with an uncleavable mutant transmembrane form of TNF alpha leads to increased expression of inflammatory cell adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and VCAM-1 (Rajashekhar et al. 2006). Even the well-known angiogenic factor basic fibroblast growth factor (bFGF) activates endothelial cells by increasing their sensitivity to inflammatory stimuli such as TNF alpha, again linking angiogenesis and inflammation through the upregulation of leukocyte adhesion molecules (Zittermann and Issekutz 2006). Interestingly, this endothelial cell activation in response to TNF alpha and the resulting enhancement in leukocyte adhesion to activated endothelial cells seems regulated in an autocrine manner by endothelial cell-derived angiopoietin-1 (Ang-2). Its lack causes a down-regulation of the TNF alpha-

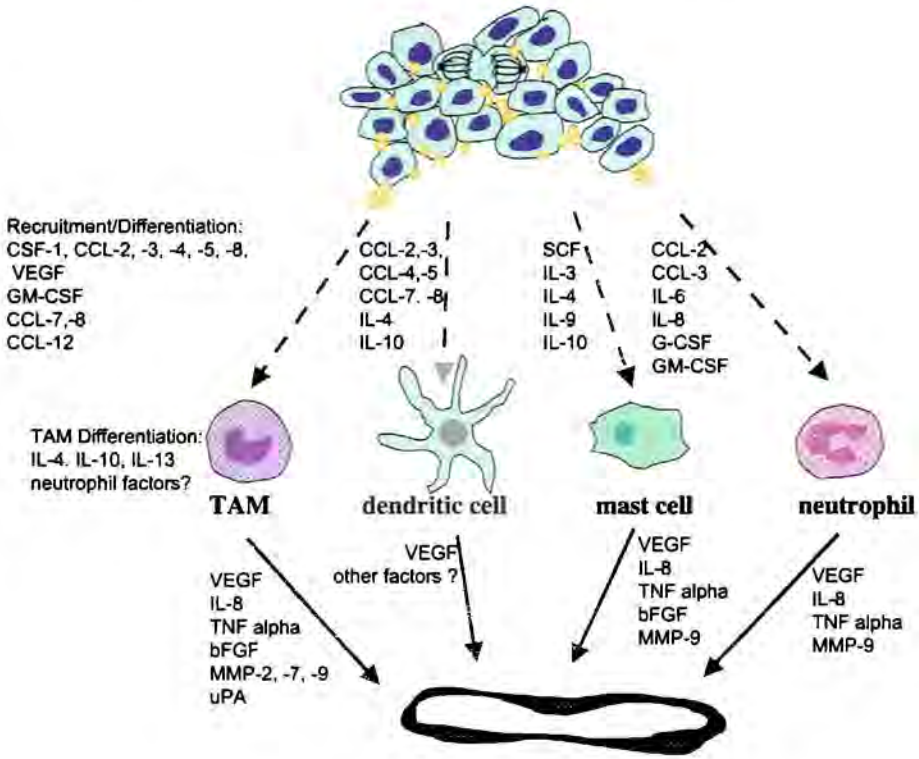


Fig. 20.1. Tumor cells secrete a number of recruitment and differentiation factors that stimulate the recruitment of inflammatory cells into the tumor microenvironment. The activated inflammatory cells contribute to angiogenesis by the secretion of potent angiogenic growth factors and of proteases

induced expression of ICAM-1 and VCAM-1, thereby resulting in defects in the adhesion of the rolling leukocytes and thus in the endothelial cell-mediated recruitment of leukocytes that is essential for the inflammatory response (Fiedler et al. 2006).

While activated endothelial cells are clearly essential for the establishment of an inflammatory response, inflammatory cells also markedly influence endothelial cell behavior and angiogenesis by multiple mechanisms, such as the release of growth factors and proteases. One example linking inflammation and angiogenesis in normal physiology is uterine vascularization in the estrous cycle, where estrogen treatment results in the enrichment of neutrophils in the endometrial vessels and subsequently stimulation of vascular proliferation by neutrophil-produced vascular endothelial growth factor (VEGF) (Heryanto et al. 2004). A second example during normal physiology is the inflammation-driven angiogenesis in the development of intestinal vessels that is mediated through the release of angiogenin 4 by intestinal Paneth cells. Here the lack of bacterial colonization in the intestine of germ-free mice blocks the normal development of the villous capillary network (Hooper et al. 2003).

Extending these observations from normal physiological processes to cancer suggests that inflammatory leukocytes in the tumor microenvironment may provide an angiogenic stimulus allowing tumor growth and progression. This concept is supported by a number of studies assigning specific proangiogenic roles to different cell types of the innate immune system.

20.3 Macrophages

Macrophages and their different functional phenotypes as wells as their role in angiogenesis and

progression of solid tumors have recently been a matter of intense research. They are derived from CD 34^+ bone marrow progenitors that continually proliferate giving rise to the promonocytes of the blood stream. These develop into monocytes that upon extravasation into the tissue differentiate into "resident" tissue macrophages (Ross and Akuger 2002). The phenotype of the resident tissue macrophages can vary considerably between different body sites; however, they all share some common functions including their ability to guard against microbial infections, to regulate normal cell turnover and tissue remodeling and to aid in the repair of tissue injuries. Macrophages are also known residents in the microenvironment of primary as well as secondary tumors (Bingle et al. 2002), and in recent years compelling evidence has accumulated that they play an important role in promoting tumor growth and progression (Pollard 2004). Indeed, like different subtypes of "resident" tissue macrophages, macrophages in the tumor microenvironment exhibit a distinct phenotype and are termed tumor-associated macrophages (TAM). Circulating monocytes are recruited into the tumor vicinity by a number of tumor-derived chemoattractants, including colony-stimulating factor-1 (CSF-1; also known as M-CSF), CC chemokines such as CCL2, CCL3, CCL4, CCL5 and CCL8, and VEGF, with the levels of many of these proteins correlating with the number of macrophages in the tumor tissue (Sica et al. 2006; Murdoch et al. 2004). The differentiation of circulating monocytes into TAMs, a cell type that is oriented towards angiogenesis and tissue remodeling (M2 phenotype) rather than being classically pro-inflammatory (M1 phenotype), is induced by IL-4, IL-10 and IL-13 (Sica et al. 2006; Mantovani et al. 2004). TAMs are poor antigen-presenting cells that can suppress T cell activation and proliferation, thereby preventing the host from mounting an effective anti-tumor response (Sica et al. 2006; Mantovani et al. 2002). At the same time TAMs considerably contribute to tumor growth and progression not only through the production of tumor-promoting factors such as epidermal growth factor (EGF), transforming growth factor beta (TGF beta), hepatocyte growth factor (HGF) and bFGF (Goswami et al. 2005; O'Sullivan et al. 1993; Lewis and Murdoch 2005) but also through the stimulation of angiogenesis. The first evidence for a role of macrophages in the modulation of angiogenesis was presented by Sunderkotter et al. (1991). Since then studies have demonstrated that the accumulation of TAMs in the tumor microenvironment is associated with the production of a number of proangiogenic factors such as VEGF, platelet-derived endothelial growth factor, IL-8, TNF alpha and bFGF (Mantovani et al. 2002; Sunderkotter et al. 1991; Lewis et al. 1995), as well as angiogenesis-modulating enzymes such as matrix metalloproteinase-2 (MMP-2), MMP-7, MMP-9, MMP-12, and urokinase plasminogen activator (uPA) (Locati et al. 2002; Lanone et al. 2002). These proteases remodel the ECM, generating reactive cleavage products of ECM molecules and activating proangiogenic factors such as VEGF and others. Furthermore, in cervical cancer TAMS were shown to produce VEGF-C and may thus contribute to peritumoral lymphangiogenesis and lymphatic dissemination of cancer cells (Schoppmann et al. 2002). The many proangiogenic functions of TAMs might be the reason for the direct correlation between the number of TAMs and vascularization that was reported for a number of tumor entities such as breast carcinoma (Leek et al. 1996), squamous cell carcinoma of the esophagus (Koide et al. 2004), bladder carcinoma (Hanada et al. 2000) and glioma (Nishie et al. 1999). Pollard and colleagues recently reported that in PyMT-induced mammary tumors macrophages are recruited to premalignant lesions immediately before the onset of angiogenesis and then induce the angiogenic switch that precedes the transition to malignancy. Depletion of these macrophages results in a significant reduction in vascular density associated with an increase in hypoxic and necrotic areas (Lewis and Pollard 2006). Interestingly, a number of studies have demonstrated that TAMs accumulate in hypoxic/necrotic areas in human endometrial, breast, prostate and ovarian carcinomas and that their accumulation correlates with increased lymph node involvement and/or poor prognosis in breast and endometrial cancer (Leek et al. 1996, 1999; Ohno et al. 2004; Negus et al. 1997). Recruitment of macrophages in

these tumor areas is most likely influenced by the hypoxic induction of macrophage chemoattractants such as EMAPII, endothelin 2 and VEGF (Murdoch et al. 2004), as well as by their phagocytic capacities. On the one hand the hypoxic environment seems to inhibit migration of macrophages, thereby triggering their accumulation in necrotic areas. On the other hand, hypoxia induces the expression of transcription factors of the hypoxia-inducible factor (HIF) family (notably HIF-1 and HIF-2) (Burke et al. 2002) that in turn will modulate the gene expression pattern of macrophages exposed to hypoxia as was demonstrated in a recent cDNA array study (White et al. 2004). TAMs express a number of angiogenic factors such as VEGF, as shown in perinecrotic areas of human breast carcinoma, and TNF alpha, which induces the expression of MMP-9, leading to the release of VEGF from the ECM (Pollard 2004; Leek et al. 2000). Additionally, as shown in human glioma xenografts, a subset of monocytes macrophages may contribute to angiogenesis through the expression of Tie-2 (Lewis and Pollard 2006). Finally, TAMs express a number of proteases such as uPA and its receptor uPAR. Expression of uPAR has been associated with high microvessel density and poor prognosis in breast cancer (Foekens et al. 2000; Hildenbrand et al. 1998). Additionally, in a hypoxic environment macrophages were also shown to express elevated levels of MMP-7. This protease has many substrates in the ECM and basement membrane and is known to stimulate endothelial cell proliferation and migration (Burke et al. 2003; Nishizuka et al. 2001). Taken together, these studies strongly suggest an important role for TAMs in the induction of tumor angiogenesis. TAMs migrate into hypoxic areas of the tumor, where they are activated to synthesize an array of angiogenic regulators including growth factors and proteases. This ultimately induces the formation of new blood vessels and allows survival of tumor cells in the hypoxic areas as well as local tumor growth.

Dendritic Cells

Dendritic cells (DCs) are the second type of professional antigen-presenting cells. They play a crucial role both in the activation of antigen-specific immunity and in the maintenance of tolerance. They participate in the regulation of the inflammatory reaction through the release of cytokines and chemokines, providing a link between adaptive and innate immunity (Banchereau and Steinman 1998). The presence of DCs within human tumors of the stomach, colon, prostate, kidney, thyroid, breast and melanoma has been reported in clinical studies (Tsujitani et al. 1990; Enk et al. 1997; Troy et al. 1998; Lespagnard et al. 1999; Schwaab et al. 1999; Bell et al. 1999; Scarpino et al. 2000). However, the functional consequence of this infiltration for tumor growth and progression remains unclear. While some studies associate DC infiltration with enhanced patient survival, others showed that tumor-associated DCs were either minimally activated (Tsujitani et al. 1990; Troy et al. 1998) or had no correlation with metastasis-free or overall patient survival (Lespagnard et al. 1999). Indeed, DCs in the tumor microenvironment often seem to be impaired in their normal function and can present an immature phenotype (Almand et al. 2000; Allavena et al. 2000). Instead, they can be converted to silence the anti-tumor response of the host (Enk et al. 1997), such that they can turn off the responding T cell and induce tolerance (Hackstein et al. 2001; Vicari and Caux 2002). Sozzani and colleagues even report that, similarly to macrophages of the M2 or the TAM phenotype, DCs can also be activated to an angiogenesis-promoting phenotype. They demonstrate that alternative activation of DCs by anti-inflammatory molecules such as calcitriol, prostaglandin E2 (PGE2) or IL-10 prompts them to secrete the potent angiogenic growth factor VEGF and inhibit their secretion of IL-12, a potent anti-angiogenic molecule that is secreted by classically activated DCs. Since solid tumors are infiltrated by DCs that lack the phenotype of classically activated DCs, the authors hypothesize that within the tumor microenvironment DCs will be activated via the

alternative mechanism to produce proangiogenic molecules and thus, like TAMs, contribute to tumor vascularization and growth (Riboldi et al. 2005).

Mast Cells

Like macrophages, mast cells (MCs) are derived from CD34+ bone marrow progenitor cells and migrate through the circulation to their tissue destination. Once they have left the vasculature they differentiate under the influence of environmental factors, finally acquiring functional maturity. MCs always accompany connective tissue: they are located in the vicinity of blood and lymphatic vessels and nerve fibers. Functionally they take part in the type 1 hypersensitivity reaction, in chronic inflammatory processes, tissue remodeling and wound healing, as well as in the pathological fibrosis of many organs such as the lungs (Nienartowicz et al. 2006). The infiltration of MCs has been found in a variety of human cancers, including breast carcinoma (Kankkunen et al. 1997), colorectal cancer (Lachter et al. 1995), basal cell carcinoma of the skin (Yamamoto et al. 1997), non-small cell lung cancer (Shijubo et al. 2003) and pulmonary adenocarcinoma (Imada et al. 2000). The influence of MCs on patient survival remains a matter of debate, with some studies associating their accumulation with enhanced tumor growth and invasion of several tumor entities (Ribatti et al. 2001a) and others correlating their presence in colorectal carcinoma with improved prognosis (Nielsen et al. 1999). Yet, early studies using animal models have already demonstrated that increasing mast cell density in the tumor promotes tumor growth (Roche 1985), while reducing their number inhibits tumor growth (Starkey et al. 1988). Subsequent analyses identified an important role for MCs in the induction of tumor angiogenesis (Hiromatsu 2003). Activated MCs produce a number of angiogenic growth factors such as VEGF, bFGF, IL-8 and TNF alpha (Hiromatsu 2003; Meininger and Zetter 1992), as well as additional angiogenic mediators like histamine and heparin, that can stimulate endothelial cell proliferation and may contribute to the leakiness of the tumor vasculature (Ribatti et al. 2001a). Indeed, it is isolated MCs and their secretory granules, but not degranulated MCs, that induce an angiogenic response in the CAM assay (Ribatti et al. 2001b). The angiogenic potential of MCs and their granules was significantly reduced by addition of neutralizing antibodies to VEGF or BFGF, supporting the idea that mast cells have potent angiogenic properties that depend on the angiogenic molecules contained in their secretory granules. Investigating the role of host MCs in angiogenesis, Starkey and colleagues were able to link MCs with tumor angiogenesis in vivo by comparing the angiogenic response of genetically MC-deficient W/W^{v} mice and MC-sufficient $^{+/+}$ litter mates to subcutaneously growing Bl6 tumors. The angiogenic response was initially slower and less intense in W/Wv mice, with fewer W/Wv developing spontaneous lung metastases. Bone marrow repair of MC deficiency restored the incidence of hematogenous metastases to the level of $^{+/+}$ mice, suggesting a role for host MCs in both tumor angiogenesis and hematologic metastases (Starkey et al. 1988). The essential role of MCs in promoting tumor angiogenesis was further substantiated by studies in a transgenic model for de novo skin carcinogenesis, namely K14-HPV16 transgenic mice, in which human papilloma virus type 16 early region genes are expressed under the control of the keratin 14 promoter. These mice develop epidermal hyperplasia that is followed by dysplasia and the formation of papillomas. Progression to malignant squamous cell carcinoma (SCC) is observed in about 20% of mice (Coussens et al. 1996). In this system MCs infiltrate hyperplastic and dysplastic lesions as well as the invasive front of tumors. There they contribute to the activation of a tumor-supporting stroma and to angiogenesis by releasing MC chymase and tryptase, as well as by activating pro-MMP-9, which is provided by MCs themselves as well as by neutrophil granulocytes. Genetic depletion of MCs (KitW/KITWWv) resulted in a clearly decreased tumor incidence, with a reduction of premalignant angiogenesis as well as a decrease in proliferation of keratinocytes and stromal fibroblasts leading to attenuated malignant

progression (Coussens et al. 1999, 2000). Additionally, by inhibiting the recruitment of innate immune cells, including MCs and neutrophils, into the premalignant tumor tissue, genetic elimination of T- and B-lymphocytes in these K14-HPV16 mice also diminished premalignant angiogenesis and reduced carcinoma incidence from 47% to 6% (de Visser et al. 2005). Taken together, the data clearly support a critical role for MCs in the regulation of angiogenesis. The K14-HPV16 model additionally suggests that an interaction of a peripheral adaptive immune response (specifically B-lymphocytes) and of soluble molecules in the serum with innate immune cells is necessary for the establishment of a chronic inflammatory response in the tumor microenvironment that ultimately contributes to an angiogenic response in the stroma.

Neutrophils

The role of neutrophils in progression of tumor angiogenesis has been somewhat neglected, perhaps due to their traditional characterization as terminally differentiated effectors of inflammation. However, neutrophils are remarkably versatile cells. They are actively involved in the regulation and resolution of inflammation and respond to a wide variety of cytokines and chemotactic molecules. As the first cells to arrive at sites of infection they are highly bactericidal. Besides their release of lytic antimicrobial peptides they can also produce, upon appropriate stimulation in vitro or in vivo, a variety of proteins including cytokines, chemotactic molecules and angiogenic growth factors (Witko-Sarsat et al. 2000; Schruefer et al. 2006). Only recently has their important and regulatory role in angiogenesis and tumor progression entered the focus of attention. The prognostic relevance of their presence in the tumor tissue is a matter of controversial debate, with some studies linking their infiltration with poorer outcome in patients with adenocarcinoma of the bronchioloalveolar carcinoma subtype (Bellocq et al. 1998) and others correlating their presence with good prognosis in gastric carcinoma (Caruso et al. 2002). Independent of these controversial data, recent studies in vitro and in animal models in vivo clearly demonstrate an angiogenesis-promoting capacity of neutrophils that seems to promote tumor progression. Neutrophils can contribute to tumor angiogenesis in vivo by releasing IL-8 and VEGF (Schaider et al. 2003). Interestingly, the neutrophil derived VEGF is thought to act on endothelial cells in a dual manner. It induces proliferation and migration of endothelial cells and thus promotes angiogenesis. Additionally, it seems to induce an upregulation of IL-8 secretion in endothelial cells, thereby augmenting the attraction of (VEGF-producing) neutrophils and ultimately leading to a paracrine feed-forward mechanism that enhances the angiogenic response (Schruefer et al. 2005). Indeed, the release of VEGF seems to be one major component in the angiogenesis-promoting capacity of neutrophils. Scapini et al. demonstrated that the angiogenic activity of CXC-ERL$^+$ cytokines, and specifically of CXCL1 (MIP-2) in vivo, is strictly dependent on neutrophils and their capacity to secrete VEGF-A. Depletion of neutrophils completely abrogated the angiogenic response, as did a deficiency in the scr family kinases Hck and Fgr ($Hck^{-/-}fgr^{-/-}$). While showing a normal neutrophil recruitment upon CXCL1/MIP-2 stimulation, the neutrophils in the kinase-deficient mice were unable to secrete VEGF-A in response to CXCL1 stimulation and thus failed to induce an angiogenic response (Scapini et al. 2004). Similarly, granulocyte colony-stimulating factor (G-CSF) induced recruitment of neutrophils into ischemic tissues, increased capillary density and provided a functional vasculature in these tissues via the release of VEGF from the neutrophils. Blockade of the VEGF pathway abrogated this G-CSF-induced angiogenesis. Interestingly, in this model the proangiogenic effect was not solely mediated by the direct stimulation of endothelial cells via neutrophil-derived VEGF but also by the VEGF-induced mobilization of endothelial progenitor cells (Ohki et al. 2005). However, neutrophils not only contribute to angiogenesis by the release of growth factors and cytokines. Additionally, they

have been identified as an important source of proteases, such as matrix metalloproteinases and elastases, in the tumor tissue (Iwatsuki et al. 2000; Scapini et al. 2002). Indeed, a number of studies demonstrate that neutrophil-derived proteases are essential for tumor angiogenesis. In the K14-HPV16 mouse model for epithelial carcinogenesis the upregulation of MMP-9, which was localized to neutrophils in the tumor stroma, was essential for early neoplastic progression. Lack of MMP-9 was associated with delayed activation of angiogenesis in the stroma of hyperplastic lesions, an effect that could be rescued by bone marrow transplantation from MMP-9 proficient mice (Coussens et al. 2000). Similarly, the lack of MMP-9-positive neutrophils as well as of MMP-2-expressing stromal cells in mice with a double deficiency for MMP-2 and MMP-9 resulted in a lack of tumor vascularization followed by a lack of tumor invasion in transplants of mouse skin SCCs (Masson et al. 2005). Reciprocally, abrogation of angiogenesis by blockade of VEGFR-2 in a heterotransplant model for human skin SCCs was associated with a significant downregulation of neutrophil-derived MMP-9 in the mouse stroma (Vosseler et al. 2005). The essential role played by infiltration of MMP-9-expressing neutrophils in tumor angiogenesis was further substantiated by studies in the Rip-Tag2 model of pancreatic islet carcinogenesis. MMP-9-expressing neutrophils were predominantly found in the angiogenic islets of dysplasias and tumors, and transient depletion of neutrophils clearly reduced the frequency of the initial angiogenic switch in the dysplasias. This was associated with a suppression of VEGF:VEGF receptor association, a result of MMP-9 activity that releases matrix-bound VEGF from the ECM (Nozawa et al. 2006). Thus, in this carcinogenesis model the infiltrating MMP-9 expressing neutrophils seem to play a crucial role in activating angiogenesis during early stages of carcinogenesis. Utilizing the HaCaT heterotransplantation model for human skin SCCs, we provided the first evidence that the infiltration of neutrophils into the tumor stroma is not only necessary to induce the initial onset of angiogenesis but also contributes to a persistent angiogenesis that is a prerequisite for tumor invasion. In a matrix-inserted surface transplantation model (Mueller and Fusenig 2002, 2004), expression of G-CSF or co-expression of G-CSF and granulocyte-macrophage colony-stimulating factor (GM-CSF) together induced malignant progression of previously benign factor-negative HaCaT tumor cells. This progression was associated with enhanced and accelerated neutrophil recruitment into the tumor vicinity that became persistent. Together with earlier and enhanced macrophage recruitment this ultimately resulted in a balance between the number of macrophages and neutrophils in the stroma of the malignant tumors. In contrast, neutrophil recruitment in benign factor-negative tumors was transient, leading to a clear excess of macrophages in the stroma of benign tumors. Generally the neutrophil recruitment preceded the induction of angiogenesis not only in the HaCaT model for skin SCCs but also in nude mouse heterotransplants of head and neck carcinomas (Obermueller et al. 2004; Gutschalk et al. 2006). Only the persistent neutrophil recruitment that was observed in malignant transplants was followed by the persistent angiogenesis that is a prerequisite for malignant tumor growth (Obermueller et al. 2004). Thus, neutrophils seem to play a dual role in the activated tumor microenvironment. They clearly promote early angiogenesis by secreting MMP-9 and VEGF (Coussens et al. 2000). Additionally, their interaction with stromal cells, specifically macrophages, might contribute to a tumor-promoting inflammatory infiltrate. Indeed, there are first indications that neutrophils can modulate the phenotype of the macrophages in the tumor vicinity via the secretion of cytokines or growth factors towards a tumor-associated macrophage phenotype (Mantovani et al. 2004; Vosseler et al. 2005; Vajkoczy et al. 2002).

Conclusion

Taken together, there is now abundant evidence that the different cell types of the innate immune system, particularly macrophages, mast cells, and

neutrophils, play an active role in enhancing tumor angiogenesis, either directly via the release of vesicle-stored growth factors, cytokines and proteolytic enzymes or indirectly via paracrine signaling cascades. Although targeting the tumor vasculature to induce tumor cell death has become an attractive concept (Ferrara et al. 2004), such treatment modalities are not always powerful enough to induce complete tumor regression (Yang et al. 2003). Given the intimate association of the inflammatory infiltrate in the tumor microenvironment with tumor angiogenesis, it seems more attractive to use the immune system itself to halt angiogenesis. There is clear evidence of a very high efficacy of anti-inflammatory therapies, specifically of COX inhibitors in chemoprevention (Clevers 2004; Turini and DuBois 2002), and a number of studies have elucidated the connection between the inflammatory mediator COX-2 and angiogenesis (Nakao et al. 2005; Wang et al. 2005). COX-2 itself has proangiogenic activity (Macarthur et al. 2004). Additionally, COX-2 upregulates the expression of Th2 cytokines, generating a Th2 environment that promotes tumor angiogenesis (Dalgleish and O'Byrne 2002). Finally, COX itself seems to be upstream of VEGF production in stromal cells (Boccaccio et al. 2005), and COX-2-catalyzed PGE2 expression seems to play a critical role in the HIF-1 alpha-mediated regulation of VEGF expression in hypoxic tumor areas (Liu et al. 2002). A number of antioxidant anti-inflammatory drugs have antiangiogenic potential, probably by acting directly on inflammation-mediated angiogenesis (Albini et al. 2005). Additionally, there are first indications of anti-inflammatory properties of angiogenesis inhibitors such as vasostatin (Huegel et al. 2006). Thus the concept of the indirect inflammation-dependent induction of angiogenesis makes inflammation a target for tumor therapy and, even better, a target for the prevention of tumor angiogenesis by anti-inflammatory agents. Ideally, chemoprevention via an anti-inflammatory approach will be able to block neovascularization before the angiogenic switch point, resulting in a significant delay in the onset of clinically relevant cancers.

References

Albini A, Tosetti F, Benelli R, Noonan DM (2005) Tumor inflammatory angiogenesis and its chemoprevention. Cancer Res 65:10637–10641

Allavena P, Sica A, Vecchi A, Locati M, Sozzani S, Mantovani A (2000) The chemokine receptor switch paradigm and dendritic cell migration: its significance in tumor tissues. Immunol Rev 177:141–149

Almand B, Resser JR, Lindman B, Nadaf S, Clark JI, Kwon ED et al (2000) Clinical significance of defective dendritic cell differentiation in cancer. Clin Cancer Res 6:1755–1766

Balkwill F (2002) Tumor necrosis factor or tumor promoting factor? Cytokine Growth Factor Rev 13:135–141

Balkwill F, Charles KA, Mantovani A (2005) Smoldering and polarized inflammation in the initiation and promotion of malignant disease. Cancer Cell 7:211–217

Banchereau J, Steinman RM (1998) Dendritic cells and the control of immunity. Nature 392:245–252

Bell D, Chomarat P, Broyles D, Netto G, Harb GM, Lebecque S et al (1999) In breast carcinoma tissue, immature dendritic cells reside within the tumor, whereas mature dendritic cells are located in peritumoral areas. J Exp Med 190:1417–1426

Bellocq A, Antoine M, Flahault A, Philippe C, Crestani B, Bernaudin JF et al (1998) Neutrophil alveolitis in bronchioloalveolar carcinoma: induction by tumor-derived interleukin-8 and relation to clinical outcome. Am J Pathol 152:83–92

Bingle L, Brown NJ, Lewis CE (2002) The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. J Pathol 196:254–265

Boccaccio C, Sabatino G, Medico E, Girolami F, Follenzi A, Reato G et al (2005) The MET oncogene drives a genetic programme linking cancer to haemostasis. Nature 434:396–400

Burke B, Giannoudis A, Corke KP, Gill D, Wells M, Ziegler-Heitbrock L et al (2003) Hypoxia-induced gene expression in human macrophages: implications for ischemic tissues and hypoxia-regulated gene therapy. Am J Pathol 163:1233–1243

Burke B, Tang N, Corke KP, Tazzyman D, Ameri K, Wells M et al (2002) Expression of HIF-1alpha by human macrophages: implications for the use of macrophages in hypoxia-regulated cancer gene therapy. J Pathol 196:204–212

Caruso RA, Bellocco R, Pagano M, Bertoli G, Rigoli L, Inferrera C (2002) Prognostic value of intratumoral neutrophils in advanced gastric carcinoma in a high-risk area in northern Italy. Mod Pathol 15:831–837

Clevers H (2004) At the crossroads of inflammation and cancer. Cell 118:671–674

Coussens LM, Hanahan D, Arbeit JM (1996) Genetic predisposition and parameters of malignant progression in K14-HPV16 transgenic mice. Am J Pathol 149:1899–1917

Coussens LM, Raymond WW, Bergers G, Laig-Webster M, Behrendtsen O, Werb Z et al (1999) Inflammatory mast cells up-regulate angiogenesis during squamous epithelial carcinogenesis. Genes Dev 13:1382–1397

Coussens LM, Tinkle CL, Hanahan D, Werb Z (2000) MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. Cell 103:481–490

Dalgleish AG, O'Byrne KJ (2002) Chronic immune activation and inflammation in the pathogenesis of AIDS and cancer. Adv Cancer Res 84:231–276

De Visser KE, Korets LV, Coussens LM (2005) De novo carcinogenesis promoted by chronic inflammation is B lymphocyte dependent. Cancer Cell 7:411–423

Enk AH, Jonuleit H, Saloga J, Knop J (1997) Dendritic cells as mediators of tumor-induced tolerance in metastatic melanoma. Int J Cancer 73:309–316

Ferrara N, Hillan KJ, Gerber HP, Novotny W (2004) Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. Nat Rev Drug Discov 3:391–400

Fiedler U, Reiss Y, Scharpfenecker M, Grunow V, Koidl S, Thurston G et al (2006) Angiopoietin-2 sensitizes endothelial cells to TNF-alpha and has a crucial role in the induction of inflammation. Nat Med 12:235–239

Foekens JA, Peters HA, Look MP, Portengen H, Schmitt M, Kramer MD et al (2000) The urokinase system of plasminogen activation and prognosis in 2780 breast cancer patients. Cancer Res 60:636–643

Goswami S, Sahai E, Wyckoff JB, Cammer M, Cox D, Pixley FJ et al (2005) Macrophages promote the invasion of breast carcinoma cells via a colony-stimulating factor-1/epidermal growth factor paracrine loop. Cancer Res 65:5278–5283

Gupta RA, Dubois RN (2001) Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. Nat Rev Cancer 1:11–21

Gutschalk CM, Herold-Mende CC, Fusenig NE, Mueller MM (2006) Granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor promote malignant growth of cells from head and neck squamous cell carcinomas in vivo. Cancer Res 66:8026–8036

Hackstein H, Morelli AE, Thomson AW (2001) Designer dendritic cells for tolerance induction: guided not misguided missiles. Trends Immunol 22:437–442

Hanada T, Nakagawa M, Emoto A, Nomura T, Nasu N, Nomura Y (2000) Prognostic value of tumor-associated macrophage count in human bladder cancer. Int J Urol 7:263–269

Hanahan D, Folkman J (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 86:353–364

Heryanto B, Girling JE, Rogers PA (2004) Intravascular neutrophils partially mediate the endometrial endothelial cell proliferative response to oestrogen in ovariectomised mice. Reproduction 127:613–620

Hildenbrand R, Glienke W, Magdolen V, Graeff H, Stutte HJ, Schmitt M (1998) Urokinase receptor localization in breast cancer and benign lesions assessed by in situ hybridization and immunohistochemistry. Histochem Cell Biol 110:27–32

Hiromatsu Y, Toda S (2003) Mast cells and angiogenesis. Microsc Res Tech 60:64–69

Hooper LV, Stappenbeck TS, Hong CV, Gordon JI (2003) Angiogenins: a new class of microbicidal proteins involved in innate immunity. Nat Immunol 4:269–273

Huegel R, Velasco P, de la Luz Sierra M, Christophers E, Schroder JM, Schwarz T et al (2006) Novel anti-inflammatory properties of the angiogenesis inhibitor vasostatin. J Invest Dermatol 127:65–74

Imada A, Shijubo N, Kojima H, Abe S (2000) Mast cells correlate with angiogenesis and poor outcome in stage I lung adenocarcinoma. Eur Respir J 15:1087–1093

Iwatsuki K, Kumara E, Yoshimine T, Nakagawa H, Sato M, Hayakawa T (2000) Elastase expression by infiltrating neutrophils in gliomas. Neurol Res 22:465–468

Kankkunen JP, Harvima IT, Naukkarinen A (1997) Quantitative analysis of tryptase and chymase containing mast cells in benign and malignant breast lesions. Int J Cancer 72:385–388

Koide N, Nishio A, Sato T, Sugiyama A, Miyagawa S (2004) Significance of macrophage chemoattractant protein-1 expression and macrophage infiltration in squamous cell carcinoma of the esophagus. Am J Gastroenterol 99:1667–1674

Korff T, Augustin HG (1999) Tensional forces in fibrillar extracellular matrices control directional capillary sprouting. J Cell Sci 112:3249–3258

Lachter J, Stein M, Lichtig C, Eidelman S, Munichor M (1995) Mast cells in colorectal neoplasias and premalignant disorders. Dis Colon Rectum 38:290–293

Lanone S, Zheng T, Zhu Z, Liu W, Lee CG, Ma B et al (2002) Overlapping and enzyme-specific contributions of matrix metalloproteinases-9 and -12 in IL-13-induced inflammation and remodeling. J Clin Invest 110:463–474

Leek RD, Lewis CE, Whitehouse R, Greenall M, Clarke J, Harris AL (1996) Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. Cancer Res 56:4625–4629

Leek RD, Landers RJ, Harris AL, Lewis CE (1999) Necrosis correlates with high vascular density and focal macrophage infiltration in invasive carcinoma of the breast. Br J Cancer 79:991–995

Leek RD, Hunt NC, Landers RJ, Lewis CE, Royds JA, Harris AL (2000) Macrophage infiltration is associated with VEGF and EGFR expression in breast cancer. J Pathol 190:430–436

Lespagnard L, Gancberg D, Rouas G, Leclercq G, de Saint-Aubain Somerhausen N, di Leo A et al (1999) Tumor-infiltrating dendritic cells in adenocarcinomas of the breast: a study of 143 neoplasms with a correlation to usual

prognostic factors and to clinical outcome. Int J Cancer 84:309–314

Lewis C, Murdoch C (2005) Macrophage responses to hypoxia: implications for tumor progression and anti-cancer therapies. Am J Pathol 167:627–635

Lewis C, Pollard JW (2006) Distinct role of macrophages in different tumor microenvironments. Cancer Res 66:605–612

Lewis C, Leek R, Harris A, McGee JO (1995) Cytokine regulation of angiogenesis in breast cancer: the role of tumor-associated macrophages. J Leukoc Biol 57:747–751

Liu XH, Kirschenbaum A, Lu M, Yao S, Dosoretz A, Holland JF et al (2002) Prostaglandin E2 induces hypoxia-inducible factor-1alpha stabilization and nuclear localization in a human prostate cancer cell line. J Biol Chem 277:50081–50086

Locati M, Deuschle U, Massardi ML, Martinez FO, Sironi M, Sozzani S et al (2002) Analysis of the gene expression profile activated by the CC chemokine ligand 5/RANTES and by lipopolysaccharide in human monocytes. J Immunol 168:3557–3562

Macarthur M, Hold GL, El-Omar EM (2004) Inflammation and cancer II. Role of chronic inflammation and cytokine gene polymorphisms in the pathogenesis of gastrointestinal malignancy. Am J Physiol Gastrointest Liver Physiol 286:G515–G520

Mantovani A, Sozzani S, Locati M, Allavena P, Sica A (2002) Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol 23:549–555

Mantovani A, Allavena P, Sica A (2004) Tumour-associated macrophages as a prototypic type II polarised phagocyte population: role in tumour progression. Eur J Cancer 40:1660–1667

Masson V, de la Ballina LR, Munaut C, Wielockx B, Jost M, Maillard C et al (2005) Contribution of host MMP-2 and MMP-9 to promote tumor vascularization and invasion of malignant keratinocytes. FASEB J 19:234–236

Meininger CJ, Zetter BR (1992) Mast cells and angiogenesis. Semin Cancer Biol 3:73–79

Mueller MM (2006) Inflammation in epithelial skin tumours: old stories and new ideas. Eur J Cancer 42:735–744

Mueller MM, Fusenig NE (2002) Tumor–stroma interactions directing phenotype and progression of epithelial skin tumor cells. Differentiation 70:486–497

Mueller MM, Fusenig NE (2004) Friends or foes - bipolar effects of the tumour stroma in cancer. Nat Rev Cancer 4:839–849

Murdoch C, Giannoudis A, Lewis CE (2004) Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues. Blood 104:2224–2234

Nakao S, Kuwano T, Tsutsumi-Miyahara C, Ueda S, Kimura YN, Hamano S et al (2005) Infiltration of COX-2-expressing macrophages is a prerequisite for IL-1 beta-induced neovascularization and tumor growth. J Clin Invest 115:2979–2991

Negus RP, Stamp GW, Hadley J, Balkwill FR (1997) Quantitative assessment of the leukocyte infiltrate in ovarian cancer and its relationship to the expression of C-C chemokines. Am J Pathol 150:1723–1734

Nielsen HJ, Hansen U, Christensen IJ, Reimert CM, Brunner N, Moesgaard F (1999) Independent prognostic value of eosinophil and mast cell infiltration in colorectal cancer tissue. J Pathol 189:487–495

Nienartowicz A, Sobaniec-Lotowska ME, Jarocka-Cyrta E, Lemancewicz D (2006) Mast cells in neoangiogenesis. Med Sci Monit 12:RA53–RA56

Nishie A, Ono M, Shono T, Fukushi J, Otsubo M, Onoue H et al (1999) Macrophage infiltration and heme oxygenase-1 expression correlate with angiogenesis in human gliomas. Clin Cancer Res 5:1107–1113

Nishizuka I, Ichikawa Y, Ishikawa T, Kamiyama M, Hasegawa S, Momiyama N et al (2001) Matrilysin stimulates DNA synthesis of cultured vascular endothelial cells and induces angiogenesis in vivo. Cancer Lett 173:175–182

Nozawa H, Chiu C, Hanahan D (2006) Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis. Proc Natl Acad Sci USA 103:12493–12398

Obermueller E, Vosseler S, Fusenig NE, Mueller MM (2004) Cooperative autocrine and paracrine functions of granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor in the progression of skin carcinoma cells. Cancer Res 64:7801–7812

Ohki Y, Heissig B, Sato Y, Akiyama H, Zhu Z, Hicklin DJ et al (2005) Granulocyte colony-stimulating factor promotes neovascularization by releasing vascular endothelial growth factor from neutrophils. FASEB J 19:2005–2007

Ohno S, Ohno Y, Suzuki N, Kamei T, Koike K, Inagawa H et al (2004) Correlation of histological localization of tumor-associated macrophages with clinicopathological features in endometrial cancer. Anticancer Res 24:3335–3342

O'Sullivan C, Lewis CE, Harris AL, McGee JO (1993) Secretion of epidermal growth factor by macrophages associated with breast carcinoma. Lancet 342:148–149

Pollard JW (2004) Tumour-educated macrophages promote tumour progression and metastasis. Nat Rev Cancer 4:71–78

Rajashekhar G, Willuweit A, Patterson CE, Sun P, Hilbig A, Breier G et al (2006) Continuous endothelial cell activation increases angiogenesis: evidence for the direct role of endothelium linking angiogenesis and inflammation. J Vasc Res 43:193–204

Ribatti D, Vacca A, Nico B, Crivellato E, Roncali L, Dammacco F (2001a) The role of mast cells in tumour angiogenesis. Br J Haematol 115:514–521

Ribatti D, Crivellato E, Candussio L, Nico B, Vacca A, Roncali L et al (2001b) Mast cells and their secretory granules are angiogenic in the chick embryo chorioallantoic membrane. Clin Exp Allergy 31:602–608

Riboldi E, Musso T, Moroni E, Urbinati C, Bernasconi S, Rusnati M, et al (2005) Cutting edge: proangiogenic properties of alternatively activated dendritic cells. J Immunol 175:2788–2792

Roche WR (1985) Mast cells and tumour angiogenesis: the tumor-mediated release of an endothelial growth factor from mast cells. Int J Cancer 36:721–728

Ross JA, Auger MJ (2002) The biology of the macrophage. In: Burke B, Lewis CE (eds) The macrophage. Oxford University Press, Oxford

Scapini P, Nesi L, Morini M, Tanghetti E, Belleri M, Noonan D et al (2002) Generation of biologically active angiostatin kringle 1–3 by activated human neutrophils. J Immunol 168:5798–5804

Scapini P, Morini M, Tecchio C, Minghelli S, di Carlo E, Tanghetti E et al (2004) CXCL1/macrophage inflammatory protein-2-induced angiogenesis in vivo is mediated by neutrophil-derived vascular endothelial growth factor-A. J Immunol 172:5034–5040

Scarpino S, Stoppacciaro A, Ballerini F, Marchesi M, Prat M, Stella MC et al (2000) Papillary carcinoma of the thyroid: hepatocyte growth factor (HGF) stimulates tumor cells to release chemokines active in recruiting dendritic cells. Am J Pathol 156:831–837

Schaider H, Oka M, Bogenrieder T, Nesbit M, Satyamoorthy K, Berking C et al (2003) Differential response of primary and metastatic melanomas to neutrophils attracted by IL-8. Int J Cancer 103:335–343

Schoppmann SF, Birner P, Stockl J, Kalt R, Ullrich R, Caucig C et al (2002) Tumor-associated macrophages express lymphatic endothelial growth factors and are related to peritumoral lymphangiogenesis. Am J Pathol 161:947–956

Schruefer R, Lutze N, Schymeinsky J, Walzog B (2005) Human neutrophils promote angiogenesis by a paracrine feedforward mechanism involving endothelial interleukin-8. Am J Physiol Heart Circ Physiol 288:H1186–H1192

Schruefer R, Sulyok S, Schymeinsky J, Peters T, Scharffetter-Kochanek K, Walzog B (2006) The proangiogenic capacity of polymorphonuclear neutrophils delineated by microarray technique and by measurement of neovascularization in wounded skin of CD18-deficient mice. J Vasc Res 43:1–11

Schwaab T, Schned AR, Heaney JA, Cole BF, Atzpodien J, Wittke F et al (1999) In vivo description of dendritic cells in human renal cell carcinoma. J Urol 162:567–573

Shijubo N, Kojima H, Nagata M, Ohchi T, Suzuki A, Abe S et al (2003) Tumor angiogenesis of non-small cell lung cancer. Microsc Res Tech 60:186–198

Sica A, Schioppa T, Mantovani A, Allavena P (2006) Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. Eur J Cancer 42:717–727

Starkey JR, Crowle PK, Taubenberger S (1988) Mast-cell-deficient W/Wv mice exhibit a decreased rate of tumor angiogenesis. Int J Cancer 42:48–52

Sugar LM (2006) Inflammation and prostate cancer. Can J Urol 13 [Suppl 1]:46–47

Sunderkotter C, Goebeler M, Schulze-Osthoff K, Bhardwaj R, Sorg C (1991) Macrophage-derived angiogenesis factors. Pharmacol Ther 51:195–216

Troy A, Davidson P, Atkinson C, Hart D (1998) Phenotypic characterisation of the dendritic cell infiltrate in prostate cancer. J Urol 160:214–219

Tsujitani S, Kakeji Y, Watanabe A, Kohnoe S, Maehara Y, Sugimachi K (1990) Infiltration of dendritic cells in relation to tumor invasion and lymph node metastasis in human gastric cancer. Cancer 66:2012–2016

Turini ME, DuBois RN (2002) Cyclooxygenase-2: a therapeutic target. Annu Rev Med 53:35–57

Vajkoczy P, Farhadi M, Gaumann A, Heidenreich R, Erber R, Wunder A et al (2002) Microtumor growth initiates angiogenic sprouting with simultaneous expression of VEGF, VEGF receptor-2, and angiopoietin-2. J Clin Invest 109:777–785

Vicari AP, Caux C (2002) Chemokines in cancer. Cytokine Growth Factor Rev 13:143–154

Virchow R (1862/1863) Die krankhaften Geschwülste: 30 Vorlesungen gehalten während des Wintersemesters 1862/1863. Hirschwald, Berlin (Vorlesungen über Pathologie; 3,1; 3,2)

Vosseler S, Mirancea N, Bohlen P, Mueller MM, Fusenig NE (2005) Angiogenesis inhibition by vascular endothelial growth factor receptor-2 blockade reduces stromal matrix metalloproteinase expression, normalizes stromal tissue, and reverts epithelial tumor phenotype in surface heterotransplants. Cancer Res 65:1294–1305

Wang W, Bergh A, Damber JE (2005) Cyclooxygenase-2 expression correlates with local chronic inflammation and tumor neovascularization in human prostate cancer. Clin Cancer Res 11:3250–3256

White JR, Harris RA, Lee SR, Craigon MH, Binley K, Price T et al (2004) Genetic amplification of the transcriptional response to hypoxia as a novel means of identifying regulators of angiogenesis. Genomics 83:1–8

Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P, Halbwachs-Mecarelli L (2000) Neutrophils: molecules, functions and pathophysiological aspects. Lab Invest 80:617–653

Yamamoto T, Katayama I, Nishioka K (1997) Expression of stem cell factor in basal cell carcinoma. Br J Dermatol 137:709–713

Yang JC, Haworth L, Sherry RM, Hwu P, Schwartzentruber DJ, Topalian SL et al (2003) A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. N Engl J Med 349:427–434

Zittermann SI, Issekutz AC (2006) Endothelial growth factors VEGF and bFGF differentially enhance monocyte and neutrophil recruitment to inflammation. J Leukoc Biol 80:247–257

Arteriovenous Malformation in Mice and Men

Nicole Revencu, Laurence Boon, Miikka Vikkula

Abstract

Arteriovenous malformations are the most dangerous vascular malformations and extremely difficult to treat. While most of them are sporadic, some are associated with autosomal dominant disorders, such as hereditary hemorrhagic telangiectasia, PTEN hamartoma tumor syndrome, and capillary malformation-arteriovenous malformation. Although important advances have been made in the diagnosis and treatment of arteriovenous malformations, the pathogenic mechanisms remain poorly understood. Yet, this is an essential step towards the development of targeted therapies. Here, we discuss the most recent insights on arteriovenous malformations, on the basis of studies on arteriovenous differentiation in animal models, and the monogenic disorders with a predisposition to arteriovenous malformations.

CONTENTS

Introduction

Vascular anomalies are a heterogeneous group of disorders subdivided into tumors (hemangiomas) and malformations. This classification was proposed in 1982 (Mulliken and Glowacki 1982) and accepted by the International Society for the Study of Vascular Anomalies (ISSVA) in 1996. In contrast to tumors, vascular malformations exhibit a normal rate of endothelial cell turnover throughout their natural history (Mulliken and Glowacki 1982). They are usually, but not always, obvious at birth and grow proportionately with the child. Based on the type of vessel affected, several groups are identified: capillary, venous, arterial, lymphatic and combined malformations. This distinction has more than

merely nosologic importance, as the prognosis, follow-up and treatment of each group are completely different. Arteriovenous malformation (AVM) is a fast-flow lesion which is either localized, affecting the brain, skin, muscles, bone, or viscera, or diffuse, affecting for instance an extremity, as in Parkes Weber syndrome. The normal capillary bed is replaced by a "nidus", via which the arterial blood from the feeding arteries is shunted into the draining veins (Figs. 21.1B and 21.2). In other cases, the communication between the artery and the vein is direct, and is called arteriovenous fistula (AVF) (Fig. 21.1C) (Lasjaunias 1997). AVMs expand slowly, but may worsen rapidly at puberty, during pregnancy, after trauma and, particularly, after incomplete treatment.

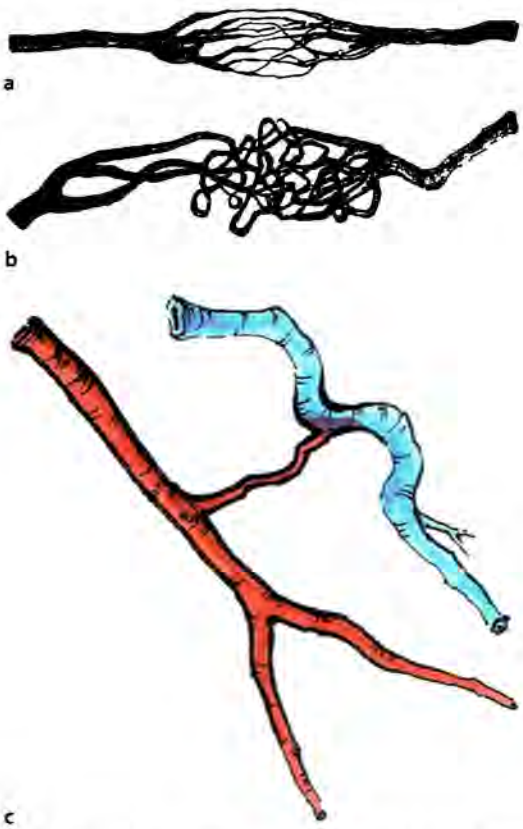


Fig. 21.1a–c. Schematic representation of **a** normal connections between arteries, capillaries and veins **b** arteriovenous malformation **c** arteriovenous fistula

The incidence of AVM is not known, as many are asymptomatic. Figures for brain AVM, based on autopsies or imaging studies, vary from 0.01% to 0.5% (Fleetwood and Steinberg 2002), sometimes even more (Choi and Mohr 2005). The evolution is variable and unpredictable, from asymptomatic to life-threatening. Clinical manifestations depend on the age of the patient and the location of the lesion. In neonates, AVMs usually present with congestive heart failure. Later symptoms are linked to the location: headache, epilepsy, hemorrhage, and focal neurological deficit in cerebral lesions; cyanosis, clubbing, polycythemia, and right-to-left shunt with cerebral abscess and embolic stroke in pulmonary lesions; heart failure, portal hypertension, and biliary disease in hepatic lesions; heaviness, pain, pulsatile mass, thrill, trophic changes, and bleeding in cutaneous, subcutaneous and muscular lesions.

The therapeutic attitude depends on lesion type, location and symptoms. The spectrum varies from conservative follow-up to aggressive treatment, such as embolization, radiotherapy and surgery. For instance, pulmonary AVM in hereditary hemorrhagic telangiectasia is actively sought and treated, while asymptomatic cerebral lesions are followed up regularly. The decision to treat or not should be made by a multidisciplinary team, including an internist, a surgeon, and an interventional radiologist. The effects of the treatment can be devastating, and should be performed only in reference centers and by trained physicians. The treatment has to be as complete as possible; otherwise, new feeding arteries are recruited and the malformation reforms.

AVM is in many cases an isolated and sporadic lesion. Nevertheless, several autosomal dominant disorders with a predisposition to AVM have been identified: hereditary hemorrhagic telangiectasia, PTEN hamartoma tumor syndrome, and capillary malformation–arteriovenous malformation. AVM can also be part of a syndrome, such as Wyburn–Mason syndrome, Cobb syndrome and Parkes Weber syndrome.

While progress has been made in management and diagnostic procedures, the origin of these vascular malformations is still mysterious. Fundamental questions such as time of occurrence (congenital

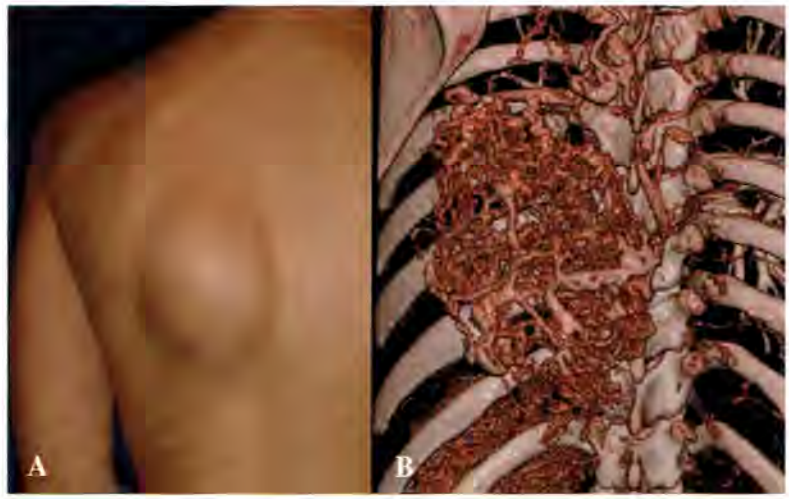


Fig. 21.2A,B.
A Adolescent girl with a pulsatile mass on the back. **B** Helical CT (3D reconstruction) with contrast injection shows a nidus-type arteriovenous malformation

or not), pathogenic mechanisms and natural history have not been completely elucidated. The currently prevailing opinion is that AVM develops during fetal life. Nevertheless, only a small proportion of brain AVMs are diagnosed prenatally, probably because the malformation is too small to be detected. A minority are symptomatic at birth; in general, symptoms appear latter. The pathogenic mechanism leading to AVM has puzzled scientists for many years. It is tempting to think that molecular pathways controlling arterial–venous (AV) differentiation play a role. Indeed, it has been shown that under- or overexpression of some of these molecules provokes AVM in animal models. More direct insight has come from identification of the causative genes for human monogenic disorders involving AVM, such as hereditary hemorrhagic telangiectasia, PTEN hamartoma tumor syndrome and capillary malformation–arteriovenous malformation.

21.2 Arterial–Venous Differentiation and Interest for AVM Pathogenesis

Arteries and veins are morphologically and functionally very different. However, in spite of significant research efforts, it is not known precisely how this distinction is established during vascular development. For about 100 years it was thought that the structural and functional differences between arteries and veins were attributable to physiological factors such as blood pressure, flow and shear stress (Thoma 1893; Murray 1926), and endothelial cells (ECs) of arteries, veins, and capillaries were considered to be a homogeneous population (Risau 1997). However, during the past few years, several molecules that are differently expressed in arterial and venous ECs, even before the onset of circulation, have been identified, suggesting that AV differentiation is governed at least in part by genetic mechanisms.

21.2.1 Genetic Factors

Arterial ECs have specific markers such as ephrinB2, neuropilin1, and members of the Notch pathway, whereas EphB4, neuropilin2, and COUP-TFII are specific for venous ECs. The function of this differential expression in AV specification has not been completely deciphered. It is thought that vein identity comes by default, yet it was recently shown that COUP-TFII, a member of the orphan nuclear receptor superfamily, is specifically expressed in vein ECs

and that its inactivation enables veins to express arterial markers (You et al. 2005).

Many molecules are involved in AV differentiation, but as their under- or overexpression does not cause AVM in animal models and they have not been involved in human AVMs, they are not discussed here. Rather, the main focus is on the Notch signaling pathway, an extremely conserved pathway across species, essential for embryonic vascular development and AV differentiation (Shawber and Kitajewski 2004). In mammals, four Notch receptors (Notch1–4) and five ligands (Dll1, 3, and 4 and Jagged-1 and-2) have been identified. Several members are expressed in ECs and/or supporting cells during development. Mutations lead to abnormalities in many organs, including the cardiovascular system.

Studies in zebrafish have indicated that Notch signaling regulates AV specification (Lawson et al. 2001; Zhong et al. 2001). Indeed, although angioblasts are spatially mixed, each of them participates in the formation of either veins or arteries, and this decision is guided via the notch-gridlock pathway (Zhong et al. 2001). Gridlock has an artery-specific expression and is encoded by grl, the homolog of the human HEY2 gene, a direct transcriptional target of the Notch pathway. Reduction of gridlock increases the expression of the venous marker EphB4 and decreases the arterial marker ephrinB2, suggesting that the Notch pathway is required to suppress venous fate in arterial endothelium. Inhibition of Notch signaling in zebrafish embryos results in AV shunts between the dorsal aorta and posterior cardinal vein (Lawson et al. 2001).

Loss of expression of artery-specific markers was also observed in mice with targeted disruption of Notch signaling players, such as *Notch1*, *Rbpsuh* (encodes Notch transcriptional mediator) and double-knockout *Hey1/Hey2* (Fischer et al. 2004; Krebs et al. 2004). Some lead to AVM, such as deletion of the *Rbpsuh* gene in ECs (Krebs et al. 2004), and heterozygous loss of the Dll4 ligand, whose expression is restricted to large arteries in murine embryos (Gale et al. 2004; Krebs et al. 2004). Expression of activated Notch4 in embryonic ECs causes failure of vascular remodeling and dilation of major vessels (Uyttendaele et al. 2001), whereas expression of constitutively active Notch4 in adult murine ECs induces blood vessel enlargement and AVM (Carlson et al. 2005). The defect in adult mice was reversible upon repression of gene expression, and overexpression of Notch4 was accompanied by ectopic venous expression of arterial marker ephrinB2. These studies suggest that activation or inhibition of the Notch pathway can cause aberrant AV specification and AVM. The observed reversibility of AVM is promising for research into targeted therapy.

In humans, mutations in *JAG1* (Jagged-1) and *NOTCH3* have been associated with Alagille syndrome and CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) respectively. Alagille syndrome is a multisystem developmental disorder with autosomal dominant inheritance. These patients do not have AVM, but a significant proportion harbor a cerebral aneurysm, which causes significant morbidity and mortality (Kamath et al. 2004). Only one CADASIL patient with a cerebral AVM has been reported (Pescini et al. 2006). Thus, the NOTCH pathway has not been directly involved in human AVM pathogenesis.

21.2.2 Non-Genetic Factors

Beside genetic factors, several studies have highlighted the importance of hemodynamic forces together with EC plasticity in AV differentiation. Thus, local factors could play a role in AVM pathogenesis. The importance of flow was shown in zebrafish by multiphoton time-lapse imaging (Isogai et al. 2003). If the primary network of the trunk was formed before the onset of circulation, the final connection pattern between this and the secondary sprouts, and thus the arterial or venous identity, was guided by flow dynamics. In chick embryo yolk sac, with the establishment of perfusion, some arteriolar branches are disconnected from the vitelline artery and subsequently reconnect to the venous system. This process is accompanied by a switch in EC markers from arterial to venous type (le Noble et al. 2004). An expression study performed on human umbilical

vein showed that exposure of ECs to mechanical or shear stress induced a modification of expression of more than 1800 genes (Andersson et al. 2005). Moreover, the response to shear stress was highly different from that to pressure, suggesting that the phenotype of ECs depends on local factors.

Studies on avian embryos have shown that ECs are endowed with plasticity in the earliest steps of development, and that this plasticity is required for AV differentiation (le Noble et al. 2004; Moyon et al. 2001). ECs from quail embryos before day 7 are able to colonize host arteries and veins with equal efficiency, regardless of their origin (Moyon et al. 2001). After an intermediate period, this plasticity is completely lost at embryonic day 11.

Insights from Monogenic Disorders Associated with AVM

Hereditary hemorrhagic telangiectasia or Rendu-Osler-Weber syndrome (HHT, ROW; OMIM 187300) is a common autosomal dominant disorder with an incidence of about 1 in 10,000, although higher incidence has been reported in specific areas (Abdalla and Letarte 2006). The syndrome is characterized by epistaxis, telangiectasias and AVM mainly in lung, liver and brain. The frequency of AVM is estimated at 30–50% in lungs, 30–40% in the liver, and 10–20% in the brain (Begbie et al. 2003; Abdalla and Letarte 2006).

HHT is genetically heterogeneous with at least four genes involved. In 1994 and 1995 two loci were identified by linkage analysis on chromosomes 9q33-q34.1 (HHT1) (McDonald et al. 1994; Shovlin et al. 1994) and 12q11-q14 (HHT2) (Vincent et al. 1995). It was shown that ENG (OMIM 131195) and *ALK1* (activin receptor-like kinase 1, *ACVRL1*; OMIM 601284) were the mutated genes, respectively (McAllister et al. 1994; Johnson et al. 1996). Since then, more than 150 different mutations have been reported in *ENG* and more than 120 in *ALK1* (Abdalla and Letarte 2006). As the encoded protein is generally either not expressed or instable, the major mechanism is haploinsufficiency. HHT1 and HHT2 are clinically similar, as all reported manifestations are known to occur in both. However, later onset and lower penetrance, fewer cerebral and pulmonary AVMs, but more liver involvement and a risk of developing pulmonary arterial hypertension are observed in HHT2. In 2004, a distinct phenotype combining HHT and juvenile polyposis (JPHT; OMIM 175050) caused by mutations in *MADH4* was described (Gallione et al. 2004). The HHT phenotype in these patients is similar to those of HHT1 and HHT2 (Gallione et al. 2004). Recently, a classical HHT family not linked to, and without mutations in, *ENG*, *ALK1*, and *MADH4* allowed identification of the HHT3 locus on chromosome 5q31.3-q32 (Cole 2005).

Interestingly, ultrastructurally, the telangiectasias are AV microfistulas (Braverman et al. 1990). The lesion begins as small dilations in postcapillary venules. The number of pericytes is increased. Progressively the venules continue to enlarge, the capillary segment disappears and a direct AV communication is formed. This entire sequence is associated with a perivascular mononuclear cell infiltrate. The histological analysis of a cerebral and a pulmonary AVM lesion in two HHT1 patients showed vessel dilation and variable thickness and disorganization of the smooth muscle cell layer (Bourdeau et al. 2000a). It has been proposed that the telangiectasias and AVM, rather than differing in kind, represent a continuum (Krings et al. 2005).

How mutations in HHT-associated genes lead to AVM is not known. *ENG*, *ALK1* and *MADH4/SMAD4* encode components of the TGF-β signaling pathway, which plays an important role in development and homeostasis of many organs, including the vascular system. In the latter, the TGF-β pathway is involved in cell proliferation, migration, extracellular matrix formation, vascular smooth muscle cell differentiation and vascular tone. The ALK1 and Endoglin proteins are primarily expressed in ECs, whereas SMAD4 is ubiquitously expressed.

ALK1 is a TGF-β receptor type I (TβRI). It is a serine-threonine kinase transmembrane receptor, recruited and activated by phosphorylation upon TGF-β binding on type II TGF-β receptor (TβRII).

ALK1 propagates signals to the nucleus via Smad 1 and 5 (Fig. 21.3). It has been proposed that, in contrast to most cell types, TGF-β signals in ECs through two types of TβRI: ALK1 and ALK5, the latter being required for ALK1 activation (Goumans et al. 2003). These two pathways would have opposite functions: TGF-β/ALK1 induces cell migration and proliferation, and TGF-β/ALK5 inhibits both processes (Fig. 21.3) (Goumans et al. 2002). The factor which tilts the balance toward ALK1 or ALK5 seems to be the level of TGF-β: a low level would favor migration and proliferation and a high level would have an opposite effect. Intriguingly, in another study, constitutively active ALK1 inhibited migration and proliferation (Lamouille et al. 2002). Moreover, it has been shown that in murine blood vessels, Alk5 is expressed in vascular smooth muscle layer, but not in the ECs (Seki et al. 2006). These unexpected results require further clarification.

Targeted Alk1 disruption in mice is lethal at midgestation (Oh et al. 2000; Urness et al. 2000). Murine *Alk1*$^{-/-}$ embryos have very few capillary vessels and display AVMs between major arteries and veins. The arterial marker *Efnb2* is down-regulated. Vascular smooth muscle cell recruitment and differentiation is deficient. Interestingly, *Alk1*$^{+/-}$ mice develop an HHT-like vascular pathology with mucocutaneous, hepatic, pulmonary and cerebral vascular lesions (Srinivasan et al. 2003).

The HHT1 protein, Endoglin (CD105; OMIM 131195), is a homodimeric transmembrane glycoprotein primarily expressed in ECs and up-regulated in tissues undergoing angiogenesis. In vitro inhibition of its expression impairs this process (Li et al. 2000). Endoglin is known to modulate cellular responses to ligands of the TGF-β superfamily. It has been proposed that its role in TGF-β signaling is to promote the TGF-β/ALK1 pathway for cell proliferation and migration (Fig. 21.3) (Lebrin et al. 2004). Indeed, cultured *Eng*$^{+/-}$ murine ECs have impaired cell proliferation, migration, and capillary tube formation and increased collagen production (Jerkic et al. 2006). This is consistent with the failure of the mature circulating ECs from HHT1 patients to form cord-like structures in vitro (Fernandez et al. 2005). Curiously, in another study *Eng*$^{-/-}$ murine ECs show a higher proliferative rate than controls (Pece-Barbara et al. 2005).

The *Eng* null mutation in mice is lethal at midgestation and shows defects in vascular and cardiac development (Bourdeau et al. 1999; Arthur et al. 2000; Sorensen et al. 2003). Vasculogenesis is normal, but the primitive vascular plexus of the yolk sac fails to mature. Vascular channels dilate and rupture with internal bleeding. Although less important and less frequent than in *Alk1*$^{-/-}$ mice, AV shunts between major arteries and veins are observed. *Eng*$^{+/-}$ mice survive and develop HHT signs, such as telangiectasias and recurrent nosebleeds (Bourdeau et al. 2000b). Some have pulmonary and hepatic vessel dilation and cerebral AVMs (Bourdeau et al. 2000b; Satomi et al. 2003). The prevalence is highly dependent on the genetic background, the 129/Ola strain being 10 times more often affected than the C57BL/6 strain (Bourdeau et al. 2001). This suggests that mice with a single Eng copy are predisposed to HHT development and that epigenetic factors and/or modifier genes could contribute to the phenotypic heterogeneity. One such factor could be the TGF-β1 level, which has been shown to be lower in wild-type 129/Ola strain than in C57BL/6, and this level was further reduced upon loss of one *Eng* allele (Bourdeau et al. 2001). Similarly, HHT1 patients have a reduced circulating level of TGF-β1 (Letarte et al. 2005). As TGF-β1 promotes recruitment and differentiation of mesenchymal cells into vascular smooth muscle cells around ECs (Carvalho et al. 2004), a decreased level of TGF-β1 could impair this process and weaken the vessels. In this scenario, 129/Ola mice would be more susceptible than C57BL/6 mice, as their TGF-β1 level is lower.

Apart its role in TGF-β signaling, it was shown that Endoglin is involved in organization of the actin cytoskeleton (Conley et al. 2004; Sanz-Rodriguez et al. 2004). Mature circulating ECs from HHT1 patients have an abnormal shape with a disorganized and depolymerized actin cytoskeleton (Fernandez et al. 2005). Another interesting function is the regulation of nitric oxide-dependent vasodilatation (Jerkic et al. 2004; Toporsian et al. 2005). However, whereas one study reported decreased endothelium-dependent vasodilatation in Eng$^{+/-}$ mice (Jerkic et al.

2004), another observed the opposite effect (Toporsian et al. 2005). This discrepancy may be linked to the small dilations observed in postcapillary venules in forming HHT lesions (Braverman et al. 1990).

MADH4 gene (OMIM 600993), mutated in the combined syndrome of juvenile polyposis and HHT, codes for SMAD4, which is the only Co-SMAD known in human (Gallione et al. 2004). In contrast with ALK1 and Endoglin, SMAD4 is expressed in many cell types, which may explain the more complex phenotype associated with mutations in *MADH4*. In ECs, it forms a complex with R-SMAD activated by ALK1 (SMAD1 and 5) or ALK5 (SMAD2 and 3) (Fig. 21.3); the complex translocates to the nucleus and activates the transcription of various genes.

MADH4 is a tumor suppressor gene with multiple roles in embryogenesis. Inactivation of the murine homolog of *MADH4*, Dpc4, causes death before day 7.5. Mutant embryos are small due to insufficient proliferation, no mesoderm is formed, and the visceral endoderm is disorganized (Sirard et al. 1998). These dramatic effects illustrate the central role played by SMAD4 in the TGF-β pathways. Heterozygous mice are normal.

PTEN hamartoma tumor syndrome (PHTS) includes several tumor-susceptibility disorders with mutations in the PTEN gene (OMIM 601728), such as Cowden syndrome (CS), Bannayan–Riley–Ruvalcaba syndrome (BRRS), and the Proteus-like syndrome (PLS). Approximately 80% of patients with CS, 60% of those with BRRS, and up to 50% of those with PLS have mutations in PTEN and are included in the PHTS group (Zbuk K, GeneReviews, http://www.geneclinics.org). Although not a recognized

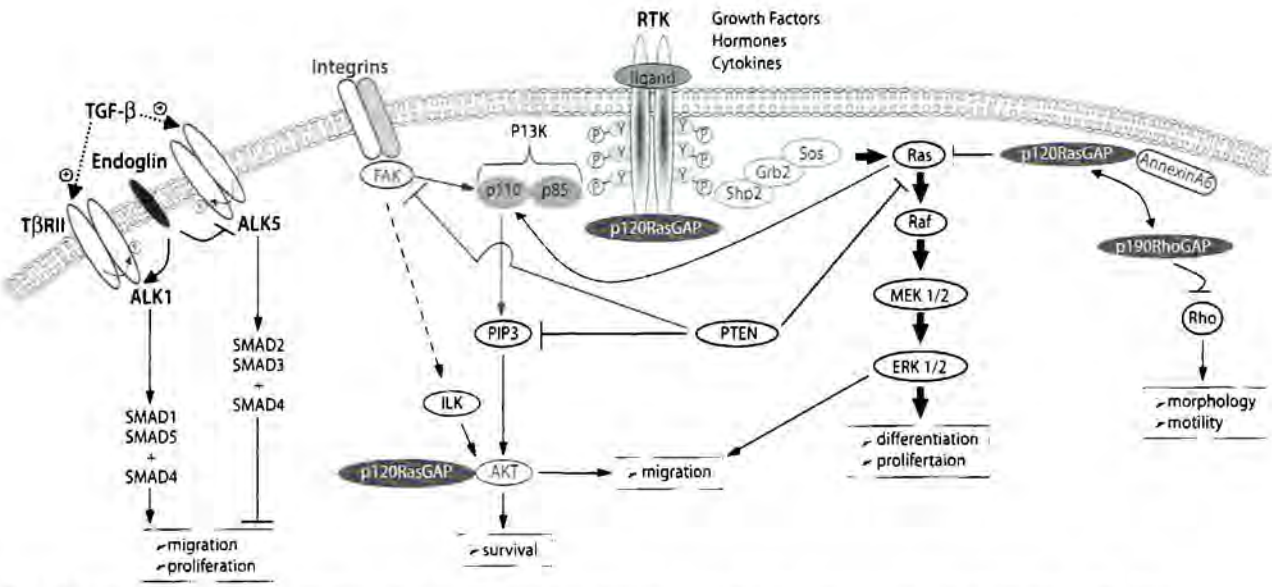


Fig. 21.3. Signaling pathways implicated in monogenic disorders associated with arteriovenous malformation. The Ras–Raf–MEK–ERK pathway is involved in differentiation, proliferation and cell migration. P120RasGAP (mutated in CM-AVM) inhibits Ras by accelerating the conversion of active Ras-GTP to inactive Ras-GDP. P120RasGAP also has the capacity to enhance AKT activity, possibly via ILK, with an antiapoptotic effect, and to interact with p190RhoGAP important for directing cell movement. PI3 K/AKT pathway is involved in cell survival. PTEN (mutated in PHTS) down-regulates this pathway. It also has an inhibitory effect on Ras/ERK pathway. By dephosphorylating FAK, PTEN could inhibit integrin-mediated signaling for cell spreading and migration. TGF-β pathway is involved in cell migration and proliferation. ALK1 (mutated in HHT2) activates SMAD1 and SMAD5, which associate with SMAD4 (mutated in JPHT). The complex is translocated to the nucleus and modulates transcription of various genes. Cell migration and proliferation are stimulated. Endoglin (mutated in HHT1) stimulates the TGF-β/ALK1 pathway and inhibits the TGF-β/ALK5 pathway

diagnostic criterion, AVM has been reported in several patients with PHTS (Turnbull et al. 2005; Zhou et al. 2000).

PTEN is an important tumor suppressor gene implicated in human cancer. It encodes a lipid/protein phosphatase. Via its lipid phosphatase activity, PTEN down-regulates the PI3K/Akt pathway and causes cell cycle arrest and apoptosis (Fig. 21.3). Indeed, its main substrate in vivo is phosphatidylinositol (3,4,5)-triphosphate (PIP3) produced by activated phosphatidylinositol-3 kinase (PI3K) (Maehama and Dixon 1998). In addition, it has been proposed that, via its protein phosphatase activity, PTEN would inhibit integrin-mediated cell spreading and migration by dephosphorylating FAK (Tamura et al. 1998, 1999a, 1999b).

The pathogenic mechanism leading from *PTEN* mutation to AVM is not known, but this association suggests PTEN to be implicated in angiogenesis. Indeed, it has been shown that PTEN down-regulates vascular endothelial growth factor (VEGF) expression, and murine Pten is indispensable for normal vascular and cardiac morphogenesis. It also has a role in tumor angiogenesis (Hamada et al. 2005; Koul et al. 2002). In vitro, heterozygous loss of Pten in murine ECs induces cell proliferation and migration in response to vascular growth factors, such as bFGF alone or in combination with either Ang-1 or VEGF-A (Hamada et al. 2005). Yet, murine embryos with $Pten^{+/-}$ ECs are viable and do not have any vascular abnormalities. At adult stage, these mice show a more rapid growth of tumors than controls, due to increased angiogenesis. Moreover, complete loss of *Pten* in EC leads to embryonic death at E11.5 due to cardiac and vascular abnormalities. The primary vascular plexus is normal, but remodeling fails. These mice have increased EC proliferation, and dilated and fewer vessels than in controls. Recruitment of pericytes and smooth muscle cells is impaired (Hamada et al. 2005).

It remains to be unraveled whether all patients with CS, BRRS and PLS with AVM have mutations in PTEN and whether particular PTEN mutations predispose to AVMs.

Capillary malformation–arteriovenous malformation (CM-AVM; OMIM 608354) is an autosomal dominant disorder associated with mutations in the *RASA1* gene (Eerola et al. 2003). Clinically, CM-AVM is characterized by particular capillary malformations, which are in general small, multiple and randomly distributed. Moreover, about one third of CM-AVM patients have AV shunts (Eerola et al. 2003; Revencu et al., unpublished). The AV shunts in CM-AVM are either localized or diffuse. Localized AVMs are seen in skin, muscle, bone and brain. The diffuse lesions are seen as part of Parkes Weber syndrome (OMIM 608355). This latter is characterized by a large cutaneous vascular stain on an extremity in association with soft tissue and skeletal hypertrophy of the affected limb, with underlying multiple AV microfistulas (Mulliken and Young 1988).

Until now, 30 different *RASA1* mutations in 32 families have been identified, most of them leading to premature stop codon (Eerola et al. 2003; Revencu et al., unpublished). *RASA1* encodes p120RasGAP (OMIM 139150), a cytoplasmic modular protein. This protein is one of the known mammalian inhibitors (GTPase-activating proteins, GAPs) for Ras. p120RasGAP also has functions independent of Ras.

As with HHT and PHTS, the mechanism by which mutations in *RASA1* cause AVMs is not known, nor whether it is Ras-dependent or Ras-independent. The Ras proteins (H-Ras, N-Ras, KiA-Ras and KiB-Ras) are small GTPases involved in cell survival and proliferation. They are central players in several signaling pathways, the best known being Raf/MAPK, leading to cell growth and proliferation, and PI3K/Akt, leading to cell survival (Fig. 21.3). Activating somatic mutations in *RAS* genes occur in up to 30% of human cancers.

In ECs, Ras is required for VEGF-induced proliferation, migration and branching morphogenesis in three-dimensional cultures (Meadows et al. 2001). The active Ras^{V12} mutant induced in primary ECs proliferation via ERK and migration via ERK and PI3K (Meadows et al. 2004). Angiopoietin 1 (Angl), acting via Tie-2 receptor, activates the MAPK pathway leading to EC migration (Yoon et al. 2003). In human umbilical vein ECs (HUVECs), the activation of EphB4 and EphB2 receptors by the artery-specific ligand, ephrinB2, inhibits the VEGF-

induced EC proliferation and migration, and Angl-induced migration (Kim et al. 2002). These activities were mediated by the recruitment of p120RasGAP to the EphB4 and EphB2 receptors. In other words, at the arterial–venous boundary, the activation of the venous Eph receptors by the arterial ephrinB2 ligand can stop venous EC migration and proliferation. Vice versa, the activation of ephrinB2 by EphB4 did not modify the VEGF and Angl effects. Thus, p120RasGAP insufficiency could lead to abnormal venous EC migration and proliferation in the presence of VEGF and Angl.

It has been shown that PTEN has the capacity to inhibit H-Ras in mouse fibroblast cell line NIH3T3. This was associated more with the inhibition of PI3K-dependent signaling cascade than with the inhibition of MAPK (Tolkacheva and Chan 2000). However, PTEN also has the capacity of inhibiting the EGF- and PDGF-mediated Ras/MAPK pathway activation, at least in glioblastoma cell line (Gu et al. 1998). Thus, we can assume that impaired function of p120RasGAP and PTEN in CM-AVM and PHTS, respectively, lead to persistent activation of Ras. This could result in activation of MAPK and/or PI3K/AKT pathways with abnormal migration, proliferation and survival, and an overlapping pathogenic mechanism for AVM in these two disorders. Yet, in vitro, in rat cardiac myocytes p120RasGAP binds to, and enhances phosphorylation and activity of, Akt with a protection against apoptosis (Yue et al. 2004). Knockdown of p120RasGAP by RNAi inhibits Akt phosphorylation. This effect is Ras-independent. Another Ras-independent p120RasGAP effect is its interaction with p190RhoGAP (a GAP for RhoGTPases). This seems to be important for directing cell movement by regulation of actin stress fibers, and focal adhesion turnover and reorientation (Kulkarni et al. 2000). The role of p120RasGAP in angiogenesis is highlighted by murine models. P120RasGAP$^{+/-}$ mice appear normal and fertile, whereas p120RasGAP$^{-/-}$ embryos die at E10.5 due to defects in vascular development (Henkemeyer et al. 1995). Vasculogenesis is normal, but subsequently ECs fail to form a highly organized vascular network. Embryos mosaic for wild-type and p120RasGAP$^{-/-}$ cells survive longer and show at E15 edema and abnormal vasculature (Henkemeyer et al. 1995).

Sporadic AVM Lesions

Although the fast-flow lesions associated with various syndromes have allowed insights into factors involved in AVM formation in man, the etiopathogenesis of isolated AVM remains unknown. A recent case–control study showed an association of a common *ALK1* polymorphism with an increased risk of cerebral AVM (Pawlikowska et al. 2005). This approach can now be used for the more recently identified AVM-associated genes: *PTEN* and *RASA1*.

Concluding Remarks

The pathogenesis of AVM has not been fully elucidated, but the associated genes in mice and men encode molecules involved in processes essential for vascular development and homeostasis, such as cell proliferation, migration, survival, morphogenesis, cell–cell interaction, and AV specification. These molecules seem to operate in different but interacting pathways, which are involved not only in AVM formation, but also in many other developmental disorders, as well as in tumor growth. Identification of the molecules involved in AVM pathogenesis permits direction of research efforts toward development of targeted therapies, which could be inhibitors/modulators of the implied signaling pathways: TGF-β signaling, Ras/Raf/MAPK, and PI3 K/AKT. Nevertheless, a better understanding of the primary versus secondary defects is needed.

Acknowledgements

N.R. is supported by Cliniques universitaires St Luc, Université catholique de Louvain, and FRSM (Fonds de la Recherche Scientifique Médicale). M.V., a Maître de Recherches du FNRS, is supported by grants from the FNRS (Fonds National de la Recherche Scientifique); ARC (Actions de Recherche Concertées – Communauté française de Belgique); Inter-

university Attraction Poles, initiated by the Belgian Federal Science Policy, networks 5/25 and 6/05; the European Commission, programme project EU FW6 Integrated Project Lymphangiogenomics LSHG-CT-2004-503573; and National Institutes of Health programme project P01 AR048564. The authors thank Ms. Liliana Niculescu for excellent secretarial help and Mr. Mircea Gotia for drawings.

References

Abdalla SA, Letarte M (2006) Hereditary haemorrhagic telangiectasia: current views on genetics and mechanisms of disease. J Med Genet 43:97–110

Andersson M, Karlsson L, Svensson PA, Ulfhammer E, Ekman M, Jernas M, Carlsson LM, Jern S (2005) Differential global gene expression response patterns of human endothelium exposed to shear stress and intraluminal pressure. J Vasc Res 42:441–52

Arthur HM, Ure J, Smith AJ, Renforth G, Wilson DI, Torsney E, Charlton R, Parums DV, Jowett T, Marchuk DA, Burn J, Diamond AG (2000) Endoglin, an ancillary TGFbeta receptor, is required for extraembryonic angiogenesis and plays a key role in heart development. Dev Biol 217:42–53

Begbie ME, Wallace GM, Shovlin CL (2003) Hereditary haemorrhagic telangiectasia (Osler-Weber-Rendu syndrome): a view from the 21st century. Postgrad Med J 79:18–24

Bourdeau A, Dumont DJ, Letarte M (1999) A murine model of hereditary hemorrhagic telangiectasia. J Clin Invest 104:1343–1351

Bourdeau A, Cymerman U, Paquet ME, Meschino W, McKinnon WC, Guttmacher AE, Becker L, Letarte M (2000a) Endoglin expression is reduced in normal vessels but still detectable in arteriovenous malformations of patients with hereditary hemorrhagic telangiectasia type 1. Am J Pathol 156:911–923

Bourdeau A, Faughnan ME, Letarte M (2000b) Endoglin-deficient mice, a unique model to study hereditary hemorrhagic telangiectasia. Trends Cardiovasc Med 10:279–285

Bourdeau A, Faughnan ME, McDonald ML, Paterson AD, Wanless IR, Letarte M (2001) Potential role of modifier genes influencing transforming growth factor-beta1 levels in the development of vascular defects in endoglin heterozygous mice with hereditary hemorrhagic telangiectasia. Am J Pathol 158:2011–2020

Braverman IM, Keh A, Jacobson BS (1990) Ultrastructure and three-dimensional organization of the telangiectases of hereditary hemorrhagic telangiectasia. J Invest Dermatol 95:422–427

Carlson TR, Yan Y, Wu X, Lam MT, Tang GL, Beverly LJ, Messina LM, Capobianco AJ, Werb Z, Wang R (2005) Endothelial expression of constitutively active Notch4 elicits reversible arteriovenous malformations in adult mice. Proc Natl Acad Sci USA 102:9884–9889

Carvalho RL, Jonker L, Goumans MJ, Larsson J, Bouwman P, Karlsson S, Dijke PT, Arthur HM, Mummery CL (2004) Defective paracrine signalling by TGFbeta in yolk sac vasculature of endoglin mutant mice: a paradigm for hereditary haemorrhagic telangiectasia. Development 131:6237–6247

Choi JH, Mohr JP (2005) Brain arteriovenous malformations in adults. Lancet Neurol 4:299–308

Cole SG, Begbie ME, Wallace GM, Shovlin CL (2005) A new locus for hereditary haemorrhagic telangiectasia (HHT3) maps to chromosome 5. J Med Genet 42:577–582

Conley BA, Koleva R, Smith JD, Kacer D, Zhang D, Bernabeu C, Vary CP (2004) Endoglin controls cell migration and composition of focal adhesions: function of the cytosolic domain. J Biol Chem 279:27440–27449

Eerola I, Boon LM, Mulliken JB, Burrows PE, Dompmartin A, Watanabe S, Vanwijck R, Vikkula M (2003) Capillary malformation-arteriovenous malformation, a new clinical and genetic disorder caused by RASA1 mutations. Am J Hum Genet 73:1240–1249

Fernandez LA, Sanz-Rodriguez F, Zarrabeitia R, Perez-Molino A, Hebbel RP, Nguyen J, Bernabeu C, Botella LM (2005) Blood outgrowth endothelial cells from hereditary haemorrhagic telangiectasia patients reveal abnormalities compatible with vascular lesions. Cardiovasc Res 68:235–248

Fischer A, Schumacher N, Maier M, Sendtner M, Gessler M (2004) The Notch target genes Hey1 and Hey2 are required for embryonic vascular development. Genes Dev 18:901–911

Fleetwood IG, Steinberg GK (2002) Arteriovenous malformations. Lancet 359:863–873

Gale NW, Dominguez MG, Noguera I, Pan L, Hughes V, Valenzuela DM, Murphy AJ, Adams NC, Lin HC, Holash J, Thurston G, Yancopoulos GD (2004) Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. Proc Natl Acad Sci USA 101:15949–15954

Gallione CJ, Repetto GM, Legius E, Rustgi AK, Schelley SL, Tejpar S, Mitchell G, Drouin E, Westermann CJ, Marchuk DA (2004) A combined syndrome of juvenile polyposis and hereditary haemorrhagic telangiectasia associated with mutations in MADH4 (SMAD4). Lancet 363:852–859

Goumans MJ, Valdimarsdottir G, Itoh S, Rosendahl A, Sideras P, ten Dijke P (2002) Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. EMBO J 21:1743–1753

Goumans MJ, Lebrin F, Valdimarsdottir G (2003) Controlling the angiogenic switch: a balance between two distinct TGF-b receptor signaling pathways. Trends Cardiovasc Med 13:301–307

Gu J, Tamura M, Yamada KM (1998) Tumor suppressor PTEN inhibits integrin- and growth factor-mediated mitogen-activated protein (MAP) kinase signaling pathways. J Cell Biol 143:1375–1383

Hamada K, Sasaki T, Koni PA, Natsui M, Kishimoto H, Sasaki J, Yajima N, Horie Y, Hasegawa G, Naito M, Miyazaki J, Suda T, Itoh H, Nakao K, Mak TW, Nakano T, Suzuki A (2005) The PTEN/PI3 K pathway governs normal vascular development and tumor angiogenesis. Genes Dev 19:2054–2065

Henkemeyer M, Rossi DJ, Holmyard DP, Puri MC, Mbamalu G, Harpal K, Shih TS, Jacks T, Pawson T (1995) Vascular system defects and neuronal apoptosis in mice lacking ras GTPase-activating protein. Nature 377:695–701

Isogai S, Lawson ND, Torrealday S, Horiguchi M, Weinstein BM (2003) Angiogenic network formation in the developing vertebrate trunk. Development 130:5281–5290

Jerkic M, Rivas-Elena JV, Prieto M, Carron R, Sanz-Rodriguez F, Perez-Barriocanal F, Rodriguez-Barbero A, Bernabeu C, Lopez-Novoa JM (2004) Endoglin regulates nitric oxide-dependent vasodilatation. FASEB J 18:609–611

Jerkic M, Rodriguez-Barbero A, Prieto M, Toporsian M, Pericacho M, Rivas-Elena JV, Obreo J, Wang A, Perez-Barriocanal F, Arevalo M, Bernabeu C, Letarte M, Lopez-Novoa JM (2006) Reduced angiogenic responses in adult Endoglin heterozygous mice. Cardiovasc Res 69:845–854

Johnson DW, Berg JN, Baldwin MA, Gallione CJ, Marondel I, Yoon SJ, Stenzel TT, Speer M, Pericak-Vance MA, Diamond A, Guttmacher AE, Jackson CE, Attisano L, Kucherlapati R, Porteous ME, Marchuk DA (1996) Mutations in the activin receptor-like kinase 1 gene in hereditary haemorrhagic telangiectasia type 2. Nat Genet 13:189–195

Kamath BM, Spinner NB, Emerick KM, Chudley AE, Booth C, Piccoli DA, Krantz ID (2004) Vascular anomalies in Alagille syndrome: a significant cause of morbidity and mortality. Circulation 109:1354–1358

Kim I, Ryu YS, Kwak HJ, Ahn SY, Oh JL, Yancopoulos GD, Gale NW, Koh GY (2002) EphB ligand, ephrinB2, suppresses the VEGF- and angiopoietin 1-induced Ras/mitogen-activated protein kinase pathway in venous endothelial cells. FASEB J 16:1126–1128

Koul D, Shen R, Garyali A, Ke LD, Liu TJ, Yung WK (2002) MMAC/PTEN tumor suppressor gene regulates vascular endothelial growth factor-mediated angiogenesis in prostate cancer. Int J Oncol 21:469–475

Krebs LT, Shutter JR, Tanigaki K, Honjo T, Stark KL, Gridley T (2004) Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. Genes Dev 18:2469–2473

Krings T, Ozanne A, Chng SM, Alvarez H, Rodesch G, Lasjaunias PL (2005) Neurovascular phenotypes in hereditary haemorrhagic telangiectasia patients according to age. Review of 50 consecutive patients aged 1 day–60 years. Neuroradiology 47:711–720

Kulkarni SV, Gish G, van der Geer P, Henkemeyer M, Pawson T (2000) Role of p120 Ras-GAP in directed cell movement. J Cell Biol 149:457–470

Lamouille S, Mallet C, Feige JJ, Bailly S (2002) Activin receptor-like kinase 1 is implicated in the maturation phase of angiogenesis. Blood 100:4495–4501

Lasjaunias PL (1997) Vascular diseases in neonates, infants and children. Springer, Berlin Heidelberg New York

Lawson ND, Scheer N, Pham VN, Kim CH, Chitnis AB, Campos-Ortega JA, Weinstein BM (2001) Notch signaling is required for arterial-venous differentiation during embryonic vascular development. Development 128:3675–3683

le Noble F, Moyon D, Pardanaud L, Yuan L, Djonov V, Matthijsen R, Breant C, Fleury V, Eichmann A (2004) Flow regulates arterial-venous differentiation in the chick embryo yolk sac. Development 131:361–375

Lebrin F, Goumans MJ, Jonker L, Carvalho RL, Valdimarsdottir G, Thorikay M, Mummery C, Arthur HM, ten Dijke P (2004) Endoglin promotes endothelial cell proliferation and TGF-beta/ALK1 signal transduction. EMBO J 23:4018–4028

Letarte M, McDonald ML, Li C, Kathirkamathamby K, Vera S, Pece-Barbara N, Kumar S (2005) Reduced endothelial secretion and plasma levels of transforming growth factor-beta1 in patients with hereditary hemorrhagic telangiectasia type 1. Cardiovasc Res 68:155–164

Li C, Hampson IN, Hampson L, Kumar P, Bernabeu C, Kumar S (2000) CD105 antagonizes the inhibitory signaling of transforming growth factor beta1 on human vascular endothelial cells. FASEB J 14:55–64

Maehama T, Dixon JE (1998) The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem 273:13375–13378

McAllister KA, Grogg KM, Johnson DW, Gallione CJ, Baldwin MA, Jackson CE, Helmbold EA, Markel DS, McKinnon WC, Murrell J, et al. (1994) Endoglin, a TGF-beta binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. Nat Genet 8:345–351

McDonald MT, Papenberg KA, Ghosh S, Glatfelter AA, Biesecker BB, Helmbold EA, Markel DS, Zolotor A, McKinnon WC, Vanderstoep JL et al (1994) A disease locus for hereditary haemorrhagic telangiectasia maps to chromosome 9q33–34. Nat Genet 6:197–204

Meadows KN, Bryant P, Pumiglia K (2001) Vascular endothelial growth factor induction of the angiogenic phenotype requires Ras activation. J Biol Chem 276:49289–49298

Meadows KN, Bryant P, Vincent PA, Pumiglia KM (2004) Activated Ras induces a proangiogenic phenotype in primary endothelial cells. Oncogene 23:192–200

Moyon D, Pardanaud L, Yuan L, Breant C, Eichmann A (2001) Plasticity of endothelial cells during arterial-venous differentiation in the avian embryo. Development 128:3359–3370

Mulliken JB, Glowacki J (1982) Classification of pediatric vascular lesions. Plast Reconstr Surg 70:120–121

Mulliken JB, Young AE (1988) Vascular birthmarks: hemangiomas and malformations. Saunders, Philadelphia

Murray P (1926) The physiological principle of minimum. The vascular system and the cost of blood volume. Proc Natl Acad Sci USA 12:207–214

Oh SP, Seki T, Goss KA, Imamura T, Yi Y, Donahoe PK, Li L, Miyazono K, ten Dijke P, Kim S, Li E (2000) Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. Proc Natl Acad Sci USA 97:2626–2631

Pawlikowska L, Tran MN, Achrol AS, Ha C, Burchard E, Choudhry S, Zaroff J, Lawton MT, Castro R, McCulloch CE, Marchuk D, Kwok PY, Young WL (2005) Polymorphisms in transforming growth factor-beta-related genes ALK1 and ENG are associated with sporadic brain arteriovenous malformations. Stroke 36:2278–2280

Pece-Barbara N, Vera S, Kathirkamathamby K, Liebner S, di Guglielmo GM, Dejana E, Wrana JL, Letarte M (2005) Endoglin null endothelial cells proliferate faster and are more responsive to transforming growth factor beta1 with higher affinity receptors and an activated Alk1 pathway. J Biol Chem 280:27800–27808

Pescini F, Sarti C, Pantoni L, Mangiafico S, Bianchi S, Dotti MT, Federico A, Inzitari D (2006) Cerebellar arteriovenous malformation and vertebral artery aneurysm in a CADASIL patient. Acta Neurol Scand 113:62–63

Risau W (1997) Mechanisms of angiogenesis. Nature 386:671–674

Sanz-Rodriguez F, Guerrero-Esteo M, Botella LM, Banville D, Vary CP, Bernabeu C (2004) Endoglin regulates cytoskeletal organization through binding to ZRP-1, a member of the Lim family of proteins. J Biol Chem 279:32858–32868

Satomi J, Mount RJ, Toporsian M, Paterson AD, Wallace MC, Harrison RV, Letarte M (2003) Cerebral vascular abnormalities in a murine model of hereditary hemorrhagic telangiectasia. Stroke 34:783–789

Seki T, Hong KH, Oh SP (2006) Nonoverlapping expression patterns of ALK1 and ALK5 reveal distinct roles of each receptor in vascular development. Lab Invest 86:116–129

Shawber CJ, Kitajewski J (2004) Notch function in the vasculature: insights from zebrafish, mouse and man. Bioessays 26:225–234

Shovlin CL, Hughes JM, Tuddenham EG, Temperley I, Perembelon YF, Scott J, Seidman CE, Seidman JG (1994) A gene for hereditary haemorrhagic telangiectasia maps to chromosome 9q3. Nat Genet 6:205–209

Sirard C, de la Pompa JL, Elia A, Itie A, Mirtsos C, Cheung A, Hahn S, Wakeham A, Schwartz L, Kern SE, Rossant J, Mak TW (1998) The tumor suppressor gene Smad4/Dpc4 is required for gastrulation and later for anterior development of the mouse embryo. Genes Dev 12:107–119

Sorensen LK, Brooke BS, Li DY, Urness LD (2003) Loss of distinct arterial and venous boundaries in mice lacking endoglin, a vascular-specific TGFbeta coreceptor. Dev Biol 261:235–250

Srinivasan S, Hanes MA, Dickens T, Porteous ME, Oh SP, Hale LP, Marchuk DA (2003) A mouse model for hereditary hemorrhagic telangiectasia (HHT) type 2. Hum Mol Genet 12:473–482

Tamura M, Gu J, Matsumoto K, Aota S, Parsons R, Yamada KM (1998) Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. Science 280:1614–1617

Tamura M, Gu J, Takino T, Yamada KM (1999a) Tumor suppressor PTEN inhibition of cell invasion, migration, and growth: differential involvement of focal adhesion kinase and p130Cas. Cancer Res 59:442–449

Tamura M, Gu J, Tran H, Yamada KM (1999b) PTEN gene and integrin signaling in cancer. J Natl Cancer Inst 91:1820–1828

Thoma R (1893) Untersuchungen über die Histogenese une Histomechanik des Gefässsystems. Enke, Stuttgart

Tolkacheva T, Chan AM (2000) Inhibition of H-Ras transformation by the PTEN/MMAC1/TEP1 tumor suppressor gene. Oncogene 19:680–689

Toporsian M, Gros R, Kabir MG, Vera S, Govindaraju K, Eidelman DH, Husain M, Letarte M (2005) A role for endoglin in coupling eNOS activity and regulating vascular tone revealed in hereditary hemorrhagic telangiectasia. Circ Res 96:684–692

Turnbull MM, Humeniuk V, Stein B, Suthers GK (2005) Arteriovenous malformations in Cowden syndrome. J Med Genet 42:e50

Urness LD, Sorensen LK, Li DY (2000) Arteriovenous malformations in mice lacking activin receptor-like kinase-1. Nat Genet 26:328–331

Uyttendaele H, Ho J, Rossant J, Kitajewski J (2001) Vascular patterning defects associated with expression of activated Notch4 in embryonic endothelium. Proc Natl Acad Sci USA 98:5643–5648

Vincent P, Plauchu H, Hazan J, Faure S, Weissenbach J, Godet J (1995) A third locus for hereditary haemorrhagic telangiectasia maps to chromosome 12q. Hum Mol Genet 4:945–949

Yoon MJ, Cho CH, Lee CS, Jang IH, Ryu SH, Koh GY (2003) Localization of Tie2 and phospholipase D in endothelial caveolae is involved in angiopoietin-1-induced MEK/ERK phosphorylation and migration in endothelial cells. Biochem Biophys Res Commun 308:101–105

You LR, Lin FJ, Lee CT, DeMayo FJ, Tsai MJ, Tsai SY (2005) Suppression of Notch signalling by the COUP-TFII transcription factor regulates vein identity. Nature 435:98–104

Yue Y, Lypowy J, Hedhli N, Abdellatif M (2004) Ras GTPase-activating protein binds to Akt and is required for its activation. J Biol Chem 279:12883–12889

Zhong TP, Childs S, Leu JP, Fishman MC (2001) Gridlock signalling pathway fashions the first embryonic artery. Nature 414:216–220

Zhou XP, Marsh DJ, Hampel H, Mulliken JB, Gimm O, Eng C (2000) Germline and germline mosaic PTEN mutations associated with a Proteus-like syndrome of hemihypertrophy, lower limb asymmetry, arteriovenous malformations and lipomatosis. Hum Mol Genet 9:765–768

Animal Models and Preclinical Anti-Angiogenic Studies

Vascular Endothelial Growth Factor Antibodies for Anti-Angiogenic Therapy

HANS-PETER GERBER, MEGAN E. BALDWIN, FARBOD SHOJAEI

Abstract

The first antibody described to block human VEGF-A is the mouse monoclonal antibody A4.6.1, originally identified based on its ability to block endothelial cell proliferation stimulated by recombinant human VEGF-A. HuMAb VEGF (bevacizumab), a humanized derivative of A4.6.1, was approved in the USA and Europe for the treatment of colorectal cancer in combination with standard chemotherapy and is currently being developed as an anti-cancer therapeutic in a large variety of human cancer types as single-agent therapy or in combination with other anti-cancer modalities, including cytotoxic compounds and radiation. Given the clinical success of the first-generation anti-VEGF antibody, second-generation anti-VEGF antibodies are likely to enter clinical development in the near future. In order to select for antibodies with improved therapeutic activities, the role of epitope binding, binding kinetics and potential effector functions will need to be investigated carefully using relevant preclinical models. One major limitation in the interpretation of data generated in preclinical animal models is the difficulty in defining the extent to which pharmacologic and physiologic responses mimic those in humans. This is particularly true when the therapeutic compounds of interest interact differentially with the human and host VEGF protein, such as in the case of first- and second-generation anti-VEGF antibodies. In this chapter, we review the efficacy, pharmacokinetic, safety and biodistribution data of first- and second-generation anti-VEGF antibodies and provide an update on the status of the pre-clinical model development to study the efficacy and safety of various compounds blocking human VEGF-A in non-primate models.

CONTENTS

Introduction

22.1.1 The Anti-angiogenic Approach as a Therapeutic Strategy to Interfere with Tumor Growth

The anti-angiogenic approach as a therapeutic strategy for the treatment of solid tumors was originally proposed based upon the observation that rapid growth of tumor explants was dependent upon the development of a rich vascular supply (Algire and Chalkley 1945). Tumor cells are often present in a dormant state and are not able to progress to more malignant states unless they undergo a process termed "the angiogenic switch." This process is characterized by the upregulation of angiogenic factors within the tumor and/or the downregulation of anti-angiogenic genes. Such a shift in the balance of angiogenic regulators towards pro-angiogenic activities results in the onset of tumor angiogenesis and tumor progression (Hanahan and Folkman 1996). Mammalian cells require oxygen and nutrients for their survival. Therefore, within solid tumors, both normal and neoplastic cells are located within 100–200 μm of blood vessels, the diffusion limit for oxygen. Without the growth of new blood vessels, tumors cannot grow beyond a critical size or metastasize to other organs. Various signals that trigger angiogenesis have been described and include metabolic stress (e.g., low pO_2, low pH, or hypoglycemia), mechanical stress (e.g., pressure generated by proliferating cells), and immune/inflammatory responses (e.g., stimuli generated by immune/inflammatory cells that have infiltrated into the tissues; Fig. 22.1). Also, genetic mutations within the tumor cells, including the activation of oncogenes or deletion of tumor-suppressor genes that control production of angiogenesis regulators, can trigger tumor angiogenesis (reviewed in Hanahan and Weinberg 2000).

22.1.2 Biological Roles of VEGF

VEGF and its two receptor tyrosine kinase receptors, VEGFR-1 [*fms*-like-tyrosine kinase (Flt-1)] and VEGFR-2 [fetal liver kinase-1 (Flk-1) in the mouse], constitute the most extensively investigated ligand–receptor system involved in the regulation of angiogenesis during physiologic and pathologic processes. VEGF is a key mitogen for endothelial cells derived from arteries, veins, and lymphatics of all organs analyzed, but it lacks significant mitogenic activity on most other cell types. The specificity in the biological activity of VEGF for endothelial cells can be attributed to the predominant expression of VEGFR-1 and VEGFR-2 on these cells. Human VEGFR-1 has the highest affinity for recombinant human VEGF ($rhVEGF_{165}$), with a K_d of approximately 10–20 pM, whereas the affinity of VEGFR-2 for $rhVEGF_{165}$ is lower (approximately 75–125 pM).

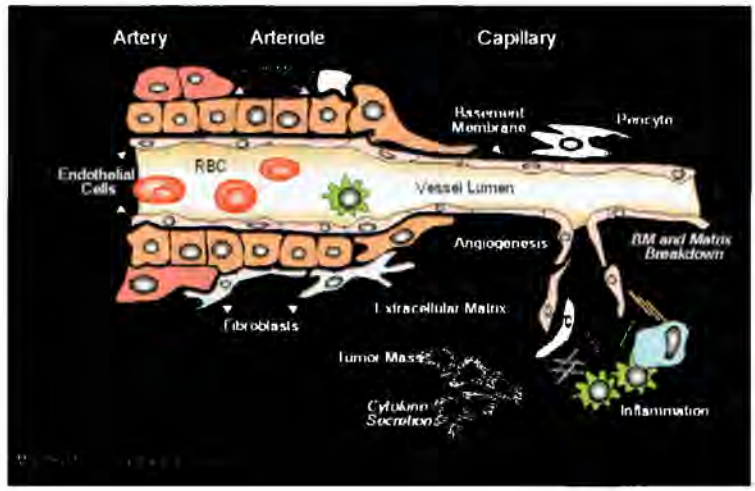


Fig. 22.1. In addition to tumor cells, a large variety of untransformed stromal cells can either directly produce VEGF or stimulate tumor cells to secrete VEGF, all contributing to tumor angiogenesis. Stromal cells producing VEGF include tumor-infiltrating stromal fibroblasts and or bone marrow-derived progenitor cells. Finally, inflammatory cells, including monocytes/macrophages, T- and B-lymphocytes, vascular leukocytes, dendritic cells, neutrophils and mast cells, are know to upregulate VEGF in tumors (reviewed in Coussens and Werb 2002).

More recently, a third VEGF receptor has been identified as neuropilin-1 (NRP1). NRP1 was shown to bind specifically to exon 7-encoded sequences of VEGF and thus fails to bind $VEGF_{121}$, which does not encode exon 7. This receptor does not appear to signal directly, but functions by facilitating binding of VEGF to VEGFR-2, in a manner that enhances the effectiveness of VEGFR-2-mediated signaling on endothelial cells (Soker et al. 1998).

Binding of VEGF to VEGFR-1 and -2 induces the homodimerization of two receptor subunits, which in turn triggers autophosphorylation of their tyrosine kinase domains located within the cytoplasm. Autophosphorylation of the tyrosine kinase domains subsequently engages a series of signal transduction events that ultimately regulate the various biological activities of VEGF on endothelial cells.

In addition to VEGF's key activity as an endothelial cell mitogen, VEGF also induces vasodilatation and, based on its ability to induce vascular leakage in the guinea pig skin, was also named vascular permeability factor (VPF) (Dvorak et al. 1995). VEGF displays chemotactic effects on endothelial cells and increases expression of proteolytic enzymes in endothelial cells involved in stromal degradation. VEGF also mediates immune effects via inhibition of maturation of antigen-presenting dendritic cells (reviewed in Ferrara 2004) and is a potent survival factor when tested in vitro on serum-starved endothelial cells isolated from different organs. The pro-survival activity of VEGF in human endothelial cells is mediated through the binding to VEGFR-2 and subsequent induction of expression of the anti-apoptotic proteins Bcl-2 and A1 (Gerber et al. 1998a). In addition, there is clear evidence that VEGFR-2 signals for survival via the PI3 kinase/Akt pathway (Gerber et al. 1998b). In support of Akt's key regulatory role in endothelial cell survival was the finding that transient expression of a constitutively active form of Akt (Akt2D), or the wild-type form of Bcl-2, was sufficient to rescue apoptotic cell death of primary human endothelial cells grown in VEGF-depleted medium. VEGFR-2 was also shown to activate other signal transduction pathways, including phospholipase C gamma and mitogen-activated kinases MAPK p44/42 (Gille et al. 2001). A survival function for VEGF on endothelial cells during neo-angiogenic events in adult animals was demonstrated (Alon et al. 1995). Targeted inactivation of the VEGF gene resulted in heterozygous embryonic lethality. $VEGF^{-/-}$ embryos are devoid of most endothelial and hematopoietic cells and die at approximately day 10 of embryonic development (Carmeliet et al. 1996; Ferrara et al. 1996). Importantly, the survival function for VEGF on endothelial cells seems to be developmentally regulated, because it is depen-

dent on the age of the animal and interference with VEGF's biological functions in adult species was well tolerated (Ryan et al. 1999; Gerber and Ferrara 2000; Malik et al. 2005).

The Role of VEGF-A in Tumor Angiogenesis

There is now compelling evidence that VEGF is indeed a major tumor angiogenesis factor and that interference with VEGF or VEGF-induced signal transduction cascades results in anti-angiogenic responses, including decreased number and diameter of tumor vessels, which ultimately results in dose-dependent inhibition of tumor growth (reviewed in Jain et al. 2006). Kim et al. (1993) provided the first direct evidence to support the hypothesis that tumor growth depends upon angiogenesis by employing the neutralizing anti-VEGF murine antibody A4.6.1, which substantially inhibited the growth of three different human tumor cell lines, when xenografted in immunodeficient mice. Following this study, many groups tested A4.6.1 and independently reported that growth of a large variety of human tumor cell lines was substantially inhibited (reviewed in Ferrara and Davis-Smyth 1997). Using a genetic mouse model of pancreatic islet carcinoma, it was demonstrated that deletion of the VEGF-A gene in early-stage tumors was sufficient to prevent adenomas from progressing to carcinomas. The failure of tumor progression occurred in the presence of all other VEGF ligand family members: VEGF-B, -C, -D, and PlGF. Thus, the data indicate that VEGF is the key regulator of the angiogenic switch and hence tumor progression in this model of experimental pancreatic carcinoma and that none of the other VEGF family members were able to compensate for the absence of VEGF-A (Inoue et al. 2002). In a model of human tumor xenografted to mice, it was demonstrated that increased expression levels of VEGF were sufficient to trigger the angiogenic switch. Stable transfection of nontumorigenic Chinese hamster ovary (CHO) cells with expression vectors for $VEGF_{165}$ or $VEGF_{121}$ conferred on these cells the ability to form rapidly growing tumors (Ferrara et al. 1993). This increase in tumor growth in vivo was associated with a strong increase in tumor vascularization but in the absence of increased tumor cell growth in vitro. These findings provided further support for the model wherein endothelial cells are the primary targets for VEGF's functions during tumor growth. However, many cancer cells express VEGFR-1 or -2 (Fan et al. 2005; Wey et al. 2005) or neuropilin 1 (NP1) (Akagi et al. 2003), and some experimental cancer cell lines were shown to depend on VEGF for survival. In these cases, VEGF inhibition may also have direct cytotoxic effects on cancer cells.

In addition, combination therapy with compounds targeting the tumor cells directly, such as chemotherapy, may potentially be complementary for the anti-angiogenic approach, based upon the different mechanisms of action underlying both strategies (Teicher 1996). Among the many therapeutic strategies designed to interfere with VEGF signaling in tumor vasculature, a humanized monoclonal antibody to human VEGF-A, which prevents binding of VEGF to its receptors, is most advanced. Key advantages of the humanized antibody strategy are a high degree of specificity for the target antigen combined with a long half-life and minimal risk of immunogenicity in humans. Furthermore, a major advantage of the anti-angiogenic strategy compared to therapeutic interventions targeting tumor cells is that the tumor vasculature is derived from local and circulating endothelial cells that are considered genetically stable and therefore, in contrast to tumor cells,are less likely to develop drug resistance during prolonged treatment periods. Thus, this novel therapeutic concept of targeting the tumor vasculature by neutralizing VEGF could potentially circumvent the development of drug resistance, which is frequently found after prolonged treatment with cytotoxic agents targeting the tumor cells. Additionally, the ratio between cancer and endothelial cells within the tumor can be very small. Such a large number of tumor cells depending upon a small number of endothelial cells might addition-

ally amplify the therapeutic effect of anti-angiogenic treatment. Finally, it is also conceivable that metastatic spread of tumor cells is dependent on the existence of a vascular system within or adjacent to the tumor mass;, thus, the inhibition of angiogenesis may reduce tumor metastasis.

Analysis of both RNA and protein indicates low to undetectable expression levels of VEGFR-1 and -2 on quiescent vasculature in most adult tissues, with the exception of renal glomeruli, where the expression of these receptors is maintained at moderate to high levels even in the adult (Jakeman et al. 1993). This is in marked contrast to the significant upregulation of both VEGF receptors found on the vasculature of many tumors (reviewed in Ferrara 2005). Similarly, increased VEGF levels were detected in most tumor sections by in situ hybridization studies and immunohistochemical analysis (Table 22.1). The cellular origin of VEGF production and secretion is not limited to neoplastic cells but extends to normal stromal cells, including fibroblasts or immune/inflammatory cell infiltrates, which are commonly found within tumor tissues. The contribution of each of these cellular compartments to the overall VEGF levels, as well as the role of other angiogenic factors, is likely to depend on the tumor type and location. Upregulation of VEGF in most human tumors relative to normal tissue and correlation between VEGF expression and tumor progression of various tumor types has been well documented (for review see Dvorak et al. 1995, Ferrara and Davis-Smyth 1997) (Table 22.1).

VEGF-A expression is regulated by differentiation and transformation as well as by oxygen deprivation (Ferrara 2002). Under hypoxic conditions, hypoxia inducible factor-1 binds to the promoter region of the VEGF-A gene and activates its transcription. Stabilization of VEGF-A mRNA also contributes to increased VEGF-A expression under conditions of limited oxygen supply. In addition to these epigenetic regulatory events, several cytokines, hormones, and growth factors upregulate the expression of VEGF mRNA and/or induce release of VEGF protein (Fig. 22.1). On normal keratinocytes, epidermal growth factor (EGF), transforming growth factor (TGF)-α and -β and keratinocyte growth factor (KGF) induce VEGF expression (Frank et al. 1995). EGF also stimulates VEGF expression in cultured glioblastoma cells (Goldman et al. 1993). Interleukin (IL)1-β induces VEGF in normal aortic smooth muscle cells (Li et al. 1995), and IL-6 has been shown to induce VEGF expression in several tumor cell lines (Cohen et al. 1996). Prostaglandin E2 and insulin-like growth factor-1 also increase VEGF-A expression and stimulate its secretion in various cell types (Ferrara and Davis-Smyth 1997).

Genetic mutations frequently found in tumors that lead to the induction of VEGF expression include a mutated form of the p53 tumor suppressor gene (Kieser et al. 1994), oncogenic mutations or amplification of ras and farnesyl transferase (Grugel et al. 1995; Rak et al. 1995), over-expression of v-raf (Grugel et al. 1995) and v-Src (Mukhopadhyay et al. 1995), and mutant forms of the tumor suppressor Von Hippel–Lindau (Siemeister et al. 1996).

Table 22.1. Selection of studies reporting correlation between vegf expression and tumor progression and/or patient surviva

Tumor location	Reference
Gastrointestinal tract	Maeda et al. 1997; Ichikura et al. 2001; Kimura et al. 2001; Karayiannakis et al. 2002
Breast	Toi et al. 1994; Gasparini et al. 1997, 1999; Eppenberger et al. 1998; Linderholm et al. 2000; reviewed in Sledge 2002
Lung	Volm et al. 1997; Yuan et al. 2001; Mall et al. 2002
Thyroid	Bunone et al. 1999
Ovary	Hazelton et al. 1999; Hata et al. 2000
Brain	Komuro et al. 2001
Prostate	Strohmeyer et al. 2000
Adrenal gland	de Fraipont et al. 2000
Liver	Poon et al. 2001
Esophagus	Shimada et al. 2001

Pharmacology of First-Generation Anti-VEGF Antibodies

22.2.1 A4.6.1 and Bevacizumab in Preclinical Models of Cancer

Bevacizumab is a human IgG1 version of the parent murine anti-human VEGF monoclonal antibody (muMAb VEGF) A4.6.1 (Kim et al. 1992), which was shown to potently suppress angiogenesis and tumor growth in a variety of human tumor cell lines transplanted into nude mice (Kim et al. 1993). While the majority of the preclinical tumor studies described in this report used A4.6.1, which neutralizes human VEGF; some of the later studies used bevacizumab. This bias towards A4.6.1 occurred primarily because A4.6.1 was available for supplying research collaborators from 1993 onward, whereas bevacizumab first became available for supply to collaborators in 1998. Bevacizumab and A4.6.1 were shown to be pharmacologically equivalent when tested with human cells, human tissues, and different human VEGF isoforms (Table 22.2 and Presta et al. 1997). The humanization of muMAb VEGF A4.6.1 was accomplished by site-directed mutagenesis of a human IgG1 framework (Presta et al. 1997). The same sequences have been successfully used in the humanization of other murine antibodies, including those targeted to HER-2 (Herceptin, trastuzumab; Carter et al. 1992) and IgE (Xolair, omalizumab; Presta et al. 1993). Overall, bevacizumab consists of about 93% human and 7% murine sequence. Bevacizumab recognizes and neutralizes all isoforms of human VEGF with a dissociation constant (Kd) of 80 pM (Presta et al. 1997) and inhibits VEGF-induced proliferation of endothelial cells in vitro and tumor growth in vivo with potency and efficacy almost identical to those of its parental murine ortholog A4.6.1 (Table 22.2).

22.2.2 Mechanism of Action of Anti-VEGF Antibodies and Preclinical Experiment to Study Potency and Efficacy

Bevacizumab binds to and neutralizes the biological activities of the human VEGF isoforms (VEGF121, 145, 165, 189, and 206) and the proteolytic cleavage product $VEGF_{110}$ (Houck et al. 1992; Kim et al. 1992). Although some of the VEGF residues that are critical for antibody binding are distinct from those important for high-affinity receptor binding, they occupy a common region on VEGF (Muller et al. 1998). The combined data from structural, biochemical, biological, and pharmacological analysis conclusively demonstrated that the neutralizing effect of bevacizumab bound to VEGF results from steric blocking of the binding of VEGF to both its receptors, VEGFR-1 and VEGFR-2 (Muller et al. 1998).

A series of experiments using preclinical tumor models was conducted to test the hypothesis that interference with tumor angiogenesis, by targeting VEGF, is a reasonable approach to inhibit tumor growth. The experiments conducted fell into four major categories:

- Assessment of the efficacy of the VEGF-neutralizing antibodies when tested as a single agent in

Table 22.2. Pharmacological characteristics of bevacizumab and A4.6.1l

	Bevacizumab	A4.6.1
Kd human VEGF165	80 pM[a]	80 pM[b]
ED_{50} ACE cell proliferation[a]	50±5 ng/ml[c]	48±8 ng/ml[c]
In vivo tumor growth inhibition[a]		
0.5 mg/kg, IP, twice weekly[d]	90%	85%
5.0 mg/kg, IP, twice weekly[d]	95%	93%

[a](Shimada et al. 2001)., [b](Kim et al. 1992)., [c]ED_{50} values based on bovine adrenal cortical endothelial cells (ACE) stimulated with 3 ng/ml rhVEGF165., [d]Percent inhibition of human rhabdomyosarcoma cell line (A673) xenografted in nude mice calculated based on tumor weight measurements 4 weeks after tumor cell implantation (Presta et al. 1997).

conjunction with more than 20 different human tumor cell lines representing 13 tumor types. These studies revealed broad and significant growth inhibition when anti-VEGF was administered simultaneously to tumor cell implantation and also when it was administered to animals bearing established tumors.

- Studies of the effects of VEGF-neutralizing antibodies when tested in experimental models of tumor metastasis. These studies demonstrated almost complete inhibition of tumor metastasis to various organs and increased survival rates of anti-VEGF treated animals.
- Studies on tumor growth in animals treated with VEGF-neutralizing antibodies in combination with cytotoxic agents or radiation therapy, which revealed additive or synergistic tumor growth inhibitory effects. Combination treatment with other anti-angiogenic compounds [matrix metalloproteinase (MMP) inhibitors] revealed less pronounced, tumor type-specific inhibitory effects.
- The analysis of vascular changes in tumors of mice treated with VEGF-neutralizing antibodies, which demonstrated a strong correlation between pharmacological effects of tumor growth inhibition and vascular changes. There was a profound reduction in the permeability of the tumor vasculature associated with a decrease in interstitial tumor pressure.

22.2.3 Broad and Significant Tumor Growth Inhibitory Response by A4.6.1 or Bevacizumab When Administered As Single Agents and Increased Survival of Treated Tumor-Bearing Animals

Bevacizumab and A4.6.1 were tested in xenotransplant models of cancer in immunodeficient mice. Robust, dose-dependent pharmacological effects were observed for all tumor types when either A4.6.1 or bevacizumab was administered as a single agent. Tumor types that displayed growth inhibition included rhabdomyosarcoma, glioblastoma multiforme, leiomyosarcoma, ovarian carcinoma, prostate carcinoma, hepatoblastoma, neuroblastoma, melanoma, pancreatic carcinoma, Wilms' tumor, colon carcinoma and breast carcinoma. For a complete review of all human tumor cell lines and the pharmacological effects of anti-VEGF treatment, see Gerber and Ferrara (2005). Dose-dependent tumor growth inhibition was observed independent of tumor location (subcutaneous, intradermal, intracranial, intrarenal, intrasplenic), and all routes of administration were effective (intravenous, intraperitoneal, and intratumoral). Inhibition of growth of primary tumors was obtained with early initiation of treatment (between days 0 and 5 after tumor implantation) and also on late intervention, i.e. when treatment was started on day 5 or later after tumor cell implantation (Kim et al. 1993; Yuan et al. 1996; Mesiano et al. 1998; Melnyk et al. 1999; Rowe et al. 2000; Fox et al. 2002; Hu et al. 2002; Bockhorn et al. 2003). The effects of anti-VEGF treatment on tumor growth was assessed by tumor size and/or weight and ranged from 25% to >95% inhibition relative to control treatment. Immunohistochemical analysis on paraformaldehyde-fixed sections of liver tumors and intravital microscopy revealed moderate to complete inhibition of tumor angiogenesis after treatment with bevacizumab or A4.6.1. In a model of experimental hepatoblastoma, a significant reduction in CD31-positive endothelial cells and concomitant increase in α-smooth muscle actin (α-SMA)-positive pericytes was identified in the tumor vasculature following 8 weeks of treatment (McCrudden et al. 2003). When tested in a rat model of intracranial glioblastoma, administration of A4.6.1 decreased tumor vascularity, increased tumor cell apoptosis, and significantly prolonged survival of tumor-bearing animals (Rubenstein et al. 2000). Importantly, administration of A4.6.1 to tumor cell lines grown in vitro had no effect on their proliferation rates (Kim et al. 1993), confirming that the primary pharmacological effects of A4.6.1 or bevacizumab are not directly on tumor cells, but the reduction of endothelial cell survival and proliferation within the tumor vasculature, ultimately targeting the blood supply to the tumor.

22.2.4 Marked Inhibition of Metastatic Spread to Various Organs After Treatment of Different Tumors with Anti-VEGF Antibodies

Tumor metastasis is associated with tumor angiogenesis; thus, interference with angiogenesis may reduce metastatic spread of solid tumors. In support of this concept, administration of A4.6.1 in tumor-bearing athymic mice led to a pronounced and significant dose-dependent inhibition of growth of subcutaneous tumor-xenografts in addition to a marked reduction in the number and size of experimental metastases. Human colon carcinoma cells, after intrasplenic injection, displayed metastatic spread into the liver of experimental mice. When tested in this model, a 90% reduction in the primary tumor mass was observed in response to A4.6.1 treatment. The average number of tumors per liver and the mean estimated tumor volume per liver were 10–18 times lower in A4.6.1-treated than in control animals. In hepatic metastases of A4.6.1-treated mice, neither blood vessels nor expression of VEGFR-2 could be demonstrated (Warren et al. 1995). Similar anti-metastatic effects were observed in murine models of established metastatic prostate cancer, where subcutaneously grown tumors metastasize into the lung. Treatment with A4.6.1 3 days after tumor cell implantation (early treatment) not only suppressed primary tumor growth but inhibited metastatic dissemination to the lung. When treatment was delayed for 7 days until the primary tumors were well established, further growth of the primary tumor was inhibited, as was the progression of metastatic disease (Melnyk et al. 1999). Finally, pronounced inhibitory effects on growth of primary tumors and metastasis were reported when A4.6.1 was tested in experimental models of prostate tumors (Hotz et al. 2003). Similar effects on tumor growth and metastasis were reported in a third study, an experimental model of anaplastic Wilms' tumor implanted into mouse kidneys. A4.6.1 treatment resulted in a greater than 95% reduction in primary tumor weight and also abolished the establishment of lung metastases (Rowe et al. 2000).

22.2.5 Bevacizumab or A4.6.1, When Administered in Combination with Cytotoxic Agents, MMP Inhibitors or Radiation Therapy, Induced Additive or Synergistic Tumor Growth Inhibitory Effects

A4.6.1 and bevacizumab were both tested in conjunction with commonly used cytotoxic agents. Combination with topotecan was tested in human Wilms' tumor (Soffer et al. 2001) and human neuroblastoma tumors (Kim et al. 2002a). Interestingly, the combination of topotecan with bevacizumab not only reduced tumor vascularity and suppressed primary tumor growth in the kidney, but also inhibited metastasis of Wilms' tumor to the lung more effectively than either agent alone (Soffer et al. 2001). In addition to the synergistic inhibitory effects on primary tumor growth, a significant reduction of tumor metastasis was observed in combination therapy, including low-dose topotecan (a topoisomerase-1 inhibitor) and A4.6.1. After intrarenal injection of the tumor cells, suppression of tumor growth and of numbers of metastases of Wilms' tumor to the lung was greater after combination treatment than after treatment with either compound alone (Soffer et al. 2001). The most pronounced inhibition of growth of a primary human tumor, neuroblastoma, occurred when combining A4.6.1 with topotecan after intrarenal implantation of tumor cells (Kim et al. 2002b).

Paclitaxel was tested in combination with bevacizumab and A4.6.1, and the effects on growth of human prostate cancer and ovarian carcinoma were analyzed (Fox et al. 2002; Hu et al. 2002). The combination of paclitaxel and bevacizumab resulted in greater inhibition of neuroblastoma tumor growth than that observed with either agent alone (Fox et al. 2002). Growth of ovarian carcinoma tumors was equally inhibited when treated with paclitaxel alone or when combined with A4.6.1. However, virtually no ascites developed in the combined treatment group or the group treated with A4.6.1 alone, while paclitaxel alone reduced ascites slightly, but not significantly (Hu et al. 2002).

Docetaxel, another cytotoxic agent, was tested in combination with A4.6.1 using an in vivo Matrigel invasion assay to determine their combined effects on angiogenesis (Sweeney et al. 2001). The combined data suggest that microenvironmental factors capable of inducing endothelial cell survival such as VEGF or basic fibroblast growth factor (FGF-2) could play a role in decreasing the anti-angiogenic effects of docetaxel. Agents directed against VEGF, such as A4.6.1, may reverse this protective effect, which may explain the additive effects on inhibiting angiogenesis in this model.

Doxorubicin, a cytotoxic agent commonly used in the treatment of breast carcinoma, reduced the growth rate of tumor cells when administered as single agent but did not significantly affect angiogenesis. Administration of doxorubicin in combination with A4.6.1 resulted in significant tumor regression. Histopathology indicated that some tumor sections in the combination group lacked viable tumor cells at the end of the 2-week observation period. The preclinical combination experiments all led to a more pronounced inhibition of tumor growth and angiogenesis or increased survival, compared to the effects induced by single-agent treatment.

In addition, A4.6.1 was tested in combination with an inhibitor of MMPs in an experimental model of human pancreatic cancer (Hotz et al. 2003). Both VEGF blockade and MMP inhibition reduced primary tumor size, metastasis, and angiogenesis, thereby increasing survival in experimental pancreatic cancer.

Finally, A4.6.1 was tested in combination with radiation therapy to assess the effects on tumor growth of two different human tumor cell lines in mice. Combination treatment induced tumor-growth delay (TGD) of glioblastoma tumors which was greater than additive; with adenocarcinoma, however, the effect was additive. The TGD occurred irrespective of the degree of hypoxia, suggesting that A4.6.1 treatment can compensate for the resistance to radiation induced by hypoxia (Lee et al. 2000). Hypoxia is a major factor contributing to the radioresistance of tumor cells. Thus the effect of A4.6.1 on oxygen levels within the tumors and the correlation with treatment response was investigated. For both tumor types, the TGD induced by the antibody was independent of oxygen levels in the tumor at the time of radiation. The increase in TGD occurred under both normoxic and hypoxic conditions, suggesting that treatment with A4.6.1 can compensate for the resistance to radiation induced by hypoxia (Lee et al. 2000).

In conclusion, these data indicate that neutralization of VEGF in combination with conventional cytotoxic agents or radiation may be superior to single treatment alone and hence beneficial in cancer treatment. Improved pharmacological effects within the combination treatment groups were observed independently of the experimental models, endpoints or anti-tumor strategies applied (radiation, cytotoxic agents, MMP inhibitors). None of the studies reported increased toxicity in the combination relative to single-treatment groups. Combined, the data provided further support for the model wherein combining anti-angiogenic compounds with anti-tumor strategies may result in increased efficacy.

22.2.6 Correlation Between Pharmacological Effects of Tumor Growth Inhibition and Vascular Changes

Intravital videomicroscopy techniques employed to study tumors treated with A4.6.1 augmented the understanding of the role of VEGF in tumorigenesis (Borgstrom et al. 1996, 1998). Noninvasive imaging of the vasculature demonstrated a nearly complete suppression of tumor-associated angiogenesis in animals treated with A4.6.1, compared to controls. These findings provided direct verification that inhibition of angiogenesis is the mechanism of tumor suppression after anti-VEGF treatment (Borgstrom et al. 1996).

Magnetic resonance imaging (MRI) enhanced with a macromolecular contrast medium (MMCM) was used to investigate the effects of VEGF on the permeability and other properties of tumor vessels (Brasch et al. 1997). In an experimental model of human ovarian cancer, MRI demonstrated robust and rapid alterations in microvascular permeability after inhibition of VEGF by A4.6.1. A significant decrease

of 97% in vascular permeability was observed as early as 24 h after administration of A4.6.1 (Brasch et al. 1997). The time course of the down-modulation of vascular permeability did not vary with the route of administration and was observed as early as 24 h after intraperitoneal (IP) or intravenous (IV) administration. Vascular imaging studies also revealed decreased vascular permeability in tumors treated for up to 4 weeks with A4.6.1; a reduction in interstitial tumor pressure was identified (Lee et al. 2000). In order to investigate the effects on tumor accumulation of two cytotoxic compounds, A4.6.1 was administered with cisplatin, a highly protein-bound cytotoxin, or 5-fluorouracil (5-FU), a small unbound cytotoxin. Dynamic MRI enhanced with a MMCM detected a similar reduction of transendothelial permeability and cisplatin, but no correlation with 5-FU was described (Daldrup-Link et al. 2004). The use of MMCM in animals inoculated intraperitoneally with human ovarian tumor cells and treated with A4.6.1 revealed accumulation of significantly smaller volumes of peritoneal ascites and significantly lower tumor microvascular permeabilities than in control animals after treatment for 10 days (Gossmann et al. 2002) or 14 days (Gossmann et al. 2000). The reduction in permeability was associated with a decrease in interstitial pressure within the tumor (Lee et al. 2000) and an almost complete inhibition of ascites formation in experimental models of human ovarian tumors (Mesiano et al. 1998; Gossmann et al. 2000). Thus, the beneficial effects of A4.6.1 treatment on neoplastic lesions located within or near the peritoneal cavity may include a decrease in peritoneal fluid accumulation in addition to the growth inhibitory effects on the primary tumor. Therefore, these noninvasive techniques confirmed the significant reduction in tumor vascularity observed by histological analysis. The reduction in tumor vascularity found in all tumor types tested correlated with the tumor growth inhibitory activities induced by A4.6.1 or bevacizumab treatment. In agreement with these preclinical observation, changes in vascular functions in patients with rectal carcinomas following a single infusion of bevacizumab have been reported, including a decrease in tumor perfusion, vascular volume, microvascular density and interstitial fluid pressure. In addition, a reduction in the number of viable, circulating endothelial cells in a small set of colorectal cancer patients was observed (Willett et al. 2004). These results are consistent with the hypothesis that new microvessels formed in response to angiogenic stimulus are hyperpermeable (Yuan et al. 1996), and support the model in which VEGF-induced hyperpermeability is a mechanistic element in tumor angiogenesis. In conclusion, the data from studies evaluating the effects of A4.6.1 or bevacizumab treatment on tumor angiogenesis by means of noninvasive techniques such as MRI and intravital microscopy in rodents and patients are consistent with a reduction in the amounts of endothelial cells and microcapillary counts within the tumor tissue. In summary, these preclinical and clinical studies conclusively demonstrate that among the many candidate angiogenic activities identified so far, interference with VEGF activity appears the most promising strategy for interference with tumor angiogenesis in a broad range of tumor types.

22.2.7 Safety Pharmacology of A4.6.1 and Bevacizumab

Because bevacizumab and A4.6.1 do not bind to murine VEGF, no formal preclinical safety pharmacology studies were conducted in preclinical murine models. Safety evaluations and toxicology tests were performed in cynomolgus monkeys, a pharmacologically reactive species. These studies revealed dose-related effects on sites of active neoangiogenesis, including an increase in the numbers of hypertrophied chondrocytes, subchondral bony plate formation, and inhibition of vascular invasion of the growth plate in young adult cynomolgus monkeys (Ryan et al. 1999). As expected, decreased ovarian and uterine weights and absence of corpora lutea were observed in female cynomolgus monkeys after treatment with bevacizumab (Ryan et al. 1999). Both the physeal and ovarian changes were reversible with cessation of treatment. In conclusion, no unexpected or significant treatment-related effects

were observed in adult animals even after prolonged treatment with high doses (26 weeks, 50 mg/kg, IV, once- or twice-weekly injections) of bevacizumab in cynomolgus monkeys. While no formal in vivo safety pharmacology studies were conducted with bevacizumab or A4.6.1, several safety endpoints were incorporated into the repeat-dose toxicology studies in cynomolgus monkeys (Ryan et al. 1999). No treatment-related effects on physical parameters, including respiration rate, blood pressure, and electrocardiographic measurement, were observed in cynomolgus monkeys administered 2–50 mg/kg of bevacizumab intravenously once or twice weekly for up to 26 weeks.

Conclusions and Future Developments

The experiments reviewed in this chapter were designed to identify potential differences between various types of tumors in their response to anti-VEGF treatment when administered as single agent or in combination with cytotoxic agents or radiation therapy. Also, the effects of early versus late onset of anti-VEGF treatment on the progression of various tumor types and their metastatic potential were investigated. Finally, vascular changes within tumors in response to anti-VEGF treatment were analyzed.

The preclinical tumor growth studies conducted with bevacizumab or its parental murine antibody, A4.6.1, have confirmed that the pharmacological activities are consistent with those expected from inhibiting VEGF. VEGF regulates multiple endothelial cell functions, including cell proliferation, adhesion, migration, survival, and vascular permeability. The experiments demonstrate that antibodies targeting human VEGF potently inhibited VEGF's survival function on endothelial cells within the tumor vasculature without interfering with endothelial cell functions in mature, quiescent vasculature in adults. Anti-VEGF antibodies (A4.6.1 and bevacizumab), when administered as single agents, displayed robust pharmacological effects and inhibited tumor angiogenesis and growth of a variety of human tumor cell lines when implanted at different locations in immunocompromised mice or rats (reviewed in Gerber and Ferrara 2005). These effects were independent of the route of administration and were observed following IP, IV, or intratumoral injections. Furthermore, the inhibitory activity of anti-VEGF antibodies on tumor angiogenesis and growth was observed irrespective of the time of onset of treatment. However, the growth inhibitory effects were less pronounced when treatment was initiated on already existing tumors. Further support for a key regulatory role of VEGF during tumor angiogenesis of multiple tumor types and locations was provided by the findings that inhibition of tumor growth in preclinical models was independent of the tumor type and site of tumor cell implantation tested. Profound inhibitory effects on tumor growth by A4.6.1 were observed when tumor cells were implanted subcutaneously, intracranially, intrarenally or intradermally.

When tested in experimental models of tumor metastasis, A4.6.1 displayed robust pharmacological activity when administered as a single agent. Anti-VEGF treatment in combination with cytotoxic agents or radiation therapy revealed additive or synergistic effects with regard to tumor inhibition and vascular regression as determined by intravital microscopy. In combination with radiation, A4.6.1 treatment reversed the radiation resistance induced by increased levels of hypoxia, which often occurs as a consequence of radiation therapy (Lee et al. 2000). Depriving the tumor endothelium of VEGF may thus represent an important strategy to increase the anti-tumor effects of radiation treatment, and may indicate that the best use of anti-VEGF therapy may be in combination with cytotoxic anti-tumor therapies.

In an experimental model of human ovarian cancer, MRI analysis demonstrated robust and rapid alterations in microvascular permeability after inhibition of VEGF by A4.6.1. These findings indicate interference with VEGF's function as a vascular permeability factor as a consequence of treatment with A4.6.1. The reduction in permeability was associated with a decrease in interstitial pressure within the tu-

mor (Lee et al. 2000). Such a decrease in interstitial pressure may allow for better tumor penetration of cytotoxic agents and thus may provide a rationale for the observed synergism between anti-VEGF and chemotherapy (Borgstrom et al. 1999). Results from studies evaluating the effects of anti-VEGF treatment on tumor angiogenesis by MRI and intravital microscopy in mice and rats are consistent with a reduction in the numbers of endothelial cells and microcapillary counts in the tumor tissue. The data obtained from MRI studies, using contrast agents to study changes in vascular permeability in anti-VEGF-treated tumors, suggested that this strategy may be used more generally in humans to assess efficacy of experimental modalities aiming at the neutralization of VEGF activity. Furthermore, the experimental findings indicate that the pharmacological effect of anti-VEGF treatment is based in part on the prevention of the leakage of proteinaceous fluid into the paratumoral interstitium, which provides an ideal matrix for the ingrowth of capillary buds.

22.3.1 Next-Generation Anti-VEGF Compounds

Two different forms of soluble VEGF receptor decoys were tested in preclinical models and one of these receptor–IgG chimeras is currently being developed clinically (Gerber et al. 2000; Holash et al. 2002). However, both constructs neutralize not only VEGF-A, but also the VEGF homologs PlGF and VEGF-B, which may play additional roles in tumor angiogenesis. Therefore, these compounds do not allow study of the effects of selective VEGF-A blockade in mice, as they have different ligand binding and pharmacological properties than antibodies. Recently, a series of novel anti-VEGF antibodies, including B20-4.1 and G6-23 and G6-31, derived from synthetic antibody phage libraries and cross-reactive between mouse and human VEGF, have been described (Fuh et al. 2005; Liang et al. 2005). All three compounds completely block growth of different human tumors xenografted to immunocompromised mice (Liang et al. 2005). In contrast, bevacizumab inhibited growth of these tumors less efficiently, presumably due to the presence of stromal cell-derived murine VEGF recruited to these tumors. Combined, these studies demonstrated the important contributions of both tumor- and stromal cell-derived VEGF-A to vascularization and growth of human tumors grown in mice.

Interestingly, these second-generation anti-VEGF antibodies target a different epitope on VEGF than bevacizumab, which matches more closely the regions bound by VEGF receptors. When tested in vitro for the ability to inhibit endothelial cell proliferation stimulated by VEGF-A, a correlation between the potency and affinity of second-generation anti-VEGF antibodies was identified (Liang et al. 2005). These in vitro findings raised the possibility, that these antibodies may exert increased potency or efficacy in pharmacodynamic experiments in vivo. However, the differences in species specificity between first- and second-generation anti-VEGF antibodies precluded direct head-to-head comparisons with first-generation MAbs, and the importance of antibody epitope and affinity for the therapeutic indexes of anti-VEGF antibodies remains to be determined.

In order to generate a preclinical model with comparable pharmacological responsiveness towards all anti-VEGF compounds, we engineered mice to express a humanized form of VEGF-A by employing Cre-LoxP technology. Homozygous knock-in mice (VEGF (mutX/mutX)) were viable and are currently used to study long-term dosing effects of various neutralizing antibodies. In addition, Mut-X ki mice were intercrossed with immunocompromised RAG2 (-/-) mice to study the effects of anti-VEGF antibodies on growth of various experimental human tumor cell lines. Although *in vitro* studies clearly showed a correlation between binding affinity and potency at blocking endothelial cell proliferation stimulated by VEGF, *in vivo* experiments failed to document any consistent correlation between antibody affinity and the ability to inhibit tumor growth and angiogenesis in most animal models. However, higher-affinity antibodies were more likely to result in glomerulosclerosis during long-term treatment. (Gerber et al, 2007)

22.3.2 Paracrine and Autocrine Signaling Mechanisms Employed by VEGF-A May Be Affected Differentially by Compounds Interfering with Different Elements of the VEGF Signaling Pathway

Genetic ablation of vegf-a in hematopoietic stem cells (HSC) and administration of small-molecule inhibitors targeting the tyrosine kinase of VEGF receptors in HSC both revealed the presence of an autocrine loop mechanism by which VEGF controls HSC survival. In contrast, experiments conducted in the presence of a ligand-blocking, soluble receptor–IgG chimeric construct did not interfere with such function. Combined, these findings suggested that inactivation of VEGF by targeting the ligand in the extracellular space, similar to using the neutralizing antibodies, had only minor effects on HSC survival and repopulation of the bone marrow after lethal irradiation (Gerber et al. 2002). The discrepancies between biochemical and genetic loss-of-function experiments suggested that the autocrine loop formed by VEGF in HSCs may not be affected by compounds targeting VEGF in the extracellular domain. These findings are characteristic for the presence of an internal or "private" autocrine loop (Gerber and Ferrara 2003). However, it is important to note that the internal autocrine function of VEGF was identified in the experimental setting of hematopoietic repopulation following lethal irradiation in mice, and additional studies will need to be conducted in order to assess the relevance of this regulatory mechanism in other models of physiological or pathological hematopoiesis or in humans. In addition, some of the small-molecule inhibitors developed to block the tyrosine kinase activity of VEGF receptor also block other tyrosine kinases, and some of them were shown to play a role during hematopoiesis. Considering these limitations, a hypothesis can be invoked that bone marrow toxicity in response to anti-VEGF therapy may be less likely to occur when employing neutralizing antibodies that display high levels of selectivity towards VEGF-A but fail to interfere with the internal autocrine loop. Finally, based on the co-expression of both VEGF-A and VEGF receptors on a variety of cell types, autocrine effector functions of VEGF may be in place on a variety of cell types (Fig. 22.2). Therefore, the

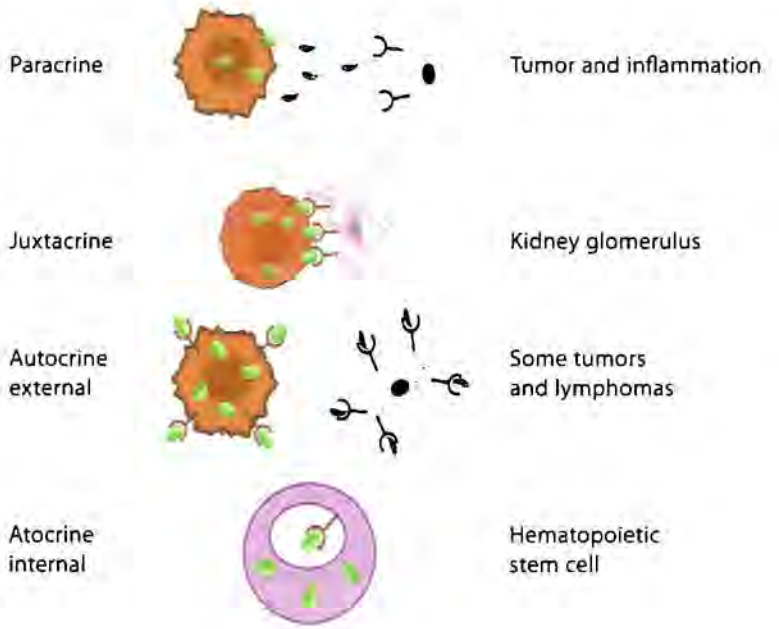


Fig. 22.2. VEGF's effector functions on endothelial cells during pathologic angiogenesis is believed to be mostly mediated via paracrine mechanisms. In organs where expression of ligands and receptor is present on cells located in close proximity, such as the kidney glomerulus, juxtacrine mechanisms may potentially be in place. Co-expression of VEGFR-1 or VEGFR-2 and VEGF-A was identified on several tumor types, including lymphomas, indicating the possibility for autocrine signaling in these tumor cells. Internal autocrine loops are potentially present in cells which are dependent on VEGF for certain biological functions, co-express VEGFR-1 or -2 and administration of ligand-blocking compounds has no effect on these functions

success of therapeutic strategies targeting VEGF in the context of solid tumors and other diseases, including hematopoietic malignancies, may depend on the relevance of autocrine and paracrine VEGF effector functions in pathological and physiological contexts. Consequently, the therapeutic window of different compounds may be determined by their potential to interfere with VEGF's regulatory functions that are critical for pathologic angiogenesis and tumor growth, while avoiding interference with physiologic roles in maintenance of adult tissues. Further investigation into the complex regulatory pathways engaged by VEGF during angiogenesis, hematopoiesis or maintenance of organ functions may help in the design of therapeutic strategies with improved therapeutic efficacies.

References

Akagi M, Kawaguchi M et al (2003) Induction of neuropilin-1 and vascular endothelial growth factor by epidermal growth factor in human gastric cancer cells. Br J Cancer 88:796–802

Algire GH, Chalkley HW (1945) Vascular reactions of normal and malignant tissues in vivo. I. Vascular reactions of mice to wounds and to normal and neoplastic transplants. J Natl Cancer Inst 6:73–85

Alon T, Hemo I et al (1995) Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. Nat Med 1:1024–1028

Bockhorn M, Tsuzuki Y et al (2003) Differential vascular and transcriptional responses to anti-vascular endothelial growth factor antibody in orthotopic human pancreatic cancer xenografts. Clin Cancer Res 9:4221–4226

Borgstrom P, Hillan KJ et al (1996) Complete inhibition of angiogenesis and growth of microtumors by anti-vascular endothelial growth factor neutralizing antibody: novel concepts of angiostatic therapy from intravital videomicroscopy. Cancer Res 56:4032–4039

Borgstrom P, Bourdon MA et al (1998) Neutralizing anti-vascular endothelial growth factor antibody completely inhibits angiogenesis and growth of human prostate carcinoma micro tumors in vivo. Prostate 35:1–10

Borgstrom P, Gold DP et al (1999) Importance of VEGF for breast cancer angiogenesis in vivo: implications from intravital microscopy of combination treatments with an anti-VEGF neutralizing monoclonal antibody and doxorubicin. Anticancer Res 19:4203–4214

Brasch R, Pham C et al (1997) Assessing tumor angiogenesis using macromolecular MR imaging contrast media. J Magn Reson Imaging 7:68–74

Bunone G, Vigneri P et al (1999) Expression of angiogenesis stimulators and inhibitors in human thyroid tumors and correlation with clinical pathological features. Am J Pathol 155:1967–1976

Carmeliet P, Ferreira V et al (1996) Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 380(6573):435–439

Carter P, Presta L et al (1992) Humanization of an anti-p185HER2 antibody for human cancer therapy. Proc Natl Acad Sci USA 89:4285–4289

Cohen T, Nahari D et al (1996) Interleukin 6 induces the expression of vascular endothelial growth factor. J Biol Chem 271:736–741

Coussens LM, Werb Z (2002) Inflammation and cancer. Nature 420:860–867

Daldrup-Link HE, Okuhata Y et al (2004) Decrease in tumor apparent permeability-surface area product to a MRI macromolecular contrast medium following angiogenesis inhibition with correlations to cytotoxic drug accumulation. Microcirculation 11:387–396

De Fraipont F, El Atifi M et al (2000) Expression of the angiogenesis markers vascular endothelial growth factor-A, thrombospondin-1, and platelet-derived endothelial cell growth factor in human sporadic adrenocortical tumors: correlation with genotypic alterations. J Clin Endocrinol Metab 85:4734–4741

Dvorak HF, Brown LF et al (1995) Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. Am J Pathol 146:1029–1039

Eppenberger U, Kueng W et al (1998) Markers of tumor angiogenesis and proteolysis independently define high- and low-risk subsets of node-negative breast cancer patients. J Clin Oncol 16:3129–3136

Fan F, Wey JS et al (2005) Expression and function of vascular endothelial growth factor receptor-1 on human colorectal cancer cells. Oncogene 24:2647–2653

Ferrara N (2002) VEGF and the quest for tumour angiogenesis factors. Nat Rev Cancer 2:795–803

Ferrara N (2004) Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev 25:581–611

Ferrara N (2005) VEGF as a therapeutic target in cancer. Oncology 69 [Suppl 3]:11–16

Ferrara N, Davis-Smyth T (1997) The biology of vascular endothelial growth factor. Endocr Rev 18:4–25

Ferrara N, Winer J et al (1993) Expression of vascular endothelial growth factor does not promote transformation but confers a growth advantage in vivo to Chinese hamster ovary cells. J Clin Invest 91:160–170

Ferrara N, Carver-Moore K et al (1996) Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 380 (6573):439–442

Fox WD, Higgins B et al (2002) Antibody to vascular endothelial growth factor slows growth of an androgen-independent xenograft model of prostate cancer. Clin Cancer Res 8:3226–3231

Frank S, Hubner G et al (1995) Regulation of VEGF expression in cultured keratinocytes. Implications for normal and impaired wound healing. J Biol Chem 270:12607–12613

Fuh G, Wu P et al (2005) Structure–function studies of two synthetic anti- vascular endothelial growth factor Fabs and comparison with the AvastinTM-Fab. J Biol Chem 281:6625–6631

Gasparini G, Toi M et al (1997) Prognostic significance of vascular endothelial growth factor protein in node-negative breast carcinoma. J Natl Cancer Inst 89:139–147

Gasparini G, Toi M et al (1999) Clinical relevance of vascular endothelial growth factor and thymidine phosphorylase in patients with node-positive breast cancer treated with either adjuvant chemotherapy or hormone therapy. Cancer J Sci Am 5:101–111

Gerber H, Ferrara N (2000) Angiogenesis and bone growth. Trends Cardiovasc Med 10:223–228

Gerber HP, Ferrara N (2003) The role of VEGF in normal and neoplastic hematopoiesis. J Mol Med 81:20–31

Gerber HP, Ferrara N (2005) Pharmacology and pharmacodynamics of bevacizumab as monotherapy or in combination with cytotoxic therapy in preclinical studies. Cancer Res 65:671–680

Gerber HP, Dixit V, Ferrara N (1998a) Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. J Biol Chem 273:13313–13316

Gerber HP, McMurtrey A et al (1998b) Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. J Biol Chem 273:30336–30343

Gerber HP, Kowalski J et al (2000) Complete inhibition of rhabdomyosarcoma xenograft growth and neovascularization requires blockade of both tumor and host vascular endothelial growth factor. Cancer Res 60:6253–6258

Gerber HP, Malik AK et al (2002) VEGF regulates haematopoietic stem cell survival by an internal autocrine loop mechanism. Nature 417:954–958

Gerber HP, Wu X et al (2007) Mice expressing a humanized form of VEGF-A may provide insights into the safety and efficacy of anti-VEGF antibodies. PNAS, 104, 9, 3478–3483.

Gille H, Kowalski J et al (2001) Analysis of biological effects and signaling properties of Flt-1 (VEGFR-1) and KDR (VEGFR-2). A reassessment using novel receptor-specific vascular endothelial growth factor mutants. J Biol Chem 276:3222–3230

Goldman C, Kim J et al (1993) Epidermal growth factor stimulates vascular endothelial growth factor production by malignant glioma cells. A model of glioblastoma multiforme pathophysiology. Mol Biol Cell 4:121–133

Gossmann A, Helbich TH et al (2000) Magnetic resonance imaging in an experimental model of human ovarian cancer demonstrating altered microvascular permeability after inhibition of vascular endothelial growth factor. Am J Obstet Gynecol 183:956–963

Gossmann A, Helbich TH et al (2002) Dynamic contrast-enhanced magnetic resonance imaging as a surrogate marker of tumor response to anti-angiogenic therapy in a xenograft model of glioblastoma multiforme. J Magn Reson Imaging 15:233–240

Grugel S, Finkenzeller G et al (1995) Both v-Ha-Ras and v-Raf stimulate expression of the vascular endothelial growth factor in NIH 3T3 cells. J Biol Chem 270:25915–25919

Hanahan D, Folkman J (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 86:353–364

Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100:57–70

Hata K, Fujiwaki R et al (2000) Expression of thymidine phosphorylase and vascular endothelial growth factor in epithelial ovarian cancer: correlation with angiogenesis and progression of the tumor. Anticancer Res 20:3941–3949

Hazelton D, Nicosia RF et al (1999) Vascular endothelial growth factor levels in ovarian cyst fluid correlate with malignancy. Clin Cancer Res 5:823–829

Holash J, Davis S et al (2002) VEGF-Trap: a VEGF blocker with potent antitumor effects. Proc Natl Acad Sci U S A 99:11393–11398

Hotz HG, Hines OJ et al (2003) Evaluation of vascular endothelial growth factor blockade and matrix metalloproteinase inhibition as a combination therapy for experimental human pancreatic cancer. J Gastrointest Surg 7:220–227; discussion 227–228

Houck KA, Leung DW et al (1992) Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. J Biol Chem 267:26031–26037

Hu L, Hofmann J et al (2002) Vascular endothelial growth factor immunoneutralization plus Paclitaxel markedly reduces tumor burden and ascites in athymic mouse model of ovarian cancer. Am J Pathol 161:1917–1924

Ichikura T, Tomimatsu S et al (2001) Prognostic significance of the expression of vascular endothelial growth factor (VEGF) and VEGF-C in gastric carcinoma. J Surg Oncol 78:132–137

Inoue M, Hager JH et al (2002) VEGF-A has a critical, nonredundant role in angiogenic switching and pancreatic beta cell carcinogenesis. Cancer Cell 1:193–202

Jain RK, Duda DG et al (2006) Lessons from phase III clinical trials on anti-VEGF therapy for cancer. Nat Clin Pract Oncol 3:24–40

Jakeman LB, Armanini M et al (1993) Developmental expression of binding sites and mRNA for vascular en-

dothelial growth factor suggests a role for this protein in vasculogenesis and angiogenesis. Endocrinology 133:848–859

Karayiannakis AJ, Syrigos KN et al (2002) Circulating VEGF levels in the serum of gastric cancer patients: correlation with pathological variables, patient survival, and tumor surgery. Ann Surg 236:37–42

Kieser A, Weich H et al (1994) Mutant p53 potentiates protein kinase C induction of vascular endothelial growth factor expression. Oncogene 9:963–969

Kim ES, Serur A et al (2002a) Potent VEGF blockade causes regression of coopted vessels in a model of neuroblastoma. Proc Natl Acad Sci USA 99:11399–11404

Kim ES, Soffer SZ et al (2002b) Distinct response of experimental neuroblastoma to combination antiangiogenic strategies. J Pediatr Surg 37:518–522

Kim KJ, Li B et al (1992) The vascular endothelial growth factor proteins: identification of biologically relevant regions by neutralizing monoclonal antibodies. Growth Factors 7:53–64

Kim KJ, Li B et al (1993) Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumor growth in vivo. Nature 362:841–844

Kimura H, Konishi K et al (2001) Prognostic significance of expression of thymidine phosphorylase and vascular endothelial growth factor in human gastric carcinoma. J Surg Oncol 76:31–36

Komuro H, Kaneko S et al (2001) Expression of angiogenic factors and tumor progression in human neuroblastoma. J Cancer Res Clin Oncol 127:739–743

Lee CG, Heijn M et al (2000) Anti-vascular endothelial growth factor treatment augments tumor radiation response under normoxic or hypoxic conditions. Cancer Res 60:5565–5570

Li J, Perrella MA et al (1995) Induction of vascular endothelial growth factor gene expression by interleukin-1 beta in rat aortic smooth muscle cells. J Biol Chem 270:308–312

Liang WC, Wu X et al (2005) Cross-species vascular endothelial growth factor (VEGF)-blocking antibodies completely inhibit the growth of human tumor xenografts and measure the contribution of stromal VEGF. J Biol Chem 281:951–961

Linderholm B, Lindh B et al (2000) p53 and vascular-endothelial-growth-factor (VEGF) expression predicts outcome in 833 patients with primary breast carcinoma. Int J Cancer 89:51–62

Maeda K, Kang SM et al (1997) Combined analysis of vascular endothelial growth factor and platelet-derived endothelial cell growth factor expression in gastric carcinoma. Int J Cancer 74:545–550

Malik AK, Baldwin ME et al (2005) Redundant roles of VEGF-B and PlGF during selective VEGF-A blockade in mice. Blood 107:550–557

Mall JW, Schwenk W et al (2002) Serum vascular endothelial growth factor levels correlate better with tumour stage in small cell lung cancer than albumin, neuron-specific enolase or lactate dehydrogenase. Respirology 7:99–102

McCrudden KW, Hopkins B et al (2003) Anti-VEGF antibody in experimental hepatoblastoma: suppression of tumor growth and altered angiogenesis. J Pedriat Surg 38:308–314

Melnyk O, Zimmerman M et al (1999) Neutralizing anti-vascular endothelial growth factor antibody inhibits further growth of established prostate cancer and metastases in a pre-clinical model. J Urol 161:960–963

Mesiano S, Ferrara N et al (1998) Role of vascular endothelial growth factor in ovarian cancer: inhibition of ascites formation by immunoneutralization. Am J Pathol 153:1249–1256

Mukhopadhyay D, Tsiokas L et al (1995) Wild-type p53 and v-Src exert opposing influences on human vascular endothelial growth factor gene expression. Cancer Res 55:6161–6165

Muller YA, Chen Y et al (1998) VEGF and the Fab fragment of a humanized neutralizing antibody: crystal structure of the complex at 2.4 A resolution and mutational analysis of the interface. Structure 6:1153–1167

Poon RT, Ng IO et al (2001) Serum vascular endothelial growth factor predicts venous invasion in hepatocellular carcinoma: a prospective study. Ann Surg 233:227–235

Presta LG, Chen H et al (1997) Humanization of an anti-VEGF monoclonal antibody for the therapy of solid tumors and other disorders. Cancer Res. 57:4593–4599

Presta LG, Lahr SJ et al (1993) Humanization of an antibody directed against IgE. J Immunol 151:2623–2632

Rak JW, St Croix BD et al (1995) Consequences of angiogenesis for tumor progression, metastasis and cancer therapy. Anticancer Drugs 6:3–18 issn: 0959–4973

Rowe DH, Huang J et al (2000) Anti-VEGF antibody suppresses primary tumor growth and metastasis in an experimental model of Wilms' tumor. J Pediatr Surg 35:30–32; discussion 32–33

Rubenstein JL, Kim J et al (2000) Anti-VEGF antibody treatment of glioblastoma prolongs survival but results in increased vascular cooption. Neoplasia 2:306–314

Ryan AM, Eppler DB et al (1999) Preclinical safety evaluation of rhuMAbVEGF, an antiangiogenic humanized monoclonal antibody. Toxicol Pathol 27:78–86

Shimada H, Takeda A et al (2001) Clinical significance of serum vascular endothelial growth factor in esophageal squamous cell carcinoma. Cancer 92:663–669

Siemeister G, Weindel K et al (1996) Reversion of deregulated expression of vascular endothelial growth factor in human renal carcinoma cells by von Hippel–Lindau tumor suppressor protein. Cancer-Res 56:2299–2301 issn: 0008–5472

Sledge GW Jr (2002) Vascular endothelial growth factor in breast cancer: biologic and therapeutic aspects. Semin Oncol 29:104–110

Soffer SZ, Moore JT et al (2001) Combination antiangiogenic

therapy: increased efficacy in a murine model of Wilms tumor. J Pediatr Surg 36:1177–1181

Soker S, Takashima S et al (1998) Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell 92:735–745

Strohmeyer D, Rossing C et al (2000) Vascular endothelial growth factor and its correlation with angiogenesis and p53 expression in prostate cancer. Prostate 45:216–224

Sweeney CJ, Miller KD et al (2001) The antiangiogenic property of docetaxel is synergistic with a recombinant humanized monoclonal antibody against vascular endothelial growth factor or 2-methoxyestradiol but antagonized by endothelial growth factors. Cancer Res 61:3369–3372

Teicher BA (1996) A systems approach to cancer therapy. (Antioncogenics + standard cytotoxics–>mechanism(s) of interaction). Cancer Metastasis Rev 15:247–272

Toi M, Hoshina S et al (1994) Association of vascular endothelial growth factor expression with tumor angiogenesis and with early relapse in primary breast cancer. Jpn J Cancer Res 85:1045–1049

Volm M, Koomagi R et al (1997) Prognostic value of vascular endothelial growth factor and its receptor Flt-1 in squamous cell lung cancer. Int J Cancer 74:64–68

Warren RS, Yuan H et al (1995) Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. J Clin Invest 95:1789–1797

Wey JS, Fan F et al (2005) Vascular endothelial growth factor receptor-1 promotes migration and invasion in pancreatic carcinoma cell lines. Cancer 104:427–438

Willett CG, Boucher Y et al (2004) Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. Nat Med 10:145–147

Yuan A, Yu CJ et al (2001) Vascular endothelial growth factor 189 mRNA isoform expression specifically correlates with tumor angiogenesis, patient survival, and postoperative relapse in non-small-cell lung cancer. J Clin Oncol 19:432–441

Yuan F, Chen Y et al (1996) Time-dependent vascular regression and permeability changes in established human tumor xenografts induced by an anti-vascular endothelial growth factor/vascular permeability factor antibody. Proc Natl Acad Sci USA 93:14765–14770

Vascular Endothelial Growth Factor

23

Receptor Tyrosine Kinase Inhibitors for the Treatment of Cancer

Stephen R. Wedge and Juliane M. Jürgensmeier

Abstract

Vascular endothelial growth factor (VEGF) signalling is a key stimulant of tumour neovascular growth, survival and capillary permeability, which are critical to solid tumour progression. The intracellular signalling responses that induce an angiogenic phenotype are dependent upon VEGF binding to specific transmembrane receptors on the endothelium and activation of intrinsic receptor tyrosine kinase activity. Attempts to inhibit this signalling pathway have included biopharmaceutical approaches that sequester ligand or obstruct its interaction with receptors. An alternative strategy is to use tyrosine kinase inhibitors that could potentially provide a more complete blockade of VEGFR signalling. These low-molecular-weight inhibitors can be selected with oral bioavailability and prepared as tablets for daily dosing, which may afford greater patient convenience. This review describes the structural features of VEGFR-2 tyrosine kinase that are exploited by ATP-competitive inhibitors to prevent its activation and thereby abrogate signalling. Preclinical data generated with a highly potent inhibitor of the VEGF receptor tyrosine kinases, AZD2171, are used to illustrate the consequences of inhibiting physiological and pathological VEGF signalling, including significant growth inhibitory activity across a wide range of tumour models in vivo. The properties of the many inhibitors in current development are also reviewed with their associated clinical findings. Whilst compounds with a range of pharmacological profiles have emerged, only inhibitors that combine high potency, selectivity and good pharmacokinetic properties are likely to test the small-molecule VEGFR signalling inhibitor concept robustly in the clinic.

CONTENTS

Vascular Endothelial Growth Factor Signalling in Cancer

Vascular endothelial growth factor (VEGF)-A-induced signalling in the endothelium is viewed as a pivotal driver of physiological and pathological angiogenesis (the generation of new blood vessels from existing vasculature) (Ferrara 2002a). In addition to regulating new capillary growth, VEGF-A can induce the mobilisation and differentiation of bone marrow-derived endothelial cell progenitors (Asahara et al. 1997; Gehling et al. 2000), is critical to neovascular survival signalling (Benjamin et al. 1999), and can influence vessel maturation by stimulating mural cell recruitment (Yamagishi et al. 1999). The permeability of the endothelium can also be increased profoundly by VEGF-A (Dvorak et al. 1995). In the context of solid tumour progression, continuous angiogenesis and maintenance of a hyper-permeable vascular network provides tumours with the oxygen and nutrients required for sustained, dysregulated growth, in addition to supplying a route for waste product removal and the intravasation of metastatic tumour cells. Inhibition of VEGF-A signalling is therefore a highly attractive target in oncology, particularly since the normal mature vasculature is effectively quiescent in the adult, with the exceptions of wound healing (Howdieshell et al. 2001) and cyclical ovarian function (Plendl 2000). In contrast, VEGF-A expression is increased disproportionately in solid tumours by a number of stimuli (Shweiki et al. 1995; Laughner et al. 2001; Zhou et al. 2004), and VEGF receptors are upregulated on angiogenic tumour vessels (Plate et al. 1994). Furthermore, VEGF-A expression correlates with poor clinical prognosis in many solid tumour types (Byrne et al. 2003; Ellis 2004; Rini and Small 2005; Langer and Natale 2005), suggesting that inhibition of VEGF-A signalling may have broad therapeutic utility.

23.1.1 VEGF Family Ligands and Receptors

Members of the VEGF receptor (VEGFR) family, VEGFR-1 (Flt-1), VEGFR-2 (KDR) and VEGFR-3 (Flt-4), are structurally similar in that they consist of an extracellular ligand-binding region, composed of seven immunoglobulin-like loop domains, attached via a short transmembrane helix to a cytoplasmic catalytic domain. VEGFR-1 and -2 are primarily located on vascular endothelium and VEGFR-3 on lymphatic endothelium. These receptors have differential binding specificities for the VEGF ligand family [VEGF-A, -B, -C, -D and placental growth factor-1 (PlGF-1) and -2] (Fig. 23.1), with an additional dependency upon alternative exon splicing or proteolytic processing of ligands (Petrova et al. 1999). Ligand binding induces receptor homo- or heterodimerisation, thereby stimulating intrinsic receptor tyrosine kinase activity, which induces the regulatory autophosphorylation required for propagation of an intracellular signalling response.

Activation of VEGFR-2 on endothelial cells is considered to be the major determinant of VEGF-A signalling, since it has been shown to be sufficient for transducing the mitogenic, motogenic and sur-

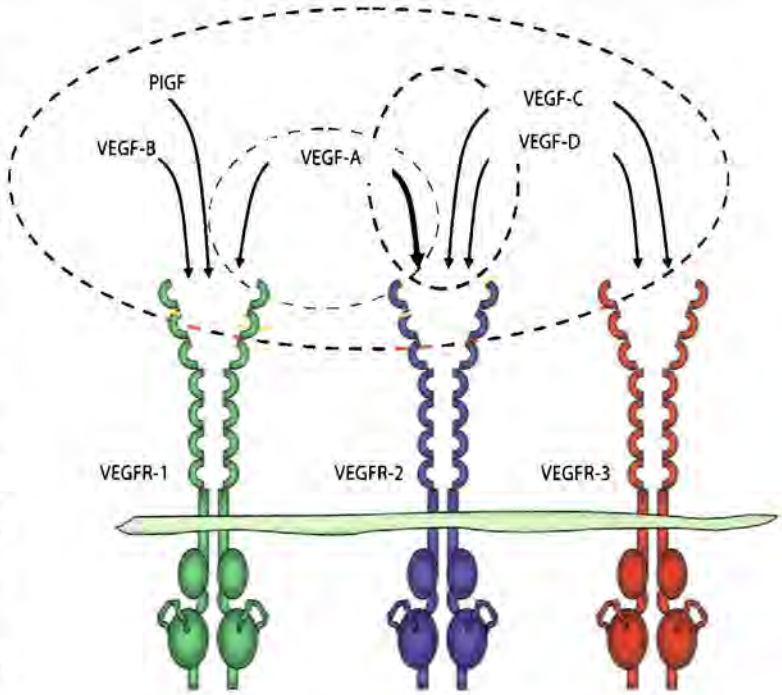


Fig. 23.1. VEGF-receptor (*VEGFR*) ligand-binding specificity and signalling inhibition. Dimeric ligands bind to the three outermost IgG loops in the extracellular domain of VEGFRs with differential selectivity, to induce receptor dimerisation and activation of the intracellular kinase domains. Signalling through VEGFR-2 is key to transducing the mitogenic, motogenic and permeability responses induced by VEGF-A in endothelial cells. The *dashed ovals* represent the potential ligand-induced signalling responses inhibited by: (1) VEGF-A ligand sequestration (*green*), with a biopharmaceutical such as bevacizumab; (2) inhibition of VEGFR-2 activation (*blue*), with a blocking antibody or a selective VEGFR-2 kinase inhibitor; (3) inhibition of VEGFR-1 and VEGFR-2 activation (*yellow*) with an VEGFR-1/-2 kinase inhibitor; and (4) inhibition of the tyrosine kinase activity associated with all three VEGF receptors (*red*) with a VEGFR-1/-2/-3 kinase inhibitor

vival signalling responses in endothelial cells, in addition to inducing vascular permeability (Meyer et al. 1999; Gille et al. 2001; Zeng et al. 2001). In contrast, VEGFR-1 has comparatively weak signalling capacity (Waltenberger et al. 1994; Rahimi et al. 2000; Hillman et al. 2001) and angiogenic phenotypes appear to have little dependency upon its activation (Waltenberger et al. 1994). VEGFR-3 does not bind VEGF-A, but is the receptor for VEGF-C and VEGF-D. It is usually restricted to lymphatic endothelium with a critical role in lymphangiogenesis (Karkkainen et al. 2004), although it has also been found on tumour vasculature (Valtola et al. 1999) and recently reported on tumour cells (Garces et al. 2006). Lymph node metastasis can be promoted by VEGF-C, or suppressed using VEGFR-3-specific antibodies, in preclinical models (Shimizu et al. 2004; Roberts et al. 2006). Furthermore, VEGF-C and -D have been identified as markers of poor prognosis in cancer patients with different tumour types (Arinaga et al. 2003; Onogawa et al. 2004; Shida et al. 2006; Jenny et al. 2006).

23.1.2 Inhibiting VEGF Signal Transduction

There are a number of distinct methods that can be used to try to perturb VEGF-A signalling. Biopharmaceutical approaches generated for this purpose are focused on preventing ligand–receptor interactions. This may be achieved through ligand sequestration, with an antibody to VEGF-A (Ferrara 2002b) or a VEGF receptor-Fc fusion construct (Holash et al. 2002), or by blocking the binding of VEGF-A to VEGFR-2 directly, using monoclonal antibodies to the receptor (Hunt 2001) or a VEGFR-2-binding adnectin (a protein based on the extracellular domains of fibronectin) (Mamluk et al. 2006). Bevacizumab (Avastin™; Genentech, South San Francisco, CA), a fully humanised monoclonal antibody to VEGF-A, is the most advanced of these approaches. It produced a significant increase in survival when combined with cytotoxic chemotherapy (irinotecan, 5-fluorouracil and leucovorin) in patients with first-line metastatic colorectal cancer (Hurwitz et

al. 2004), providing the first confirmatory evidence that inhibition of VEGF signalling can confer a survival benefit in cancer. Significant improvement in overall survival has been reported in clinical studies of bevacizumab in combination with cytotoxic chemotherapy in first-line advanced non-squamous non-small-cell lung cancer (NSCLC) (Sandler et al. 2005), and an increase in progression-free survival (PFS) observed in advanced breast cancer (Miller et al. 2005).

An alternative method (the subject of this review) is to use small synthetic molecules to inhibit VEGFR tyrosine kinase activation, thereby blocking downstream intracellular signal transduction. There are a number of potential advantages to this approach, not least that an inhibitor of VEGFR-2 tyrosine kinase has the potential for differentiated activity, given that it should block all signalling from this receptor, irrespective of the activating ligand concentration or type. Since the fully mature forms of VEGF-C and -D can also bind to and activate VEGFR-2 (Joukov et al. 1996a, 1996b; Achen et al. 1998), inhibiting these additional signalling responses with a kinase inhibitor may conceivably offer advantages over selective VEGF-A sequestration. Furthermore, because of the high degree of homology within the VEGFR kinase domains, inhibitors of VEGFR-2 are frequently found to have additional activity versus VEGFR-1 and/or -3 tyrosine kinases. Whilst the contribution of signalling from VEGFR-1 and -3 to tumour progression is unclear, both of these have been reported to be expressed on some types of tumour cells and may contribute to reduced chemosensitivity, tumour progression or metastasis (Fan et al. 2005; Garces et al. 2006). Simultaneous inhibition of all VEGF receptors may therefore offer added therapeutic benefit. Low-molecular-weight inhibitors of VEGFR-2 tyrosine kinase may also have pharmacokinetic advantages, in that they can be selected to be orally bioavailable, preferably as a once-daily oral tablet for patient convenience. Chronic administration of a kinase inhibitor and attainment of steady-state plasma levels could provide more consistent inhibition of VEGF-A signalling than biopharmaceuticals (administered intravenously or subcutaneously), which may be more heavily influenced by the heterogeneous haemodynamics and irregular vascular architecture encountered within solid tumours. Furthermore, the antiangiogenic and antivascular effects of VEGF-A signalling inhibition may be reversed more rapidly once dosing of a kinase inhibitor is stopped, in comparison to a monoclonal antibody approach with a long plasma half-life (bevacizumab has a plasma half-life of approximately 3 weeks). This could be an advantage if there is a need to transiently avoid compromising wound healing (e.g. a sudden requirement for surgical intervention). Given that small molecules have potentially attractive features and VEGFR-2 transduces the angiogenic effects of VEGF-A, inhibitors of VEGFR-2 tyrosine kinase have been avidly sought.

VEGFR-2 (KDR) Tyrosine Kinase: Structure and Function

Cytoplasmic receptor tyrosine kinase domains are composed of a small N-terminal lobe, a regulatory activation loop and a C-terminal lobe. The kinase domains of the VEGFR family also contain a non-catalytic kinase insert, which spans 68 hydrophilic residues (N933 to L1000) in VEGFR-2 (McTigue et al. 1999).

23.2.1 ATP Binding

ATP binding and phosphotransfer requires tremendous structural flexibility within the kinase domain. ATP binds at the hinge region at the cleft between the N- and C-terminal lobes. In VEGFR-2 this involves two hydrogen bonding interactions between the NH2 and N1 atoms of the adenine ring with, respectively, the C=O of Glu917 and the NH of Cys919 in the kinase backbone (McTigue et al. 1999). There is also a requirement for magnesium cofactor to chelate to ATP, which aligns the phosphate residues in an orientation suitable for phosphotransfer. Mag-

nesium binding at an additional site on the kinase also assists catalysis (Parast et al. 1998). Transfer of the terminal γ-phosphate of ATP to a substrate tyrosine residue is then mediated by transfer of hydrogen from the tyrosine hydroxyl group to an invariant aspartic acid residue (D1028) in the kinase catalytic domain (McTigue et al. 1999). Finally, the kinase undergoes a further conformational change to release the phosphorylated tyrosine residue, ADP and magnesium. The kinase is then available for further ATP/magnesium binding and catalysis, as the hydrogen atom bound to the aspartic acid residue readily dissociates into solution.

23.2.2 VEGFR-2 Tyrosine Kinase Activation and Signalling

The events involved in VEGFR-2 kinase activation and signalling are shown schematically in Fig. 23.2. Receptor tyrosine kinase activity is regulated by conformational constraints that are relieved as a consequence of receptor dimerisation. In particular, a regulatory activation loop in VEGFR-2 (D1046-E1075) adopts a flexible disordered conformation, which, in a monomeric receptor, can sterically hinder ATP and substrate binding to the catalytic site. Dimeric VEGF ligand binding to VEGFR-2 monomers and the induction of receptor dimerisation is followed by transphosphorylation of two tyrosine residues, Y1054 and Y1059, in the regulatory activation loop of the adjoining receptor partner (Parast et al. 1998). When phosphorylated, these residues serve to stabilise the loop in a conformation that permits greater ATP and substrate binding, thereby increasing catalytic activity (Dougher and Terman 1999; Kendall et al. 1999). Phosphorylation of additional tyrosine residues in the intracellular VEGFR-2 domain is then enhanced significantly. There are three other major autophosphorylation sites in VEGFR-2 in addition to the two tyrosine residues in the activation loop: Y951 in the kinase insert sequence and Y1175 and Y1214 in the C-terminal tail (Matsumoto et al. 2005). Phosphorylation of Y951 has been shown recently to induce binding of an adapter molecule (termed TSAd) that activates Src kinase-mediated migratory signalling (Matsumoto et al. 2005). Phosphorylated Y1175 is considered a key epitope for mediating VEGF-induced proliferative signalling in endothelial cells. Phospholipase Cγ1 binds to this residue, becomes phosphorylated and activated, and subsequently induces a protein kinase C/Raf/mitogen-activated protein kinase (MAPK) signalling response (Takahashi et al. 2001). This is in contrast to other receptor tyrosine kinases which utilise Ras to activate MAPK. The Shb adapter protein also binds to phosphorylated Y1175, and stimulates the phosphatidylinositol-3-kinase-dependent phosphorylation of focal adhesion kinase, thereby regulating stress fibre formation and migratory signalling (Holmqvist et al. 2004). The importance of Y1175 in vasculogenic VEGFR-2 signalling has been demonstrated via a genetic knock-in strategy, to replace the corresponding murine tyrosine residue with phenylalanine (Sakurai et al. 2005). This modification results in embryo lethality with defective endothelial and haematopoietic development: a phenotype comparable to that of murine VEGFR-2 (*Flk-1*) null mice (Shalaby et al. 1995). In contrast, phenylalanine replacement of the residue corresponding to Y1214 in VEGFR-2 results in viable, fertile homozygous mice. The role of this autophosphorylation site in pathological signalling remains to be elucidated (Sakurai et al. 2005).

23.2.3 Inhibition of VEGFR-2 Tyrosine Kinase with Small-Molecule Approaches

There are a reported 518 human kinases (Manning et al. 2002) that are responsible for the majority of eukaryotic cellular signal transduction, which mediate a variety of physiological responses and homeostatic regulatory mechanisms. Given that kinases have a high degree of phylogenetic conservation within the structural elements that are involved in catalysis, it was initially assumed that developing ATP-competitive small-molecule inhibitors with any degree of selectivity would be impractical. However, despite the high catalytic homology, crystallographic meth-

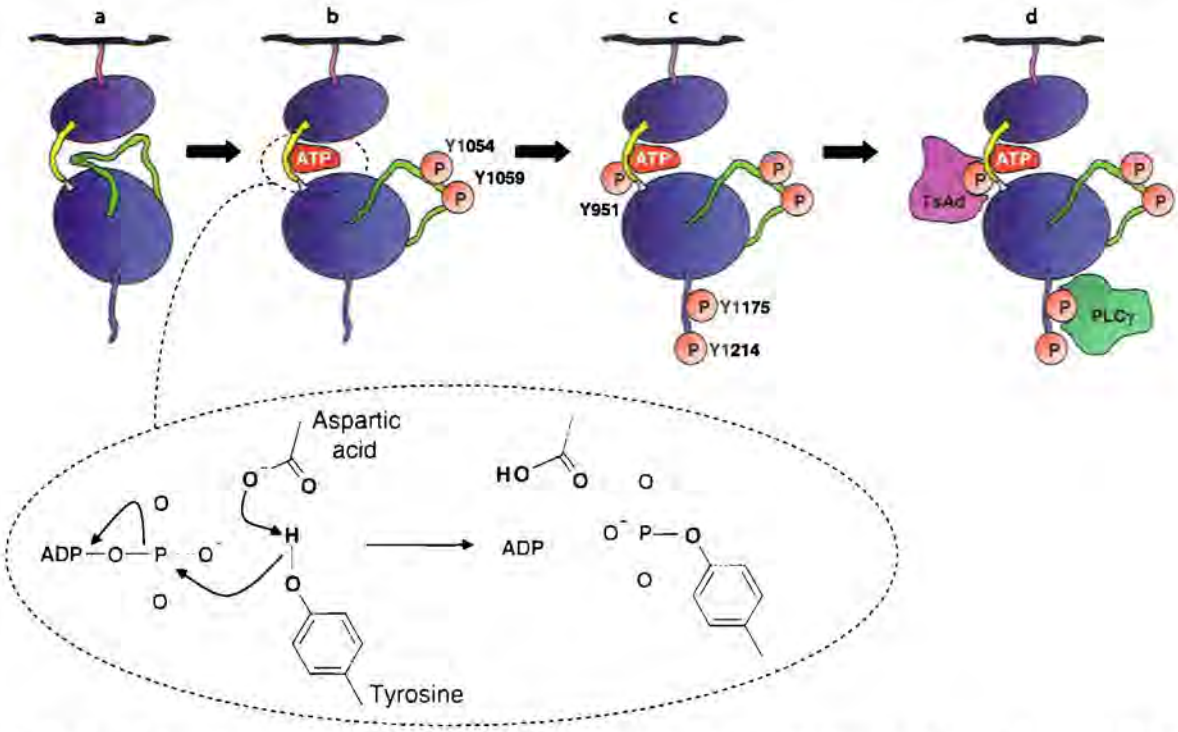


Fig. 23.2a–d. Schematic representation of VEGFR-2 activation. a The intracellular domain of VEGFR-2 consists of the terminal section of the transmembrane helix (shown in *pink*), attached to an N-terminal lobe and C-terminal lobe, that are separated by a hinge region (shown in *yellow*) and a non-catalytic kinase insert (shown in *grey*). The kinase also contains a disordered activation loop (shown in *green*) that may hinder ATP binding. b Extracellular ligand stimulates receptor dimerisation (extracellular events and receptor binding partner not shown), relieving a number of conformational restraints for activation. ATP binding within the kinase hinge region leads to the phosphorylation of key tyrosine residues in the receptor, mediated by an aspartic acid residue in the kinase domain (*inset diagram*). ADP is removed and the kinase can bind further ATP. Phosphorylation of two tyrosines in the activation loop leads to its displacement and stabilisation away from the hinge region, facilitating additional ATP binding. c There are three other major phosphorylation sites in VEGFR-2: a tyrosine residue in the kinase insert and two in the C-terminus. d Phosphorylation of these sites serves as the stimulus for specific adapter molecule binding and the induction of intracellular signalling responses. See text for further details

ods revealed that subtle differences in kinase structure can result in markedly differentiated spatial features, making kinases attractive targets against which small-molecule inhibitors can be developed. In particular, a non-conserved hydrophobic region adjacent to the ATP-binding site that varies in size and shape between kinases is frequently referred to as the 'selectivity pocket', its exploitation by inhibitors being a major determinant of selectivity (Fig. 23.3a).

ATP-competitive VEGFR-2 kinase inhibitors generally mimic binding of the ATP adenine ring to the kinase backbone and exploit further interactions within the selectivity pocket. For example, a proposed binding mode for many quinazoline-based VEGFR-2 inhibitors (based on homology modelling), such as AZD2171, is the N1 of the quinazoline group forming a single H-bond at Cys919 on the kinase backbone, with the C6 and C7 substituents pointing towards solution space at the entrance of the ATP binding cleft, and binding of the fluoro-, methyl-indole group into an adjacent hydrophobic region not utilised by ATP (Fig. 23.3b). Such a molecule has much higher binding affinity for VEGFR-2

than native ATP, and therefore prevents the access of ATP to the catalytic site. Consequently, the receptor cannot participate in phosphorylation-dependent signal transduction and is rendered functionally inactive while the inhibitor is bound.

Preclinical Validation of the VEGFR Tyrosine Kinase Inhibitor Approach

AZD2171 (AstraZeneca) represents an appropriate example by which to illustrate the preclinical potential of a low-molecular-weight VEGF signalling inhibitor, given it has high potency versus VEGFR tyrosine kinases, good selectivity versus a wide range of other kinases and is suitable for once-daily oral dosing.

23.3.1 AZD2171

AZD2171(4-[(4-fluoro-2-methyl-1*H*-indol-5-yl)oxy]-6-methoxy-7-[3-(pyrrolidin-1-yl)propoxy]quinazoline) inhibits the tyrosine kinase activity of all three VEGF receptors in recombinant enzyme assays with IC_{50} values of ≤5 nM (Wedge et al. 2005). AZD2171 retains high potency for VEGFR-2 tyrosine kinase in human umbilical vein endothelial cells (HUVEC), inhibiting receptor phosphorylation with an IC_{50} of 0.5 nM. Similarly, in phenotypic cell assays, AZD2171 prevents VEGF-driven HUVEC proliferation with an IC_{50} of 0.4 nM, and inhibits tubule branching, area and length in an in vitro HUVEC/fibroblast angiogenesis model, with IC_{50} values of ≤0.2 nM. In contrast, AZD2171 is not likely to inhibit the proliferation of tumour cells directly in vivo at the doses examined in human tumour xenograft experiments: concentrations of 3–7.5 μM are required to inhibit tumour cell line proliferation in vitro (between 7,500 and 18,500 times greater than is needed to similarly inhibit VEGF-induced HUVEC proliferation). This margin can be attributed to the high degree of selectivity that this compound has for inhibition of VEGFR signalling versus a range of other kinases (Wedge et al. 2005). Malignancies driven by aberrant stem cell factor receptor (c-Kit) signalling, such as gastrointestinal stromal tumours (GIST) or acute myeloid leukaemia, are an exception, since AZD2171 also inhibits c-Kit tyrosine kinase activity in cells with an IC_{50} of ≤0.2 nM (Jürgensmeier et al. 2005).

23.3.2 Inhibition of Physiological VEGF Signalling

Although physiological angiogenesis is limited in healthy adult animals, there are a number of preclinical settings in which the consequences of inhibiting VEGF-A signalling can be examined.

Whilst growth plates are closed in adults, VEGF-A signalling has a major regulatory role in bone morphogenesis during growth. Expression of VEGF-A by hypertrophic chondrocytes in long bones stimulates vascular invasion of the metaphyseal growth plate (Horner et al. 1999; Hall et al. 2006), which is required to induce terminal chondrocyte differentiation and apoptosis. VEGF-A also regulates osteoclast and osteoblast activity directly (Nakagawa et al. 2000; Mayr-Wohlfart et al. 2002). This complex, orchestrated regulation of multiple cell types is critical to permit the formation of endochondral bone from expanding cartilage at the head of the bone shaft. Specific sequestration of VEGF-A with an antibody (Ryan et al. 1999) or a soluble receptor construct (Gerber et al. 1999) has been shown to prevent the ossification of growing bone, leading to expansion of the hypertrophic chondrocyte zone. This phenotype has also been induced by conditional knockout of VEGF-A in cartilage (Haigh et al. 2000). Consistent with potent inhibition of VEGF signalling in vivo, once-daily oral administration of AZD2171 to growing rats (6 weeks of age) for 28 days also induces a comparable growth plate dysplasia, which can be reversed completely following an equivalent period of AZD2171 withdrawal (Wedge et al. 2005).

Cyclical formation of corpora lutea in the ovary is also highly dependent upon VEGF-A signalling to

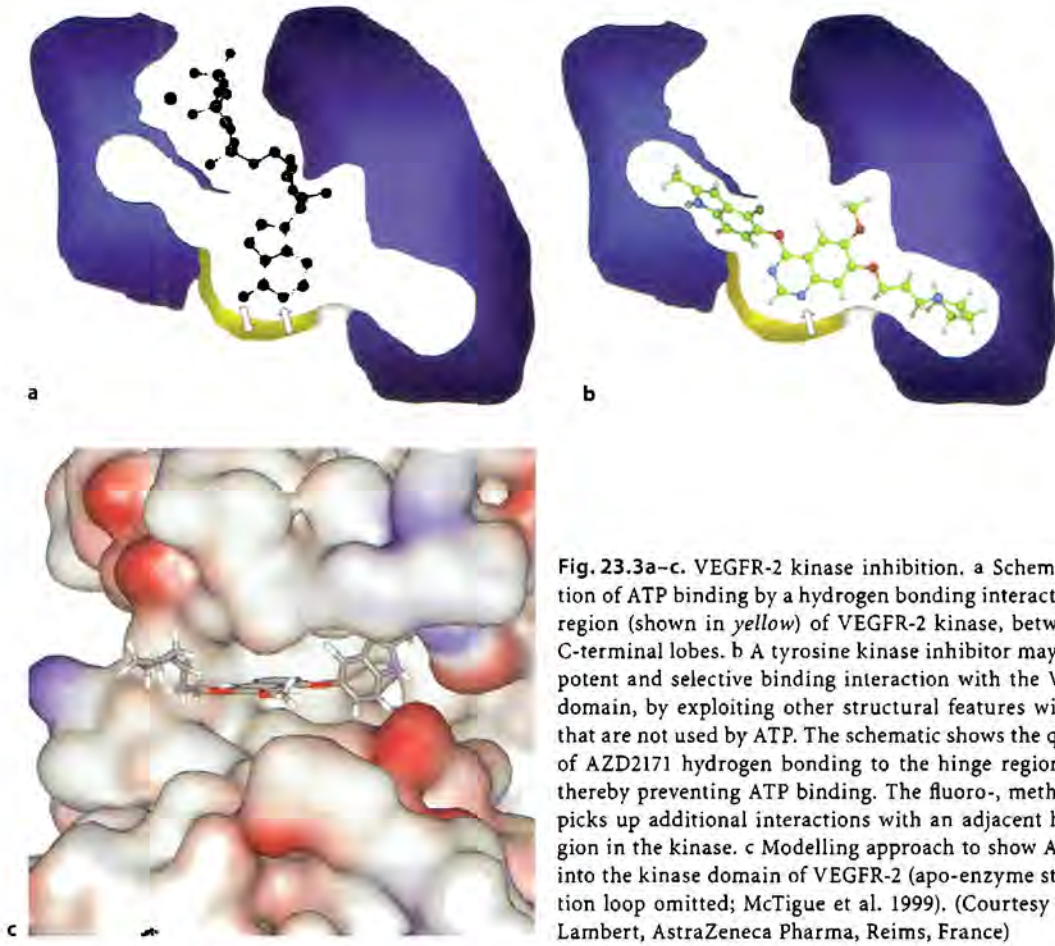


Fig. 23.3a–c. VEGFR-2 kinase inhibition. a Schematic representation of ATP binding by a hydrogen bonding interaction to the hinge region (shown in *yellow*) of VEGFR-2 kinase, between the N- and C-terminal lobes. b A tyrosine kinase inhibitor may achieve a more potent and selective binding interaction with the VEGFR-2 kinase domain, by exploiting other structural features within the kinase that are not used by ATP. The schematic shows the quinazoline core of AZD2171 hydrogen bonding to the hinge region of the kinase, thereby preventing ATP binding. The fluoro-, methyl-indole group picks up additional interactions with an adjacent hydrophobic region in the kinase. c Modelling approach to show AZD2171 docked into the kinase domain of VEGFR-2 (apo-enzyme structure, activation loop omitted; McTigue et al. 1999). (Courtesy of Dr Christine Lambert, AstraZeneca Pharma, Reims, France)

induce rapid blood vessel growth and meet the oxygen and nutrient demands of the developing follicle. Chronic once-daily oral administration of AZD2171 (5 mg/kg/day) has been shown to restrain luteal development in the ovaries of rats (Wedge et al. 2005). Similar effects have been documented in mice using a soluble VEGF-A receptor construct (Ferrara et al. 1998), and in primate using antibody approaches to either sequester VEGF-A (Ryan et al. 1999) or block VEGF ligand binding to VEGFR-2 (Zimmermann et al. 2002).

VEGF-A signalling also has a homeostatic role in the maintenance of blood pressure, via its vasodilatory properties. Accordingly, approaches that inhibit VEGF-A signalling, including bevacizumab, have been found to induce hypertension in man. Using conscious, unrestrained, radiotelemetered rats, oral administration of 0.1–1 mg/kg/day AZD2171 for 3 days produced a mild hypertensive effect (+10 mmHg). Administration of 3 mg/kg/day AZD2171 for 3 days overcame homeostatic blood pressure regulation and induced a more marked hypertensive change of 35–50 mmHg (Curwen et al. 2005). Encouragingly, the marked hypertensive change was reversed with nifedipine, a direct vasodilator, and co-administration of this antihypertensive agent did not impact negatively upon the antitumour activity of AZD2171 in a human tumour xenograft model in rat (Curwen et al. 2005).

23.3.3 Inhibition of Pathological VEGF Signalling in Model Systems

The Matrigel plug assay is a recognised method for examining antiangiogenic activity directly in vivo (Akhtar et al. 2002). In this assay, angiogenic factors such as VEGF-A are introduced into liquid Matrigel, which, after subcutaneous injection into mice, solidifies and supports penetration by host endothelial cells, leading to new blood vessel formation. VEGF-A-induced vessel formation over an 8-day period can be observed macroscopically and in immunofluorescent images after CD31 staining for microvessels. Consistent with inhibition of VEGF-A signalling and angiogenesis, once-daily oral administration of AZD2171 for 8 days completely abolished VEGF-A-induced vessel formation in Matrigel plugs (Wedge et al. 2005) (Fig. 23.4a, b).

In addition to its ability to induce angiogenesis, VEGF-A plays a key role in neovascular survival, and fragile, newlyformed vasculature within tumours is critically dependent on VEGFR signalling (Liu et al. 2002). In a Calu-6 lung xenograft model, once-daily oral dosing of AZD2171 (6 mg/kg) for 3 weeks inhibited tumour growth by >60% (Wedge et al. 2005). Immunohistochemical analysis of microvessel density (MVD; CD31 staining) in tumours revealed that after just over 2 days of treatment (three doses; last dose 4 h before sampling), a reduction in vessel number of 47% (normalised to area) was observed, increasing to a reduction of >70% by day 21 with continued daily dosing (Fig. 23.4c) (Wedge et al. 2005). While the vessel number normalised to area remained constant in control tumours, irrespective of tumour volume or time, the relatively rapid reduction in vessel density observed in tumours from treated animals indicates that AZD2171 can cause vascular regression. These findings are consistent with potent inhibition of VEGF-A signalling and a direct effect of AZD2171 on tumour endothelium. Similar effects have been shown using a VEGFR Fc-fusion construct, VEGF-trap, and an anti-VEGFR-2 antibody in human tumour xenograft and transgenic models (Kim et al. 2002; Inai et al. 2004; Kiessling et al. 2004). The potent antivascular action observed with these inhibitors suggests that VEGF signalling inhibitors can do more than simply inhibit new vessel growth.

VEGF-A also greatly enhances the permeability of tumour endothelium via VEGFR-2 activation and increases vasodilation (Bates et al. 1999). Blood vessels that result from pathological angiogenesis, including those in solid tumours, are comparatively dilated, hyper-permeable, and have a tortuous architecture (Jain 2003). These abnormal functional and structural features are frequently attributed to the effect of VEGF-A, which was also termed 'vascular permeability factor' because of its profound permeabilising effect on vasculature (Dvorak et al. 1995). Since relatively acute changes in tumour haemodynamic parameters may conceivably result from inhibition of VEGF-A signalling, dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) has been used to examine the effects of VEGF-A signalling inhibitors, both preclinically (Drevs et al. 2002; Checkley et al. 2003; Bradley et al. 2007) and clinically (Morgan et al. 2003; Drevs et al. 2007). Using a macromolecular contrast agent (gadomelitol) to examine human SW620 colorectal tumour xenografts in nude rat, administration of two doses of AZD2171 (3 mg/kg at 0 h and 22 h; imaging at 24 h) was found to reduce the mean permeability surface area product per unit volume of tissue by 80% and the fractional plasma volume by nearly 70% (Bradley et al. 2007). This dose of AZD2171 inhibits growth of SW620 xenografts in nude rat by 89% when administered chronically, once daily, for 21 days. Significantly reduced uptake of the contrast agent gadopentetate has also been observed using MRI in the liver metastases of patients treated with AZD2171 (Drevs et al. 2007; Chap. 37 in this book).

23.3.4 Inhibition of Tumour Growth

That inhibition of VEGFR signalling by AZD2171 compromises tumour growth significantly has been demonstrated in a wide range of preclinical tumour models.

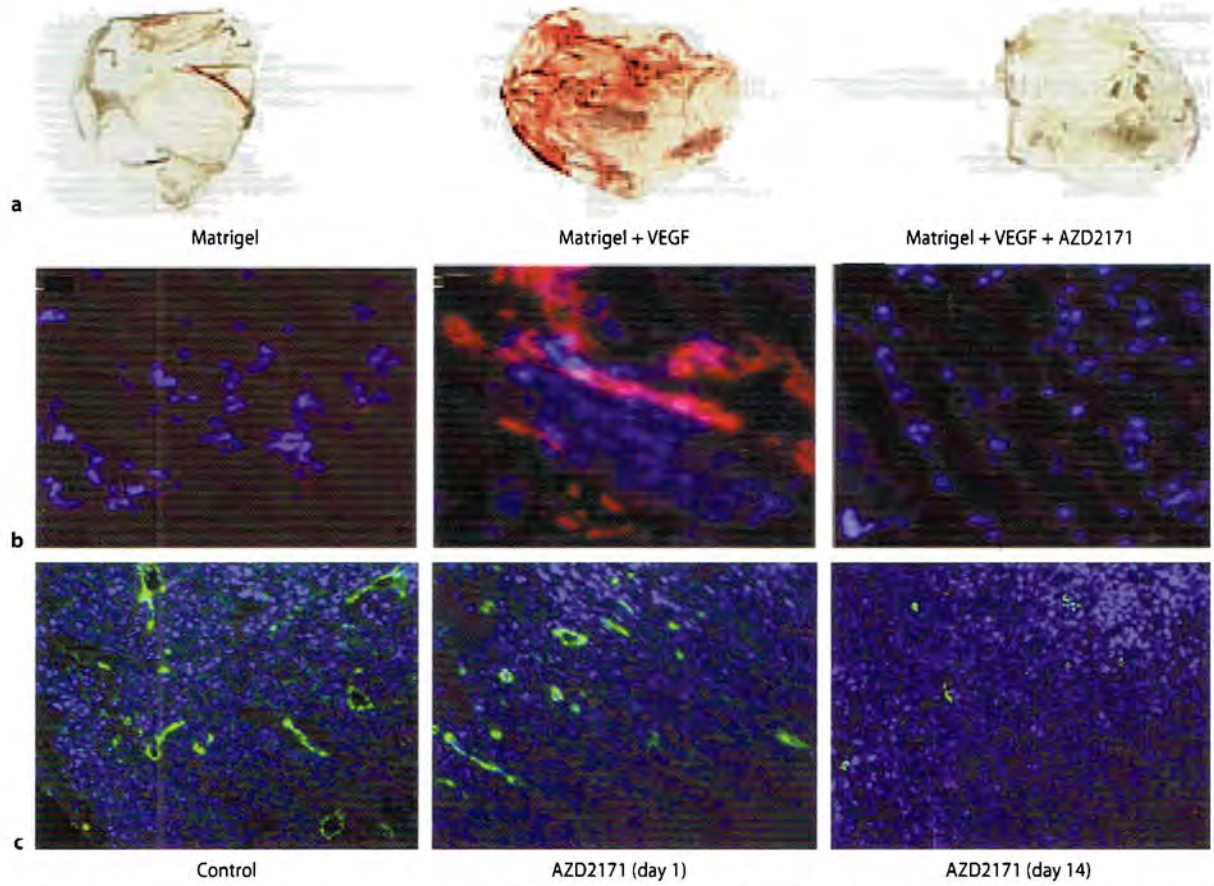


Fig. 23.4a–c. Inhibition of experimental and pathological angiogenesis by VEGFR-2 tyrosine kinase inhibition. Matrigel plugs containing VEGF elicit an angiogenic response over an 8-day period when implanted subcutaneously in mice. Oral administration of AZD2171 (6 mg/kg/day) for the duration of implantation abolishes the VEGF-induced angiogenesis. a Representative plug retrieved at day 8. b Immunofluorescent images of vascular CD31 (platelet/endothelial cell adhesion molecule 1) staining (*red* is CD31 staining and *blue* is a DAPI counterstain). A highly significant reduction in vessel density is also observed in Calu-6 human lung tumour xenografts grown in nude mice, following chronic AZD2171 treatment (6 mg/kg/day) for 2 weeks. c Immunofluorescent images of representative sections from control tumours or those treated with AZD2171 (*green* is CD31 and *blue* a DAPI counterstain). All images adapted with permission from Wedge et al. (2005)

23.3.4.1 Tumour Xenografts

Chronic once-daily oral administration of AZD2171 to nude mice dose-dependently inhibited the growth of established human tumour xenografts of histologically distinct origin (colon, lung, breast, ovarian and prostate) (Wedge et al. 2005) (Fig. 23.5).Administration of 1.5 mg/kg/day AZD2171 produced a statistically significant inhibition of tumour growth in each model, and 6 mg/kg/day of AZD2171 (24–28 days continuous dosing) inhibited their growth by >90% (Wedge et al. 2005). AZD2171 also demonstrated good inhibition of tumour growth in further studies in vulval, glial, gastric, and head and neck xenograft models (Wedge et al. 2004; Bozec et al. 2006; Takeda et al. 2006) and again demonstrated good inhibition of tumour growth. This broad-

spectrum activity is consistent with an inhibition of VEGFR signalling and an effect on tumour vasculature rather than a direct antiproliferative effect on tumour cells.

23.3.4.2 Orthotopic Tumour Models

Tumour xenografts are routinely implanted into a subcutaneous site, which is easily accessible and permits the sequential assessment of tumour growth by calliper measurement. However, orthotopic implantation of tumours (i.e. implantation into the tissue of histological origin) has been proposed to represent a more clinically relevant tumour model, since this has been found to more accurately replicate tumour–host interactions and can significantly influence the neovascular architecture and functionality (Fukumura et al. 1997; Suggitt and Bibby 2005).

In an orthotopic human lung adenocarcinoma model (NCI-H441), which closely mimics the patterns of growth and metastasis observed in man, once-daily oral dosing of AZD2171 (6 mg/kg) suppressed tumour growth and pleural effusion significantly, a finding which was accompanied by a marked reduction in MVD (Wu et al. 2005). In orthotopic breast tumours, AZD2171 similarly inhibited growth of established MCF-7 tumours and induced regression in VEGF-transfected MCF-7 tumours (Miller et al. 2006). AZD2171 has also been shown to inhibit the

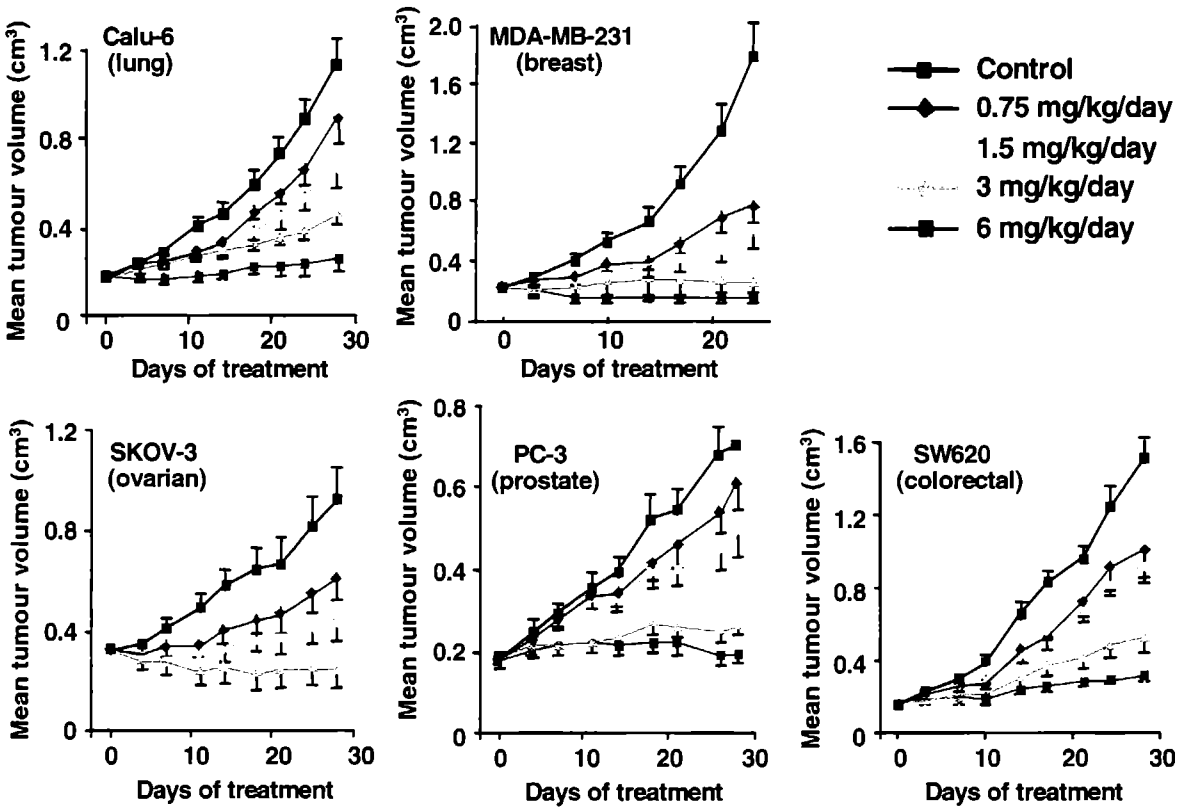


Fig. 23.5. VEGFR-2 tyrosine kinase inhibition confers broad-spectrum antitumour activity in tumour xenograft models. Human tumour xenografts of diverse histological origin were established (0.1–0.5 cm^3 volume) in nude mice and treated orally, once daily, with AZD2171 (0.7–6 mg/kg/day) or vehicle [a 1% (w/v) solution of polyoxyethylene (20) sorbitan monooleate in deionised water]. AZD2171 inhibited the growth of each tumour, dose-dependently, with >90% inhibition being evident in each model following 24–28 days of treatment at 6 mg/kg/day

growth of an orthotopically implanted metastasizing murine renal cell carcinoma (RENCA) model, with a corresponding reduction in tumour MVD and number of lung metastases formed (Drevs et al. 2004).

23.3.4.3 Transgenic and Spontaneous Tumour Models

Transgenic tumour models are of interest since tumour development occurs in situ, enabling therapeutic intervention to be examined at relatively early, and more advanced, stages of disease (Suggitt and Bibby 2005). However, their use is challenging, as tumour development is asynchronous and the rate of tumour development is typically slow. AZD2171 has been examined in transgenic MMTVneu mice (Klinowska et al. 2004), which express an activated form of the rat neu (erbB2) oncogene under the control of the MMTV promoter (Muller et al. 1988). After two pregnancies these mice spontaneously develop multiple tumours within each mammary gland. AZD2171 was administered to MMTVneu transgenic mice using two alternative dosing protocols. To examine the impact of treatment on the development of early lesions, animals were dosed from 14 to 19 weeks of age with AZD2171 (0.75–6 mg/kg/day). Treatment with AZD2171 reduced total tumour burden in the mammary glands, but did not influence the number of tumour foci. This restriction of tumour growth, but not of formation of tumour foci, is consistent with an antiangiogenic mechanism of action. In well-established tumours, AZD2171 treatment again inhibited tumour growth dose-dependently, but doses of 3 and 6 mg/kg/day were found to induce marked tumour regression compared to the pre-treatment tumour volume of $\geq 0.4\ cm^3$.

AZD2171 has also been studied in a mouse model of multiple intestinal neoplasia ($Apc^{Min/+}$). These mice inherit a mutant copy of the adenomatous polyposis coli (APC) gene and develop numerous benign polyps in the gastrointestinal (GI) tract following loss heterozygosity of APC in GI epithelial cells (Goodlad et al. 2006). The mutation in APC is analogous to that in patients with familial adenomatous polyposis (FAP), although mutations in APC have also been described in many sporadic colorectal cancers. AZD2171 significantly reduced the number and size of polyps in the early stage of polyp formation. In 6-week-old mice, AZD2171 reduced polyp number in the small bowel and colon by 46% and 62%, respectively and reduced mean tumour burden (the product of polyp number and volume) in the small bowel by 85%. In 10-week-old mice, AZD2171 did not alter polyp number in the small bowel, but reduced polyp diameter, resulting in a 46% decrease in tumour burden, again consistent with an antiangiogenic mechanism of action. These results suggest that VEGFR signalling plays a key role in the development of intestinal adenomas, and that inhibiting this activity can reduce tumour burden.

Collectively the preclinical data generated with AZD2171 (once-daily oral dosing) indicate that a VEGFR tyrosine kinase inhibitor is an effective method for constraining physiological and pathological VEGF-A signalling in vivo. As a consequence of the inhibition of VEGFR signalling, angiogenesis, neovascular survival and tumour growth are inhibited, irrespective of the tumour origin or site of growth.

23.4 Clinical Development of VEGFR Tyrosine Kinase Inhibitors

23.4.1 Optimal Criteria for a VEGFR Tyrosine Kinase Inhibitor

A multidisciplinary effort containing a highly skilled medicinal chemistry component is required to determine whether an appropriate balance of features can be found in one molecule, that can then be considered worthy of investigation in man as a potential therapeutic agent. A number of key criteria need to be considered in the selection of a VEGFR kinase inhibitor:

- *Potency*
  High potency against VEGFR-2 tyrosine kinase in endothelial cells should help to reduce the total dose of compound that has to be administered to inhibit VEGF-A signalling. Consequently, a small molecule for oral therapy can be manufactured as a comparatively small tablet, making it easier to swallow and potentially reducing variability in its dissolution and absorption characteristics. High potency may also accompany improved selectivity (see following bullet point on selectivity), as binding against the VEGFR-2 catalytic domain is optimised preferentially.
- *Selectivity*
  One of the perceived benefits of targeting the tumour vasculature is minimal toxicity to the host, given the paucity of physiological angiogenesis in healthy adults. It can therefore be argued that a VEGFR inhibitor must have its primary pharmacological activity against VEGFR-2 tyrosine kinase, in order to achieve robust or maximal inhibition of this target at well-tolerated doses. Additional activity against many different kinases, or unrelated (i.e. non-kinase) targets, may have undesired toxic consequences and potentially limit the dose or schedule of compound administration. An added concern is that a broad inhibitory profile may limit a compound's suitability for combination with cytotoxic chemotherapy, a foundation of cancer treatment, particularly if there are overlapping toxicity profiles (e.g. myelosuppression). However, it can also be argued that if the inhibitor has additional activity versus one or two select kinases that are also important in tumour progression, this may benefit the overall therapeutic profile (e.g. the additional activity of ZD6474 against epidermal growth factor receptor (EGFR) and RET tyrosine kinases). This is still an area of much controversy (Arteaga 2003), with a number of VEGFR kinase inhibitors in the field reportedly having activity against a spectrum of kinases (Table 23.1).
- *Physical properties*
  Identifying inhibitors with good physical properties can be challenging given that the VEGFR-2 kinase domain represents a hydrophobic target, and molecules best placed to exploit lipophilic interactions are less likely to have good 'drug-like' characteristics (e.g. high aqueous solubility or appreciable free plasma levels). Poor physical properties can hinder oral bioavailability significantly. Consequently, it may be necessary to examine an appropriate salt form, or investigate a prodrug version of the compound, in an attempt to mitigate such limitations.
- *Pharmacokinetics*
  Pharmacokinetics is a term that describes drug residence in vivo, dictated by the absorption, distribution, metabolism and elimination characteristics of a molecule.

Data from preclinical tumour models with VEGFR signalling inhibitors suggests that prolonged plasma exposure is required for continual constraint of angiogenesis, to deliver maximal inhibition of tumour growth (Wedge et al. 2000). To be compatible with oral administration, therefore, the inhibitor should attain pharmacologically relevant plasma levels that are maintained for the duration of the dosing interval, with a relatively small ratio between the maximum and minimum plasma concentrations. Ideally this should be achieved following once-daily dosing, not only for patient convenience but to facilitate increased compliance. The inhibitor should also produce a proportionally greater exposure with increasing dose, and not interfere with major drug elimination pathways (e.g. inhibition of liver cytochrome P450 isoforms) that could lead to adverse drug reactions with concomitant medication. Furthermore, the compound should not increase the level of liver enzymes involved in its own elimination (a process termed autoinduction), since its plasma exposure may be diminished significantly during chronic administration.

23.4.2 VEGFR Tyrosine Kinase Inhibitors in Current Clinical Development

A large number of VEGFR kinase inhibitors have been described using divergent structural templates, with a range of potencies, physical properties and

pharmacokinetics. These may be categorised on the basis of their reported selectivity against kinases. Inhibitors of the VEGFR tyrosine kinases demonstrate varying degrees of selectivity for members of the platelet-derived growth factor receptor (PDGFR) tyrosine kinase family (Table 23.1). This is perhaps not surprising given that that the PDGFR family of tyrosine kinases contain significant structural homology with VEGFR-1, -2 and -3, including the presence of a non-catalytic insert in their tyrosine kinase domain. The PDGFR family consists of PDGFR-α and -β, c-Kit, the colony-stimulating factor 1 receptor (CSF-1R) and the Fms-like tyrosine kinase-3 (Flt-3).

VEGFR tyrosine kinase inhibitors have also been described with additional activity versus the fibroblast growth factor receptors (FGFR), the erbB family of receptors, the cytoplasmic Raf kinases and the cyclin-dependent kinases. In general, the more selective VEGFR tyrosine kinase inhibitors would be expected to be well tolerated, particularly for a cancer therapy, the most common adverse effect being the induction of hypertension (a consequence of inhibiting VEGF-A-induced vasodilation), with some incidence of fatigue, headache and diarrhoea.

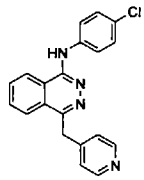
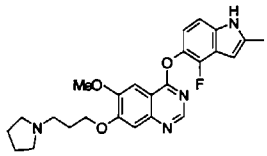
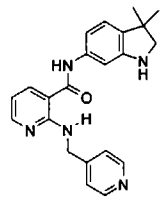
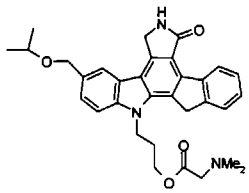
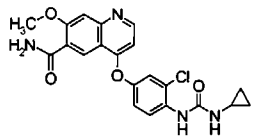
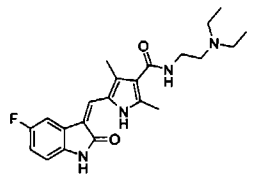
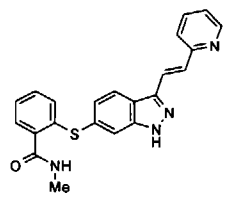
23.4.2.1 Selective VEGFR Tyrosine Kinase Inhibitors (± c-Kit Activity)

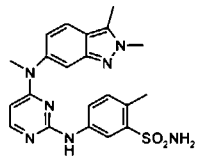
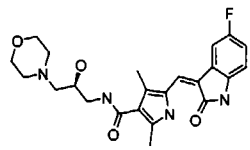
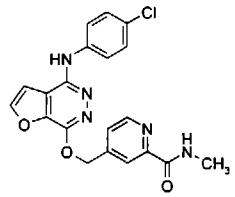
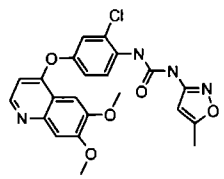
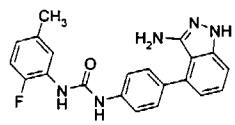
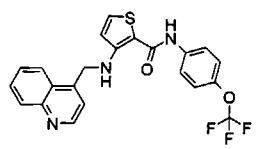
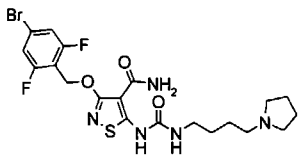
A number of the clinically advanced and selective VEGFR tyrosine kinase inhibitors also appear to have some activity against c-Kit tyrosine kinase. However, activity against c-Kit does not appear to impart any major tolerability issues, and may confer added therapeutic benefit in c-Kit-dependent malignancies. VEGFR inhibitors with a comparatively selective profile are summarised in Table 23.1(a).

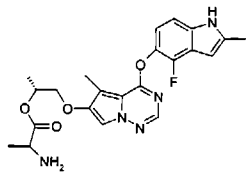
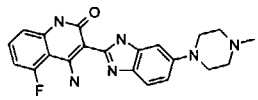
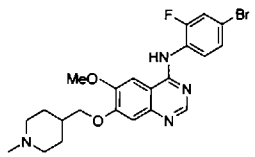
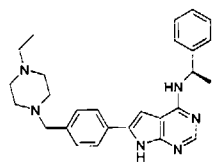
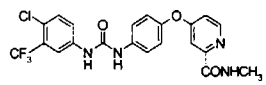
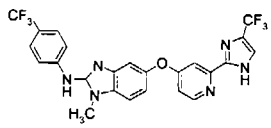
Vatalanib (PTK787; Novartis), a phthalazine derivative, was one of the first VEGFR kinase inhibitors to enter clinical development. Although widely referred to as an inhibitor of VEGFR-1 and -2 tyrosine kinase with additional activity against c-Kit and PDGFR-β, it demonstrates appreciable selectivity against the latter two kinases (Wood et al. 2000). DCE-MRI detected changes in contrast uptake by liver metastases following vatalanib treatment of patients with colon cancer (Morgan et al. 2003). Elevated plasma VEGF (Drevs et al. 2005) was also observed during the first 28-day cycle of treatment. However, vatalanib had a short plasma half-life in man (2–6 h) and when dosed once daily (at ≥1000 mg using 250-mg tablets) induced light-headedness with reports of ataxia, presumably attributable to a rapid high peak plasma concentration 1–2 h after dosing (Thomas et al. 2005). The pharmacokinetic profile of this compound was further compromised by hepatic autoinduction, thought to be mediated by the CYP3A4 isozyme, which, following chronic vatalanib administration for 28 days, resulted in 30–40% lower exposure (area under the curve) than at day 1 (Morgan et al. 2003; Thomas et al. 2005). Nonetheless, vatalanib progressed to phase III trials in colorectal cancer in combination with 5-fluorouracil/leucovorin/oxaliplatin (FOLFOX4) but failed to meet the PFS endpoint, although there was a trend towards improved PFS with vatalanib/FOLFOX compared with the FOLFOX arm of the trial (Hecht et al. 2005). This limited activity may be a consequence of the compound's pharmacokinetic profile, and emphasises the need to identify compounds with consistent, sustained exposure at therapeutic concentrations, or the need to exploit multiple daily dosing schedules.

Of the more selective VEGFR kinase inhibitors, AZD2171 (AstraZeneca) combines high potency with particularly desirable pharmacokinetic features: a plasma half-life of approximately 20 h is evident in man following administration of AZD2171, necessitating only once-daily dosing. The compound produces dose-dependent increases in plasma exposure, and there is no evidence of autoinduction or altered clearance upon chronic administration (Drevs et al. 2007). AZD2171 also produced highly significant changes in DCE-MRI changes in liver metastases (up to 80% reduction in contrast agent uptake), and time- and dose-dependent reductions in soluble VEGFR-2 circulating in plasma. In a phase I monotherapy trial once-daily oral dosing of AZD2171 up to 45 mg was generally well tolerated, with doses >20 mg showing encouraging signs of antitumour activity. Preliminary evidence of efficacy included

Table 23.1. VEGFR tyrosine kinase inhibitors in clinical development

Name[1] (Company)	Phase[2]	Structure	Relative[3] potency	Selectivity profile[4]	Human PK[5]	Refs
(a) Selective VEGFR tyrosine kinase inhibitors (+/- c-Kit activity)						
Vatalanib PTK787 (Novartis)	III		B	VEGFR (1, 2)	t½ = 2–6 h q.d. (≥1000 mg/d)	Wood et al. 2000; Morgan et al. 2003
AZD2171 (Astra Zeneca)	II/III		A	VEGFR (1, 2, 3) c-kit	t½ ~ 20 h q.d. (30–45 mg/d)	Wedge et al. 2005; Drevs et al. 2007
AMG-706 (Amgen)	II		B	VEGFR (1, 2, 3) c-kit	t½ = 5–7 h q.d. (125 mg)/b.i.d.	Polverino et al. 2006; Herbst et al. 2004
CEP-7055 (Cephalon)	II		B	VEGFR (1, 2, 3) c-kit	b.i.d./t.i.d.	Ruggeri et al. 2003
E7080 (Eisai)	I		A	VEGFR (1, 2, 3)	–	Matsui et al. 2003
(b) VEGFR tyrosine kinase inhibitors with additional activity versus PDGFR kinases						
Sunitinib SU11248 (Pfizer)	Approved for GIST and RCC		B	VEGFR (1, 2, 3) c-Kit PDGFRα/β CSF-1R Flt-3	t½ = 60 h q.d. (50 mg/d; 3 weeks on, 1 week off)	Mendel et al. 2003; O'Farrell et al. 2003; Bello et al 2005;
Axitinib AG-013736 (Pfizer)	II		A	VEGFR (1, 2, 3) PDGFRβ c-Kit	t½ = 2–5h b.i.d. (fasted) (5 mg)	Wickman et al 2003; Rugo et al. 2005

Name[1] (Company)	Phase[2]	Structure	Relative[3] potency	Selectivity profile[4]	Human PK[5]	Refs
Pazopanib GW-786034 (Glaxo-Wellcome)	II		B	VEGFR (1, 2, 3) PDGFRα/β c-Kit	t½ ~ 35 h q.d. (800–2000 mg)	Suttle et al. 2004; Hurwitz et al. 2005; Kumar et al 2005
SU14813 (Pfizer)	II		B	VEGFR (1, 2) PDGFRα/β c-Kit Flt-3 CSF-1R	t½ = 13–23 h q.d. (200 mg; 4 weeks on, 1 week off)	Patyna et al 2006; Fiedler et al. 2005
Telatinib BAY 57-9352 (Bayer)	I/II		B	VEGFR (2, 3) c-Kit PDGFRβ	t½ = 5–12 h b.i.d. (1800 mg)	Chang et al. 2005; Strumberg et al. 2006
KRN-951 (AV-951: Kirin Brewery and AVEO pharmaceuticals)	I		A	VEGR (1, 2, 3) c-kit PDGFRβ	–	Nakamura et al, 2006
ABT-869 (Abbott)	I		A	VEGFR(1, 2), PDGFRβ CSF-1R Flt-3 c-Kit	t½ = 16 h q.d. (10mg)	Albert et al 2005; Goh et al, 2006
OSI-930 (OSI)	I		B	VEGFR(1, 2) c-Kit CSF-1R, PDGFRβ	q.d.	Garton et al. 2006
(c) VEGFR tyrosine kinase inhibitors with additional activity versus FGFR kinases						
CP-547,632 (Pfizer)	II		B	VEGFR-2, FGFR1	t½ = 29h q.d. (<300mg)	Beebe et al. 2003; Tolcher et al. 2002
BIBF-1120 (Boehringer Ingelheim)	II	Indolinone derivative Full structure undisclosed	B	VEGFR (1, 2, 3) PDGFRα/β, FGFR-1	t½ ~ 15h q.d. (250 mg)	Hilberg et al. 2004; Mross et al. 2004

Name[1] (Company)	Phase[2]	Structure	Relative[3] potency	Selectivity profile[4]	Human PK[5]	Refs
BMS 582664 (Bristol Myers Squibb)	I		B	VEGFR(1, 2) FGFR-1	-	Fargnoli et al. 2005
CHIR-258 (TKI-258; Novartis/ Chiron Corp.)	I		B		-	Lee et al. 2005; Sarker et al. 2006
(d) VEGFR tyrosine kinase inhibitors with additional activity versus EGFR kinase						
ZD6474 Zactima™ (AstraZeneca)	III + ODS in thyroid		B	VEGFR (2, 3) RET EGFR	t½ = 120 h q.d. (100–300 mg)	Wedge et al. 2002; Carlomagno et al. 2002
AEE-788 (Novartis)	II		C	EGFR ErbB2 VEGFR(1, 2)	q.d. (≤ 400mg)	Traxler et al. 2004; Reardon et al. 2005
(e) VEGFR tyrosine kinase inhibitors with additional activity versus Raf kinases						
BAY 43-9006 Sorafenib (Bayer/ Onyx)	Approved in RCC		B	VEGFR(2, 3) PDGFRβ C and B-Raf Flt-3, c-Kit	t½ ~ 20–38 h b.i.d. (800 mg)	Wilhelm et al. 2004; Clark et al. 2005
CHR-265 (Novartis/ Chiron Corp.)	I		C	C and B-Raf VEGFR2 PDGFRβ c-Kit	-	Renhow et al. 2006
(f) VEGFR tyrosine kinase inhibitor with additional activity versus cyclin dependent kinases						
ZK-304709 (Bayer Schering Pharma AG)	I	Structure not disclosed	B	VEGFR (1, 2, 3) PDGFRβ CDK(2,1,4)	-	Siemester et al. 2005

[1] Ranked in order of Phase of development, then potency versus VEGFR-2, selectivity and pharmacokinetics. [2] Highest reported phase of clinical development. GIST; Gastrointestinal stromal tumour. RCC; renal cell cancer. ODD; orphan drug designation. [3] Relative potency versus VEGFR-2 tyrosine kinase in cells (VEGFR-2 phosphorylation or VEGF-induced HUVEC proliferation) from literature values (A: 0.5 to ≤5 nM; B: 5 to 50 nM; C: >50nM). For the majority of compounds, a good correlation exists between values generated against both parameters. [4] Based on reported selectivity in literature. [5] t½= plasma half-life in man following a single dose. q.d. = dosed once-daily, b.i.d. = dosed twice-daily, t.i.d. = dosed three times per day, (total daily dose)

22 reports of stable disease and two confirmed partial responses, in 63 evaluable patients with a variety of advanced solid tumour types. The effects on tumour size appeared to be dose related (Drevs et al. 2007).

AZD2171 has also been shown to rapidly inhibit vasogenic brain oedema in patients with recurrent glioblastoma, leading to reduced corticosteroid usage (Batchelor et al. 2007). Phase II/III trials are currently underway in a number of tumour types.

Less potent compounds with reportedly similar selectivity include AMG-706 (Amgen) (Herbst et al. 2004; Polverino et al. 2006) and CEP-7055 (Ruggeri et al. 2003). AMG-706 has a plasma half-life of 4–7 h in man (Herbst et al. 2004) and is being examined with different dosing schedules (Rosen et al. 2005). In a phase I study in patients with advanced solid tumours refractory to standard therapy, some stable disease and partial responses were reported (Herbst et al. 2004; Rosen et al. 2005). AMG706 is now being examined in phase II trials. CEP-7055 (Cephalon) is an N,N-dimethyl glycine ester prodrug of CEP-5214 (Ruggeri et al. 2003) and is used to increase the oral bioavailability of the latter, although this is still reportedly low in mice (15%). The relationship between dose and plasma exposure for CEP-5214 was approximately linear between 10 and 40 mg twice daily, but appeared to plateau at 80 and 120 mg twice daily in a phase I study (Pili et al. 2003).

23.4.2.2 VEGFR Tyrosine Kinase Inhibitors with Additional Activity Versus PDGFR Kinases

A combination of activity against VEGFR and PDGFR-β signalling has been proposed as a potentially interesting therapeutic profile, given that simultaneous targeting of the latter may help to destabilise pericyte–endothelial cell interactions. A significant proportion of this hypothesis stems from preclinical experiments conducted in transgenic Rip-Tag mice, which develop pancreatic islet tumours (Bergers et al. 2003). However, VEGF sequestration alone has been also found to exert highly significant antitumour activity in this model (Inai et al. 2004), and it is unclear whether in the clinical setting, chronic inhibition of PDGFR-β signalling is required in the presence of marked VEGF inhibition.

VEGFR kinase inhibitors are shown in Table 23.1(b). Of those VEGFR tyrosine kinase inhibitors with claimed selectivity for PDGFR versus the other PDGFR family members, axitinib (AG-013736; Pfizer Inc.) has demonstrated significant DCE-MRI changes in a phase I study in patients with advanced solid tumours (Liu et al. 2005) and yielded encouraging phase II data in second-line renal cell carcinoma (RCC) (Rini and Small 2005). RCC may have a particular sensitivity to VEGF-signalling inhibitors, given that mutations in the von Hippel–Lindau (VHL) tumour suppressor gene are common in this disease and result in VEGF over-expression (Rini and Small 2005). However, axitinib has a short plasma half-life, necessitating twice-daily dosing, and pharmacokinetic interactions with CYP3A inhibitors and food have been reported, the latter necessitating fasting prior to dosing (Rugo et al. 2005). In addition to hypertension, the phase I safety profile of axitinib included haemoptysis, thromboembolism, skin rash, stomatitis and increased liver transaminases. Pazopanib (GW-786034; GSK), a tyrosine kinase inhibitor of VEGFR-1,-2 and -3, PDGFR-α and -β, and c-Kit, also reported tumour shrinkage in three patients with RCC treated at >800 mg during phase I and some incidence of hypertension (Hurwitz et al. 2005). However, the characteristics of this compound are such that its absorption is saturated at doses of 800 mg, there being little evidence of increased exposure at doses of 2000 mg; this may limit its future potential.

Of compounds with broad activity, the most advanced is sunitinib (SU11248; Pfizer Inc.), which was approved for use in two indications during early 2006. The first of these is for the treatment of GIST patients who are intolerant of imatinib (Glivec™) or who have progressed during imatinib treatment (Demetri et al. 2006). This activity is most likely to be attributable to sunitinib's inhibition of c-Kit tyrosine kinase, since it has a different binding conformation to imatinib and may therefore not be susceptible to kinase mutations that commonly confer imatinib resistance (Chen et al. 2005). Sunitinib has

also been approved for the treatment of metastatic RCC based on response rates and the duration of responses in patients who had failed cytokine therapy (Motzer et al. 2006), although there are currently no randomised trial data to show a survival increase. The encouraging antitumour activity of this agent is accompanied by significant toxicity, notably grade 3 fatigue, asthenia, myelosuppression (neutropenia, thrombocytopenia and lymphopenia), mucositis, hair and skin depigmentation and skin coloration, in addition to hypertension and bleeding (Faivre et al. 2006; Motzer et al. 2006). In addition, patients receiving sunitinib should be carefully monitored for clinical signs and symptoms of cardiac heart failure, and baseline and periodic evaluations of left ventricular ejection fraction should be considered. Management of this side effect profile currently requires an intermittent dosing schedule to be used (4 weeks on, 2 weeks off), and it may be likely to be less widely used in combination regimens (Faivre et al. 2006). A follow-up compound, SU14813, which retains activity against a spectrum of kinases, appears to have an improved therapeutic index: a 4-weeks-on, 1-week-off regimen is tolerated, and chronic continuous once-daily administration is now being explored in phase II (Fiedler et al. 2005; Patyna et al. 2006).

Other VEGF tyrosine kinase inhibitors with additional activity versus PDGFR kinases that are presently being evaluated in phase I clinical studies include KRN-951 (AV-951; Kirin Brewery and AVEO Pharmaceuticals), ABT-869 (Abbott), OSI-930 (OSI Pharmaceuticals) and telatinib (BAY 57-9352; Bayer) (Table 23.1(b)).

23.4.2.3 VEGFR Tyrosine Kinase Inhibitors with Additional Activity Versus Structurally Distinct Kinases

Fibroblast Growth Factor Receptor Tyrosine Kinases

Angiogenesis is a complex biological process that relies on a variety of growth factors. Whilst VEGF signalling is considered pivotal for angiogenesis, FGFR signalling is also thought to be implicated. However, there is no discernable benefit of having this additional activity in cancer treatment, to date. The most advanced molecule combining activity versus VEGFR-2 and FGFR-1 is CP-547,632 (Pfizer Inc.) (Beebe et al. 2003), which has shown some evidence of stable disease for >8 weeks as monotherapy in 7 out of 22 evaluable patients with advanced solid tumours (Tolcher et al. 2002) and is presently being investigated in a phase I study in NSCLC in combination with paclitaxel and carboplatin (Cohen et al. 2004). At doses of 200 mg/day, dose-limiting toxicities were rash and diarrhoea. However, there have been no recent reports of the clinical progress of this drug. Other compounds with additional activity versus FGFR include BMS-582664 (Bristol-Myers Squibb), an L-alanine ester prodrug that improves the solubility and oral bioavailability of the active drug BMS-540215 (Fargnoli et al. 2005), which is in phase I, and BIBF-1120 (Boehringer Ingelheim) (Hilberg et al. 2004). BIBF-1120 has been explored with once-daily dosing, and subsequently twice-daily dosing to increase drug exposure, although elevated liver enzymes were found to be a dose-limiting toxicity on both dosing schedules (Mross et al. 2004, 2005). Early evidence of disease stabilisation was observed together with reduced contrast agent uptake, measured by DCE-MRI. However, it remains unclear whether these biomarker changes and disease stabilisation are due to a combined inhibition of VEGFR-2 and FGFR, or simply to inhibition of VEGF signalling alone. CHIR-258 (TKI-258, GFKI-258; Novartis/Chiron) is a multi-targeted inhibitor of receptor tyrosine kinases (Lee et al. 2005; Goldbeck et al. 2005). A phase I study in patients with advanced malignancies with intermittent and continuous dosing schedules is under way and showed early evidence of stable disease in several patients, including GIST patients (Sarker et al. 2006), that was most likely attributable to c-Kit inhibition. Adverse events included hypertension, anaemia and rash.

Epidermal Growth Factor Tyrosine Kinase

EGFR over-expression by tumour cells has been found to correlate with a poor prognosis in a range of malignancies. EGFR can be activated by a range of structurally related ligands that can be produced

by epithelial-derived tumour cells and result in autocrine stimulation of tumour cell proliferation (Woodburn 1999). EGFR signalling has also been implicated in other processes driving tumour progression, which include migration, apoptosis and angiogenesis (Musallam et al. 2001; Alper et al. 2001; Ciardiello et al. 2001).

ZD6474 (Zactima™; AstraZeneca) has a unique activity profile that distinguishes it from other molecular targeting agents: in addition to inhibiting VEGF signalling, ZD6474 is also a potent inhibitor of the tyrosine kinases associated with EGFR and the oncoprotein RET (Carlomagno et al. 2002; Wedge et al. 2002). While aberrant EGFR signalling is implicated in a number of solid tumours (Harari 2004), constitutively active RET is involved in the development of several neuroendocrine diseases, including carcinomas of the thyroid (Ichihara et al. 2004). ZD6474 consequently offers the opportunity to inhibit key signalling pathways in tumour progression, either via inhibition of VEGF-dependent tumour angiogenesis indirectly or by targeting tumour growth directly. As part of an ongoing phase II programme, the efficacy of ZD6474 alone and in combination with standard chemotherapy regimens is being investigated in a range of tumour types. Phase I and II studies have shown ZD6474 to be generally well tolerated with a pharmacokinetic profile supportive of once-daily oral dosing. Preliminary evidence of efficacy was observed in patients with NSCLC in a Japanese phase I study (Minami et al. 2003), and ZD6474 demonstrated significant improvements in PFS during phase II evaluation in NSCLC, as a monotherapy versus gefitinib (Iressa™) (Natale et al. 2005), and when combined with docetaxel, versus docetaxel alone (Heymach et al. 2005). Phase III evaluation of ZD6474 has been initiated in second-line NSCLC. ZD6474 has shown encouraging evidence of activity in hereditary medullary thyroid cancer and clinical evaluation is ongoing (Wells et al. 2006). ZD6474 has been granted Fast Track status and Orphan Drug designation by the US Food and Drug Administration (FDA) for the treatment of patients with follicular, medullary, anaplastic, and locally advanced and metastatic papillary thyroid cancer. The EU Committee for Orphan Medicinal Products (COMP) have granted Orphan Drug designation for the treatment of medullary thyroid cancer. Common adverse events were diarrhoea, rash and asymptomatic QTc prolongation, all of which responded to standard management. (For further information on ZD6474 see also Chap. 41 of this book.)

AEE788 (Novartis) is another small-molecule tyrosine kinase inhibitor in development that primarily targets EGFR, but has some additional activity against the v-erb-b2 avian erythroblastic leukaemia viral oncogene homologue 2 (ErbB2) and VEGFR-2, and weak inhibitory activity versus RET (Traxler et al. 2004). Besides diarrhoea and rash, which are likely to be associated with EGFR pharmacology, adverse events included grade 3 and 4 AST/ALT elevation and possible drug-induced hepatitis (Reardon et al. 2005; Martinelli et al. 2005). AEE788 exposure was low in glioblastoma patients receiving enzyme-inducing anticonvulsants, suggesting that AEE788 is sensitive to the effects of enzyme-inducing agents (Reardon et al. 2005).

Raf Kinases

The Ras/Raf signalling pathway is important for tumour cell proliferation and angiogenesis. Raf kinases are serine/threonine protein kinases that function as downstream effector molecules of Ras and include A-Raf, B-Raf and Raf-1. B-Raf is reportedly mutated in 70% of malignant melanomas, in 33% of papillary thyroid carcinomas and to a lesser frequency in other tumours (Davies et al. 2002; Rajagopalan et al. 2002; Kimura et al. 2003). There is also evidence to suggest that Raf-1 and B-Raf can play a role in the regulation of endothelial cell apoptosis (Alavi et al. 2003). Multi-targeted inhibitors that display activity against mutant B-Raf together with inhibition of VEGFR-2 kinase could therefore have a direct antiproliferative effect in certain malignancies (e.g. malignant melanoma) in addition to targeting angiogenesis.

Sorafenib (Bay 43-9006; Bayer) is a bi-aryl urea that has been shown to inhibit Raf-1, B-Raf, the V559E B-Raf mutant and VEGFR-2, VEGFR-3, PDGFR-β and c-Kit (Wilhelm et al. 2004). In phase I

studies, the pharmacokinetics of this compound was non-linear with a large inter-individual variability after single and multiple dosing. The peak plasma concentration occurred between 2.5 and 12.5 h after dosing, and the mean plasma half-life ranged from 24 to 38 h, with substantial accumulation following chronic twice-daily dosing (Strumberg et al. 2005). Sorafenib has been shown to inhibit CYP2B6 and CYP2C8, and may therefore increase the exposure of compounds that are cleared by these enzymes when dosed concomitantly. Adverse events include skin rash, hand–foot syndrome and elevation of liver enzymes. Sorafenib has demonstrated preliminary antitumour activity in patients with RCC, hepatocellular carcinoma, melanoma and sarcoma (Strumberg et al. 2005). Recently, sorafenib was approved for treatment of patients with advanced RCC based on an improvement in PFS versus placebo (Escudier et al. 2005), which is likely to be attributable to inhibition of VEGF signalling.

Another inhibitor of Raf kinases and VEGFRs has been described recently, CHIR-265 (Novartis/Chiron Corp.) (Amiri et al. 2006). This compound has increased potency versus B-Raf mutants when compared with B-Raf and is beginning clinical trials in melanoma patients.

Cyclin-dependent Kinases

ZK-304709 (Bayer Schering Pharma AG) combines activity versus VEGFRs and PDGFR-β tyrosine kinase with inhibition of the serine/threonine kinases, cyclin-dependent kinases 2, 1 and 4. Consequently, evidence of cell-cycle arrest and antiangiogenic effects have been shown in preclinical models (Siemeister et al. 2005). This novel spectrum of activity confers a much narrower therapeutic index in preclinical studies than more selective VEGFR tyrosine kinase inhibitors.

Conclusions

VEGFR tyrosine kinase inhibitors hold great promise as convenient therapeutic agents for solid tumour treatment. However, only those inhibitors possessing a good balance of features (Sect. 23.4.1) are likely to achieve optimal inhibition of VEGF signalling, whilst retaining suitability for use in combination regimens with existing therapeutic modalities. To date, the most successfully advanced tyrosine kinase inhibitors with anti-VEGF activity have been approved for use in RCC, which appears to have a particularly marked dependency on VEGF signalling, largely attributable to mutations in the VHL gene. In addition, promising signals have been seen in indications where activities versus additional targets are likely to have provided benefit (e.g. the activity of sunitinib versus c-Kit tyrosine kinase in imatinib-resistant GIST, and ZD6474 activity versus EGFR and RET tyrosine kinases in NSCLC and thyroid cancer, respectively). More recent compounds such as AZD2171, which combines high potency and good physical properties with an attractive pharmacokinetic profile, have shown encouraging signs of activity as monotherapy alone. Such compounds should now enable the small-molecule VEGFR signalling inhibitor concept to be tested robustly.

References

Achen MG, Jeltsch M, Kukk E, Makinen T, Vitali A, Wilks AF, Alitalo K, Stacker SA (1998) Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). Proc Natl Acad Sci USA 95:548–553

Akhtar N, Dickerson EB, Auerbach R (2002) The sponge/Matrigel angiogenesis assay. Angiogenesis 5:75–80

Alavi A, Hood JD, Frausto R, Stupack DG, Cheresh DA (2003) Role of Raf in vascular protection from distinct apoptotic stimuli. Science 301:94–96

Albert DH, Tapang P, Magoc TJ, Pease LJ, Reuter DR, Li J, Guo J, Ghoreishi-Haack NS, Brooks JV, Bukofzer GT, Wang YC, Stavropoulos JA, Hartandi K, Niquette AL,

Wei RQ, McCall JO, Bouska JJ, Luo L, Donawho CK, Dai Y, Marcotte PA, Glaser KB, Michaelides MR, Davidsen SK (2005) Preclinical activity of ABT-869, a multi-targeted receptor tyrosine kinase inhibitor. Proc Am Assoc Cancer Res abstr 676

Alper O, Bergmann-Leitner ES, Bennett TA, Hacker NF, Stromberg K, Stetler-Stevenson WG (2001) Epidermal growth factor receptor signaling and the invasive phenotype of ovarian carcinoma cells. J Natl Cancer Inst 93:1375–1384

Amiri P, Aikawa ME, Dove J, Stuart DD, Poon D, Pick T, Ramurthy S, Subramanian S, Levine B, Costales A, Harris A, Paul R (2006) CHIR-265 is a potent selective inhibitor of c-Raf/B-Raf/mutB-Raf that effectively inhibits proliferation and survival of cancer cell lines with Ras/Raf pathway mutations. Proc Am Assoc Cancer Res 47 abstr 4855

Arinaga M, Noguchi T, Takeno S, Chujo M, Miura T, Uchida Y (2003) Clinical significance of vascular endothelial growth factor C and vascular endothelial growth factor receptor 3 in patients with nonsmall cell lung carcinoma. Cancer 97:457–464

Arteaga CL (2003) Molecular therapeutics: is one promiscuous drug against multiple targets better than combinations of molecule-specific drugs? Clin Cancer Res 9:1231–1232

Asahara T, Murohara T, Sullivan A, Silver M, van der ZR, Li T, Witzenbichler B, Schatteman G, Isner JM (1997) Isolation of putative progenitor endothelial cells for angiogenesis. Science 275:964–967

Batchelor TT, Sorensen AG, di Tomaso E, Zhang W-T, Duda DG, Cohen KS, Kozak KR, Cahill DP, Chen P-J, Zhu M, Ancukiewicz M, Mrugala MM, Plotkin S, Drappatz J, Louis DN, Ivy P, Scadden DT, Benner T, Loeffler JS, Wen PY, Jain RK (2007) AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor, normalizes tumor vasculature and alleviates edema in glioblastoma patients. Cancer Cell 11:83–95

Bates DO, Lodwick D, Williams B (1999) Vascular endothelial growth factor and microvascular permeability. Microcirculation 6:83–96

Beebe JS, Jani JP, Knauth E, Goodwin P, Higdon C, Rossi AM, Emerson E, Finkelstein M, Floyd E, Harriman S, Atherton J, Hillerman S, Soderstrom C, Kou K, Gant T, Noe MC, Foster B, Rastinejad F, Marx MA, Schaeffer T, Whalen PM, Roberts WG (2003) Pharmacological characterization of CP-547,632, a novel vascular endothelial growth factor receptor-2 tyrosine kinase inhibitor for cancer therapy. Cancer Res 63:7301–7309

Bello C, Laurie S, Zhou J, Verkh L, Smeraglia J, Mount J, Klamerus K (2005) Food does not effect the pharmacokinetics of sunitinib malate (SU11248), a multi-targeted receptor tyrosine kinase inhibitor, in healthy subjects. Clin Cancer Res 11 abstr B175

Benjamin LE, Golijanin D, Itin A, Pode D, Keshet E (1999) Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. J Clin Invest 103:159–165

Bergers G, Song S, Meyer-Morse N, Bergsland E, Hanahan D (2003) Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. J Clin Invest 111:1287–1295

Bozec A, Formento P, Fischel J-L, Hofman P, Etienne M-C, Milano G (2006) Combined effect of EGFR targeting with antiangiogenesis and irradiation. Proc Am Assoc Cancer Res 47 abstr 232

Bradley DP, Tessier JL, Checkley D, Kuribayashi H, Waterton JC, Kendrew J, Wedge SR (2007) The effects of AZD2171 and ZD6474 (ZACTIMA™) on haemodynamic parameters in an SW620 human colon tumour model: an investigation using dynamic contrast-enhanced magnetic resonance imaging and the rapid clearance blood pool contrast agent gadomelitol. NMR Biomed (in press)

Byrne AT, Ross L, Holash J, Nakanishi M, Hu L, Hofmann JI, Yancopoulos GD, Jaffe RB (2003) Vascular endothelial growth factor-trap decreases tumor burden, inhibits ascites, and causes dramatic vascular remodeling in an ovarian cancer model. Clin Cancer Res 9:5721–5728

Carlomagno F, Vitagliano D, Guida T, Ciardiello F, Tortora G, Vecchio G, Ryan AJ, Fontanini G, Fusco A, Santoro M (2002) ZD6474, an orally available inhibitor of KDR tyrosine kinase activity, efficiently blocks oncogenic RET kinases. Cancer Res 62:7284–7290

Chang YS, Cortes C, Polony B, Brink C, Elting JJ (2005) Preclinical chemotherapy with the VEGFR-2 and PDGFR inhibitor, BAY 57–9352, in combination with capecitabine and paclitaxel. Proc Am Assoc Cancer Res abstr 2030

Checkley D, Tessier JJ, Kendrew J, Waterton JC, Wedge SR (2003) Use of dynamic contrast-enhanced MRI to evaluate acute treatment with ZD6474, a VEGF signalling inhibitor, in PC-3 prostate tumours. Br J Cancer 89:1889–1895

Chen LL, Sabripour M, Andtbacka RH, Patel SR, Feig BW, Macapinlac HA, Choi H, Wu EF, Frazier ML, Benjamin RS (2005) Imatinib resistance in gastrointestinal stromal tumors. Curr Oncol Rep 7:293–299

Ciardiello F, Caputo R, Troiani T, Borriello G, Kandimalla ER, Agrawal S, Mendelsohn J, Bianco AR, Tortora G (2001) Antisense oligonucleotides targeting the epidermal growth factor receptor inhibit proliferation, induce apoptosis, and cooperate with cytotoxic drugs in human cancer cell lines. Int J Cancer 93:172–178

Clark JW, Eder JP, Ryan D, Lathia C, Lenz HJ (2005) Safety and pharmacokinetics of the dual action Raf kinase and vascular endothelial growth factor receptor inhibitor, BAY 43-9006, in patients with advanced, refractory solid tumors. Clin Cancer Res 11:5472–5480

Cohen RB, Simon G, Langer CJ, Schol JR, McHale J, Eisenberg P, Hainsworth JD, Liau KF, Healey D (2004) Phase I trial of oral CP-547,632 (VEGFR2) in combination with paclitaxel (P) and carboplatin (C) in advanced non-small cell lung cancer (NSCLC). J Clin Oncol 22 abstr 3014

Curwen JO, Musgrove HL, Graham GA, Kendrew J, Richmond GHP, Ogilvie DJ, Wedge SR (2005) Management of hypertension induced by AZD2171, an oral, highly potent and reversible VEGFR signaling inhibitor, in rat. Clin Cancer Res 11 abstr B9

Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R, Jayatilake H, Gusterson BA, Cooper C, Shipley J, Hargrave D, Pritchard-Jones K, Maitland N, Chenevix-Trench G, Riggins GJ, Bigner DD, Palmieri G, Cossu A, Flanagan A, Nicholson A, Ho JW, Leung SY, Yuen ST, Weber BL, Seigler HF, Darrow TL, Paterson H, Marais R, Marshall CJ, Wooster R, Stratton MR, Futreal PA (2002) Mutations of the BRAF gene in human cancer. Nature 417:949–954

Demetri G, van Oosterom AT, Garrett C, Blackstein M, Shah M, Verweij JJ, McArthur G, Judson I, Baum C, Casali P (2006) Improved survival and sustained clinical benefit with SU11248 (SU) in pts with GIST after failure of imatinib mesylate (IM) therapy in a Phase III trial. Proc Am Soc Clin Oncol abstr 8

Dougher M, Terman BI (1999) Autophosphorylation of KDR in the kinase domain is required for maximal VEGF-stimulated kinase activity and receptor internalization. Oncogene 18:1619–1627

Drevs J, Muller-Driver R, Wittig C, Fuxius S, Esser N, Hugenschmidt H, Konerding MA, Allegrini PR, Wood J, Hennig J, Unger C, Marme D (2002) PTK787/ZK 222584, a specific vascular endothelial growth factor-receptor tyrosine kinase inhibitor, affects the anatomy of the tumor vascular bed and the functional vascular properties as detected by dynamic enhanced magnetic resonance imaging. Cancer Res 62:4015–4022

Drevs J, Esser N, Wedge SR, Ryan AJ, Oglivie DJ, Marme D (2004) Effect of AZD2171, a highly potent VEGF receptor tyrosine kinase inhibitor, on primary tumor growth, metastasis and vessel density in murine renal cell carcinoma. Proc Am Assoc Cancer Res 45:1051

Drevs J, Zirrgiebel U, Schmidt-Gersbach CI, Mross K, Medinger M, Lee L, Pinheiro J, Wood J, Thomas AL, Unger C, Henry A, Steward WP, Laurent D, Lebwohl D, Dugan M, Marme D (2005) Soluble markers for the assessment of biological activity with PTK787/ZK 222584 (PTK/ZK), a vascular endothelial growth factor receptor (VEGFR) tyrosine kinase inhibitor in patients with advanced colorectal cancer from two phase I trials. Ann Oncol 16:558–565

Drevs J, Siegert P, Medinger M, Strecker R, Zirrgiebel U, Harder J, Blum H, Robertson J, Jürgensmeier JM, Puchalski TA, Young H, Saunders O, Unger C (2007) Phase I clinical study of AZD2171, an oral, highly potent VEGF signaling inhibitor, in patients with advanced solid tumors. J Clin Oncol (in press)

Dvorak HF, Detmar M, Claffey KP, Nagy JA, van de WL, Senger DR (1995) Vascular permeability factor/vascular endothelial growth factor: an important mediator of angiogenesis in malignancy and inflammation. Int Arch Allergy Immunol 107:233–235

Ellis LM (2004) Preclinical data targeting vascular endothelial growth factor in colorectal cancer. Clin Colorectal Cancer 4 [Suppl 2]:S55–S61

Escudier B, Szczylik C, Eisen T, Stadler WM, Schwartz B, Shan M, Bukowski RM (2005) Randomized Phase III trial of the Raf kinase and VEGFR inhibitor sorafenib (BAY 43-9006) in patients with advanced renal cell carcinoma (RCC). Proc Am Soc Clin Oncol abstr 4510

Faivre S, Delbaldo C, Vera K, Robert C, Lozahic S, Lassau N, Bello C, Deprimo S, Brega N, massimini G, Armand JP, Scigalla P, Raymond E (2006) Safety, pharmacokinetic, and antitumor activity of SU11248, a novel oral multitarget tyrosine kinase inhibitor, in patients with cancer. J Clin Oncol 24:25–35

Fan F, Wey JS, McCarty MF, Belcheva A, Liu W, Bauer TW, Somcio RJ, Wu Y, Hooper A, Hicklin DJ, Ellis LM (2005) Expression and function of vascular endothelial growth factor receptor-1 on human colorectal cancer cells. Oncogene 24:2647–2653

Fargnoli J, Bhide R, Lombardo L, Hunt JT, Cai ZW, Barrish J, Galbraith S, Jeyaseelan R, Mortillo S, Kukral D, Wautlet B, Krishnan B, Lewin A, Henley B (2005) Preclinical studies of BMS-582664, an alanine prodrug of BMS-540215, a potent, dual inhibitor of VEGFR-2 and FGFR-1 kinases. Proc Am Assoc Cancer Res abstr 3033

Ferrara N (2002a) VEGF and the quest for tumour angiogenesis factors. Nat Rev Cancer 2:795–803

Ferrara N (2002b) Role of vascular endothelial growth factor in physiologic and pathologic angiogenesis: therapeutic implications. Semin Oncol 29:10–14

Ferrara N, Chen H, Davis-Smyth T, Gerber HP, Nguyen TN, Peers D, Chisholm V, Hillan KJ, Schwall RH (1998) Vascular endothelial growth factor is essential for corpus luteum angiogenesis. Nat Med 4:336–340

Fiedler W, Giaccone G, Lasch P, van der Horst I, Brega NM, Gentile A, Ljubimir V, Pithavala YK, Bokemeyer C, Boven E (2005) Phase I study of SU014813, a novel oral multitargeted receptor tyrosine kinase (RTK) inhibitor. Proc AACR-NCI-EORTC abstr B119

Fukumura D, Yuan F, Monsky WL, Chen Y, Jain RK (1997) Effect of host microenvironment on the microcirculation of human colon adenocarcinoma. Am J Pathol 151:679–688

Garces CA, Kurenova EV, Golubovskaya VM, Cance WG (2006) Vascular endothelial growth factor receptor-3 and focal adhesion kinase bind and suppress apoptosis in breast cancer cells. Cancer Res 66:1446–1454

Garton AJ, Crew AP, Franklin M, Cooke AR, Wynne GM, Castaldo L, Kahler J, Winski SL, Franks A, Brown EN, Bittner MA, Keily JF, Briner P, Hidden C, Srebernak MC, Pirrit C, O'Connor M, Chan A, Vulevic B, Henninger D,

Hart K, Sennello R, Li AH, Zhang T, Richardson F, Emerson DL, Castelhano AL, Arnold LD, Gibson NW (2006) OSI-930: a novel selective inhibitor of Kit and kinase insert domain receptor tyrosine kinases with antitumor activity in mouse xenograft models. Cancer Res 66:1015–1024

Gehling UM, Ergun S, Schumacher U, Wagener C, Pantel K, Otte M, Schuch G, Schafhausen P, Mende T, Kilic N, Kluge K, Schafer B, Hossfeld DK, Fiedler W (2000) In vitro differentiation of endothelial cells from AC133-positive progenitor cells. Blood 95:3106–3112

Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N (1999) VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat Med 5:623–628

Gille H, Kowalski J, Li B, LeCouter J, Moffat B, Zioncheck TF, Pelletier N, Ferrara N (2001) Analysis of biological effects and signaling properties of Flt-1 (VEGFR-1) and KDR (VEGFR-2). A reassessment using novel receptor-specific vascular endothelial growth factor mutants. J Biol Chem 276:3222–3230

Goldbeck C, Shephard LB, Louie SG, Lee SH, Lea A, Heise. C.C. (2005) Development of pharmacodynamic endpoint assays for use in clinical trials of CHIR-258, a multitargeted receptor tyrosine kinase inhibitor. Proc Am Assoc Cancer Res abstr 465

Goh BC, Thng CH, Sukri N, Wong CI, Soo R, Carr R, Lee SC, Chen CS, Humerickhouse R (2006) Phase I study of ABT869, a multiple receptor tyrosine kinase inhibitor, in patients with refractory solid malignancies. Eur J Cancer [Suppl] 4(12) abstr 108

Goodlad RA, Ryan AJ, Wedge SR, Pyrah IT, Alferez D, Poulsom R, Smith NR, Mandir N, Watkins AJ, Wilkinson RW (2006) Inhibiting vascular endothelial growth factor receptor-2 signaling reduces tumor burden in the Apc-Min/+ mouse model of early intestinal cancer. Carcinogenesis 10: 2133–2139

Haigh JJ, Gerber HP, Ferrara N, Wagner EF (2000) Conditional inactivation of VEGF-A in areas of collagen2a1 expression results in embryonic lethality in the heterozygous state. Development 127:1445–1453

Hall AP, Westwood FR, Wadsworth PF (2006) Review of the effects of anti-angiogenic compounds on the epiphyseal growth plate. Toxicol Pathol 34:131–147

Harari PM (2004) Epidermal growth factor receptor inhibition strategies in oncology. Endocr Relat Cancer 11:689–708

Hecht JR, Trarbach T, Jaeger E, Hainsworth J, Wolff R, Lloyd K, Bodoky G, Borner M, Laurent D, Jacques C (2005) A randomized, double-blind, placebo-controlled, phase III study in patients (Pts) with metastatic adenocarcinoma of the colon or rectum receiving first-line chemotherapy with oxaliplatin/5-fluorouracil/leucovorin and PTK787/ZK 222584 or placebo (CONFIRM-1). Proc Am Soc Clin Oncol abstr 3

Herbst R, Kurzrock R, Parson M, Benjamin R, Chen L, Ng C, Ingram M, Wong S, Chang D, Rosen L (2004) AMG 706 first in human, open-label, dose-finding study evaluating the safety and pharmacokinetics (PK) in subjects with advanced solid tumors. EJC Suppl 2:48

Heymach JV, Johnson BE, Rowbottom JA, Fidias P, Lu C, Prager D, Roubec J, Csada E, Dimery I, Herbst RS (2005) A randomized, placebo-controlled Phase II trial of ZD6474 plus docetaxel, in patients with NSCLC. Proc Am Soc Clin Oncol abstr 3023

Hilberg F, Tontsch-Grunt U, Colbatzky F, Heckel A, Lotz R, van Meel JCA, Roth GJ (2004) BIBF1120 a novel, small molecule triple angiokinase inhibitor: profiling as a clinical candidate for cancer therapy. EJC Suppl 2:50

Hillman NJ, Whittles CE, Pocock TM, Williams B, Bates DO (2001) Differential effects of vascular endothelial growth factor-C and placental growth factor-1 on the hydraulic conductivity of frog mesenteric capillaries. J Vasc Res 38:176–186

Holash J, Davis S, Papadopoulos N, Croll SD, Ho L, Russell M, Boland P, Leidich R, Hylton D, Burova E, Ioffe E, Huang T, Radziejewski C, Bailey K, Fandl JP, Daly T, Wiegand SJ, Yancopoulos GD, Rudge JS (2002) VEGF-Trap: a VEGF blocker with potent antitumor effects. Proc Natl Acad Sci USA 99:11393–11398

Holmqvist K, Cross MJ, Rolny C, Hagerkvist R, Rahimi N, Matsumoto T, Claesson-Welsh L, Welsh M (2004) The adaptor protein shb binds to tyrosine 1175 in vascular endothelial growth factor (VEGF) receptor-2 and regulates VEGF-dependent cellular migration. J Biol Chem 279:22267–22275

Horner A, Bishop NJ, Bord S, Beeton C, Kelsall AW, Coleman N, Compston JE (1999) Immunolocalisation of vascular endothelial growth factor (VEGF) in human neonatal growth plate cartilage. J Anat 194:519–524

Howdieshell TR, Callaway D, Webb WL, Gaines MD, Procter CD Jr, Sathyanarayana, Pollock JS, Brock TL, McNeil PL (2001) Antibody neutralization of vascular endothelial growth factor inhibits wound granulation tissue formation. J Surg Res 96:173–182

Hunt S (2001) Technology evaluation: IMC-1C11, ImClone Systems. Curr Opin Mol Ther 3:418–424

Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R, Kabbinavar F (2004) Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 350:2335–2342

Hurwitz H, Dowlati A, Savage S, Fernando N, Lasalvia S, Whitehead B, Suttle B, Collins D, Ho P, Pandite L (2005) Safety, tolerability and pharmacokinetics of oral administration of GW786034 in pts with solid tumors. Proc Am Soc Clin Oncol abstr 3012

Ichihara M, Murakumo Y, Takahashi M (2004) RET and neuroendocrine tumors. Cancer Lett 204:197–211

Inai T, Mancuso M, Hashizume H, Baffert F, Haskell A, Baluk P, Hu-Lowe DD, Shalinsky DR, Thurston G, Yancopoulos

GD, McDonald DM (2004) Inhibition of vascular endothelial growth factor (VEGF) signaling in cancer causes loss of endothelial fenestrations, regression of tumor vessels, and appearance of basement membrane ghosts. Am J Pathol 165:35–52

Jain RK (2003) Molecular regulation of vessel maturation. Nat Med 9:685–693

Jenny B, Harrison J, Baetens D, Tille JC, Burkhardt K, Mottaz H, Kiss J, Dietrich PY, De Tribolet N, Pizzolato G, Pepper M (2006) Expression and localization of VEGF-C and VEGFR-3 in glioblastomas and haemangioblastomas. J Pathol 209:34–43

Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, Saksela O, Kalkkinen N, Alitalo K (1996a) A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. EMBO J 15:1751

Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, Saksela O, Kalkkinen N, Alitalo K (1996b) A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. EMBO J 15:290–298

Jürgensmeier JM, Brave SR, James NH, Wedge SR, Oglivie DJ, Barry ST (2005) AZD2171, a highly potent inhibitor of VEGF receptor signaling, has additional activity versus the c-Kit receptor tyrosine kinase. Proc Am Assoc Cancer Res 46 abstr 5834

Karkkainen MJ, Haiko P, Sainio K, Partanen J, Taipale J, Petrova TV, Jeltsch M, Jackson DG, Talikka M, Rauvala H, Betsholtz C, Alitalo K (2004) Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. Nat Immunol 5:74–80

Kendall RL, Rutledge RZ, Mao X, Tebben AJ, Hungate RW, Thomas KA (1999) Vascular endothelial growth factor receptor KDR tyrosine kinase activity is increased by autophosphorylation of two activation loop tyrosine residues. J Biol Chem 274:6453–6460

Kiessling F, Farhan N, Lichy MP, Vosseler S, Heilmann M, Krix M, Bohlen P, Miller DW, Mueller MM, Semmler W, Fusenig NE, Delorme S (2004) Dynamic contrast-enhanced magnetic resonance imaging rapidly indicates vessel regression in human squamous cell carcinomas grown in nude mice caused by VEGF receptor 2 blockade with DC101. Neoplasia 6:213–223

Kim ES, Serur A, Huang J, Manley CA, McCrudden KW, Frischer JS, Soffer SZ, Ring L, New T, Zabski S, Rudge JS, Holash J, Yancopoulos GD, Kandel JJ, Yamashiro DJ (2002) Potent VEGF blockade causes regression of coopted vessels in a model of neuroblastoma. Proc Natl Acad Sci USA 99:11399–11404

Kimura ET, Nikiforova MN, Zhu Z, Knauf JA, Nikiforov YE, Fagin JA (2003) High prevalence of BRAF mutations in thyroid cancer: genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signaling pathway in papillary thyroid carcinoma. Cancer Res 63:1454–1457

Klinowska TC, Jackson JA, Farrington PM, Smith DS, Barnett S, Whittaker L, Bigley AL, Wadsworth PF, Oglivie DJ, Wedge SR (2004) AZD2171, a highly potent inhibitor of VEGF receptor tyrosine kinase activity, inhibits the growth of spontaneous mammary tumors in the MMTV-neu transgenic mouse. Proc Am Assoc Cancer Res 45:1048

Kumar R, Harrington LE, Hopper TM, Miller CG, Onori JA, Cheung M, Stafford JA, Epperly AH, Gilmer TM (2005) Correlation of anti-tumor and anti-angiogenic activity of VEGFR inhibitors with inhibition of VEGFR2 phosphorylation in mice. Proc Am Soc Clin Oncol abstr 9537

Langer CJ, Natale RB (2005) The emerging role of vascular endothelial growth factor receptor tyrosine kinase inhibitors. Semin Oncol 32:S23–S29

Laughner E, Taghavi P, Chiles K, Mahon PC, Semenza GL (2001) HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol Cell Biol 21:3995–4004

Lee SH, Lopes dM, Vora J, Harris A, Ye H, Nordahl L, Garrett E, Samara E, Aukerman SL, Gelb AB, Heise C (2005) In vivo target modulation and biological activity of CHIR-258, a multitargeted growth factor receptor kinase inhibitor, in colon cancer models. Clin Cancer Res 11:3633–3641

Liu W, Reinmuth N, Stoeltzing O, Parikh AA, Fan F, Ahmad SA, Jung YD, Ellis LM (2002) Antiangiogenic therapy targeting factors that enhance endothelial cell survival. Semin Oncol 29:96–103

Liu G, Rugo HS, Wilding G, McShane TM, Evelhoch JL, Ng C, Jackson E, Kelcz F, Yeh BM, Lee FT Jr, Charnsangavej C, Park JW, Ashton EA, Steinfeldt HM, Pithavala YK, Reich SD, Herbst RS (2005) Dynamic contrast-enhanced magnetic resonance imaging as a pharmacodynamic measure of response after acute dosing of AG-013736, an oral angiogenesis inhibitor, in patients with advanced solid tumors: results from a Phase I study. J Clin Oncol 23:5464–5473

Mamluk R, Storek MJ, Gokemeijer J, Bates JM, Morse BM, Robinson JJ, Gosselin ML, Harris AS (2006) CT-322: a novel protein therapeutic with favorable pharmacokinetics and biodistribution. Proc Am Assoc Cancer Res 47 abstr 230

Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S (2002) The protein kinase complement of the human genome. Science 298:1912–1934

Martinelli E, Takimoto CH, van Oosterom AT, Tabernero J, Rowinsky E, Schoffski P, Huang J, Casado E, Mita A, Dumez H (2005) AEE788, a novel multitargeted inhibitor of ErbB and VEGF receptor family tyrosine kinases: preliminary phase 1 results. Proc Am Soc Clin Oncol abstr 3039

Matsui J, Obaishi H, Watanabe T, Yamaguchi A, Suzuki S, Yamamoto Y, Nakamura K, Nomoto K, Mimura F, Funahashi Y, Matsushima T, Miyazaki K, Haneda T, Fukuda Y,

Kamata J, Takahashi K, Matsukura M, Tsuruoka, Asada M, Yoshimatsu K (2003) E7080(ER-203492-00), a novel VEGF receptor tyrosine kinase inhibitor-I. Characterisation a an angiogenesis inhibitor. Proc Am Assoc Cancer Res abstr 51

Matsumoto T, Bohman S, Dixelius J, Berge T, Dimberg A, Magnusson P, Wang L, Wikner C, Qi JH, Wernstedt C, Wu J, Bruheim S, Mugishima H, Mukhopadhyay D, Spurkland A, Claesson-Welsh L (2005) VEGF receptor-2 Y951 signaling and a role for the adapter molecule TSAd in tumor angiogenesis. EMBO J 24:2342–2353

Mayr-Wohlfart U, Waltenberger J, Hausser H, Kessler S, Gunther KP, Dehio C, Puhl W, Brenner RE (2002) Vascular endothelial growth factor stimulates chemotactic migration of primary human osteoblasts. Bone 30:472–477

McTigue MA, Wickersham JA, Pinko C, Showalter RE, Parast CV, Tempczyk-Russell A, Gehring MR, Mroczkowski B, Kan CC, Villafranca JE, Appelt K (1999) Crystal structure of the kinase domain of human vascular endothelial growth factor receptor 2: a key enzyme in angiogenesis. Structure 7:319–330

Mendel DB, Laird AD, Xin X, Louie SG, Christensen JG, Li G, Schreck RE, Abrams TJ, Ngai TJ, Lee LB, Murray LJ, Carver J, Chan E, Moss KG, Haznedar JO, Sukbuntherng J, Blake RA, Sun L, Tang C, Miller T, Shirazian S, McMahon G, Cherrington JM (2003) In vivo antitumor activity of SU11248, a novel tyrosine kinase inhibitor targeting vascular endothelial growth factor and platelet-derived growth factor receptors: determination of a pharmacokinetic/pharmacodynamic relationship. Clin Cancer Res 9:327–337

Meyer M, Clauss M, Lepple-Wienhues A, Waltenberger J, Augustin HG, Ziche M, Lanz C, Buttner M, Rziha HJ, Dehio C (1999) A novel vascular endothelial growth factor encoded by Orf virus, VEGF-E, mediates angiogenesis via signalling through VEGFR-2 (KDR) but not VEGFR-1 (Flt-1) receptor tyrosine kinases. EMBO J 18:363–374

Miller KD, Wang M, Gralow J, Dickler M, Cobleigh MA, Perez EA, Shenkler TN, Davidson NE (2005) A randomized Phase III trial of paclitaxel versus paclitaxel plus bevacizumab as first-line therapy for locally recurrent or metastatic breast cancer. ASCO Oral presentation: no abstract available

Miller KD, Miller M, Mehrotra S, Agarwal B, Mock BH, Zheng QH, Badve S, Hutchins GD, Sledge GW Jr (2006) A physiologic imaging pilot study of breast cancer treated with AZD2171. Clin Cancer Res 12:281–288

Minami H, Ebi H, Tahara M, Sasaki Y, Yamamoto N, Yamada Y, Tamura T, Saijo N (2003) A Phase I study of an oral VEGF receptor tyrosine kinase inhibitor ZD6474, in Japanese patients with solid tumors. Proc Am Soc Clin Oncol 194

Morgan B, Thomas AL, Drevs J, Hennig J, Buchert M, Jivan A, Horsfield KM, Ball HA, Lee L, Mietlowski W, Fuxius S, Unger C, O'Byrne KJ, Henry A, Cherryman GR, Leurent D, Dugan M, Marme D, Steward WP (2003) Dynamic contrast-enhanced magnetic resonance imaging as a biomarker for the pharmacological response of PTK787/ZK222584, an inhibitor of the vascular endothelial growth factor receptor tyrokine kinases, in patients with advanced colorectal cancer and liver metastases: results from two Phase I studies. J Clin Oncol 21:3955–3964

Motzer RJ, Michaelson MD, Redman BG, Hudes GR, Wilding G, Figlin RA, Ginsberg MS, Kim ST, Baum CM, DePrimo SE, Li JZ, Bello CL, Theuer CP, George DJ, Rini BI (2006) Activity of SU11248, a multitargeted inhibitor of vascular endothelial growth factor receptor and platelet-derived growth factor receptor, in patients with metastatic renal cell carcinoma. J Clin Oncol 24:16–24

Mross K, Baas F, Medinger M, Drevs J, Strecker R, Hennig J, Wolters M, Stehle G, Stefanic M (2004) A clinical phase I dose escalation, pharmacokinetic (PK) and pharmacodynamic (PD) study of BIBF 1120 in advanced cancer patients. EJC Suppl 2:44

Mross KB, Gmehling D, Frost A, Baas F, Strecker R, Hennig J, Stopfer P, Stefanic M, Stehle G, de Rossi L (2005) A clinical phase I, pharmacokinetic (PK), and pharmacodynamic study of twice daily BIBF 1120 in advanced cancer patients. Proc Am Soc Clin Oncol abstr 3031

Muller WJ, Sinn E, Pattengale PK, Wallace R, Leder P (1988) Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. Cell 54:105–115

Musallam L, Ethier C, Haddad PS, Bilodeau M (2001) Role of EGF receptor tyrosine kinase activity in antiapoptotic effect of EGF on mouse hepatocytes. Am J Physiol Gastrointest Liver Physiol 280:G1360–G1369

Nakagawa M, Kaneda T, Arakawa T, Morita S, Sato T, Yomada T, Hanada K, Kumegawa M, Hakeda Y (2000) Vascular endothelial growth factor (VEGF) directly enhances osteoclastic bone resorption and survival of mature osteoclasts. FEBS Lett 473:161–164

Nakamura K, Taguchi E, Miura T, Yamamoto A, Takahashi K, Bichat F, Guilbaud N, Hasegawa K, Kubo K, Fujiwara Y, Suzuki R, Kubo K, Shibuya M, Isoe T (2006) KRN951, a highly potent inhibitor of vascular endothelial growth factor receptor tyrosine kinases, has antitumour activities and affects functional vascular properties. Cancer Res 66: 9134–9142

Natale R, Bodkin D, Govindan R, Sleckman B, Rizvi N, Capo A, Germonpre P, Dimmery I, Webster A, Ranson M (2005) A comparison of the antitumour efficacy of ZD6474 and gefitinib (Iressa) in patients with NSCLC: results of a randomized, double-blind Phase II study. Lung Cancer 49 [Suppl 2]:S37

O'Farrell AM, Abrams TJ, Yuen HA, Ngai TJ, Louie SG, Yee KW, Wong LM, Hong W, Lee LB, Town A, Smolich BD, Manning WC, Murray LJ, Heinrich MC, Cherrington JM (2003) SU11248 is a novel FLT3 tyrosine kinase inhibitor with potent activity in vitro and in vivo. Blood 101:3597–3605

Onogawa S, Kitadai Y, Tanaka S, Kuwai T, Kimura S, Chayama K (2004) Expression of VEGF-C and VEGF-D at the invasive edge correlates with lymph node metastasis and prognosis of patients with colorectal carcinoma. Cancer Sci 95:32–39

Parast CV, Mroczkowski B, Pinko C, Misialek S, Khambatta G, Appelt K (1998) Characterization and kinetic mechanism of catalytic domain of human vascular endothelial growth factor receptor-2 tyrosine kinase (VEGFR2 TK), a key enzyme in angiogenesis. Biochemistry 37:16788–16801

Patyna S, Laird AD, Mendel DB, O'Farrell A-M, Liang C, Guan H, Vojkovsky T, Vasile S, Wang X, Chen J, Grazzini M, Yang CY, Haznedar JÖ, Sukbuntherng J, Zhong W-Z, Cherrington JM, Hu-Lowe D (2006) SU14813: a novel multiple tyrosine kinase inhibitor with potent antiangiogenic and antitumor activity. Mol Cancer Ther 5:1774–1782

Petrova TV, Makinen T, Alitalo K (1999) Signaling via vascular endothelial growth factor receptors. Exp Cell Res 253:117–130

Pili P, Carducci M, Robertson P, Brown P, Russel L, Hurwitz H (2003) A phase I study of the pan-VEGFR tyrosine kinase inhibitor, CEP-7055, in patients with advanced malignancy. Proc Am Soc Clin Oncol abstr 831

Plate KH, Breier G, Weich HA, Mennel HD, Risau W (1994) Vascular endothelial growth factor and glioma angiogenesis: coordinate induction of VEGF receptors, distribution of VEGF protein and possible in vivo regulatory mechanisms. Int J Cancer 59:520–529

Plendl J (2000) Angiogenesis and vascular regression in the ovary. Anat Histol Embryol 29:257–266

Polverino A, Coxon A, Starnes C, Diaz Z, DeMelfi T, Wang L, Bready J, Estrada J, Cattley R, Kaufman S, Chen D, Gan Y, Kumar G, Meyer J, Neervannan S, Alva G, Talvenheimo J, Montestruque S, Tasker A, Patel V, Radinsky R, Kendall R (2006) AMG 706, an oral multikinase inhibitor that selectively targets vascular endothelial growth factor, platelet-derived growth factor, and Kit receptors, potently inhibits angiogenesis and induces regression in tumour xenografts. Cancer Res 66:8715–8721

Rahimi N, Dayanir V, Lashkari K (2000) Receptor chimeras indicate that the vascular endothelial growth factor receptor-1 (VEGFR-1) modulates mitogenic activity of VEGFR-2 in endothelial cells. J Biol Chem 275:16986–16992

Rajagopalan H, Bardelli A, Lengauer C, Kinzler KW, Vogelstein B, Velculescu VE (2002) Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. Nature 418:934

Reardon D, Cloughesy T, Conrad C, Prados M, Xia J, Mietlowski W, Dugan M, Mischel P, Friedman H, Yung A (2005) A phase I study of AEE788, a novel multitargeted inhibitor of ErbB and VEGF receptor family tyrosine kinases, in recurrent GBM patients. Proc Am Soc Clin Oncol abstr 3063

Rini BI, Small EJ (2005) Biology and clinical development of vascular endothelial growth factor-targeted therapy in renal cell carcinoma. J Clin Oncol 23:1028–1043

Roberts N, Kloos B, Cassella M, Podgrabinska S, Persaud K, Wu Y, Pytowski B, Skobe M (2006) Inhibition of VEGFR-3 activation with the antagonistic antibody more potently suppresses lymph node and distant metastases than inactivation of VEGFR-2. Cancer Res 66:2650–2657

Rosen L, Kurzrock R, Jackson E, Wathen L, Parson M, Eschenberg M, Mulay M, Purdom M, Yan L, Herbst RS (2005) Safety and pharmacokinetics of AMG 706 in patients with advanced solid tumors. J Clin Oncol (meeting abstracts) 23:3013

Ruggeri B, Singh J, Gingrich D, Angeles T, Albom M, Chang H, Robinson C, Hunter K, Dobrzanski P, Jones-Bolin S, Aimone L, Klein-Szanto A, Herbert JM, Bono F, Schaeffer P, Casellas P, Bourie B, Pili R, Isaacs J, Ator M, Hudkins R, Vaught J, Mallamo J, Dionne C (2003) CEP-7055: a novel, orally active pan inhibitor of vascular endothelial growth factor receptor tyrosine kinases with potent antiangiogenic activity and antitumor efficacy in preclinical models. Cancer Res 63:5978–5991

Rugo HS, Herbst RS, Liu G, Park JW, Kies MS, Steinfeldt HM, Pithavala YK, Reich SD, Freddo JL, Wilding G (2005) Phase I trial of the oral antiangiogenesis agent AG-013736 in patients with advanced solid tumors: pharmacokinetic and clinical results. J Clin Oncol 23:5474–5483

Ryan AM, Eppler DB, Hagler KE, Bruner RH, Thomford PJ, Hall RL, Shopp GM, O'Neill CA (1999) Preclinical safety evaluation of rhuMAbVEGF, an antiangiogenic humanized monoclonal antibody. Toxicol Pathol 27:78–86

Sakurai Y, Ohgimoto K, Kataoka Y, Yoshida N, Shibuya M (2005) Essential role of Flk-1 (VEGF receptor 2) tyrosine residue 1173 in vasculogenesis in mice. Proc Natl Acad Sci USA 102:1076–1081

Sandler AB, Gray R, Brahmer J, Dowlati A, Schiller JH, Perry MC, Johnson DH (2005) Randomized phase II/III trial of paclitaxel (P) plus carboplatin (C) with or without bevacizumab (NSC # 704865) in patients with advanced nonsquamous non-small cell lung cancer (NSCLC): an Eastern Cooperative Oncology Group (ECOG) Trial – E4599. Proc Am Soc Clin Oncol abstr LBA4

Sarker D, Evans TRJ, Hardie M, Molife R, Marriott C, Morrison R, Fox JA, Heise C, Xu X, Louie S, Aziz N, Garzon F, Skouteris G, Michelson G, Judson I, Jadayel D, Scott K, Liu W, DeBono JS (2006) CHIR-258: preliminary data from a first in human phase 1 dose escalating trial of an oral, selectively targeted receptor tyrosine kinase inhibitor in patients with solid tumours. Proc Am Soc Clin Oncol abstr 3043

Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 376:62–66

Shida A, Fujioka S, Kobayashi K, Ishibashi Y, Nimura H, Mitsumori N, Yanaga K (2006) Expression of vascular endothelial growth factor (VEGF)-C and -D in gastric carcinoma. Int J Clin Oncol 11:38–43

Shimizu K, Kubo H, Yamaguchi K, Kawashima K, Ueda Y, Matsuo K, Awane M, Shimahara Y, Takabayashi A, Yamaoka Y, Satoh S (2004) Suppression of VEGFR-3 signaling inhibits lymph node metastasis in gastric cancer. Cancer Sci 95:328–333

Shweiki D, Neeman M, Itin A, Keshet E (1995) Induction of vascular endothelial growth factor expression by hypoxia and by glucose deficiency in multicell spheroids: implications for tumor angiogenesis. Proc Natl Acad Sci USA 92:768–772

Siemeister G, Briem H, Brumby T, Jautelat R, Krueger M, Luecking U, Reichel A, Schaefer M, Mumberg D, Bosslet K (2005) ZK-304709, the oral Multitarget Tumor Growth InhibitorTM, acts via inhibition of cell cycle progression and tumor-induced angiogenesis. Proc Am Assoc Cancer Res abstr 5842

Strumberg D, Richly H, Hilger RA, Schleucher N, Korfee S, Tewes M, Faghih M, Brendel E, Voliotis D, Haase CG, Schwartz B, Awada A, Voigtmann R, Scheulen ME, Seeber S (2005) Phase I clinical and pharmacokinetic study of the Novel Raf kinase and vascular endothelial growth factor receptor inhibitor BAY 43-9006 in patients with advanced refractory solid tumors. J Clin Oncol 23:965–972

Strumberg D, Christensen O, Strecker R, Scheulen ME, Frost A, Rajagopalan P, Voliotis D, Zuehlsdorf M, Wensing G, Mross K (2006) Clinical and biomarker responses in a phase I study of BAY 57 9352 – a VEGFR-2 inhibitor – administered as continuous dosing in patients with advanced solid tumours. Eur J Cancer [Suppl] 4(12) abstr 41

Suggitt M, Bibby MC (2005) 50 years of preclinical anticancer drug screening: empirical to target-driven approaches. Clin Cancer Res 11:971–981

Suttle AB, Hurwitz H, Dowlati A, Fernando N, Savage S, Coviello K, Dar M, Ertel P, Whitehead B, Pandite L (2004) Pharmacokinetics (PK) and tolerability of GW786034, a VEGFR tyrosine kinase inhibitor, after daily oral administration to patients with solid tumors. Proc Am Soc Clin Oncol abstr 3054

Takahashi T, Yamaguchi S, Chida K, Shibuya M (2001) A single autophosphorylation site on KDR/Flk-1 is essential for VEGF-A-dependent activation of PLC-gamma and DNA synthesis in vascular endothelial cells. EMBO J 20:2768–2778

Takeda M, Arao T, Yokote H, Sakai K, Kimura H, Yanagihara K, Sasaki H, Tamura T, Saijo N, Nishio K (2006) AZD2171 shows potent anti-tumor activity against gastric cancer expressing variant K-SAM/FGFR2. Proc Am Assoc Cancer Res 47 abstr 3785

Thomas AL, Morgan B, Horsfield MA, Higginson A, Kay A, Lee L, Masson E, Puccio-Pick M, Laurent D, Steward WP (2005) Phase I study of the safety, tolerability, pharmacokinetics, and pharmacodynamics of PTK787/ZK 222584 administered twice daily in patients with advanced cancer. J Clin Oncol 23:4162–4171

Tolcher AW, O'Leary JJ, DeBono JS, Caulkins JD, Molpus K, Sutula K, Ferrante KJ, Gualberto A, Noe DA, Huberman M, Healey D (2002) A phase I and biologic correlative study of an oral vascular endothelial growth factor receptor-2 (VEGFR-2) tyrosine kinase inhibitor, CP-547,632, in patients (pts) with advanced solid tumors. Proc Am Soc Clin Oncol abstr 334

Traxler P, Allegrini PR, Brandt R, Brueggen J, Cozens R, Fabbro D, Grosios K, Lane HA, McSheehy P, Mestan J, Meyer T, Tang C, Wartmann M, Wood J, Caravatti G (2004) AEE788: a dual family epidermal growth factor receptor/ErbB2 and vascular endothelial growth factor receptor tyrosine kinase inhibitor with antitumor and antiangiogenic activity. Cancer Res 64:4931–4941

Valtola R, Salven P, Heikkila P, Taipale J, Joensuu H, Rehn M, Pihlajaniemi T, Weich H, deWaal R, Alitalo K (1999) VEGFR-3 and its ligand VEGF-C are associated with angiogenesis in breast cancer. Am J Pathol 154:1381–1390

Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M, Heldin CH (1994) Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. J Biol Chem 269:26988–26995

Wedge SR, Ogilvie DJ, Dukes M, Kendrew J, Curwen JO, Hennequin LF, Thomas AP, Stokes ES, Curry B, Richmond GH, Wadsworth PF (2000) ZD4190: an orally active inhibitor of vascular endothelial growth factor signaling with broad-spectrum antitumor efficacy. Cancer Res 60:970–975

Wedge SR, Ogilvie DJ, Dukes M, Kendrew J, Chester R, Jackson JA, Boffey SJ, Valentine PJ, Curwen JO, Musgrove HL, Graham GA, Hughes GD, Thomas AP, Stokes ES, Curry B, Richmond GH, Wadsworth PF, Bigley AL, Hennequin LF (2002) ZD6474 inhibits vascular endothelial growth factor signaling, angiogenesis, and tumor growth following oral administration. Cancer Res 62:4645–4655

Wedge S, Kendrew J, Valentine PJ, Brave SR, Barnett S, Jürgensmeier JM, Hennequin LF, Oglivie DJ (2004) AZD2171, a potent VEGF receptor tyrosine kinase inhibitor, combined with mechanistically distinct antitumor therapies in vivo. EJC Suppl 2:52

Wedge SR, Kendrew J, Hennequin LF, Valentine PJ, Barry ST, Brave SR, Smith NR, James NH, Dukes M, Curwen JO, Chester R, Jackson JA, Boffey SJ, Kilburn LL, Barnett S, Richmond GH, Wadsworth PF, Walker M, Bigley AL, Taylor ST, Cooper L, Beck S, Jürgensmeier JM, Ogilvie DJ (2005) AZD2171: a highly potent, orally bioavailable, vascular endothelial growth factor receptor-2 tyrosine kinase inhibitor for the treatment of cancer. Cancer Res 65:4389–4400

Wells S, You YN, Lakhani V, Hou J, Langmuir P, Headley D, Skinner M, Morse M, Burch W, Schlumberger M (2006) A Phase II trial of ZD6474 in patients with hereditary metastatic medullary thyroid cancer. Proc Am Soc Clin Oncol abstr 5533

Wickman G, Hallin M, Dillon R, Amundson K, Acena A, Grazzini M, Hermann M, Vekich S, McTigue M, Kania R, Bender S, Shalinsky DR, Hu-Lowe DD (2003) Further characterisation of the potent VEGF/PDGT receptor-tyrosine

kinase inhibitor, AG013736, in preclinical tumour models for its antiangiogenesis and antitumour activity. Proc Am Assoc Cancer Res abstr 3780

Wilhelm SM, Carter C, Tang L, Wilkie D, McNabola A, Rong H, Chen C, Zhang X, Vincent P, McHugh M, Cao Y, Shujath J, Gawlak S, Eveleigh D, Rowley B, Liu L, Adnane L, Lynch M, Auclair D, Taylor I, Gedrich R, Voznesensky A, Riedl B, Post LE, Bollag G, Trail PA (2004) BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. Cancer Res 64:7099-7109

Wood JM, Bold G, Buchdunger E, Cozens R, Ferrari S, Frei J, Hofmann F, Mestan J, Mett H, O'Reilly T, Persohn E, Rosel J, Schnell C, Stover D, Theuer A, Towbin H, Wenger F, Woods-Cook K, Menrad A, Siemeister G, Schirner M, Thierauch KH, Schneider MR, Drevs J, Martiny-Baron G, Totzke F (2000) PTK787/ZK 222584, a novel and potent inhibitor of vascular endothelial growth factor receptor tyrosine kinases, impairs vascular endothelial growth factor-induced responses and tumor growth after oral administration. Cancer Res 60:2178-2189

Woodburn JR (1999) The epidermal growth factor receptor and its inhibition in cancer therapy. Pharmacol Ther 82:241-250

Wu W, Fujitaka K, Mandal J, Imagumbai T, Ryan A, Jürgensmeier JM, Fidler IJ, O'Reilly MS, Herbst RS (2005) AZD2171, an oral, highly potent VEGFR signaling inhibitor, in combination with gefitinib or paclitaxel: results of a study in an orthotopic human lung adenocarcinoma model. Clin Cancer Res 11 abstr B7

Yamagishi S, Yonekura H, Yamamoto Y, Fujimori H, Sakurai S, Tanaka N, Yamamoto H (1999) Vascular endothelial growth factor acts as a pericyte mitogen under hypoxic conditions. Lab Invest 79:501-509

Zeng H, Sanyal S, Mukhopadhyay D (2001) Tyrosine residues 951 and 1059 of vascular endothelial growth factor receptor-2 (KDR) are essential for vascular permeability factor/vascular endothelial growth factor-induced endothelium migration and proliferation, respectively. J Biol Chem 276:32714-32719

Zhou YJ, Xiong YX, Wu XT, Shi D, Fan W, Zhou T, Li YC, Huang X (2004) Inactivation of PTEN is associated with increased angiogenesis and VEGF overexpression in gastric cancer. World J Gastroenterol 10:3225-3229

Zimmermann RC, Xiao E, Bohlen P, Ferin M (2002) Administration of antivascular endothelial growth factor receptor 2 antibody in the early follicular phase delays follicular selection and development in the rhesus monkey. Endocrinology 143:2496-2502

Vascular Endothelial Growth Factor Receptor Antibodies for Anti-Angiogenic Therapy

24

PETER BOHLEN, ZHENPING ZHU, DANIEL J. HICKLIN

Abstract

Vascular endothelial cell growth factors (VEGFs) and their receptors are key molecules in the development and maintenance of the vascular and lymphatic systems in mammals. Inappropriate regulation of vascular growth is associated with various pathological states, especially with tumor growth. As cancer growth is supported in large part by excessive tumor-induced vascularization, interference with VEGF signaling has emerged as an important anti-angiogenic strategy to combat cancer. Here we review the state of the art with regard to one powerful such approach – the efficient blockage of VEGF receptor function with fully human monoclonal antibodies. There are three types of VEGF receptors: VEGFR2 and VEGFR3, which are expressed highly selectively on vascular and lymphatic endothelial cells, respectively, and VEGFR1, which is expressed in many cell types, including endothelial cells, inflammatory cells, and many tumor cells. Antibodies against each of these receptors can interfere with VEGF/VEGF receptor interactions in a highly receptor-specific manner, which prevents VEGF-induced signaling in VEGF receptor-positive cells, and results in impairment of essential functions of endothelial and other cells that support tumor growth and, ultimately, by tumor growth inhibition. The mechanisms of action of these antibodies differ widely, reflecting the unique distribution and biological roles of each of the VEGF receptors. There is abundant preclinical evidence that antibody-mediated VEGF receptor blockage can cause powerful inhibition of tumor growth in animals. However, as tumor growth control is achieved by mechanisms that are primarily cytostatic, cessation of treatment causes tumor re-growth. A preferred treatment modality is, therefore, to combine antibody treatment with cytotoxic (chemo- or radiation) therapy. Various forms of such combination therapy have been successful in treating many types of experimental tumors, even under conditions when single-agent treatments are ineffective. Anti-VEGFR1 and -R2 antibodies are currently being investigated as cancer therapeutics in clinical trials. In view of the potential therapeutic usefulness of these antibodies we also discuss possible advantages and disadvantages of anti-VEGFR antibodies and other approaches of VEGF signaling inhibition [antibodies against VEGF (Avastin®), small-molecule kinase inhibitors] with respect to differential efficacy and adverse effect profiles.

CONTENTS

Introduction: The VEGF/VEGFR System

The vascular endothelial growth factor (VEGF) family of endothelial growth factors consists of at least 11 structurally related glycoproteins that are derived from six genes (VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, PlGF) by expression of alternatively spliced mRNA (see recent reviews by Ferrara 2004; Hicklin and Ellis 2005). The VEGFs are variously produced by many different cell types. They bind to and activate three types of VEGF receptors [VEGFR1 (flt-1), VEGFR2 (flk-1/KDR), and VEGFR3 (flt-4)] in a selective but redundant manner. The binding relationship between ligands and receptors is shown in Fig. 24.1. VEGF receptors are structurally related transmembrane proteins that are comprised of seven extracellular immunoglobulin-like domains and an intracellular tyrosine kinase domain. Like other tyrosine kinase receptors, they undergo dimerization upon binding of ligand to their extracellular domain. Receptor dimerization allows the intracellular kinase domains to come into close proximity, such that in their dimeric state the kinase domains will catalyze the cross-phosphorylation of multiple tyrosine residues on each other's intracellular portions. Various specialized proteins can then bind to these phosphorylated sites and enable the process of signal transduction from the outside of the cell (ligand binding) to the nucleus (DNA synthesis, activation of various cell functions, including proliferation). In addition, two other transmembrane proteins, neuropilin-1 and -2, are thought to serve as ligand-selective co-receptors for VEGFR1, VEGFR2, and VEGFR3, respectively. The VEGF receptors are primarily expressed in endothelial cells of the vasculature and the lymphatic systems, and their functions are still poorly understood.

In mammals, the VEGFs and their receptors have long been recognized as crucial components of regulatory systems that govern the formation and maintenance of blood (VEGFR1, VEGFR2, VEGFR3) and lymphatic (VEGFR3) vessels under physiological and pathological conditions, including cancer. Rapid tumor growth is dependent on the formation of a sustaining vasculature, and the VEGF/VEGFR systems are key to achieve this. Most tumor cells express and secrete high levels of VEGF, and they also produce enzymes (e.g., metalloproteinases, heparanase) that liberate VEGFs and certain other heparan sulfate-bound endothelial mitogens (e.g., FGFs) from the extracellular matrix of tissues where much of these highly active heparin-binding growth factors are stored in sequestered, inactive form. VEGFs, upon binding to VEGF receptors, activate endothelial cells such that a cellular program is set in motion to support the overall process of new blood vessel formation and maturation. As a result, endothelial cells divide rapidly in the vicinity of tumors, and migrate to organize into vascular tubes leading to rapid vascularization of tumor tissue that can sustain growth of that tissue. Activation of endothelial cells also manifests itself by (a) modulation of the expression of proteins that help to promote endothelial motility (e.g., cytoskeleton components), migration and invasion (e.g., proteases); (b) upregulation of survival mechanisms; and (c) increased receptiv-

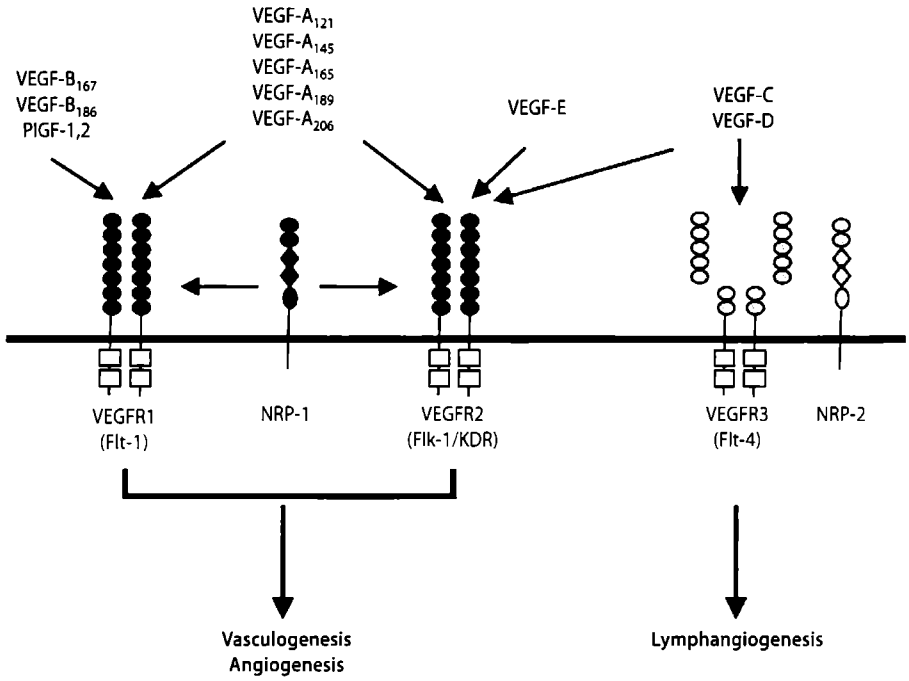


Fig. 24.1. Binding relationships between VEGFs and VEGF receptors. *VEGF*, vascular endothelial growth factor; *PlGF*, placenta growth factor; *VEGFR*, VEGF receptor; *NRP*, neuropilin. Reproduced from Hicklin and Ellis (2005), with permission from the American Society of Clinical Oncology

ity to additional activation signals (e.g., various cytokine receptors) that further help the cells to adopt to rapid expansion. Additionally, some VEGFs potently induce vascular permeability, thus aiding in providing an "inflammatory" environment conducive to the stimulation of tumor cell proliferation. While tumor cell-induced endothelial activation is not only driven by VEGF, this growth factor is a dominant player in the process, as demonstrated by the obligatory role in vasculogenesis during development and the profound effects that inhibitors of VEGF signaling have on angiogenesis.

Given the prominent biological relevance of the VEGF/VEGFR pathways for tumor angiogenesis and tumor-associated lymphangiogenesis, it is not surprising that inhibition of VEGF signaling for therapeutic purposes has attracted major interest. As a result, many ways to block tumor angiogenesis via interference with VEGF signaling in endothelial cells have been developed. The best-studied approaches include (a) neutralization of VEGF ligand with antibodies or soluble receptors (VEGF trap), (b) inhibition of the kinase function of VEGF receptors using small-molecule kinase inhibitors, and (c) inhibition of receptor function with antibodies that bind with high affinity to the ligand-binding sites of the VEGF receptors and thereby competitively prevent receptor activation and ligand-induced signaling. Each of these approaches has unique features that may be relevant to the success of therapy (e.g., specificity, toxicity). The concept of angiogenesis inhibition as a therapy for the treatment of tumors has been validated with the regulatory approval of an anti-VEGF antibody for the treatment of colorectal cancer [Avastin® (bevacizumab)]. Drugs involving other approaches are in various stages of clinical development, and it remains to be seen how the distinguishing features of such drugs will translate into distinct differences in efficacy and/or safety. The drug industry has so far focused largely on blocking tumor angiogenesis by inhibition of VEGFR2 signaling. Evidence suggests, however, that inhibition of VEGF-induced signaling through other VEGF receptors as well may have therapeutic potential.

The concept that function-blocking antibodies can be deployed as highly effective cancer therapeu-

tics has been validated with the regulatory approval of the monoclonal antibodies Herceptin®, (trastuzumab), Erbitux® (cetuximab), and bevacizumab, which inhibit the functions of the Her-2 receptor, the EGF receptor, and the VEGF-A ligand, respectively. These successes have spawned increased efforts to develop additional antibodies to block the function of receptors essential in various stages of the malignant process, among them the VEGF receptors. Here we review the current research and development status of function blocking anti-VEGFR antibodies.

Anti-VEGFR2 Antibodies

24.2.1 Anti-murine VEGFR2 Antibodies: Proof-of-Concept Studies

24.2.1.1 Inhibition of Tumor Angiogenesis, Tumor Growth and Metastasis

The concept of targeting VEGFR2 was first pursued by scientists at ImClone Systems, who created an anti-VEGFR2 function-blocking monoclonal antibody by immunizing rats with a recombinant protein corresponding to the extracellular domain of murine VEGFR2 (soluble VEGFR2). Using standard hybridoma technology, antibodies were identified that competed with the binding of soluble VEGFR2 to VEGF immobilized to ELISA plates (Rockwell et al. 1995). One of the resulting monoclonal antibodies, DC101, was shown to possess potent in vivo anti-angiogenic activity in various mouse models, and to block tumor angiogenesis and tumor growth in mice bearing murine or human xenograft tumors (Skobe et al. 1997; Witte et al. 1998; Prewett et al. 1999). DC101 has since been used by many investigators in a large variety of tumor models to further characterize the effects of VEGFR2 inhibitors on tumor angiogenesis and tumor growth. Tables 24.1 and 24.2 summarize published reports that describe the use of DC101 in human tumor xenograft and syngeneic mouse tumor models, respectively. Following the initial development and testing of DC101, other anti-murine VEGFR2 antibodies have been generated and tested (Ran et al. 2003; Li et al. 2004a) showing similar effects on tumor angiogenesis and inhibition of tumor growth.

As expected, based on the predicted mechanism of action (inhibition of endothelial cell proliferation), DC101 was consistently found to strongly inhibit new blood vessel formation in tissues undergoing angiogenesis. Treatment with DC101 caused reduction in the density of microvessels in tumors or other tissues undergoing angiogenesis. As demonstrated in many studies using standard human xenograft tumors in immunodeficient mice, this effect translated into powerful inhibition of tumor growth. Even when administered alone, DC101 frequently caused complete inhibition of tumor growth, and tumor regression was sometimes observed. Typical examples of anti-tumor activity with DC101 are shown in Fig. 24.2. Tumor inhibition was sustained during continuous treatment for long periods (over 3 months) but resumed with normal kinetics after cessation of antibody treatment (Prewett et al. 1999). DC101 was also shown to reduce the number and growth of metastases. Similarly, inhibition of tumor angiogenesis, tumor growth and metastasis was also reported for various murine tumors (Table 24.2). It must be noted, however, that, DC101 effects were generally not as profound in these models as those induced in immunodeficient mice. This is not surprising: the reduced efficacy in syngeneic murine models is probably attributable to the development of an neutralizing anti-DC101 (a rat antibody) immune response in immunocompetent mice used in these studies.

These profound effects on human or mouse tumor tissue are solely caused by the interaction of the antibody with mouse VEGFR2 located on mouse endothelium. The antibody does not cross-react with human VEGFR2; therefore, direct effects on (rare) VEGFR2-positive human tumors can be excluded. The antibody also does not cross-react with mouse VEGFR1 or VEGFR3 or other more distantly related

Table 24.1. Treatment of human tumors grown as xenografts in immunodeficient (nude, SCID) mice with anti-murine VEGFR2 antibody DC101

Tumor type	Model type	Combination treatment	Tumor growth inhibition	Angiogenesis inhibition	Metastasis inhibition	Other	Reference
Bladder, 253 J B-V	*o*	paclitaxel	yes	yes	yes	*7*	Inoue et al. (2000)
Bladder, 253 J B-V	*o*	no	nd	yes	–	*1,3*	Davis et al. (2004)
Colorectal	*sc*	no	yes	yes	yes		Bruns et al. (2000)
Colorectal, HCT116	*sc*	no	yes	nd	–	*6*	Yu et al. (2002)
Colorectal, KM12L4	*o*	EGFR mab	yes	yes	yes	*2*	Shaheen et al. (2001a)
Colorectal, LS174T	*sc*	no	nd	nd	–	*1*	Tong et al. (2004)
Epidermoid, A431	*sc*	no	yes	yes	–		Prewett et al. (1999)
Gastric, TMK-1	*o*	EGFR mab	yes	yes	–		Jung et al. (2002)
Glioblastoma, G55	*o*	EGFR mab	yes	nd	–		Lamszus et al. (2005)
Glioblastoma, G55	*o*	no	yes	yes	–		Kunkel et al. (2001)
Glioblastoma, GBM-18	*sc*	no	yes	nd	–		Prewett et al. (1999)
Glioblastoma, U87	*sc*	no	nd	nd	–	*1*	Tong et al. (2004)
Glioblastoma, U87	*o*	radiation	nd	yes	–	*1,2*	Winkler et al. (2004)
Glioblastoma, U87	*sc*	radiation	yes	nd	–		Kozin et al. (2001)
Leukemia, HL60	*iv*	no	yes	nd	yes	*7*	Dias et al. (2001)
Lung, small cell, 54A	*sc*	no	nd	nd	–	*1*	Tong et al. (2004)
Lung, small cell, 54A	*sc*	radiation	yes	nd	–		Kozin et al. (2001)
Lymphoma, NHL, DLBCL	*sc*	VEGFR1 mab	yes	yes	–		Wang et al. (2004)
Lymphoma, NHL, DLBCL	*sc*	CD20 mab	yes	yes	–		Wang et al. (2004)
Lymphoma, NHL, DLBCL	*sc*	methotrexate	yes	yes	–		Wang et al. (2004)
Mammary, MD22	*o*	Adriamycin	yes	yes	–	*5*	Klement et al. (2002)
Mammary, MDA-CDDP54	*o*	Cisplatin	yes	yes	–	*5*	Klement et al. (2002)
Mammary, MDA-MB231	*o*	CTX	yes	nd	–	*5*	Man et al. (2002)
Mammary, MPAHS	*o*	vinblastine	yes	yes	–	*5*	Klement et al. (2002)
Mammary, MVB9	*o*	vinblastine	yes	yes	–	*5*	Klement et al. (2002)
Neuroblastoma	*sc*	radiation	yes	yes	–		Gong et al. (2003)
Neuroblastoma, SK-NMC	*sc*	vinblastine	yes	yes	–	*5*	Klement et al. (2000)
Pancreas	*o*	gemcitabine	yes	yes	yes		Bruns et al. (2002)
Pancreas, BxPC-3	*sc*	no	yes	nd	–		Prewett et al. (1999)

Tumor type	Model type	Combination treatment	Tumor growth inhibition	Angiogenesis inhibition	Metastasis inhibition	Other	Reference
Prostate, PC-3M-MM2	*o*	no	yes	yes	yes		Sweeney et al. (2002)
Prostate, LNCaP-LN3	*o*	no	yes	yes	yes		Sweeney et al. (2002)
Renal, SK-RC-29	*sc*	no	yes	nd	–	*2*	Prewett et al. (1999)
Sarcoma, leiomyo	*sc*	doxorubicin	yes	yes	–	*5*	Zhang et al. (2002)
Sarcoma, rhabdomyo	*sc*	doxorubicin	yes	yes	–	*5*	Zhang et al. (2004)
Squamous cell	*sc*	no	nd	yes	–		Kiessling et al. (2004)
Squamous cell	*sc*	radiation	yes	yes	–		Li et al. (2005)
Squamous cell	*sc*	no	nd	yes	–		Skobe et al. (1997)
Squamous cell, A5-RT3	*o*	no	nd	yes	–		Krix et al. (2003)
Squamous cell, A5-RT3	*o*	no	nd	yes	–	*1,4*	Vosseler et al. (2005)
Squamous cell, A5-RT3	*o*	no	nd	yes	–	*1,4*	Miller et al. (2005)

Abbreviations: *iv*, intravenous; *o*, orthotopically implanted tumor; *sc*, subcutaneously implanted tumor; *s*, spontaneous tumor; *c*, chemically induced tumor. Codes for significant results or experimental approaches listed in the column "Other:" *1*, vascular normalization; *2*, vascular permeability; *3*, in vivo signaling; *4*, stromal normalization; *5*, combination with metronomic (continuous low-dose) chemotherapy; *6*, DC101 resistance; *7*, long-term survival or tumor rejection; 8, drug scheduling

growth factor receptors. In contrast, other common approaches to inhibit VEGF signaling (anti-VEGF antibodies, small-molecule kinase inhibitors) generally lack this level of specificity, making data interpretation more difficult in situations where species or pathway specificity do play a role (see below). In this context it should also be noted that VEGFR2, although expressed with higher frequency in angiogenic (tumor) endothelium, is also expressed appreciably in the vasculature of normal tissues (e.g., kidney), and even in some other cell types (e.g., neurons; see below). Expression in normal endothelium may give rise to certain side effects, as will be discussed later.

24.2.1.2 Mechanism of Action

VEGF exerts its action on VEGFR2-expressing endothelial cells by binding to the ligand-binding site of the receptor. VEGF, being a dimeric molecule comprised of two identical disulfide-linked subunits, can bind two receptor molecules and thereby bring two receptors together in close proximity on the cell surface. This process, referred to also as receptor dimerization, enables the kinase domains of each dimer partner to phosphorylate a number of tyrosine residues of the other partner's intracellular domain. This cross-phosphorylation of receptors starts the transduction of the exterior VEGF signal into the interior of the cell by allowing various specialized docking proteins to bind to phosphorylated sites on the receptor. Receptor-bound docking proteins transduce the original signal by acting as kinases or attracting kinases which, in several steps, phosphorylate further kinases or other proteins in a cascade-like manner. Eventually, the phosphorylation state of the last member of the signal transduction chain will determine whether a transcription factor or a transcription inhibitor will bind to DNA and induce or suppress gene expression. In endothelial cells, VEGFR2 prominently activates signaling via

Table 24.2. Treatment of murine tumors with anti-murine VEGFR2 antibody DC101

Tumor type	Model type	Combination treatment	Tumor growth inhibition	Angiogenesis inhibition	Metastasis inhibition	Other	Reference
Adenocarcinoma, s.c.	s	no	yes	yes	-		Izumi et al. (2003)
Colon, CT26	o	MuLV vacc + CTLA4 mab	nd	nd	-	7	Pedersen et al. (2005)
Colon, CT26	o	no	yes	yes	-		Shaheen et al. (2001b)
Colon, CT26	o	no	yes	yes	-		Hicklin and Ellis (2001)
Hepatocellular	c	VEGFR1 mab	yes	yes	yes		Yoshiji et al. (2004a)
Hepatocellular	o	No	Yes	Yes	-	3,8	Yoshiji et al. (1999)
Hepatocellular, MH134	sc	no	nd	nd	-	2	Yoshiji et al. (2001)
Hepatocellular, MH134	sc	no	yes	nd	-		Yoshiji et al. (2002)
Hepatocellular, MH134	sc	no	yes	yes	-		Yoshiji et al. (2004b)
Leukemia, C1498 AML	iv	no	yes	yes	-		Reichert et al. (2005)
Lung, Lewis	sc	no	yes	yes	yes		Prewett et al. (1999)
Mammary	s	no	yes	yes	-	8	Fenton et al. (2004a)
Mammary, 4T1	sc	IL-12 GT	nd	yes	yes		Rakhmilevich et al. (2004)
Mammary, 4T1	sc	no	yes	nd	-		Prewett et al. (1999)
Mammary, Mca4, MCa35	sc	no	yes	yes	-		Fenton et al. (2004a)
Mammary, Mca4, MCa35	sc	radiation	yes	yes	-		Fenton et al. (2004b)
Mammary, MCaIV	sc	no	nd	nd	-	1	Tong et al. (2004)
Mammary, Shionogi	sc	doxo/CTX	yes	yes	-		Hansen-Algenstaedt et al. (2001)
Melanoma, B16	sc	no	yes	nd	-		Prewett et al. (1999)
Pancreas islet, Rip-Tag	s	no	yes	yes	-	6	Casanovas et al. (2005)
Sarcoma	s	no	nd	nd	-	2	Stoelcker et al. (2000)
Sarcoma, s.c.	s	no	yes	yes	-		Izumi et al. (2003)

Abbreviations: s, spontaneous tumor; c, chemically-induced tumor; GT, gene therapy; for other abbreviations and table content explanations see legend of Table 24.1

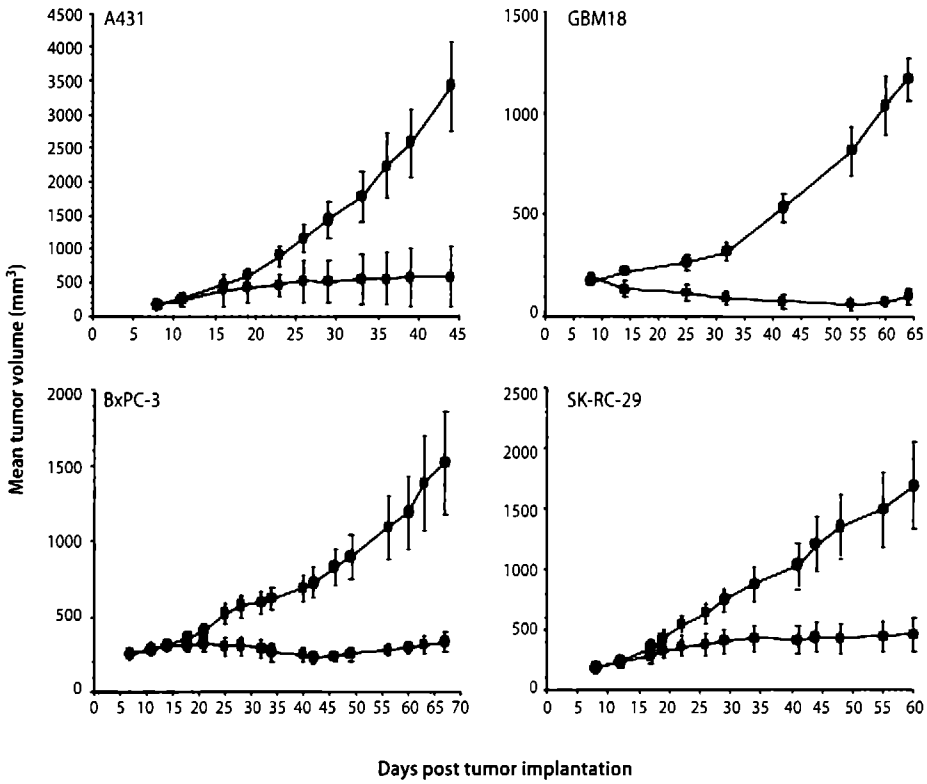


Fig. 24.2. Tumor growth inhibition with single-agent DC101 therapy. Subcutaneous tumors were established by injecting athymic nude mice with tumor cells. Treatment of mice with DC101 (27 mg/kg) (*red*) or control antibody (*blue*) was started when subcutaneous tumors were palpable (150–250 mm^3). DC101 was injected twice weekly for the duration of the experiments. *A431*, human epidermoid carcinoma; *GBM18*, glioblastoma; *BxPC-3*, pancreatic carcinoma; *SK-RC-29*, renal cell carcinoma. For additional details see Prewett et al. (1999). Reproduced from Prewett et al. (1999), with permission from the American Association of Cancer Research

the phosphatidylinositide-3 kinase (PI3-kinase)/akt pathway (which controls the expression of the genes involved in cell survival) and the ras/raf/map-kinase pathway (which regulates cell proliferation). DC101 prevents these signaling pathways from being activated by binding to the ligand-binding site of the receptor such that VEGF cannot bind to the receptor and induce its dimerization. Without signaling induced by VEGF, endothelial cells remain in an inactive state, and new vessel formation does not take place.

The expected outcome of anti-VEGFR2 action is cytostatic arrest of endothelial cells in G0, followed by inhibition of incremental tumor growth due to lack of sufficient blood supply. However, DC101 was also found to strongly induce apoptosis in tumor endothelium, followed by death of existing tumor tissue (Bruns et al. 2000; Shaheen et al. 2001b). But how can this antibody with a primarily cytostatic mechanism also be cytotoxic to endothelial as well as tumor cells? The reason is that VEGF, together with other tumor-secreted factors (cytokines, enzymes), broadly "activates" endothelium within the tumor (see above). A critical part of this activation is the triggering of the PI3-kinase/akt signaling pathway that helps to ensure cell survival under adverse conditions. Agents like DC101 that inhibit VEGF-induced survival will therefore promote apoptosis of stressed cells. This vulnerability can even be observed in cell culture. When endothelial cells are induced to proliferate with VEGF under non-optimal (low serum) conditions, addition of DC101 not only inhibits further proliferation but also causes cells to die from apoptosis (Bruns et al. 2000). In a tumor environment, rapidly proliferating endothelial cells that attempt to organize into vessels are stressed and thus vulnerable while the vessel network is formed and maturation of new structures has not yet occurred. These cells rely on survival mechanisms. When a major VEGF-induced survival pathway,

PI3-kinase/akt signaling, is blocked by a VEGFR2 antagonist, tumor endothelial cells undergo massive apoptosis (Shaheen et al. 2001b). This is even more the case when tumors are treated with chemotherapy or radiation and tumor endothelium is further stressed. Indeed, DC101 can strongly synergize with the apoptotic action of cytotoxic therapy on tumor endothelium (Bruns et al. 2002; Zhang et al. 2002). DC101-induced endothelial cell apoptosis causes vessel regression which results in nutrient restriction and hypoxia in existing tumor tissue, followed by widespread apoptotic tumor cell death in undervascularized areas (Bruns et al. 2000; Shaheen et al. 2001b).

Interestingly, the collective experience with using DC101 in varied tumor models points to a broader role of VEGF and its receptor VEGFR2 than endothelial proliferation and survival. Excessive tumor endothelial cell proliferation results in vessels that are largely abnormal, unstable and inefficient. While the activated state of endothelial cells supports their proliferation and facilitates their migration and tissue invasion through increased activity of enzymes such as metalloproteinases, cathepsins, and heparanase, it does not promote the stabilization of new vessel structures. Tumor vessels grow chaotically, are often tortuous, uneven in size and shape, and reach abnormally high densities. They do not produce a normal basement membrane and lack association with vessel-supporting pericytes. As a consequence, tumor vessels are leaky and unstable. Vascular permeability increases interstitial pressure in tumors, and elevated interstitial pressure often causes unstable vessels to collapse, leading to interrupted blood flow. In short, tumor vasculature functions inefficiently, a deficit that can be overcome only by even more tumor angiogenesis. Abnormal tumor vessel permeability has long been recognized as a VEGF-induced phenomenon (Senger et al. 1993), and VEGFR2 is now known to be the receptor that regulates vessel permeability through interaction with adhesion molecules such as β3-integrins and VE-cadherin, and src-like tyrosine kinases (Weis and Cheresh 2005).

Studies with DC101 have provided much evidence that VEGF, via action on VEGFR2, is also responsible for other abnormal characteristics of tumor vessels. DC101 normalizes tumor vasculature broadly by (a) reducing vessel permeability (Prewett et al. 1999); (b) reducing ascites formation associated with the growth of some tumors (Yoshiji et al. 2001; Shaheen et al. 2001a; Stoelcker et al. 2000); (c) decreasing interstitial fluid pressure (Tong et al. 2004); (d) increasing oxygenation (Winkler et al. 2004; Tong et al. 2004); (e) regression of tumor vessels (pruning), largely at the expanding periphery of tumors (Davis et al. 2004; Tong et al. 2004; Vosseler et al. 2005); (f) increasing coverage of tumor vessels with vessel-supporting pericytes (Tong et al. 2004; Vosseler et al. 2005); and (g) normalizing stroma, as evidenced by decreased stromal metalloproteinase production (Vosseler et al. 2005; Miller et al. 2005) and improved basement membrane formation in the tumor microenvironment (Tong et al. 2004; Vosseler et al. 2005; Miller et al. 2005).

The "malfunction" of tumor vasculature is also thought to interfere with therapy and to contribute to tumor metastasis. High interstitial pressure reduces the diffusion rate of chemotherapeutics into the tumor tissue, and hypoxia renders radiation therapy less effective because oxygen is a radiosensitizer (Jain et al. 2006). Increased tumor vessel permeability may contribute to accelerated intravasation and dissemination of metastases (Weis and Cheresh 2005). Again, DC101 strongly reverses these abnormalities. Treatment with antibody was shown to enhance the effects of radiation therapy by increasing tissue oxygenation (Li et al. 2005), to increase drug penetration by reducing intratumoral pressure (Winkler et al. 2004; Tong et al. 2004), and to greatly reduce metastasis formation (Tables 24.1 and 24.2).

24.2.1.3 Combination Therapy

While tumor growth control with DC101 is often impressive and long-term in animal models of human tumors, and tumor cell death invariably occurs (often associated with tumor regression) as a consequence of angiogenesis inhibition, antibody treatment alone does not cure the disease.

Invariably, treatment with DC101 leads to extensive tumor necrosis, but a surviving population of tumor cells remains supplied by existing, VEGF-independent vessels. Thus, upon cessation of antibody therapy, tumors eventually resume growth from this surviving tumor cell population in a manner indistinguishable from the growth of non-treated tumors.

Therefore, the concept of combining anti-angiogenic and cytotoxic therapy has been widely tested. DC101 and chemo- and radiation therapy can act synergistically because both modalities can act, directly or indirectly, on both endothelial and tumor cells. As shown above, DC101 can cause tumor cell apoptosis by depriving tumors of the blood vessels they need. On the other hand, cytotoxic drugs or radiation that kill tumor cells by interfering with the cell cycle can also inhibit angiogenesis by killing activated and rapidly proliferating endothelial cells (Bruns et al. 2002). In addition, DC101 prevents growth of new tumor blood vessels during recovery periods between doses of chemo- or radiation therapy. Thus, in combination, DC101 and cytotoxic agents or radiation enhance each other's tumor-controlling effects. There are many studies demonstrating the superior effect of DC101 treatment in combination with chemo- or radiation therapy (see Tables 24.1 and 24.2). Significant combinatorial antitumor effects have been observed for chemo- and radiation therapy even under conditions where either single agent produced little effect (Zhang et al. 2002; Fenton et al. 2004b). An example of the power of combined DC101 and chemotherapy is shown in Fig. 24.3.

Anti-angiogenic therapy with DC101 has also been used in combination with continuous low-dose, or metronomic, chemotherapy. The rationale for metronomic chemotherapy suggests that the synergistic effect of anti-angiogenic and low-dose chemotherapy should produce a sustained anti-tumor effect comparable to that of standard chemotherapy with maximally tolerated doses, without the severe toxicities seen with standard chemotherapy, and possibly without rapid induction of drug resistance. Indeed, remarkable results have been observed with combination treatments in several tumor models using different drug and treatment modalities. At best, combination of DC101 with chemotherapy has resulted in significant tumor regression and/or long-term survival, or produced better tumor inhibition than standard chemotherapy without the severe toxicity associated with standard chemotherapy (Klement et al. 2000, 2002; Zhang et al. 2002; Man et al. 2002).

With increasing knowledge about biological mechanisms of cancer progression, and the growing realization that the optimal clinical benefit for cancer patients is likely to incorporate newer targeted therapies with similar agents or conventional cytotoxins, combination therapy is now being investigated broadly. DC101 has been tested in a variety of such combinations, including combinations with other antibodies against tumor growth factor receptors (VEGFR1, EGFR), cell surface proteins (CD20), immunomodulatory receptors (CTLA-4), and others (see Tables 24.1 and 24.2). In general, combination therapy proved to be more effective than single-agent therapy in these biological combinations as well; in one study with a vaccine (Pedersen et al. 2005), tumor rejection rates of 80% were observed.

24.2.1.4 Other Findings

One original hypothesis of anti-angiogenesis therapy was that it may not exhibit the problem of drug resistance because the target of the therapy, the endothelial cell, is genetically stable. In support of this hypothesis, evidence that treated animals acquire resistance to anti-angiogenic agents is largely absent. Several longer-term studies have not suggested that human cancers in xenograft mouse models develop resistance to DC101 treatment. However, the clinical evidence with the VEGF antibody bevacizumab clearly shows that tumors eventually progress while patients are on anti-angiogenic therapy. Further preclinical and clinical studies are needed to determine whether or not the apparent long-term failure of anti-angiogenesis therapy (Jain et al. 2006) is caused by resistance of endothelial cells. It is more likely that tumors become resistant to angiogenesis inhibitors such as bevacizumab because, during tumor

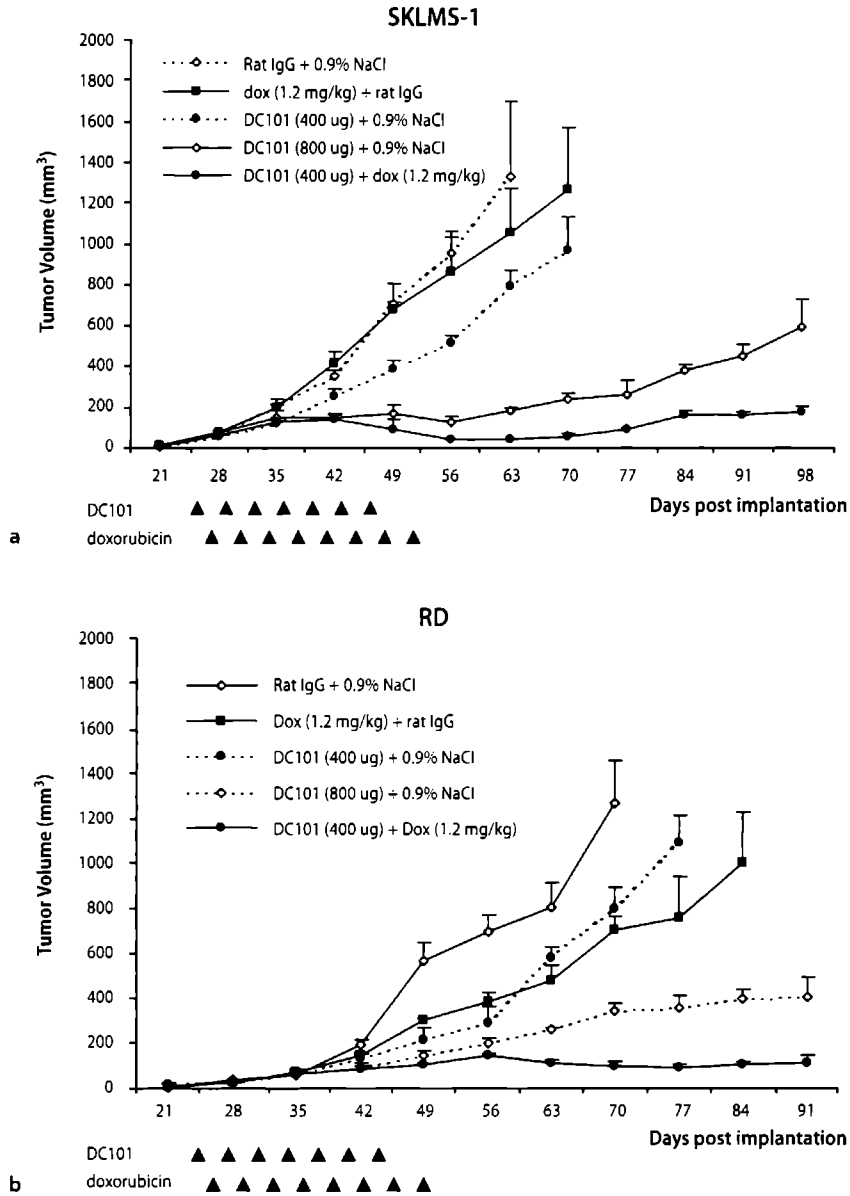


Fig. 24.3a,b. Antitumor effect of combined DC101 and continuous low-dose doxorubicin against human soft tissue sarcoma xenografts SKLMS-1 (a) and RD (b). *Arrowheads*, days on which treatment was administered. Results are given as mean tumor volume of 10 mice/group. Bar, standard deviation. a, $P<0.05$ vs control; b, $P<0.001$ vs control; c, $P<0.01$ vs DC101 (400 μg) alone; d, $P<0.01$ vs continuous low-dose doxorubicin alone. Reproduced from Zhang et al. (2002), with permission from the American Association of Cancer Research

progression, tumors switch to other mechanisms to sustain tumor angiogenesis and tumor growth. Evidence to support this hypothesis is now emerging. For example, a recent study shows that late-stage spontaneous pancreatic islet tumors in the transgenic Rip-Tag mice lose their sensitivity to DC101 by switching to alternative pro-angiogenic growth factor systems (FGF), thereby evading the VEGFR2 blockade (Casanovas et al. 2005). Other studies (Hansen-Algenstaedt et al. 2000; Yu et al. 2002), which showed apparent resistance to DC101 treatment, may be interpreted accordingly, although underlying mechanisms have not been documented.

Another hypothesis is that anti-angiogenic therapy is most effective early during tumor growth. Intuitively, it seems reasonable that anti-angiogenesis therapy would be most effective in the more rapid, earlier growth phase of tumors, rather than later on when tumors have a good supply of larger and more mature vessels. In one study, Fenton et al. (2004a) showed that early but not late administration of DC101 inhibited tumor angiogenesis. However, it is not clear whether or not this finding was related to resistance as discussed above, i.e. progression of tumors to states less dependent on VEGF. In other studies, late administration of DC101 to animals bearing large tumors proved to be effective in controlling tumor growth (Yoshiji et al. 1999; Izumi et al. 2003). The issue of whether large tumors can be effectively treated with anti-angiogenic therapy may require further preclinical and clinical validation.

Although almost all preclinical work with anti-VEGFR2 antibodies has been done with DC101, another anti-VEGFR2 antibody, RAFL, has also been tested in animals (Ran et al. 2003). In addition, an antibody that binds to murine VEGFR2 (but does not neutralize VEGF action) has been used as a targeting vehicle to deliver yttrium-90 nanoparticles selectively to tumor vasculature in several murine tumor models (Li et al. 2004a). With this approach, significant tumor growth delay has been achieved.

24.2.2 Anti-human VEGFR2 Antibodies: the Path to Clinical Studies

Proof-of-principle studies with the rat anti-mouse antibody DC101 indicated the high promise of blocking VEGFR2 for cancer therapy. Since DC101 does not recognize the human VEGFR2 receptor, scientists at ImClone developed a number of anti-human VEGFR2 antibodies. The first of these antibodies that eventually progressed to clinical testing was generated by screening a single-chain antibody phage display library obtained from a mouse immunized with the human receptor and fusing the variable domains of the selected scFv fragment to the constant region of human IgG1 (Zhu et al. 1998, 1999). The resulting mouse/human chimeric antibody, 1C11, binds to human VEGFR2 and inhibits VEGF-induced signaling, proliferation, and migration of human endothelial cells with potency similar to that of DC101 (Zhu et al. 1998, 1999; Li et al. 2005)). However, testing antibody 1C11 in vivo in standard tumor models was not possible because of a lack of cross-reactivity with murine VEGFR2. Investigation of antibody cross-reactivity with other species indicated that the antibody cross-reacts with dog and monkey VEGFR2. When administered to these animals, 1C11 was shown to neutralize pathological and physiological angiogenesis in models of dog retinopathy (McLeod et al. 2002) and rhesus monkey ovarian follicle development (Zimmermann et al. 2002). Furthermore, 1C11 inhibited VEGFR2 signaling in human VEGFR2-positive leukemia cells. In a mouse xenograft model of human leukemia, 1C11 prolonged survival by acting directly on the VEGFR2-positive leukemia cells and inhibiting their growth (Dias et al. 2000, 2001). Collectively, these in vivo studies provided convincing evidence that 1C11 is capable of potently blocking VEGFR2 signaling in vivo and thus can be expected to inhibit tumor angiogenesis in cancer patients. ImClone Systems entered 1C11 into a phase 1 clinical trial with patients suffering from advanced solid tumors. The results showed that the 1C11 antibody had an acceptable pharmacokinetic and safety profile in humans up to the highest dose tested (8 mg/kg) (Posey et al. 2003). In parallel, ImClone Systems also developed a second generation of fully human anti-VEGFR2 antibodies using human Fab antibody libraries for identifying suitable candidates. From this work emerged antibody 2C6 (Lu et al. 2003b; Zhang et al. 2004). The binding affinity and potency of 2C6 was further improved by light-chain shuffling (Lu et al. 2003b). The resulting fully human antibody 1121B was found to be several times more potent in vitro and in vivo than 1C11 and 2C6 (Lu et al. 2003b; Zhu et al. 2003) and entered clinical phase 1 trials in January 2005.

The antibody-binding site of 1C11 and 1121B is located within the first three N-terminal Ig-like domains of the receptor, where VEGF is known to bind

(Lu et al. 2000, 2002a). Binding to Ig-like domain III of the receptor was found to be necessary and sufficient for effective neutralizing antibody action (Lu et al. 2002a; Li et al. 2004b).

UCB Celltech is developing another VEGFR2 antibody for cancer indications. This antibody, CDP-791, is a humanized $F(ab')_2$ antibody fragment conjugated to polyethylene glycol for longer serum half-life. It is currently in phase II clinical testing in patients with non-small-cell lung carcinoma, in combination with standard chemotherapy (www.clinicaltrials.gov).

A limited number of human tumor cells have been reported to express VEGFR2 (e.g., melanoma, leukemia, mesothelioma). The role of VEGFR2 in these tumor cells is unclear. However, it appears that at least in leukemia and mesothelioma VEGFR2 can be functional and that inhibition of this function can delay tumor growth (Dias et al. 2000, 2001; Masood et al. 2003). Treatment of VEGFR2-positive tumors with VEGFR2 inhibitors may therefore be particularly beneficial because of the expected dual action of these inhibitors: tumor growth would be inhibited by the inhibitor's paracrine action on tumor endothelium and by its autocrine action on tumor cells (Fiedler et al. 1997; Dias et al. 2000, 2001; List et al. 2004). Dias et al. (2000, 2001) showed elegantly, by using combination therapy with anti-mouse DC101 (for paracrine anti-angiogenic action) and anti-human 1C11 or 1121B (for autocrine anti-tumor action), that survival of immunodeficient mice xenotransplanted with VEGFR2-positive leukemia was prolonged strongly with the combination of paracrine and autocrine effects. VEGFR2 pathway inhibitors such as antibody 1121B could therefore be agents of choice to treat VEGFR2-positive human leukemias because of their ability to simultaneously inhibit both mechanisms.

Others have also created human anti-VEGFR2 antibodies for various purposes. These include non-neutralizing antibodies or antibody scFv fragments for targeting KDR-positive vasculature with cytotoxic agents or liposomes (Backer and Backer 2001; Backer et al. 2002, 2004; Popkov et al. 2004; Rubio-Demirovic et al. 2005), non-neutralizing scFv fragments for the isolation of VEGFR2-positive cells such as circulating endothelial cells or bone marrow-derived endothelial progenitor cells (Boldicke et al. 2001), and a neutralizing antibody for cell signaling studies (Kanno et al. 2000).

Anti-VEGFR1 Antibodies

VEGFR1 and VEGFR2 have much in common: they have similar structures, share the same activating ligand, are both expressed in endothelial cells, and are both involved in blood vessel formation in major ways. Nevertheless, elucidation of the precise functions of VEGFR1, unlike those of VEGFR2, in the angiogenic process proved to be rather difficult. While the major function of VEGFR2 was quickly recognized as that of a typical growth factor receptor, namely to govern endothelial cell proliferation, a clear role for VEGFR1 in endothelial cell function remains elusive because of seemingly contrasting findings. For example, how can it be that VEGFR1 gene deletion causes death in utero because normal vasculature is not formed, but transgenic mice expressing a functionally defective VEGFR1 lacking the intracellular kinase domain develop normally? Or, how is it possible that VEGFR1-specific ligands such as PlGF or VEGF-B can induce angiogenesis, and neutralizing antibodies against VEGFR1 can block angiogenesis, yet evidence suggests that signaling through VEGFR1 in endothelial cells is significantly lower than that of VEGFR2? Despite such contradictory findings, efforts to define VEGFR1 function have led to an emerging picture that reveals unexpected complexity of VEGFR1 function. In contrast to VEGFR2, whose angiogenic role is largely restricted to endothelial cell function, VEGFR1 is expressed in other cell types where it may function to regulate cellular activities that support the process of neovascularization.

Much of the work that has contributed to this emerging understanding of VEGFR1 biology was enabled by the availability of function-blocking antibodies against VEGFR1. As with anti-VEGFR2

antibodies, scientists at ImClone Systems generated the antibodies MF1 and 6.12 against murine and human VEGFR1, respectively, and made them widely available for biological investigation.

Many investigators, using a variety of animal models, have found that function-blocking anti-VEGFR1 antibodies (MF1 and others) inhibit angiogenesis (Wu et al. 2001; Carmeliet et al. 2001; Lyden et al. 2001; Luttun et al. 2002; Silvestre et al. 2003; Wang et al. 2004; Yoshiji et al. 2004a; Ohki et al. 2005; Heissig et al. 2005). MF1 treatment inhibited angiogenesis as determined by reduction of microvascular density in tumor or Matrigel implant models. Moreover, MF1 inhibited the growth of human tumor xenografts in mice. This effect was attributable solely to the anti-angiogenic action of MF1, because the anti-mouse antibody does not cross-react with human VEGFR1 and therefore cannot act directly on human tumor cells in these models. Typically, MF1 effects on various measures of angiogenesis are qualitatively similar to those seen with DC101, although MF1 potency was often found to be inferior to that of DC101 (Wu et al. 2001; Luttun et al. 2002; Wang et al. 2004). For the most part, these studies did not address the question of whether the antibody blocked VEGFR1 function in angiogenic endothelium. Instead, several studies provide evidence for indirect mechanisms. MF1 appears to block the mobilization into the general circulation of various types of bone marrow-derived cells, such as hematopoietic stem cells, endothelial progenitor cells (EPCs), and inflammatory cells (Stefanik et al. 2001; Lyden et al. 2001; Luttun et al. 2002; Heissig et al. 2005; Ohki et al. 2005). The concept of VEGFR1-mediated inhibition of bone marrow cell mobilization is consistent with the findings that MF1 reduced influx of inflammatory cells into inflamed tissue, e.g., in models of atherosclerosis (Luttun et al. 2002), arthritis (Luttun et al. 2002; de Bandt et al. 2003), and psoriasis-like skin inflammation (Kunstfeldt et al. 2004). MF1-induced inhibition of bone marrow-derived cell mobilization is also consistent with the delayed hematopoietic reconstitution in mice after myelosuppression induced by 5-FU treatment (Hattori et al. 2002), with reduced macrophage infiltration in tumor tissue (Stefanik et al. 2001), and with disease amelioration seen in a genetic model of arthritis dependent on bone marrow-derived osteoclasts (de Bandt et al. 2003).

The notion that tumor-derived VEGF may stimulate tumor angiogenesis via VEGFR1 by enabling the recruitment of bone marrow-derived cells implies two possible mechanisms for VEGFR1 antibody action. One mechanism is based on the recruitment of EPCs into the general circulation. These cells are VEGFR1-positive, but it is unclear whether or not inhibition of mobilization by antibodies is caused by direct action on EPCs (inducing their mobilization) or on other VEGFR1-positive cells in the bone marrow that facilitate the mobilization of EPC. While the origin, identity and precise function of EPCs remains unclear, there is good evidence from bone marrow transplantation experiments in mice that mobilized EPCs mature into so-called circulating endothelial cells (CEPs), which are integrated into tumor vessels during angiogenesis (Ribatti 2004; Hicklin and Ellis 2005). It seems, however, that blood concentration of EPCs/CEPs and the extent of integration vary widely, depending on disease and models studied, from zero (Gothert et al. 2004) to <0.1–50% (Ribatti 2004; Yamamoto et al. 2004). The molecular mechanism(s) that govern EPC release from bone marrow are not well understood. It is thought to be regulated, at least in part, by the activation of metalloproteinases that induce the release of Kit ligand to facilitate egress of cells from the marrow to the circulation (Hiratsuka et al. 2002; Rabbany et al. 2003). The other mechanism is based on the recruitment of inflammatory cell precursors from the bone marrow. Inflammatory/immune cells (e.g., macrophages, monocytes, neutrophils, mast cells) infiltrate inflammatory (angiogenic) sites including tumors, where they release VEGF and other angiogenic factors in order to stimulate neovascularization (Stefanik et al. 2001; Yu and Rak 2003; Robinson and Coussens 2005; Lewis and Murdoch 2005; Heissig et al. 2005; Ohki et al. 2005; Sho et al. 2005; Lewis and Pollard 2006). Inhibition of VEGF-dependent mobilization of such cells from the bone marrow with MF1, but not VEGFR2 antagonists, will reduce the influx of inflammatory cells to angio-

genic sites, with concomitant reduction in availability of pro-angiogenic cytokines (Luttun et al. 2002; de Bandt et al. 2003).

These mechanisms explain how VEGFR1 inhibition can reduce tumor angiogenesis. It is not clear, however, to what extent these mechanisms contribute to overall angiogenesis in a tumor. Available evidence suggests that a larger fraction of newly generated tumor endothelium derives from VEGFR2-driven endothelial cell proliferation. This is consistent with findings that angiogenesis and associated tumor growth are generally inhibited to a lesser degree by VEGFR1 inhibitors than by VEGFR2 inhibitors and that the inhibitory effect of anti-VEGFR1 therapy can be enhanced with VEGFR2 inhibitors (Yoshiji et al. 2004b; Wang et al. 2004). These mechanisms also do not address the question of the role of endothelial VEGFR1 in tumor vessels. Does VEGF stimulate these cells by activating not only VEGFR2 but also VEGFR1 in tumor endothelium, and if so, what is the cellular response to VEGFR1 stimulation? While there is evidence for a signaling role of VEGFR1 in angiogenic endothelium (Seetharam et al. 1995; Kanno et al. 2000), there are also conflicting results (Koolwijk et al. 2001; Cai et al. 2003), making data difficult to interpret. More recent studies are interesting as they reveal complex aspects of VEGFR1 signaling in endothelial cells. Studies with the VEGFR1-specific ligands PlGF or VEGF-B revealed that VEGFR1 can trigger the PI3-kinase/akt signaling pathway, thereby possibly supporting angiogenesis by prolonging endothelial survival signals and enabling vascular maintenance during angiogenesis (Cai et al. 2003). RNA interference studies showed that VEGFR1 inhibition caused decreased expression of VEGFR2, suggesting that VEGFR1 signaling determines VEGFR2 abundance and the associated ability of endothelial cells to proliferate (Kou et al. 2005). Furthermore, PlGF was found to induce phosphorylation of VEGFR2 (although it cannot bind to this receptor), implying intermolecular cross-phosphorylation of VEGFR2 by VEGFR1 within a VEGFR1/2 heterodimer receptor complex (Autiero et al. 2003). Further evidence for a signaling heterodimer receptor derives from studies with anti-VEGFR oligonucleotides (Neagoe et al. 2005). In conclusion, the emerging picture of how VEGFR1 helps to regulate angiogenesis is complex. It is plausible to suggest, based on currently available data, that VEGFR1 plays a role in tumor angiogenesis both in the tumor endothelium and via its function on tumor-associated myeloid cells. In view of potential clinical applications of anti-VEGFR1 antibodies, a better understanding of the underlying mechanisms of VEGFR1-mediated angiogenesis is important.

VEGFR1 is also expressed on various non-endothelial cells. There is increasing evidence that the receptor is prevalent on human tumor cells, e.g., in breast (Wu et al. 2003, 2006), colorectal (Fan et al. 2005), and pancreas carcinoma (Wey et al. 2005), as well as in leukemia (Fragoso et al. 2006), myeloma (Vincent et al. 2005), and lymphoma (Wang et al. 2004). There is also clear evidence that VEGFR1-specific agents such as PlGF induce signaling in VEGFR1-positive tumor cells via the map kinase, jnk, and akt pathways and that the anti-VEGFR1 antibody 6.12 inhibits signaling and resulting tumor cell functions such as proliferation, migration, and invasion (Wu et al. 2003, 2006; Wang et al. 2004; Fan et al. 2005; Vincent et al. 2005; Wey et al. 2005). Antibody 6.12 also inhibited epithelial-to-mesenchymal transition in pancreatic carcinoma cells (Yang et al. 2006). Since 6.12 is specific for human VEGFR1 and does not cross-react with the murine receptor and therefore cannot inhibit tumor angiogenesis in these mouse models, the data suggest that the antibody may inhibit tumor growth in xenograft tumor models by direct inhibition on tumor cells. Indeed, the antibody showed strong tumor growth inhibitory activity in breast cancer and lymphoma models (Wu et al. 2003, 2006; Wang et al. 2004). Even better anti-tumor effects were achieved with human xenograft tumor models when animals were simultaneously treated with 6.12 (against human tumor cells) and MF1 (against murine tumor vasculature) (Wu et al. 2003, 2006; Wang et al. 2004). As with the treatment of certain VEGFR2-positive tumors with VEGFR2 inhibitors (Dias et al. 2000, 2001), the dual action of anti-VEGFR1 may be clinically exploited for treatment of VEGFR1-positive tumors. Since the frequency of VEGFR1 expression on human tumors

is more extensive than that of VEGFR2, treatment of such tumors with anti-VEGFR1 agents may provide a desirable therapeutic avenue. Clinical studies will have to show to what extent the anti-angiogenic/anti-tumor dual action of VEGFR1 antibodies will translate into real therapeutic benefit for patients with VEGFR1-positive tumors.

A fully human anti-VEGFR1 antibody, 18F1, has been generated (Wu et al. 2004), and phase I clinical trials have been initiated (www.imclone.com) to treat patients with VEGFR1-positive tumors, such as breast carcinoma, via a dual anti-tumor/anti-angiogenesis action (Wu et al. 2005).

Anti-VEGFR3 Antibodies

VEGFR3, the third member of the VEGFR family, and its ligands VEGF-C and VEGF-D are recognized as playing a major roles in the development and maintenance of lymphatic vasculature and in the regulation of abnormal lymphangiogenesis in cancer and other diseases (for reviews see Jussila and Alitalo 2002; Stacker et al. 2002; Alitalo and Carmeliet 2002; Pepper et al. 2003; Alitalo et al. 2005; He et al. 2004). Stimulation of VEGFR3 with VEGF-C/D or inhibition of VEGFR3 function is thought to affect primarily the lymphatic system. However, VEGF-C/D can also interact with VEGFR3 or VEGFR2 expressed in normal angiogenic vasculature (Kubo et al. 2002; Patila et al. 2006) and tumor endothelium (Karpanen et al. 2001; Tsurusaki et al. 1999; Kubo et al. 2000) to stimulate angiogenesis. Recent evidence is also suggests that VEGFR3 may have hitherto unknown functions in other tissues (e.g., neural progenitor cells, Le Bras et al. 2006).

Since the role of the lymphatic system in metastasis is well established, VEGFR3 is considered a potential therapeutic target for inhibiting metastasis. Scientists from ImClone Systems began to explore possible therapeutic applicability of VEGFR3 inhibitors by creating function-blocking antibodies against mouse and human VEGFR3. These antibodies were shown to potently block the binding of VEGF-C to VEGFR3. The anti-mouse antibody mF4-31C1 inhibited regeneration of lymphatic vessels after ablation in a mouse tail skin model without affecting new blood vessel formation (Pytowski et al. 2005). It also completely suppressed the growth of lymphatic vessels, but not blood vessels, in a mycoplasma-induced airway inflammation model (Baluk et al. 2005). In contrast, another function-blocking rat anti-mouse antibody, AFL-4, has been shown to inhibit blood vessel formation in corneal angiogenesis and tumor growth models (Kubo et al. 2000, 2002). Antibody mF4-31C1 also blocked tumor growth in renal and colorectal carcinoma xenograft models, and in the mouse Lewis lung carcinoma (Tonra et al. 2005) to a considerable extent. In the renal carcinoma model, the antibody strongly suppressed density of lymph vessels, by 78%, but also reduced tumor microvessel density by 30%, suggesting that tumor growth inhibition is mediated by VEGFR3 in the tumor microvasculature (Tonra et al. 2005). As expected, the antibody inhibited metastases in a VEGF-C-overexpressing breast carcinoma xenograft model (Roberts et al. 2006) and in Lewis lung carcinoma (Tonra et al. 2005). Interestingly, in the study by Roberts et al. (2006), the antibody was more effective when animals were treated in a preventive mode (start of therapy at time of tumor inoculation) than in an interventional mode (start of therapy after establishment of metastases). In contrast, other investigators (Tonra et al. 2005) found no such difference using the Lewis lung carcinoma model. This suggests that the antibody may reduce the growth of metastases by inhibiting metastasis neovascularization rather than by inhibiting the metastatic process itself. It remains to be determined, therefore, whether VEGFR3 inhibition constitutes a true anti-metastatic therapeutic avenue. As discussed earlier, the VEGFR2 inhibitor DC101 also inhibits metastasis. In one metastasis model where the two antibodies were compared (Roberts et al. 2006), the VEGFR3 antibody was more effective than the VEGFR2 antibody. However, in these studies tumor cells were engineered to overexpress VEGF-C, so the model may be biased towards VEGFR3-mediated function. Again, it is unclear to

what extent inhibition of lymphangiogenesis rather than angiogenesis contributed to the inhibition of metastatic growth or development.

The fully anti-human VEGFR3 antibody, hF4-3C5, was shown to have potent and specific inhibitory activity against VEGFR3 signaling in human and bovine endothelial cells (both expressing VEGFR3) and to prevent mitogenesis, migration and vitro tube formation of endothelial cells (Persaud et al. 2004; Nilsson et al. 2004). Since in vivo efficacy of this antibody will be difficult if not impossible to study in normal laboratory animals, because of lack of species cross-reactivity, these in vitro tests may be crucial in defining the usefulness of this or other non-cross-reacting antibodies in human exploratory anti-VEGFR3 therapeutic studies.

Outlook

24.5.1 Comparison of Approaches to Block VEGF Pathway Signaling

Now that the ongoing clinical experience with the VEGF antibody bevacizumab is defining a new standard for future anti-angiogenic agents, an important question is what one may expect from the anti-VEGFR2 antibodies and small molecules now in all stages of clinical development. Can lessons learned from recent phase III trials with bevacizumab (Jain et al. 2006) be applied to new anti-angiogenesis agents currently under development? Although we cannot answer these questions today, since experience with bevacizumab is still limited and the VEGFR2 inhibitors have not progressed sufficiently in the clinical setting, it is tempting to consider potential relative advantages and disadvantages of anti-VEGF and anti-VEGFR agents. All these agents share a principal mechanism of action, the inhibition of mitogenic and survival signaling in endothelial cells, and are potent inhibitors of tumor growth in animal models. It is therefore not expected that these agents would substantially differ in efficacy. Nevertheless, there are differences between the two approaches that could potentially have a distinguishing effect in therapy. Anti-VEGF antibodies do not only block theVEGFR2 pathway, they also interfere with VEGFR1 signaling in endothelial and/or other cells. Therefore, since VEGFR1 contributes to angiogenesis by several mechanisms that are not shared with VEGFR2, a VEGF antagonist could be a more effective angiogenesis inhibitor. On the other hand, blockade of an additional pathway could give rise to pathway toxicities, thus rendering an anti-VEGF agent less desirable. Another potential difference between anti-ligand and anti-receptor antibodies derives from how VEGFs are delivered to its receptors on endothelium. VEGFs are produced and secreted abundantly by tumor cells. As heparin-binding growth factors, they are sequestered in large part by heparan sulfate proteoglycans in the interstitial or pericellular space where freely soluble, active VEGFs exist in dynamic equilibrium with various forms of VEGF/heparan sulfate complexes (soluble or insoluble, active or inactive). It may be difficult for an antibody to completely neutralize all ligand forms of VEGF efficiently unless the antibody is present in large excess. In contrast, VEGFR2 is expressed in low abundance on the abluminal side of endothelial cells even in activated tumor endothelium. Antibodies can easily reach these receptors, especially in the leaky tumor vessels, and block VEGF access to the receptors. As theoretical as these arguments are, it is possible that such differences – and others, such as those associated with unique antibody affinities and kinetics – could render one antibody type advantageous over the other. No obvious differences have become apparent in animals studies; however, but it should be appreciated that careful comparison of antibodies has not been undertaken. Differences may be difficult to pick up in clinical studies, given that preclinical data have not as yet steered investigators towards appropriate and measurable pharmacodynamic parameters.

Potential differences in efficacy and/or safety between VEGFR2 inhibitory antibodies and small-molecule VEGFR2 kinase inhibitors are also not

known because appropriate comparative studies have not been carried out. Although antibodies and kinase inhibitors are both highly efficacious and without overt signs of toxicity in laboratory animals, it is more likely that differences in efficacy and/or safety will be observed as the development various agents progresses into more advanced phases of clinical study. There are several differentiating factors. Most importantly, small molecules generally lack strict target specificity; they tend to also inhibit the activity of closely related kinases (such as VEGFR1 and VEGFR3), and they even affect functionally more distant kinases in rather unpredictable manner. Such lack of specificity clearly increases the potential for adverse effects. However, drug developers now increasingly see the lack of specificity for small-molecule kinase inhibitors as a potential benefit, since potent VEGFR2 kinase inhibitors may serendipitously target other cancer-relevant kinases. Such molecules are now considered as potentially more effective anti-cancer drugs, and the number of dual- or even multi-targeted kinase inhibitors entering clinical trials is increasing. Whether benefits from multi-targeting outweigh increased toxicity liability cannot be assessed at this time, due to our persisting poor understanding of the side-effect issues of broader kinase inhibition. Another distinguishing factor between antibodies and small molecules is off-target toxicity due to unpredictable drug interference with mechanisms unrelated to the intended mechanism of action. In this respect, small molecules must be considered as being potentially disadvantageous. Toxicity from antibodies is usually mechanism-based; there are few known occurrences of random toxicities other than occasional hypersensitivity/anaphylactoid reactions. In contrast, small molecules carry a larger risk of toxicity because of their higher propensity to randomly interact with proteins and thus potentially interfere with unintended biological regulatory systems. Most likely, the relative advantages and disadvantages of anti-VEGFR2 antibodies and small molecule inhibitors will be determined in advanced clinical trials, and winners or losers will emerge randomly for some time to come. Antibodies, due to their specificity and relative lack of toxicity will probably advance through clinical trials more predictably, and increasingly establish their presence in cancer therapy. However, the clinical and commercial success of Gleevec® (imatinib mesylate), a multi-targeted kinase inhibitor of the bcr-abl, kit, and PDGFR kinases for treatment of chronic myeloid leukemia and gastrointestinal stromal tumors, has inspired drug developers who now seek to duplicate this success with other compounds against other cancer-relevant kinases, including angiogenic kinases.

24.5.2 Potential for Adverse Effects

VEGFR pathway inhibitors and all other angiogenesis inhibitors share a potential for obvious adverse effects with regard to required blood vessel formation, e.g., during wound healing or the female reproductive cycle. Bevacizumab has been reported to impair surgical wound healing in colorectal patients (Scappaticci et al. 2005) and bone growth in monkeys (Ryan et al. 1999), and anti-VEGF and -VEGFR2 antibodies inhibit angiogenesis-dependent female reproductive functions in monkeys (Zimmermann et al. 2001b, 2002) and mice (Zimmermann et al. 2001a, 2003; Pauli et al. 2005). These side effects are generally of little clinical concern because situations that would give rise to them are usually avoidable or manageable. From the now considerable experience with adverse effects from bevacizumab treatment it is apparent, however, that certain similar toxicities are likely to occur with VEGFR2 pathway inhibitors as well, including hypertension, proteinuria, and bleeding. There is evidence that hypertension and proteinuria are mechanism-based toxicities. Hypertension is believed to be caused by inhibition of VEGFR2-mediated release of nitrous oxide from endothelial cells (Jin et al. 2003). Proteinuria may be a result of interfering with normal VEGFR2-regulated permeability function of fenestrated endothelium in the kidney (Kitamoto et al. 2001; Sugimoto et al. 2003). These adverse effects were generally mild to moderate with bevacizumab and reversible upon cessation of therapy (Gordon and Cunningham 2005).

More importantly, the trials with bevacizumab revealed rare and sometimes severe adverse events, including gastrointestinal perforations, thromboembolic events and life-threatening or lethal bleeding. The mechanisms that lead to minor bleeding and gastrointestinal perforations are not clear. The cause of thromboembolism in patients undergoing combination therapy involving anti-VEGF antibodies is also unclear. Severe bleeding events, mostly seen in small-cell lung cancer where they tend to occur in the larger airways, may be related to destabilization and sudden failure of large but immature blood vessels in the core of tumors when the tumor regresses quickly in response to therapy. There is now also concern about increased risk of congestive heart failure (Jain et al. 2006) caused by combination treatments involving bevacizumab. Although rare, this toxicity is potentially life-threatening, and it seems to be mechanism-based. Inhibition of both VEGFR1 and VEGFR2 pathways has been shown to impair collateral blood vessel formation in animal models of ischemia (Luttun et al. 2002; Fernandez et al. 2004). Cancer patients undergoing treatment with anti-VEGF/R therapy who also have ischemic heart disease may therefore have an elevated risk of related adverse events.

Will VEGFR2 inhibitors have adverse event profiles similar to those of bevacizumab? Our understanding of VEGFR biology would suggest that there is at least the potential for comparable toxicity. Perhaps VEGFR2-based therapies may not show certain toxicities if these are based on a mechanism involving VEGF-R1. This could be true, for example, with cancer patients who have ischemic heart disease. As mentioned above, collateral vessel formation may be dependent not only on VEGFR2, but also on VEGFR1. The VEGFR1-specific ligand VEGF-B is expressed strongly in myocardial tissue (Oloffson et al. 1996) and appears to be involved in normal cardiac and coronary vessel function (Bellomo et al. 2000; Aase et al. 2001). It is therefore possible that treatment with bevacizumab, which blocks both the VEGFR1 and VEGFR2 pathways, would have a greater detrimental effect than therapy with a pure VEGFR2 inhibitor in patients with heart ischemia. VEGFR1 also mediates bone marrow reconstitution after chemotherapy-induced myelosuppression, and it is thus conceivable that drugs interfering with VEGFR1 signaling might cause clinically undesirable impairment of bone marrow recovery in certain therapeutic regimens involving myelosuppressive chemotherapy. If so, an anti-VEGFR2 inhibitor might provide a therapeutic advantage. It must be pointed out, though, that there is currently no evidence that such theoretical arguments have any base in clinical reality.

As our knowledge grows, new findings may alert us to additional causes of potential toxicity. Recently, it has been established that VEGF also acts on neurons and other CNS cells. As with vascular cells, VEGF provides a survival signal to these CNS cells. In test systems in vitro and in vivo, VEGFs have been shown to have neuroprotective properties which seem to be mediated by all VEGF receptors (Zachary 2005; Le Bras et al. 2006). It is currently unknown whether and how such activities could affect clinical safety. Related adverse effects have not been reported so far and may not be of much concern with therapy based on antibodies, because antibodies do not generally penetrate the blood–brain barrier unless it is locally compromised by tumors and events of acute ischemia (stroke). However, interference with VEGF-mediated neuroprotection might be an issue for patients with clinical signs of neurodegeneration who undergo chronic treatment of malignancies with small-molecule VEGFR inhibitors (which cross the blood–brain barrier). Again, this is a purely speculative issue, and it would probably be difficult to demonstrate such risks in the clinic. However, it is possible that animal studies could address this potential risk and provide a basis for assessing the relevant risks of drug classes and individual drugs.

24.5.3 Future Trends in Antibody Technology

Broad acceptance of monoclonal antibodies in cancer therapy has required a long-term, persistent effort to overcome many significant technical hurdles from the discovery of monoclonal antibodies

in mice in the 1970s to the large-scale commercial manufacturing of fully human antibodies and their approval for human therapy by health regulatory agencies worldwide. Today, antibodies are being recognized as highly efficacious and safe drugs, and they are increasingly penetrating the cancer drug market. Antibodies are not without shortcomings, however. They are not orally active and require weekly or biweekly infusion in the setting of cancer therapy; they are difficult and expensive to manufacture, as their production requires specialized technology; and they generate significant cost burdens for consumers and health care systems (costs can be as high as $100,000 per patient-year for some widely used anti-cancer antibodies (*New York Times*, 15 February 2006).

Therefore, the use of antibodies in the clinic will continue to evolve. For example, additional function-blocking antibodies against other cancer targets will win regulatory approval. Monoclonal antibodies or fragments thereof will also increasingly be used as the "magic bullets" for delivery of cytotoxic agents to tumor cells with high specificity. While the magic-bullet concept constituted the original therapeutic promise of antibodies, and a few such antibodies are now marketed, progress has been slow in this area because of difficulties with cytotoxic agents as well as limited target choices in the pre-genome environment. With improvements in technology, target selection, and the rather finite availability of opportunities for highly effective function-blocking antibodies, efforts to generate magic bullets are continuing. In the context of tumor angiogenesis, VEGFR2 has attracted interest as a target for magic bullets owing to its selective expression on tumor endothelium (Backer and Backer 2001; Backer et al. 2002; Renno et al. 2004). It is questionable, however, whether potentially significant toxicity hurdles posed by the presence of background levels of VEGFR2 in normal vasculature and high expression in some specialized vascular beds (e.g., in kidney glomeruli) can be overcome by most approaches.

Increasingly, there is a perception of potentially increased therapeutic value for therapies that combine two biological treatments (e.g., two antibodies against two tumor targets), or are based on multi-targeted small molecules. Antibody engineering techniques allow the combination of different target-recognizing antibody domains (scFv or fab fragments) in a single molecule in various ways (e.g., Zuo et al. 2000; Lu et al. 2002b, 2003a). With such bispecific antibodies a single molecule can simultaneously inhibit two different functions of a tumor cells, thus increasing its anti-tumor activity. Bispecific tetravalent antibodies have been tested in vivo in mouse xenograft tumor models and in some instances shown to be as efficacious as the treatment with the two corresponding monospecific antibodies, and more efficacious than each mono-specific antibody alone (Lu et al. 2005). In proof-of-concept studies, several bispecific antibodies related to VEGF receptors have been created in various formats, combining VEGFR1/R2 (Lu et al. 2001) and VEGFR2/R3 (Jimenez et al. 2005). They possess the expected in vitro characteristics (comparable potency in regard to monospecific antibodies); however, they are directed against human receptors and thus largely unsuitable for study in standard animal tumor models for assessment of their supposedly superior efficacy. Furthermore, the production of large quantities of such antibodies is still difficult. Regardless, bispecific antibodies may be of considerable clinical interest since they offer the potential to increase the clinical utility of antibody drugs.

Improved gene transfer methods targeting specific cell types may lead to increased generation and in vivo testing of novel engineered antibodies, the so-called intrabodies. Intrabodies are expressed in cells of interest after therapeutic transfer of antibody genes. These antibodies can be made in various formats, ranging from single-domain antibodies to bispecific diabodies. Importantly, intrabodies can be targeted against intracellular targets, for example, phosphorylated or unphosphorylated mediators of signal transduction, nuclear receptors and transcription factors (Kontermann 2004; Paz et al. 2005; Stocks 2005). In the context of tumor angiogenesis and VEGF receptors, there are several encouraging studies which demonstrate the feasibility of this approach (Jendreyko et al. 2003;

Wheeler et al. 2003; Popkov et al. 2005; Boldicke et al. 2005). If technical hurdles, e.g., therapeutic gene transfer, can be overcome, intrabodies may offer attractive opportunities for the development of safe drugs against many targets that are currently only reachable with small-molecule drugs, as well as intracellular protein targets that cannot readily be attacked with small-molecule drugs.

Finally, as standard production of monoclonal antibodies is expensive, efforts to develop more cost-effective production methods will be important. In this respect, transgenic methods for large-scale protein expression in the milk of farm animals, in chicken eggs, or in the roots, leaves, or seed of various plants offer promise. Antibodies have been produced in these organisms and studied in considerable detail, but development remains slow due to significant regulatory hurdles. The feasibility of high-level expression of a full IgG therapeutic antibody in corn seed has been demonstrated (Ludwig et al. 2004). In this study, the function-blocking anti-EGFR antibody cetuximab was produced in aglycosylated and glycosylated forms and compared to the mammalian cell culture-produced and FDA-approved parent antibody cetuximab. The two forms of antibody expressed at high levels in corn seed were indistinguishable in potency in inhibiting tumor cell proliferation in vitro, and both possessed strong anti-tumor activity in vivo. Acute-dose primate pharmacokinetic studies, however, revealed a marked increase in clearance for the glycosylated corn antibody, while the aglycosylated antibody possessed in vivo kinetics similar to those of conventionally produced cetuximab. The study established that corn-derived function-blocking monoclonal antibodies possess comparable efficacy to mammalian cell culture-derived antibody, and offer a cost-effective alternative to large-scale mammalian cell culture production.

Conclusions

Antibodies are superior tools for in vivo biological investigation because of their nearly absolute specificity for the intended target. Function-blocking antibodies against the VEGF ligand and its receptors have proved to be uniquely valuable tools for the elucidation of the rather complex biology of VEGF and its receptors in tumor development and other physiological or pathological conditions. Function-blocking anti-receptor antibodies have also proved to be important in defining the pharmacology of VEGFR signaling inhibition to an extent difficult or impossible to achieve with other means such as anti-ligand antibodies or small-molecule VEGFR kinase inhibitors.

Antibodies have come a long way from being research tools to becoming major therapies for human disease. The regulatory approval of the anti-VEGF antibody bevacizumab has provided important clinical validation of the larger concept of inhibiting tumor angiogenesis as a novel way to help combat cancer. It has also validated the VEGF/VEGFR signaling as a target of choice. The validation of the hypothesis that function-blocking antibodies could be used as therapeutics has contributed to important shifts in the approach to treatment of cancer, namely the addition to standard cytotoxic therapy of less toxic agents that interfere with tumor mechanisms. Although now widely recognized as promising, the field of anti-angiogenic cancer therapeutics is still in an early stage of development. Only a few antibodies are approved for therapeutic use or nearing such regulatory clearance, and approval is still limited to relatively few indications. VEGFR2 pathway inhibitory antibodies show broad therapeutic promise. It remains to be seen how other antibodies against additional angiogenesis targets will add benefits over anti-VEGFR2 therapies.

References

Aase K, von Euler G, Li X, Ponten A, Thoren P, Cao R, Cao Y, Oloffson B, Gebre-Medhin S, Pekay M, Alitalo K (2001) Vascular endothelial growth factor B-deficient mice display an atrial conduction defect. Circulation 104:358–364

Alitalo K, Carmeliet P (2002) Molecular mechanisms of lymphangiogenesis in health and disease. Cancer Cell 1:219–227

Alitalo K, Tammela T, Petrova T (2005) Lymphangiogenesis in development and human disease. Nature 438:946–953

Autiero M, Waltenberger J, Communi D, Kranz A, Moons L, Lambrechts D, Kroll J, Plaisance S, De Mol M, Bono F, Kliche S, Fellbrich G, Ballmer-Hofer K, Maglione D, Mayr-Beyrle U, Dewerchin M, Dombrowski S, Stanimirovic D, Van Hummelen P, Dehio C, Hicklin D, Persico G, Herbert J, Communi D, Shibuya M, Collen D, Conway E, Carmeliet P (2003) Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. Nat Med 9:936–943

Backer M, Backer J (2001) Targeting endothelial cells overexpressing VEGFR-2: selective toxicity of Shiga-like toxin-VEGF fusion proteins. Bioconjug Chem 12:1066–1073

Backer M, Aloise R, Przekop K, Stoletov K, Backer J (2002) Molecular vehicles for targeted drug delivery. Bioconjug Chem 13:462–467

Backer M, Elliot J, Gaynutdinov T, Backer J (2004) Assembly of targeting complexes driven by a single chain antibody. J Immunol Methods 289:37–45

Baluk P, Tammela T, Ator E, Lyubynska N, Achen M, Hicklin D, Jeltsch M, Petrova T, Pytowski B, Stacker S, Yla-Herttuala S, Jackson D, Alitalo K, McDonald D (2005) Pathogenesis of persistent lymphatic vessel hyperplasia in chronic airway inflammation. J Clin Invest 115:247–257

Bellomo D, Headrick J, Silins G, Paterson C, Thomas P, Gartside M, Mould A, Cahill M, Tonks I, Grimmond S, Townson S, Wells C, Little M, Cummings M, Hayward N, Kay G (2000) Mice lacking the vascular endothelial growth factor B gene (Vegfb) have smaller hearts, dysfunctional coronary vasculature, and impaired recovery from cardiac ischemia. Circ Res 86:E29–E35

Boldicke T, Tesar M, Griesel C, Rohde M, Grone HJ, Waltenberger J, Kollet O, Lapidot T, Yayon A, Weich H (2001) Anti-VEGFR-2 scFvs for cell isolation. Single-chain antibodies recognizing the human vascular endothelial growth factor receptor-2 (VEGFR-2/flk-1) on the surface of primary endothelial cells and preselected CD34+ cells from cord blood. Stem Cells 19:24–36

Boldicke T, Weber H, Mueller P, Barleon B, Bernal M (2005) Novel highly efficient intrabody mediates complete inhibition of cell surface expression of the human vascular endothelial growth factor receptor-2 (VEGFR-2/KDR). J Immunol Methods 300:146–159

Bruns C, Liu W, Davis D, Shaheen, McConkey D, Wilson M, Bucana C, Hicklin D, Ellis L (2000) Vascular endothelial growth factor is an in vivo survival factor for tumor endothelium in a murine model of colorectal carcinoma liver metastases. Cancer 89:488–499

Bruns C, Shrader M, Harbison M, Portera C, Solorzano C, Jauch K, Hicklin D, Radinsky R, Ellis L (2002) Effect of the vascular endothelial growth factor receptor-2 antibody DC101 plus gemcitabine on growth, metastasis and angiogenesis of human pancreatic cancer growing orthotopically in nude mice. Int J Cancer 102:101–108

Cai J, Ahmad S, Jiang W, Huang J, Kontos C, Boulton M, Ahmed A (2003) Activation of vascular endothelial growth factor receptor-1 sustains angiogenesis and Bcl-2 expression via the phosphatidylinositol 3-kinase pathway in endothelial cells. Diabetes 52:2959–2968

Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De Mol M, Wu Y, Bono F, Devy L, Beck H, Scholz D, Acker T, DiPalma T, Dewerchin M, Noel A, Stalmans I, Barra A, Blacher S, Vandendriessche T, Ponten A, Eriksson U, Plate K, Foidart J, Schaper W, Charnock-Jones D, Hicklin D, Herbert JM, Collen D, Persico M (2001) Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat Med 7:575–583

Casanovas O, Hicklin DJ, Bergers G, Hanahan D (2005) Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. Cancer Cell 8:299–309

Davis D, Inoue K, Dinney C, Hicklin D, Abbruzzese J, McConkey D (2004) Regional effects of an antivascular endothelial growth factor receptor monoclonal antibody on receptor phosphorylation and apoptosis in human 253 J B-V bladder cancer xenografts. Cancer Res 64:4601–4610

De Bandt M, Ben Mahdi MH, Ollivier V, Grossin M, Dupuis M, Gaudry M, Bohlen P, Lipson K, Rice A, Wu Y, Gougerot-Pocidalo M, Pasquier C (2003) Blockade of vascular endothelial growth factor receptor I (VEGF-RI), but not VEGF-RII, suppresses joint destruction in the K/BxN model of rheumatoid arthritis. J Immunol 171:4853–4859

Dias S, Hattori K, Zhu Z, Heissig B, Choy M, Lane W, Wu Y, Chadburn A, Hyjek E, Gill M, Hicklin D, Witte L, Moore M, Rafii S (2000) Autocrine stimulation of VEGFR-2 activates human leukemic cell growth and migration. J Clin Invest 106:511–521

Dias S, Hattori K, Heissig B, Zhu Z, Wu Y, Witte L, Hicklin D, Tateno M, Bohlen P, Moore MA, Rafii S (2001) Inhibition of both paracrine and autocrine VEGF/ VEGFR-2 signaling pathways is essential to induce long-term remission of xenotransplanted human leukemias. Proc Natl Acad Sci USA 98:10857–10862

Fan F, Wey J, McCarty M, Belcheva A, Liu W, Bauer T, Somcio R, Wu Y, Hooper A, Hicklin D, Ellis L (2005) Expression and function of vascular endothelial growth factor

receptor-1 on human colorectal cancer cells. Oncogene 24:2647–2653

Fenton B, Paoni S, Ding I (2004a) Effect of VEGF receptor-2 antibody on vascular function and oxygenation in spontaneous and transplanted tumors. Radiother Oncol 72:221–230

Fenton B, Paoni S, Ding I (2004b) Pathophysiological effects of vascular endothelial growth factor receptor-2-blocking antibody plus fractionated radiotherapy on murine mammary tumors. Cancer Res 64:5712–5719

Fernandez M, Vizzutti F, Garcia-Pagan J, Rodes J, Bosch J (2004) Anti-VEGF receptor-2 monoclonal antibody prevents portal-systemic collateral vessel formation in portal hypertensive mice. Gastroenterology 126:886–894

Ferrara N (2004) Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev 25:581–611

Fiedler W, Graeven U, Ergun S, Verago S, Kilic N, Stockschlader M, Hossfeld D (1997) Vascular endothelial growth factor, a possible paracrine growth factor in human acute myeloid leukemia. Blood 89:1870–1875

Fragoso R, Pereira T, Wu Y, Zhu Z, Cabecadas J, Dias S (2006) VEGFR-1 (FLT-1) activation modulates acute lymphoblastic leukemia localization and survival within the bone marrow, determining the onset of extramedullary disease. Blood 107:1608–1616 (erratum in: Blood 2006; 107:3057)

Gong H, Pottgen C, Stuben G, Havers W, Stuschke M, Schweigerer L (2003) Arginine deiminase and other antiangiogenic agents inhibit unfavorable neuroblastoma growth: potentiation by irradiation. Int J Cancer 106:723–728

Gordon M, Cunningham D (2005) Managing patients treated with bevacizumab combination therapy. Oncology 69 [Suppl 3]:25–33

Gothert J, Gustin S, van Eekelen J, Schmidt U, Hall M, Jane S, Green A, Gottgens B, Izon D, Begley C (2004) Genetically tagging endothelial cells in vivo: bone marrow-derived cells do not contribute to tumor endothelium. Blood 104:1769–1777

Hansen-Algenstaedt N, Stoll B, Padera T, Dolmans D, Hicklin D, Fukumura D, Jain R (2000) Tumor oxygenation in hormone-dependent tumors during vascular endothelial growth factor receptor-2 blockade, hormone ablation, and chemotherapy. Cancer Res 60:4556–4560 (erratum in: Cancer Res 2001; 61:6304)

Hattori K, Heissig B, Wu Y, Dias S, Tejada R, Ferris B, Hicklin D, Zhu Z, Bohlen P, Witte L, Hendrikx J, Hackett N, Crystal R, Moore M, Werb Z, Lyden D, Rafii S (2002) Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. Nat Med 8:841–849

He Y, Karpanen T, Alitalo K (2004) Role of lymphangiogenic factors in tumor metastasis. Biochim Biophys Acta 1654:3–12

Heissig B, Rafii S, Akiyama H, Ohki Y, Sato Y, Rafael T, Zhu Z, Hicklin D, Okumura K, Ogawa H, Werb Z, Hattori K (2005) Low-dose irradiation promotes tissue revascularization through VEGF release from mast cells and MMP-9-mediated progenitor cell mobilization. J Exp Med 202:739–750

Hicklin D, Ellis L (2001) Effects of an antibody to vascular endothelial growth factor receptor-2 on survival, tumor vascularity, and apoptosis in a murine model of colon carcinomatosis. Int J Oncol 18:221–226

Hicklin D, Ellis L (2005) Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. J Clin Oncol 23:1011–1027

Hiratsuka S, Nakamura K, Iwai S, Murakami M, Itoh T, Kijima H, Shipley J, Senior R, Shibuya M (2002) MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis. Cancer Cell 2:289–300

Inoue K, Slaton J, Davis D, Hicklin D, McConkey D, Karashima T, Radinsky R, Dinney C (2000) Treatment of human metastatic transitional cell carcinoma of the bladder in a murine model with the anti-vascular endothelial growth factor receptor monoclonal antibody DC101 and paclitaxel. Clin Cancer Res 6:2635–2643

Izumi Y, Di Tomaso E, Hooper A, Huang P, Huber J, Hicklin D, Fukumura D, Jain R, Suit H (2003) Responses to antiangiogenesis treatment of spontaneous autochthonous tumors and their isografts. Cancer Res 63:747–751

Jain R, Duda D, Clark J, Loeffler J (2006) Lessons from phase III clinical trials on anti-VEGF therapy for cancer. Nat Clin Pract Oncol 3:24–40

Jendreyko N, Popkov M, Beerli R, Chung J, McGavern D, Rader C, Barbas C (2003) Intradiabodies: bispecific, tetravalent antibodies for the simultaneous functional knockout of two cell surface receptors. J Biol Chem 278:47812–47819

Jimenez X, Lu D, Brennan L, Persaud K, Liu M, Miao H, Witte L, Zhu Z (2005) A recombinant, fully human, bispecific antibody neutralizes the biological activities mediated by both vascular endothelial growth factor receptors 2 and 3. Mol Cancer Ther 4:427–434

Jin Z, Ueba H, Tanimoto T, Lungu A, Frame M, Berk B (2003) Ligand-independent activation of vascular endothelial growth factor receptor 2 by fluid shear stress regulates activation of endothelial nitric oxide synthase. Circ Res 93:354–363

Jung Y, Mansfield P, Akagi M, Takeda A, Liu W, Bucana C, Hicklin D, Ellis L (2002) Effects of combination anti-vascular endothelial growth factor receptor and anti-epidermal growth factor receptor therapies on the growth of gastric cancer in a nude mouse model. Eur J Cancer 38:1133–1140

Jussila L, Alitalo K (2002) Vascular growth factors and lymphangiogenesis. Physiol Rev 82:673–700

Kanno S, Oda N, Abe M, Terai Y, Ito M, Shitara K, Tabayashi K, Shibuya M, Sato Y (2000) Roles of two VEGF receptors, Flt-1 and KDR, in the signal transduction of VEGF

effects in human vascular endothelial cells. Oncogene 19:2138–2146

Karpanen T, Egeblad M, Karkkainen M, Kubo H, Yla-Herttuala S, Jaattela M, Alitalo K (2001) Vascular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. Cancer Res 61:1786–1790

Kiessling F, Farhan N, Lichy M, Vosseler S, Heilmann M, Krix M, Bohlen P, Miller D, Mueller M, Semmler W, Fusenig N (2004) Dynamic contrast-enhanced magnetic resonance imaging rapidly indicates vessel regression in human squamous cell carcinomas grown in nude mice caused by VEGF receptor 2 blockade with DC101. Neoplasia 6:213–223

Kitamoto Y, Takeya M, Tokunaga H, Tomita K (2001) Glomerular endothelial cells are maintained by vascular endothelial growth factor in the adult kidney. Tohoku J Exp Med 195:43–54

Klement G, Baruchel S, Rak J, Man S, Clark K, Hicklin D, Bohlen P, Kerbel R (2000) Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. J Clin Invest 105:R15–R24

Klement G, Huang P, Mayer B, Green SK, Man S, Bohlen P, Hicklin D, Kerbel R (2002) Differences in therapeutic indexes of combination metronomic chemotherapy and an anti-VEGFR-2 antibody in multidrug-resistant human breast cancer xenografts. Clin Cancer Res 8:221–232

Kontermann R (2004) Intrabodies as therapeutic agents. Methods 34:163–170

Koolwijk P, Peters E, van der Vecht B, Hornig C, Weich H, Alitalo K, Hicklin D, Wu Y, Witte L, van Hinsbergh V (2001) Involvement of VEGFR-2 (kdr/flk-1) but not VEGFR-1 (flt-1) in VEGF-A and VEGF-C-induced tube formation by human microvascular endothelial cells in fibrin matrices in vitro. Angiogenesis 4:53–60

Kou R, SenBanerjee S, Jain M, Michel T (2005) Differential regulation of vascular endothelial growth factor receptors (VEGFR) revealed by RNA interference: interactions of VEGFR-1 and VEGFR-2 in endothelial cell signaling. Biochemistry 44:15064–15073

Kozin S, Boucher Y, Hicklin D, Bohlen P, Jain R, Suit H (2001) Vascular endothelial growth factor receptor-2-blocking antibody potentiates radiation-induced long-term control of human tumor xenografts. Cancer Res 61:39–44

Krix M, Kiessling F, Vosseler S, Farhan N, Mueller M, Bohlen P, Fusenig N, Delorme S (2003) Sensitive noninvasive monitoring of tumor perfusion during antiangiogenic therapy by intermittent bolus-contrast power Doppler sonography. Cancer Res 63:8264–8270

Kubo H, Fujiwara T, Jussila L, Hashi H, Ogawa M, Shimizu K, Awane M, Sakai Y, Takabayashi A, Alitalo K, Yamaoka Y, Nishikawa S (2000) Involvement of vascular endothelial growth factor receptor-3 in maintenance of integrity of endothelial cell lining during tumor angiogenesis. Blood 96:546–553

Kubo H, Cao R, Brakenhielm E, Makinen T, Cao Y, Alitalo K (2002) Blockade of vascular endothelial growth factor receptor-3 signaling inhibits fibroblast growth factor-2-induced lymphangiogenesis in mouse cornea. Proc Natl Acad Sci USA 99:8868–8873

Kunkel P, Ulbricht U, Bohlen P, Brockmann MA, Fillbrandt R, Stavrou D, Westphal M, Lamszus K (2001) Inhibition of glioma angiogenesis and growth in vivo by systemic treatment with a monoclonal antibody against vascular endothelial growth factor receptor-2. Cancer Res 61:6624–6628

Kunstfeld R, Hirakawa S, Hong YK, Schacht V, Lange-Asschenfeldt B, Velasco P, Lin C, Fiebiger E, Wei X, Wu Y, Hicklin D, Bohlen P, Detmar M (2004) Induction of cutaneous delayed-type hypersensitivity reactions in VEGF-A transgenic mice results in chronic skin inflammation associated with persistent lymphatic hyperplasia. Blood 104:1048–1057

Lamszus K, Brockmann M, Eckerich C, Bohlen P, May C, Mangold U, Fillbrandt R, Westphal M (2005) Inhibition of glioblastoma angiogenesis and invasion by combined treatments directed against vascular endothelial growth factor receptor-2, epidermal growth factor receptor, and vascular endothelial-cadherin. Clin Cancer Res 11:4934–4940

Le Bras B, Barallobre MJ, Homman-Ludiye J, Ny A, Wyns S, Tammela T, Haiko P, Karkkainen MJ, Yuan L, Muriel MP, Chatzopoulou E, Breant C, Zalc B, Carmeliet P, Alitalo K, Eichmann A, Thomas J (2006) VEGF-C is a trophic factor for neural progenitors in the vertebrate embryonic brain. Nat Neurosci 9:340–348

Lewis C, Murdoch C (2005) Macrophage responses to hypoxia: implications for tumor progression and anti-cancer therapies. Am J Pathol 167:627–635

Lewis C, Pollard J (2006) Distinct role of macrophages in different tumor microenvironments. Cancer Res 66:605–612

Li J, Huang S, Armstrong E, Fowler JF, Harari P (2005) Angiogenesis and radiation response modulation after vascular endothelial growth factor receptor-2 (VEGFR2) blockade. Int J Radiat Oncol Biol Phys 62:1477–1485

Li L, Wartchow C, Danthi S, Shen Z, Dechene N, Pease J, Choi H, Doede T, Chu P, Ning S, Lee D, Bednarski M, Knox S (2004a) A novel antiangiogenesis therapy using an integrin antagonist or anti-Flk-1 antibody coated 90Y-labeled nanoparticles. Int J Radiat Oncol Biol Phys 58:1215–1227

Li R, Xiong D, Shao X, Liu J, Xu Y, Xu Y, Liu H, Zhu Z, Yang C (2004b) Production of neutralizing monoclonal antibody against human vascular endothelial growth factor receptor II. Acta Pharmacol Sin 25:1292–1298

List A, Glinsmann-Gibson B, Stadheim C, Meuillet E, Bellamy W, Powis G (2004) Vascular endothelial growth factor receptor-1 and receptor-2 initiate a phosphatidylinositide 3-kinase-dependent clonogenic response in acute myeloid leukemia cells. Exp Hematol 32:526–535

Lu D, Kussie P, Pytowski B, Persaud K, Bohlen P, Witte L, Zhu Z (2000) Identification of the residues in the extracellular region of KDR important for interaction with vascular endothelial growth factor and neutralizing anti-KDR antibodies. J Biol Chem 275:14321–14330

Lu D, Jimenez X, Zhang H, Wu Y, Bohlen P, Witte L, Zhu Z (2001) Complete inhibition of vascular endothelial growth factor (VEGF) activities with a bifunctional diabody directed against both VEGF kinase receptors, fms-like tyrosine kinase receptor and kinase insert domain-containing receptor. Cancer Res 61:7002–7008

Lu D, Jimenez X, Zhang H, Bohlen P, Witte L, Zhu Z (2002a) Selection of high affinity human neutralizing antibodies to VEGFR2 from a large antibody phage display library for antiangiogenesis therapy. Int J Cancer 97:393–399

Lu D, Jimenez X, Zhang H, Bohlen P, Witte L, Zhu Z (2002b) Fab-scFv fusion protein: an efficient approach to production of bispecific antibody fragments. J Immunol Methods 267:213–226

Lu D, Jimenez X, Zhang H, Atkins A, Brennan L, Balderes P, Bohlen P, Witte L, Zhu Z (2003a) Di-diabody: a novel tetravalent bispecific antibody molecule by design. J Immunol Methods 279:219–232

Lu D, Shen J, Vil M, Zhang H, Jimenez X, Bohlen P, Witte L, Zhu Z (2003b) Tailoring in vitro selection for a picomolar affinity human antibody directed against vascular endothelial growth factor receptor 2 for enhanced neutralizing activity. J Biol Chem 278:43496–43507

Lu D, Zhang H, Koo H, Tonra J, Balderes P, Prewett M, Corcoran E, Mangalampalli V, Bassi R, Anselma D, Patel D, Kang X, Ludwig DL, Hicklin D, Bohlen P, Witte L, Zhu Z (2005) A fully human recombinant IgG-like bispecific antibody to both the epidermal growth factor receptor and the insulin-like growth factor receptor for enhanced antitumor activity. J Biol Chem 280:19665–19672

Ludwig D, Witte L, Hicklin D, Prewett M, Bassi R, Burtrum D, Pereira D, Jimenez X, Fox F, Saxena B, Zhou Q, Ma Y, Kang X, Patel D, Barry M, Kussie P, Zhu Z, Russell D, Peterson W, Jury T, Gaitan-Gaitan F, Moran D, Delannay X, Storrs B, Tou J, Zupec M, Gustafson K, McIntyre J, Tarnowski J, Bohlen P (2004) Conservation of receptor antagonist antitumor activity by epidermal growth factor receptor antibody expressed in transgenic corn seed. Hum Antibodies 13:81–90

Luttun A, Tjwa M, Moons L, Wu Y, Angelillo-Scherrer A, Liao F, Nagy J, Hooper A, Priller J, de Klerck B, Compernolle V, Daci E, Bohlen P, Dewerchin M, Herbert J, Fava R, Matthys P, Carmeliet G, Collen D, Dvorak H, Hicklin D, Carmeliet P (2002) Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. Nat Med 8:831–840

Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L, Chadburn A, Heissig B, Marks W, Witte L, Wu Y, Hicklin D, Zhu Z, Hackett N, Crystal R, Moore M, Hajjar K, Manova K, Benezra R, Rafii S (2001) Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat Med 7:1194–1201

Man S, Bocci G, Francia G, Green S, Jothy S, Hanahan D, Bohlen P, Hicklin D, Bergers G, Kerbel R (2002) Antitumor effects in mice of low-dose (metronomic) cyclophosphamide administered continuously through the drinking water. Cancer Res 62:2731–2735

Masood R, Kundra A, Zhu S, Xia G, Scalia P, Smith D, Gill P (2003) Malignant mesothelioma growth inhibition by agents that target the VEGF and VEGF-C autocrine loops. Int J Cancer 104:603–610

McLeod D, Taomoto M, Cao J, Zhu Z, Witte L, Lutty G (2002) Localization of VEGF receptor-2 (KDR/Flk-1) and effects of blocking it in oxygen-induced retinopathy. Invest Ophthalmol Vis Sci 43:474–482

Miller D, Vosseler S, Mirancea N, Hicklin D, Bohlen P, Volcker H, Holz F, Fusenig N (2005) Rapid vessel regression, protease inhibition, and stromal normalization upon short-term vascular endothelial growth factor receptor 2 inhibition in skin carcinoma heterotransplants. Am J Pathol 167:1389–1403

Neagoe PE, Lemieux C, Sirois MG (2005) Vascular endothelial growth factor (VEGF)-A165-induced prostacyclin synthesis requires the activation of VEGF receptor-1 and -2 heterodimer. J Biol Chem 280:9904–9912

Nilsson I, Rolny C, Wu Y, Pytowski B, Hicklin D, Alitalo K, Claesson-Welsh L, Wennstrom S (2004) Vascular endothelial growth factor receptor-3 in hypoxia-induced vascular development. FASEB J 18:1507–1515

Ohki Y, Heissig B, Sato Y, Akiyama H, Zhu Z, Hicklin D, Shimada K, Ogawa H, Daida H, Hattori K, Ohsaka A (2005) Granulocyte colony-stimulating factor promotes neovascularization by releasing vascular endothelial growth factor from neutrophils. FASEB J 19:2005–2007

Oloffson B, Pajusola K, Kaipainen A, von Euler G, Joukov V, Saksela O, Orpana A, Petterson R, Alitalo K, Eriksson U (1996) Vascular endothelial growth factor B, a novel growth factor for endothelial cells. Proc Natl Acad Sci USA 93:2576–2581

Pauli S, Tang H, Wang J, Bohlen P, Posser R, Hartman T, Sauer M, Kitajewski J, Zimmermann R (2005) The vascular endothelial growth factor (VEGF)/VEGF receptor 2 pathway is critical for blood vessel survival in corpora lutea of pregnancy in the rodent. Endocrinology 146:1301–1311

Patila T, Ikonen T, Rutanen J, Ahonen A, Lommi J, Lappalainen K, Krogerus L, Ihlberg L, Partanen T, Lahteenoja L, Virtanen K, Alitalo K, Yla-Herttuala S, Harjula A (2006) Vascular endothelial growth factor C-induced collateral formation in a model of myocardial ischemia. J Heart Lung Transplant 25:206–213

Paz K, Brennan L, Iacolina M, Doody J, Hadari Y, Zhu Z (2005) Human single-domain neutralizing intrabodies

directed against Etk kinase: a novel approach to impair cellular transformation. Mol Cancer Ther 4:1801–1809

Pedersen A, Buus S, Claesson M (2005) Treatment of transplanted CT26 tumour with dendritic cell vaccine in combination with blockade of vascular endothelial growth factor receptor 2 and CTLA-4. Cancer Lett May 28 (Epub ahead of print)

Pepper MS, Tille JC, Nisato R, Skobe M (2003) Lymphangiogenesis and tumor metastasis. Cell Tissue Res 314:167–177

Persaud K, Tille JC, Liu M, Zhu Z, Jimenez X, Pereira D, Miao H, Brennan L, Witte L, Pepper M, Pytowski B (2004) Involvement of the VEGF receptor 3 in tubular morphogenesis demonstrated with a human anti-human VEGFR-3 monoclonal antibody that antagonizes receptor activation by VEGF-C. J Cell Sci 117:2745–2756

Popkov M, Jendreyko N, Gonzalez-Sapienza G, Mage R, Rader C, Barbas C (2004) Human/mouse cross-reactive anti-VEGF receptor 2 recombinant antibodies selected from an immune b9 allotype rabbit antibody library. J Immunol Methods 288:149–164

Popkov M, Jendreyko N, McGavern D, Rader C, Barbas C (2005) Targeting tumor angiogenesis with adenovirus-delivered anti-Tie-2 intrabody. Cancer Res 65:972–981

Posey J, Ng T, Yang B, Khazaeli M, Carpenter M, Fox F, Needle M, Waksal H, LoBuglio A (2003) A phase I study of anti-kinase insert domain-containing receptor antibody, IMC-1C11, in patients with liver metastases from colorectal carcinoma. Clin Cancer Res 9:1323–1332

Prewett M, Huber J, Li Y, Santiago A, O'Connor W, King K, Overholser J, Hooper A, Pytowski B, Witte L, Bohlen P, Hicklin J (1999) Antivascular endothelial growth factor receptor (fetal liver kinase 1) monoclonal antibody inhibits tumor angiogenesis and growth of several mouse and human tumors. Cancer Res 59:5209–5218

Pytowski B, Goldman J, Persaud K, Wu Y, Witte L, Hicklin D, Skobe M, Boardman K, Swartz M (2005) Complete and specific inhibition of adult lymphatic regeneration by a novel VEGFR-3 neutralizing antibody. J Natl Cancer Inst 97:14–21

Rabbany S, Heissig B, Hattori K, Rafii S (2003) Molecular pathways regulating mobilization of marrow-derived stem cells for tissue revascularization. Trends Mol Med 9:109–117

Rakhmilevich A, Hooper A, Hicklin D, Sondel P (2004) Treatment of experimental breast cancer using interleukin-12 gene therapy combined with anti-vascular endothelial growth factor receptor-2 antibody. Mol Cancer Ther 3:969–976

Ran S, Huang X, Downes A, Thorpe P (2003) Evaluation of novel antimouse VEGFR2 antibodies as potential antiangiogenic or vascular targeting agents for tumor therapy. Neoplasia 5:297–307

Reichert F, Barak V, Tarshis M, Prindull G, Tarshis E, Ben-Ishay Z (2005) Anti-angiogenic effects and regression of localized murine AML produced by anti-VEGF and anti-Flk-1 antibodies. Eur J Haematol 75:41–46

Renno R, Terada Y, Haddadin M, Michaud N, Gragoudas E, Miller J (2004) Selective photodynamic therapy by targeted verteporfin delivery to experimental choroidal neovascularization mediated by a homing peptide to vascular endothelial growth factor receptor-2. Arch Ophthalmol 122:1002–1011

Ribatti D (2004) The involvement of endothelial progenitor cells in tumor angiogenesis. J Cell Mol Med 8:294–300

Roberts N, Kloos B, Cassella M, Podgrabinska S, Persaud, K, Wu Y, Pytowski B, Skobe M (2006) Inhibition of VEGFR-3 activation with the antagonistic antibody more potently suppresses lymph node and distant metastases than inactivation of VEGFR-2. Cancer Res 66:2650–2657

Robinson S, Coussens L (2005) Soluble mediators of inflammation during tumor development. Adv Cancer Res 93:159–187

Rockwell P, Neufeld G, Glassmann A, Caron D, Goldstein N (1995) In vitro neutralization of vascular endothelial growth factor activation of Flk-1 by a monoclonal antibody. Mol Cell Diff 3:91–109

Rubio Demirovic A, Marty C, Console S, Zeisberger SM, Ruch C, Jaussi R, Schwendener R, Ballmer-Hofer K (2005) Targeting human cancer cells with VEGF receptor-2-directed liposomes. Oncol Rep 13:319–324

Ryan A, Eppler D, Hagler K, Bruner R, Thomford P, Hall R, Shopp G, O'Neill C (1999) Preclinical safety evaluation of rhuMAbVEGF, an antiangiogenic humanized monoclonal antibody. Toxicol Pathol 27:78–86

Scappaticci F, Fehrenbacher L, Cartwright T, Hainsworth J, Heim W, Berlin J, Kabbinavar F, Novotny W, Sarkar S, Hurwitz H (2005) Surgical wound healing complications in metastatic colorectal cancer patients treated with bevacizumab. J Surg Oncol 91:173–180

Seetharam L, Gotoh N, Maru Y, Neufeld G, Yamaguchi S, Shibuya M. (1995) A unique signal transduction from FLT tyrosine kinase, a receptor for vascular endothelial growth factor VEGF. Oncogene 10:135–147

Senger D, van de Water L, Brown L, Nagy J, Yeo K, Yeo T, Berse B, Jackman R, Dvorak A, Dvorak H. (1993) Vascular permeability factor (VPF, VEGF) in tumor biology. Cancer Metastasis Rev 12:303–324

Shaheen R, Ahmad S, Liu W, Reinmuth N, Jung Y, Tseng W, Drazan K, Bucana C, Hicklin D, Ellis L (2001a) Inhibited growth of colon cancer carcinomatosis by antibodies to vascular endothelial and epidermal growth factor receptors. Br J Cancer 85:584–589

Shaheen R, Tseng W, Vellagas R, Liu W, Ahmad S, Jung Y, Reinmuth N, Drazan K, Bucana C, Hicklin D, Ellis L (2001b) Effects of an antibody to vascular endothelial growth factor receptor-2 on survival, tumor vascularity, and apoptosis in a murine model of colon carcinomatosis. Int J Oncol 18:221–226

Sho M, Akashi S, Kanehiro H, Hamada K, Kashizuka H,

Ikeda N, Nomi T, Kuzumoto Y, Tsurui Y, Yoshiji H, Wu Y, Hicklin D, Briscoe DM, Nakajima Y (2005) Function of the vascular endothelial growth factor receptors Flt-1 and Flk-1/KDR in the alloimmune response in vivo. Transplantation 80:717–722

Silvestre JS, Tamarat R, Ebrahimian T, Le-Roux A, Clergue M, Emmanuel F, Duriez M, Schwartz B, Branellec D, Levy B (2003) Vascular endothelial growth factor-B promotes in vivo angiogenesis. Circ Res 93:114–123

Skobe M, Rockwell P, Goldstein N, Vosseler S, Fusenig N (1997) Halting angiogenesis suppresses carcinoma cell invasion. Nat Med 3:1222–1227

Stacker S, Achen M, Jussila L, Baldwin M, Alitalo K (2002) Lymphangiogenesis and cancer metastasis. Nat Rev Cancer 2:573–583

Stefanik D, Fellows W, Rizkalla L, Rizkalla W, Stefanik P, Deleo A, Welch W (2001) Monoclonal antibodies to vascular endothelial growth factor (VEGF) and the VEGF receptor, FLT-1, inhibit the growth of C6 glioma in a mouse xenograft. J Neurooncol 55:91–100

Stocks M (2005) Intrabodies as drug discovery tools and therapeutics. Curr Opin Chem Biol 9:359–365

Stoelcker B, Echtenacher B, Weich H, Sztajer H, Hicklin D, Mannel D (2000) VEGF/Flk-1 interaction, a requirement for malignant ascites recurrence. J Interferon Cytokine Res 20:511–517

Sugimoto H, Hamano Y, Charytan D, Cosgrove D, Kieran M, Sudhakar A, Kalluri R (2003) Neutralization of circulating vascular endothelial growth factor (VEGF) by anti-VEGF antibodies and soluble VEGF receptor 1 (sFlt-1) induces proteinuria. J Biol Chem 278:12605–12608

Sweeney P, Karashima T, Kim S, Kedar D, Mian B, Huang S, Baker C, Fan Z, Hicklin D, Pettaway C, Dinney C (2002) Anti-vascular endothelial growth factor receptor 2 antibody reduces tumorigenicity and metastasis in orthotopic prostate cancer xenografts via induction of endothelial cell apoptosis and reduction of endothelial cell matrix metalloproteinase type 9 production. Clin Cancer Res 8:2714–2724

Tong R, Boucher Y, Kozin S, Winkler F, Hicklin D, Jain R (2004) Vascular normalization by vascular endothelial growth factor receptor 2 blockade induces a pressure gradient across the vasculature and improves drug penetration in tumors. Cancer Res 64:3731–3736

Tonra J, Deevi D, Joynes C, Li H, Rong L, Bassi R, Persaud K, Prewett M, Carrick F, Steiner P, Hicklin D, Pytowski B (2005) Effects of an antibody to VEGFR-3, mF4-31C1, on tumor growth in mouse models. Clin Cancer Res 11:9042s (abstract B44)

Tsurusaki T, Kanda S, Sakai H, Kanetake H, Saito Y, Alitalo K, Koji T. (1999) Vascular endothelial growth factor-C expression in human prostatic carcinoma and its relationship to lymph node metastasis. Br J Cancer 80:309–313

Vincent L, Jin D, Karajannis M, Shido K, Hooper A, Rashbaum W, Pytowski B, Wu Y, Hicklin D, Zhu Z, Bohlen P, Niesvizky R, Rafii S (2005) Fetal stromal-dependent paracrine and intracrine vascular endothelial growth factor-a/vascular endothelial growth factor receptor-1 signaling promotes proliferation and motility of human primary myeloma cells. Cancer Res 65:3185–3192

Vosseler S, Mirancea N, Bohlen P, Mueller M, Fusenig N (2005) Angiogenesis inhibition by vascular endothelial growth factor receptor-2 blockade reduces stromal matrix metalloproteinase expression, normalizes stromal tissue, and reverts epithelial tumor phenotype in surface heterotransplants. Cancer Res 65:1294–1305

Wang E, Teruya-Feldstein J, Wu Y, Zhu Z, Hicklin D, Moore M (2004) Targeting autocrine and paracrine VEGF receptor pathways inhibits human lymphoma xenografts in vivo. Blood 104:2893–2902

Weis S, Cheresh D (2005) Pathophysiological consequences of VEGF-induced vascular permeability. Nature 437:397–504

Wey J, Fan F, Gray M, Bauer T, McCarty M, Somcio R, Liu W, Evans D, Wu Y, Hicklin D, Ellis L (2005) Vascular endothelial growth factor receptor-1 promotes migration and invasion in pancreatic carcinoma cell lines. Cancer 104:427–438

Wheeler Y, Kute T, Willingham M, Chen S, Sane D (2003) Intrabody-based strategies for inhibition of vascular endothelial growth factor receptor-2: effects on apoptosis, cell growth, and angiogenesis. FASEB J 17:1733–1735

Winkler F, Kozin S, Tong R, Chae S, Booth M, Garkavtsev I, Xu L, Hicklin D, Fukumura D, di Tomaso E, Munn L, Jain R (2004) Kinetics of vascular normalization by VEGFR2 blockade governs brain tumor response to radiation: role of oxygenation, angiopoietin-1, and matrix metalloproteinases. Cancer Cell 6:553–563

Witte L, Hicklin D, Zhu Z, Pytowski B, Kotanides H, Rockwell P, Bohlen P (1998) Monoclonal antibodies targeting the VEGF receptor-2 (FLK-1/KDR) as an anti-angiogenic therapeutic strategy. Cancer Metastasis Rev 17:155–161

Wu Y, Abdullah R, Bassi R, Hooper A, Overholser J, Jimenez X, Navarro E, Persaud K, Pytowski B, Witt, L, Bohlen P, Hicklin D (2001) Inhibition of tumor growth and angiogenesis in a mouse model by a neutralizing anti-flt1 monoclonal antibody. Proc Am Assoc Cancer Res 42 abstr 4436

Wu Y, Zhong Z, Hooper A, Li H, Finnerty B, Bassi R, Zhu Z, Witte L, Bohlen P, Rafii S, Hicklin D (2003) Monoclonal antibody against VEGFR1 directly inhibits growth of human breast tumors. Proc Am Assoc Cancer Res 44 abstr 6340

Wu Y, Zhong Z, Li H, Huber,J, Bassi R, Hooper A, Finnerty B, Jimenez X, Koo H, Balderes P, Ludwig D, Kussie P, Witte L. Bohlen P, Hicklin D (2004) A fully human antibody against VEGFR1 inhibits growth of human breast cancers. Proc Am Assoc Cancer Res 45 abstr 3005

Wu Y, Zhong Z, Huber J, Bassi R, Finnerty B, Carrick F, Tonra, J, Hicklin D (2005) Preclinical assessment of a fully hu-

man antibody against vascular endothelial growth factor receptor 1 as a therapeutic agent for treating breast cancer. Clin Cancer Res 11:9144s (abstr C180)

Wu Y, Hooper AT, Zhong Z, Witte L, Bohlen P, Rafii S, Hicklin DJ (2006) The vascular endothelial growth factor receptor (VEGFR-1) supports growth and survival of human breast carcinoma. Int J Cancer 119:1519–1529

Yamamoto K, Kondo T, Suzuki S, Izawa H, Kobayashi M, Emi N, Komori K, Naoe T, Takamatsu J, Murohara T (2004) Molecular evaluation of endothelial progenitor cells in patients with ischemic limbs: therapeutic effect by stem cell transplantation. Arterioscler Thromb Vasc Biol 24:192–196

Yang A, Camp E, Fan F, Shen L, Gray M, Liu W, Somcio R, Bauer T, Wu Y, Hicklin D, Ellis L (2006) Vascular endothelial growth factor receptor-1 activation mediates epithelial to mesenchymal transition in human pancreatic carcinoma cells. Cancer Res 66:46–51

Yoshiji H, Kuriyama S, Hicklin D, Huber J, Yoshii J, Miyamoto Y, Kawata M, Ikenaka Y, Nakatani T, Tsujinoue H, Fukui H. (1999) KDR/Flk-1 is a major regulator of vascular endothelial growth factor-induced tumor development and angiogenesis in murine hepatocellular carcinoma cells. Hepatology 30:1179–1186

Yoshiji H, Kuriyama S, Hicklin DJ, Huber J, Yoshii J, Ikenaka Y, Noguchi R, Nakatani T, Tsujinoue H, Fukui H (2001) The vascular endothelial growth factor receptor KDR/Flk-1 is a major regulator of malignant ascites formation in the mouse hepatocellular carcinoma model. Hepatology 33:841–847

Yoshiji H, Kuriyama S, Yoshii J, Ikenaka Y, Noguchi R, Hicklin D, Huber J, Nakatani T, Tsujinoue H, Yanase K, Imazu H, Fukui H (2002) Synergistic effect of basic fibroblast growth factor and vascular endothelial growth factor in murine hepatocellular carcinoma. Hepatology 35:834–842

Yoshiji H, Kuriyama S, Yoshii J, Ikenaka Y, Noguchi R, Hicklin DJ, Wu Y, Yanase K, Namisaki T, Kitade M, Yamazaki M, Tsujinoue H, Masaki T, Fukui H (2004a) Halting the interaction between vascular endothelial growth factor and its receptors attenuates liver carcinogenesis in mice. Hepatology 39:1517–1524

Yoshiji H, Kuriyama S, Yoshii J, Ikenaka Y, Noguchi R, Yanase K, Namisaki T, Kitade M, Yamazaki M, Tsujinoue H, Masaki T, Fukui H (2004b) Involvement of the vascular endothelial growth factor receptor-1 in murine hepatocellular carcinoma development. J Hepatol 41:97–103

Yu J, Rak J (2003) Host microenvironment in breast cancer development: inflammatory and immune cells in tumour angiogenesis and arteriogenesis. Breast Cancer Res 5:83–88

Yu J, Rak J, Coomber B, Hicklin D, Kerbel R (2002) Effect of p53 status on tumor response to antiangiogenic therapy. Science 295:1526–1528

Zachary I (2005) Neuroprotective role of vascular endothelial growth factor: signalling mechanisms, biological function, and therapeutic potential. Neurosignals 14:207–221

Zhang L, Yu D, Hicklin D, Hannay JA Ellis L, Pollock R (2002) Combined anti-fetal liver kinase 1 monoclonal antibody and continuous low-dose doxorubicin inhibits angiogenesis and growth of human soft tissue sarcoma xenografts by induction of endothelial cell apoptosis. Cancer Res 62:2034–2042

Zhang H, Li Y, Li H, Bassi R, Jimenez X, Witte L, Bohlen P, Hicklin D, Zhu Z (2004) Inhibition of both the autocrine and the paracrine growth of human leukemia with a fully human antibody directed against vascular endothelial growth factor receptor 2. Leuk Lymph 45:1887–1897

Zhu Z, Rockwell P, Lu D, Kotanides H, Pytowski B, Hicklin D, Bohlen P, Witte L (1998) Inhibition of vascular endothelial growth factor-induced receptor activation with anti-kinase insert domain-containing receptor single-chain antibodies from a phage display library. Cancer Res 58:3209–3214

Zhu Z, Lu D, Kotanides H, Santiago A, Jimenez X, Simcox T, Hicklin D, Bohlen P, Witte L (1999) Inhibition of vascular endothelial growth factor induced mitogenesis of human endothelial cells by a chimeric anti-kinase insert domain-containing receptor antibody. Cancer Lett 13:203–213

Zhu Z, Hattori K, Zhang H, Jimenez X, Ludwig DL, Dias S, Kussie P, Koo H, Kim H, Lu D, Liu M, Tejada R, Friedrich M, Bohlen P, Witte L, Rafii S (2003) Inhibition of human leukemia in an animal model with human antibodies directed against vascular endothelial growth factor receptor 2. Correlation between antibody affinity and biological activity. Leukemia 17:604–611

Zimmermann RC, Hartman T, Bohlen P, Sauer MV, Kitajewski J (2001a) Preovulatory treatment of mice with anti-VEGF receptor 2 antibody inhibits angiogenesis in corpora lutea. Microvasc Res 62:15–25

Zimmermann R, Xiao E, Husami N, Sauer M, Lobo R, Kitajewski J, Ferin M (2001b) Short-term administration of antivascular endothelial growth factor antibody in the late follicular phase delays follicular development in the rhesus monkey. J Clin Endocrinol Metab 86:768–772

Zimmermann RC, Xiao E, Bohlen P, Ferin M (2002) Administration of antivascular endothelial growth factor receptor 2 antibody in the early follicular phase delays follicular selection and development in the rhesus monkey. Endocrinology 143:2496–2502

Zimmermann R, Hartman T, Kavic S, Pauli S, Bohlen P, Sauer M, Kitajewski J (2003) Vascular endothelial growth factor receptor 2-mediated angiogenesis is essential for gonadotropin-dependent follicle development. J Clin Invest 112:659–669

Zuo Z, Jimenez X, Witte L, Zhu Z (2000) An efficient route to the production of an IgG-like bispecific antibody. Protein Eng 13:361–367

Angiopoietin-2 Antagonists for Anti-Angiogenic Therapy

25

PAUL E. HUGHES, ANTHONY POLVERINO, JONATHAN D. OLINER, RICHARD L. KENDALL

Abstract

Interactions between Tie2, an endothelial cell-specific receptor tyrosine kinase, and its ligands the angiopoietins have been shown to play critical roles in multiple steps of angiogenesis and vascular remodeling. These include the stabilization of immature vessels, the destabilization of mature vessels, and endothelial cell migration and survival. Angiopoietin 1 has been shown to be a Tie2 agonist, stimulating autophosphorylation and the activation of downstream signaling pathways responsible for the maturation and stabilization of the developing vasculature. In contrast, angiopoietin 2 is largely thought to function as a context-dependent antagonist of Tie2 signaling, inducing vascular plasticity and sensitivity to pro-angiogenic factors such as VEGF-A. Furthermore, Ang2 exhibits broad expression in the vasculature of human tumors but limited expression in normal tissues, suggesting it could be an attractive target for safe and effective anti-angiogenic therapy. Potent and specific antibodies and peptide-Fc fusion proteins neutralizing the interaction between Ang2 and Tie2 have now been developed. In a variety of nonclinical studies in rodents, treatment with these agents resulted in an inhibition of the growth of human tumor xenografts and an inhibition of VEGF-stimulated corneal angiogenesis. In addition, mechanism of action studies suggest that Ang2 antagonists achieve efficacy by targeting the tumor vasculature. Data in support of the clinical utility of Ang2 inhibition in the oncology setting is currently being collected, with AMG 386, an Ang2 inhibitor, having been advanced into phase I oncology clinical trials in patients with solid tumors.

CONTENTS

Introduction

It is well established that tumors must acquire the ability to stimulate the formation of new blood vessels if they are to progress from a small, localized growth with limited oxygen and nutrient supply to a well-vascularized established, tumor. This has led to an extensive effort to identify targets and agents for anti-angiogenic therapy. While numerous pro-angiogenic factors have been described, extensive work performed over the last decade has established a critical role for vascular endothelial growth factor (VEGF) in the regulation of tumor angiogenesis. Promising results have been observed in clinical trials with agents targeting the VEGF–VEGFR axis (Ferrara 2005; Ferrara and Kerbel 2005; Jain et al. 2006). Bevacizumab, a humanized anti-VEGF-A monoclonal antibody, gained FDA approval for the treatment of metastatic colorectal cancer in combination with 5-fluorouracil (FU)-based chemotherapy (Hurwitz et al. 2004). More recently, Nexavar and Sutent, ATP-competitive small-molecule inhibitors of several receptor tyrosine kinases, including VEGFR2, were approved for the treatment of metastatic renal cancer, and GIST in the case of Sutent (Strumberg 2005). Several additional small-molecule tyrosine kinase inhibitors targeting VEGF receptors, including AMG 706 and PTK787, are at various stages of clinical development for the treatment of a variety of solid tumors (Ferrara and Kerbel 2005; Jain et al. 2006).

Given the complexity and heterogeneity of solid tumors, it comes as no surprise to find that not all tumors respond to inhibition of VEGF signaling, suggesting that alternative pathways can drive tumor angiogenesis in certain situations. In the clinical setting there is evidence of acquired resistance to inhibition of the VEGF pathway (Ferrara and Kerbel 2005; Jain et al. 2006). Proposed mechanisms of resistance include a shift from VEGF-A to other pro-angiogenic growth factors (Sweeney et al. 2003; Ferrara and Kerbel 2005), the selection of tumor cell populations that are resistant to hypoxia induced by anti-angiogenic therapy (Yu et al. 2002), and a change in the nature of tumor vasculature with resistant tumors being served by mature vessels that are not responsive to VEGF blockade (Ferrara and Kerbel 2005). A recent preclinical study by Casanovas et al. (2005) suggests that hypoxia-mediated induction of alternate pro-angiogenic factors could be a plausible mechanism by which tumors could develop resistance to the inhibition of VEGF signaling. Fibroblasts and macrophages associated with the tumor microenvironment may also play a key role in the induction of tumor angiogenesis following inhibition of VEGF axis by providing a source of pro-angiogenic and endothelial cell survival factors. Hypoxia has been shown to lead to the recruitment of tumor-associated macrophages, which are thought to be a rich source of pro-angiogenic factors (Lewis and Murdoch 2005). Tumor-associated fibroblasts could also contribute to VEGF-independent tumor angiogenesis, via the release of stromal cell-derived factor (SDF-1), which is proposed to stimulate the recruitment of endothelial progenitor cells to the tumor vasculature (Orimo et al. 2005).

The emerging issue of resistance to VEGF inhibition has led to a considerable effort in the angiogenesis field to derive a more complete understanding of other pathways that may modulate tumor angiogenesis, as well as efforts to identify other pro-angiogenic pathways that may provide alternative sources of targets for anti-angiogenic therapies.

Like the VEGF–VEGFR axis, the angiopoietin–Tie2 pathway is another largely endothelial cell-specific system that has been shown, primarily through studies in knockout and transgenic mice, to play a critical role in vascular development (Thurston 2002; Eklund and Olsen 2005). Considerable progress has been made in investigating the therapeutic potential of blocking angiopoietin–Tie2 signaling as an anti-angiogenic strategy. In this chapter we will review recent work characterizing the angiopoietin–Tie2 axis in the developing and adult vasculature and discuss recent observations suggesting that inhibition of angiopoietin-2 may represent an effective strategy for treating pathologic angiogenesis in a number of disease settings, including cancer, arthritis and macular degeneration.

Angiopoietin–Tie2 Signaling in the Adult and Developing Vasculature

The angiopoietins consist of a family of four extracellular ligands that specifically recognize and bind to the extracellular domain of Tie2, a largely endothelial cell-specific receptor tyrosine kinase (Thurston 2002; Eklund and Olsen 2005). Correct regulation of Tie2 signaling is required for normal vascular homeostasis and maturation, as well as regulating vascular remodeling and destabilization (Thurston 2002; Eklund and Olsen 2005). In addition to a central role in vascular development further studies, have suggested a role for Tie2 and the angiopoietins in lymphangiogenesis and hematopoiesis (Arai et al. 2004; Alitalo et al. 2005).

Of the four angiopoietins, angiopoietin-1 (Angl) and angiopoietin-2 (Ang2) are the most thoroughly characterized. Angl acts as a Tie2 agonist, stimulating the receptor's phosphorylation and activating downstream signaling pathways (Davis et al. 1996). In contrast, Ang2 is thought to function primarily to antagonize Tie2 phosphorylation (Maisonpierre et al. 1997). The downstream consequences of the ligation of Tie2 by both Angl and Ang2 are depicted in Fig. 25.1.

Much of what we know about the Tie2–angiopoietin system comes from the study of genetically modified mice (Dumont et al. 1994; Sato et al. 1995; Suri et al. 1996; Maisonpierre et al. 1997; Gale et al. 2002). Tie2 deficiency results in embryonic lethality at E10.5, and is associated with cardiac failure and vascular abnormalities (Dumont et al. 1994; Sato et al. 1995). The vessels of Tie2 null embryos exhibit fewer endothelial cells, decreased contact between the endothelial cells and the surrounding perivascular cells (pericytes and smooth muscle cells) and failure to remodel the vasculature into large and small vessels. This phenotype suggests a role for Tie2 in the maturation and stabilization of the developing vasculature. Angl was originally identified as a novel ligand for Tie2, that was capable of inducing the tyrosine phosphorylation of Tie2 (Davis et al. 1996). Mice deficient for Angl (Suri et al. 1996) die by embryonic day 12.5, and display a vascular phenotype similar to that of Tie2-deficient mice. This similarity in phenotypes suggests that Angl is indeed a Tie2 agonist, and mediates vascular remodeling and vessel stabilization via the recruitment of perivascular support cells. It is thought that the role of the Angl–Tie2 axis in maintaining vessel stability is conserved throughout embryonic development into the adult.

Considerable effort has gone into increasing our understanding of the consequences of the activation of Tie2 by Angl. In vitro it has been shown that Angl can act as a survival factor, but not a mitogen for endothelial cells, and Angl is also capable of inducing endothelial cell motility and sprouting (Eklund and Olsen 2005). In an effort to understand how Tie2 mediates its pro-angiogenic effects, the identification of downstream targets of Tie2 has been a subject of intense study. Ligand binding to the extracellular domain of Tie2 is thought to result in receptor dimerization that facilitates activation of the kinase domain and autophosphorylation of specific tyrosine residues. The phosphorylated tyrosine residues act as docking sites for a number of effectors, including the p85 subunit of PI3-K, the protein tyrosine phosphatase SHP2, and the adapter proteins GRB2 and Dok-R (Huang et al. 1995; Jones and Dumont 1998; Kontos et al. 1998; Master et al. 2001). The interaction of signaling molecules with phosphotyrosine residues leads ultimately to activation of the mitogen-activated protein kinase (MAPK) pathway, the serine–threonine kinase Akt, and the members of the Rho family of small GTP-binding proteins (Eklund and Olsen 2005). However, it remains to be fully determined as to how signals from distinct Tie2 effectors are integrated to influence angiogenesis and vascular stability.

Ang2 was originally found to bind to Tie2, but not stimulate autophosphorylation in endothelial cells (Maisonpierre et al. 1997). Transgenic mice overexpressing Ang2 have a phenotype consistent with that observed in Tie2- and Angl-null mice, leading to the paradigm that Ang2 acts predominantly as a natural antagonist of Angl (Maisonpierre et al. 1997). Unlike Angl, the postnatal expression of Ang2 is relatively dynamic and is highest at sites undergoing active vascular remodeling, such as the female reproductive tract and the tumor vasculature (Maisonpierre

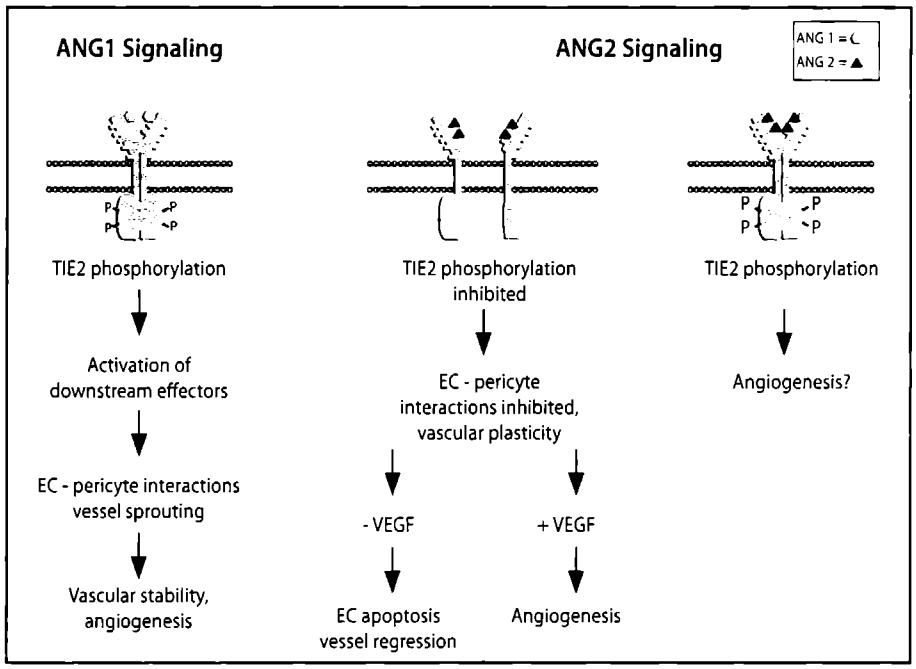


Fig. 25.1. A schematic representation of Angiopoietin-Tie2 signaling, and subsequent biological effects of Tie2 ligation by either Ang1 or Ang2

et al. 1997; Tait and Jones 2004). An example of the high levels of Ang2 present in the tumor vasculature is illustrated in Fig. 25.2. However, further study suggests that Ang2 can indeed stimulate the phosphorylation of Tie2 in endothelial cells in certain contexts. For example, when used at high concentrations and extended durations in endothelial cells, Ang2 can induce Tie2 phosphorylation and act as an endothelial cell survival factor (Kim et al. 2000).

Ang2 is expressed by endothelial cells, with high levels of Ang2 combined with high levels of VEGF-A inducing angiogenic sprouting in a number of different experimental conditions (Asahara et al. 1998; Holash et al. 1999; Lobov et al. 2002; Machein et al. 2004). In contrast, a low level of VEGF-A relative to high levels of Ang2 is associated with vascular regression (Lobov et al. 2002). The published observations in a variety of experimental systems are consistent with a paradigm in which binding of Ang2 does not provide a specific pro-angiogenic signal, but rather interferes with the Angl-induced vascular stability signal and, as a result, makes vessels plastic and sensitive to the actions of other angiogenic factors such as VEGF-A. In support of this model are the observations that Ang2 promotes VEGF-induced angiogenesis in a variety of experimental systems, including models of corneal angiogenesis and in transgenic mice overexpressing Ang2 and VEGF-A in cardiac muscle (Visconti et al. 2002).

The results of ectopic overexpression of Ang2 in tumor cells provide additional support for the role of Ang2, rather than Angl, as a pro-angiogenic factor. In contrast to Angl, Ang2 overexpression in tumor cells has been reported by a number of investigators to promote tumor growth and vascularization in tumor xenograft models, whereas Angl overexpression has been shown to inhibit tumor growth (Tanaka et al. 1999; Ahmad et al. 2001; Etoh et al. 2001; Machein et al. 2004). Machein et al. (2004) reported more numerous, highly branched vessels with an extensive pericyte coverage in the vasculature of tumors derived from cells overexpressing Angl, which would suggest improved tumor perfusion. In contrast, tumors derived from Ang2-expressing cells were smaller, and contained a poorly defined vascular network with poor pericyte coverage (Machein et al. 2004). However, these experiments should be interpreted with a degree of caution, as Ang2 overexpres-

sion has also been reported to inhibit tumor growth (Yu and Stamenkovic 2001).

Ang2-deficient mice have a complex phenotype (Gale et al. 2002). Following birth the pups appear normal, but as the result of a lymphatic defect quickly develop chylous ascites and edema, which often results in morbidity by day 14. Furthermore, Ang2-knockout mice display early postnatal vascular remodeling defects in the eye, where the hyaloid vasculature fails to regress and the retinal vasculature fails to form. Significantly, the lymphatic, but not the ocular, phenotype can be largely rescued by replacing the Ang2 gene with the cDNA for Angl, suggesting distinct functions for Angl and Ang2 in vascular remodeling of the eye, but redundant roles in lymphatic development, where both Angl and Ang2 may function as agonists (Gale et al. 2002). In addition, further study of Ang2-null mice shows phenotypic changes in the vascular capillaries found in the kidney (Pitera et al. 2004), suggesting that Ang2 may have a more widespread role than initially thought in the development of the vasculature. However, it should be noted that in more recent studies it was observed that severe lymphatic and vascular defects associated with the Ang2 knockout were dependent on the genetic background of the knockout mice (Fiedler et al. 2006).

In summary, the endothelial cell-specific expression of Ang2, and its observed functions, suggest that Ang2 functions in an autocrine fashion to regulate angiogenesis by controlling endothelial cell quiescence and vascular plasticity. These data combined with the largely normal phenotype of the Ang2-knockout mice, suggest that inhibition of Ang2 may represent a viable and safe strategy for inhibiting tumor angiogenesis.

Inhibition of Angiopoietin–Tie2 Signaling

The utilization of selective inhibitors has proved to be a powerful tool for elucidating the role of particular cytokines or signaling pathways in blood vessel growth. Strategies that have proven to be broadly effective include: soluble receptors; inhibitory antibodies directed against receptors or ligands; RNA-aptamers; and selective small-molecule tyrosine kinase inhibitors. A combination of such approaches has been particularly effective in demonstrating a critical role for members of the VEGF family and their receptors in normal and pathological angiogenesis.

Efforts using similar approaches to inhibit the angiopoietin–Tie2 signaling systems have shown that interference of this axis can result in the inhibition of angiogenesis. The therapeutic utility of blocking Tie2 signaling was initially demonstrated by Lin et al. Following systemic administration of an adenoviral vector expressing soluble Tie2, inhibition of the growth and metastasis of two different murine tumors was reported (Lin et al. 1997, 1998). These studies were followed by additional reports showing that blocking angiopoietin signaling via sequestration with a soluble Tie2 receptor has an inhibitory effect on both tumor angiogenesis and corneal angiogenesis (Siemeister et al. 1999; Hangai et al. 2001; Das et al. 2003; Fathers et al. 2005). Approaches using peptides to block Tie2–angiopoietin interactions and anti-Tie2 intrabodies have provided further insights into the role of Tie2 signaling in pathological angiogenesis, and support the potential utility of anti-angiogenic agents that target Tie2-angiopoietin signaling (Tournaire et al. 2004; Popkov et al. 2005). In addition, the numerous studies with soluble Tie2 receptors would suggest that systemic inhibition of the Tie2–angiopoietin axis is not overtly toxic, at least in rodents.

Identification and Characterization of Selective Angiopoietin-2 Inhibitors

For many of the published studies using inhibitors of the angiopoietin–Tie2 axis, experimental interpretation has been limited, as the molecular tools used lacked specificity and therefore could not be used

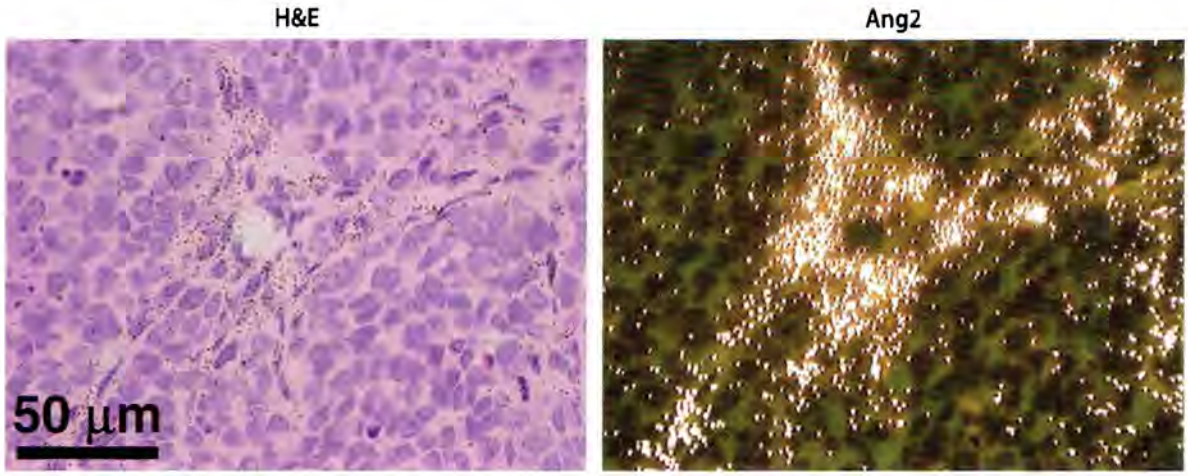


Fig. 25.2. Paired bright-field and dark-field images of an Ang2 in situ hybridization illustrating high levels of Ang2 mRNA expression in the endothelial cells lining a vessel within the body of an A431 tumor xenograft. The *horizontal bars* indicate scale. Reproduced from Oliner et al. (2004) with permission from Elsevier Press

to determine the contribution of each of the different angiopoietins to the resulting phenotype. The studies with soluble Tie2 receptors are particularly difficult to interpret for this reason. It is known that all four of the angiopoietins are capable of binding Tie2 and as a result it is unclear whether the observed activity could be attributed solely to inhibition of Ang2, or is the result of combined inhibition of multiple angiopoietins. Initial attempts to selectively neutralize Ang2 in vivo using RNA aptamers resulted in modest inhibition of bFGF-induced corneal angiogenesis (White et al. 2003). The RNA aptamers used in this study were not optimized for in vivo studies, and there was no pharmacodynamic readout to determine the extent of Ang2 inhibition, thus leaving unanswered the question of whether Ang2 inhibition alone is sufficient to fully inhibit angiogenesis. Nonetheless, this study was significant, as it provided further evidence that Ang2 is an indirect pro-angiogenic factor and demonstrated that specific inhibition of Ang2 is anti-angiogenic.

In an effort to more clearly define the effects of an inhibition of Ang2 function on angiogenesis, Oliner et al. (2004) generated peptide–Fc fusion proteins (peptibodies) and fully human antibodies that potently and selectively neutralized the interaction between human Ang2 and Tie2, with IC_{50} values ranging from 23 to 140 pM. The three inhibitors described by Oliner et al. exhibited between 30-fold and >4500-fold selectivity for Ang2 over other angiopoietin family members and had similar IC_{50} values against Ang2 from rat and mouse (Table 25.1). In addition, these agents displayed favorable pharmacokinetic properties, supporting daily to weekly administration via subcutaneous administration. The combined potency, selectivity and pharmacokinetic profiles of these molecules made them excellent tools to evaluate the effect of Ang2 inactivation on tumor angiogenesis in a variety of preclinical models.

Oliner et al. observed that administration of three distinct Ang2 inhibitors to mice bearing subcutaneous A431 human epidermoid, Colo205 human colorectal, and HT29 human colorectal xenografts resulted in inhibition of tumor growth, demonstrating that the effects of Ang2 inhibition were not reagent- or tumor model-dependent. Figure 25.2 illustrates the effect of the Ang2 antagonist 2×Con4(C) on the growth rate of Colo205 xenografts and its effects on tumor cell viability. In the experiment illustrated in Fig. 25.3, Colo205 tumors were allowed to grow for 28 days before dosing with 2×Con4(C) was initiated. Separation in growth

Table 25.1. Potency and selectivity of the peptide–Fc fusion proteins and antibodies used by Oliner et al. as competitive inhibitors of angiopoietin–Tie2 interactions

	*h*Ang2	*m*Ang2	*r*Ang2	*h*Ang1
	IC_{50}(nM)	IC_{50}(nM)	IC_{50}(nM)	IC_{50}(nM)
2×Con4(C)	0.023	0.021	0.049	0.9
L1-7(N)	0.054	0.071	0.160	>100
Ab536	0.140	0.061	0.270	>100
IgG1 Fc	>100	>100	>100	>100

Peptide–Fc fusion proteins, antibody and human IgG1 were evaluated by ELISA for their ability to block the interactions between Tie2 and human (*h*), rat (*r*) and mouse (*m*) angiopoietins

rates between the vehicle and treated groups was observed after 3 days of treatment, with the volume of the 2×Con4(C)-treated tumors remaining constant over the 21-day study period. At the conclusion of the study, hematoxylin-stained histological sections of bisected tumors revealed a significantly smaller viable tumor fraction in the group of animals treated with an Ang2 inhibitor than in those treated with a vehicle control (Fig. 25.3). Therefore, analysis of tumor viability would suggest that changes in tumor volume underestimate the anti-tumor effects of Ang2 inhibition, at least in Colo205 tumor xenografts.

Further tumor xenograft studies revealed that Ang2 inhibition was able to rapidly induce regression of large established tumors, with tumor regrowth appearing when pharmacokinetic modeling predicted that serum Ang2 inhibitor concentrations fell below optimal levels. Resumption of dosing with an Ang2 inhibitor again resulted in a sustained inhibition of tumor growth, indicating that the tumors had not acquired resistance to Ang2 inhibition (Oliner et al. 2004).

Experiments aimed at elucidating the mechanism of action of the Ang2 antagonists suggested that the reduction in tumor cell growth was consistent with an anti-angiogenic mechanism. FACS analysis of cells isolated from 2×Con4(C)-treated tumors revealed a sustained and progressive reduction in endothelial cell proliferation (Oliner et al. 2004), a phenomenon that has now been observed using other Ang2 inhibitors (Oliner et al., unpublished observations). Furthermore, treatment with 2×Con4(C) produced a dose-dependent inhibition of VEGF-stimulated neovascularization in a rat corneal model of angiogenesis, suggesting that Ang2 inhibition can elicit an anti-angiogenic effect outside the context of a tumor. Interestingly, in situ hybridization analysis of Ang2 expression during VEGF-induced corneal angiogenesis revealed that Ang2 expression was detected in newly sprouting vessels. These observations are consistent with the paradigm of Ang2 up-regulation facilitating vascular plasticity and a positive response to pro-angiogenic cytokines.

Thickening of the epiphyseal growth plate was observed in male rats following dosing of the Ang2 inhibitor 2×Con4(C). Epiphyseal plate thickening is thought to be an on-mechanism consequence of anti-angiogenic therapy, and has been observed in cynomolgus monkeys treated with VEGF neutralizing antibodies and immature rodents treated with Flt-1(1–3)-IgG (Gerber et al. 1999; Ferrara et al. 2005). The observations that Ang2 inhibition does not lead to significant effects on the developed vasculature is consistent with the phenotype of the Ang2-knockout mice in which no significant vascular defects were observed (Gale et al. 2002).

The study by Oliner et al. has considerable therapeutic implications; it provides compelling evidence to suggest that Ang2 inhibition may represent a new therapeutic strategy for treating pathological angiogenesis. An additional finding of this study was the observed inhibition of endothelial cell proliferation followed by a presumed secondary effect on tumor cell viability. These data suggest that Ang2 antagonists, like those targeting VEGF and its receptors, derive efficacy by initially targeting the tumor vasculature. However, Ang2 antagonists, unlike VEGF pathway antagonists, do not appear to induce apoptosis of tumor endothelial cells (Oliner et al., unpublished observations). To test the applicability of Ang2 inhibition in treating human tumors, AMG386, a peptibody selectively targeting Ang2, has been advanced into clinical development in patients with solid tumors.

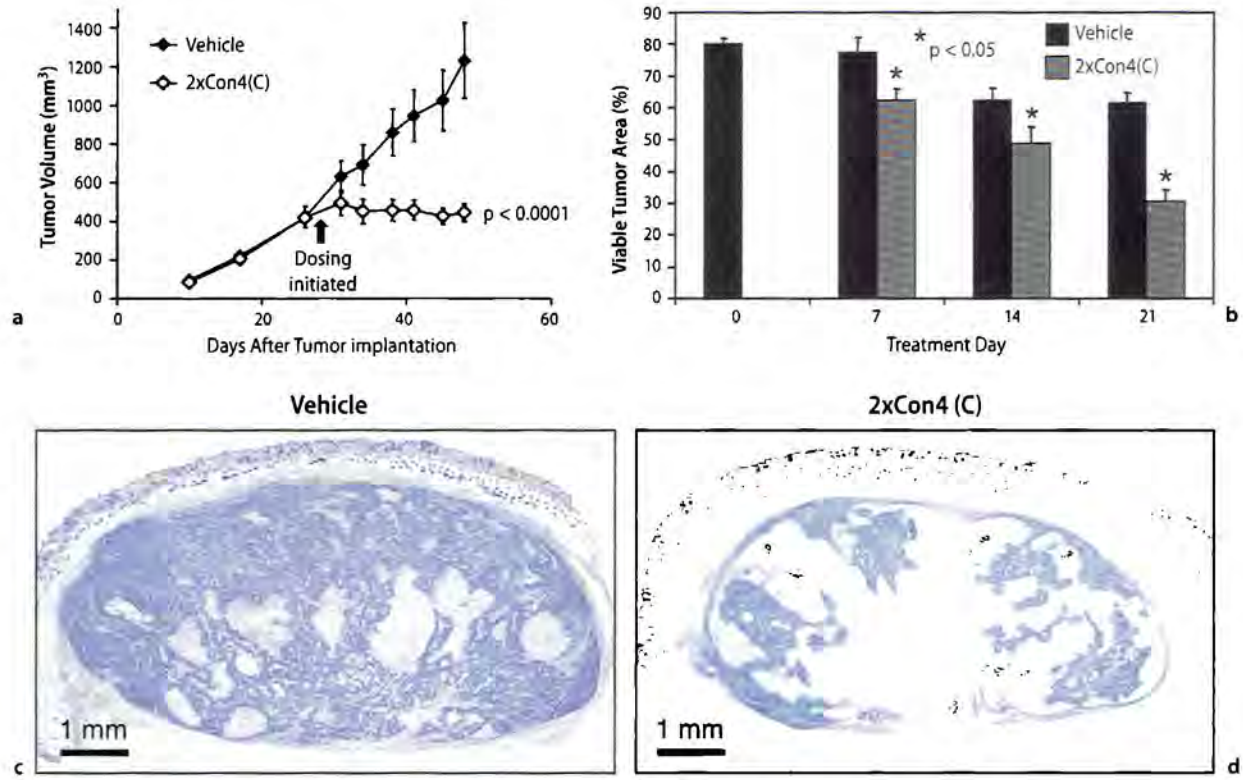


Fig. 25.3a–d. Effect of an Ang2 inhibitor on the growth and viable tumor fraction of tumor xenografts. a Ang2 inhibition clearly prevents the growth of established Colo205 xenografts. Mice were treated twice weekly with 15 mg/kg 2×Con4(C), starting on day 28 post injection of Colo205 tumor cells. b Histological analysis of tumor viability, which revealed a significant reduction in the viable tumor fraction in mice treated with an Ang2 inhibitor compared to mice treated with a vehicle control. c, d Examples of representative hematoxylin-stained histological sections of bisected tumors following 21 days of treatment with vehicle or 2×Con4(C), respectively. Note the considerable necrosis observed in the section from the 2×Con4(C)-treated tumor. The *horizontal bars* indicate scale. Reproduced from Oliner et al. (2004) with permission from Elsevier Press

25.5 Summary: Unanswered Questions and Future Developments

A wealth of evidence demonstrates that the angiopoietin–Tie2 signaling axis is critical for blood vessel formation, principally regulating vascular remodeling and maturity. Recent studies have gone a long way to illuminating the biology around the Tie2–angiopoietin axis and have led to the generally accepted view that Angl functions as the major agonist for Tie2, whereas Ang2 most likely acts as an antagonist, or as a context-dependent agonist.

The cessation of angiogenesis induced by Ang2 neutralization raises a number of interesting questions around the cellular and molecular consequences of selective Ang2 inhibition. It appears that Ang2 neutralization results in the inhibition of endothelial cell proliferation. Lobov et al. (2002) demonstrated that ectopically administered Ang2 induced endothelial cell proliferation and vascular sprouting in the papillary membrane of the eye, with these effects being associated with increased capillary diameter, basal lamina remodeling and endothelial cell migration. It is reasonable to speculate that these effects might be opposed by Ang2

inhibition. It is possible that by neutralizing Ang2, the vascular stabilizing signals of Angl remain intact, and, as a result, the endothelium is less responsive to the proliferative signals from pro-angiogenic factors such as VEGF (Holash et al. 1999; Lobov et al. 2002). Studies on the nature of both tumor and normal vessels following Ang2 inhibition promise to be particular useful in addressing this question. Of particular interest is the effect of systemic Ang2 inhibition on the coverage of capillaries with pericytes and other types of vascular supporting cells, as Angl is thought to promote these interactions and Ang2 oppose them.

At the molecular level, it remains to be determined whether Ang2 inhibition affects Tie2 phosphorylation, either globally or in individual vessels actively participating in the angiogenic process, and whether the inhibition of Tie2 phosphorylation is critical for efficacy. A definitive answer to these important questions in a physiologically relevant setting would be extremely useful in elucidating the biochemical role of Ang2. Attempts to address these challenging questions have yet to be successful. Reagents, such as phospho-specific Tie2 antibodies, of sufficient sensitivity to determine whether Ang2 inhibition induces changes in Tie2 phosphorylation have yet to be developed. Furthermore, the activity of highly selective small-molecule Tie2 kinase inhibitors in preclinical models remains to be thoroughly tested. Such molecules would be very useful tools for answering key questions around the role of Tie2 phosphorylation. It would be particularly insightful to determine whether they had an efficacy and mechanism of action profile consistent with or distinct from that observed by Ang2 inhibitors.

Many challenges and open questions remain to be addressed when considering the future strategy for development of Ang2 antagonists. Among them is the question of what represents the ideal selectivity profile for an angiopoietin antagonist to produce maximal anti-angiogenic activity. Oliner et al. (2004) demonstrated that the Ang2 inhibitor 2×Con4(C), which displays some inhibitory activity against Angl, had an activity similar to that of selective Ang2 inhibitors, suggesting that Ang2 may be the dominant angiopoietin in promoting angiogenesis, at least in models tested in that particular study. However, given that Ang2 and Angl are involved in distinct phases of angiogenesis, Ang2 in vascular remodeling and Angl in vessel stability, it is possible there are the advantages to dual Angl/2 inhibition over selective Ang2 inhibition. The generation of potent and selective Angl inhibitors may enable answers to be obtained for some of the outstanding questions around the therapeutic utility of Angl inhibition, either alone or in combination with Ang2 inhibition.

When considering the clinical development of Ang2 antagonists, it is worth noting that it appears likely that anti-angiogenic cancer therapy, with a few notable exceptions, will be combinatorial. In light of this it is worth considering possible combinations with other effective anti-angiogenic agents, such as those targeting VEGF and VEGF receptors. The VEGF axis plays a key role in vessel sprouting and endothelial cell proliferation, whereas the angiopoietin–Tie2 system plays roles in vascular remodeling and maturation. Given the complementary roles of the two systems it is attractive to speculate that the combined neutralization of Ang2 and inhibition of VEGF receptor signaling will produce a more robust anti-angiogenic effect than inhibiting each one alone. Indeed, in tumor xenograft models the combination of an Ang2 antagonist with agents targeting the VEGF axis results in a greater inhibition of tumor growth than that conferred by each agent alone (Leal J, Coxon A, Oliner J, Polverino A et al., unpublished observations). Additional combination studies with Ang2 inhibitors might include studies with chemotherapeutics. Combinations of anti-angiogenic agents targeting the VEGF axis and chemotherapeutic agents have been observed to have additive or even synergistic anti-tumor effects compared to chemotherapy alone (Jain et al. 2006).

In summary, data from preclinical studies suggests that Ang2 inhibition appears represents a viable strategy for treating pathological angiogenesis associated with tumor growth. However, Ang2 inhibitors may also have applicability for treating a range of human diseases, as Ang2 up-regulation has also been observed in indications other than cancer, including age-related macular degeneration (AMD),

rheumatoid arthritis, osteoarthritis, and psoriasis. The evaluation of an Ang2 inhibitor in AMD is perhaps the most attractive of these possibilities for several reasons. Unlike rheumatoid arthritis, AMD is a largely unmet medical need, and strategies targeting VEGF-A have shown efficacy in AMD, validating the utility of an anti-angiogenic approach in this setting. Data in support of the clinical utility of Ang2 inhibition in the oncology setting are currently being collected. AMG 386, an Ang2 inhibitor, has been advanced into oncology clinical trials in patients with solid tumors.

Acknowledgements

The authors would like to thank Chris Storgard, Angela Coxon and Jim Bready for their constructive and critical review of the manuscript. The authors would also like to thank Sean Caenepeel for his help in generating and formatting figures.

References

Ahmad SA, Liu W, Jung YD, Fan F, Wilson M, Reinmuth N, Shaheen RM, Bucana CD, Ellis LM (2001) The effects of angiopoietin-1 and -2 on tumor growth and angiogenesis in human colon cancer. Cancer Res 61:1255–1259

Alitalo K, Tammela T, Petrova TV (2005) Lymphangiogenesis in development and human disease. Nature 438:946–953

Arai F, Hirao A, Ohmura M, Sato H, Matsuoka S, Takubo K, Ito K, Koh GY, Suda T (2004) Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell 118:149–161

Asahara T, Chen D, Takahashi T, Fujikawa K, Kearney M, Magner M, Yancopoulos GD, Isner JM (1998) Tie2 receptor ligands, angiopoietin-1 and angiopoietin-2, modulate VEGF-induced postnatal neovascularization. Circ Res 83:233–240

Casanovas O, Hicklin DJ, Bergers G, Hanahan D (2005) Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. Cancer Cell 8:299–309

Das A, Fanslow W, Cerretti D, Warren E, Talarico N, McGuire P (2003) Angiopoietin/Tek interactions regulate mmp-9 expression and retinal neovascularization. Lab Invest 83:1637–1645

Davis S, Aldrich TH, Jones PF, Acheson A, Compton DL, Jain V, Ryan TE, Bruno J, Radziejewski C, Maisonpierre PC, Yancopoulos GD (1996) Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. Cell 87:1161–1169

Dumont DJ, Gradwohl G, Fong GH, Puri MC, Gertsenstein M, Auerbach A, Breitman ML (1994) Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. Genes Dev 8:1897–1909

Eklund L, Olsen BR (2005) Tie receptors and their angiopoietin ligands are context-dependent regulators of vascular remodeling. Exp Cell Res 312:630–641

Etoh T, Inoue H, Tanaka S, Barnard GF, Kitano S, Mori M (2001) Angiopoietin-2 is related to tumor angiogenesis in gastric carcinoma: possible in vivo regulation via induction of proteases. Cancer Res 61:2145–2153

Fathers KE, Stone CM, Minhas K, Marriott JJ, Greenwood JD, Dumont DJ, Coomber BL (2005) Heterogeneity of Tie2 expression in tumor microcirculation: influence of cancer type, implantation site, and response to therapy. Am J Pathol 167:1753–1762

Ferrara N (2005) VEGF as a therapeutic target in cancer. Oncology 69 [Suppl 3]:11–16

Ferrara N, Kerbel RS (2005) Angiogenesis as a therapeutic target. Nature 438:967–974

Ferrara N, Hillan KJ, Novotny W (2005) Bevacizumab (Avastin), a humanized anti-VEGF monoclonal antibody for cancer therapy. Biochem Biophys Res Commun 333:328–335

Fiedler U, Reiss Y, Scharpfenecker M, Grunow V, Koidl S, Thurston G, Gale NW, Witzenrath M, Rosseau S, Suttorp N, Sobke A, Herrmann M, Preissner KT, Vajkoczy P, Augustin HG (2006) Angiopoietin-2 sensitizes endothelial cells to TNF-alpha and has a crucial role in the induction of inflammation. Nat Med 12:235–239

Gale NW, Thurston G, Hackett SF, Renard R, Wang Q, McClain J, Martin C, Witte C, Witte MH, Jackson D, Suri C, Campochiaro PA, Wiegand SJ, Yancopoulos GD (2002) Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by angiopoietin-1. Dev Cell 3:411–423

Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N (1999) VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat Med 5:623–628

Hangai M, Moon YS, Kitaya N, Chan CK, Wu DY, Peters KG, Ryan SJ, Hinton DR (2001) Systemically expressed soluble Tie2 inhibits intraocular neovascularization. Hum Gene Ther 12:1311–1321

Holash J, Maisonpierre PC, Compton D, Boland P, Alexander CR, Zagzag D, Yancopoulos GD, Wiegand SJ (1999) Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. Science 284:1994–1998

Huang L, Turck CW, Rao P, Peters KG (1995) GRB2 and SH-PTP2: potentially important endothelial signaling molecules downstream of the TEK/TIE2 receptor tyrosine kinase. Oncogene 11:2097–2103

Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hain-

sworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R, Kabbinavar F (2004) Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 350:2335–2342

Jain RK, Duda DG, Clark JW, Loeffler JS (2006) Lessons from phase III clinical trials on anti-VEGF therapy for cancer. Nat Clin Pract Oncol 3:24–40

Jones N, Dumont DJ (1998) The Tek/Tie2 receptor signals through a novel Dok-related docking protein, Dok-R. Oncogene 17:1097–1108

Kim I, Kim JH, Moon SO, Kwak HJ, Kim NG, Koh GY (2000) Angiopoietin-2 at high concentration can enhance endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Oncogene 19:4549–4552

Kontos CD, Stauffer TP, Yang WP, York JD, Huang L, Blanar MA, Meyer T, Peters KG (1998) Tyrosine 1101 of Tie2 is the major site of association of p85 and is required for activation of phosphatidylinositol 3-kinase and Akt. Mol Cell Biol 18:4131–4140

Lewis C, Murdoch C (2005) Macrophage responses to hypoxia: implications for tumor progression and anti-cancer therapies. Am J Pathol 167:627–635

Lin P, Polverini P, Dewhirst M, Shan S, Rao PS, Peters K (1997) Inhibition of tumor angiogenesis using a soluble receptor establishes a role for Tie2 in pathologic vascular growth. J Clin Invest 100:2072–2078

Lin P, Buxton JA, Acheson A, Radziejewski C, Maisonpierre PC, Yancopoulos GD, Channon KM, Hale LP, Dewhirst MW, George SE, Peters KG (1998) Antiangiogenic gene therapy targeting the endothelium-specific receptor tyrosine kinase Tie2. Proc Natl Acad Sci USA 95:8829–8834

Lobov IB, Brooks PC, Lang RA (2002) Angiopoietin-2 displays VEGF-dependent modulation of capillary structure and endothelial cell survival in vivo. Proc Natl Acad Sci USA 99:11205–11210

Machein MR, Knedla A, Knoth R, Wagner S, Neuschl E, Plate KH (2004) Angiopoietin-1 promotes tumor angiogenesis in a rat glioma model. Am J Pathol 165:1557–1570

Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, Yancopoulos GD (1997) Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 277:55–60

Master Z, Jones N, Tran J, Jones J, Kerbel RS, Dumont DJ (2001) Dok-R plays a pivotal role in angiopoietin-1-dependent cell migration through recruitment and activation of Pak. EMBO J 20:5919–5928

Oliner J, Min H, Leal J, Yu D, Rao S, You E, Tang X, Kim H, Meyer S, Han SJ, Hawkins N, Rosenfeld R, Davy E, Graham K, Jacobsen F, Stevenson S, Ho J, Chen Q, Hartmann T, Michaels M, Kelley M, Li L, Sitney K, Martin F, Sun JR, Zhang N, Lu J, Estrada J, Kumar R, Coxon A, Kaufman S, Pretorius J, Scully S, Cattley R, Payton M, Coats S, Nguyen L, Desilva B, Ndifor A, Hayward I, Radinsky R, Boone T, Kendall R (2004) Suppression of angiogenesis and tumor growth by selective inhibition of angiopoietin-2. Cancer Cell 6:507–516

Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL, Weinberg RA (2005) Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell 121:335–348

Pitera JE, Woolf AS, Gale NW, Yancopoulos GD, Yuan HT (2004) Dysmorphogenesis of kidney cortical peritubular capillaries in angiopoietin-2-deficient mice. Am J Pathol 165:1895–1906

Popkov M, Jendreyko N, McGavern DB, Rader C, Barbas CF 3rd (2005) Targeting tumor angiogenesis with adenovirus-delivered anti-Tie-2 intrabody. Cancer Res 65:972–981

Sato TN, Tozawa Y, Deutsch U, Wolburg-Buchholz K, Fujiwara Y, Gendron-Maguire M, Gridley T, Wolburg H, Risau W, Qin Y (1995) Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. Nature 376:70–74

Siemeister G, Schirner M, Weindel K, Reusch P, Menrad A, Marme D, Martiny-Baron G (1999) Two independent mechanisms essential for tumor angiogenesis: inhibition of human melanoma xenograft growth by interfering with either the vascular endothelial growth factor receptor pathway or the Tie-2 pathway. Cancer Res 59:3185–3191

Strumberg D (2005) Preclinical and clinical development of the oral multikinase inhibitor sorafenib in cancer treatment. Drugs Today (Barc) 41:773–784

Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, Sato TN, Yancopoulos GD (1996) Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. Cell 87:1171–1180

Sweeney CJ, Miller KD, Sledge GW Jr (2003) Resistance in the anti-angiogenic era: nay-saying or a word of caution? Trends Mol Med 9:24–29

Tait CR, Jones PF (2004) Angiopoietins in tumours: the angiogenic switch. J Pathol 204:1–10

Tanaka S, Mori M, Sakamoto Y, Makuuchi M, Sugimachi K, Wands JR (1999) Biologic significance of angiopoietin-2 expression in human hepatocellular carcinoma. J Clin Invest 103:341–345

Thurston G (2002) Complementary actions of VEGF and angiopoietin-1 on blood vessel growth and leakage. J Anat 200:575–580

Tournaire R, Simon MP, le Noble F, Eichmann A, England P, Pouyssegur J (2004) A short synthetic peptide inhibits signal transduction, migration and angiogenesis mediated by Tie2 receptor. EMBO Rep 5:262–267

Visconti RP, Richardson CD, Sato TN (2002) Orchestration of angiogenesis and arteriovenous contribution by angio-

poietins and vascular endothelial growth factor (VEGF). Proc Natl Acad Sci USA 99:8219–8224

White RR, Shan S, Rusconi CP, Shetty G, Dewhirst MW, Kontos CD, Sullenger BA (2003) Inhibition of rat corneal angiogenesis by a nuclease-resistant RNA aptamer specific for angiopoietin-2. Proc Natl Acad Sci USA 100:5028–5033

Yu JL, Rak JW, Coomber BL, Hicklin DJ, Kerbel RS (2002) Effect of p53 status on tumor response to antiangiogenic therapy. Science 295:1526–1528

Yu Q, Stamenkovic I (2001) Angiopoietin-2 is implicated in the regulation of tumor angiogenesis. Am J Pathol 158:563–570

Anti-Angiogenic Therapy with Thrombospondins

26

JACK LAWLER

Abstract

Thrombospondin-1 (TSP-1) is an extracellular protein that regulates the behavior of many cell types, including endothelial cells, during the tissue remodeling that is associated with angiogenesis, development, synaptogenesis, wound healing and neoplasia. The physiological angiogenesis that occurs during processes such as wound healing, mammary gland involution and cycling of the hair follicle requires endogenous inhibitors to complete the remodeling process. During tumor progression, the spatial and temporal coordination of pro- and anti-angiogenic signaling is lost and the endothelial cells receive conflicting signals from the constituents of the tumor microenvironment. The importance of suppression of angiogenesis by TSP-1 is underscored by the observation that TSP-1-null mice exhibit increased tumor angiogenesis and the fact that TSP-1-based therapeutics are currently in clinical trials for the inhibition of angiogenesis. TSP-1 and -2 inhibit angiogenesis through direct effects on endothelial cell migration and apoptosis, and through indirect effects on the various growth factors, cytokines and proteases that stimulate angiogenesis. Using synthetic peptides and recombinant proteins, several investigators have shown that the direct effects of TSP-1 are mediated by the interaction of the three type 1 repeats (3TSR) with the endothelial cell membrane protein CD36. The detailed understanding of the mechanisms of action of TSP-1 and -2 has facilitated the design of therapeutic strategies to optimize these activities for the inhibition of tumor progression.

CONTENTS

Introduction

Thrombospondin-1 (TSP-1) was first identified in 1981 as a high-molecular-weight glycoprotein that is rapidly secreted from platelets in response to thrombin. The TSP-1 that is secreted by platelets participates in the regulation of the various steps that are involved in wound healing. Subsequent studies have shown that TSP-1 is widely expressed by a variety of tissues during development. In addition, four other genes that are homologous to TSP-1 have been identified as members of the thrombospondin gene family. Of these proteins, TSP-1 and -2 have equivalent domain structure and are potent inhibitors of angiogenesis.

The expression of TSP-1 in adult tissue is limited for the most part to sites of tissue remodeling. At these sites, TSP-1 acts in the pericellular space to regulate cellular phenotype and extracellular matrix structure. Virtually every domain of TSP-1 has a receptor on the cell surface. The specific repertoire of receptors that a given cell expresses may determine its response to TSP-1. Whereas TSP-1 promotes the migration of vascular smooth muscle cells, it is a potent inhibitor of endothelial cell migration. TSP-1 modulates extracellular matrix structure by binding to matrix proteins such as fibronectin and collagen, and by modulating the activity of extracellular proteinase such as matrix metalloproteinases (MMPs) and plasmin. These properties enable TSP-1 and -2 to be key regulators of the tissue remodeling that is associated with development, wound healing, mammary gland involution, and synaptogenesis. They are also involved in the pathological tissue remodeling that is associated with neoplasia and tumor angiogenesis.

Like most large proteins, the TSPs can be divided into structural domains that reflect exon shuffling during evolution (Chen et al. 2000) (Fig. 26.1). TSP-1 and -2 have an N-terminal domain of approximately 200 amino acids that contains a high-affinity-binding site for heparan sulfate proteoglycans. This domain also mediates the uptake and clearance of the TSPs through a low-density lipoprotein receptor-related (LRP)-dependent mechanism. Three copies of the thrombospondin type 1 repeat (TSR) and three copies of the epidermal growth factor (EGF) repeat are found in the middle of TSP-1 and -2. The TSRs are found in approximately 100 proteins in the human genome (Tucker 2004). The TSRs of TSP-1 bind to β1 integrins, CD36 and transforming growth factor (TGF) β. Whereas TSP-1 and -2 probably have similar functions, they differ in their ability to activate TGFβ. Activation of TGFβ requires the sequence RFK between the first and second TSR of TSP-1 (Young and Murphy-Ullrich 2004b). Since this sequence is not found in TSP-2, it is not able to activate TGFβ. The TSRs have been shown to inhibit tumor angiogenesis and growth (Lawler and Detmar 2004). Furthermore, a therapeutic designated ABT-510 is based on an eight-amino-acid sequence within the second TSR (Haviv et al. 2005). The C-terminal domain of TSP-1 and -2 is composed of a series of contiguous calcium binding sites that are wrapped around a β sandwich structure that is formed by the last 200 amino acids of the proteins (Carlson et al. 2005). The C-terminal domain is highly conserved in all five members of the thrombospondin gene family and thus has been designated the thrombospondin signature domain. This domain appears to bind 30 calcium ions, suggesting that the TSPs are involved in calcium homeostasis within the cell; however, this function of the thrombospondins has not been explored in detail. The cell biology of TSP-1 has been previously reviewed (Chen et al. 2000). In this chapter, the inhibition of angiogenesis by TSP-1 and -2 will be discussed, with a focus on the potential therapeutic applications of these proteins.

Mechanisms of Inhibition of Angiogenesis by TSP-1 and -2

Numerous in vitro and in vivo approaches have been used to identify multiple mechanisms by which TSP-1 and -2 can inhibit angiogenesis. These mechanisms can be broadly characterized as having direct

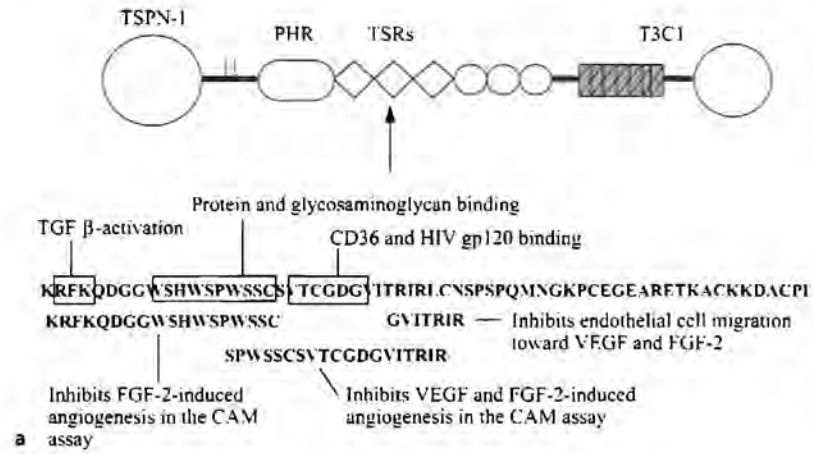
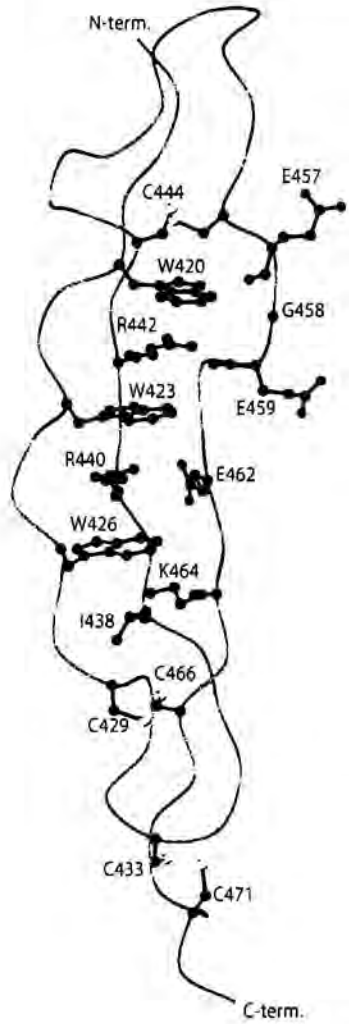


Fig. 26.1a,b. The structure of TSP-1 and the TSRs. a The domain structure of TSP-1(top) and the amino acid sequence of the second type 1 repeat (TSR+RFK). Active amino acid sequences and the anti-angiogenic peptides are indicated. b Schematic representation of the second TSR of TSP-1. Residues that are involved in forming the layered structure are drawn in the ball-and-stick representation. Two anti-angiogenic sequences interdigitate to define a positively charged patch on one surface of the TSR



effects on endothelial cell migration and apoptosis, or indirect effects on the various growth factors, cytokines and proteases that regulate angiogenesis (Table 26.1). A detailed understanding of the mechanisms of action of TSP-1 and -2 has facilitated the design of therapeutic strategies to optimize these activities.

26.2.1 Direct Effects of TSP-1 on Endothelial Cell Function

TSP-1 and -2 are potent inhibitors of endothelial cell migration that is induced by VEGF or bFGF. This activity has been mapped to the TSRs (Fig. 26.2) (Lawler and Detmar 2004). In an early study, a second sequence in the procollagen homology region was also reported to inhibit migration; however, the function of this sequence has not been characterized further (Tolsma et al. 1993). Three different synthetic peptides from the TSRs inhibit endothelial cell migration (Iruela-Arispe et al. 1999). Together, they comprise a continuous region in the N-terminal half of the second TSR of TSP-1. Whereas TSP-2 has not been studied as extensively, the active sequences in TSP-1 are conserved in TSP-2. In the structure of the TSRs, the active sequences fall in two anti-parallel β-strands and the intervening turn (Tan et al. 2002). The

Table 26.1. Inhibition of angiogenesis by TSP-1

Direct effects	Indirect effects
Inhibition of endothelial cell migration	Inhibition of activation of MMPs
Induction of endothelial cell apoptosis	Clearance of MMPs Sequestration of VEGF, bFGF and hepatocyte growth factor Binding to endothelial cell proteoglycans Activation of TGFβ

arginines within the second β-strand orient their side chains so that they interdigitate with three tryptophans on the first β-strand. In this way, the three active sequences are arranged in a positively charged surface patch on the second TSR. This surface patch is thought to represent the binding site for the various ligands of the TSRs, including TGFβ and CD36. The anti-angiogenic therapeutic ABT-510 is based on the sequence GVITRIR in the second β-strand of TSR2 (Reiher et al. 2002; Westphal 2004). This sequence has been modified in several ways to improve its stability in circulation. The N- and C-terminals have been capped, the isoleucine is replaced by D-allo-isoleucine and the first arginine is replaced by norvaline. The second arginine is essential for the activity of ABT-510 (Reiher et al. 2002). The TSRs and ABT-510 reportedly inhibit endothelial cell migration through their interaction with CD36 (Simantov and Silverstein 2003). CD36 is an 88,000-Da membrane protein that mediates the uptake of oxidized lipids and the anti-angiogenic activity of TSP-1 and -2 (Dawson et al. 1997). CD36 is enriched in cholesterol-rich lipid rafts, where it associates with the src family kinases Fyn, Lyn and Yes (Simantov and Silverstein 2003). As discussed below, Fyn is in the signaling pathway that leads to induction of endothelial cell apoptosis in response to TSP-1. CD36 also associates with integrins and the tetraspanin, CD9 (Miao et al. 2001b). The physiological significance of this association is not known; however, β1 integrins have recently been reported to mediate the inhibition of large-vessel endothelial cell migration by the TSRs (Short et al. 2005). This effect is specific for β1 integrins, because integrin β3-siRNA has no effect. Antagonists of α3 and α5 integrin subunits and phosphoinositol 3 kinase (PI3K) block the inhibition of large vessel endothelial cell migration by the TSRs. Taken together, the data suggest that CD36 and β1 integrins may collaborate to form a receptor complex for the TSRs that functions to inhibit endothelial cell migration.

The interaction of the TSRs with CD36 also results in the induction of endothelial cell apoptosis (Jimenez et al. 2000). Fyn and c-Jun N-terminal kinase (JNK) appear to be essential for this process, as TSP-1 is inactive in Fyn-null or JNK-1-null mice (Jimenez et al. 2000, 2001). This pathway also involves p38 MAPK and the up-regulation of Fas ligand (Volpert et al. 2002a). Quiescent endothelial cells express low levels of Fas receptor; however, its expression is increased in response to stimulation by VEGF. The increase in Fas ligand in response to TSP-1 leads to signals that counter the pro-survival signals of VEGF. These data suggest that TSP-1 potentiates the normal vessel regression that is associated with decreased VEGF expression. This conclusion is consistent with the observation that vessel density is increased in TSP-1-null mice during the catagen phase of the hair follicle cycle (Yano et al. 2003). Since tumor blood vessels are formed in response to VEGF and other growth factors and cytokines, the up-regulation of Fas ligand may target these new blood vessels in particular. A threefold increase in tumor endothelial cell apoptosis is observed within pancreatic tumors when mice are systemically treated with the TSRs (Zhang et al. 2005). Whereas various proteins have been shown to be involved in TSP-1-induced apoptosis of endothelial cells, the details of the signaling pathway

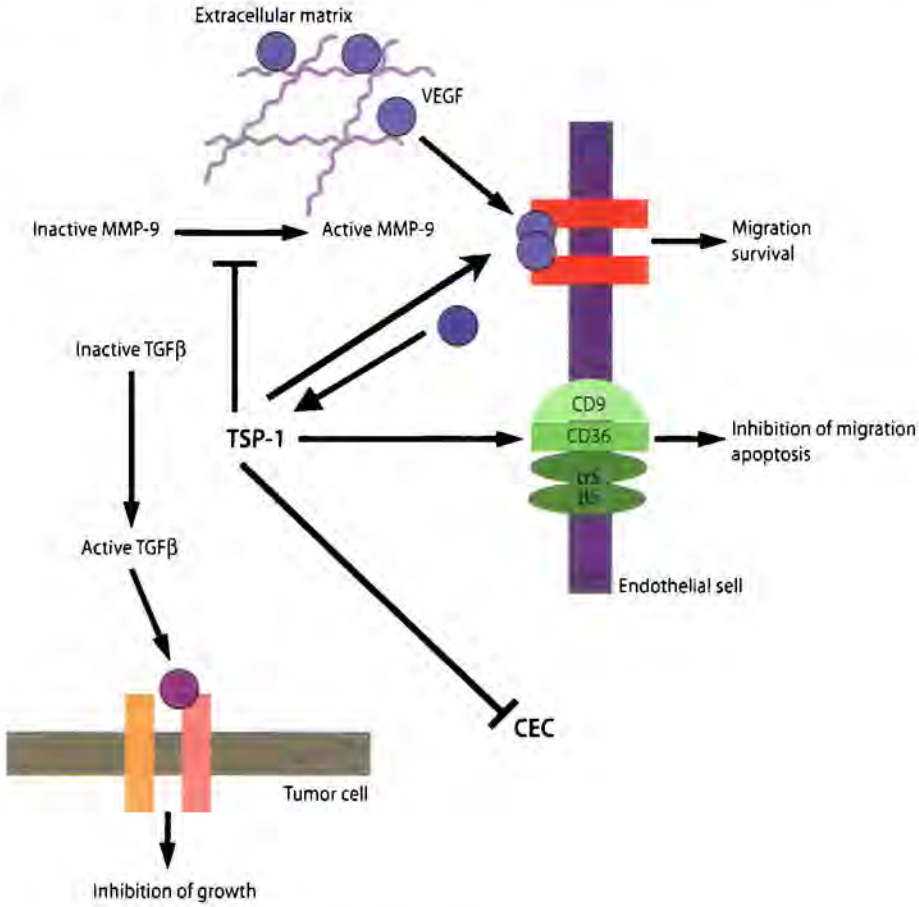


Fig. 26.2. Schematic representation of the functions of TSP-1 in the tumor microenvironment

remain to be determined. A better understanding of this pathway would facilitate the design of optimal therapeutic approaches.

Some portions of the endothelial cells that form the tumor microvasculature are reportedly derived from circulating endothelial progenitor cells (Rafii et al. 2002; Shaked et al. 2005). These cell populations are approximately two times higher in TSP-1-null mice, suggesting that endogenous levels of TSP-1 suppress production and/or survival of these circulating endothelial progenitor cells. In addition, ABT-510 suppresses the elevated levels of circulating endothelial cells in TSP-1-null mice. These data suggest that TSP-1- and TSR-based therapeutics may inhibit angiogenesis by decreasing the level of circulating endothelial cells.

26.2.2 Indirect Effects of TSP-1 on Endothelial Cell Function

TSP-1 inhibits angiogenesis by antagonizing VEGF mobilization from the extracellular matrix and by binding directly to VEGF (Gupta et al. 1999; Rodriguez-Manzaneque et al. 2001). Inhibition of VEGF activity involves the TSRs and other domains of TSP-1. The N-terminal heparin-binding domain of TSP-1 reportedly competes with VEGF for binding sites on endothelial cell proteoglycans (Gupta et al. 1999; Margosio et al. 2003). TSP-1 also binds to fibroblast growth factor-2 and hepatocyte growth factor/scatter factor, suggesting that it functions as a scavenger for angiogenic growth factors (Margosio et al. 2003).

The precise domain for growth factor binding has not been identified.

Matrix-bound VEGF is released by the proteolytic activity of MMPs. TSP-1 and -2 reportedly down-regulate the activity and increase the rate of clearance of MMPs (Bein and Simons 2000; Yang et al. 2001). The level of active MMP-9 in mammary tumor tissue is inversely correlated to the amount of TSP-1 (Rodriguez-Manzaneque et al. 2001). When TSP-1 is over-expressed, the amount of VEGF that is associated with VEGF receptor-2 (VEGFR-2) is decreased and the vessel size and number is decreased within the mammary tumors. In gliomas, the concomitant binding of TSP-1, MMP-2 and LRP results in decreased angiogenesis (Fears et al. 2005). The original yeast-two-hybrid data indicates that the TSRs bind to the type II fibronectin repeats of MMP-2 (Bein and Simons 2000). Thus, TSP-1 inhibits the activation of MMPs and facilitates their uptake and clearance.

TSP-1 also indirectly influences angiogenesis through the activation of TGFβ. Whereas the precise mechanism underlying the activation of TGFβ by TSP-1 is not fully understood, the amino acid sequence arginine–phenylalanine–lysine (RFK) between the first and second TSRs of TSP-1 is essential (Young and Murphy-Ullrich 2004a). Since the other TSPs do not have this sequence, TSP-1 is the only member of the thrombospondin family that can activate TGFβ. The effect of TGFβ on angiogenesis is complex, involving both positive and negative effects (Bertolino et al. 2005). In A431 experimental tumors, over-expression of the TSRs results in an increase in active TGFβ (Yee et al. 2004). Only constructs that contain the RFK sequence increase active TGFβ and decrease vessel size, suggesting that the TGFβ that is activated by TSP-1 inhibits angiogenesis. Mammary tumors that form in TSP-1-null mice display decreased levels of collagen (Yee et al. 2006). By contrast, TSP-1 reportedly decreases collagen types 1α1, 1α2 and IIIα1 in muscle explants growing in three-dimensional culture (Zhou et al. 2006). Since these changes correspond to decreased angiogenesis, the authors posited that TSP-1 inhibits angiogenesis through regulation of collagen gene expression.

Therapeutic Applications

Whereas oncogenes tend to suppress TSP-1 expression, tumor suppressor genes commonly increase TSP-1 synthesis. Thus, tumor tissue acts to promote angiogenesis by increasing stimulators of angiogenesis, like VEGF, and by decreasing endogenous inhibitors of angiogenesis. Hyperactivation of oncogenic Ras leads to activation of PI3K, Rho, Rho-associated kinase (ROCK) and Myc, and decreased TSP-1 expression (Watnick et al. 2003). Down-regulation of TSP-1 by Myc appears to involve increased TSP-1 mRNA turnover more than suppression of transcription (Janz et al. 2000). The tumor cells also appear to secrete factors that result in decreased TSP-1 expression in neighboring stromal fibroblasts (Kalas et al. 2005). Ras-transformed fibrosarcoma cells secrete low-molecular-weight, heat-labile, trypsin-resistant factors that down-regulate TSP-1 in nontumorigenic dermal fibroblast cell lines (Kalas et al. 2005). In this way, the tumor cells create a microenvironment that is permissive for growth factor-induced angiogenesis.

The fact that TSP-1 is a potent endogenous inhibitor of angiogenesis has prompted several groups to explore therapeutic applications of TSP-1. These efforts fall into two basic approaches, the identification of strategies to up-regulate endogenous TSP-1, and the delivery of recombinant TSRs or synthetic peptides that contain sequences from the TSRs.

26.3.1 Strategies for Up-Regulation of TSP-1 or -2

The continuous administration of low doses of chemotherapeutics is sometimes referred to as metronomic dosing or anti-angiogenic chemotherapy (Browder et al. 2000). Metronomic dosing with cyclophosphamide increases the circulating levels of TSP-1 (Bocci et al. 2003). The source of the TSP-1 is controversial in that one group identifies endothelial cells as the source, and another proposes that the tumor cells secrete elevated TSP-1 (Bocci et al. 2003;

Hamano et al. 2004). To show that the inhibition of tumor growth is due to increased TSP-1, the efficacy of metronomic dosing has been compared in wild-type and TSP-1-null mice. Whereas metronomic dosing with cyclophosphamide inhibits the growth of experimental Lewis lung carcinomas in wild-type mice, it is not effective in TSP-1-null mice (Bocci et al. 2003). Since metronomic dosing therapy depends on TSP-1 up-regulation, circulating levels of TSP-1 can be used as a marker for its efficacy.

Low doses of cyclophosphamide, cisplatin or docetaxel also reportedly increase endothelial cell Fas receptor (Yap et al. 2005). TSP-1 and ABT-510 increase the level of Fas ligand on endothelial cells (Volpert et al. 2002b; Yap et al. 2005). Thus, the combination of chemotherapy agents and TSP-1 or ABT-510 induces endothelial cell apoptosis and inhibits tumor angiogenesis.

The implantation of a biodegradable polymer that contains TSP-2 over-expressing fibroblasts into the ovarian pedicle produces elevated systemic levels of TSP-2 for at least 5 weeks (Streit et al. 2002). The increased TSP-2 inhibited the growth of squamous cell carcinomas, melanomas and Lewis lung carcinomas. The decreased tumor growth was associated with a decrease in tumor vessel size and an increase in tumor cell apoptosis. Nor and co-workers (Nor et al. 2000) have described the production of a recombinant portion of TSP-2 that includes the N-terminal domain through the TSRs. Systemic delivery of this protein inhibits the growth of experimental squamous cell carcinomas.

26.3.2 TSR-based Therapeutics

Systemic injection of purified platelet TSP-1 inhibits B16F10 experimental tumor growth (Miao et al. 2001a). Since TSP-1 is a large molecule, several groups have sought to define a region of the molecule that is anti-angiogenic and more amenable to therapeutic use. Synthetic peptides that include sequences from the procollagen homology region and from the TSRs inhibit angiogenesis (Dawson and Bouck 1999; Iruela-Arispe et al. 1999; Reiher et al. 2002; Tolsma et al. 1993). In addition, systemic injections of a recombinant version of all three TSRs of TSP-1, designated 3TSR, inhibits experimental pancreatic tumors, melanoma and Lewis lung carcinoma (Miao et al. 2001a; Zhang et al. 2005). Inhibition of pancreatic tumor growth is associated with decreased vessel size and increased endothelial cell apoptosis (Zhang et al. 2005). To identify essential amino acids for the inhibition of tumor growth by TSP-1, we have prepared a recombinant version of the second TSR of TSP-1 with (designated TSR2+RFK) and without (designated TSR2) the RFK sequence that is essential for the activation of TGFβ (Miao et al. 2001a). TSR2 is a potent inhibitor of angiogenesis and growth of experimental and Lewis lung carcinoma tumors. Inclusion of the RFK sequence results in the activation of TGFβ and a further inhibition of B16F10 melanoma, but not Lewis lung carcinoma. This difference results from the fact that the B16F10 melanoma cells are responsive to the growth inhibitory effects of TGFβ, while the Lewis lung carcinomas are not.

The safety, pharmacokinetics and pharmacodynamic results of a phase I clinical trial of ABT-510 have been reported (Hoekstra et al. 2005). ABT-510 was administrated subcutaneously as a bolus injection either once or twice daily with dose escalation beginning with 100 mg/24 h. Patients in a continuous-infusion arm of the study developed erythema and edema of the skin at the infusion site which was sometimes painful. As a result, this arm of the study was discontinued. Of the 35 patients who received bolus injections of ABT-510, three adverse events were considered potentially related to the drug treatment: a fetal intracranial hemorrhage, a transient ischemic attack and a case of new-onset diabetes mellitus. In all three cases, a causal link between ABT-510 treatment and the adverse event could not be unequivocally made. ABT-510 has a half-life of 1.1±0.2 h in circulation (Hoekstra et al. 2005). These data suggest that multiple treatment during a fixed period of time will be more effective than increasing a single dose. Whereas the phase I clinical trial is not designed to determine efficacy, some patients appeared to benefit from ABT-510 treatment. Prolonged stable disease was observed in patients with sarcoma, renal cell carcinoma, carcinoma of the

cervix, colorectal carcinoma or a germ cell tumor. Phase II clinical trials are currently underway with ABT-510 as a single agent or in combination with chemotherapy for the treatment of soft tissue sarcoma, renal cell carcinoma, lymphoma and non-small-cell lung cancer.

CD36 expression on endothelial cells is increased by peroxisome proliferating-activated receptor (PPARγ) ligands, such as troglitazone. Since CD36 is reportedly the receptor for ABT-510, the effect of a combination of troglitazone and ABT-510 on experimental bladder carcinomas has been investigated (Huang et al. 2004). Troglitazone (50 mg/kg/day) increased CD36 expression in tumor-associated capillaries. Whereas ABT-510 or troglitazone alone inhibit tumor growth by about 30%, the combination of these reagents inhibited tumor growth by 74%. As anticipated, the mean vessel density and the degree of endothelial cell apoptosis are significantly greater with the combination therapy than with ABT-510 alone.

A peptide therapeutic, designated ABT-526, has been used to treat cancer in pet dogs (Khanna et al. 2002). ABT-526 is equivalent to ABT-510 except that the D-allo-isoleucine is replaced by D-isoleucine. ABT-510 is more soluble in water and has a longer half-life in primate circulation than ABT-526 (Westphal 2004). Eleven of 56 dogs with various types of tumors that were treated with ABT-526 were found to have stable disease, six displayed partial remission, and complete remission was observed in two dogs (Khanna et al. 2002).

Taken together, the data indicate that ABT-510 has the potential to be an effective therapeutic with low toxicity. However, it should be noted that the preclinical and clinical trials have not involved treatment for long periods of time. The receptor for ABT-510, CD36, is expressed on platelets, monocytes, hematopoietic cells and breast epithelial cells. It is important to assess the effect of long-term ABT-510 treatment on these cell types. This is especially true for treatment strategies that sensitize the tumor microvasculature to ABT-510 by up-regulating CD36. In addition, the effect of ABT-510 on physiological angiogenesis, for example during wound healing, needs to be fully evaluated.

Combination therapy using TSP-1 or ABT-510 with other treatment modalities or other anti-angiogenic reagents has been shown to be effective in various murine models of cancer. Endostatin and TSP-1 act through different receptors and affect the expression of different genes, suggesting that they have distinct modes of action (Cline et al. 2002). Thus, a combination of endostatin and a TSR-based peptide that is a predecessor of ABT-510 effectively inhibits angiogenesis and experimental Lewis lung carcinoma growth (Cline et al. 2002). Similarly, treatment with TSP-1 has been shown to improve the efficacy of radiation therapy (Rofstad et al. 2003). When TSP-1 is injected before radiation treatment, it increases endothelial cell apoptosis in the tumor microvasculature and reduces the fraction of radiobiologically hypoxic cells. Continued TSP-1 treatment after radiation inhibits the outgrowth of D-12 melanoma metastases.

Future Considerations for the Development of TSP-1-based Therapies

The data obtained to date indicate that TSP-1 is a potent inhibitor of tumor angiogenesis with considerable therapeutic promise. TSP-1 levels can be increased by direct delivery of the protein or by reagents that up-regulate its expression in tissue. The anti-angiogenic activity of TSP-1 can be mimicked by recombinant proteins or peptides that contain TSR sequences. These reagents are considerably easier and less expensive to produce than the intact TSP-1 molecule. Since multiple TSR sequences seem to be important, and because the tertiary structure of the TSRs may be necessary for optimal orientation of these sequences, the recombinant proteins may be more active than the synthetic peptides.

Whereas the structure of the TSRs has been elucidated, very little is known about the structure of CD36. An understanding of the structure of CD36, and the nature of its interaction with the TSRs, will facilitate the identification of small-molecule ligands

of CD36 that can mimic the effect of TSP-1 binding. In addition, a more complete understanding of the signaling pathways that are affected by CD36 will help to identify novel targets. It would be very helpful to identify points of crosstalk between the CD36 signaling pathway and that of VEGF. Recent studies have implicated β1 integrins as a receptor system involved in the inhibition of endothelial cell migration by the TSRs (Short et al. 2005). This observation identifies two areas of further investigation that will be important for the development of TSR-based therapeutics. The relative importance of CD36 and β1 integrins for inhibition of angiogenesis by TSP-1 needs to be determined. Furthermore, since β1 integrins are widely expressed, it will be important to carefully search for side effects of long-term TSR treatment.

Drug resistance is a common occurrence in chemotherapy because the genome of the cancer cell is unstable. Since the endothelial cell is the target of anti-angiogenesis therapy, it was possible that drug resistance would not limit this approach; however, tumor angiogenesis results from a dialog between the cancer cell and the endothelium. To stimulate angiogenesis, tumor cells express VEGF, and other pro-angiogenic factors, and down-regulate TSP-1. The decrease in TSP-1 also serves to relieve the growth-inhibitory effect of TGFβ. Fibrosarcomas have been shown to overcome the inhibitory effects of TSP-1 by increasing VEGF expression and by becoming resistant to TGFβ (Filleur et al. 2001). Although over-expression of TSP-1 in the fibrosarcoma cells initially inhibits tumor growth, eventually the tumors do grow. Cells from these tumors grow rapidly when they are harvested and re-injected into rats. Thus, in the presence of TSP-1, it appears that a population of tumor cells that were resistant to its inhibitory effects was selected. Significant inhibition of tumor growth can be achieved by concomitant up-regulation of TSP-1 and down-regulation of VEGF (Filleur et al. 2003).

Whereas the role of TSP-1 as an inhibitor of primary tumor growth is well documented, its role in tumor metastasis is controversial. TSP-1 expression correlates with a non-metastatic phenotype in two clones that were derived from the MDA-MB-435 breast cancer cell line (Urquidi et al. 2002). The non-metastatic cell line NM-2C5 displays a 15-fold increase in TSP-1 expression compared to the metastatic cell line M-4A4. In another study, Transfection of full-length TSP-1 into MDA-MB-435 cells decreased the growth of orthotopic experimental tumors and decreased the number of pulmonary metastases by approximately 50% (Weinstat-Saslow et al. 1994). However, in vitro studies suggest that TSP-1 promotes MDA-MB-435 cell migration and invasion (Albo et al. 1998). Over-expression of TSP-1 results in an approximately 50% increase in cell-associated plasmin. TSP-1 also increases the migration of invasive breast cancer cell lines through collagen gels, but does not affect the migration of a noninvasive cell line (Wang et al. 1996). A similar correlation is seen with squamous cell carcinoma cells (Yabkowitz et al. 1993). If TSP-1 is indeed prometastatic, it will be important to develop strategies for the inhibition of angiogenesis and primary tumor growth that do not promote metastatic spread.

Acknowledgements

I would like to thank Lydia Gregg and Sami Lawler for help in preparing the manuscript. This work was supported by NIH grant HL68003.

References

Albo B, Berger DH, Tuszynski GP (1998) The effect of thrombospondin-1 and TGF-β1 on pancreatic cancer cell invasion. J Surg Res 76:86–90

Bein K, Simons M (2000) Thrombospondin type 1 repeats interact with matrix metalloproteinase 2. Regulation of metalloproteinase activity. J Biol Chem 275:32167–32173

Bertolino P, Deckers M, Lebrin F, ten Dijke P (2005) Transforming growth factor-beta signal transduction in angiogenesis and vascular disorders. Chest 128:585S–590S

Bocci G, Francia G, Man S, Lawler J, Kerbel RS (2003) Thrombospondin 1, a mediator of the antiangiogenic effects of low-dose metronomic chemotherapy. Proc Natl Acad Sci USA 100:12917–12922

Browder T, Butterfield CE, Kraling BM, Shi B, Marshall B, O'Reilly MS, Folkman J (2000) Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. Cancer Res 60:1878–1886

Carlson CB, Bernstein DA, Annis DS, Misenheimer TM, Hannah BL, Mosher DF, Keck JL (2005) Structure of the calcium-rich signature domain of human thrombospondin-2. Nat Struct Mol Biol 12:910–914

Chen H, Herndon ME, Lawler J (2000) The cell biology of thrombospondin-1. Matrix Biol 19:597–614

Cline EI, Bicciato S, DiBello C, Lingen MW (2002) Prediction of in vivo synergistic activity of antiangiogenic compounds by gene expression profiling. Cancer Res 62:7143–7148

Dawson DW, Bouck NP (1999) Thrombospondin as an inhibitor of angiogenesis. In: Teicher BA (ed) Antiangiogenic agents in cancer therapy. Humana, Totowa, NJ, pp 185–203

Dawson DW, Pearce SFA, Zhong R, Silverstein RL, Frazier WA, Bouck NP (1997) CD36 mediates the in vitro inhibitory effects of thrombospondin-1 on endothelial cells. J Cell Biol 138:707–717

Fears CY, Grammer JR, Stewart JE Jr, Annis DS, Mosher DF, Bornstein P, Gladson CL (2005) Low-density lipoprotein receptor-related protein contributes to the antiangiogenic activity of thrombospondin-2 in a murine glioma model. Cancer Res 65:9338–9346

Filleur S, Volpert OV, Degeorges A, Voland C, Reiher F, Clezardin P, Bouck N, Cabon F (2001) In vivo mechanisms by which tumors producing thrombospondin 1 bypass its inhibitory effects. Genes Dev 15:1373–1382

Filleur S, Courtin A, Ait-Si-Ali S, Guglielmi J, Merle C, Harel-Bellan A, Clezardin P, Cabon F (2003) SiRNA-mediated inhibition of vascular endothelial growth factor severely limits tumor resistance to antiangiogenic thrombospondin-1 and slows tumor vascularization and growth. Cancer Res 63:3919–3922

Gupta K, Gupta P, Wild R, Ramakrishnan S, Hebbel RP (1999) Binding and displacement of vascular endothelial growth factor (VEGF) by thrombospondin: effect on human microvascular endothelial cell proliferation and angiogenesis. Angiogenesis 3:147–158

Hamano Y, Sugimoto H, Soubasakos MA, Kieran M, Olsen BR, Lawler J, Sudhakar A, Kalluri R (2004) Thrombospondin-1 associated with tumor microenvironment contributes to low-dose cyclophosphamide-mediated endothelial cell apoptosis and tumor growth suppression. Cancer Res 64:1570–1574

Haviv F, Bradley MF, Kalvin DM, Schneider AJ, Davidson DJ, Majest SM, McKay LM, Haskell CJ, Bell RL, Nguyen B, Marsh KC, Surber BW, Uchic JT, Ferrero J, Wang YC, Leal J, Record RD, Hodde J, Badylak SF, Lesniewski RR, Henkin J (2005) Thrombospondin-1 mimetic peptide inhibitors of angiogenesis and tumor growth: design, synthesis, and optimization of pharmacokinetics and biological activities. J Med Chem 48:2838–2846

Hoekstra R, de Vos FY, Eskens A, Gietema JA, van der Gaast A, Groen HJ, Knight RA, Carr RA, Humerickhouse RA, Verweij J, de Vries EG (2005) Phase I safety, pharmacokinetic, and pharmacodynamic study of the thrombospondin-1-mimetic angiogenesis inhibitor ABT-510 in patients with advanced cancer. J Clin Oncol 23:5188–5197

Huang H, Campbell SC, Bedford DF, Nelius T, Veliceasa D, Shroff EH, Henkin J, Schneider, A, Bouck N, Volpert OV (2004) Peroxisome proliferator-activated receptor gamma ligands improve the antitumor efficacy of thrombospondin peptide ABT510. Mol Cancer Res 2:541–550

Iruela-Arispe ML, Lombardo M, Krutzsch HC, Lawler J, Roberts DD (1999) Inhibition of angiogenesis by thrombospondin-1 is mediated by 2 independent regions within the type 1 repeats. Circulation 100:1423–1431

Janz A, Sevignani C, Kenyon K, Ngo CV, Thomas-Tikhonenko A (2000) Activation of the myc oncoprotein leads to increased turnover of thrombospondin-1 mRNA. Nucleic Acids Res 28:2268–2275

Jimenez B, Volpert OV, Crawford SE, Febbraio M, Silverstein RL, Bouck N (2000) Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. Nat Med 6:41–48

Jimenez B, Volpert OV, Reiher F, Chang L, Munoz A, Karin M, Bouck N (2001) c-Jun N-terminal kinase activation is required for the inhibition of neovascularization by thrombospondin-1. Oncogene 20:3443–3448

Kalas W, Yu JL, Milsom C, Rosenfeld J, Benezra R, Bornstein P, Rak J (2005) Oncogenes and angiogenesis: down-regulation of thrombospondin-1 in normal fibroblasts exposed to factors from cancer cells harboring mutant ras. Cancer Res 65:8878–8886

Khanna A, Plummer M, Bromberek C, Bresnahan B, Hariharan S (2002) Expression of TGF-beta and fibrogenic genes in transplant recipients with tacrolimus and cyclosporine nephrotoxicity. Kidney Int 62:2257–2263

Lawler J, Detmar M (2004) Tumor progression: the effects of thrombospondin-1 and -2. Int J Biochem Cell Biol 36:1038–1045

Margosio B, Marchetti D, Vergani V, Giavazzi R, Rusnati M, Presta M, Taraboletti G (2003) Thrombospondin 1 as a scavenger for matrix-associated fibroblast growth factor 2. Blood 102:4399–4406

Miao W, Seng WL, Duquette M, Laus C, Detmar M, Lawler J (2001a) Thrombospondin-1 type 1 repeat recombinant proteins inhibit tumor growth. Cancer Res 97:1689–1696

Miao WM, Vasile E, Lane WS, Lawler J (2001b) CD36 associates with CD9 and integrins on human blood platelets. Blood 97:1689–1696

Nor JE, Mitra RS, Sutorik MM, Mooney DJ, Castle VP, Polverini PJ (2000) Thrombospondin-1 induces endothelial cell apoptosis and inhibits angiogenesis by activating the caspase death pathway. J Vasc Res 37:209–218

Rafii S, Lyden D, Benezra R, Hattori K, Heissig B (2002) Vascular and haematopoietic stem cells: novel targets for anti-angiogenesis therapy? Nat Rev Cancer 2:826–835

Reiher FK, Volpert OV, Jimenez B, Crawford SE, Dinney CP, Henkin J, Haviv F, Bouck NP, Campbell SC (2002) Inhibition of tumor growth by systemic treatment with thrombospondin-1 peptide mimetics. Int J Cancer 98:682–689

Rodriguez-Manzaneque JC, Lane TF, Ortega MA, Hynes RO, Lawler J, Iruela-Arispe ML (2001) Thrombospondin-1 supresses tumor growth by a novel mechanism that includes blockade of matrix metalloproteinase-9 activation. Proc Natl Acad Sci USA 98:12485–12490

Rofstad EK, Henriksen K, Galappathi K, Mathiesen B (2003) Antiangiogenic treatment with thrombospondin-1 enhances primary tumor radiation response and prevents growth of dormant pulmonary micrometastases after curative radiation therapy in human melanoma xenografts. Cancer Res 63:4055–4061

Shaked Y, Bertolini F, Man S, Rogers MS, Cervi D, Foutz T, Rawn K, Voskas D, Dumont DJ, Ben-David Y, Lawler J, Henkin J, Huber J, Hicklin DJ, D'Amato RJ, Kerbel RS (2005) Genetic heterogeneity of the vasculogenic phenotype parallels angiogenesis: implications for cellular surrogate marker analysis of antiangiogenesis. Cancer Cell 7:101–111

Short S, Derrien A, Narsimhan R, Lawler J, Ingber D, Zetter B (2005) Thrombospondin type 1 repeats inhibit endothelial cell migration via b1 integrins by a CD36-independent mechanism. J Cell Biol 168:643–653

Simantov R, Silverstein RL (2003) CD36: a critical anti-angiogenic receptor. Front Biosci 8:s874

Streit M, Stephen AE, Hawighorst T, Matsuda K, Lange-Asschenfeldt B, Brown LF, Vacanti JP, Detmar M (2002) Systemic inhibition of tumor growth and angiogenesis by thrombospondin-2 using cell-based antiangiogenic gene therapy. Cancer Res 62:2004–2012

Tan K, Duquette M, Liu JH, Dong Y, Zhang R, Joachimiak A, Lawler J, Wang JH (2002) Crystal structure of the TSP-1 type 1 repeats: a novel layered fold and its biological implication. J Cell Biol 159:373–382

Tolsma SS, Volpert OV, Good DJ, Frazier WA, Polverini PJ, Bouck N (1993) Peptides derived from two separate domains of the matrix protein thrombospondin-1 have anti-angiogenic activity. J Cell Biol 122:497–511

Tucker RP (2004) The thrombospondin type 1 repeat superfamily. Int J Biochem Cell Biol 36:969–974

Urquidi V, Sloan D, Kawai K, Agarwal D, Woodman AC, Tarin D, Goodison S (2002) Contrasting expression of thrombospondin-1 and osteopontin correlates with absence or presence of metastatic phenotype in an isogenic model of spontaneous human breast cancer metastasis. Clin Cancer Res 8:61–74

Volpert OV, Pili R, Sikder HA, Nelius T, Zaichuk T, Morris C, Shiflett CB, Devlin MK, Conant K, Alani RM (2002a) Id1 regulates angiogenesis through transcriptional repression of thrombospondin-1. Cancer Cell 2:473–483

Volpert OV, Zaichuk T, Zhou W, Reiher F, Ferguson TA, Stuart PM, Amin M, Bouck NP (2002b) Inducer-stimulated Fas targets activated endothelium for destruction by anti-angiogenic thrombospondin-1 and pigment epithelium-derived factor. Nat Med 8:349–357

Wang TN, Qian X, Granick MS, Solomon MP, Rothman VL, Berger DH, Tuszynski GP (1996) Thrombospondin-1 (TSP-1) promotes the invasive properties of human breast cancer. J Surg Res 63:39–43

Watnick RS, Cheng YN, Rangarajan A, Ince TA, Weinberg RA (2003) Ras modulates Myc activity to repress thrombospondin-1 expression and increase tumor angiogenesis. Cancer Cell 3:219–231

Weinstat-Saslow DL, Zabrenetzky VS, VanHoutte K, Frazier WA, Roberts DD, Steeg PS (1994) Transfection of thrombospondin 1 complementary DNA into a human breast carcinoma cell line reduces primary tumor growth, metastatic potential and angiogenesis. Cancer Res 54:6504–6511

Westphal JR (2004) Technology evaluation: ABT-510, Abbott. Curr Opin Mol Ther 6:451–457

Yabkowitz R, Dixit VM, Guo N, Roberts DD, Shimizu Y (1993) Activated T-cell adhesion to thrombospondin is mediated by the alpha 4 beta 1 (VLA-4) and alpha 5 beta 1 (VLA-5) integrins. J Immunol 151:149–158

Yang Z, Strickland DK, Bornstein P (2001) Extracellular matrix metalloproteinase 2 levels are regulated by the low density lipoprotein-related scavenger receptor and thrombospondin 2. J Biol Chem 276:8403–8408

Yano K, Brown LF, Lawler J, Miyakawa T, Detmar M (2003) Thrombospondin-1 plays a critical role in the induction of hair follicle involution and vascular regression during the catagen phase. J Invest Dermatol 120:14–19

Yap R, Veliceasa D, Emmenegger U, Kerbel RS, McKay LM, Henkin J, Volpert OV (2005) Metronomic low-dose chemotherapy boosts CD95-dependent antiangiogenic effect of the thrombospondin peptide ABT-510: a complementation antiangiogenic strategy. Clin Cancer Res 11:6678–6685

Yee KO, Streit M, Hawighorst T, Detmar M, Lawler J (2004) Expression of the type-1 repeats of thrombospondin-1 inhibits tumor growth through activation of transforming growth factor-beta. Am J Pathol 165:541–552

Yee KO, Connolly CM, Pines M, Lawler J (2006) Halofuginone inhibits tumor growth in the polyoma middle T antigen mouse via a thrombospondin-1 independent mechanism. Cancer Biol Ther 5:218–224

Young GD, Murphy-Ullrich JE (2004a) Molecular interactions that confer latency to transforming growth factor-beta. J Biol Chem 279:38032–38039

Young GD, Murphy-Ullrich JE (2004b) The tryptophan-rich motifs of the thrombospondin type 1 repeats bind VLAL motifs in the latent transforming growth factor-beta complex. J Biol Chem 279:47633–47642

Zhang X, Galardi E, Duquette M, Delic M, Lawler J, Parangi S (2005) Antiangiogenic treatment with three thrombospondin-1 type 1 repeats recombinant proteins in an orthotopic human pancreatic cancer model. Clin Cancer Res 11:1–8

Zhou L, Isenberg JS, Cao Z, Roberts DD (2006) Type 1 collagen is a molecular target for inhibition of angiogenesis by endogenous thrombospondin-1. Oncogene 25:536–545

The Use of Orthotopic Models to Validate Antivascular Therapies for Cancer

27

ROBERT R. LANGLEY, SUN-JIN KIM, ISAIAH J. FIDLER

Abstract

The growth and survival of malignant tumors are dependent on an adequate vascular supply. A growing body of preclinical evidence suggests that therapeutic targeting of tumor-associated blood vessels may be an effective means to control tumor growth and limit metastatic spread. Unfortunately, the success of vascular targeting approaches for the treatment of human cancer has been less than remarkable. One likely explanation for the discrepancy between the preclinical findings and results observed in patients is related to the tumor models utilized in preclinical studies. Indeed, most reports seeking to validate the use of antivascular agents for the treatment of cancer have drawn conclusions from tumors implanted into the subcutaneous space and thus have neglected to account for the impact of the specific organ microenvironment on the regulation of tumor growth. While it is difficult to replicate several facets of human tumor growth using animal models, microenvironmental concerns may be easily addressed through the use of orthotopically implanted tumors. Differences between orthotopic and ectopic models are considered, and advances in antivascular therapy for cancer are discussed.

CONTENTS

27.1 Introduction

The progressive growth and survival of malignant tumors are dependent on tumor cell proximity to a vascular supply. Experimental evidence suggests that tumor cell division takes place within 75 μm of the nearest blood vessel, and that cells located beyond 150 μm from a vessel are destined for programmed cell death (Fidler et al. 2002). This latter figure closely approximates the diffusion coefficient of oxygen (Brown and Giaccia 1998); therefore, simple diffusion is sufficient to support the metabolic requirements of tumors with a mass less than 0.5 mm, but growth beyond 0.5 mm can be sustained only by an increase in vascular density.

Persistent growth factor signaling and the loss of cell cycle regulation promote tumor cell proliferation that, in turn, leads to an imbalance between the metabolic demands of the expanding mass and blood flow delivery. An extensive body of evidence has collectively determined that the primary compensatory mechanism employed by tumor cells to offset increasing metabolic pressures involves the recruitment of resident microvascular endothelial cells to form new blood vessels (i.e., angiogenesis) (Folkman 1989). Investigations conducted over recent decades have identified several of the pathways that signal for angiogenesis in tumor tissues, and the molecular effectors that regulate these cascades have emerged as principal targets for therapeutic intervention.

Initial evaluations of anti-angiogenic agents in animal models created a sense of optimism among investigators by demonstrating that inhibiting the growth of new capillary networks could repress tumor growth and metastasis. Unfortunately, to date, the dramatic observations reported in preclinical studies have not been reproduced in the clinical setting. The disappointing results of the clinical trials have prompted a more intensified investigation of the biology of tumor angiogenesis in an attempt to improve patient therapy. As an outgrowth of these efforts, recent studies have identified several potential issues that may account for some of the limited responses observed in the clinic. For example, early reports argued that pharmacological targeting of tumor endothelium might be superior to attempts directed toward the malignant cell population because the blood vessels perfusing tumor tissue were considered genetically stable and, therefore, not inclined to develop resistance to therapy. However, more recent systematic examinations performed on tumor-associated endothelial cells indicate that these cells are, in fact, susceptible to genetic abnormalities (Hida et al. 2004). The cellular and molecular mechanisms responsible for the genetic instability observed in the tumor vascular endothelium remain largely unknown, as do the consequences for anti-angiogenic initiatives. In addition, studies have also determined that certain subsets of tumors are capable of continued growth without invoking angiogenesis (Leenders et al. 2002). Instead of recruiting endothelial cells to form new vascular networks, these tumors meet their metabolic requirements by residing in the vicinity of preexisting blood vessels. Similar data suggest that tumors may revert to an angiogenesis-independent pattern of growth when challenged with anti-angiogenic therapy (Leenders et al. 2004). Angiogenesis-independent tumor growth has been reported in murine models of melanoma brain metastases (Kusters et al. 2002), glioma (Bernsen et al. 2005) and in human non-small-cell lung tumors (Pezzella et al. 1997) and, intuitively, this phenotype should not be responsive to interventions designed to inhibit formation of new blood vessels. Another dilemma facing cancer therapies that are directed toward the neovasculature is the recent observation that when therapy is designed to inhibit signaling initiated by a single endothelial cell mitogen, tumors respond by elaborating secondary, i.e., redundant, factors capable of sustaining endothelial cell growth and survival (Casanovas et al. 2005).

While the aforementioned results pose significant challenges for the successful development and utilization of anti-angiogenic therapies, a more fundamental issue may be responsible for several of the inadequate responses observed in the clinic. Specifically, the vast majority of preclinical studies are performed in subcutaneous tumor models and, consequently, fail to account for the impact of the organ microenvironment on the regulation of angiogenesis and tumor growth. The introduction of tumor cells (regardless of their origin) into the subcutaneous space requires considerably less manual dexterity from the investigator and also provides a visible, noninvasive means to monitor tumor growth. Despite these advantages, evaluations of anti-angiogenic therapies in subcutaneous tumor models are informative only with respect to the agent's actions on dermal endothelial cells. The exploitation of subcutaneous tumor models to evaluate treatment strategies that target tumor-associated endothelial cells is somewhat surprising given the voluminous collection of literature documenting heterogeneity

of endothelial cells from different anatomic regions (Gerritsen 1987; Thorin and Shreeve 1998; Langley et al. 2003). It is also unrealistic that a clinician would choose an anti-angiogenic agent over surgery or radiation to treat tumors of the skin. Death due to cancer most often stems from an inability to effectively control the growth of metastases; the preferred target organs of which are bone, brain, liver, and lung. A more focused investigation on the vascular beds that support the outgrowth of metastases using tumor models that more closely approximate the clinical setting may generate more meaningful clinical results. In the following sections, we review how the microenvironment regulates tumor metastasis and angiogenesis, and then describe how anti-angiogenic therapy was used in an orthotopic model of prostate cancer to produce therapy of tumor.

Metastasis

Tumor metastasis refers to the transfer of malignant cells from one organ to another (distant) organ. The metastatic process consists of a complex series of interconnected steps that is initiated when tumor cells downregulate homotypic intercellular adhesion molecules (e.g., E-cadherin), allowing them to detach from the primary mass and invade adjacent tissue. Invasive tumor cells release degradative enzymes that digest a pathway through restrictive basement membranes and surrounding matrix in a step that signifies the transition from a benign carcinoma to a malignant invasive tumor. Tumor cells access the systemic circulation directly by penetrating through small blood vessels, or indirectly by trafficking through the lymphatic system. While in the vascular compartment, tumor cells must be able to withstand turbulent flow patterns and avoid hostile encounters with components of the immune system. Tumor cells arrest in distant microvascular blood vessels by passive (i.e., steric hindrance) or active (e.g., selective adhesive interactions) mechanisms. Finally, adherent tumor cells must extravasate into the underlying tissue parenchyma and activate angiogenesis to ensure their growth.

Each step of the metastatic cascade is considered rate limiting inasmuch as failure to complete any step effectively terminates the process. Overall, metastasis is considered a highly inefficient pathological process, as evidenced by the fact that less than 0.01% of disseminating cells are able to survive and form secondary growths (Fidler 1970). Tumor cells that are successful in generating metastases are believed to be the progeny of a highly specialized subpopulation of cells that emerge from a heterogeneous primary tumor (Fidler and Kripke 1977). It is thought that these cells are endowed with phenotypic characteristics that are advantageous for metastatic spread.

Recent advances in molecular biology are beginning to provide an improved understanding of the genetic determinants that are critical for the formation of metastases. Much of the new information results from the development of various DNA microarray platforms that allow users to simultaneously evaluate transcription of thousands of genes. Kang and colleagues recently applied such an approach to create transcriptional profiles on parental MDA-MB-231 breast cancer cells and several derivative subpopulations that possessed inherent differences in metastatic potential (Kang et al. 2003). A comparison of gene expression patterns between parental cells and a bone-colonizing variant revealed an underlying gene expression signature that could explain the organ tropism to bone. Bone-colonizing tumor cells expressed elevated levels of matrix metalloproteinase-1 (MMP-1), interleukin-11 (IL-11), osteopontin, connective tissue growth factor (CTGF), and the chemokine receptor CXCR-4. The coordinated expression of this gene set was sufficient to explain homing to bone (CXCR-4), as well as activation of proteolysis (MMP-1), angiogenesis (CTGF), and osteoclastogenesis (IL-11, osteopontin).

Seed and Soil Hypothesis

That some tumors exhibit a predilection for spread to certain organs has been recognized for over a century now. Paget was among the first to describe the organ preference pattern of metastases following his examination of autopsy records that had been assembled from more than 700 women with breast cancer (Paget 1889). Paget recorded a nonrandom pattern of visceral and bone metastasis that allowed him to conclude that certain tumor cells (the "seed") grew preferentially in the microenvironment of selected organs (the "soil"). Hence, metastases result when the appropriate seed is implanted in its suitable soil. Paget's contention that the microenvironment plays a critical role in metastasis is supported by a number of experimental observations. In a pioneering study, Kinsey implanted fragments of lung and several other tissues at ectopic locations in syngeneic mice and noted that lung-homing melanoma cells metastasized to normal lung and ectopically placed lung, but not to other tissues (Kinsey 1960). Kinsey noted that the tissue specificity was retained irrespective of the route of tumor cell injection. Schackert and Fidler demonstrated that some tumor cells exhibit metastatic growth in different regions within a *single* organ (Schackert and Fidler 1998). These investigators noted that injection of murine K-1735 melanoma cells into the internal carotid artery of mice produced metastases only in the brain parenchyma. However, when the experiment was repeated using murine B16 melanoma cells, metastases were confined to the meningeal region of the brain.

Several lines of evidence indicate that the organ-preference patterns of metastasis observed in some tumors are the result of selective adhesive interactions that take place between circulating tumor cells and the microvascular endothelium of the target organ. Specifically, a number of reports have demonstrated that the cytokine-inducible endothelial cell adhesion molecules, E-selectin and vascular cell adhesion molecule-1 (VCAM-1), mediate the adhesion of a number of tumor cells to the vascular wall. E-selectin and VCAM-1 expression are primarily restricted to activated endothelium, and their appearance requires cytokine stimulation and protein synthesis. During an inflammatory response, E-selectin and VCAM-1 are responsible for directing the respective localization of neutrophils and lymphocytes to the site of tissue injury. Studies examining the contribution of endothelial cell adhesion molecules to malignant disease have shown that the entry of colorectal carcinoma cells into the hepatic circulation stimulates cytokine production from Kupffer cells that leads to de novo synthesis of E-selectin by the hepatic sinusoidal endothelium (Khatib et al. 1999). Metastasizing colorectal carcinoma cells utilize their tetrasaccharide ligands, sialyl Lewis x (sLe^x) and sialyl Lewis a (sLe^a), to form adhesive bonds with E-selectin and promote their retention in the liver. Expression of sLe^x and sLe^a antigens by colorectal carcinoma cells is positively correlated with their metastatic potential (Sato et al. 1997), and blockade of E-selectin expression in the liver microcirculation significantly reduces the frequency of liver metastases in experimental animal models (Brodt et al. 1997). In addition to facilitating the adherence of colorectal tumor cells to the hepatic sinusoidal endothelium, there is also evidence that suggests that prostate tumor cells may exploit E-selectin to form stable adhesive contacts with microvascular endothelial cells in the bone (Dimitroff et al. 2004). The bone microcirculation differs from vascular beds found in other tissues in that E-selectin is constitutively expressed on endothelial cells, where it mediates the homing of hematopoietic progenitor cells to the bone. Hence, it is likely that the adherence between prostate tumor cells and bone endothelial cells may be independent of cytokine production. Most of the evidence implicating VCAM-1 in metastasis has come from studies examining malignant melanoma. Expression levels of VLA-4, the integrin receptor for VCAM-1, are upregulated on metastatic melanomas in situ in comparison to benign melanocytes, and this is negatively associated with disease-free interval and survival (Schadendorf et al. 1995). VCAM-1 becomes selectively upregulated in preferred target organs (i.e., lung, brain, liver, and heart) during melanoma me-

tastasis (Langley et al. 2001), and antibody blockade strategies directed toward either VCAM-1 or VLA-4 significantly attenuates metastatic burden in animal models (Okahara et al. 1994).

Angiogenesis and the Tumor Microenvironment

Angiogenesis refers to the development of new blood vessels from the existing vascular bed. The formation of new blood vessels is essential for embryonic development, maintenance of the female reproductive cycle, and wound repair (Folkman 1995). In addition, angiogenesis is widely regarded as a critical cofactor in the progression of a number of pathophysiologic processes, including arthritis, diabetic retinopathy, macular degeneration, and tumor growth. Angiogenesis associated with tumor growth differs from that which accompanies highly regulated biologic processes in that the vascularization of tumors is unremitting. For this reason, tumors have been referred to as "wounds that do not heal" (Dvorak 1986).

Current evidence suggests that angiogenesis is the product of an imbalance between inhibitor and stimulator molecules. Normal tissues are bathed in an excess of factors that maintain the vascular endothelium in a non-proliferating state. Indeed, estimates derived from kinetic studies examining cell proliferation in different tissues indicate that the turnover time for normal endothelial cells may be measured in years (Hobson and Denekamp 1984). The transition from an avascular tumor to an angiogenic tumor is a rate-limiting step in tumor progression and is referred to as the "angiogenic switch". Induction of the angiogenic switch may occur at any stage of tumor development, but usually coincides with increasing metabolic pressures, oncogene activation, or mutation of genes that encode tumor suppressor proteins (Bergers and Benjamin 2003). The acquisition of an angiogenic phenotype elicits a number of biological responses from local microvascular endothelial cells, including directional migration, invasion, cell division, proteolysis, upregulation of anti-apoptotic proteins and, ultimately, new capillary formation. Reports suggest that the intensity of the angiogenic response may vary considerably among different types of tumors. For example, measurements of endothelial cell proliferation performed on a broad panel of human cancers indicate that angiogenesis associated with glioblastoma and renal cell carcinoma growth is much more profound than the blood vessel development that accompanies lung or prostate tumor growth (Eberhard et al. 2000).

Regardless of the intensity of angiogenic response, the rate of tumor cell proliferation is several orders of magnitude greater than the rate of neovascularization, and blood flow delivery to tumors is usually compromised and a fraction of that found in normal tissues. Tumor blood flow measurements recorded in animals bearing experimental ovarian tumors were 50 times less than that present in normal ovarian tissue (Gullino and Grantham 1961). The reduction in blood flow significantly impairs oxygen delivery to tumor cells, and therefore the tumor microenvironment is frequently hypoxic. In fact, current estimates predict that up to 50–60% of locally advanced solid tumors exhibit hypoxic or anoxic regions that are heterogeneously distributed within the tumor mass (Vaupel and Mayer 2004). For head and neck carcinomas, the decline in oxygen tension has been shown to function as a selection pressure that leads to proliferation of tumor cells with enhanced metastatic potential (Brizel et al. 1997).

Many tumors respond to low oxygen availability by upregulating hypoxia-inducible factor (HIF)-1α, a key transcriptional regulator that modulates cellular responses to low oxygen tension (Semenza 2002). HIF-1α has been localized to hypoxic regions of tumors, and overexpression of HIF-1α has been reported in several primary tumors and their metastases. HIF-1α is stabilized by declining oxygen tension and then redistributed to the cell nucleus, where it heterodimerizes with HIF-1β to activate a number of genes implicated in angiogenesis and metastasis. One of the primary target genes of HIF-1α activation is vascular endothelial cell growth factor/

vascular permeability factor (VEGF/VPF). Initial investigations of VEGF focused on its ability to induce protein extravasation in tumor tissue, and further studies have shown that VEGF is essential for both physiologic and pathologic angiogenesis. To date, five VEGF ligands (VEGFA to -E) have been identified, and these proteins mediate their effects by initiating signaling from three distinct VEGFR receptors: VEGFR1 (Flt1), VEGFR2 (KDR/Flk-1), and VEGFR3 (Flt-4). VEGFA isoforms are widely regarded as key regulators of angiogenesis by virtue of their ability to elicit all of the endothelial cell processes necessary for the development of new blood vessels. Signaling through VEGFR2 also activates survival programs in endothelial cells through stimulation of the phosphatidylinositol-3 kinase/Akt pathway and upregulation the anti-apoptotic proteins Bcl-2 and A1 (Gerber et al. 1998). VEGFC and VEGFD preferentially bind to VEGFR3 and have been implicated in adult lymphangiogenesis.

Recent studies have demonstrated that the dependency of tumors on HIF-1α activation for tumor growth may be determined by the physical location of the tumor. To study how HIF-1α inactivation affected tumor growth in different tissues, Blouw and coworkers generated HIF-1α-deficient astrocytes and implanted the cells ectopically (subcutis) or orthotopically (brain) in nude mice (Blouw et al. 2003). These experiments revealed that loss of HIF-1α significantly impaired the growth of ectopically placed astrocytomas but had no effect on cells growing in the brain. The investigators reported that HIF-1α-deficient cells lacked the ability to mobilize VEGF and, hence, were unable to recruit new vascular networks in the inherently blood vessel-poor subcutaneous space. In contrast, the HIF-1α-deficient cells implanted in the brain were able to support their growth by exploiting the preexisting rich vascular networks. These results caution against attempts to convey information generated from ectopic tumors to those residing in their natural tissue of origin.

A number of additional reports examining the growth of tumors in different anatomic regions conclude that the microenvironment has an important regulatory role in determining expression levels of pro-angiogenic proteins. Takahashi and colleagues implanted gastric cancer cells in the stomachs of nude mice and compared VEGF expression with the identical cells implanted in the subcutaneous space (Takahashi et al. 1996). Tumors growing in the stomach expressed significantly more VEGF and were supported by a greater number of blood vessels than the ectopically placed tumors. Moreover, only tumors implanted into the stomach were capable of producing metastases. Studies evaluating the growth of human colon cancer in the cecum and subcutis produced similar results (Jung et al. 2000). Another growth factor whose expression is regulated by the microenvironment is basic fibroblast growth factor (bFGF), a cytokine that controls the angiogenic switch of some tumors (Kandel et al. 1991). In a report in which human renal cell carcinomas were implanted into different organ microenvironments in nude mice, the expression of bFGF was shown to be 10–20 times higher in tumors implanted into the kidney than in those implanted into subcutaneous tissues (Singh et al. 1994). The subcutaneously implanted tumors contained fewer vessels and were characterized by a stroma rich in the angiostatic protein interferon-β (IFN-β), while no IFN-β was detected in renal cell carcinomas implanted in the kidney.

In addition to modulating expression levels of angiogenic stimulators, the tumor microenvironment may also have an important role in determining the functional role of pro-angiogenic proteins. Several independent lines of investigation have shown that the contribution of platelet-derived growth factor (PDGF) to the tumor vascularization process is dependent on the anatomical location of the tumor. At present, four known PDGF polypeptides have been identified that can combine to form five PDGF isoforms: PDGF-AA, -AB, -BB, -CC, and -DD. These isoforms mediate their effects by binding to two protein tyrosine kinase receptors designated PDGFR-α and PDGFR-β. Studies from our laboratory have demonstrated that PDGF-BB functions as a survival factor for blood vessels in the bone that supply prostate tumors (Langley et al. 2004), whereas tumors growing in the skin rely on PDGF-BB to regulate the level of interstitial fluid pressure (Pietras et al. 2001). In pancreatic tumors, PDGF assumes a completely

different role and functions to provide integrity to developing blood vessels by recruiting mural cells that lend support to the immature vascular wall (Bergers and Benjamin 2003). PDGF-BB contributes to angiogenesis in central nervous system tumors by stimulating the release of VEGF from the tumor endothelium (Guo et al. 2003).

Microenvironmental Modulation of Cancer Therapy

Empirical evidence from clinical reports suggests that the organ microenvironment is a critical factor in determining the responsiveness of tumors to therapy. For example, metastases residing in the lymph nodes and skin of women with breast cancer are more responsive to systemic chemotherapy than either lung or bone metastases (Slack and Bross 1975). To provide a molecular basis for the differential effects of chemotherapy observed between the skin and lung, we implanted CT-26 murine colon cancer cells into the subcutaneous tissue or inoculated the cells intravenously to generate lung metastases (Wilmanns et al. 1992). We noted that CT-26 tumor cells growing in the lungs of syngeneic mice were refractory to systemic administration of doxorubicin, whereas tumor cells residing in subcutaneous tissues were sensitive to the drug. CT-26 colon tumor cells harvested from lung metastases exhibited a greater resistance to doxorubicin than cells harvested from subcutaneous tissues. It was determined that there was a direct correlation between resistance of CT-26 cells and expression of the multi-drug-resistant protein P-glycoprotein and *mdr-1*. To determine whether the increased resistance to doxorubicin resulted from selection of a resistant subpopulation, we injected the CT-26 cells from lung metastases into subcutis of syngeneic mice and noted that the resulting tumors were sensitive to doxorubicin. Parallel studies in which CT-26 cells were harvested from the subcutis and then inoculated to generate lung metastases led to the generation of tumors with increased resistance to doxorubicin. The resistant phenotype of the CT-26 cells harvested from lung was diminished after a short period of time in culture conditions. These experiments demonstrate that factors in the lung microenvironment alter gene expression patterns of some tumors, leading to an increased resistance to therapy.

While most of the studies evaluating the differential effects of drug based on tumor location have been performed with chemotherapeutic agents, there is some evidence to suggest that the effectiveness of anti-angiogenic agents may also be determined by the anatomical location of the tumor. In a report evaluating the effect of TNP-470 on the growth of human glioblastoma growth in nude mice, Lund et al found that TNP-470 produced an 80% reduction in the tumor volume of subcutaneous implants, but had no effect on growth of tumors implanted into the brain (Lund et al. 2000). Collectively, the results from the preceding sections illustrate that there are vast differences in the expression patterns of angiogenic regulators between subcutaneously implanted tumors and orthotopically implanted tumors and that, moreover, subcutaneously growing tumors are more sensitive to therapeutic agents.

Anti-angiogenic Therapy in an Orthotopic Model of Prostate Cancer Bone Metastases

Cancer of the prostate is the most common cancer affecting men in North America and is the second leading cause of cancer-related deaths. Mortality from prostate cancer usually results from the metastasis of hormone-refractory cancer cells. Reports examining the pattern of metastasis in advanced prostate cancer indicate that dissemination to bone and lymph nodes occurs in over 80% of cases (Garnick and Fair 1996). The pathophysiology of prostate cancer bone metastases is complex and involves several different cell popu-

lations (i.e., tumor cells, osteoblasts, osteoclasts, endothelial cells) and an assortment of regulatory proteins (e.g., steroid hormones, cytokines, and growth factors).

To clarify those factors that are critical for growth of prostate cancer cells in the bone, we established a murine model of hormone-refractory prostate cancer bone metastasis. To generate prostate cancer growth in the bone, we performed a percutaneous intraosseal injection on nude mice by inserting a 27-gauge needle into the tibia immediately proximal to the tuberositas tibiae (Uehara et al. 2003). After penetrating the cortical bone, we deposited 20 μl of tumor cell suspension (2×10^5 androgen-independent PC3-MM2 cells) in the bone cortex with the use of a calibrated, push button-controlled dispensing device. Five weeks later, we resected the tumor-bearing leg and performed an extensive immunohistochemical survey of the bone lesions in an effort to identify potential factors that may be involved in the regulation of prostate tumor cell growth. A preliminary immunohistochemical evaluation revealed robust tumor cell expression of bFGF, VEGF, IL-8, PDGF-BB, and its receptor PDGFR-β. Expression of these proteins was most pronounced in tumors that were growing adjacent to the bone. In contrast, in those tumors that had lysed the bone and extended their growth to include the surrounding muscle, we detected only minimal levels of the angiogenic proteins, suggesting that factors within the bone environment were influencing the phenotype of the tumor cells.

A more comprehensive examination of the distribution pattern of PDGFR-β revealed that PDGFR-β was present both on prostate tumor cells and on tumor-associated endothelium and that, moreover, this receptor tyrosine kinase was activated. Phosphorylated PDGFR-β was not found either in the contralateral non-tumor leg or in tumor cells growing away from the bone, i.e., in the muscle. These findings indicate that the PDGF-BB produced by tumor cells was acting in an autocrine manner to stimulate tumor cells and in paracrine fashion to convey information to tumor-associated endothelial cells. The expression pattern of activated PDGFR-β in the bone metastases suggested that it might be a good candidate for therapy in that inhibition of this signaling cascade could potentially affect both the malignant cell population and the blood vessels necessary for their growth. To test this hypothesis, we devised a therapeutic strategy to treat mice with experimental bone metastasis using the small-molecule inhibitor of PDGFR-β, STI571 (imatinib mesylate, Gleevec). We found that treatment of mice with STI571 or the combination of STI571 plus paclitaxel led to induction of significant apoptosis of endothelial cells and tumor cells that resulted in smaller tumors, fewer lymphatic metastases, and a pronounced reduction in bone lysis (Uehara et al. 2003). These experiments demonstrated that tumor-associated endothelial cells express phosphorylated PDGFR when they encounter tumor cells that express PDGF, and that inhibition of this activation with a PDGFR tyrosine kinase inhibitor, particularly in combination with chemotherapy, can produce a significant therapeutic effect.

To provide a molecular mechanism for the anti-angiogenic effects observed on the tumor-associated endothelial cells in vivo, we established cultures of murine bone microvascular endothelial cells and examined their response to stimulation with PDGF-BB ligand and to blockade of PDGFR signaling with STI571 (Langley et al. 2004). Cultured bone endothelial cells expressed PDGFR-β, and the PDGF-BB-induced phosphorylation on these cells could be inhibited by STI571 in a dose-dependent manner. Stimulation of the bone endothelial cells with PDGF-BB resulted in a profound upregulation of Akt and ERK1/2, and this effect could be completely abrogated by STI571. In addition, we noted that bone endothelial cells respond to PDGF-BB by increasing their cell division and upregulating the anti-apoptotic protein Bcl-2. We then examined the response of bone endothelial cells to treatment with STI571 and paclitaxel (Taxol). Treatment of bone endothelial cells with only a single agent produced little effect. However, the combined treatment with STI571 and Taxol resulted in a significant increase in the number of cells expressing activated caspase-3 and a concomitant decline in Bcl-2. Consistent with these results, we found that when

bone endothelial cells were confronted with both STI571 and low levels of Taxol, there was a threefold increase in their cytotoxicity (Fig. 27.1).

When considered in aggregate, our data suggest that a primary target for the STI571 and Taxol therapy may well be the blood vessels that perfuse the tumor tissue. To test this hypothesis, we established a multi-drug-resistant prostate cell line by chronically exposing PC3-MM2 cells to increasing concentrations of Taxol (Kim et al. 2006). The resulting cell line, PC3-MM2-MDR, is 70 times more resistant to paclitaxel in vitro, and the growth of the cells is not affected by treatment with paclitaxel or the combination of paclitaxel and STI571. When the PC3-MM2-MDR cells were implanted into the bone microenvironment, they displayed the same angiogenic profile as the parental cell line. In vivo, we found that the PC3-MM2-MDR bone lesions were responsive to the systemic administration of STI571 and paclitaxel. An examination of these lesions following 14 days of treatment revealed that apoptosis (as determined by TUNEL-positive cells) was largely confined to the tumor-associated endothelial cells. This suggested that the first wave of apoptosis takes place in the tumor vasculature. However, after 4 weeks of treatment with STI571 and paclitaxel, we observed significant apoptosis in both tumor-associated endothelial cells and in the tumor cells. The resulting lesions were characterized by significant necrosis.

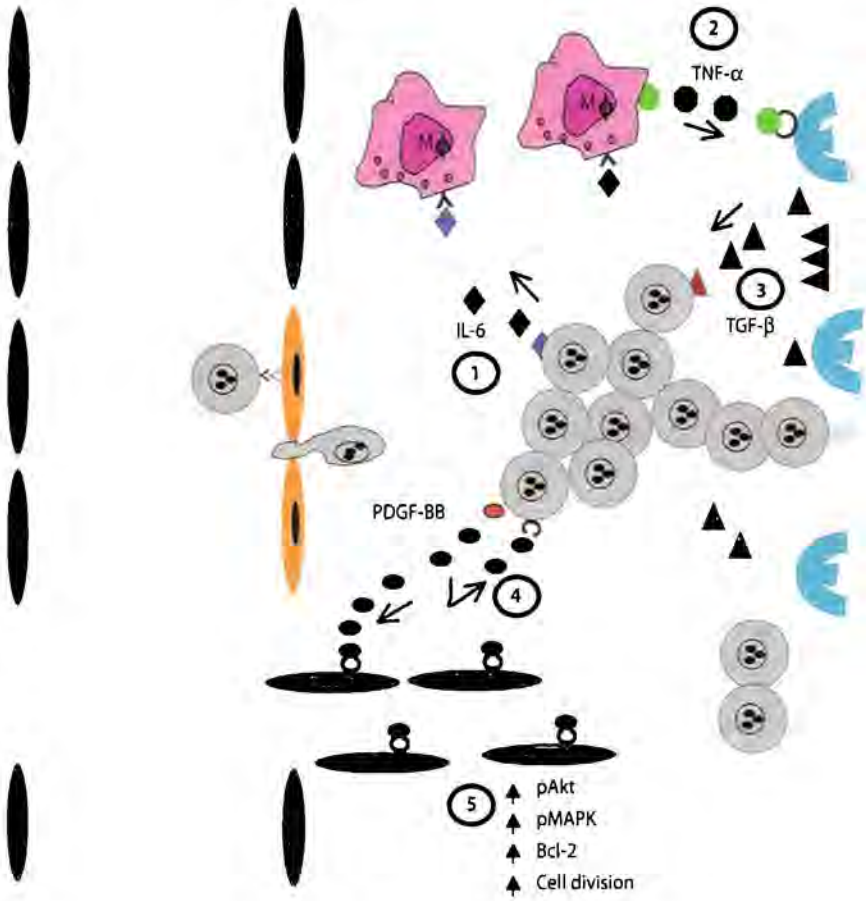


Fig. 17.1. Prostate tumor cells residing in the bone microenvironment release interleukin-6, which results in recruitment of macrophages (*1*). Tumor necrosis factor-alpha (TNF-α) produced by activated macrophages (*2*) stimulates the bone remodeling process, which releases transforming growth factor beta (TGF-β) from the bone matrix (*3*). Binding of TGF-β to its receptors on tumor cells enhances secretion of PDGF-BB, which acts in an autocrine manner to phosphorylate PDGFR-β on tumor cells (*4*) and in paracrine fashion to signal activation of tumor-associated endothelial cells (*5*). Signaling on tumor endothelial cells leads to activation of programs that regulate survival and proliferation

Heterogeneity of Microvascular Endothelial Cells

The vascular endothelium is comprised of a single layer of cells that lines the intimal surface of all blood vessels. In addition to serving as a barrier at the blood-tissue interface, the endothelium also functions to maintain vascular homeostasis through regulation of macromolecule and fluid exchange, hemostasis, coagulation, inflammation, and immunologic responses. While several of these biologic properties are intrinsic to all endothelial cells, the vascular endothelium is widely regarded as a functionally heterogeneous structure (Gerritsen 1987). The molecular basis underlying endothelial cell diversity remains incompletely understood, but is believed to be the product of both genetic (Mikawa and Fischman 1992) and environmental (Aird et al. 1997) influences.

Studies have shown that endothelial cells from different vascular beds exhibit differences in their phenotype, antigenic composition, production of vasoactive factors, metabolic properties, response to growth factors, and susceptibility to pathological conditions (Thorin and Shreeve 1998). Much of the recent interest in endothelial cell diversity has focused on the characterization of differentially distributed endothelial determinants that may be exploited for therapeutic targeting. Examinations of the vascular surface using phage display peptide libraries have demonstrated that the blood vessels perfusing both normal (Rajotte et al. 1998; Essler and Ruoslahti 2002) and pathologic (Arap et al. 1998) tissues express unique endothelial cell receptors and that, moreover, these surface specializations can support site-directed delivery of drug (Arap et al. 1998). Biochemical and structural differences have also been detected in the transport vesicles residing in different endothelial cells. McIntosh and coworkers reported that the caveolae of pulmonary microvascular endothelial cells were distinct from those present in other regional circulations and that this discriminating feature could be exploited to selectively transport immunotoxin to the lung (McIntosh et al. 2002). A separate, but equally important, area of investigation has sought to determine those factors that promote endothelial cell proliferation in different tissues. Results generated from this area of study indicate that the efficacy of VEGF to promote neovascularization varies considerably among different anatomic regions (Pettersson et al. 2000). Additional data indicate that some organs synthesize unique endothelial mitogens that possess activity only for select tissues (LeCouter et al. 2001).

To advance our understanding of how endothelial cells from different organs contribute to angiogenesis and metastasis, we established a panel of microvascular endothelial cells lines from different organs of *H-2 K^b-tsA58* transgenic mice (Langley et al. 2003). Each of the different endothelial cell lines possess a temperature-sensitive SV40 large T antigen that is coupled to the MHC class I promoter. This unique feature allows the investigator to regulate SV40 large T protein expression and thus control the level of cell differentiation. Examinations conducted on these cells indicate that there are significant differences among organ-derived endothelial cells with respect to expression of receptor tyrosine kinases, chemokine receptors, and proteins that mediate the efflux of toxic substrates. For example, cerebral endothelial cells express measurable levels of PDGFR-β, the chemokine receptor CXCR-2, and P-glycoprotein. However, endothelial cells derived from the pulmonary circulation do not express any of these proteins. The organ-derived endothelial cells also show vast differences in their response to stimulation with known endothelial cell mitogens. For brain and liver endothelial cells, bFGF promotes the greatest increase in cell division, whereas EGF is the most dominant mitogen for endothelial cells originating from uterus and lung. We have also noted that endothelial cells from some organs appear to have redundant receptors that signal for cell division and allow them to respond to a number of endothelial cell growth factors. Endothelial cells that were derived from the brain exhibited a significant increase in cell division in response to every growth factor that we evaluated (e.g., bFGF, EGF, PDGF-BB, IL-6, IL-8). It is tempting to speculate that the redundant growth factor signaling pathways present in brain endothelial cells are a reflection of the deleterious consequences that ensue upon cessation of cerebral blood flow. Regardless, this finding

underscores the challenges faced when attempting to inhibit the neovascularization response in this organ.

Molecular profiling of tumor-associated endothelial cells using serial analysis of gene expression indicates that these cells differ from endothelial cells found in normal blood vessels (St. Croix et al. 2000). A recent examination of tumor endothelial cells determined that one distinguishing feature of tumor endothelia is their tendency to express the epidermal growth factor receptor (EGF-R) (Amin et al. 2006). We recently demonstrated that EGF-R is activated on tumor blood vessels both in man and in xenograft models when tumor cells express the EGF-R ligands, transforming growth factor alpha (TGF-α) or epidermal growth factor (EGF) (Yokoi et al. 2005). EGF-R signaling on tumor blood vessels appears to play a critical role in malignant progression, since pharmacologic suppression of this pathway impedes growth of primary lesions and, more importantly, reduces the incidence of metastasis (Baker et al. 2002; Yokoi et al. 2005). To pattern the phenotype of tumor endothelial cells and determine the effects of perpetual stimulation of EGF-R on endothelial cells, we created a novel, constitutively active chimeric EGF-R by fusing the entire intracellular domain of the EGF-R to the N-terminus of the CD3ζ component of the T-cell receptor signaling complex (Cheng et al. 2005). The chimeric receptor, CD3-EGFR, was then stably introduced into brain endothelial cells, where it signaled for enhanced migration, synthesis of MMP-9, invasion, and aggressive growth. An examination of the intracellular signaling pathways in CD3-EGFR brain endothelial cells revealed that signal transducer and activator of transcription 3 (Stat3) was responsible for mediating the pro-angiogenic phenotype and that this phenotype could be reverted with EGF-R tyrosine kinase inhibitor PKI166.

Stat proteins are members of a family of transcription factors that are activated in response to the binding of an assortment of ligands (e.g., growth factors, cytokines, hormones) to their respective receptors. Stat activation leads to the generation of homodimers and heterodimers that enter the cell nucleus and bind to target gene promoters. Constitutively activated Stat3 expression has been reported in a variety of tumors, including those arising from the head and neck, brain, breast, lung, and other human tissues (Turkson and Jove 2000). Several of these tumors are dependent on Stat3 signaling for sustaining their growth and survival and, as a result, Stat3 is rapidly becoming an important target for therapeutic intervention. Our observation that Stat3 initiates activation of angiogenic programs in some endothelial cells suggests that targeting of Stat3 in tumors that produce EGF or TGF-α may control tumor growth by affecting tumor cells and also by limiting the angiogenic response of the tumor vasculature.

Conclusion

Over the past several years, there has been an appreciable increase in our understanding of the cellular and molecular mechanisms that regulate the progression of tumors. Many of the key pathways that signal for angiogenesis in tumors have been identified, and much effort has been expended toward developing agents that interrupt these networks. Similarly, the genetic determinants responsible for the spread of malignant tumor cells are becoming increasingly known, and this, too, will most likely lead to development of new therapeutic interventions. The development of successful cancer therapeutics requires the use of appropriate model systems. The evidence presented here suggests that utilization of models in which the tumor is placed into an ectopic location may lead to optimistic yet erroneous conclusions. The collective results generated in our laboratory regarding the use of anti-angiogenic agents in orthotopic models suggest that the tumor vascular phenotype can, to a large extent, be predicted by examining the expression patterns of proteins that are produced by the tumor. As demonstrated here, when tumors produce PDGF-BB, then it is likely that the PDGFR is activated on the tumor-associated endothelium and, hence, can serve as a true molecular target to produce therapy of tumors.

References

Aird WC, Edelberg JM, Weiler-Guettler H, Simmons WW, Smith TW, Rosenberg RD (1997) Vascular bed-specific expression of an endothelial cell gene is programmed by the tissue microenvironment. J Cell Biol 138:1117–1124

Amin DN, Hida K, Bielenberg DR, Klagsbrun M (2006) Tumor endothelial cells express epidermal growth factor receptor (EGFR) but not Erb3 and are responsive to EGF and EGFR kinase inhibitors. Cancer Res 66:2173–2180

Arap W, Pasqualini R, Ruoslahti E (1998) Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. Science 279:377–380

Baker CH, Kedar D, McCarty MF, Tsan R, Weber KL, Bucana CD, Fidler IJ (2002) Blockade of epidermal growth factor receptor signaling on tumor cells and tumor-associated endothelial cells for therapy of human carcinomas. Am J Pathol 161:929–938

Bergers G, Benjamin LE (2003) Tumorigenesis and the angiogenic switch. Nat Rev Cancer 3:401–410

Bernsen H, van der Laak J, Kusters B, van der Ven A, Wesseling P (2005) Gliomatosis cerebri: quantitative proof of vessel recruitment by cooption instead of angiogenesis. 103:702–706

Blouw B, Song H, Tihan T, Bosze J, Ferrara N, Gerber HP, Johnson RS, Bergers G (2003) The hypoxic response of tumors is dependent on their microenvironment. Cancer Cell 4:133–146

Brizel DM, Sibley GS, Prosnitz LR, Scher RL, Dewhirst MW (1997) Tumor hypoxia adversely affects the prognosis of carcinoma of the head and neck. Int J Radiat Oncol Biol Phys 38:285–289

Brodt P, Fallavollita L, Bresalier RS, Meterissian S, Norton CR, Wolitzky BA (1997) Liver endothelial E-selectin mediates carcinoma cell adhesion and promotes liver metastasis. Int J Cancer 71:612–619

Brown JM, Giaccia AJ (1998) The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. Cancer Res 58:1408–1416

Casanovas O, Hicklin DJ, Bergers G, Hanahan D (2005) Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. Cancer Cell 8:299–309

Cheng H, Langley RR, Wu Q, Wu W, Feng J, Tsan R, Fan D, Fidler IJ (2005) Construction of a novel constitutively active chimer EGFR to identify new targets for therapy. Neoplasia 7:1065–1072

Dimitroff CJ, Lechpammer M, Long-Woodward D, Kutok JL (2004) Rolling of human bone-metastatic prostate tumor cells on human bone marrow endothelium under shear flow is mediated by E-selectin. Cancer Res 64:5261–5269

Dvorak HF (1986) Tumors: wounds that do not heal: similarities between tumor stroma generation and wound healing. N Engl J Med 315:1650–1659

Eberhard A, Kahlert S, Goede V, Hemmerlein B, Plate KH, Augustin HG (2000) Heterogeneity of angiogenesis and blood vessel maturation in human tumors: implications for antiangiogenic therapy. Cancer Res 60:1388–1393

Essler M, Ruoslahti E (2002) Molecular specialization of breast vasculature: a breast-homing phage-displayed peptide binds to aminopeptidase P in breast vasculature. Proc Natl Acad Sci USA 99:2252–2257

Fidler IJ (1970) Metastasis: quantitative analysis of distribution and fate of tumor emboli labeled with 125I-5-iodo-2′-deoxyuridine. J Natl Cancer Inst 45:773–782

Fidler IJ, Kripke ML (1977) Metastasis results from preexisting variant cells within a malignant tumor. Science 197:893–895

Fidler IJ, Uano S, Zhang R, Fujimaki T, Bucana CD (2002) The seed and soil hypothesis: vascularization and brain metastases. Lancet Oncol 3:53–57

Folkman J (1989) What is the evidence that tumors are angiogenesis dependent? J Natl Cancer Inst 82:4–6

Folkman J (1995) Angiogenesis in cancer, vascular, rheumatoid, and other diseases. Nat Med 2:727–732

Garnick MB, Fair WR (1996) Prostate cancer: emerging concepts (part II). Ann Intern Med 125:205–212

Gerber HP, Dixit V, Ferrara N (1998) Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. J Biol Chem 273:13313–13316

Gerritsen ME (1987) Functional heterogeneity of vascular endothelial cells. Biochem Pharmacol 36:2701–2711

Gullino PM, Grantham FH (1961) Studies on the exchange of fluids between host and tumor. The blood flow of hepatomas and other tumors in rats and mice. J Natl Cancer Inst 27:1465–1491

Guo P, Hu B, Gu W, Wang D, Huang HJ, Cavenee WK, Cheng SY (2003) Platelet-derived growth factor B enhances glioma angiogenesis by stimulating vascular endothelial growth factor expression in tumor endothelia and by promoting pericytes recruitment. Am J Pathol 162:1083–1093

Hida K, Hida Y, Amin DN, Flint AF, Panigraphy D, Morton CC, Klagsbrun M (2004) Tumor-associated endothelial cells with cytogenic abnormalities. Cancer Res 64:8249–8255

Hobson B, Denekamp J (1984) Endothelial proliferation in tumours and normal tissues: continuous labeling studies. Br J Cancer 49:405–413

Jung YD, Ahmad SA, Akagi Y, Takahashi Y, Liu W, Reinmuth N, Shaheen RM, Fan F, Ellis LM (2000) Role of the tumor microenvironment in mediating response to anti-angiogenic therapy. Cancer Metastasis Rev 19:147–157

Kandel J, Bossy-Wetzel E, Radvanyi F, Klagsbrun M, Folkman J, Hanahan D (1991) Neovascularization is associated with a switch in the export of bFGF in the multistep development of fibrosarcoma. Cell 66:1095–1104

Kang Y, Siegal PM, Shu W, Drobnjak M, Kakonen SM, Cordón-Cardo C, Guise TA, Massagué J (2003) A multigenic program mediating breast cancer metastasis to bone. Cancer Cell 3:537–549

Khatib AM, Kontogiannea M, Fallavollita L, Jamison B, Meterissian S, Brodt P (1999) Rapid induction of cytokine and E-selectin expression in the liver in response to metastatic tumor cells. Cancer Res 59:1356–1361

Kim SJ, Uehara H, Yazici S, Busby JE, Nakamura T, He J, Maya M, Logothetis C, Mathew P, Wang X, Do KA, Fan D, Fidler IJ (2006) Targeting platelet-derived growth factor receptor on endothelial cells of multidrug-resistant prostate cancer. J Natl Cancer Inst 98:783–793

Kinsey DL (1960) An experimental study of preferential metastasis. Cancer 13:675–676

Kusters B, Leenders WP, Wesseling P, Smits D, Verrijp K, Ruiter DJ, Peters JP, van der Kogel AJ, de Wall RM (2002) Vascular endothelial cell growth factor-A (165) induces progression of melanoma brain metastases without induction of sprouting angiogenesis. Cancer Res 62:341–345

Langley RR, Carlisle R, Ma L, Gerritsen ME, Granger DN (2001) Endothelial expression of vascular cell adhesion molecule-1 (VCAM-1) correlates with metastatic pattern in spontaneous melanoma. Microcirculation 8:335–345

Langley RR, Ramirez KM, Tsan RZ, Van Arsdall M, Nilsson MB, Fidler IJ (2003) Tissue-specific microvascular endothelial cell lines from H-Kb-tsA58 mice for studies of angiogenesis and metastasis. Cancer Res 63:2971–2976

Langley RR, Fan D, Tsan RZ, Rebhun R, He J, Kim SJ, Fidler IJ (2004) Activation of platelet-derived growth factor receptor enhances survival of murine bone endothelial cells. Cancer Res 64:3727–3730

LeCouter J, Kowalski J, Foster J, Hass P, Zhang Z, Dillard-Telm L, Frantz G, Rangell L, DeGuzman L, Keller GA, Peale F, Gurney A, Hillan KJ, Ferrara N (2001) Identification of an angiogenic mitogen selective for endocrine gland endothelium. Nature 412:877–884

Leenders WP, Kusters B, de Wall RM (2002) Vessel co-option: how tumors obtain blood supply in the absence of sprouting angiogenesis. Endothelium 9:83–87

Leenders WPJ, Küsters B, Verrijp K, Maass C, Wesseling P, Heerschap A, Ruiter D, Ryan A, deWall R (2004) Antiangiogenic therapy of cerebral melanoma metastases results in sustained tumor progression via vessel co-option. Clin Cancer Res 10:6222–6230

Lund EL, Bastholm L, Kristjansen PB (2000) Therapeutic synergy of TNP-470 and ionizing radiation: effects on tumor growth, vessel morphology, and angiogenesis in human glioblastoma multiform xenografts. Clin Cancer Res 6:071–978

McIntosh DP, Tan XY, Oh P, Shnitzer JE (2002) Targeting endothelium and its dynamic caveolae for tissue-specific transcytosis in vivo: a pathway to overcome cell barriers to drug and gene delivery. Proc Natl Acad Sci USA 99:1996–2001

Mikawa T, Fischman DA (1992) Retroviral analysis of cardiac morphogenesis: discontinuous formation of coronary vessels. Proc Natl Acad Sci USA 89:9504–9508

Okahara H, Yagita H, Miyaka K, Okumura K (1994) Involvement of very late activation antigen 4 (VLA-4) and vascular cell adhesion molecule 1 (VCAM-1) in tumor necrosis factor-alpha enhancement of experimental metastasis. Cancer Res 54:3233–3236

Paget S (1889) The distribution of secondary growths in cancer of the breast. Lancet 1:571–573

Pettersson A, Nagy JA, Brown LF, Sundberg C, Morgan E, Jungles S, Carter R, Krieger JE, Manseau EJ, Harvey VS, Eckelhoefer IA, Feng D, Dvorak AM, Mulligan RC, Dvorak HF (2000) Heterogeneity of the angiogenic response induced in different normal adult tissues by vascular permeability factor/vascular endothelial cell growth factor. Lab Invest 80:99–115

Pezzella F, Pasorino U, Tagliabue E, Andreola S, Sozzi G, Gasparini G, Menard S, Gatter KC, Harris AL, Fox S, Buyse M, Pilotti S, Pierotti M, Rilke F (1997) Non-small-cell lung carcinoma without morphological evidence of neo-angiogenesis. Am J Pathol 151:1417–1123

Pietras K, Östman A, Sjöguist M, Buchdunger E, Reed RK, Heldin CH, Rubin K (2001) Inhibition of platelet-derived growth factor receptors reduces interstitial hypertension and increases transcapillary transport in tumors. Cancer Res 61:2929–2934

Rajotte D, Arap W, Hagedorn M, Koivunen E, Pasqualini R, Ruoslahti E (1998) Molecular heterogeneity of the vascular endothelium revealed by in vivo phage display. J Clin Invest 102:430–437

Sato M, Narita T, Kimura N, Zenita K, Hashimoto T, Manabe T, Kannagi R (1997) The association of sialyl Lewis (a) antigen with the metastatic potential of human colon cancer cells. Anticancer Res 17:3505–3511

Schackert G, Fidler IJ (1998) Site-specific metastasis of mouse melanomas and fibrosarcoma in the brain or the meninges of syngeneic animals. Cancer Res 48:3478–3483

Schadendorf D, Heidel J, Gawlik C, Suter L, Czarnetzi BM (1995) Association with clinical outcome of expression of VLA-4 in primary cutaneous malignant melanoma as well as P-selectin and E-selectin on intratumoral vessels. J Natl Cancer Inst 87:366–371

Semenza GL (2002) HIF-1 and tumor progression: pathophysiology and therapeutics. Trends Mol Med 8:S62-S67

Singh RK, Bucana CD, Gutman M, Fan D, Wilson MR, Fidler IJ (1994) Organ site-dependent expression of basic fibroblast growth factor in human renal carcinoma cells. Am J Pathol 145:365–374

Slack NH, Bross JDJ (1975) The influence of site of metastasis on tumor growth and response to chemotherapy. Br J Cancer 32:78–86

St Croix B, Ragio C, Velculescu V, Traverso G, Romans KE, Montgomery E, Lal A, Riggins GJ, Lengauer C, Vogelstein B, Kinzler KW (2000) Genes expressed in human tumor endothelium. Science 18:1197–1202

Takahashi Y, Mai M, Wilson MR, Kitadi Y, Bucana CD, Ellis LM (1996) Site-dependent expression of vascular endothelial cell growth factor, angiogenesis and proliferation in human gastric carcinoma. Int J Oncol 8:701–705

Thorin E, Shreeve SM (1998) Heterogeneity of vascular endothelial cells in normal and disease states. Pharmacol Ther 78:155–166

Turkson J, Jove R (2000) STAT proteins: novel molecular targets for cancer drug discovery. Oncogene 19:6613–6626

Uehara H, Kim SJ, Karashima T, Shepherd DL, Fan D, Tsan R, Killion JJ, Logothetis C, Mathew P, Fidler IJ (2003) Effects of blocking platelet-derived growth factor-receptor signaling in a mouse model of experimental prostate cancer bone metastasis. J Natl Cancer Inst 95:558–570

Vaupel P, Mayer A (2004) Hypoxia and anemia: effects on tumor biology and treatment resistance. Transfusion Clin Biol 12:5–10

Wilmanns C, Fan D, O'Brian CA, Bucana C, Fidler IJ (1992) Orthotopic and ectopic organ environments differentially influence the sensitivity of murine colon carcinoma cells to doxorubicin and 5-fluorouracil. Int J Cancer 52:98–104

Yokoi K, Thaker PH, Yazici S, Rebhun R, Nam DH, He J, Kim SJ, Abruzzese JL, Hamilton SR, Fidler IJ (2005) Dual inhibition of epidermal growth factor receptor and vascular endothelial growth factor receptor phosphorylation by AEE788 reduces tumor growth and metastasis of human colon carcinoma in an orthotopic nude mouse model. Cancer Res 65:3716–3725

Vascular Tumor Targeting

28

DRAGAN GRABULOVSKI and DARIO NERI

Abstract

To overcome the hurdles of conventional tumor therapy, efforts have been directed towards the development of anti-angiogenic agents, since the growth of solid tumors is dependent on their capacity to acquire a blood supply. More recently, it has become apparent that the targeted destruction of the established tumor vasculature or the delivery of bioactive agents to the new blood vessels may open complementary exciting therapeutic opportunities. In this chapter, we present evidence that vascular targeting is an effective antitumor strategy in animal models, describe known targets and strategies for identifying them and discuss future prospects for vascular targeting applications in the clinical setting.

CONTENTS

Concept and Definitions

The treatment of solid tumors with most chemotherapeutic drugs relies on the expectation that the drugs will preferentially kill rapidly dividing tumor cells, rather than normal cells. However, the lack of selectivity towards tumor cells leads to toxicities in normal tissues with enhanced proliferation rates, such as the bone marrow, gastrointestinal tract and hair follicles. As the high interstitial pressure and the irregular vasculature of the tumor impair the accumulation of the active agents at the tumor site, the efficiency of conventional therapy is further decreased (Bosslet et al. 1998). Moreover, the activity of multi-drug resistance proteins minimizes drug uptake and leads often to failure of the therapy (Ramachandran and Melnick 1999). A promising approach to circumvent the hurdles of tumor therapy is the emerging field of vascular targeting.

Vascular tumor targeting aims at the rapid and selective shutdown or damage of the established tumor vasculature. This strategy will lead to tumor cell death as the blood supply to these cells has been cut off. Potential advantages of this strategy over attacking tumor cells are:

- Occluding one blood vessel will trigger the death of a large number of tumor cells which depend on it for supply of nutrients and oxygen.
- One single vascular targeting agent could in principle be used to treat a wide range of solid tumors.
- Endothelial cells and cells of the surrounding stroma are genetically more stable than tumor cells, so therapy resistance is less likely to occur. Moreover, these cells are usually easily accessible for any compound.

A broader definition of vascular targeting, which is not limited to the thrombosis of neovasculature, may be "the targeted delivery of bioactive agents to new blood vessels". Figure 28.1 illustrates the overall concept.

Vascular tumor targeting is only possible because the endothelium and the surrounding stroma in tumors differ from that in normal tissue; this fact has long been known, but only recently have these differences begun to be characterized at the molecular level. Compared with the vasculature in normal tissue, the tumor vasculature is strikingly disorganized and tortuous (Eberhard et al. 2000; Konerding et al. 2001; McDonald and Choyke 2003). The flow of blood through the tumor capillaries is frequently sluggish, and at times might be stationary or even experience a reversal in direction (Tozer et al. 1990, 2005). The microenvironment – including blood and the endothelium lining the vessels – is profoundly hypoxic (Helmlinger et al. 1997). In this environment, the endothelial cell proliferates rapidly and contributes to active angiogenesis (Denekamp and Hobson 1982). The tumor is also nutrient starved, acidic and under oxidative stress (Toyokuni et al. 1995; Brown and Bicknell 2001); it has been shown that the endothelial cell responds transcriptionally to these stimuli and remodeling activities, giving rise to the production of new proteins either on their surface or in the surrounding extracellular matrix. Some of these specific markers can be used as targets for vascular-targeting approaches.

We will first review the classes of compounds that are currently used for the targeting. The best-characterized markers of angiogenesis will then be analyzed and the technologies for finding them will be discussed. The last two sections deal with imaging and therapy applications based on vascular tumor targeting strategies.

Classes of Molecules For Vascular Tumor Targeting Applications

In pharmaceutical biotechnology, antibodies are indisputably the best-established class of binding molecules for tumor diagnosis and therapy. Nevertheless, even these blockbusters exhibit certain drawbacks, such as the requirement for an expensive mammalian cell production system, low expression yields, dependence on disulfide bonds for stability,

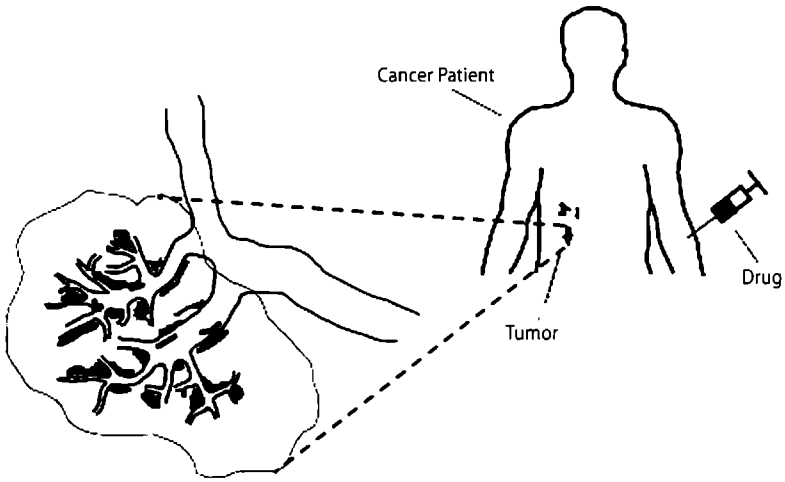


Fig. 28.1. Illustration of the concept of vascular targeting. The targeted drug is delivered intravenously and homes to the tumor-induced antigen that may be either on the endothelial cell or in the perivascular space

and the tendency to aggregate, especially when fused to additional domains or proteins (e.g. cytokines). For this reason, small globular proteins, peptides, aptamers and specific small organic molecules as binding reagents are currently being investigated for tumor-targeting applications.

28.2.1 Antibodies

At present, monoclonal antibodies (and possibly aptamers) are the only general class of binding molecules that can be rapidly isolated with high affinity and specificity to virtually any target. Rodent monoclonal antibodies produced by hybridoma cells were obtained as early as 1975 (Kohler and Milstein 1975). In order to circumvent the immunogenicity of rodent antibodies, also human monoclonal antibodies could be produced either by fusing B-cells with an appropriate partner cell to obtain hybridomas (Kozbor et al. 1982; Karpas et al. 2001) or by immortalizing B-cells with Epstein–Barr virus (Steinitz et al. 1977; Kozbor and Roder 1981). Moreover, in order to improve efficiency diverse other strategies have been developed for the production of human antibodies of desired specificity. These strategies include humanization of murine monoclonal antibodies through protein engineering (Jones et al. 1986), selection of antibodies from phage-display libraries of human origin (McCafferty et al. 1990; Viti et al. 2000; Silacci et al. 2005) and immunization of transgenic mice carrying human immunoglobulin loci, followed by production of monoclonal antibodies using hybridoma technology (Green 1999). In 1999, ribosome display has also been proposed as a fully in vitro avenue for the isolation and affinity maturation of human antibodies (Schaffitzel et al. 1999).

Monoclonal IgG antibodies exhibit slow elimination from the blood and accumulate in the liver. For these reasons, rapidly clearing antibody fragments are typically preferred for imaging applications in nuclear medicine. By contrast, intact immunoglobulins continue to represent the antibody format of choice for many therapeutic applications (Brekke and Sandlie 2003), especially for those which rely on the antibody ability to interfere with signaling events or to activate antibody-dependent cellular cytotoxicity mechanisms or complement.

In our experience, antibody phage technology represents an extremely efficient avenue for producing good-quality human monoclonal antibodies. The methodology yields monoclonal antibody fragments (typically in scFv or Fab format), which can easily be reformatted into IgG format by transplanting the genes coding for the variable antibody domains into suitable expression vectors.

28.2.2 Small Globular Proteins as Binding Reagents

Already in the 1990s several research groups have focused their research on the development of small globular proteins as substitutes for antibody-based drugs. In this approach, the concept of a universal binding site from the antibody structure is transferred to alternative protein frameworks, the so-called scaffolds. For example, a peptide with known affinity towards a certain target can be inserted as a loop into the structure of a carrier protein. In this way, the desired favorable characteristics of the scaffold (e.g. cheap to manufacture, stability, solubility, pharmacokinetic profile etc.) are combined with the binding properties of the peptide (Ali et al. 1999). Alternatively, certain amino acid residues of a globular protein can be combinatorially mutated, followed by screening for variants with specific binding properties towards the target. Until now, more than 40 scaffolds have been used for the generation of protein binders (reviewed in Binz et al. 2005). Mainly of academic interest at first, this approach has been pursued by small and medium-sized companies, some of which now have binders that are already in phase II trials for several diseases (reviewed in Hey et al. 2005).

As therapeutics, small globular proteins are particularly interesting if the neutralization of a target protein is the only desired pharmacological effect (in contrast to a full-length antibody, where the Fc portion may stimulate immune processes) or as fusion proteins, for the targeted delivery of bioactive molecules to sites of disease. Beside pharmacokinetic aspects for small proteins, such as rapid clearance, a crucial issue for these therapeutics will be their immunogenicity profile. Since it has been reported that even fully human proteins could induce immune responses (Mirick et al. 2004), similar problems might arise with globular proteins. Preclinical and clinical trials will show which scaffolds will be good enough for pharmaceutical application.

28.2.3 Peptides

Phage display libraries of linear and disulfide constraint peptides (Felici et al. 1995) are commercially available and can be used for the isolation of peptidic binders to target proteins of interest. In general, success rates are not as high as in the case of antibody phage technologies, and affinities are rarely better than micromolar [higher only in exceptional cases, although avidity can be improved by multimerization (Terskikh et al. 1997; Wrighton et al. 1997)]. Ribosome display (Mattheakis et al. 1994) and other technologies for the construction of very large peptide libraries and for their molecular evolution have been proposed (Collins et al. 2001), but the isolation of high-affinity peptidic binders remains a formidable challenge. In addition, the limited in vivo stability of linear peptides may compromise their pharmaceutical application and remains a cause of concern.

The groups of Pasqualini and Ruoslahti (Pasqualini and Ruoslahti 1996; Trepel et al. 2002) used peptide phage libraries for in vivo panning. This avenue has been pursued by other groups (Arap et al. 1998; Finger et al. 2002), but the real imaging and therapeutic potential of these phage-derived peptides remains to be investigated in advanced animal models, as well as in the clinic.

A number of internalizing peptides, specific to receptors which are overexpressed in tumor cells, have been used for the imaging of tumors and for the selective delivery of therapeutic radionuclides to neoplastic lesions. The somatostatin analog octreotide (Kowalski et al. 2003), for example, has been approved in Europe and USA for the imaging of tumors. Several other reagents are in development (Behr et al. 2001; Kowalski et al. 2003), such as integrin-binding peptides (RGD peptides; Chen et al. 2004) and bombesin peptide analogue (Okarvi and al-Jammaz 2003).

The successful imaging results obtained with somatostatin analogues suggest that cyclic peptides

may be used for the molecular targeting of angiogenesis, particularly if one can select for peptides which are rapidly internalized by the proliferating endothelial cells, but not by the quiescent endothelium.

28.2.4 Aptamers

Besides antibodies, aptamers (single-stranded nucleic acids, DNA or RNA, capable of adopting a complex three-dimensional structure) are possibly the only other class of molecules from which specific binding molecules against a variety of target proteins can be isolated. Aptamer technology relies on the fact that it is possible to generate large ($>10^{12}$ members) libraries of single-stranded nucleic acids which can be panned against the macromolecular target of interest. The nucleic acids captured in this procedure can then be amplified using PCR-based techniques and used to generate single-stranded material for further cycles of panning. Aptamers have to be stabilized for in vivo applications. A number of strategies have been proposed for this purpose, including notably Spiegelmer technology (Vater and Klussmann 2003).

Recently, the first aptamer (Macugen™) was approved for the treatment of age-related macular degeneration (Doggrell 2005). Macugen™ binds to VEGF-165 (but not to smaller VEGF-A isoforms) and inhibits ocular angiogenesis, following intravitreal administration.

Promising imaging studies of rodent models of cancer with radiolabeled aptamers specific to tenascin-C have been described (Hauff et al. 2003). The potential of aptamers for tumor-targeting applications is now being investigated in the clinical setting.

28.2.5 Small Organic Drugs

The vast majority of drugs on the market are small organic compounds. In typical cases, oral availability, ease of manufacture, lack of immunogenicity and the ability to penetrate into cells represent some of the advantages that small organic compounds have over biopharmaceuticals.

In most cases, small organic drugs on the market interact with a component of one of four main protein classes: enzymes, receptors, ion channels and carrier molecules. In contrast to antibody technology, the isolation of high-affinity small molecules binding to proteins lacking well-defined pockets or that modulate protein–protein interactions is difficult (Arkin and Wells 2004). An increasing number of experimental findings provide evidence that high-affinity and specific binders can be achieved by linking two or more organic compounds which recognize adjacent epitopes of the target protein, thus exploiting the chelate effect. Methods for the identification of such bidentate ligands include SAR-by-NMR (Shuker et al. 1996), dynamic combinatorial chemistry (Ramstrom and Lehn 2002) and tethering approaches (Erlanson et al. 2003). Our laboratory recently developed a novel technology termed encoded self-assembling chemical library technology (ESACHEL; Melkko et al. 2004). In this technology, every compound of the library is covalently coupled to an oligonucleotide, which mediates the self-assembly of the library and carries an identification DNA tag for every pharmacophore. Similar to antibody phage display libraries, ESACHEL libraries can be panned in solution, thus enriching bidentate ligands towards the target of interest. The decoding of the selected compounds can take place by a number of experimental techniques (e.g. hybridization on DNA chips, by a modified PCR followed by sequencing). We have described the isolation of ESACHEL-derived bidentate molecules with nanomolar affinity to carbonic anhydrase II (Melkko et al. 2004). Isoenzymes of carbonic anhydrases turned out to be relevant targets, e.g. carbonic anhydrase IX shows an expression only under hypoxic conditions and in some tumors (Chrastina 2003; Grabmaier et al. 2004).

More in general, it appears that combinatorial chemistry methodologies such as SpeedScreen (Zehender et al. 2004) and DNA-encoded chemical library technology (Gartner et al. 2004) may allow the screening of chemical libraries of unprecedented

size. It remains to be seen whether these approaches can rival the efficiency of recombinant antibody technology for the generation of specific binders to protein targets of interest.

Classes of Vascular Targets

A number of protein targets expressed either in the vessel or in the adjacent matrix of the vessel have been characterized as targets for the selective delivery of compounds to the tumor neovasculature.

28.3.1 Extra-Domain B of Fibronectin

Fibronectin is a large glycoprotein that is present in large amounts in the plasma and tissues. Extra-domain B (EDB) is a 91-amino-acid type III homology domain that becomes inserted into the fibronectin molecule under tissue-remodeling conditions by a mechanism of alternative splicing at the level of the primary transcript (Zardi et al. 1987). EDB is essentially undetectable in healthy adult individuals, whereas EDB-containing fibronectin is abundant in many aggressive solid tumors and displays either predominantly vascular or diffuse stromal patterns of expression, depending on the tumor type (Carnemolla et al. 1989). Despite its very restricted expression, EDB does not seem to be indispensable, as mice lacking the EDB exon develop normally (Fukuda et al. 2002). The EDB sequence is identical in mouse, rat, rabbit, dog, monkey and man. This feature facilitates animal experiments in immunocompetent syngeneic settings, but has, so far, prevented the isolation of anti-EDB antibodies using hybridoma technology, probably due to tolerance.

A few years ago, using phage display technology (Winter et al. 1994) and other methods (Giovannoni et al. 2001), our group, in collaboration with the group of L. Zardi (Genoa, Italy), succeeded in isolating a number of human monoclonal antibodies to EDB (Carnemolla et al. 1996; Neri et al. 1997; Pini et al. 1998). These include the high-affinity human antibody L19, which has been shown to efficiently localize to tumor blood vessels in animal models (Tarli et al. 1999; Viti et al. 1999; Demartis et al. 2001) and in patients with cancer (Santimaria et al. 2003) following intravenous injection. A large number of therapeutic derivatives of the L19 antibody have been produced and tested in animals, including conjugates to fluorophores and photosensitizers (Birchler et al. 1999a, 1999b), therapeutic radionuclides (Borsi et al. 2002; Berndorff et al. 2005), liposomes (Marty et al. 2002), procoagulant agents (Nilsson et al. 2001), cytokines (Carnemolla et al. 2002; Halin et al. 2002a, 2002b; Borsi et al. 2003; Ebbinghaus et al. 2005), enzymes (Heinis et al. 2004) and other proteins (Melkko et al. 2002; Niesner et al. 2002). Importantly, the anti-EDB antibody L19, in homodimeric single-chain Fv format and labeled with iodine-123, has been studied in over 40 patients with cancer. The results obtained in the first 20 patients have recently been described (Santimaria et al. 2003) and confirm the ability of the antibody to localize to tumor masses exhibiting rapid growth. L19-interleukin 2 (L19-IL2), L19-tumor necrosis factor (L19-TNF) and small-immuno-protein [SIP]-L19-I131 are three therapeutic derivatives of the L19 antibody which are currently in clinical development (Menrad and Menssen 2005).

28.3.2 Large Tenascin-C Isoforms

Tenascin-C, a polymorphic high-molecular-mass extracellular matrix glycoprotein exists in several isoforms which are generated as a result of different patterns of alternative splicing in the region between domains A1 and D (Siri et al. 1991). These large isoforms of tenascin-C, containing extra domains, have long been known to be tumor-associated antigens. Though not completely absent in healthy tissues, they show a more restricted pattern of expression than the isoforms without extra domains (Borsi et al. 1992). Especially the C domain of tenascin-C shows the most

restricted expression pattern: while being undetectable in normal human tissues and only barely detectable in most carcinomas, it is extremely abundant in high-grade astrocytoma (grade III and glioblastoma), particularly around vascular structures and proliferating cells (Carnemolla et al. 1999).

A critical immunohistochemical analysis of the expression pattern of the different isoforms in various cancer types is needed to evaluate their potential as targets for biomolecular intervention. Radiolabeled derivatives of monoclonal antibodies to domains A1 and D of tenascin-C have been used for imaging and radioimmunotherapy in patients with cancer for over a decade (Paganelli et al. 1999; Riva et al. 1999a, 1999b; Leins et al. 2003; Bartolomei et al. 2004). The pattern of staining of these antibodies varies among different tumors, the two extremes being a predominantly vascular and a diffuse stromal staining. Recently, there has been renewed interest in the use of antibodies specific to the small isoform of tenascin-C for tumor-targeting applications (Petronzelli et al. 2005).

28.3.3 Phosphatidyl Serine

Phosphatidyl serine (PS) phospholipids are major components of the cell membrane which are preferentially found in the inner leaflet of the lipid bilayer. However, under conditions of cellular stress, apoptosis, platelet activation and endothelial cell proliferation in tumors, PS becomes exposed on the outer leaflet of the cell membrane (Bucki et al. 2001; Ran et al. 2002). Annexin V and monoclonal antibodies have been used to confirm the surface accessibility of the PS moiety on endothelial cells in vitro (for example, after treatment with hydrogen peroxide) and in vivo (Ran et al. 2002, 2005). The impressive microscopic analysis of tumor-targeting performance by monoclonal antibodies to PS has not yet been complemented by a quantitative biodistribution analysis, but the 9D2 and 3G4 antibodies displayed potent antitumor activities even when used as naked antibody in rodent models of cancer. Recently it was shown that the vascular targeting antibody, 3G4, significantly enhances the therapeutic efficacy of docetaxel against the growth and dissemination to the lungs of MDA-MB-435 human breast tumors in mice without concomitant increase in host toxicity (Huang et al. 2005). A chimeric version of 3G4 has been developed and is scheduled to enter clinical trials now.

28.3.4 Annexin A1

Annexins are cytosolic proteins that can associate with cell membranes in a calcium-dependent manner. Some annexins may translocate the lipid bilayer to the external cell surface. Annexin A1 was discovered as a tumor endothelial target by Schnitzer and co-workers (Oh et al. 2004). A monoclonal antibody to this antigen has been used for the radioimmunoscintigraphic detection of solid tumor lesions in a rat model. Furthermore, relatively low radioactive doses of the same antibody labeled with iodine-125 have shown therapeutic benefit in rats.

28.3.5 Integrins

During vascular remodeling and angiogenesis, endothelial cells show increased expression of several cell-surface molecules that potentiate cell invasion and proliferation. One such molecule is integrin-$\alpha v\beta 3$, which has a key role in endothelial cell survival during angiogenesis in vivo and which might serve as a target for therapeutic molecules, particularly those that require internalization in endothelial cells. Monoclonal antibodies to integrin-$\alpha v\beta 3$ have been shown to display anti-angiogenic activities and to preferentially stain tumor blood vessels. However, expression of integrin-$\alpha_v\beta_3$ and other integrins has been reported in several normal tissues.

Efforts to influence the biology of blood vessels by gene delivery have been pursued using cationic nanoparticles coupled to an integrin-$\alpha_v\beta_3$ targeting ligand for the selective gene delivery to angiogenic blood vessels, with a substantial therapeutic benefit in tumor-bearing mice (Hood et al. 2002).

A high-affinity humanized anti-$\alpha_v\beta_3$ antibody is in clinical development as an anti-angiogenic therapeutic (Gutheil et al. 2000); to date, however, its tumor-targeting performance for cancer has been unsatisfactory (Posey et al. 2001).

Anti-$\alpha_v\beta_3$ antibodies have been shown to preferentially localize to tumor blood vessels using ex vivo fluorescence microscopy detection (Baluk et al. 2003). Furthermore, a paramagnetic contrast agent targeted to the LM609 monoclonal antibody, which is specific to $\alpha_v\beta_3$, has been described for the in vivo imaging of angiogenesis using magnetic resonance (Sipkins et al. 1998).

28.3.6 Vascular Endothelial Growth Factors and Their Receptors

Vascular endothelial growth factors (VEGFs) represent a class of proteins that mediate angiogenesis. The overexpression of VEGFs and their receptors in tumors (Brekken et al. 1998; Oh et al. 2004) makes them attractive targets. Especially, the recent approval of the humanized anti-VEGF monoclonal antibody bevacizumab for first-line cancer treatment (Ferrara 2004; Ferrara et al. 2004) has highlighted the contribution of VEGF-A to cancer progression. In addition, the selective localization of monoclonal antibodies to VEGF-A, VEGF receptor 2 and the VEGF-A/VEGF receptor 2 complex has been studied (Ke et al. 1996; Prewett et al. 1999; Zhu and Witte 1999; Brekken et al. 2000; Cooke et al. 2001). The targeting efficiencies reported so far were modest, which possibly reflects kinetic limitations in the targeting of low or medium abundance antigens, even when they are readily accessible to binding agents injected into the bloodstream (Halin et al. 2002a).

28.3.7 Prostate-specific Membrane Antigen

Prostate-specific membrane antigen (PSMA) is a membrane glycoprotein with proteolytic activity. It is predominantly expressed in the prostate, and serum concentrations are often elevated in patients with prostate cancer (Bostwick et al. 2000). The interest for vascular targeting applications of PSMA has been stimulated by the observation that PSMA is over-expressed in the neovasculature of several solid tumors (Liu et al. 1997; Chang et al. 1999), whereas expression around blood vessels in normal tissues is limited to breast, kidney, duodenum and prostate. Specific antibody derivatives to PSMA with alpha-emitting radionuclides in rodent cancer models have been reported (Li et al. 2002). More recently, the radiolabeled antibody J591 has been used for tumor imaging (Bander et al. 2003) and is currently being evaluated for therapeutic applications (Milowsky et al. 2004; Bander et al. 2005).

28.3.8 Endoglin

Endoglin (CD105) is a transforming growth factor-β (TGF-β) co-receptor that is overexpressed in tumor neovasculature (Wang et al. 1993; Burrows et al. 1995). Even though immunohistochemical analysis has shown endoglin expression in normal adult tissues (Matsubara et al. 2000; Balza et al. 2001), monoclonal antibodies to endoglin have been used in biodistribution studies and for imaging in rodents and dogs (Bredow et al. 2000; Fonsatti et al. 2000).

28.3.9 Nucleolin

Ruoslahti and colleagues reported a 31-amino-acid synthetic peptide (F3) that accumulates in the nuclei of tumor endothelial cells and tumor cells (Porkka et al. 2002). The cell surface protein that is recognized by F3 was then identified as nucleolin (Christian et al. 2003). The internalization of F3 takes place in a nucleolin-dependent manner, as antinucleolin antibodies that were previously injected inhibited F3 cellular uptake. The restricted expression pattern of nucleolin and its ability to internalize binding agents make it an attractive target for directed tumor therapy.

28.3.10 Other Possible Targets

Over the past few years, a range of antigens have been proposed as vascular targets for imaging and for the selective delivery of drugs to the tumor.

Peptides to the endothelial antigen CD13 have been isolated (Pasqualini et al. 1997, 2000), and peptide fusions with tumor necrosis factor (TNF) have shown to markedly increase the therapeutic ratio of this biopharmaceutical (Curnis et al. 2000), even at low doses (Curnis et al. 2002).

Radiolabeled monoclonal antibodies against a CD44 isoform, which is a cell-surface receptor, have performed impressively in tumor-targeting experiments in animal models (Wakai et al. 2000).

Magic roundabout or ROBO4, a member of the roundabout receptor family, is a promising target as it is absent in adult tissues except at sites of angiogenesis (Huminiecki et al. 2002). This gene product is believed to be intimately involved in the development of the vasculature. Biodistribution studies, e.g. with radiolabeled antibodies, will give more information about the suitability of ROBO-4 as a vascular tumor target.

Developmental endothelial locus-1 (Del-1) is a unique integrin ligand produced by endothelial cells, thus mediating autocrine signals. It has been reported to mediate angiogenesis in the adult (Aoka et al. 2002). Biodistribution studies are needed for the evaluation of the suitability of Del-1 as a target.

Endothelial-specific protein disulfide isomerase (EndoPDI) is a hypoxia-induced gene that is predominantly expressed by the endothelium (Sullivan et al. 2003). Functionally, EndoPDI has been shown to protect the endothelium from apoptosis during hypoxia-induced stress. Use of RNA interference has shown to that EndoPDI is required for the folding of endothelial-protective molecules, including endothelin-1 (EDN1), adrenomedullin (ADM) and CD105, which are produced when endothelial cells are exposed to hypoxic stress. Inhibition or ablation of EndoPDI should, therefore, make the hypoxic tumor endothelium more sensitive to apoptosis, particularly if it is concurrently stressed by a cytotoxic drug or radiotherapy.

Other tumor endothelial markers (TEMs) will be discussed below.

Methodologies for the Discovery of Novel Vascular Tumor Targets

Until recently, most attempts to identify tumor vascular targets were based on the study of in vitro primary cultures of endothelial cells, exposed to conditions which would mimic cell proliferation or quiescence. Another popular approach has been to raise antibodies [typically by immunization (Borsi et al. 1987) or by antibody phage technology (Mutuberria et al. 2004)] to different endothelial cultures. Both efforts failed to identify differentially expressed proteins at the molecular level, although differences in gene expression could be observed. However, real advances have come with the advent of techniques that allow full genome analysis. In particular, mRNA-based serial analysis of gene expression (SAGE) or microarray analyses combined with bioinformatics analyses on the wealth of expression data that are now available in the public domain have been particularly fruitful.

28.4.1 Bioinformatics

In 1995, Adams and colleagues described an initial assessment of human gene diversity and expression patterns based on 83 million nucleotides of DNA sequence (Adams et al. 1995). They noted that the endothelial cell is one of the richest transcriptional sites, which indicates that there could be several genes for which expression is restricted to the endothelium. Tumor endothelial markers were selected by screening the expression of endothelial specific genes in normal and tumor tissues. One such approach applied a subtractive algorithm to

the expressed sequence tag expression data that are available in the public databases to identify novel endothelial-specific genes (Huminiecki and Bicknell 2000). These were then screened for expression by in situ hybridization, which identified, for example, ROBO-4 and EndoPDI as tumor endothelial markers.

28.4.2 Transcriptomic Profiling

The SAGE technique allows simultaneous and quantitative analysis of a large number of transcripts (Velculescu et al. 1995). SAGE is based on the serial sequencing of 15-bp tags that are unique to each and every gene. These gene-specific tags are produced by a series of molecular biological manipulations and then concatenated for automated sequencing. An advantage of SAGE is that the method is unbiased by experimental conditions, so direct comparison of data sets is possible. St. Croix and colleagues used the SAGE technique to gain a molecular understanding of tumor angiogenesis (St. Croix et al. 2000). After isolation of endothelium from normal and cancerous colon, SAGE libraries were constructed and a direct comparison identified genes that were upregulated in the tumor endothelium. This led to the identification of several novel tumor endothelial markers (TEMs). As vascular targeting is more straightforward if the molecular target is expressed on the cell surface, further work has concentrated on four of these genes that encode proteins with predicted transmembrane proteins (Carson-Walter et al. 2001; Nanda and St. Croix 2004). TEM1, TEM7 and TEM8 show single-pass transmembrane domains. TEM5 is an orphan seven-pass transmembrane G-protein-coupled receptor (GPCR) with a long extracellular amino-terminal domain and belongs to the family of the so-called adhesion family of GPCRs. Mouse orthologs have been identified and their expression in normal and tumor tissues investigated. TEM1, TEM5 and TEM8 showed strong tumor-endothelial expression but were essentially absent from normal tissue (Carson-Walter et al. 2001). The subsequent molecular cloning of endosialin showed it to be identical to TEM1 (Christian et al. 2001). Recent work has shown conclusively that endosialin/TEM1 is predominantly expressed by fibroblasts and a subset of pericytes associated with tumor vessels, and not by the tumor endothelium itself (MacFadyen et al. 2005), indicating that the isolated tumor endothelial cells from which the SAGE library was made were contaminated with these closely associated cells.

TEM8 has been shown to be an anthrax-toxin receptor, and binding of the toxin to TEM8 expressed on tumor endothelium, followed by endothelial death, might explain the antitumor activity of the toxin (Duesbery et al. 2001).

28.4.3 Perfusion with Silica Beads

One of the most direct ways to discover markers of angiogenesis may consist in the in vivo labeling of vascular structures, followed by recovery and comparative proteomic analysis. Schnitzer and co-workers have used a subtractive proteomic mapping strategy to identify proteins that are differentially expressed on the endothelial surface in normal and tumor tissue. Colloidal silica is used for the in vivo coating of the vasculature, followed by subcellular fractionation to directly isolate luminal endothelial cell plasma membranes (Durr et al. 2004). The isolated plasma membranes are then analyzed using two-dimensional gel electrophoresis or multiple multidimensional mass spectrometry techniques to produce high-resolution protein maps. Differential spot analysis followed by mass spectrometry of tryptic peptides, database searching and immunoblotting allowed the characterization of differentially expressed proteins. Recently, the analysis of the endothelium of normal rat lung tissue compared to lungs with metastatic breast adenocarcinomas led to the identification of 15 proteins that were upregulated on the endothelium (Oh et al. 2004).

28.4.4 In Vivo and Ex Vivo Biotinylation

More recently, the in vivo perfusion of tumor-bearing mice with active ester derivatives of biotin has been reported (Rybak et al. 2005). This approach involves the reaction of biotin ester derivatives with primary amino groups of proteins which are readily accessible from the bloodstream. Biotinylated proteins can then be purified on a streptavidin column in the presence of SDS from homogenized tissue, digested on resin and identified using LC-MS/MS methodologies (Rybak et al. 2004). The biotinylation of mice with F9 subcutaneous tumors or orthotopic kidney tumors revealed both quantitative and qualitative differences in the recovery of biotinylated proteins, as compared with normal tissues (Rybak et al. 2005). More recently, this approach has been extended to the ex vivo biotinylation of surgically resected human organs with cancer (Castronovo et al. 2006).

Imaging Applications of Vascular Tumor Targeting

One of the most straightforward biomedical applications of ligands (typically monoclonal antibodies) capable of selective localization around tumor vascular structures may reside in the macroscopic in vivo imaging of sites of disease. The visualization of the homing of ligands to vascular structures imposes two main requirements:

- Appropriate chemical modification to make the ligand visible
- Macromolecular targets that are abundant enough to counterbalance the small contribution of vascular structures to the overall solid tumor mass

In principle, four main chemical modification strategies can be envisaged for the molecular imaging of angiogenesis-related diseases:

- The radioactive labeling of ligands, for SPECT or PET imaging modalities (Dzik-Jurasz 2003; Fukumoto 2004; Verel et al. 2005)
- The use of near-infrared (NIR) fluorophores, which can be detected using epi-illumination (Neri et al. 1997; Birchler et al. 1999a), optical coherence tomography (Podoleanu 2005) or diffuse optical tomography (Ntziachristos et al. 2000; Weissleder and Ntziachristos 2003)
- The use of ligands for the targeted delivery of microbubbles, to be used as contrast agents for sonographic imaging applications (Joseph et al. 2004; Weller et al. 2005)
- The use of magnetic nanoparticles (Weissleder et al. 2005)

The ability of radioactively or fluorescently labeled antibodies to image tumors in vivo is by now well established. For macroscopic imaging applications, NIR fluorescence imaging may be limited by the reduced light penetration of tissues (Wan et al. 1981; Birchler et al. 1999a). However, fluorescently labeled antibodies may facilitate the microscopic imaging of superficial lesions (e.g., using endoscopic methods: Pelegrin et al. 1991) and of transparent structures within the body (e.g., angiogenesis-related ocular disorders; Birchler et al. 1999a; Matter et al. 2004). Furthermore, the use of NIR dyes may open novel angiographic imaging opportunities for solid tumors and other conditions, such as atherosclerosis (Weissleder and Ntziachristos 2003; Matter et al. 2004).

It is much more debatable whether antibody-modified microbubbles and nanoparticles can efficiently extravasate and reach abluminal antigens in tumors and other diseases. Promising tumor imaging results with antibody–quantum dot conjugates suggest that even large particles can selectively accumulate at the tumor site. This contrasts with experience of our group, suggesting that highly charged antibody derivatives (Halin et al. 2002a; Melkko et al. 2002; Niesner et al. 2002) and large antibody derivatives (Halin et al. 2003) may completely abrogate the tumor-targeting ability of the parental antibody in vivo, while retaining unperturbed antigen-binding properties.

28.6 Therapeutic Applications of Vascular Tumor Targeting

The following discussion is limited to derivatives of tumor-targeting antibodies, a class of therapeutic agents for which a substantial amount of preclinical experimental data is now available. To a certain extent, some of the considerations made in this section may be applicable for other classes of ligands (e.g., small globular proteins and peptides).

Figure 28.2 illustrates some of the main classes of antibody-derivatives which have been considered for tumor-targeting applications. Some of these strategies (e.g., antibody–photosensitizer and antibody–procoagulant conjugates) appear to be ideally suited for vascular targeting applications, since they may lead to intravascular blood coagulation, causing an avalanche of tumor cell death (Thorpe and Ran 2000; Fabbrini et al. 2005).

Many of the antibody functionalization strategies depicted in Fig. 28.2 have been applied both to full immunoglobulin and to antibody fragments. In most cases, we prefer the use of recombinant antibody fragments because of easy expression, rapid blood clearance and lack of Fc (which avoids the undesired targeting of bioactive moieties to cells bearing Fc receptors). However, some therapeutic strategies, such as antibody–drug conjugates, may benefit from the long blood circulation times of antibodies in the IgG format.

Antibody fragments have successfully been coupled to cytokines (Carnemolla et al. 2002; Halin et

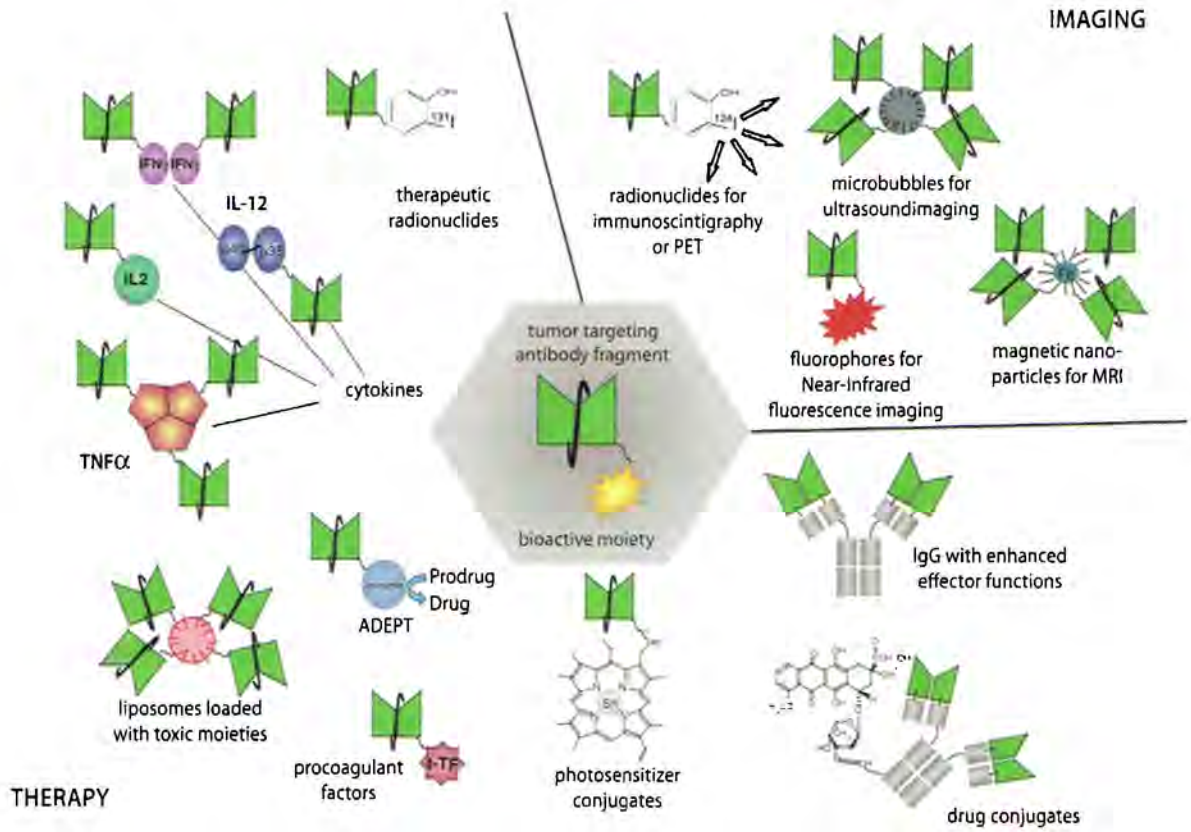


Fig. 28.2. Antibody derivatives that could be considered either for tumor imaging or therapy

al. 2002a, 2002b; Borsi et al. 2003; Ebbinghaus et al. 2005), chemokines (Li et al. 2003), fluorophores and photosensitizers (Birchler et al. 1999a, 1999b; Fabbrini et al. 2005), drugs (Damle 2004), pro-coagulant factors (Thorpe and Ran 2000), enzymes for pro-drug activation (Bagshawe et al. 2004) radionuclides (Brack et al. 2004) and liposomes (Marty and Schwendener 2005), but also to more exotic functional moieties such as uranium-loaded ferritin for neutron capture therapy (Hainfeld 1992).

Figure 28.3a shows the selective accumulation at the tumor site of the SIP-L19 antibody labeled with the infrared fluorophore Cy7. Figure 28.3b illustrates a striking therapeutic effect, observed in orthotopic

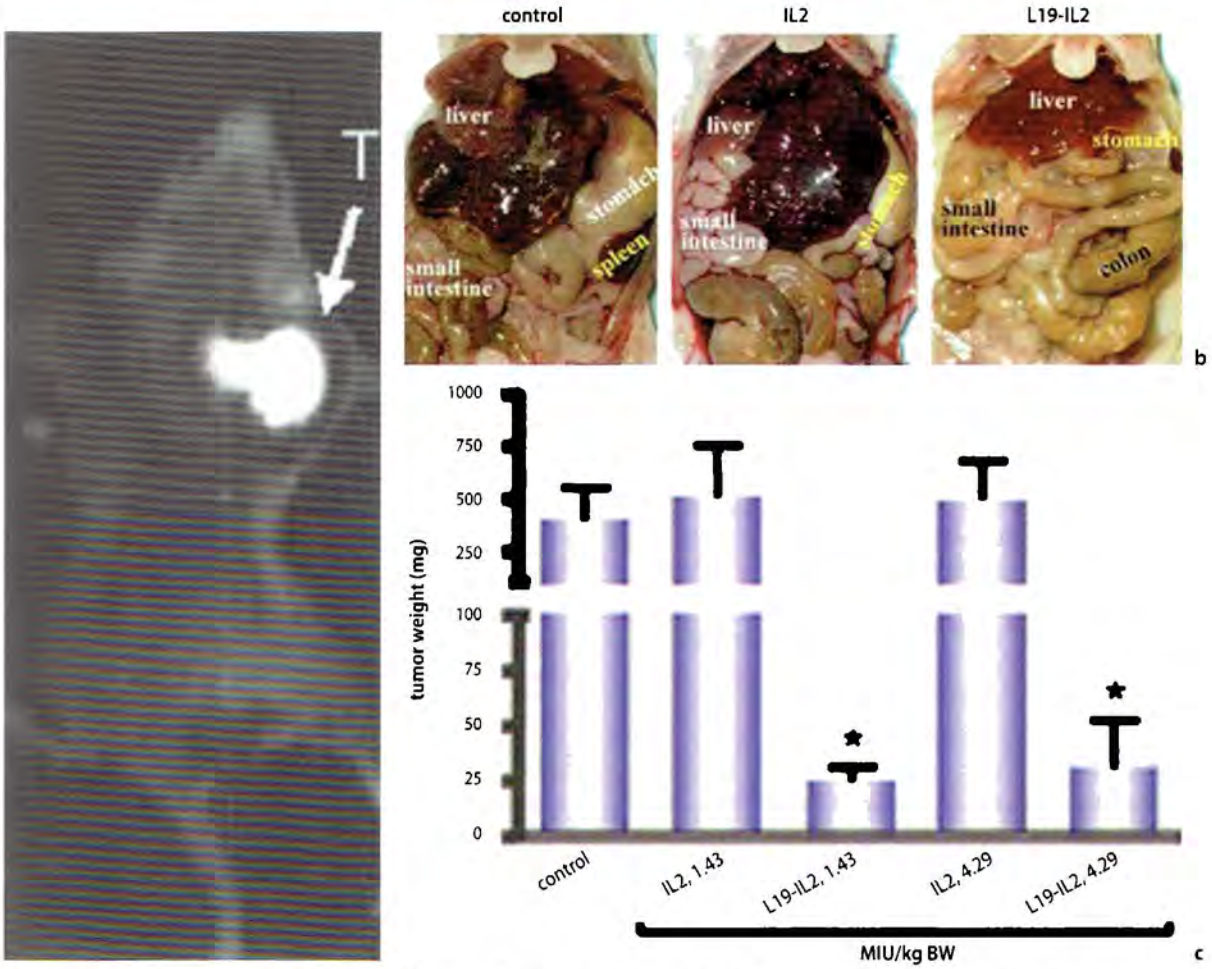


Fig. 28.3. a Selective accumulation of L19 in the tumor. Twenty-four-hour near-infrared image of a 129SvEv mouse subcutaneously grafted with F9 teratocarcinoma, i.v. injected with SIP (L19), labeled with the infrared fluorophore Cy7. The tumor is indicated by an *arrow*. b The therapeutic efficacy of L19-IL2 in the orthotopic HuH7 mouse model for hepatocellular carcinoma. HuH7 tumors (tumor volume 70–100 mm^3 before treatment) were treated with two treatment cycles consisting of five consecutive daily i.v. bolus injections of L19-IL2 or Proleukine (free IL2; two dose levels for each drug) followed by two drug-free days. After the second treatment cycle, the animals were killed and tumor volumes were measured. Compared with the non-targeted IL2 (Proleukine), L19-IL2 showed superior efficacy

animal models of hepatocellular carcinoma, using the fusion protein L19-IL2. As mentioned above, the vascular targeting properties of the L19 antibody, specific to the EDB domain of fibronectin, can be used to dramatically improve the tumor accumulation of the pro-inflammatory cytokine IL2, thus enhancing the therapeutic index of this anti-cancer biopharmaceutical.

Most of the studies performed with antibody derivatives suggest that the tumor-targeting properties of judiciously chosen antibodies can substantially potentiate the therapeutic action of the bioactive moiety chosen for antibody coupling.

Concluding Remarks

Vascular markers selectively expressed on tumor blood vessels are attractive for ligand-based vascular targeting approaches due to their accessibility from the blood stream and to the therapeutic options that they allow (e.g., induction of coagulation or recruitment of immune cells).

Tumor-induced antigens can be located either on the endothelial cells (luminal) or in the perivascular space. Luminal antigens are readily accessible for drugs circulating in the blood stream, but this advantage is counterbalanced by the generally low abundance of these molecules (Halin et al. 2002a). Therefore, abundant antigens, typically located in the extracellular matrix or on tumor cells, have proven to be a good choice for ligand-mediated targeting of solid tumors. However, the perivascular location and the elevated tumor interstitial pressure limit the absolute amount of antibody uptake in the tumor. In general, abundance as well as the restricted pattern of expression are important criteria for the selection of suitable target proteins.

Some of the markers of angiogenesis considered for vascular targeting applications are not only found around tumor blood vessels. For example, the EDB domain of fibronectin is found in certain tumors only around vascular structures (e.g., glioblastoma), while for other tumors and for metastases a diffuse stromal staining is often observed. In spite of these considerations, some antibodies which exhibit a broad staining pattern in the solid tumor mass may preferentially target vascular structures in vivo because of the antigen barrier effect (Adams et al. 2001).

As some of the vascular targeting antibody derivatives are now being investigated in clinical trials, we are anxiously waiting to learn more about their pharmaceutical potential and, in a broader context, about the potential and limitations associated with the use of tumor-targeting antibodies for improving the therapeutic index of bioactive agents. Some of the issues which will be clarified only in the clinical setting include the potential immunogenicity of antibody derivatives and, in the case of immunocytokines, the presence or absence of "sequestering" effects by cytokine receptors in circulating cells (Ebbinghaus et al. 2005). If, as we hope, vascular targeting antibodies prove to be efficacious for the therapy of certain types of cancer, they may find applications also in therapeutic areas outside oncology and will certainly trigger new investigations into innovative functionalization strategies which convert the preferential localization of an antibody at a site of disease into a therapeutic benefit for the patient.

References

Adams GP et al (2001) High affinity restricts the localization and tumor penetration of single-chain fv antibody molecules. Cancer Res 61:4750–4755

Adams MD et al (1995) Initial assessment of human gene diversity and expression patterns based upon 83 million nucleotides of cDNA sequence. Nature 377 [Suppl]:3–174

Ali SA et al (1999) Transferrin trojan horses as a rational approach for the biological delivery of therapeutic peptide domains. J Biol Chem 274:24066–24073

Aoka Y et al (2002) The embryonic angiogenic factor Dell accelerates tumor growth by enhancing vascular formation. Microvasc Res 64:148–161

Arap W et al (1998) Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. Science 279:377–380

Arkin MR, Wells JA (2004) Small-molecule inhibitors of

protein-protein interactions: progressing towards the dream. Nat Rev Drug Discov 3:301–317
Bagshawe KD et al (2004) Antibody-directed enzyme prodrug therapy (ADEPT) for cancer. Expert Opin Biol Ther 4:1777–1789
Baluk P et al (2003) Abnormalities of basement membrane on blood vessels and endothelial sprouts in tumors. Am J Pathol 163:1801–1815
Balza E et al (2001) Lack of specificity of endoglin expression for tumor blood vessels. Int J Cancer 94:579–585
Bander NH et al (2003) Targeted systemic therapy of prostate cancer with a monoclonal antibody to prostate-specific membrane antigen. Semin Oncol 30:667–676
Bander NH et al (2005) Phase I trial of 177lutetium-labeled J591, a monoclonal antibody to prostate-specific membrane antigen, in patients with androgen-independent prostate cancer. J Clin Oncol 23:4591–4601
Bartolomei M et al (2004) Combined treatment of glioblastoma patients with locoregional pre-targeted 90Y-biotin radioimmunotherapy and temozolomide. Q J Nucl Med Mol Imaging 48:220–228
Behr TM et al (2001) Imaging tumors with peptide-based radioligands. Q J Nucl Med 45:189–200
Berndorff D et al (2005) Radioimmunotherapy of solid tumors by targeting extra domain B fibronectin: identification of the best-suited radioimmunoconjugate. Clin Cancer Res 11:7053s–7063s
Binz HK et al (2005) Engineering novel binding proteins from nonimmunoglobulin domains. Nat Biotechnol 23:1257–1268
Birchler M et al (1999a) Infrared photodetection for the in vivo localisation of phage-derived antibodies directed against angiogenic markers. J Immunol Methods 231:239–248
Birchler M et al (1999b) Selective targeting and photocoagulation of ocular angiogenesis mediated by a phage-derived human antibody fragment. Nat Biotechnol 17:984–889
Borsi L et al (1987) Monoclonal antibodies in the analysis of fibronectin isoforms generated by alternative splicing of mRNA precursors in normal and transformed human cells. J Cell Biol 104:595–600
Borsi L et al (1992) Expression of different tenascin isoforms in normal, hyperplastic and neoplastic human breast tissues. Int J Cancer 52:688–592
Borsi L et al (2002) Selective targeting of tumoral vasculature: comparison of different formats of an antibody (L19) to the ED-B domain of fibronectin. Int J Cancer 102:75–85
Borsi L et al (2003) Selective targeted delivery of TNFalpha to tumor blood vessels. Blood 102:4384–4392
Bosslet K et al (1998) Elucidation of the mechanism enabling tumor selective prodrug monotherapy. Cancer Res 58:1195–1201
Bostwick DG et al (2000) Prognostic factors in prostate cancer. College of American Pathologists Consensus Statement 1999. Arch Pathol Lab Med 124:995–1000
Brack SS et al (2004) Molecular targeting of angiogenesis for imaging and therapy. Eur J Nucl Med Mol Imaging 31:1327–1341
Bredow S et al (2000) Imaging of tumour neovasculature by targeting the TGF-beta binding receptor endoglin. Eur J Cancer 36:675–681
Brekke OH, Sandlie I (2003) Therapeutic antibodies for human diseases at the dawn of the twenty-first century. Nat Rev Drug Discov 2:52–62
Brekken RA et al (1998) Vascular endothelial growth factor as a marker of tumor endothelium. Cancer Res 58:1952–1959
Brekken RA et al (2000) Selective inhibition of vascular endothelial growth factor (VEGF) receptor 2 (KDR/Flk-1) activity by a monoclonal anti-VEGF antibody blocks tumor growth in mice. Cancer Res 60:5117–5124
Brown NS, Bicknell R (2001) Hypoxia and oxidative stress in breast cancer. Oxidative stress: its effects on the growth, metastatic potential and response to therapy of breast cancer. Breast Cancer Res 3:323–327
Bucki R et al (2001) Involvement of phosphatidylinositol 4,5-bisphosphate in phosphatidylserine exposure in platelets: use of a permeant phosphoinositide-binding peptide. Biochemistry 40:15752–15761
Burrows FJ et al (1995) Up-regulation of endoglin on vascular endothelial cells in human solid tumors: implications for diagnosis and therapy. Clin Cancer Res 1:1623–1634
Carnemolla B et al (1989) A tumor-associated fibronectin isoform generated by alternative splicing of messenger RNA precursors. J Cell Biol 108:1139–1148
Carnemolla B et al (1996) Phage antibodies with pan-species recognition of the oncofoetal angiogenesis marker fibronectin ED-B domain. Int J Cancer 68:397–405
Carnemolla B et al (1999) Identification of a glioblastoma-associated tenascin-C isoform by a high affinity recombinant antibody. Am J Pathol 154:1345–1352
Carnemolla B et al (2002) Enhancement of the antitumor properties of interleukin-2 by its targeted delivery to the tumor blood vessel extracellular matrix. Blood 99:1659–1665
Carson-Walter EB et al (2001) Cell surface tumor endothelial markers are conserved in mice and humans. Cancer Res 61:6649–6655
Castronovo V et al (2006) Accessible proteins expressed in kidney cancer. Mol Cell Proteomics 5:2083–2091
Chang SS et al (1999) Five different anti-prostate-specific membrane antigen (PSMA) antibodies confirm PSMA expression in tumor-associated neovasculature. Cancer Res 59:3192–3198
Chen X et al (2004) MicroPET imaging of brain tumor angiogenesis with 18F-labeled PEGylated RGD peptide. Eur J Nucl Med Mol Imaging 31:1081–1089
Chrastina A (2003) High cell density-mediated pericellular hypoxia is a crucial factor inducing expression of the intrinsic hypoxia marker CA IX in vitro in HeLa cells. Neoplasma 50:251–256
Christian S et al (2001) Molecular cloning and characteriza-

tion of endosialin, a C-type lectin-like cell surface receptor of tumor endothelium. J Biol Chem 276:7408–7414
Christian S et al (2003) Nucleolin expressed at the cell surface is a marker of endothelial cells in angiogenic blood vessels. J Cell Biol 163:871–878
Collins J et al (2001) Cosmix-plexing: a novel recombinatorial approach for evolutionary selection from combinatorial libraries. J Biotechnol 74:317–338
Cooke SP et al (2001) A strategy for antitumor vascular therapy by targeting the vascular endothelial growth factor: receptor complex. Cancer Res 61:3653–3659
Curnis F et al (2000) Enhancement of tumor necrosis factor alpha antitumor immunotherapeutic properties by targeted delivery to aminopeptidase N (CD13). Nat Biotechnol 18:1185–1190
Curnis F et al (2002) Improving chemotherapeutic drug penetration in tumors by vascular targeting and barrier alteration. J Clin Invest 110:475–482
Damle NK (2004) Tumour-targeted chemotherapy with immunoconjugates of calicheamicin. Expert Opin Biol Ther 4:1445–1452
Demartis S et al (2001) Selective targeting of tumour neovasculature by a radiohalogenated human antibody fragment specific for the ED-B domain of fibronectin. Eur J Nucl Med 28:534–539
Denekamp J, Hobson B (1982) Endothelial-cell proliferation in experimental tumours. Br J Cancer 46:711–720
Doggrell SA (2005) Pegaptanib: the first antiangiogenic agent approved for neovascular macular degeneration. Expert Opin Pharmacother 6:1421–1423
Duesbery NS et al (2001) Suppression of ras-mediated transformation and inhibition of tumor growth and angiogenesis by anthrax lethal factor, a proteolytic inhibitor of multiple MEK pathways. Proc Natl Acad Sci USA 98:4089–4094
Durr E et al (2004) Direct proteomic mapping of the lung microvascular endothelial cell surface in vivo and in cell culture. Nat Biotechnol 22:985–992
Dzik-Jurasz AS (2003) Molecular imaging in vivo: an introduction. Br J Radiol 76 (Spec No 2):S98–S109
Ebbinghaus C et al (2005) Engineered vascular-targeting antibody-interferon-gamma fusion protein for cancer therapy. Int J Cancer 116:304–313
Eberhard A et al (2000) Heterogeneity of angiogenesis and blood vessel maturation in human tumors: implications for antiangiogenic tumor therapies. Cancer Res 60:1388–1393
Erlanson DA et al (2003) In situ assembly of enzyme inhibitors using extended tethering. Nat Biotechnol 21:308–314
Fabbrini M et al (2006) Selective occlusion of tumor blood vessels by targeted delivery of an antibody-photosensitizer conjugate. Int J Cancer 118:1805–1813
Felici F et al (1995) Peptide and protein display on the surface of filamentous bacteriophage. Biotechnol Annu Rev 1:149–183
Ferrara N(2004) Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev 25:581–611
Ferrara N et al (2004) Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. Nat Rev Drug Discov 3:391–400
Finger AN et al (2002) Scavenger receptor block as strategy for the identification of bone marrow homing phages by panning in vivo random peptide phage displayed libraries. J Immunol Methods 264:173–186
Fonsatti E et al (2000) Endoglin is a suitable target for efficient imaging of solid tumors: in vivo evidence in a canine mammary carcinoma model. Clin Cancer Res 6:2037–2043
Fukuda T et al (2002) Mice lacking the EDB segment of fibronectin develop normally but exhibit reduced cell growth and fibronectin matrix assembly in vitro. Cancer Res 62:5603–5610
Fukumoto M(2004) Single-photon agents for tumor imaging: 201Tl, 99mTc-MIBI, and 99mTc-tetrofosmin. Ann Nucl Med 18:79–95
Gartner ZJ et al (2004) DNA-templated organic synthesis and selection of a library of macrocycles. Science 305:1601–1605
Giovannoni L et al (2001) Isolation of anti-angiogenesis antibodies from a large combinatorial repertoire by colony filter screening. Nucleic Acids Res 29:E27
Grabmaier K et al (2004) Strict regulation of CAIX(G250/MN) by HIF-1alpha in clear cell renal cell carcinoma. Oncogene 23:5624–5631
Green LL (1999) Antibody engineering via genetic engineering of the mouse: XenoMouse strains are a vehicle for the facile generation of therapeutic human monoclonal antibodies. J Immunol Methods 231:11–23
Gutheil JC et al (2000) Targeted antiangiogenic therapy for cancer using Vitaxin: a humanized monoclonal antibody to the integrin alphavbeta3. Clin Cancer Res 6:3056–3061
Hainfeld JF (1992) Uranium-loaded apoferritin with antibodies attached: molecular design for uranium neutron-capture therapy. Proc Natl Acad Sci USA 89:11064–11068
Halin C et al (2002a) Tumor-targeting properties of antibody-vascular endothelial growth factor fusion proteins. Int J Cancer 102:109–116
Halin C et al (2002b) Enhancement of the antitumor activity of interleukin-12 by targeted delivery to neovasculature. Nat Biotechnol 20:264–269
Halin C et al (2003) Synergistic therapeutic effects of a tumor targeting antibody fragment, fused to interleukin 12 and to tumor necrosis factor alpha. Cancer Res 63:3202–3210
Hauff P et al (2003) New imaging probes. Excerpta Medica Medical Communications BV, The Netherlands
Heinis C et al (2004) Engineering a thermostable human prolyl endopeptidase for antibody-directed enzyme prodrug therapy. Biochemistry 43:6293–6303
Helmlinger G et al (1997) Interstitial pH and pO2 gradients in solid tumors in vivo: high-resolution measurements reveal a lack of correlation. Nat Med 3:177–182

Hey T et al (2005) Artificial, non-antibody binding proteins for pharmaceutical and industrial applications. Trends Biotechnol 23:514–522

Hood JD et al (2002) Tumor regression by targeted gene delivery to the neovasculature. Science 296:2404–2407

Huang X et al (2005) A monoclonal antibody that binds anionic phospholipids on tumor blood vessels enhances the antitumor effect of docetaxel on human breast tumors in mice. Cancer Res 65:4408–4416

Huminiecki L, Bicknell R (2000) In silico cloning of novel endothelial-specific genes. Genome Res 10:1796–1806

Huminiecki L et al (2002) Magic roundabout is a new member of the roundabout receptor family that is endothelial specific and expressed at sites of active angiogenesis. Genomics 79:547–552

Jones PT et al (1986) Replacing the complementarity-determining regions in a human antibody with those from a mouse. Nature 321:522–525

Joseph S et al (2004) A real-time in vitro assay for studying functional characteristics of target-specific ultrasound contrast agents. Pharm Res 21:920–926

Karpas A et al (2001) A human myeloma cell line suitable for the generation of human monoclonal antibodies. Proc Natl Acad Sci USA 98:1799–1804

Ke L et al (1996) Vascular targeting of solid and ascites tumours with antibodies to vascular endothelial growth factor. Eur J Cancer 32A:2467–2473

Kohler G, Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495–497

Konerding MA et al (2001) 3D microvascular architecture of pre-cancerous lesions and invasive carcinomas of the colon. Br J Cancer 84:1354–1362

Kowalski J et al (2003) Evaluation of positron emission tomography imaging using [68 Ga]-DOTA-D Phe(1)-Tyr(3)-Octreotide in comparison to [111In]-DTPAOC SPECT. First results in patients with neuroendocrine tumors. Mol Imaging Biol 5:42–48

Kozbor D, Roder JC (1981) Requirements for the establishment of high-titered human monoclonal antibodies against tetanus toxoid using the Epstein–Barr virus technique. J Immunol 127:1275–1280

Kozbor D et al (1982) Human anti-tetanus toxoid monoclonal antibody secreted by EBV-transformed human B cells fused with murine myeloma. Hybridoma 1:323–328

Leins A et al (2003) Expression of tenascin-C in various human brain tumors and its relevance for survival in patients with astrocytoma. Cancer 98:2430–2439

Li J et al (2003) LEC/chTNT-3 fusion protein for the immunotherapy of experimental solid tumors. J Immunother 26:320–331

Li Y et al (2002) In vitro and preclinical targeted alpha therapy of human prostate cancer with Bi-213 labeled J591 antibody against the prostate specific membrane antigen. Prostate Cancer Prostatic Dis 5:36–46

Liu H et al (1997) Monoclonal antibodies to the extracellular domain of prostate-specific membrane antigen also react with tumor vascular endothelium. Cancer Res 57:3629–3634

MacFadyen JR et al (2005) Endosialin (TEM1, CD248) is a marker of stromal fibroblasts and is not selectively expressed on tumour endothelium. FEBS Lett 579:2569–2575

Marty C, Schwendener RA (2005) Cytotoxic tumor targeting with scFv antibody-modified liposomes. Methods Mol Med 109:389–402

Marty C et al (2002) Cytotoxic targeting of F9 teratocarcinoma tumours with anti-ED-B fibronectin scFv antibody modified liposomes. Br J Cancer 87:106–112

Matsubara S et al (2000) Analysis of endoglin expression in normal brain tissue and in cerebral arteriovenous malformations. Stroke 31:2653–2660

Matter CM et al (2004) Molecular imaging of atherosclerotic plaques using a human antibody against the extra-domain B of fibronectin. Circ Res 95:1225–1233

Mattheakis LC et al (1994) An in vitro polysome display system for identifying ligands from very large peptide libraries. Proc Natl Acad Sci USA 91:9022–9036

McCafferty J et al (1990) Phage antibodies: filamentous phage displaying antibody variable domains. Nature 348:552–554

McDonald DM, Choyke PL (2003) Imaging of angiogenesis: from microscope to clinic. Nat Med 9:713–725

Melkko S et al (2002) An antibody-calmodulin fusion protein reveals a functional dependence between macromolecular isoelectric point and tumor targeting performance. Int J Radiat Oncol Biol Phys 54:1485–1490

Melkko S et al (2004) Encoded self-assembling chemical libraries. Nat Biotechnol 22:568–574

Menrad A, Menssen HD (2005) ED-B fibronectin as a target for antibody-based cancer treatments. Expert Opin Ther Targets 9:491–500

Milowsky MI et al (2004) Phase I trial of yttrium-90-labeled anti-prostate-specific membrane antigen monoclonal antibody J591 for androgen-independent prostate cancer. J Clin Oncol 22:2522–2531

Mirick GR et al (2004) A review of human anti-globulin antibody (HAGA, HAMA, HACA, HAHA) responses to monoclonal antibodies. Not four letter words. Q J Nucl Med Mol Imaging 48:251–257

Mutuberria R et al (2004) Isolation of human antibodies to tumor-associated endothelial cell markers by in vitro human endothelial cell selection with phage display libraries. J Immunol Methods 287:31–47

Nanda A, St. Croix B (2004) Tumor endothelial markers: new targets for cancer therapy. Curr Opin Oncol 16:44–49

Neri D et al (1997) Targeting by affinity-matured recombinant antibody fragments of an angiogenesis associated fibronectin isoform. Nat Biotechnol 15:1271–1275

Niesner U et al (2002) Quantitation of the tumor-targeting properties of antibody fragments conjugated to cell-permeating HIV-1 TAT peptides. Bioconjug Chem 13:729–736

Nilsson F et al (2001) Targeted delivery of tissue factor to the ED-B domain of fibronectin, a marker of angiogenesis, mediates the infarction of solid tumors in mice. Cancer Res 61:711–716

Ntziachristos V et al (2000) Concurrent MRI and diffuse optical tomography of breast after indocyanine green enhancement. Proc Natl Acad Sci USA 97:2767–2772

Oh P et al (2004) Subtractive proteomic mapping of the endothelial surface in lung and solid tumours for tissue-specific therapy. Nature 429:629–635

Okarvi SM, al-Jammaz I (2003) Synthesis, radiolabelling and biological characteristics of a bombesin peptide analog as a tumor imaging agent. Anticancer Res 23:2745–2750

Paganelli G et al (1999) Antibody-guided three-step therapy for high grade glioma with yttrium-90 biotin. Eur J Nucl Med 26:348–357

Pasqualini R, Ruoslahti E (1996) Organ targeting in vivo using phage display peptide libraries. Nature 380:364–366

Pasqualini R et al (1997) Alpha v integrins as receptors for tumor targeting by circulating ligands. Nat Biotechnol 15:542–546

Pasqualini R et al (2000) Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis. Cancer Res 60:722–727

Pelegrin A et al (1991) Antibody–fluorescein conjugates for photoimmunodiagnosis of human colon carcinoma in nude mice. Cancer 67:2529–37

Petronzelli F et al (2005) Improved tumor targeting by combined use of two antitenascin antibodies. Clin Cancer Res 11:7137s–7145s

Pini A et al (1998) Design and use of a phage display library. Human antibodies with subnanomolar affinity against a marker of angiogenesis eluted from a two-dimensional gel. J Biol Chem 273:21769–21776

Podoleanu AG (2005) Optical coherence tomography. Br J Radiol 78:976–988

Porkka K et al (2002) A fragment of the HMGN2 protein homes to the nuclei of tumor cells and tumor endothelial cells in vivo. Proc Natl Acad Sci US 99:7444–7449

Posey JA et al (2001) A pilot trial of Vitaxin, a humanized anti-vitronectin receptor (anti alpha v beta 3) antibody in patients with metastatic cancer. Cancer Biother Radiopharm 16:125–132

Prewett M et al (1999) Antivascular endothelial growth factor receptor (fetal liver kinase 1) monoclonal antibody inhibits tumor angiogenesis and growth of several mouse and human tumors. Cancer Res 59:5209–5218

Ramachandran C, Melnick SJ (1999) Multidrug resistance in human tumors – molecular diagnosis and clinical significance. Mol Diagn 4:81–94

Ramstrom O, Lehn JM (2002) Drug discovery by dynamic combinatorial libraries. Nat Rev Drug Discov 1:26–36

Ran S et al (2002) Increased exposure of anionic phospholipids on the surface of tumor blood vessels. Cancer Res 62:6132–6140

Ran S et al (2005) Antitumor effects of a monoclonal antibody that binds anionic phospholipids on the surface of tumor blood vessels in mice. Clin Cancer Res 11:1551–1562

Riva P et al (1999a) Loco-regional radioimmunotherapy of high-grade malignant gliomas using specific monoclonal antibodies labeled with 90Y: a phase I study. Clin Cancer Res 5[Suppl]:3275s–3280s

Riva P et al (1999b) 131I radioconjugated antibodies for the locoregional radioimmunotherapy of high-grade malignant glioma – phase I and II study. Acta Oncol 38:351–359

Rybak JN et al (2004) Purification of biotinylated proteins on streptavidin resin: a protocol for quantitative elution. Proteomics 4:2296–2299

Rybak JN et al (2005) In vivo protein biotinylation for identification of organ-specific antigens accessible from the vasculature. Nat Methods 2:291–298

Santimaria M et al (2003) Immunoscintigraphic detection of the ED-B domain of fibronectin, a marker of angiogenesis, in patients with cancer. Clin Cancer Res 9:571–579

Schaffitzel C et al (1999) Ribosome display: an in vitro method for selection and evolution of antibodies from libraries. J Immunol Methods 231:119–135

Shuker SB et al (1996) Discovering high-affinity ligands for proteins: SAR by NMR. Science 274:1531–1534

Silacci M et al (2005) Design, construction, and characterization of a large synthetic human antibody phage display library. Proteomics 5:2340–2350

Sipkins DA et al (1998) Detection of tumor angiogenesis in vivo by alphaVbeta3-targeted magnetic resonance imaging. Nat Med 4:623–626

Siri A et al (1991) Human tenascin: primary structure, pre-mRNA splicing patterns and localization of the epitopes recognized by two monoclonal antibodies. Nucleic Acids Res 19:525–531

St. Croix B et al (2000) Genes expressed in human tumor endothelium. Science 289:1197–1202

Steinitz M et al (1977) EB virus-induced B lymphocyte cell lines producing specific antibody. Nature 269:420–422

Sullivan DC et al (2003) EndoPDI, a novel protein-disulfide isomerase-like protein that is preferentially expressed in endothelial cells acts as a stress survival factor. J Biol Chem 278:47079–47088

Tarli L et al (1999) A high-affinity human antibody that targets tumoral blood vessels. Blood 94:192–198

Terskikh AV et al (1997) "Peptabody": a new type of high avidity binding protein. Proc Natl Acad Sci USA 94:1663–1668

Thorpe PE, Ran S (2000) Tumor infarction by targeting tissue factor to tumor vasculature. Cancer J 6 [Suppl 3]: S237–S244

Toyokuni S et al (1995) Persistent oxidative stress in cancer. FEBS Lett 358:1–3

Tozer GM et al (1990) The relationship between regional

variations in blood flow and histology in a transplanted rat fibrosarcoma. Br J Cancer 61:250–257

Tozer GM et al (2005) Intravital imaging of tumour vascular networks using multi-photon fluorescence microscopy. Adv Drug Deliv Rev 57:135–152

Trepel M et al (2002) In vivo phage display and vascular heterogeneity: implications for targeted medicine. Curr Opin Chem Biol 6:399-404

Vater A, Klussmann S (2003) Toward third-generation aptamers: Spiegelmers and their therapeutic prospects. Curr Opin Drug Discov Dev 6:253–261

Velculescu VE et al (1995) Serial analysis of gene expression. Science 270:484–487

Verel I et al (2005) The promise of immuno-PET in radioimmunotherapy. J Nucl Med 46 [Suppl 1]:164S–171S

Viti F et al (1999) Increased binding affinity and valence of recombinant antibody fragments lead to improved targeting of tumoral angiogenesis. Cancer Res 59:347–352

Viti F et al (2000) Design and use of phage display libraries for the selection of antibodies and enzymes. Methods Enzymol 326:480–505

Wakai Y et al (2000) Effective cancer targeting using an antitumor tissue vascular endothelium-specific monoclonal antibody (TES-23). Jpn J Cancer Res 91:1319–1325

Wan S et al (1981) Transmittance of nonionizing radiation in human tissues. Photochem Photobiol 34:679–681

Wang JM et al (1993) A monoclonal antibody detects heterogeneity in vascular endothelium of tumours and normal tissues. Int J Cancer 54:363–70

Weissleder R, Ntziachristos V (2003) Shedding light onto live molecular targets. Nat Med 9:123–128

Weissleder R et al (2005) Cell-specific targeting of nanoparticles by multivalent attachment of small molecules. Nat Biotechnol 23:1418–1423

Weller GE et al (2005) Ultrasonic imaging of tumor angiogenesis using contrast microbubbles targeted via the tumor-binding peptide arginine-arginine-leucine. Cancer Res 65:533–539

Winter G et al (1994) Making antibodies by phage display technology. Annu Rev Immunol 12:433–455

Wrighton NC et al (1997) Increased potency of an erythropoietin peptide mimetic through covalent dimerization. Nat Biotechnol 15:1261–1265

Zardi L et al (1987) Transformed human cells produce a new fibronectin isoform by preferential alternative splicing of a previously unobserved exon. EMBO J 6:2337–2342

Zehender H et al (2004) SpeedScreen: The "missing link" between genomics and lead discovery. J Biomol Screen 9:498–505

Zhu Z, Witte L (1999) Inhibition of tumor growth and metastasis by targeting tumor-associated angiogenesis with antagonists to the receptors of vascular endothelial growth factor. Invest New Drugs 17:195–212

Molecular Imaging of Targets and Therapeutics in Tumour Angiogenesis

29

James P.B. O'Connor, Daniela D. Rosa, Alan Jackson, Gordon C. Jayson

Abstract

Various quantitative imaging techniques are now frequently used as biomarkers in the evaluation of novel anti-angiogenic and vascular disrupting compounds in clinical trials. Positron emission tomography (PET) techniques have been utilised in a small number of studies to evaluate changes in specific molecular pathways following administration of angiogenesis inhibitors, and can also quantify changes in perfusion and vascular volume. However, technical considerations have, to date, limited the widespread application of PET. Dynamic contrast-enhanced imaging biomarkers have shown evidence of therapeutic effect, dose-dependent response and change in progression-free survival in some trials. However, image acquisition and analysis methods in computed tomography and magnetic resonance techniques are complex and solely reflect changes in physiological processes, rather than measurement of therapeutic effects on specific molecular pathways. In this chapter we review the critical issues that influence data acquisition, image analysis and application of PET, MRI and CT imaging techniques in the assessment of angiogenesis targets and therapeutics. Finally the current clinical trial findings and important areas for future development are discussed.

CONTENTS

Introduction

More than one third of the population of Europe and North America will develop cancer at some stage of their lives, and around one in four people will die from the disease (Office of National Statistics 2005; Parkin et al. 2002; American Cancer Society 2005). Despite considerable improvements in cancer treatment over the past few decades, with advances in the conventional treatment options of surgery, radiotherapy, cytotoxic chemotherapy and hormonal therapy, survival rates for many common cancers remain poor (Department of Health 2004). Advances in molecular cell biology and genomics have produced an increasingly detailed understanding of the biological basis to malignant transformation, survival and progression (Hanahan and Weinberg 2000), which has in turn provided various novel targets for oncology drug development (Kelloff et al. 2005). One major area of interest focuses on the process of angiogenesis during malignancy, where tumours develop vascular networks of their own, enabling them to survive, grow (Carmeliet and Jain 2000) and metastasise (Fidler 2001).

Tumour blood vessels are both structurally and functionally abnormal. They are typically dilated, tortuous and exhibit increased permeability. Local vascular networks have aberrant branching and shunts, so that blood flow is chaotic and lacks the tight physiological control demonstrated in normal tissue (Carmeliet and Jain 2000; Folkman 1971). Therapeutics that selectively inhibit cell signalling pathways can disrupt the formation of new vessels (anti-angiogenesis) or destroy existing vessels (vascular disruption). Recent studies have supported this strategy and provided evidence that targeting tumour vasculature can improve clinical outcome in patients with advanced solid tumours (Hurwitz et al. 2004; Yang 2004; Johnson et al. 2004; Miller et al. 2005).

New agents undergoing early clinical trial evaluation require assessment of pharmacokinetic and pharmacodynamic (biological) endpoints to evaluate whether or not a drug should progress into phase II/III trials (Workman et al. 2006). Anti-angiogenic and vascular disrupting agents create several challenges for phase I/II trial design since they reduce tumour growth and/or prevent metastases through predominantly cytostatic mechanisms. Biopsy and bio-fluids remain important surrogate endpoints in trial evaluation, but there has been increasing interest in the use of non-invasive imaging as a means of extracting biomarkers of dose, drug schedule and efficacy (Collins 2003). Conventional imaging assessments of reduction in tumour size may not be adequate for assessing these agents in clinical studies. For these reasons, imaging strategies that extract more physiological measures of vasculature function are desirable (Jayson and Waterton 2005). This chapter reviews the current use of imaging methods in the evaluation of angiogenesis and its inhibition by conventional therapy and novel anti-cancer drugs in early phase clinical trials.

Imaging

29.2.1 General Considerations

Imaging techniques are attractive for assessing drug therapy within tumour vasculature since they are non- or minimally invasive and can be used to evaluate large regions or the entire volume of a tumour repeatedly. However, spatial resolution is limited by the voxel size, which is in the order of 0.5 to 5 mm for all imaging modalities, which makes validation against histopathology difficult. It also means that most techniques, with the exception of some positron emission tomography (PET), are inherently geared towards evaluating biomarkers that represent a composite of physiological processes, rather than measuring histological markers of angiogenesis, such as microvascular density (MVD), or assays

of molecules such as soluble vascular endothelial growth factor (VEGF) or its receptor (VEGF-R) (Jayson and Waterton 2005).

Modalities available for clinical evaluation of angiogenesis include PET, single photon emission computed tomography (SPECT), X-ray computed tomography (CT), magnetic resonance imaging (MRI) and spectroscopy (MRS), Doppler ultrasound and optical imaging (Miller et al. 2005). Each imaging modality has strengths and weaknesses, so that no one technique is ideal in all circumstances (Table 29.1). While the molecular imaging afforded by PET is of particular interest, it has several practical limitations. In contrast, dynamic MRI (which produces "less physiological" parameters) has been successfully applied to a larger number of clinical trials of anti-angiogenic and vascular disrupting agents. Further promising results have been demonstrated by dynamic CT, but other modalities remain largely a research interest (MRS, ultrasound, SPECT, optical imaging), with few successful applications in human studies to date. Therefore, this review focuses mainly on MRI, CT and PET methods and applications.

Table 29.1. The strengths, weaknesses and current use of imaging modalities in the assessment of angiogenesis. *CA*, contrast agent (other abbreviations explained in text)

Modality	Contrast agent	Voxel size	Strengths	Weaknesses	Current use
MRI	Gadolinium	1–4 mm	No ionising radiation Low CA toxicity Versatility in sequences Can perform alongside anatomical imaging	Non-linear relationship of signal intensity to [CA] hence requires complex data modelling Complex physiologic meaning of parameters Susceptible to motion Complex/specialist analysis	Most widely used imaging tool Performed on routine scanner hardware Published guidelines on use in cancer Poor at imaging/thoracic lesions
PET	^{15}O-H_2O ^{15}O-CO ^{18}F-FDG ^{124}I-VEGF	3–4 mm	Emission directly proportional to [CA] Direct measurement of blood volume Truly capable of molecular imaging	Radionuclide $t_{1/2}$ in minutes Ionising radiation Poor spatial resolution and signal-to-noise ratio	Practical limitations – require on-site cyclotron Requires methodology developments to increase use in angiogenesis studies
CT	Iodinated agents	0.5–2 mm	Attenuation directly proportional to [CA] Can perform alongside anatomical imaging	Ionising radiation CA toxicity Low sensitivity to contrast Dynamic imaging limited to small volumes	Performed on routine scanner hardware Ionising radiation limits repeated imaging
Ultrasound	Microbubble	0.5 mm	Doppler shift used to assess flow Can assess perfusion with microbubble CA	Not truly quantitative Cannot measure permeability Doppler ineffective in vessels of ≤100 μm Operator dependant	Not widely used to assess angiogenesis

29.2.2 Magnetic Resonance Imaging

Dynamic contrast-enhanced MRI (DCE-MRI) provides a non-invasive method of investigating the structure and function of tumour blood vessels by imaging low-molecular-weight paramagnetic contrast agents as they traverse the tumour microvasculature. Blood flow, vessel permeability and the volumes of both the vascular and extracellular extravascular compartments can be measured. The technique is safe, does not use ionising radiation, and can be performed using T_2/T_2^*- or T_1-weighted sequences on standard 1.5-tesla clinical systems. In these respects, it has several practical advantages over PET. DCE-MRI provides good spatial resolution with voxels in the range of 1–4 mm and may be combined with conventional anatomical imaging and other sequences including ^{1}H MRS, diffusion-weighted imaging and blood oxygen level-dependent imaging. However, unlike PET, the parameters derived from DCE-MRI are relative to values for normal tissue, rather than absolute measurements.

29.2.2.1 T_2*-weighted Image Acquisition: Brain Studies

T_2 and T_2^* (susceptibility-mediated) techniques have been used to evaluate brain perfusion and microvascular structure (Aronen et al. 1994; Li et al. 2000), but their use in cancer imaging has been largely restricted to cerebral tumours due to technical limitations in peripheral tissues. The signal loss resulting from passage of the contrast agent bolus on T_2^*-weighted images can be used to calculate the change in contrast concentration occurring in each pixel, since the change in transverse relaxation rate R_2^* is related to signal intensity. Thus, an estimate of the relative cerebral blood volume (rCBV) can be made:

$$rCBV = \int_{t0}^{te} \Delta R_2(t)dt$$

where t_0 is the time of first arrival of contrast agent and t_e is the time at which the change in R_2 returns to baseline values (Jackson 2004). An approximate measure of the mean transit time (MTT) can be made from the width of the curve; for example, the full width at half maximum height (FWHM) and then the relative blood flow (rCBF) can then be calculated using the central volume theorem:

$$rCBF = \frac{rCBV}{MTT}$$

Because of the initially high concentration of contrast agent during the first pass, T_2^*-weighted imaging is sensitive to flow and blood volume. The technique is not easily applied to thoracic, abdominal or pelvic tumours, which experience motion and permeability across the vasculature. In contrast, T_1-weighted techniques have been extensively applied to tumours outside the brain and form the basis for the remainder of this review.

29.2.2.2 T_1-weighted Image Acquisition: the Importance of the Arterial Input Function

A series of equivalent images are acquired every few seconds in conventional T_1-weighted DCE-MRI, over a period of around 6–10 min. Variations in protocols reflect the innate trade-offs between spatial resolution, the volume of tumour covered, and temporal resolution for the acquisitions (typically every 1–5 s, but sometimes longer). Radiofrequency-spoiled fast field gradient echo sequences are preferred since these provide rapid data acquisition, good contrast medium sensitivity, high signal-to-noise ratio, and adequate temporal resolution. The dynamic series is typically preceded by survey and anatomical images and sequences to allow calculation of baseline T_1 values prior to contrast administration (multiple flip angle, inversion recovery, or proton density-based techniques) which are used in the subsequent data analysis to transform measured signal changes into the changes in contrast agent concentration (Buckley and Parker 2005).

A bolus of contrast agent enters the tumour vascular bed following rapid intravenous injection (usually through an automated injector pump).

Gadolinium ions are paramagnetic due to the presence of unpaired outer shell electrons. They therefore enhance the loss of energy through spin–spin relaxation, thus shortening the T_1 relaxation times of tissue protons in proximity with contrast agent, producing signal enhancement. The degree of signal enhancement measured from each voxel on T_1-weighted imaging reflects both physiological and physical factors, including blood perfusion, the delivery of contrast agent per unit time, capillary surface area and permeability and the volume of the extracellular extravascular space (EES). The signal obtained is also affected by native tissue T_1-relaxation times, the dose of contrast agent, the precise imaging parameters and machine variables.

The amount of gadolinium contrast agent that passes through normal tissue and tumour vessels depends on the injected dose and local perfusion. The concentration–time course of contrast agent entering the artery supplying the vascular bed is termed the arterial input function (AIF). Accurate characterisation of the early part of the AIF requires very high temporal resolution in the region of around 1 s, which imposes severe limitations on the spatial resolution of the imaging sequence and the volume of tissue that can be encompassed in any given acquisition (Henderson et al. 1998). For this reason, many investigators either sample the AIF every 2–6 s (sufficiently reproducible for most studies), or use an idealised mathematical approximation (Weinmann et al. 1984; Fritz-Hansen et al. 1996) that removes restrictions on temporal resolution (imposed by the need to sample the initial peak), slice position (imposed by the need to obtain the AIF from a major vessel) and sequence choice (imposed by the need to avoid flow artefacts in the AIF).

The amount of intravascular contrast agent within each given voxel at any time will reflect the proportion of the voxel formed by perfused blood vessels. The rate and quantity of contrast agent leak into the EES reflect the difference in concentration between the blood plasma and the EES, the product of endothelial membrane permeability and surface area, and the size of the EES. T_1-weighted DCE-MRI analysis aims to identify surrogate markers that represent one of, or combinations of, these individual biological features, and unlike T_2^*-weighted sequences, it is heavily dependent on contrast agent permeability as well as flow and blood volume (Jackson 2003).

29.2.2.3 Data Extraction and Semiquantitative T_1-derived Parameters

Calculation of microvascular parameters from DCE-MRI data is complex. Several distinct analysis methods have been used. Data can be evaluated by analysis of the signal intensity–time curve or the related gadolinium concentration–time curve (in both cases without fitting a model to the data). Components of the original signal intensity–time curve such as curve shape, gradient or time to maximum enhancement are simple qualitative descriptors that were used in early studies of tumour angiogenesis (Daniel et al. 1998; Dowlati et al. 2002). They are seldom used in current study design since they are prone to noise, less reproducible and experience considerable variation among scanner manufacturers and examination settings, thus making direct comparisons between patients and trials near impossible (Parker and Buckley 2005).

More robust analyses require conversion of signal enhancement into information concerning contrast agent concentration. The relationship between R_1 ($1/T_1$) and the contrast agent concentration [C] is given by

$$\frac{1}{T_1} = \frac{1}{T_{1(0)}} + \alpha[C]$$

where $1/T_1$ is the measured R_1 after contrast, $1/T_1(0)$ is the baseline R_1 and α is a constant. Thus signal intensity data must be converted into R_1 in order to accurately produce the contrast agent concentration–time curve, unlike in dynamic CT, where a simple linear relationship between density and contrast concentration holds true (Jackson 2003; Miles 1999).

Some parameters describe the shape of the contrast concentration–time curve without requiring modelling. Of these parameters, termed semiquantitative, the most widely used is the initial area under

the gadolinium concentration-time curve (IAUGC), derived from the signal intensity-time curve, defined as

$$IAUGC_t = \int_0^t [CA](t')dt'$$

where [CA] (t') represents the concentration of contrast agent measured in the tissue at time t'. IAUGC is a reasonably reliable and reproducible quantity, reflecting a combination of flow, blood volume, vessel permeability and EES volume, and is widely used as a surrogate biomarker in clinical studies (Evelhoch 1999).

29.2.2.4
T_1 Methods Requiring Pharmacokinetic Modelling

Pharmacokinetic modelling techniques of varying complexity enable *estimates* of physiological characteristics such as flow and capillary endothelial permeability to be calculated. Measured data (the tumour concentration-time curve and an AIF) is 'fitted' to mathematical equations from which parameters that reflect microvascular function are derived (Parker et al. 2005). The parameters produced are, in theory, independent of acquisition protocol and should only reflect tissue characteristics, enabling their use in multicentre studies employing varying image acquisition protocols and equipment. The choice of analysis technique is crucial, since it will determine not only the range of parameters available, but also their *precise meaning*. In all cases, there is an inherent trade-off between extracting relatively simple parameters with poor specificity versus physiologically congruent but less statistically stable parameters. Despite their wide use, there is little evidence that any of the various techniques employed accurately estimate absolute values of processes such as perfusion, permeability and blood volume (Buckley 2002). Commonly used standard terms are listed in Table 29.2.

The simple Tofts and Kermode model describes the concentration of contrast agent within each voxel, thus allowing calculation of the size of the EES (v_e), and the bulk transfer coefficient (K^{trans}):

$$v_e \frac{dC_e(t)}{dt} = K^{trans}\left[C_p(t) - C_e(t)\right]$$

where $C_e(t)$ is the concentration of agent in v_e, C_p is the concentration of agent in the plasma volume (v_p) and K^{trans} is the volume transfer constant between v_p and v_e (Tofts and Kermode 1991). Other similar models have been derived from the same general kinetic model, but vary in their terminology and in some details (Brix et al. 1991; Larsson et al. 1990). Crucially, changes in blood flow (F), blood volume, endothelial permeability and endothelial surface area can all produce changes in measurements of K^{trans}, and the specific contribution of each of these components cannot be separated. Thus when contrast agent delivery is ample ($F>>PS$), K^{trans} represents the permeability surface area product per unit volume of tissue, for transendothelial transport between plasma and EES (K^{trans}~PS). This assumption is not true in cases when the delivery of contrast agent is compromised ($PS>>F$). Here, K^{trans} represents the blood plasma flow per unit volume of tissue [K^{trans}~F(1–haematocrit)] (Tofts et al. 1999). In practice, the situation is seldom entirely one or the other, and the contribution of each scenario varies in time and space throughout the tumour.

This illustrates one of the most important limitations of DCE-MRI – an inability to produce a true quantitative measure of vascular parameters. Despite this caveat, these basic pharmacokinetic models are commonly used in clinical trials, leading to the recommendation that volume transfer constant (K^{trans}) and the fractional volume of extravascular extracellular space per unit volume of tissue (EES or v_e), should be a standard outcome measures in drugs trials, along with IAUGC (Leach et al. 2005) (see Fig. 29.1).

An extension of the Tofts and Kermode model (Tofts 1997) allows calculation of v_p in addition to v_e, and K^{trans},

$$C_t(t) = v_p C_p(t) + K \exp\left(\frac{-Kt}{v_e}\right) \int_0^t C_p(t') \exp\left(\frac{-Kt'}{v_e}\right) dt'$$

where v_p is the plasma volume, but once again, flow and permeability are inextricably combined within

Table 29.2. List of abbreviations and standard terms used in DCE-MRI pharmacokinetic analyses (adapted from Tofts et al. 1999)

Symbol	Short name	Unit	End point	Notes
K^{trans}	Volume transfer constant between EES and plasma	min^{-1}	Primary	
k_{ep}	Rate constant between EES and plasma	min^{-1}	Secondary	
K_i	Uni-directional influx constant	min^{-1}	-	Larsson model measure of PS
C_p	Tracer concentration in arterial blood plasma	mM	-	
C(*t*)	Concentration of contrast agent at time t in each voxel	mM	-	
TV	Tumour volume	ml	-	Should measure
VV	Vascularised volume	ml	-	Should calculate
v_e	Volume of EES per unit volume tissue	None	Secondary	Value $0 \leq v_e \leq 1$
v_p	Blood plasma volume	ml	Secondary	
E	Initial extraction ratio	-	-	
F	Flow	ml gm^{-1} min^{-1}	-	
P	Total capillary wall permeability	cm min^{-1}	-	
PS	Permeability-surface area product per unit mass of tissue	ml min^{-1}	-	
EES	Extracellular extravascular space	None	-	
IAUGC	Initial area under gadolinium concentration - time curve	mM Gd min	Primary	

the model itself. A more complex model described by St. Lawrence and Lee allows direct estimation of local tissue blood flow, EES, v_p and K^{trans} (St. Lawrence and Lee 1998):

$$C_t(t) = F_p \int_0^t C_p(t-t')\,dt' + EF_p \int_\tau^t C_p(t-t') \exp\left(\frac{-EF_p(t'-\tau)}{v_e}\right) dt'$$

and quantifies the extraction fraction (E), flow and mean capillary transit time (τ), so that K^{trans} effectively represents the permeability-surface area product. However, this model is far from easy to apply and is associated with significantly increased noise and measurement variation, despite requiring more accurate and reliable curve fitting and much higher temporal resolution data (Parker and Buckley 2005). Consequently its use in clinical studies has been limited. In none of these pharmacokinetic models does K^{trans} purely equate to capillary endothelial permeability, as often assumed. Instead, the exact meaning of each parameter depends on the pharmacokinetic model employed.

29.2.2.5 Practical Considerations

Despite uncertainties over the precise physiologic meaning of its parameters, DCE-MRI has been used more widely than any other functional imaging technique in the investigation of conventional (Mayr et al. 1996; Lankester et al. 2005; Padhani et al. 2006) and novel anti-angiogenic (Jayson et al. 2002, 2005; Eder et al. 2002; Morgan et al. 2003; Thomas et al. 2003; Medved et al. 2004; Xiong et al. 2004; Conrad et al. 2004; O'Donnell et al. 2005; Thomas et al. 2005; Mross et al. 2005a,b; Liu et al. 2005; O'Dwyer et la. 2005; Drevs et al. 2005; Watson et al. 2006; Wedam et al. 2006; Padhani et al. 2006) and

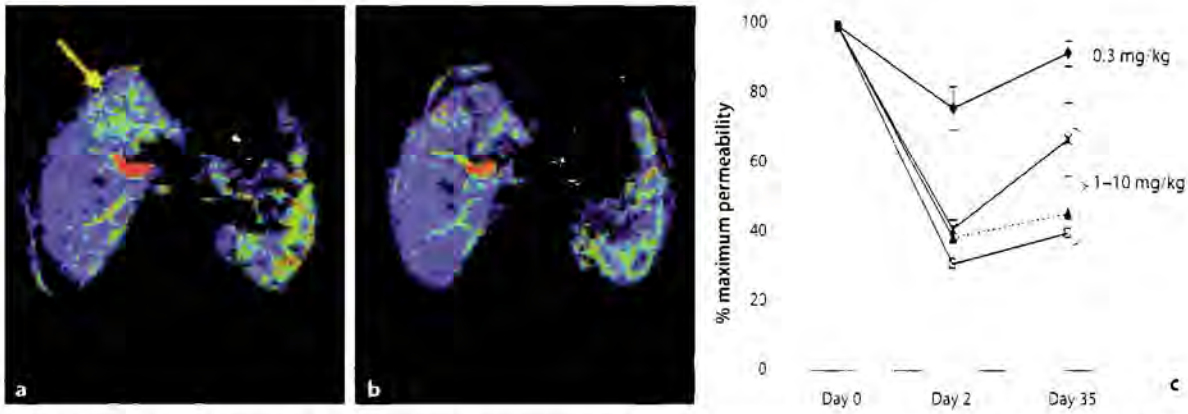


Fig. 29.1a–c. DCE-MRI map of K^{trans} in a hepatic metastasis (*yellow arrow*) in the left lobe of the liver, a before and b 48 h after treatment with the humanized anti-angiogenic vascular endothelial growth factor antibody HuMV833 (1 mg/kg). *Green* and *blue* areas represent high and low vascular permeability, respectively. *Red* and *yellow* pixels represent high (artefact) measurements in the hepatic vein. c The K^{trans} of representative tumours shown for all patients before treatment, 2 days after first treatment, and 35 days after the first treatment was initiated. Data expressed as the mean percentage change relative to the value before treatment for each different treatment dose level, with 95% confidence intervals. Each dose level is represented by a different symbol: *diamonds*, 0.3 mg/kg; *squares*, 1 mg/kg; *triangles*, 3 mg/kg; *crosses*, 10 mg/kg. (Reproduced from Jayson et al. 2002)

vascular disrupting (Dowlati et al. 2002; Galbraith et al. 2002a, 2003; Stevenson et al. 2003; Evelhoch et al. 2004; McKeage et al. 2006) therapies. Consensus guidelines for use of DCE-MRI in trials of angiogenesis inhibitors have been published (Leach et al. 2005), although to date, considerable variation is seen in image acquisition and analysis. Single-site DCE-MRI studies require considerable technical expertise, with specialist image acquisition protocols and image analysis tailored towards each trial. The AIF and region of interest (ROI) for analysis must be defined and data quality checks performed, necessitating collaboration between basic science, oncology, radiology, and technical experts. Multicentre trials not only require all of the above, but also need uniform image acquisition and analysis, favouring reproducible machine-independent protocols and modelling. A recent trial of the tyrosine kinase inhibitor AG-013736 (Pfizer, New York, NY, USA) (Liu et al. 2005) successfully recruited from three centres, demonstrating that multicentre DCE-MRI acquisition is feasible.

Most trials performed to date have used cohorts of 8–35 patients with mixed tumour histology, size and site. The marked variation in tumour size (Evelhoch et al. 2004), anatomy and pathophysiology creates significant difficulties for image analysis. In practice, lesions need to be large enough to avoid partial volume artefacts (typically at least 2–3 cm in minimum diameter) and free from significant motion artefact (thus excluding lung nodules from evaluation). Lesion variation may also mask subtle drug effects and has prompted discussion as to whether stricter selection criteria should be used for patient inclusion (Thomas et al. 2005; O'Dwyer et al. 2005), or whether intra-patient dose escalation designs should be employed so that each subject can act as their own control (Jayson et al. 2002). Some compounds have moved into phase II evaluation in specific clinical situations, for example, to determine optimum dose and schedule for primary or metastatic disease in combination or single-agent therapy (Wedam et al. 2006), before translation into multicentre prospective randomised phase III clinical trials.

In DCE-MRI, the degree of change from baseline values within individual patients is more meaningful than absolute values. Consequently, baseline scans

must be performed routinely (Leach et al. 2005). The likely timing and duration of drug effect should influence image acquisition and potential scheduling. Antivascular effects typically occur within a few hours after drug administration, but are lost within 24 h, thus providing a rationale for imaging at baseline, 4–6 h and 24 h, in contrast to protocols for anti-angiogenic agents, whose effects occur within days to weeks and may persist for weeks to months. A second baseline scan may be performed to establish intra-patient reproducibility and is recommended as standard practice in phase I/II trials of anti-angiogenic agents where imaging is used (Leach et al. 2005; Jayson et al. 2005; Padhani et al. 2006; Galbraith et al. 2003). The particular choice of analysis parameter is important. The lack of physiological specificity of the parameters K^{trans} and IAUGC has some advantage for early-phase trial analysis: since they reflect composites of flow and permeability, they have the potential to detect changes induced by a wide variety of signalling mechanisms. They may be less useful in detecting specific mechanisms of drug action in phase III trials.

ROI definition should be performed on subtraction or non-contrast images. It can be manual or automated and may be drawn from 2D slices or 3D (volume of interest) datasets. Several different strategies for evaluating a ROI have been employed by investigators when evaluating angiogenesis inhibitors. The ROI can encompass part or all of the tumour cross-sectional area or volume, from which a single average enhancement curve is extracted and then evaluated to produce values of parameters of interest (such as IAUGC or K^{trans}), before the same parameters are compared following therapy (Dowlati et al. 2002; Morgan et al. 2003; Thomas et al. 2005; Mross et al. 2005a; Liu et al. 2005). Alternatively, summary statistics such as the mean and standard deviation, or median and interquartile range, may be derived from individual voxels within the ROI (Jayson et al. 2002, 2005; Galbraith et al. 2003; Evelhoch et al. 2004). Both methods are commonplace, relatively simple and allow readers to compare two numbers. Unfortunately, the assumption that such figures offer a meaningful summary of measured values and/or modelled quantities within each voxel is incorrect, since most malignant tumours demonstrate heterogeneous enhancement due to spatial variation in growth factor expression and signalling, and macroscopically in areas of fibrosis, hypoxia and necrosis (Jackson 2003). Simple data analysis that quotes mean or median values of IAUGC or K^{trans} across the entire tumour ROI oversimplifies biological realities such as the rim-core action differential of antivascular agents (Walker-Samuel et al. 2006). More subtle pixel-based analysis with histograms have been applied to animal models (Checkley et al. 2003) and in an increasing number of human studies (Watson et al. 2006; Hayes et al. 2002). Future application of more complex mathematical tools may increase the application of DCE-MRI in the rational assessment of anti-angiogenic and antivascular compounds within clinical trials.

29.2.2.6 Clinical Findings

A full and comprehensive discussion of the clinical application of DCE-MRI to both conventional therapies and to trials of anti-angiogenic and antivascular agents is beyond the scope of this review, but is considered elsewhere (O'Connor et al. 2007). Both T_1 and T_2^* DCE-MRI sequences have been successfully applied in the differentiation of benign and malignant pathology (Kvistad et al. 1999), non-invasive tumour grading (Aronen et al. 1994), staging cancer (Barentsz et al. 1996), guiding stereotactic biopsy (Knopp et al. 1999), monitoring response to treatment (Mayr et al. 1996; Padhani et al. 2006; Hayes et al. 2002) and identifying early tumour relapse (Gilles et al. 1993). In practice, few indications for DCE-MRI have permeated routine practice, although some centres use T_2^* sequences to assist diagnosis and biopsy of some brain tumours.

DCE-MRI biomarkers can provide effective and informative evaluation of novel vascular therapies. Three related studies of the multiple receptor tyrosine kinase inhibitor vatalanib (PTK787/ZK222584; Novartis, Basel, Switzerland) have shown reduction in K_i (a parameter similar to K^{trans}) of ≥40% (considered significant) from baseline to day 2. The reduction was dose-dependent, with greatest response

with doses of 1000 mg a day. In addition, responders were distinguished from non-responders; patients whose K_i fell below 50% had stable or better disease. Thus K_i was considered a useful marker of drug activity capable of suggesting biologically active doses and predicting clinical response (Morgan et al. 2003; Thomas et al. 2005; Mross et al. 2005a). However, two randomised, double blind phase III trials assessed patients with metastatic colorectal carcinoma receiving oral PTK/ZK (1250 mg qds) or placebo along with oxaloplatin/5-fluorouracil/leucovorin (FOLFOX4) as first-line (CONFIRM-1) (Hecht et al. 2005) or second-line (CONFIRM-2) therapy (Koehne et al. 2006). Both failed to demonstrate significant changes in progression-free survival, overall survival or response rates. Thus, it may be that DCE-MRI parameter changes are necessary but not sufficient biomarkers of drug efficacy.

Recently, a significant correlation of reduction in median K^{trans} with response rate ($p<0.004$) and progression-free survival ($p<0.001$) was reported in a trial of the tyrosine kinase inhibitor, sorafenib (BAY43-9006; Bayer, Leverkusen, Germany) (O'Dwyer et al. 2005). Finally, encouraging results have also been shown in a phase II of the monoclonal antibody bevacizumab (Avastin; Genetech, San Francisco, CA, USA) in combination chemotherapy with doxorubicin and docetaxel. Significant reduction of 34% in K^{trans} was demonstrated after cycle 1 (21 days) ($p=0.003$) and maintained by the end of cycle 7 (Wedam et al. 2006) ($p=0.0001$). Thus, the data derived from a significant number of trials with VEGF inhibitors using DCE-MRI have shown that there is an association between dose and reduction in the particular DCE-MRI vascular parameter. Secondly, other results have shown a relationship between the changes in DCE-MRI and clinical benefit, and these were associated with molecular changes (Jayson et al. 2002) that were consistent with the inhibition of VEGF.

DCE-MRI has also been used to evaluate other anti-angiogenic modes of action. For instance, when a specific anti-PDGF antibody was administered to patients, a rapid increase in the vascularised fraction in the tumour was found. As PDGF regulates tumour interstitial pressure, this observation was consistent with the mode of action of the drug: a reduction in interstitial pressure would allow vessels that were closed by the pressure to become patent again (Jayson et al. 2005).

29.2.3 Computed Tomography

X-ray computed tomography is more widely applied to both clinical investigation and research studies of cancer than any other imaging modality. It forms the basis of follow-up assessment of tumour response by imaging criteria, including the World Health Organisation (WHO) and Response Evaluation Criteria in Solid Tumours (RECIST) recommendations (Therasse et al. 2000), where relapse, stability or response are evaluated by simple one- and/or two-dimensional measurements. Dynamic CT sequences can easily be added to conventional anatomical sequences or response evaluation scans, and for this reason, dynamic CT is in one sense an attractive option for imaging angiogenesis. Change in density on CT imaging is linearly related to the concentration of contrast agent. Hence estimates of blood volume are absolute, and parameters that represent perfusion can be extracted without recourse to complex pharmacokinetic modelling algorithms.

29.2.3.1 CT Image Acquisition and Data Analysis

Dynamic contrast-enhanced CT (DCE-CT) images can be acquired on conventional multidetector commercial scanners (Miles and Griffiths 2003). Following non-contrast baseline images, an intravenous injection of iodinated contrast medium is administered, which acts as an effective absorber of energy via the photoelectric effect, thus significantly increasing attenuation of the X-ray beam. A dynamic series is then acquired over a period of around 10 min, from which an attenuation–time course curve is derived. This curve can be converted into a concentration–time curve by a relatively simple process, since the relationship between attenuation and contrast agent concentration is effectively linear. The function of

contrast agent concentration can then be analysed to derive two main types of parameter. Data from the first 45–60 s of the series, when the contrast agent is predominantly intravascular, reflect perfusion. Data collected after this point (usually 2–10 min after injection) reflect contrast agent leakage and can be taken to reflect vessel permeability.

As with DCE-MRI, protocol variations reflect trade-offs between image quality, practicalities, and, in the case of DCE-CT, radiation dose. Increasing the tube current produces more photons (better signal-to-noise ratio), but increases dose. Increasing the number of images acquired per unit time results in better data (more sampling points in the attenuation–time curve) at the expense of increased dose and limited volume coverage, since high temporal resolution limits the time available for table movement, restricting the volume covered to the detector width (approximately 2 cm on current multidetector CT systems). In practice, this limits fast imaging to one anatomical site. Two adjacent 10-mm slices or four adjacent 5-mm slices are commonly acquired. Thinner slices produce unacceptable image noise. Greater volume coverage can be achieved by spiral acquisitions at the expense of reducing image frequency from every 1–2 s to approximately every 15–30 s, and limiting the subsequent options for data analysis. Respiratory motion is a source of artefact and limits data quality, and is counteracted by breath holding for short acquisitions or quiet breathing during longer sequences (Miles 2003).

Perfusion data can be achieved through a variety of relatively simple data processing steps. Semiquantitative measures such as peak enhancement suffer from the same uncertainties of meaning and variation between examinations seen in DCE-MRI, but can be easily adapted to a more 'physiological' parameter, the standardised perfusion value (SPV) from the dose of contrast agent and patient weight (Miles et al. 2001). Analogous to the standardised uptake value used in PET, the SPV is a ratio of tumour perfusion to mean whole body perfusion and has been shown to reduce interobserver measurement error. Variations on this theme exist, and are available in most commercial scanner software packages. Measures of permeability are less straightforward, and in practice are performed infrequently. A full account of these methods is beyond the scope of this review, but they are discussed elsewhere (Miles 1999).

29.2.3.2 Practical Considerations and Clinical Findings

Several studies have shown promising applications for DCE-CT. It is superior at demonstrating pulmonary lesions (Yamashita et al. 1995), is capable of demonstrating occult metastases that are undetectable on conventional qualitative CT assessment (Platt et al. 1997; Blomley et al. 1995; van Beers et al. 2001) and can predict and stratify tumour stage and grade and response to cytotoxic chemotherapy (Dugdale et al. 1999) and radiotherapy (Harvey et al. 2001). However, DCE-CT suffers from two main limitations. Volume coverage is limited compared to DCE-MRI, and the significant dose of ionising radiation makes serial scanning protocols less desirable. Consequently, DCE-CT has only been applied to a limited number of studies investigating angiogenesis inhibitors (Thomas et al. 2003; Xiong et al. 2004; Willett et al. 2004; McNeel et al. 2005). Three of these studies failed to demonstrate any significant clinical findings. However, Willett and colleagues used dynamic CT to provide direct evidence of the anti-angiogenic actions of bevacizumab in rectal cancer, in a study of major significance. Here, MVD interstitial fluid pressure (IFP), circulating biomarkers, FDG-PET and dynamic CT were all used to assess the effect of a single infusion of bevacizumab. Significant reductions in perfusion by 40–44% ($p<0.05$) and blood volume by 16–39% ($p<0.05$) were measured by dynamic CT at 12 days, and were accompanied by significant reductions in MVD and IFP (Willett et al. 2004).

29.2.4 Positron Emission Tomography

PET is a sensitive and quantitative nuclear medicine technique that detects differences in tissue function and metabolism. The main advantage of PET is its functional sensitivity and specificity and its

ability to characterise molecular pathways as well as providing truly quantitative and absolute measurements of physiological processes such as tumour regional blood flow. To this end, it has two major benefits over the MRI and CT methods described above. It is therefore an important tool for the non-invasive assessment of tumour angiogenesis. However, unlike MRI methods, PET imaging produces ionising radiation, and the short-lived nature of isotopes such as ^{15}O introduces further complexity with radio-tracer preparation and administration.

29.2.4.1 PET Image Acquisition

PET imaging requires the administration of a labelled compound consisting of both radio-tracer and biologically active elements. After injection or inhalation of a radio-labelled tracer, the radio-isotope decays and emits a positron (positively charged electron) that travels a distance of around a millimetre within tissue before colliding with a negatively charged electron. The interaction produced results in annihilation of both particles and the production of two 511-keV photons at approximately 180° separation. These gamma-ray photons can be detected within a short time frame, typically around 20 ns, by an array of photoelectric crystals within a dedicated PET scanner detector. Raw data are then processed by computer reconstruction algorithms to map the spatial and temporal distribution of photon production and thus positron emission. The dual emission of PET confers an improvement on the spatial resolution offered by conventional nuclear medicine techniques, typically 3–8 mm, but current anatomical resolution is still considerably poorer than in clinical and functional CT and MRI techniques (Tarantola et al. 2003). Developments in PET-CT fusion may improve anatomical resolution and reduce scan time by removing the need for PET attenuation-correction sequences.

29.2.4.2 Data Acquisition and Extraction of Parameters

In contrast with DCE-MRI, where models of varying complexity can generate fewer or greater numbers of similar parameters, PET techniques tend to each produce a variation in one parameter, and can be combined into a single protocol for each patient visit. As with dynamic MRI and CT techniques, biomarkers of vascular function can represent the net result of several molecular pathways, rather than the activity or inhibition of a particular molecule, such as vascular endothelial growth factor (VEGF) or its receptor, VEGF-R. Several tracers have been used to investigate microvascular function. While the tracer ^{18}F-fluorodeoxyglucose (FDG) is employed for nearly all clinical PET studies in tumour detection, it is less useful in assessing the tumour vasculature. One potential confounding factor is that while an anti-angiogenic agent might reduce metabolism and therefore reduce FDG uptake, the consequent hypoxia can cause up-regulation of glucose transport and therefore an increase in FDG uptake, leading to an ambiguous imaging result.

Most PET studies of tumour perfusion use ^{15}O-labelled water. This requires the direct piping of gas to the scanner since ^{15}O has a half-life of just 123 s. Following intravenous injection of a bolus of ^{15}O-H_2O, PET data are acquired for approximately 10 min along with a peripherally acquired AIF. The change of tissue concentration over time can then be modelled as

$$\frac{dC_t(t)}{dt} = P \cdot C_a(t) - \left(\frac{P}{V_D} + \lambda\right) \cdot C_t(t)$$

where $C_t(t)$ is the tissue concentration of ^{15}O-water at time t, $C_a(t)$ is the arterial concentration of ^{15}O-water at time t, both in units of Bq per ml of tissue, P is perfusion (millilitres of blood per minute per millilitre of tissue), VD is the 'volume of distribution' or proportion of region of interest containing radio-labelled isotope and λ is the radioactive decay constant for ^{15}O. By applying a convolution technique to this model, solutions for perfusion (P) and V_D can be found (Anderson and Price 2002). This technique is independent of equipment and hardware and is considered the most accurate method for measuring regional perfusion (Alpert et al. 1984) (see Fig. 29.2).

Tumour vascular volume can be imaged by inhaling a fixed (safe) dose of ^{15}O-labelled carbon monoxide through a face mask followed by 1–2 min of room

or medical air to allow the labelled carbon monoxide to irreversibly bind to haemoglobin, forming $C^{15}O$-Hb (carboxyhaemoglobin). While labelled water is able to freely diffuse between compartments, $C^{15}O$-Hb remains entirely within the vasculature and is therefore an accurate measure of true vascular volume. PET and arterial $C^{15}O$-Hb data are sampled simultaneously over a period of around 10 min, and the tissue vascular volume can be calculated by

$$V_v = \frac{V_t \cdot \int C_t}{R \cdot \int C_a}$$

where V_v and V_t are the vascular and tissue volumes respectively (measured in ml) and R is the ratio of small-vessel to large-vessel haematocrit (assumed to be 1) (Laking and Price 2003; Wilson et al. 1992).

An alternative strategy is to use PET to image changes within specific molecular pathways, for example the activity of VEGF. Although they are in early stages of development, these strategies present a promising area for imaging angiogenesis. The binding and distribution of the anti-VEGF antibody HuMV833 has been successfully imaged within a phase I trial (Jayson et al. 2002). Animal models suggest that other molecules involved in signalling pathways that mediate angiogenesis, such as $\alpha_v\beta_3$-integrin, can be successfully quantified and imaged with PET isotopes (Chen et al. 2004).

29.2.4.3 Practical Considerations

PET has several advantages over other techniques for imaging angiogenesis as outlined above. However, practical considerations have limited its use in clinical trials of conventional and novel therapeutic agents. To date, seven trials of anti-angiogenic and vascular disrupting agents have incorporated PET into their studies (Jayson et al. 2002; Kurdziel et al. 2000; Herbst et al. 2002; Anderson et al. 2003a, 2003b; Logan et al. 2002; Lara et al. 2003). The technique is expensive, time consuming and requires careful collaboration between radio-pharmacists, nuclear medicine radiographers, basic scientists and clinicians. Few centres possess the necessary cyclotron and delivery systems that are capable of processing such short-lived isotopes, restricting use of ^{15}O-based PET imaging. The

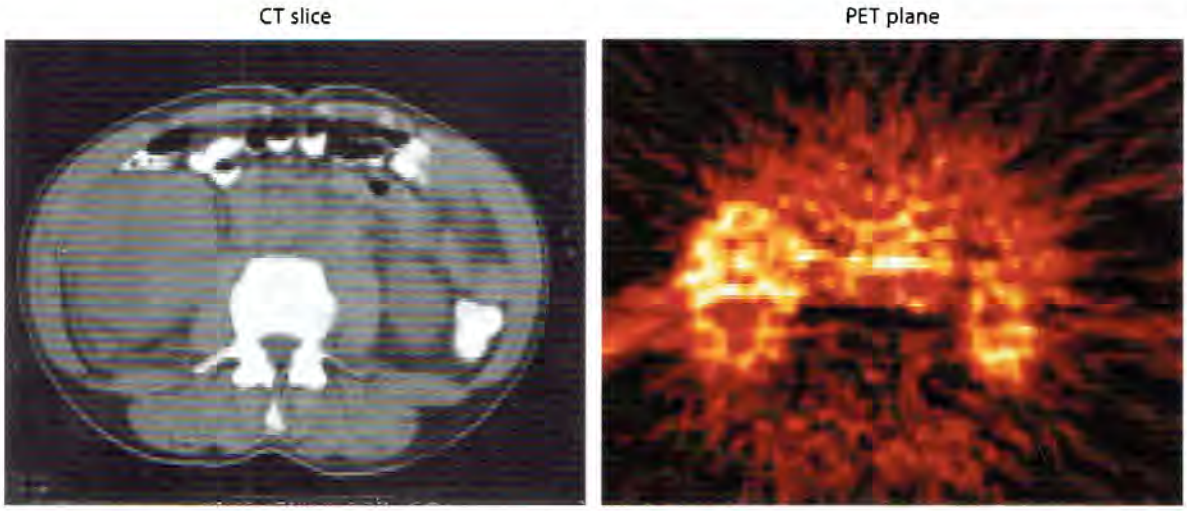


Fig. 29.2. Axial CT scan through the mid-abdomen (*left*) and corresponding image from a summed PET ^{15}O-labelled H_2O study (*right*). Areas of increased brightness on the PET scan represent increased perfusion (the whiter the area, the higher the perfusion). The large right renal mass seen on the CT image represents a primary renal cell carcinoma. This is seen on the PET image as a bright area with a black centre representing a necrotic core. Vascular structures centrally, such as the aorta, are also bright. (Reproduced from Anderson et al. 2003a)

dose from an examination varies subject to local protocols, but is significant and typically in the region of 8–10 mSv.

In contrast to MRI dynamic series, where a variety of parameters may be derived from the same acquisition, different PET tracers produce different parameters. More thorough acquisition protocols may consequently take considerably longer to perform, and patient visits can be demanding. Depending on the local procedure and the particular study protocol, the entire preparation and image acquisition may take anywhere between 1 h and 3 h in total, as demonstrated in the effective but lengthy protocol used by Anderson and colleagues in a phase-I trial of CA-4-P (Anderson et al. 2003b). PET can detect changes as small as a few millimetres, but, as with MRI, practical imaging is restricted to a ROI for lesions of at least 2 cm to avoid partial volume effect.

29.2.4.4 Clinical Findings

The feasibility of PET as a means of measuring angiogenesis inhibition has been demonstrated in a study of CA-4-P. Images acquired 30 min after drug administration showed a 30–50% reduction in flow from baseline values (^{15}O-water; p=0.001) and a 15% reduction in tumour vascular volume (^{15}O-CO; p=0.007). Vascular recovery was seen at 24 h, although residual significant changes in perfusion (p=0.13) remained (Anderson et al. 2003b). A study of thalidomide in prostate metastases has shown some evidence of correlation of blood volume and metabolism (measured by FDG-PET) (Kurdziel et al. 2000). Other studies have shown less encouraging results, but further demonstrate the possibility of incorporating PET regimens into clinical trials.

Future Directions and Conclusions

At present only a small minority of early-phase clinical trials employ any of the methods reviewed as endpoints (Workman et al. 2006). This reflects both lack of appreciation of the benefits of imaging and an awareness of the cost (in both time and money) and complexity of many of the procedures, which frequently limit techniques to specialist research centres. However, functional imaging techniques undoubtedly provide useful information in studies of tumour angiogenesis. Careful attention must be paid to the methodology employed in each study, since the particular choice of data acquisition and analysis can radically alter not only the value, but also the precise meaning of the parameter measured.

Further work is required to validate the use of DCE-MRI, dynamic CT and PET biomarkers as surrogates for clinical endpoints, although some encouraging studies report correlation of imaging biomarkers with some clinical endpoints, histological markers (e. g. MVD) and assays of growth factor expression (Miller et al. 2005). It is also essential that measurements are not only sensitive and specific, but are repeatable, in order to calculate necessary sample sizes for trials. Imaging parameters are prone to biological variation, random error and systemic error, all of which can cause day-to-day variation in measured values.

It is important to appreciate that the parameters described vary in their degree of reproducibility. For example, the amount of change needed for significance for K^{trans} and v_e on T_1-weighted DCE-MRI within a cohort of 16 patients has been reported as −14% to +16% and ±1.9 ml/ml (6%) respectively for pixel-based analysis. Whole-tumour ROI analysis had the same value for v_e but a slightly wider interval of −16% to +19% for K^{trans}. However, trials of anti-angiogenic and vascular disrupting compounds tend to analyse patient data individually rather than in moderately sized cohorts; thus, equivalent figures for single-patient reproducibility are more relevant, reported here as −45 to +83% for K^{trans} and ±7.6 ml/ml (24%) for ve (Galbraith et al. 2002b). Less variation has been reported in brain lesions, using T_2^* sequences, which are subject to different physical and physiological variation (Jackson et al. 2003).

The current challenge is to accurately select appropriate imaging techniques that are capable of

addressing specific questions in both early- and later-phase clinical trials. This requires accurate, robust and repeatable protocols (involving one or more modalities) that enable us to identify positive drug effects and provide confidence that a negative result (not demonstrating a positive drug effect) is truly negative. Further validation with other surrogate and clinical outcome measures is required. Once this is achieved, PET, dynamic MRI, dynamic CT and other functional imaging modalities may become more widely used to investigate angiogenesis in oncology.

Acknowledgements

P. B. is funded by a Cancer Research UK Clinical Research Training Fellowship Grant, ref C19221/A6086. Cancer Research UK is a UK registered charity, no. 1089464.

References

Alpert NM, Eriksson L, Chang JY et al (1984) Strategy for the measurement of regional cerebral blood flow using short-lived tracers and emission tomography. J Cereb Blood Flow Metab 4:28–34

American Cancer Society (2005) Cancer facts and figures. American Cancer Society, Atlanta

Anderson H, Price P (2002) Clinical measurement of blood flow in tumours using positron emission tomography: a review. Nucl Med Commun 23:131–138

Anderson H, Yap JT, Wells P et al (2003a) Measurement of renal tumour and normal tissue perfusion using positron emission tomography in a phase II clinical trial of razoxane. Br J Cancer 89:262–267

Anderson H, Yap JT, Miller MP, Robbins A, Jones T, Price PM (2003b) Assessment of pharmacodynamic vascular response in a phase I trial of combretastatin A4 phosphate. J Clin Oncol 21:2823–2830

Aronen HJ, Gazit IE, Louis DN et al (1994) Cerebral blood volume maps of gliomas: comparison with tumor grade and histologic findings. Radiology 191:41–51

Barentsz JO, Jager GJ, van Vierzen PB et al (1996) Staging urinary bladder cancer after transurethral biopsy: value of fast dynamic contrast-enhanced MR imaging. Radiology 201:185–193

Blomley MJ, Coulden R, Dawson P et al (1995) Liver perfusion studied with ultrafast CT. J Comput Assist Tomogr 19:424–433

Brix G, Semmler W, Port R, Schad LR, Layer G, Lorenz WJ (1991) Pharmacokinetic parameters in CNS Gd-DTPA enhanced MR imaging. J Comput Assist Tomogr 15:621–628

Buckley DL (2002) Uncertainty in the analysis of tracer kinetics using dynamic contrast-enhanced T1-weighted MRI. Magn Reson Med 47:601–606

Buckley DL, Parker GJM (2005) Measuring contrast agent concentration in T1-weighted dynamic contrast-enhanced MRI. In: Jackson A, Buckley DL, Parker GJM (eds) Dynamic contrast-enhanced magnetic resonance imaging in oncology. Springer, Berlin Heidelberg New York, pp 69–79

Carmeliet P, Jain RK (2000) Angiogenesis in cancer and other diseases. Nature 407:249–257

Checkley D, Tessier JJ, Kendrew J, Waterton JC, Wedge SR (2003) Use of dynamic contrast-enhanced MRI to evaluate acute treatment with ZD6474, a VEGF signalling inhibitor, in PC-3 prostate tumours. Br J Cancer 89:1889–1895

Chen X, Liu S, Hou Y et al (2004) MicroPET imaging of breast cancer alphav-integrin expression with 64Cu-labeled dimeric RGD peptides. Mol Imaging Biol 6:350–359

Collins JM (2003) Functional imaging in phase I studies: decorations or decision making? J Clin Oncol 21:2807–2809

Conrad C, Friedman H, Reardon D et al (2004) A phase I/II trial of single-agent PTK 787/ZK 222584 (PTK/ZK), a novel, oral angiogenesis inhibitor, in patients with recurrent glioblastoma multiforme (GBM). J Clin Oncol (Meetings Abstracts) 22 (14S):1512

Daniel BL, Yen YF, Glover GH et al (1998) Breast disease: dynamic spiral MR imaging. Radiology 209:499–509

Department of Health (2004) The NHS cancer plan and the new NHS: providing a patient-centred service. Department of Health, London

Dowlati A, Robertson K, Cooney M et al (2002) A phase I pharmacokinetic and translational study of the novel vascular targeting agent combretastatin A-4 phosphate on a single-dose intravenous schedule in patients with advanced cancer. Cancer Res 62:3408–3416

Drevs J, Medinger M, Mross K et al (2005) Phase I clinical evaluation of AZD2171, a highly potent VEGF receptor tyrosine kinase inhibitor, in patients with advanced tumors J Clin Oncol (Meetings Abstracts) 23:3002

Dugdale PE, Miles KA, Bunce I, Kelley BB, Leggett DA (1999) CT measurement of perfusion and permeability within lymphoma masses and its ability to assess grade, activity, and chemotherapeutic response. J Comput Assist Tomogr 23:540–547

Eder JP Jr, Supko JG, Clark JW et al (2002) Phase I clinical trial of recombinant human endostatin administered as a short intravenous infusion repeated daily. J Clin Oncol 20:3772–3784

Evelhoch JL (1999) Key factors in the acquisition of contrast kinetic data for oncology. J Magn Reson Imaging 10:254–259

Evelhoch JL, LoRusso PM, He Z et al (2004) Magnetic res-

onance imaging measurements of the response of murine and human tumors to the vascular-targeting agent ZD6126. Clin Cancer Res 10:3650–3657

Fidler IJ (2001) Angiogenic heterogeneity: regulation of neoplastic angiogenesis by the organ microenvironment. J Natl Cancer Inst 93:1040–1041

Folkman J (1971) Tumor angiogenesis: therapeutic implications. N Engl J Med 285:1182–1186

Fritz-Hansen T, Rostrup E, Larsson HB, Sondergaard L, Ring P, Henriksen O (1996) Measurement of the arterial concentration of Gd-DTPA using MRI: a step toward quantitative perfusion imaging. Magn Reson Med 36:225–231

Galbraith SM, Rustin GJ, Lodge MA et al (2002a) Effects of 5,6-dimethylxanthenone-4-acetic acid on human tumor microcirculation assessed by dynamic contrast-enhanced magnetic resonance imaging. J Clin Oncol 20:3826–3840

Galbraith SM, Lodge MA, Taylor NJ et al (2002b) Reproducibility of dynamic contrast-enhanced MRI in human muscle and tumours: comparison of quantitative and semi-quantitative analysis. NMR Biomed 15:132–142

Galbraith SM, Maxwell RJ, Lodge MA et al (2003) Combretastatin A4 phosphate has tumor antivascular activity in rat and man as demonstrated by dynamic magnetic resonance imaging. J Clin Oncol 21:2831–2842

Gilles R, Guinebretiere JM, Shapeero LG et al (1993) Assessment of breast cancer recurrence with contrast-enhanced subtraction MR imaging: preliminary results in 26 patients. Radiology 188:473–478

Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100:57–70

Harvey CJ, Blomley MJ, Dawson P et al (2001) Functional CT imaging of the acute hyperemic response to radiation therapy of the prostate gland: early experience. J Comput Assist Tomogr 25:43–49

Hayes C, Padhani AR, Leach MO (2002) Assessing changes in tumour vascular function using dynamic contrast-enhanced magnetic resonance imaging. NMR Biomed 15:154–163

Hecht J, Trarbach T, Jaeger E et al (2005) A randomized, double-blind, placebo-controlled, phase III study in patients (Pts) with metastatic adenocarcinoma of the colon or rectum receiving first-line chemotherapy with oxaliplatin/5-fluorouracil/leucovorin and PTK787/ZK 222584 or placebo (CONFIRM-1). J Clin Oncol (Meetings Abstracts) 23:3

Henderson E, Rutt BK, Lee TY (1998) Temporal sampling requirements for the tracer kinetics modeling of breast disease. Magn Reson Imaging 16:1057–1073

Herbst RS, Mullani NA, Davis DW et al (2002) Development of biologic markers of response and assessment of antiangiogenic activity in a clinical trial of human recombinant endostatin. J Clin Oncol 20:3804–3814

Hurwitz H, Fehrenbacher L, Novotny W et al (2004) Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 350:2335–2342

Jackson A (2003) Imaging microvascular structure with contrast enhanced MRI. Br J Radiol 76:S159–173

Jackson A (2004) Analysis of dynamic contrast enhanced MRI. Br J Radiol 77:S154–S166

Jackson A, Jayson GC, Li KL et al (2003) Reproducibility of quantitative dynamic contrast-enhanced MRI in newly presenting glioma. Br J Radiol 76:153–162

Jayson GC, Parker GJ, Mullamitha S et al (2005) Blockade of platelet-derived growth factor receptor-beta by CDP860, a humanized, PEGylated di-Fab', leads to fluid accumulation and is associated with increased tumor vascularized volume. J Clin Oncol 23:973–981

Jayson GC, Waterton JC (2005) Applications of dynamic contrast-enhanced MRI in oncology drug development. In: Jackson A, Buckley D, Parker G (eds) Dynamic contrast-enhanced magnetic resonance imaging in oncology. Springer, Berlin Heidelberg New York, pp 281–297

Jayson GC, Zweit J, Jackson A et al (2002) Molecular imaging and biological evaluation of HuMV833 anti-VEGF antibody: implications for trial design of antiangiogenic antibodies. J Natl Cancer Inst 94:1484–1493

Johnson DH, Fehrenbacher L, Novotny WF et al (2004) Randomized phase II trial comparing bevacizumab plus carboplatin and paclitaxel with carboplatin and paclitaxel alone in previously untreated locally advanced or metastatic non-small-cell lung cancer. J Clin Oncol 22:2184–2191

Kelloff GJ, Krohn KA, Larson SM et al (2005) The progress and promise of molecular imaging probes in oncologic drug development. Clin Cancer Res 11:7967–7985

Knopp EA, Cha S, Johnson G et al (1999) Glial neoplasms: dynamic contrast-enhanced T2*-weighted MR imaging. Radiology 211:791–798

Koehne C, Bajetta E, Lin E et al (2006) Results of an interim analysis of a multinational randomized, double-blind, phase III study in patients (pts) with previously treated metastatic colorectal cancer (mCRC) receiving FOLFOX4 and PTK787/ZK 222584 (PTK/ZK) or placebo (CONFIRM 2). J Clin Oncol (Meetings Abstracts) 24:3508

Kurdziel K, Bacharach S, Carrasquillo J et al (2000) Using PET 18F-FDG, 11CO, and 15O-water for monitoring prostate cancer during a phase II anti-angiogenic drug trial with thalidomide. Clin Positron Imaging 3:144

Kvistad KA, Lundgren S, Fjosne HE, Smenes E, Smethurst HB, Haraldseth O (1999) Differentiating benign and malignant breast lesions with T2*-weighted first pass perfusion imaging. Acta Radiol 40:45–51

Laking GR, Price PM (2003) Positron emission tomographic imaging of angiogenesis and vascular function. Br J Radiol 76:S50–S59

Lankester KJ, Taylor NJ, Stirling JJ et al (2005) Effects of platinum/taxane based chemotherapy on acute perfusion in human pelvic tumours measured by dynamic MRI. Br J Cancer 93:979–985

Lara PN Jr, Quinn DI, Margolin K et al (2003) SU5416 plus interferon alpha in advanced renal cell carcinoma: a phase

II California Cancer Consortium Study with biological and imaging correlates of angiogenesis inhibition. Clin Cancer Res 9:4772–4781

Larsson HB, Stubgaard M, Frederiksen JL, Jensen M, Henriksen O, Paulson OB (1990) Quantitation of blood-brain barrier defect by magnetic resonance imaging and gadolinium-DTPA in patients with multiple sclerosis and brain tumors. Magn Reson Med 16:117–131

Leach MO, Brindle KM, Evelhoch JL et al (2005) The assessment of antiangiogenic and antivascular therapies in early-stage clinical trials using magnetic resonance imaging: issues and recommendations. Br J Cancer 92:1599–1610

Li KL, Zhu XP, Waterton J, Jackson A (2000) Improved 3D quantitative mapping of blood volume and endothelial permeability in brain tumors. J Magn Reson Imaging 12:347–357

Liu G, Rugo HS, Wilding G et al (2005) Dynamic contrast-enhanced magnetic resonance imaging as a pharmacodynamic measure of response after acute dosing of AG-013736, an oral angiogenesis inhibitor, in patients with advanced solid tumors: results from a phase I study. J Clin Oncol 23:5464–5473

Logan TF, Jadali F, Egorin MJ et al (2002) Decreased tumor blood flow as measured by positron emission tomography in cancer patients treated with interleukin-1 and carboplatin on a phase I trial. Cancer Chemother Pharmacol 50:433–444

Mayr NA, Yuh WT, Magnotta VA et al (1996) Tumor perfusion studies using fast magnetic resonance imaging technique in advanced cervical cancer: a new noninvasive predictive assay. Int J Radiat Oncol Biol Phys 36:623–633

McKeage MJ, Fong P, Jeffery M et al (2006) 5,6-Dimethylxanthenone-4-acetic acid in the treatment of refractory tumors: a phase I safety study of a vascular disrupting agent. Clin Cancer Res 12:1776–1784

McNeel DG, Eickhoff J, Lee FT et al (2005) Phase I trial of a monoclonal antibody specific for alphavbeta3 integrin (MEDI-522) in patients with advanced malignancies, including an assessment of effect on tumor perfusion. Clin Cancer Res 11:7851–7860

Medved M, Karczmar G, Yang C et al (2004) Semiquantitative analysis of dynamic contrast enhanced MRI in cancer patients: variability and changes in tumor tissue over time. J Magn Reson Imaging 20:122–128

Miles KA (1999) Tumour angiogenesis and its relation to contrast enhancement on computed tomography: a review. Eur J Radiol 30:198–205

Miles KA (2003) Perfusion CT for the assessment of tumour vascularity: which protocol? Br J Radiol 76:S36–S42

Miles KA, Griffiths MR (2003) Perfusion CT: a worthwhile enhancement? Br J Radiol 76:220–231

Miles KA, Griffiths MR, Fuentes MA (2001) Standardized perfusion value: universal CT contrast enhancement scale that correlates with FDG PET in lung nodules. Radiology 220:548–553

Miller JC, Pien HH, Sahani D, Sorensen AG, Thrall JH (2005) Imaging angiogenesis: applications and potential for drug development. J Natl Cancer Inst 97:172–187

Miller KD, Chap LI, Holmes FA et al (2005) Randomized phase III trial of capecitabine compared with bevacizumab plus capecitabine in patients with previously treated metastatic breast cancer. J Clin Oncol 23:792–799

Morgan B, Thomas AL, Drevs J et al (2003) Dynamic contrast-enhanced magnetic resonance imaging as a biomarker for the pharmacological response of PTK787/ZK 222584, an inhibitor of the vascular endothelial growth factor receptor tyrosine kinases, in patients with advanced colorectal cancer and liver metastases: results from two phase I studies. J Clin Oncol 21:3955–3964

Mross K, Drevs J, Muller M et al (2005a) Phase I clinical and pharmacokinetic study of PTK/ZK, a multiple VEGF receptor inhibitor, in patients with liver metastases from solid tumours. Eur J Cancer 41:1291–1299

Mross K, Gmehling D, Frost A et al (2005b) A clinical Phase I, pharmacokinetic (PK), and pharmacodynamic study of twice daily BIBF 1120 in advanced cancer patients J Clin Oncol (Meetings Abstracts) 23:3031

O'Connor JP, Jackson A, Parker GJ, Jayson GC (2007) DCE-MRI biomarkers in the clinical evaluation of antiangiogenic and vascular disrupting agents. Br J Cancer 96:189–195

O'Donnell A, Padhani A, Hayes C et al (2005) A Phase I study of the angiogenesis inhibitor SU5416 (semaxanib) in solid tumours, incorporating dynamic contrast MR pharmacodynamic end points. Br J Cancer 93:876–883

O'Dwyer PJ, Rosen M, Gallagher M, Schwartz B, Flaherty KT (2005) Pharmacodynamic study of BAY 43–9006 in patients with metastatic renal cell carcinoma. J Clin Oncol (Meetings Abstracts) 23:3005

Office of National Statistics (2005) Cancer statistics registrations. HMSO, London

Padhani AR, Hayes C, Assersohn L et al (2006) Prediction of clinicopathologic response of breast cancer to primary chemotherapy at contrast-enhanced MR imaging: initial clinical results. Radiology 239:361–374

Padhani AR, Taylor NJ, d'Arcy JA et al (2006) Dynamic MRI evaluation of the triple receptor tyrosine kinase inhibitor BIBF 1120 in patients with advanced solid tumours. Proc Int Soc Mag Res Med 14:765

Parker GJM, Buckley DL (2005) Tracer kinetic modelling for T1-weighted DCE-MRI. In: Jackson A, Buckley DL, Parker GJM (eds) Dynamic contrast-enhanced magnetic resonance imaging in oncology. Springer, Berlin Heidelberg New York, pp 81–92

Parkin DM, Bray F, Ferlay J, Pisani P (2005) Global cancer statistics, 2002. CA Cancer J Clin 55:74–108

Platt JF, Francis IR, Ellis JH, Reige KA (1997) Liver metastases: early detection based on abnormal contrast material enhancement at dual-phase helical CT. Radiology 205:49–53

St. Lawrence KS, Lee TY (1998) An adiabatic approximation to the tissue homogeneity model for water exchange in the brain. I. Theoretical derivation. J Cereb Blood Flow Metab 18:1365–1377

Stevenson JP, Rosen M, Sun W et al (2003) Phase I trial of the antivascular agent combretastatin A4 phosphate on a 5-day schedule to patients with cancer: magnetic resonance imaging evidence for altered tumor blood flow. J Clin Oncol 21:4428–4438

Tarantola G, Zito F, Gerundini P (2003) PET instrumentation and reconstruction algorithms in whole-body applications. J Nucl Med 44:756–769

Therasse P, Arbuck SG, Eisenhauer EA et al (2000) New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 92:205–216

Thomas AL, Morgan B, Horsfield MA et al (2005) Phase I study of the safety, tolerability, pharmacokinetics, and pharmacodynamics of PTK787/ZK 222584 administered twice daily in patients with advanced cancer. J Clin Oncol 23:4162–4171

Thomas JP, Arzoomanian RZ, Alberti D et al (2003) Phase I pharmacokinetic and pharmacodynamic study of recombinant human endostatin in patients with advanced solid tumors. J Clin Oncol 21:223–231

Tofts PS (1997) Modeling tracer kinetics in dynamic Gd-DTPA MR imaging. J Magn Reson Imaging 7:91–101

Tofts PS, Kermode AG (1991) Measurement of the blood-brain barrier permeability and leakage space using dynamic MR imaging. 1. Fundamental concepts. Magn Reson Med 17:357–367

Tofts PS, Brix G, Buckley DL et al (1999) Estimating kinetic parameters from dynamic contrast-enhanced T(1)-weighted MRI of a diffusable tracer: standardized quantities and symbols. J Magn Reson Imaging 10:223–232

Van Beers BE, Leconte I, Materne R, Smith AM, Jamart J, Horsmans Y (2001) Hepatic perfusion parameters in chronic liver disease: dynamic CT measurements correlated with disease severity. AJR Am J Roentgenol 176:667–673

Walker-Samuel S, Taylor NJ, Padhani AR, Leach MO, Collins DJ (2006) The effect of heterogeneous tumour enhancement on the assessment of response to treatment. Proc Int Soc Mag Res Med 14:761

Watson Y, Cheung S, Roberts C et al (2006) Prognostic power of DCE-MRI heterogeneity analysis in patients with advanced solid tumors. Proc Int Soc Mag Res Med 14:755

Wedam SB, Low JA, Yang SX et al (2006) Antiangiogenic and antitumor effects of bevacizumab in patients with inflammatory and locally advanced breast cancer. J Clin Oncol 24:769–777

Weinmann HJ, Laniado M, Mutzel W (1984) Pharmacokinetics of GdDTPA/dimeglumine after intravenous injection into healthy volunteers. Physiol Chem Phys Med NMR 16:167–172

Willett CG, Boucher Y, di Tomaso E et al (2004) Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. Nat Med 10:145–147

Wilson CB, Lammertsma AA, McKenzie CG, Sikora K, Jones T (1992) Measurements of blood flow and exchanging water space in breast tumors using positron emission tomography: a rapid and noninvasive dynamic method. Cancer Res 52:1592–1597

Workman P, Aboagye EO, Chung YL et al (2006) Minimally invasive pharmacokinetic and pharmacodynamic technologies in hypothesis-testing clinical trials of innovative therapies. J Natl Cancer Inst 98:580–598

Xiong HQ, Herbst R, Faria SC et al (2004) A phase I surrogate endpoint study of SU6668 in patients with solid tumors. Invest New Drugs 22:459–466

Yamashita K, Matsunobe S, Tsuda T et al (1995) Solitary pulmonary nodule: preliminary study of evaluation with incremental dynamic CT. Radiology 194:399–405

Yang JC (2004) Bevacizumab for patients with metastatic renal cancer: an update. Clin Cancer Res 10:6367S–6370S

Imaging of Tumor Angiogenesis and Antiangiogenesis

30

FABIAN KIESSLING and MARTIN KRIX

Abstract

Due to the exquisite importance of angiogenesis for tumor development and cure, imaging methods that elucidate morphological, functional and molecular characteristics of tumor angiogenesis are evident. This chapter gives an overview on current applications of MRI, CT, ultrasound and nuclear medicine techniques to image neovasculature. This includes functional methods to assess surrogate markers such as relative blood volume, perfusion and vessel permeability. While these methods principally have shown potential to predict early therapy response of antiangiogenic tumor therapies, some possible pitfalls in data interpretation also have to be addressed. Also innovative strategies to depict tumor vessel anatomy using MRI and CT are discussed. In this context, in vivo MRI and experimental CT techniques have the potential to resolve tumor vessels less than 100–200 μm in diameter. Ultrasmall vessels of less than 50 μm, however, have been visualized almost exclusively on images of excised tissue specimens, which were recorded by micro-CT taking into account high X-ray doses and long scan times. In the last part of the chapter the use of targeted imaging compounds to non-invasively assess the expression of molecular angiogenesis markers by endothelial cells is introduced.

CONTENTS

Impact of Non-invasive Imaging

Antiangiogenic tumor therapy has recently entered clinical evaluation, and some of these treatment strategies have already proven to efficiently inhibit tumor progression. However, to increase efficacy of treatment individualized therapy regimens are required which provide a close and efficient observation of the patient with non-invasive imaging methods. This gives the observer the opportunity to assess therapy response early or react fast if another therapy is required.

In clinical routine, response to anti-tumor therapy is usually assessed purely by measuring the size of the tumor. However, often there is a delay between start of therapy and decrease in tumor size, and in some cases tumors even increase in size after initiation of therapy before they start shrinking.

Therefore, more specific imaging strategies are required which provide insight into the vitality of the tumor. Beside data on tumor metabolism, apoptosis and the expression of molecular markers, functional information about tumor vascularization is desirable, the latter being particularly important for monitoring antiangiogenic therapies.

Unfortunately, the functional consequences of tumor vascularization and its impact for tumor characterization and the assessment of therapy response are still poorly understood. Therefore, it is mandatory to combine basic research on the cellular mechanisms of angiogenesis with research on new non-invasive imaging strategies at the preclinical level. At this level molecular biology elucidates proteomics and genomics in the regulation of angiogenesis and can thereby provide new targets for specific diagnostic and therapeutic agents. Non-invasive imaging allows the study of functional changes in tumor vascularization after modulation of molecular regulators of angiogenesis and, consequently, the preclinical evaluation of new therapeutics in animal experiments. During this process, imaging concepts for consecutive clinical trials can be developed which are individualized for the specific drug and which improve the translation of basic to preclinical research.

In this chapter, important modalities and methods for non-invasive imaging of angiogenesis are introduced. Important findings regarding the functional effects of antiangiogenic therapy on tumor vascularization are reported and discussed in the context of early clinical trials.

In addition, an overview is given on the current status of non-invasive "molecular imaging" methods, including specific contrast agents and the non-invasive tracking of cells that contribute to the angiogenesis process.

Magnetic Resonance Imaging

30.2.1 Dynamic Contrast-Enhanced Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) is performed by measuring the relaxation of nuclei (usually protons) in the tissue after excitation by an external electromagnetic pulse. The term relaxation denotes the process of dissipation of previously stored energy. One distinguishes two different relaxation processes: the longitudinal T_1 relaxation and the transverse T_2 relaxation, which are tissue-dependent processes. Since the T_2 relaxation is usually faster than the T_1 relaxation, the two processes can be investigated separately. This gives the observer the opportunity to quantify T_1 and T_2 relaxation times and to produce images that are more influenced by T_1 (T_1-weighted image) or by T_2 (T_2-weighted image). Depending on this weighting, the tissue contrast is different.

MRI contrast agents considerably influence the relaxation times of the surrounding tissues, creating an additionally positive or negative contrast effect. Clinically, low-molecular-weight contrast agents are used to delineate tumors and other pathologies. The enhancement in the lesion is related to high extravasation of the contrast material due to increased vessel permeability, the increased size of the inter-

stitial space, delayed redistribution into vessels, and the abnormal perfusion.

In the clinical routine, usually one to three contrast-enhanced scans of the same region of the body are obtained. In most cases this is sufficient to detect a pathologic lesion and to get a rough idea of the underlying disease. For a more detailed characterization of a pathologic vascularization by means of functional data such as tissue blood volume, perfusion and vessel permeability, however, a high number of contrast-enhanced scans are required at the same position at short intervals. This measurement procedure is termed dynamic contrast-enhanced (DCE) imaging.

DCE MRI is usually performed using T_1-weighted or T_2^*-weighted imaging. While the injection of contrast agent induces a signal enhancement in T_1-weighted scans, a darkening occurs in T_2^*-weighted DCE MRI (Padhani 2003; Akella et al. 2004). A major difference between these techniques is that T_2^*-weighted imaging is usually performed with high temporal resolution in order to catch the first pass of contrast medium in the vessels, providing data on tissue blood volume, perfusion and blood flow. In contrast, T_1-weighted DCE MRI scans are usually performed with lower temporal resolution and the measured signal intensity change derives mostly from contrast medium which is extravasated into the interstitial space. Vascularization descriptors can be extracted from the behavior of the signal enhancement over time on the T_1-weighted DCE MRI scans, using mathematical interpolations. Alternatively, pharmacokinetic models can be applied that provide physiological data about tissue blood volume, perfusion and vessel permeability.

Frequently used pharmacokinetic models are the two-compartment models developed by Brix (Brix et al. 1991) and Tofts (Tofts and Kermode 1991). In both models compartment 1 is considered to be the intravascular and compartment 2 the interstitial space. After injection of contrast medium there is an exchange of the contrast agent between the two compartments, described by the parameters k_{12} (k_{pe}) and k_{21} (k_{ep}) (Fig. 30.1). The exchange rate constant k_{21} is considered mainly to reflect vessel permeability. However, it has to be considered that in the case of low tissue perfusion and high extravasation this parameter will also become highly influenced by perfusion. Another relevant parameter is the amplitude, which describes the maximum increase in signal intensity in the tissue after administration of contrast medium. This parameter is a relative measure of the tissue blood volume. However, for its interpretation, one has to consider that most of the recorded signal derives from contrast material accumulated in the interstitial space.

It is obvious that a model distinguishing only two tumor compartments does not completely consider the tumor biology in all its complexity. Therefore, multi-compartment models have been developed (Port et al. 1999) that display the in vivo situation to a greater extent. However, in particular for MRI noninvasive imaging is always a compromise between temporal and spatial resolution in order to achieve a sufficiently high signal-to-noise ratio (SNR). The SNR is proportional to the field strength of the MR scanner, to the chosen voxel size and to the scan time. Complex pharmacokinetic models usually require an arterial input function of the target tissue, and high temporal resolution is prerequisite for accurate measurement of peak contrast agent inflow. Thus, complex models often cannot reliably be applied in small animals, where high circulation times come together with the demand for high-resolution imaging. In addition, particularly in tumors a singular arterial input function does often not exist.

30.2.2 Functional Vascularization Parameters Derived from DCE MRI

Tissue *blood volume* proved to be a sensitive indicator of therapy response (Kiessling et al. 2004a; Akella et al. 2004). In experimental tumors in nude mice, which were treated with an antiangiogenic inhibitory vascular endothelial growth factor (VEGF)-receptor blocking antibody, a reduction in tumor blood volume was observed even before tumor size reduction (Fig. 30.2) (Kiessling et al. 2004a).

Also in patients with brain tumors examined using T_2^*-weighted DCE MRI the response to

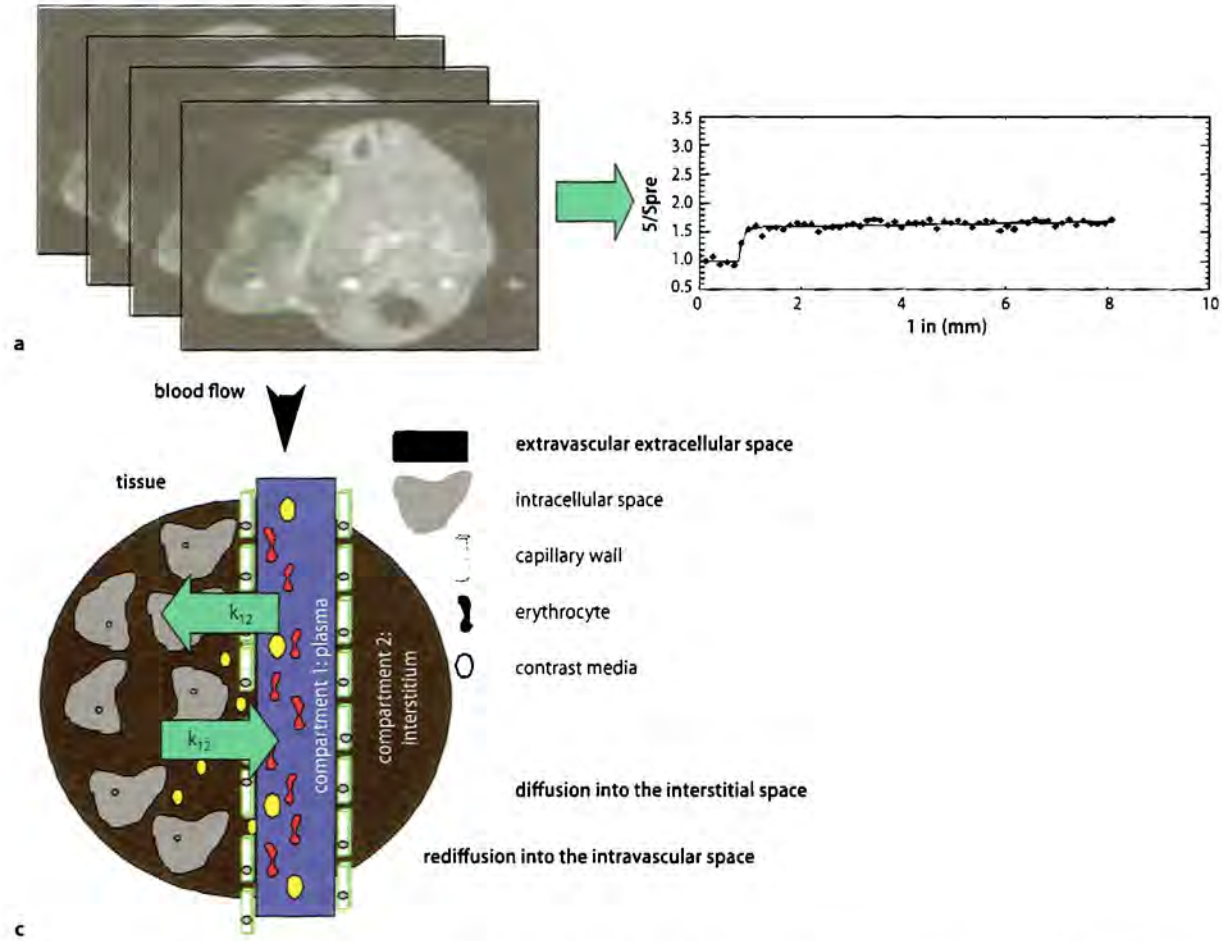


Fig. 30.1a–c. Principle of DCE MRI and postprocessing using a pharmacokinetic two-compartment model. a Repeated scans are performed at identical position after injection of contrast medium. b Regions of interest (ROI) are defined on the images and signal intensity–time curves are calculated. c Postprocessing of these signal intensity–time curves can be done using different pharmacokinetic models. Exemplarily the principle of the two-compartment model of Brix is shown, which considers the inflow of contrast medium into the target tissue and the bidirectional exchange (k12 and k21) between an intravascular and an extravascular extracellular compartment

antiangiogenic drugs was reflected in a reduction of tumor blood volume and tumor blood flow (Akella et al. 2004). However, the use of tissue blood volume as an indicator of therapy response can become problematic when tumors start to decrease in size. It may hapen that vessel density decreases faster than the tumor shrinks. In this case a decrease in tissue blood volume will be observed, and as a consequence, functional perfusion imaging could be a valuable monitoring tool. However, it may also happen that the decreases in vessel density and tumor volume are balanced, which would lead to no change in tumor blood volume. In the worst case the tumor volume decreases faster than the vessel volume, causing a relative increase in tumor blood volume. This would be the case if large mature vessels

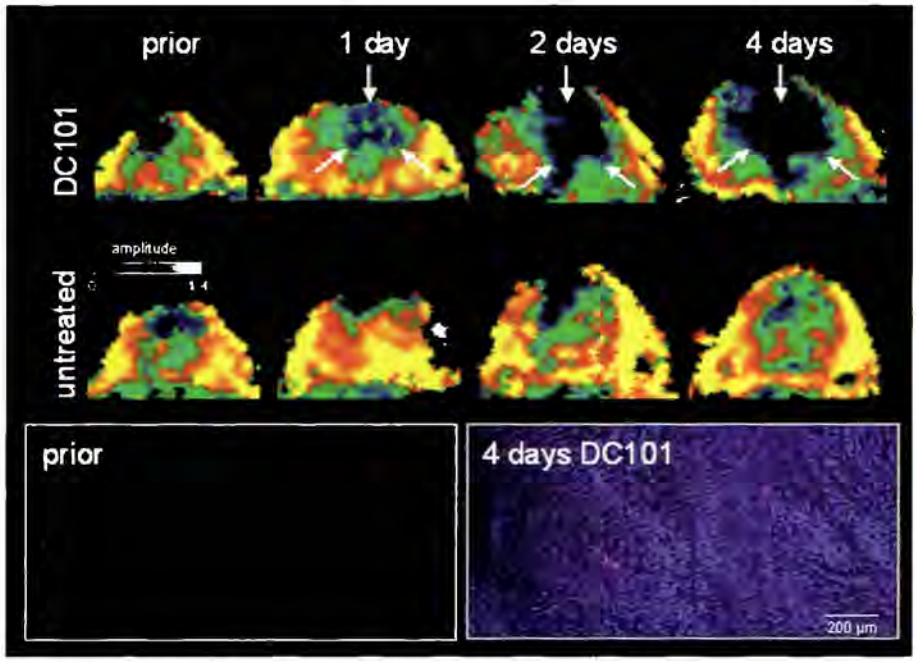


Fig. 30.2. Color-coded parameter maps of MR amplitude (reflecting relative blood volume) of two subcutaneous squamous cell carcinoma xenografts in nude mice during follow-up. The tumor in the top row was treated with the VEGF-R2-blocking antibody DC101, while the tumor in the middle row was untreated. The MR parameter maps indicate a rapid decrease in central tumor blood volume in the treated tumor, which occurs prior to size reduction. Central regressive decrease in blood volume is also observed in the control tumor, but this is much less pronounced. Immunofluorescence images of vessels (bottom row; CD31, red) confirm the central decrease in vessel density during therapy (Kiessling et al. 2004a)

of the tumor periphery, which carry high amounts of blood, are less affected by the antiangiogenic therapy and draw closer due to shrinkage of the tumors. Without knowing these mechanisms, an increase in tumor blood volume could be misinterpreted as relapse.

In summary, tumor blood volume can be considered as an appropriate measure of early antiangiogenic therapy response (as long as tumors do not markedly decrease in size). This means for clinical purposes that optimal timing between therapy initiation and non-invasive imaging may be mandatory, necessitating accurate preclinical and clinical evaluation of therapy effects for each tumor type.

A second important parameter of vessel function is the *permeability*. Due to the high leakiness of immature vessels, permeability can be considered as an indicator of the degree of vessel maturity. In particular, tumor vessels regularly show large fenestrations which make them permeable for plasma proteins and other macromolecules. Furthermore, vessel permeability is highly influenced by angiogenic factors such as VEGF, and it has been shown that vessel permeability can be used as a measure of tumor malignancy (Yang et al. 2003; Hawighorst et al. 1999). In patients with colorectal cancer and liver metastases, treated with an inhibitor of VEGF receptor tyrosine kinases, tumor vessel permeability as determined by DCE MRI was even capable of predicting clinical response to the treatment (Morgan et al. 2003).

Unfortunately, DCE MRI in combination with small clinically approved gadolinium chelates often fails to show effects of antiangiogenic therapy on vessel permeability. This is true even for highly effective treatments against VEGF and its receptors (Kiessling

et al. 2004a; Preda et al. 2004). There is a simple explanation for this unexpected result. Depending on the tumor type, tumor vessel permeability can be so high that the extravasation of the contrast agent occurs almost immediately after it arrives in the vessels. The short time between the arrival of the contrast medium in the vessel and its extravasation cannot be assessed by the MRI due to limitations in temporal resolution. Thus, the resulting parameters of vessel permeability actually reflect tissue perfusion more than vessel permeability. The use of macromolecular or protein-binding contrast agents is an appropriate solution to overcome this problem.

Indeed, it has been shown that using blood-pool contrast agents, permeability becomes a suitable indicator of tumor response to antivascular therapy (Preda et al. 2004; Daldrup-Link et al. 2004; Brasch and Turetschek 2000; Marzola et al. 2004). Attempts have been made to evaluate an optimal size range for MR contrast agents to assess vessel permeability (Brasch and Turetschek 2000). In general, a molecular weight range between 5 kDa and 60 kDa seems to be suitable. However, this can vary depending on the charge, mobility and three-dimensional structure of the contrast agents. For a systematic analysis of vessel permeability, polymers that contain gadolinium or other lanthanides are suited because their molecular weight can be modified without highly influencing the charge and constitution of the probes (Weissleder et al. 2001; Kiessling et al. 2006).

Dendritic polymers containing gadolinium chelates of intermediate molecular weight have found their way into clinical evaluation (Kobayashi and Brechbiel 2003). These have primarily been developed as blood-pool contrast agents for MR angiography but are also valuable for studying vessel permeability (Kobayashi et al. 2004).

A further way of studying vessel permeability is the labeling of plasma proteins such as albumin with gadolinium chelates. Albumin extravasates slowly in tumors, is deposited in the interstitial space, trapped in necrotic areas (Pathak et al. 2005) and partially phagocytosed and metabolized by the tumor cells (Kiessling et al. 2002). Since the extravasation of gadolinium-DTPA-labeled albumin in tumors is mostly attributed to immature hyperpermeable vessels, it is suited for discrimination of mature and immature vessels and to show the regulation of tumor vessel permeability in vivo (Gilad et al. 2005).

There are also contrast agents that temporarily bind to plasma proteins and which have been developed mainly for MR angiography in the clinical use (Knopp et al. 1999). It has been shown that these are principally also suited for DCE MRI studies on vessel permeability (Preda et al. 2004). However, the unbound fraction of the contrast agent and fractions bound to various plasma proteins make pharmacokinetic analysis difficult and suggest the use of descriptive models.

30.2.3 Contrast-enhanced MR Angiography

Visualization of small vessels in tumors by MRI requires long scan times (>10 min), and the use of blood-pool contrast agents is mandatory due to fast extravasation of the small gadolinium-containing compounds that are used clinically. In principle, most of the above-mentioned blood-pool contrast agents can be used for MR angiography.

Using clinical MR scanners, MR angiography is capable of visualizing tumor vessels down to diameters of approximately 100 μm (Kobayashi et al. 2001). Capillaries and small immature vessels, however, which are of the highest interest for the imaging of angiogenesis, cannot be resolved. It has to be shown in the future down to what size tumor vessels can be displayed using high-field MR scanners, which provide high SNR at high spatial resolution but lower contrast for gadolinium-containing contrast agents due to longer T_1 relaxation times.

30.2.4 MRI of Vessel Morphology and Function Using Intrinsic Contrast

There are methods to visualize vessels and assess functional parameters of tissue vascularization without injecting contrast agents. One of those is "blood oxygenation level-dependent" (BOLD) MRI.

BOLD imaging uses the fact that gradient-echo MR sequences are sensitive to changes in blood flow and the level of oxygenated hemoglobin. In detail, this means that oxygenation of hemoglobin reduces T_2^* effects and thus increases the signal in the tissue. Local changes in tissue vascularization, e.g. dilatation of vessels and increased perfusion, can be assessed. The effect can be enhanced by increasing the amount of oxygen in the breathed air. In the context of tumor angiogenesis it was shown by Michal Neeman and co-workers that mature and immature vessels can be differentiated by studying the reactivity of vessels to hyperoxia and hypercapnia using BOLD imaging (Neeman et al. 2001; Abramovitch et al. 1999). For this purpose, during MR imaging the animals were exposed consecutively to ambient air, a mixture of air (95%) and CO_2 (5%), and a mixture of O_2 (95%) and CO_2 (5%). The BOLD signal was then highly dependent on the amounts of reactive and non-reactive vessels. Using this method in an experimental tumor model with inducible overexpression of VEGF, the response of tumor vessels to VEGF was non-invasively assessed (Abramovitch et al. 1999).

In this context, it is important to note that the above-mentioned MR application and also other applications using intrinsic contrast, such as "time of flight" (TOF) angiography and "arterial spin labeling" (to measure perfusion and blood flow) require high SNRs and in small animals often can only reliably performed with dedicated MR scanners with higher field strengths (>4.7 tesla).

Computed Tomography

In contrast to MRI, computed tomography (CT) generates images based on the absorption of X-rays. Most CT scanners are equipped with an X-ray source and a experimental (CT scanners) detector system at the opposite side of the gantry, both rotating around the examined object. The object, lying on a table, is moved through the gantry and scanned slice by slice or continuously in a spiral mode. In contrast, modern clinical CT scanners use fixed multirow detector rings allowing the scanning of 4–64 slices (some prototypes; up to 256 slices) simultaneously. This allows continuous coverage of more than 2 cm in the longitudinal axis of the object. However, while a resolution of about 200 μm pixel length can be resolved in the transverse plane, a reduction of the slice thickness under 500 μm is usually not feasible.

Flat panel-equipped CT (fpVCT) devices use amorphous silicate flat panel detectors, which allow large z-coverage with high isotropic resolution (Kiessling et al. 2004b).

As contrast agents small iodine-containing substances are used which, like small gadolinium chelates, show fast extravasation. Attempts have been made to design blood-pool CT contrast agents using iodine-containing, liposomal-like substances. However, these do not yet fulfill the requirements to image microvessels, due to the minor vessel enhancement that can be achieved compared with the bolus enhancement of clinical contrast agents. Therefore, the best way to morphologically visualize small vessels by CT is a fast scan during the first pass of the contrast agent.

Using clinical CT scanners, larger central vessels of mice and rats such as the aorta, the pulmonary arteries and veins, the veins of the liver and some vessels of the extremities can be resolved; however, small tumor vessels have not yet been visualized. In contrast, fpVCT has proven capable of depicting smaller vessels like the suprarenal arteries and cardiac veins in mice (Greschus et al. 2005). It has also been shown that the development of vessels in experimental tumors of nude mice, which had diameters clearly smaller than 100 μm, could be visualized (Fig. 30.3) and monitored longitudinally (Kiessling et al. 2004b). In this study the iodine doses administered to visualize the tumor vessels were high; however, no side effects on the tumor-bearing animals were observed during the examination period.

There are two major limitations of this imaging method that can influence the assessment of angiogenesis and antiangiogenic effects: Although the scans were performed during the first pass of the contrast agent, it remains unclear to what degree contrast medium that was extravasated early into

the interstitial space supported the visualization of the small tumor vessels. In addition, like in MR angiography no capillaries and small immature vessels, on which the most pronounced effects of antiangiogenic therapy are expected, were visualized.

Beside the morphologic visualization of tumor vessels, dynamic contrast-enhanced experiments to assess functional data on perfusion can also be performed using fpVCT scanners. Initial experience with perfusion measurements of the rat brain show that reliable data can be determined (Greschus et al. 2005). In comparison with DCE MRI, it is an advantage that larger volumes of the animal can be measured dynamically in less than 3 s and that the CT data can be quantified directly. However, it is a disadvantage that the animals are exposed to X-rays and that the X-ray dose for a dynamic scan is high. In particular, in follow-up studies with several dynamic fpVCT scans on the same animal, the administered doses accumulate and thus the total dose should be considered carefully before starting the trial, in order to avoid any influence of the CT measurement on tumor biology and animal health.

In contrast to fpVCT scanners, μCT scanners are designed to provide higher resolution with less than 10 μm pixel length but usually need long scan times (>10 min) and high X-ray doses, which are not tolerated by animals. Even if the X-ray dose can be decreased to a level that allows survival of the animals, an influence on the biological process under consideration, e.g. tumor growth, cannot be excluded. Thus, most high-resolution μCT scanners are more suited for the examination of tissue specimens than of living animals. In tissue samples, however, it has been shown that tumor vessels with diameters less than 30 μm can be visualized and reconstructed three-dimensionally (Maehara 2003). In excised VX2 tumors of rabbits, small immature vessels, filled with barium sulfate, could be detected only 3 days after tumor cell transplantation.

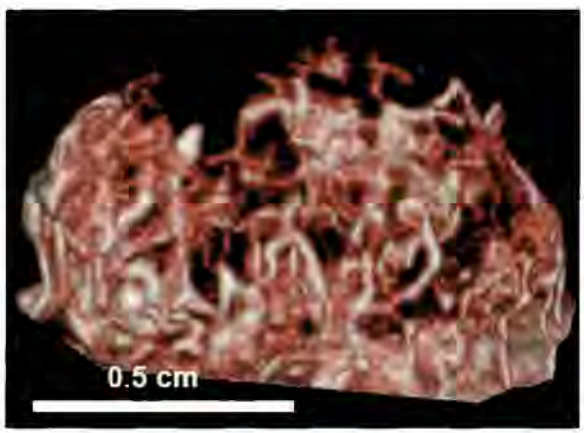


Fig. 30.3. Three dimensional reconstruction of a contrast-enhanced fpVCT scan of a nude mouse, showing the vessels of a subcutaneous squamous cell carcinoma xenograft approximately 1 cm in diameter

30.4 Contrast-Enhanced Ultrasound

Ultrasound has some advantages over other non-invasive imaging modalities. It is a real-time method with high temporal and spatial resolution, even in the detection of tissue vascularization. Furthermore, it is widely available and inexpensive. However, in principle it is only a two-dimensional method, and the image quality depends on the acoustic characteristics of the examined tissue. Thus, the technical adequacy is examiner and examination dependent, and may vary considerably.

The use of ultrasound contrast agents that consist of small microbubbles (1–10 μm in diameter) enables the visualization of perfusion. In principle, each single microbubble can be detected using contrast-specific ultrasound techniques, thus providing exquisite sensitivity, much higher than that of MRI.

Microbubbles can be destroyed using high – but clinically applied – ultrasound pulses. This destruction of the bubbles creates a large ultrasound signal (stimulated acoustic emission), which can then be detected by the ultrasound device. Thus, high-energy techniques are very sensitive in detecting ultrasound contrast agents. However, it has to be taken into account that each ultrasound pulse destroys the bubbles in the region of interest. Therefore, the contrast agent might not be able to enter the smallest vessels. In order to

visualize also capillary perfusion, the pulse repetition rate of the ultrasound device must be reduced to give the microbubbles enough time to enter the capillaries. In previous studies this was often not considered. This is one possible explanation why in several studies no correlation was found between ultrasound perfusion parameters and histological microvessel density (Schroeder et al. 2001; Forsberg et al. 2002; Iordanescu et al. 2002), in contrast to recent studies using more dedicated technical procedures (Pollard et al. 2002; Krix et al. 2003b).

Novel ultrasound techniques with higher specificity to ultrasound contrast agents use the fact that microbubbles show a non-linear behavior of the vibrations that are generated by the acoustic field. Since microbubbles expand more readily in phases of negative acoustic pressure than they contract with high pressure, the backscatter characteristics of the contrast agent will be non-linear. These non-linear signals are amplified in contrast-specific techniques, while the background signal from the tissue is reduced. Using these techniques, higher spatial resolution in contrast-enhanced ultrasound is possible. Furthermore, these techniques work at a low energy level (low mechanical index). Thus, the destruction of the microbubbles is considerably reduced, which results in the ability to visualize perfusion in real time. Recent studies have shown the potential of these ultrasound techniques, which yielded sensitive and detailed non-invasive imaging of tumor microvascularization (Fig. 30.4), but detailed results in clinical or preclinical applications are still lacking.

For quantification of tissue perfusion in ultrasound the dynamics of the signal enhancement after injection of contrast medium have to be measured in regions of interest (ROI), similar to other radiological modalities. An important assumption for the quantification of contrast-enhanced ultrasound signals is that the measured signal is proportional to the microbubble concentration. Several signal parameters have been used, and high-frequency raw data are considered as the gold standard. Video signal parameters such as the color pixel density or the mean color value (e.g. of a dynamic power Doppler examination) are more easily obtained and have shown to be useful (Wei et al. 1998; Krix et al. 2003b; Kiessling et al. 2003).

Compared to other modalities, complex pharmacokinetic models describing the extravasation of the contrast agent are not necessary using contrast-enhanced ultrasound, since the microbubbles remain strictly intravascular. Therefore, perfusion values can be measured accurately. On the other hand, it is not possible to obtain information about the permeability of the examined vessels.

Mathematical parameters of the perfusion curve (ultrasound signal intensity-time curve) such as the maximum, the initial increase, the start of the increase, or the area under the whole curve have been used to indirectly assess tissue perfusion. Since the perfusion curve describes the wash-in and wash-out dynamics of the intravasal contrast agent, these parameters reflect the tissue vascularization to a certain degree. In several studies the degree of vascularization and therapeutic effects could be monitored using such perfusion parameters derived from contrast-enhanced ultrasound in preclinical studies (Schroeder et al. 2001; Forsberg et al. 2002; Iordanescu et al. 2002; Denis et al. 2002). However, it has to be kept in mind that these parameters merely indirectly describe perfusion. They are not derived from an underlying model, which could allow for direct correlation with physiological perfusion quantities such as blood volume or blood flow.

Such a direct quantification of perfusion is possible using a specific ultrasound method, which is called replenishment analysis. This technique uses the destruction of microbubbles by high-energy ultrasound pulses ("flash") and measures the consecutive refilling of contrast medium from outside the ROI where the bubbles are still present. The initial increase of the replenishment curve (ultrasound signal intensity over time after destruction) reflects the mean blood flow velocity inside the ROI. The higher the mean blood flow, the higher the gradient of the replenishment curve will be. If the ultrasound beam width is known, the blood flow velocity can be absolutely calculated. The replenishment curve asymptotically reaches a plateau when all vessels inside the ROI are completely refilled with microbubbles. This plateau can be considered as a parameter proportional to the blood volume in the ROI. A parameter proportional to the perfusion can be then calculated by the product of blood volume and

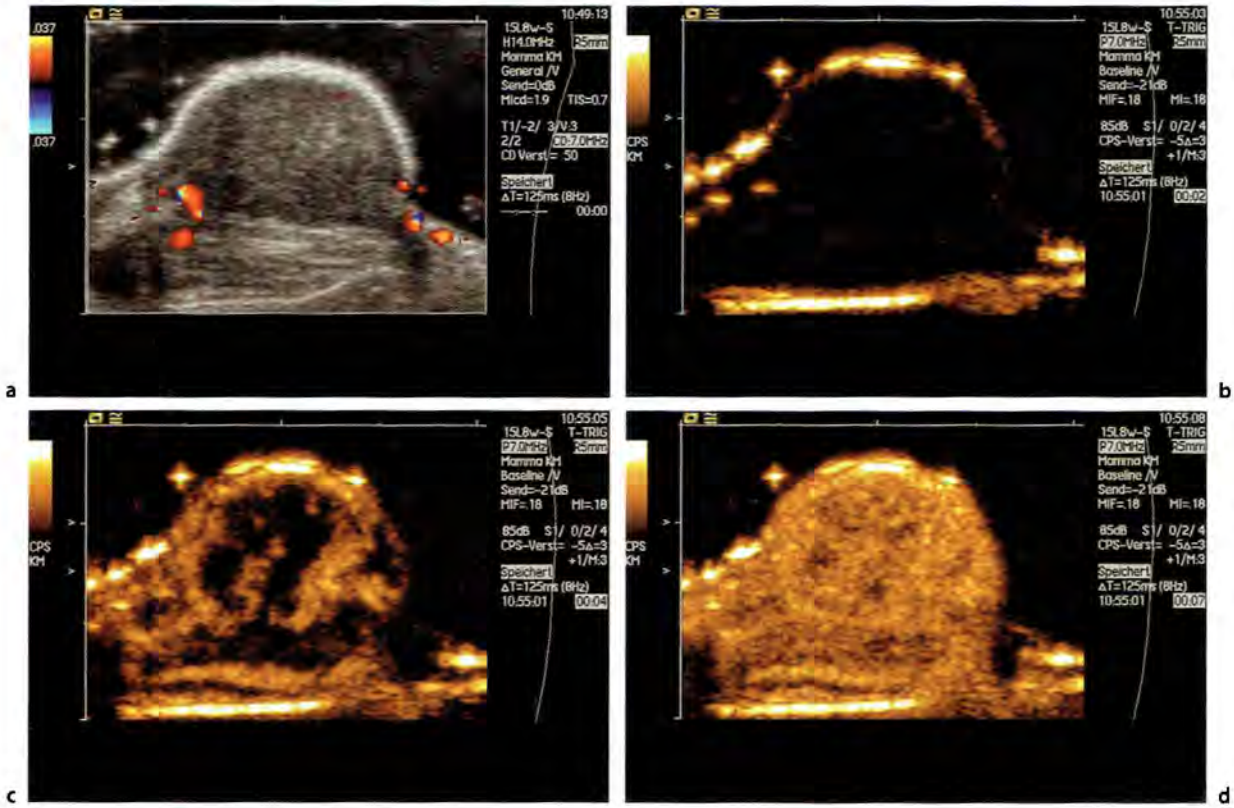


Fig. 30.4a–d. Ultrasound examination of a murine subcutaneous squamous cell carcinoma (HaCaT-ras). In contrast to conventional Doppler sonography (a; blood flow is visualized color-coded), contrast-specific ultrasound techniques (after injection of 100 µl Sonovue®, Bracco, Milan, Italy) sensitively detects strong and nearly homogeneous tumor vascularization: b before contrast enhancement; c during wash-in; d at maximum enhancement, 7 s after injection

blood flow velocity according to a model proposed by Wei (Wei et al. 1998). Several studies have proven the validity and accuracy of this detailed quantification of perfusion (Wei et al. 1998; Seidel et al. 2000; Rim et al. 2001; Krix et al. 2003b). A significant correlation with histologically assessed microvessel density, perfusion parameters from DCE MRI, and ultrasound perfusion parameters was found (Kiessling et al. 2003), and clinical application has yielded promising results (Krix et al. 2004).

However, Wei's model assumed a constant velocity of refilling and neglected the fact that the contrast agent re-entry will depend on the alignment of the vessels (varying caliber and direction). It was chosen for mainly practical reasons, i.e., because of its simplicity, but was far from being a consistent description of the refilling process. Therefore, other models have been proposed attempting a more physiological analysis of replenishment kinetics (Lucidarme et al. 2003; Pollard et al. 2002). One example is a multivessel model which was previously developed to calculate tumor perfusion (Krix et al. 2003a). Using this model one can also calculate a "vessel distribution coefficient," which helps to determine the degree of uniformity of the blood flow velocities inside all vessels in the ROI. This may be used as additional information to assess functional changes, e.g., in tumors. In addition, this multivessel model can be used with a single bolus injection instead of the usually required continuous infusion

of contrast agent when analyzing replenishment kinetics. This enabled the examination of small animals in preclinical research (Krix et al. 2003a, 2003b), where an infusion is not feasible in small animals like nude mice.

After the initial studies evaluating contrast-enhanced ultrasound for use in assessing tumor vascularization, treatment monitoring was performed in several studies. Echocardiography played an important role in the development of new contrast-enhanced ultrasound methods (Wei et al. 1998). Proangiogenic effects of VEGF-121 in the myocardium could be assessed using contrast echocardiography (Villanueva et al. 2002). Antiangiogenic drugs have gained importance, and consequently most preclinical treatment monitoring studies using contrast-enhanced ultrasound have been performed in the context of antiangiogenic therapy. Several tumor models, such as subcutaneous prostate, breast and squamous cell carcinomas, have been used. Contrast-enhanced ultrasound successfully showed changes in the perfusion after treatment with antiangiogenic agents such as VEGF antibody or protamine (Iordanescu et al. 2002; Krix et al. 2003b; Tang et al. 2003). As in MRI it was found that early follow-up measurement may be necessary in ultrasound to comprehensively assess treatment related changes in tumor perfusion (Krix et al. 2003b) (Fig. 30.5).

30.5 Positron Emission Tomography and Single Photon Emission Tomography

In Positron emission tomography (PET) and single photon emission tomography (SPECT), gamma ray photons derived directly or indirectly from injected radionuclides are measured by rotating scintillation detectors. Advantages of these modalities are that the data can be analyzed quantitatively and that the sensitivity for detection of radioactive tracers is much higher than with MRI and CT. In contrast to radionuclides used in SPECT, in which the gamma ray photons are emitted directly from the radioisotope, in PET the positron moves about 1 mm in the tissue until it combines with a normal electron, causing the emission of two 511-keV photons at an angle of almost 180°.

While a broad range of SPECT tracers are available, varying in half-life between minutes and days, most PET tracers have very short half-life, between 2 min and 20 min. Thus, in order to generate the isotopes for PET a cyclotron has to be available.

Compared with MRI, CT and ultrasound, nuclear medicine techniques are limited by the lack of intrinsic tissue contrast and the low spatial

Fig. 30.5a–c. Ultrasound images of a treated tumor at the time of maximal enhancement after bolus injection of contrast agent. a When the tumor was 3 weeks old. b One week after the commencement of therapy with antibody to VEGF receptor, the tumor has become larger but the vascularization is strongly decreased. c During the following week, this resulted in a reduction of tumor volume (inversely correlated with tumor perfusion at the week before).

resolution. This, however, can be balanced using image fusion with CT or MRI scans and explains increasing clinical interest in PET with the introduction of PET-CT combination scanners.

Of the numerous possible PET techniques for assessment of surrogate markers of tissue vascularization, $H_2{}^{15}O$-PET is most frequently used to determine tissue perfusion and $C^{15}O$-carbon monoxide-PET is most frequently employed to determine tissue blood volume (Laking and Price 2003). To determine tissue perfusion, the accumulation of $H_2{}^{15}O$ is measured in the target tissue and in an arterial input function after intravenous injection. Pharmacokinetic modeling of the concentration over the course of time then allows the extraction of physiological parameters. In line with the post-processing of DCE MRI scans, pharmacokinetic one- to four-compartment models can be applied (Laking and Price 2003).

In order to determine tissue blood volume one uses the fact that $C^{15}O$-carbon monoxide irreversibly binds to hemoglobin. Thus, this tracer remains strictly intravascular, which allows ready determination of the relative vascular volume in the tissue after determination of the tracer concentration in the blood (Laking and Price 2003).

Several clinical studies on tumors and metastases of patients with advanced neoplastic disease have indicated that $H_2{}^{15}O$-perfusion and $C^{15}O$-carbon monoxide PET are able to assess cytostatic therapy effects (Laking and Price 2003; Herbst et al. 2002; Logan et al. 2002; Koh et al. 2003). In this context, Herbst and co-workers published initial results on patients with different types of cancer who were treated with a human recombinant endostatin, and observed a dose-dependent decrease in tumor blood flow after therapy (Herbst et al. 2002). However, in line with the results from MRI, a reduction of perfusion and vascular volume was not found in all PET studies after therapy; in some cases no change, or even an increase, in perfusion and blood flow was observed (Gupta et al. 2003). This does not indicate weakness of the method but clearly demonstrates the complexity of tumor vessel function and the need for a comprehensive and systematical analysis.

Molecular Imaging Approaches

Molecular imaging is defined as the non-invasiv assessment of molecular characteristics and th visualization of cell function. In the context o angiogenesis, molecular imaging yields importan insights into the characteristics and formation o vessels and allows specific follow-up of antiangio genic therapy.

Two major tasks will be discussed in this sec tion: (1) the non-invasive tracking of cells in orde to study the cellular assembly of new vessels and the role of progenitor cells in vessel formation; (2) the use of specific contrast agents, which usually consist of a specific ligand bound to a signaling molecule (Weissleder and Mahmood 2001).

Several modalities are suited for this approach. The highest sensitivity for specific probes is achieved using nuclear medicine techniques such as SPECT and PET. Optical imaging also yields high sensitivity; however, scattering limits the tissue penetration. The visualization of targets located deeper in the tissue can be improved using near-infrared dyes and optical tomography techniques (Ntziachristos et al. 2003). Compared with the nuclear medicine techniques, it is a limitation of optical imaging that absolute quantification of the signals is still challenging.

Compared to optical and nuclear medicine techniques the sensitivity of MRI to contrast agents is lower by a factor of more than 1000. On the other hand, MRI provides excellent tissue contrast and resolution, which is important to localize the area of interest in vivo. In particular, high spatial resolution and excellent tissue contrast are desirable for the tracking of labeled cells and the localization of angiogenic sites in tumors. Therefore, it is worth developing molecular imaging approaches for MRI; however, the low sensitivity has to be considered in the choice of the target and the signaling molecule: One might choose surface receptors that trigger receptor-mediated endocytosis of the contrast agents and cause them to accumulate in the lysosomes of the cells.

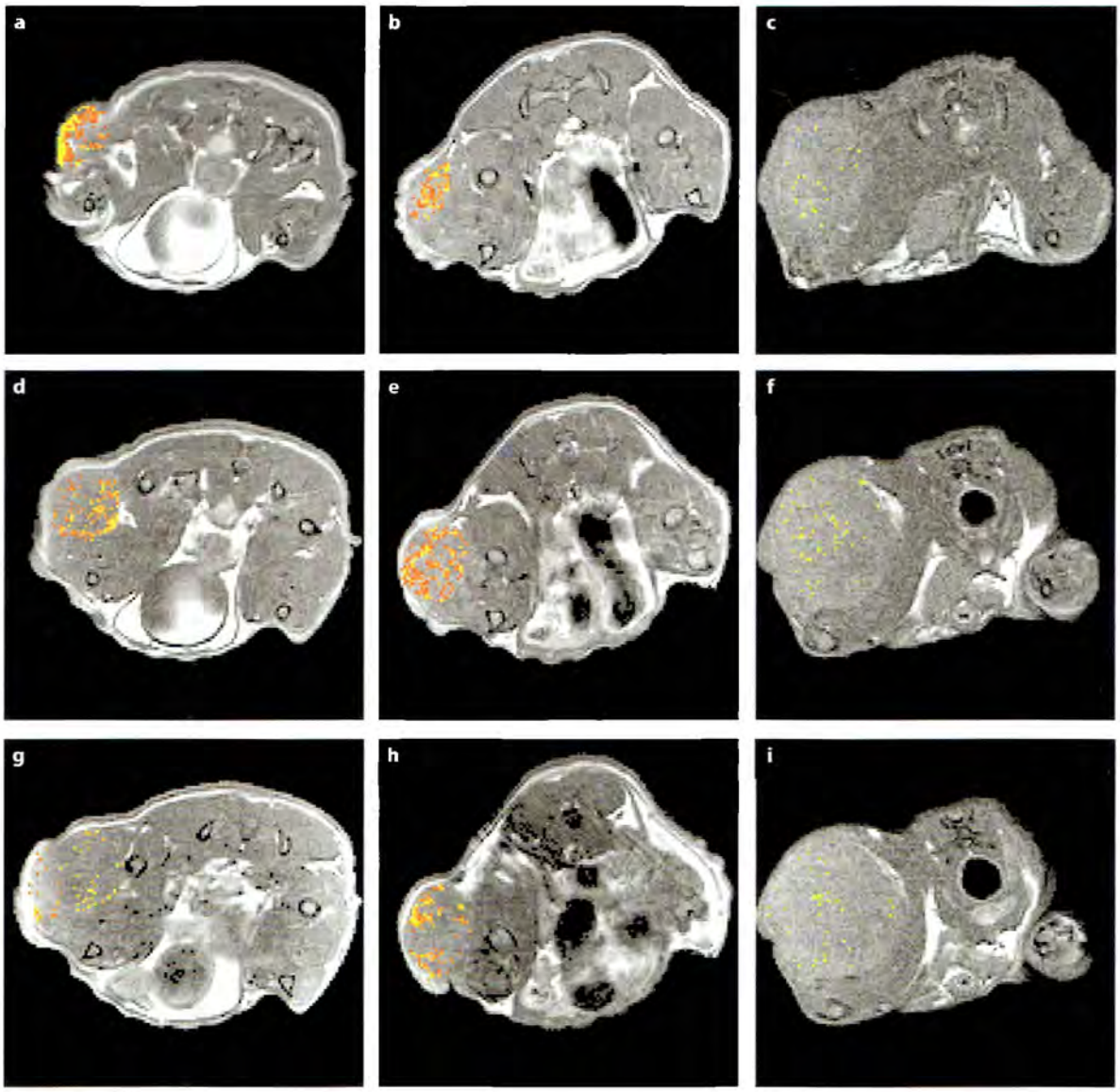


Fig. 30.6.a–c. Magnetic resonance images of slices through a tumor of an animal 35 min after it was injected with paramagnetic RGD liposomes. a A slice through the periphery of the tumor showed a large amount of imaging voxels with significant signal enhancement. b Within an MRI slice, the signal enhancement was also mainly found at the edge of the tumor. c A slice through the middle of the tumor showed a low fraction of imaging voxels with signal enhancement, predominantly in the rim of the tumor. d–f Magnetic resonance images of slices through the tumor of an animal 35 min after injection with paramagnetic RAD liposomes. Signal enhancement was observed throughout the tumor at all slice positions. g–i Slices through the tumor of an animal that was injected first with nonparamagnetic RGD liposomes to block the $\alpha v\beta 3$-integrin sites, and 35 min thereafter with paramagnetic RGD liposomes. Only a small number of voxels showed contrast enhancement. The color indicates the percentage of signal enhancement according to the pseudo-color scale on the right

Furthermore, sensitivity can be increased using "negative contrast agents" instead of gadolinium chelates, which cause a strong reduction of T_2 and T_2^* relaxation times and produce local susceptibility effects in the tissue. This leads to a local signal intensity decrease, and black spots occur on the T_2- and T_2^*-weighted images. Usually these "negative contrast agents" consist of a superparamagnetic core of iron oxides (magnetite and maghemite) and a coating layer of dextran, citrate, or silicate. Larger superparamagnetic iron oxide particles (SPIOs) are distinguished from ultrasmall superparamagnetic iron oxide particles (USPIOs) with diameters of less than 60 nm.

Cells can be incubated with these particles and phagocytose them unspecifically. When this happens, larger particles with negative surface charge usually show stronger uptake. Uptake can be increased by using transfection agents or by binding small peptides which trigger the transport through the plasma membrane, such as HIV-Tat, to the surface of the particles. In vitro experiments proved that even single cells that are loaded with such superparamagnetic particles can be detected (Foster-Gareau et al. 2003). In the context of tumor angiogenesis, in vivo studies showed that MRI can track the accumulation in tumors of USPIO-labeled progenitor cells, which differentiate there to endothelial cells, as indicated by subsequent histological evaluation (Anderson et al. 2005).

Ultrasound can also be used in molecular imaging approaches to angiogenesis. Specific ligands are bound to the microbubbles that are used as contrast agents in ultrasound. However, since microbubbles do not extravasate due to their large size, only intravascular targets can be addressed. The sensitivity of modern ultrasound devices for microbubbles is high, and even single microbubbles can be detected, particularly if new contrast-specific techniques are used (Hauff et al. 2004). In studies on arteriosclerosis it has already been shown that specific imaging of blood and lymphatic vessels can be successful using ligands like ICAM and L-selectin (Hauff et al. 2004).

Frequently used targets for molecular imaging of angiogenesis by MRI and ultrasound are VEGF, VEGF receptors, ICAM, E-selectin, and αvβ3-integrin (Miller et al. 2005). In particular for the αvβ3-integrins there are publications with all mentioned modalities (Miller et al. 2005; Neeman 2002; Sipkins et al. 1998; Mulder et al. 2005). Beside antibodies (Sipkins et al. 1998), cyclic RGD peptides are often used as ligands to this marker, and in a recent publication the presence of αvβ3-integrins on angiogenic tumor vessels could even be visualized by MRI in a positive contrast using RGD liposomes containing Gd-DTPA (Mulder et al. 2005) (Fig. 30.6).

In summary, these initial results indicate that molecular imaging of angiogenesis works in small animals. However, in particular for MRI no specific diagnostic compounds to angiogenic targets have entered clinical evaluation so far. Thus, it has to be elucidated whether these compounds can also be used successfully in patients and whether tumor characterization and assessment of therapy response can be improved.

References

Abramovitch R, Dafni H, Smouha E, Benjamin LE, Neeman M (1999) In vivo prediction of vascular susceptibility to vascular susceptibility endothelial growth factor withdrawal: magnetic resonance imaging of C6 rat glioma in nude mice. Cancer Res 59:5012–5016

Akella SN, Twieg DB, Mikkelsen T et al (2004) Assessment of brain tumor angiogenesis inhibitors using perfusion magnetic resonance imaging: quality and analysis results of a phase I trial. J Magn Reson Imaging 20:913–922

Anderson SA, Glod J, Arbab AS et al (2005) Noninvasive MR imaging of magnetically labeled stem cells to directly identify neovasculature in a glioma model. Blood 105:420–425

Brasch R, Turetschek K (2000) MRI characterization of tumors and grading angiogenesis using macromolecular contrast media: status report. Eur J Radiol 34:148–155

Brix G, Semmler W, Port R et al (1991) Pharmacokinetic parameters in CNS Gd-DTPA enhanced MR imaging. J Comput Assist Tomogr 15:621–628

Daldrup-Link HE, Okuhata Y, Wolfe A et al (2004) Decrease in tumor apparent permeability-surface area product to a MRI macromolecular contrast medium following angiogenesis inhibition with correlations to cytotoxic drug accumulation. Microcirculation 11:387–396

Denis F, Bougnoux P, de Poncheville L et al (2002) In vivo quantitation of tumour vascularisation assessed by Doppler sonography in rat mammary tumours. Ultrasound Med Biol 28:431–437

Forsberg F, Dicker AP, Thakur ML et al (2002) Comparing contrast-enhanced ultrasound to immunohistochemical markers of angiogenesis in a human melanoma xenograft model: preliminary results. Ultrasound Med Biol 28:445–451

Foster-Gareau P, Heyn C, Alejski A et al (2003) Imaging single mammalian cells with a 1.5 T clinical MRI scanner. Magn Reson Med 49:968–971

Gilad AA, Israely T, Dafni H et al (2005) Functional and molecular mapping of uncoupling between vascular permeability and loss of vascular maturation in ovarian carcinoma xenografts: the role of stroma cells in tumor angiogenesis. Int J Cancer 117:202–211

Greschus S, Kiessling F, Lichy M et al (2005) Potential applications of flat-panel volumetric CT in morphological and functional small animal imaging. Neoplasia 7:730–740

Gupta N, Saleem A, Osman S et al (2003) Carbogen and nicotinamide selectively increase blood flow index (BFI) and 5 fluorouracil (5FU) delivery in human tumours. Proc Am Soc Clin Oncol 21:Abstract #493

Hauff P, Reinhardt M, Briel A et al (2004) Molecular targeting of lymph nodes with L-selectin ligand-specific US contrast agent: a feasibility study in mice and dogs. Radiology 231:667–673

Hawighorst H, Knapstein PG, Knopp MV et al (1999) Cervical carcinoma: standard and pharmacokinetic analysis of time–intensity curves for assessment of tumor angiogenesis and patient survival. MAGMA 8:55–62

Herbst RS, Mullani NA, Davis DW et al (2002) Development of biologic markers of response and assessment of antiangiogenic activity in a clinical trial of human recombinant endostatin. J Clin Oncol 20:3804–3814

Iordanescu I, Becker C, Zetter B et al (2002) Tumor vascularity: evaluation in a murine model with contrast-enhanced color Doppler US effect of angiogenesis inhibitors. Radiology 222:460–467

Kiessling F, Fink C, Hansen M et al (2002) Magnetic resonance imaging of nude mice with heterotransplanted high-grade squamous cell carcinomas: use of a low-loaded, covalently bound Gd-Hsa conjugate as contrast agent with high tumor affinity. Invest Radiol 37:193–198

Kiessling F, Krix M, Heilmann M et al (2003) Comparing dynamic parameters of tumor vascularization in nude mice revealed by MRI and contrast enhanced intermittent power Doppler sonography. Invest Radiol 38:516–524

Kiessling F, Farhan N, Lichy M et al (2004a) Dynamic contrast enhanced magnetic resonance imaging rapidly indicates vessel regression in human squamous cell carcinomas grown in nude mice caused by VEGF-receptor 2 blockade with DC101. Neoplasia 6:213–223

Kiessling F, Greschus S, Lichy MP et al (2004b) Volumetric computed tomography (VCT): a new technology for noninvasive, high-resolution monitoring of tumor angiogenesis. Nat Med 10:1133–1138

Kiessling F, Heilmann M, Lammers T et al (2006) Synthesis and characterization of HE-24.8: a polymeric contrast agent for magnetic resonance angiography. Bioconjug Chem 17:42–51

Knopp MV, von Tengg-Kobligk H, Floemer F et al (1999) Contrast agents for MRA: future directions. J Magn Reson Imaging 10:314–316

Kobayashi H, Brechbiel MW (2003) Dendrimer-based macromolecular MRI contrast agents: characteristics and application. Mol Imaging 2:1–10

Kobayashi H, Sato N, Kawamoto S et al (2001) 3D MR angiography of intratumoral vasculature using a novel macromolecular MR contrast agent. Magn Reson Med 46:579–585

Kobayashi H, Reijnders K, English S et al (2004) Application of a macromolecular contrast agent for detection of alterations of tumor vessel permeability induced by radiation. Clin Cancer Res 10:7712–7720

Koh T, Taniguchi H, Yamagishi H (2003) Oxygen-15 positron emission tomography for predicting selective delivery of a chemotherapeutic agent to hepatic cancers during angiotensin II-induced hypertension. Cancer Chemother Pharmacol 51:349–358

Krix M, Kiessling F, Farhan N et al (2003a) A new multivessel model describing replenishment kinetics of ultrasound contrast agent for quantification of tissue perfusion. Ultrasound Med Biol 29:1421–1430

Krix M, Kiessling F, Vosseler S et al (2003b) Sensitive noninvasive monitoring of tumor perfusion during antiangiogenic therapy by intermittent, bolus-contrast power Doppler sonography. Cancer Res 63:8264–8270

Krix M, Plathow C, Kiessling F et al (2004) Quantification of perfusion of liver tissue and metastases using a multivessel model for replenishment kinetics of ultrasound contrast agents. Ultrasound Med Biol 30:1355–1363

Laking GR, Price PM (2003) Positron emission tomographic imaging of angiogenesis and vascular function. Br J Radiol 76:50–59

Logan TF, Jadali F, Egorin MJ et al (2002) Decreased tumor blood flow as measured by positron emission tomography in cancer patients treated with interleukin-1 and carboplatin on a phase I trial. Cancer Chemother Pharmacol 50:433–444

Lucidarme O, Franchi-Abella S, Correas JM et al (2003) Blood flow quantification with contrast-enhanced US: "entrance in the section" phenomenon – phantom and rabbit study. Radiology 228:473–479

Maehara N (2003) Experimental microcomputed tomography study of the 3D microangioarchitecture of tumors. Eur Radiol 13:1559–1565

Marzola P, Degrassi A, Calderan L et al (2004) In vivo assessment of antiangiogenic activity of SU6668 in an

experimental colon carcinoma model. Clin Cancer Res 10:739–750
Miller JC, Pien HH, Sahani D et al (2005) Imaging angiogenesis: applications and potential for drug development. J Natl Cancer Inst 97:172–187
Morgan B, Thomas AL, Drevs J (2003) Dynamic contrast-enhanced magnetic resonance imaging as a biomarker for the pharmacological response of PTK787/TK 222584, an inhibitor of the vascular endothelial growth factor receptor tyrosine kinases, in patients with advanced colorectal cancer and liver metastases: results from two phase I studies. J Clin Oncol 21:3955–3964
Mulder WJ, Strijkers GJ, Habets JW et al (2005) MR molecular imaging and fluorescence microscopy for identification of activated tumor endothelium using a bimodal lipidic nanoparticle. FASEB J 4:S1530–S6860
Neeman M (2002) Functional and molecular MR imaging of angiogenesis: seeing the target, seeing it work. J Cell Biochem [Suppl] 39:11–17
Neeman M, Dafni H, Bukhari O, (2001) In vivo BOLD contrast MRI mapping of subcutaneous vascular function and maturation: validation by intravital microscopy. Magn Reson Med 45:887–898
Ntziachristos V, Bremer C, Weissleder R (2003) Fluorescence imaging with near-infrared light: new technological advances that enable in vivo molecular imaging. Eur Radiol 13:195–208
Padhani AR (2003) MRI for assessing antivascular cancer treatments. Br J Radiol 76:60–80
Padhani AR, Gapinski CJ, Macvicar DA et al (2000) Dynamic contrast enhanced MRI of prostate cancer: correlation with morphology and tumour stage, histological grade and PSA. Clin Radiol 55:99–109
Pathak AP, Artemov D, Ward BD et al (2005) Characterizing extravascular fluid transport of macromolecules in the tumor interstitium by magnetic resonance imaging. Cancer Res 65:1425–1432
Pollard RE, Sadlowski AR, Bloch SH et al (2002) Contrast-assisted destruction-replenishment ultrasound for the assessment of tumor microvasculature in a rat model. Technol Cancer Res Treat 1:459–470
Port RE, Knopp MV, Hoffmann U, Milker Zabel S, Brix G (1999) Multicompartment analysis of gadolinium chelate kinetics: blood-tissue exchange inmammary tumors as monitored by dynamic MR imaging. J Magn Reson Imaging 10:233–241
Preda A, Novikov V, Möglich M et al (2004) MRI monitoring of avastin antiangiogenesis therapy using B22956/1, a new blood pool contrast agent in an experimental model of human ca ncer. J Magn Reson Imaging 20:865–873
Rim SJ, Leong-Poi H, Lindner JR et al (2001) Quantification of cerebral perfusion with "real-time" contrast-enhanced ultrasound. Circulation 104:2582–2587
Schroeder R, Hauff P, Bartels T et al (2001) Tumor vascularization in experimental melanomas: correlation between unenhanced and contrast enhanced power doppler imaging and histological findings. Ultrasound Med Biol 27:761–771
Seidel G, Algermissen C, Christoph A et al (2000) Visualization of brain perfusion with harmonic gray scale and power doppler technology: an animal pilot study. Stroke 31:1728–1734
Sipkins DA, Cheresh DA, Kazemi MR et al (1998) Detection of tumor angiogenesis in vivo by alphaVbeta3-targeted magnetic resonance imaging. Nat Med 4:623–626
Stehle G, Sinn H, Wunder A et al (1997) The loading rate determines tumor targeting properties of methotrexate-albumin conjugates in rats. Anticancer Drugs 8:677–685
Tang J, Li S, Li J et al (2003) Evaluation of the effect of protamine on human prostate carcinoma PC-3m using contrast enhanced Doppler ultrasound. J Urol 170:611–614
Tofts PS, Kermode AG (1991) Measurement of the blood-brain barrier permeability and leakage space using dynamic MR imaging. 1. Fundamental concepts. Magn Reson Med 17:357–367
Villanueva FS, Abraham JA, Schreiner GF et al (2002) Myocardial contrast echocardiography can be used to assess the microvascular response to vascular endothelial growth factor-121. Circulation 105:759–765
Wei K, Jayaweera AR, Firoozan S et al (1998) Quantification of myocardial blood flow with ultrasound-induced destruction of microbubbles administered as a constant venous infusion. Circulation 97:473–483
Weissleder R, Mahmood U (2001) Molecular imaging. Radiology 219:316–333
Weissleder R, Bogdanov A, Tung CH et al (2001) Size optimization of synthetic graft copolymers for in vivo angiogenesis imaging. Bioconjug Chem 12:213–219
Yang S, Law M, Zagzag D (2003) Dynamic contrast-enhanced perfusion MR imaging measurements of endothelial permeability: differentiation between atypical and typical meningiomas. Am J Neuroradiol 24:1554–1559

Visualization of Microcirculation and Anti-Angiogenic Tumor Therapy

31

Marcus Czabanka and Peter Vajkoczy

Abstract

Mechanistic analysis of processes involved in tumor angiogenesis and vascularization requires sophisticated in vivo experimental models and techniques. Intravital microscopy allows for direct assessment of tumor angiogenesis, microcirculation and overall perfusion. Furthermore, recent advances in intravital microscopy now enable detailed analysis of tumor blood vessel permeability, tumor oxygenation and imaging of blood vessel-specific epitopes at the molecular level, as well as intra- and extravascular cell–cell interactions. With the recent advances in the field of bioluminescence and fluorescent reporter genes, appropriate for in vivo imaging, the intravital fluorescent microscopic approach has to be considered a powerful tool to study microvascular, cellular and molecular mechanisms of tumor growth.

CONTENTS

31.1 Introduction

Today, critical insights into the biology of cancer are increasingly derived from experimental study designs that move away from the single cell level and focus rather on systems biology. This represents an important step, since neoplastic tissues consist of at least three distinct compartments, which in concert influence tumor biology, and may be classified as cellular, interstitial or vascular in nature. Especially the tumor microcirculation and tumor interstitium have moved into the center of attention with respect to tumor growth, metastasis, tumor diagnosis and anti-tumor therapy. Angiogenesis and continuous remodeling of tumor microvasculature are considered essential for adequate tissue oxygenation and nutritional supply. Without angiogenesis most tumors will not progress to a clinically

relevant size nor will they metastasize to distant organs via the bloodstream. Just as tumors rely on their microvascular system for growth, metastasis and nutritional supply, therapeutic strategies like radiotherapy, chemotherapy and immunotherapy critically depend upon the adequate delivery of molecules and oxygen, a process governed by tumor blood flow and interstitial transport. In the light of the complexity and the dynamics of tumor microcirculation and interstitial transport of molecules, it has also become apparent that the petri dish or conventional histomorphological techniques fail to adequately reflect the full dimensions of local and systemic feedback loops underlying tumor microcirculation in vivo. This notion prompted the search for in vivo experimental models and techniques, preferentially non- or minimally invasive, that allow for a detailed and sophisticated approach to the vascular compartment of a tumor.

In this respect, intravital epifluorescence microscopy is currently the imaging modality of choice, representing a versatile experimental tool for the direct in vivo evaluation of vascular, cellular and molecular mechanisms underlying tumor angiogenesis and microcirculation. This chapter will focus on the intravital microscopic visualization of the tumor microcirculation and its application in the study of anti-angiogenic tumor therapies.

Experimental Models

In order to assess a tumor by intravital microscopic means it has to be directly accessible for the optical system, which necessitates a microsurgical preparation of the tumor-bearing organ or tissue. Basically, there are acute and chronic preparations available for an intravital microscopic analysis of tumor angiogenesis and microcirculation.

The acute intravital microscopic approach is most frequently performed on the liver, since it can be easily exposed by a transverse laparotomy and externalization on a plastic disc held by an adjustable stage (Lehr et al. 1999; Vollmar et al. 1994). Using this experimental model, liver metastases can be induced by tumor cell injection into the femoral/cecal vein and may subsequently be analyzed by intravital microscopy. In this way, Kan and coworkers were able to analyze the microvasculature of liver metastases, placing emphasis on the individual roles of the hepatic artery and portal vein for the blood supply of these tumors (Kan et al. 1993). In addition, refined microsurgical techniques have also made other organs amenable for acute intravital microscopic analysis, such as the brain or spine (Vajkoczy et al. 2001). However, all of these acute animal preparations are limited by one major drawback: they do not allow monitoring of angiogenic and microcirculatory events during tumor growth and progression over a prolonged period of time; rather, they represent a snapshot of tumor microcirculation. To overcome this limitation, acute models require the study of a considerable number of animals at different time points of tumor growth. This limitation is further aggravated by the considerable biological inter- and intraindividual variability among tumors, again necessitating a large number of experimental animals when studied by intravital microscopy in acute animal preparations.

Consequently, for long-term intravital microscopic study of tumor microcirculation and, more importantly, the consequences of anti-angiogenic therapies, chronic models are superior to acute preparations. Currently, the most common chronic models are the dorsal skinfold chamber and the chronic cranial window. The dorsal skinfold chamber consists of two symmetrical titanium frames, which are used to embed the dorsal skinfold of the animal. Fixation of the frame is achieved by sutures and screws, one of them securing the top of the frames while the other two perforate the extended double layer of the skin on the bottom of the frames, without injuring the observed vessel or compromising the animal's breathing. Within the area of the observation window, one layer of skin is completely removed in a circular area of 15 mm diameter and the remaining layer, consisting of striated muscle, subcutaneous tissue and epidermis, is covered with a glass cover slip which is incorporated into one of

the frames and fixed with an elastic ring (Lehr et al. 1993; Vajkoczy et al. 1995). In order to exclude preparations with trauma-induced or inflammation-associated changes of tissue, a recovery period of 48 h is allowed to elapse before the cover slip is temporarily removed and tumor cell suspensions, tumor spheroids or intact tumor specimens are implanted by directly placing them onto the striated muscle (Leunig et al. 1992; Vajkoczy et al. 1998). The obvious drawback of the dorsal skinfold chamber is that it does not represent an orthotopic implantation site for most tumor entities (except melanoma or mammary carcinoma). Thus, in order to study the microcirculation of brain tumors in an orthotopic model the chronic cranial window preparation has been developed (Yuan et al. 1994). A 6×7-mm bone flap is created bilaterally over the frontal and parietal regions of the skull and freed from the underlying dura and sagittal sinus. The dura overlying each hemisphere is removed, avoiding any damage to the sinus and bridging veins (Fig. 31.1A). Finally, the tumor cell suspension or a tumor cell spheroid is directly placed onto the pial surface of one of the hemispheres and the window is sealed with a glass cover slip by sticking it to the bone using a histocompatible glue (Fig. 31.1B).

Using these two models, intravital microscopy represents a highly sophisticated and versatile experimental technique to investigate the functional and structural characteristics of tumor microcirculation in vivo. Nevertheless, the models described above also feature distinct limitations. Since the implanted tumors are generated from transplantable cell lines or grafted tumor specimens, the experimental tumors may respond differently to anti-angiogenic treatment modalities than primary tumors (Field et al. 1991). This obstacle could be overcome by transgenic animal tumor models allowing for in vivo analysis of spontaneous tumor entities (Field et al. 1991). Another disadvantage is the limited life span of the in-vivo preparations, which do not allow for an extended analysis of tumor angiogenesis and microcirculation beyond an observation period of 3–4 weeks, thus restricting the observation period to the early stages of tumor growth.

Tumor Angiogenesis and Microcirculation

As previously described, blood vessels may grow by different means (Carmeliet 2003). While vasculogenesis refers to the formation of blood vessels by endothelial progenitor cells, angiogenesis refers to vascular sprouting and subsequent stabilization of these sprouts by mural cells (Carmeliet 2003). In this context tumor angiogenesis as observed by intravital microscopy represents a multi-step process. The first phase, an avascular phase, occurring on days 0–6 following tumor implantation, is characterized by the formation of initial vessel sprouts originating from the venular segments of the host microvasculature without significant tumor growth. The next phase is characterized by the development of a functional microvascular network and tumor microcirculation (days 6–14), accompanied by the initiation of tumor growth. On days 14–21, an advanced phase can be observed with high neo-angiogenic activity in the periphery and active microvascular remodeling within the center of the growing tumor (Vajkoczy et al. 1998).

A more detailed description of the tumor angiogenic process demands quantitative analysis of multiple microcirculatory parameters. This is best achieved with the support of a computer-assisted image analysis system. The length of all newly formed microvessels per tumor area is referred to as "total vascular density". Although this parameter gives an impression of the tumor's angiogenic potential (similar to histological vessel density), it does not reveal information about the perfusion status of the tumor, which is critical for its nutritional blood supply. Intravital microscopic analysis offers the unique advantage that it visualizes not only the morphology of the tumor vasculature but also functional aspects of tumor microcirculation. For this purpose the parameter "functional vascular density", defined as the length of red blood cell-perfused microvessels per tumor area, was introduced, providing perfusion information on the tumor vasculature (Vajkoczy et al. 1998, 1999b). In order to additionally specify the efficacy of the tumor an-

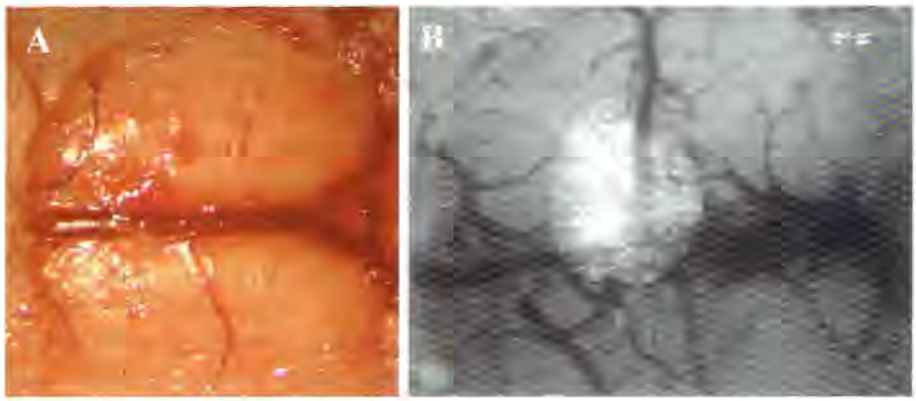


Fig. 31.1. A Macroscopic view of the cranial window preparation before its sealing with the glass cover slip. B Intravital fluorescence microscopic view of the chronic cranial window preparation following implantation of a tumor cell suspension. Tumor cells are labeled with Fast Blue

giogenic process, the ratio between functional and total vascular density can be calculated, representing the microvascular perfusion index (Vajkoczy et al. 1998). By determination of these parameters, intravital microscopic analyses in fact demonstrated a low functional efficacy of the tumor angiogenic process, since tumor microcirculation is characterized by a perfusion index of only 50%–70% as compared to 95% in normal tissue (Vajkoczy et al. 1998). Thus, even though many neoplasms are strongly vascularized and highly angiogenic tumors, tissue perfusion is often found to be lower and more variable than in the host tissue under physiological conditions (Groothuis et al. 1983). Clearly, insights of this kind are of major importance for future antitumor therapies.

Furthermore, an in-depth analysis of tumor microcirculation using intravital microscopy may also include an assessment of microvascular volume, vascular surface area and mean blood vessel diameter. Similarly, functional parameters such as pressure gradients, microvascular hematocrit and hemorheology may be included in the analysis. Finally, intravital microscopy is also capable of visualizing the high heterogeneity in tumor angioarchitecture, tumor vessel differentiation and functional blood supply. This heterogeneity in tumor angioarchitecture is the result of an immaturity of tumor blood vessels compared to the hierarchically organized vascular system of regular organs (Verheul et al. 2004). Unlimited tumor expansion and therefore the continuous stimulation of vessel outgrowth prevent endothelial cells from generating a mature and organized vasculature, but instead continuously stimulate them to expand the vascular compartment of the growing tumor (Verheul et al. 2004). Consequently, tumors contain tortuous and maximally dilated vessels, shunts, variable intervascular spaces and large avascular areas (Leunig et al. 1992; Vajkoczy et al. 1998).

Using intravital microscopy tumor microcirculation can be classified into two different compartments. The peritumoral compartment refers to the area outside the tumor and adjacent to the tumor edges, whereas the intratumoral compartment is the solid tumor mass. Both compartments are characterized by a distinct angiogenic activity, expression of angiogenic growth factors and cognate receptors as well as vascular density (Fukumura et al. 1998; Vajkoczy et al. 1998, 1999b). However, microcirculation of tumors is not solely dependent on the apparent vascular density. In addition to the vascular length (≈ functional vessel length per area), tissue perfusion (ml/100 g/min) is further determined by mean vessel diameter, mean red blood cell velocity and intervascular spacing (Intaglietta and Zweifach 1974). Additionally, these parameters allow the calculation of blood flow rate (nl/s), vascular surface area (%) and geometrical resistance, providing further information about the microcirculatory features of the tumor (Baish et al. 1996; Leunig et al. 1992; Vollmar et al. 1997b).

Apart from the above-mentioned aspects, the angioarchitecture and its dynamic changes during tu-

mor growth may also affect tumor microcirculation. Most knowledge of tumor angioarchitecture is only descriptive and qualitative in nature, like the analysis of vascular branching orders and angles, vessel morphology, vessel length and diameter (Konerding et al. 1999; Less et al. 1997). Yet, there are a growing number of quantitative approaches to assess functional data in this respect. A detailed computer reconstruction of intravital microscopic images, visualizing small regions of tumor microvasculature, was applied to elucidate the complex microvascular arrangements (Secomb et al. 1993). By evaluating the fractal characteristics of vascular networks of tumors implanted into dorsal skinfold chambers in nude mice, the influence of tumor angioarchitecture on perfusion and transport may be assessed (Baish et al. 1996). Using an invasion percolation-based network model, the importance of tortuosity, vascular spacing, vessel diameter and viscosity for nutritional and drug transport is emphasized (Baish et al. 1996). Taking all findings together, tumor microcirculation is influenced by multiple morphological, functional and hemorheological parameters, and intravital microscopy has contributed significantly to unraveling this complexity in vivo.

Molecular Imaging

Newly formed blood vessels display specific molecular characteristics that are distinct from those of mature blood vessels. Molecules capable of selectively targeting epitopes that are specific for tumor angiogenesis, either within the blood vessel wall or the tumor stroma, may thus provide unique opportunities for future diagnosis and therapy of tumors. For example, the ED-B domain of fibronectin represents a possible molecular target, since it specifically accumulates around neovascular structures, whereas it cannot be detected on mature blood vessels (Castellani et al. 1994). In order to better understand the mechanisms and dynamics of this tumor blood vessel and/or tumor stroma targeting strategy, intravital fluorescence microscopy may be applied as an unique technique for experimental molecular imaging. By using fluorescently labeled high-affinity antibody fragments or small molecules directed against the epitope of interest, intravital microscopy provides the opportunity to analyze the distinct tumor blood vascular/stromal binding of the antibody, taking advantage of the high spatial resolution and high signal-to-noise ratio of the technique. Additionally, the time course and dynamics of the targeting process may be determined due to the high temporal resolution of the intravital microscopic approach. Finally, intravital microscopy also enables the correlation of the targeting process with the determinants of vessel perfusion. Hypothetically, high perfusion rates may lead to enhanced molecular binding to the vessel wall due to increased delivery of the antibody. On the other hand, high perfusion rates may also result in reduced targeting due to minimized contact time with the vessel wall. Clearly, molecular imaging by intravital microscopy represents the technique of choice in order to shed light on these controversies in the future and to provide valuable pre-clinical information for future clinical applications of vascular or stromal targeting strategies.

Cell Interactions with the Tumor Blood Vessel Wall

Using various in-vivo and ex-vivo labeling techniques, intravital microscopy allows visualization of the interplay between circulating cells, endothelial cells and tumor cells. In combination with the simultaneous in vivo imaging of tumor blood vessels, this multi-fluorescent technique offers an approach to elucidate the roles that these cells play in controlling angiogenesis, tumor microcirculation, metastasis and anti-tumor immune responses.

For in-vivo labeling of circulating cells, such as leukocytes or platelets, several fluorochromes have been proposed. Especially fluorescent DNA mark-

ers like Rhodamine 6G and Acridine Orange represent useful tools for this purpose (Menger et al. 1991). Additionally, in order to prevent artifacts due to phototoxicity or interference of the fluorescent tracer with cellular metabolism, intravital microscopic set-ups and protocols have been standardized and optimized (Saetzler et al. 1997). Using these techniques, a detailed analysis of leukocyte–endothelium interactions has been performed in experimental set-ups for inflammation and ischemia/reperfusion. In this context, intravital microscopic analyses have revealed the multistep nature of leukocyte–endothelium interactions and their implications for leukocyte recruitment from the microvasculature to the interstitium: fast-moving leukocytes in the bloodstream tether and roll ("rollers") on activated endothelium (von Andrian et al. 1991). Chemokines and other proinflammatory mediators released by various cells activate rolling leukocytes, resulting in firm adhesion ("stickers") (Kunkel et al. 1997; Ley 1994). Transmigration across the endothelial barrier may then occur after firm leukocyte adhesion (Lawrence and Springer 1991). While this multi-step process has been proven in various inflammatory and ischemia/reperfusion experiments, the significance of leukocyte–endothelium interactions for tumor angiogenesis and microcirculation is still a matter of controversy. On the one hand, in vivo fluorescence microscopic studies showed reduced leukocyte–endothelium interactions within tumor blood vessels compared with normal control vessels, indicating immuno-incompetence of the host (Fukumura et al. 1995; Jain et al. 1996). This effect may be explained by a reduced flux of leukocytes in angiogenic vessels or an inability of the new endothelium to upregulate the respective adhesion molecules. On the other hand, a large number of immunohistological studies in both human and experimental tumors have clearly shown the presence of leukocytes as well as the microvascular expression of cell adhesion molecules (Jain et al. 1996; Suzuki et al. 1995). Tumor-associated immune/inflammatory cells produce a myriad of pro-angiogenic cytokines and growth factors (Rosenkilde and Schwartz 2004), that act not only on endothelial cells but also stimulate migration and attraction of leukocytes into the tumor area, potentially forming self-perpetuating positive feedback loops (Rosenkilde and Schwartz 2004). Therefore, the role of leukocyte–endothelium interactions in tumor angiogenesis and microcirculation needs further exploration, and intravital microscopy should be the appropriate tool for those future studies.

Local tumor cell invasion is a hallmark of the diffuse infiltrative growth behavior of many human neoplasms (e.g. gliomas, squamous cell carcinoma, breast tumor). A number of experimental reports have causally linked tumor cell invasion to tumor angiogenesis and microcirculation (Giese and Westphal 1996; Skobe et al. 1997). Invading tumor cells have been shown to be characterized by a high affinity to the perivascular basal membrane, suggesting that vessels growing from the host tissue towards the tumor might provide a trail for local tumor dissemination via the perivascular space (Giese and Westphal 1996). In order to investigate the detailed mechanisms underlying this relationship, fluorescently labeled glioma spheroids, instead of tumor cell suspensions, can be implanted into both the dorsal skinfold chamber and the cranial window. Via intravital microscopy a direct and repetitive evaluation of tumor cell migration in vivo can be performed, offering an approach for concurrent visualization of the dynamics of tumor cell spread and tumor angiogenesis. This unique technique will enable future studies to elucidate the association between tumor cell invasion and angiogenesis (Vajkoczy et al. 1999a).

In the light of rapidly evolving stem cell research, the investigation of the specific function of endothelial progenitor cell biology in the field of tumor angiogenesis has become of special interest. Intravital microscopy displays a versatile method to elucidate this complex biological issue. Using murine embryonic endothelial progenitor cells, the multi-step process of endothelial progenitor cell homing and incorporation into functional tumor blood vessels has been recently defined (Vajkoczy et al. 2003). High temporal and spatial resolution of intravital microscopy has contrib-

uted to the successful visualization of the homing process of circulating endothelial progenitor cells to the tumor microvasculature, followed by transendothelial migration into the interstitial space and incorporation into a functional microvasculature (Vajkoczy et al. 2003). As endothelial progenitor cells contribute to tumor angiogenesis and vessel formation in vivo, the exact role of endothelial progenitor cell biology in tumor angiogenesis remains unclear and needs further investigation. Using intravital microscopic techniques, upcoming questions on this topic may be answered in future studies.

Tumor Metabolism

The regulation of tissue metabolism and homeostasis is the ultimate goal of tumor angiogenesis and microcirculation. Over the years, major indicators reflecting the metabolic status of a tumor, such as oxygenation, mitochondrial redox state, pH, and oxidative stress, have become amenable to intravital fluorescence videomicroscopy by applying advanced signal detection techniques. Accordingly, the assessment of intracellular NADH fluorescence allows for quantification of the mitochondrial redox state, which represents an indicator of cellular oxygen in the presence of sufficient substrate and phosphate (Chance et al. 1962; Vollmar et al. 1997a). Furthermore, oxygenation can be assessed noninvasively within intra- and extravascular areas using the phosphorescence decay technique with palladium-porphyrins bound to albumin as an in vivo indicator (Torres Filho and Intaglietta 1993). Also quantitative pH measurement within tumor tissue can be realized by intravital fluorescence ratio imaging microscopy using BCECF as a H^+-sensitive fluorochrome (Helmlinger et al. 1997). Finally, the formation of reactive oxygen species has been visualized with in vivo microscopic techniques using hydroperoxide-sensitive fluorescent probes that are trapped within viable cells (Suematsu et al. 1993).

Intravital Laser Scanning Confocal Microscopy

One drawback of intravital epifluorescence microscopy is its limitation of the penetration depth to 100 µm, together with a sometimes impaired visualization with low-contrast images due to a contamination of light from out-of-focus optical planes. One way to overcome this limitation is the extension of laser scanning confocal microscopy to intravital applications. Laser scanning confocal microscopy enables the investigator to obtain systematically 2D images from different depths in the specimen with high contrast and clarity (Wallen et al. 1988). In this way, for example, the relationship and distribution of microglia, astrocytes and vasculature within the brain could be demonstrated (Dailey and Waite 1999). Additionally, the application of laser scanning confocal microscopy provides the opportunity to generate 3D images of the sample (Jiang et al. 2005). Regarding tumor angiogenesis it allows the description of the 3D architecture of tumor vessels, including their relationship to tumor cells and other tissue structures (Jiang et al. 2005). In combination with a versatile fluorescent labeling strategy, laser scanning confocal microscopy has multiple advantages over epifluorescence microscopy. It represents a multi-fluorescent approach generating exceptional multiprobe imaging of samples, due to a large variety of excitation wave lengths on a single microscope set-up (Wright et al. 1989). Furthermore, a subcellular resolution can be achieved for a rather large area of interest. Additionally, the optical sectioning capabilities coupled with the ability to record multiple focus stacks make it possible to reconstruct 3D images (Amos and White 2003). In this context it should be noted that the possibility of time-lapse laser scanning confocal microscopy offers the capability of providing information about the dynamics of cellular morphological changes, cellular locomotion or extravasation of circulating macromolecules (Fig. 31.2) (O'Rourke and Fraser 1990).

As a consequence, laser scanning confocal microscopy represents a valuable complementary

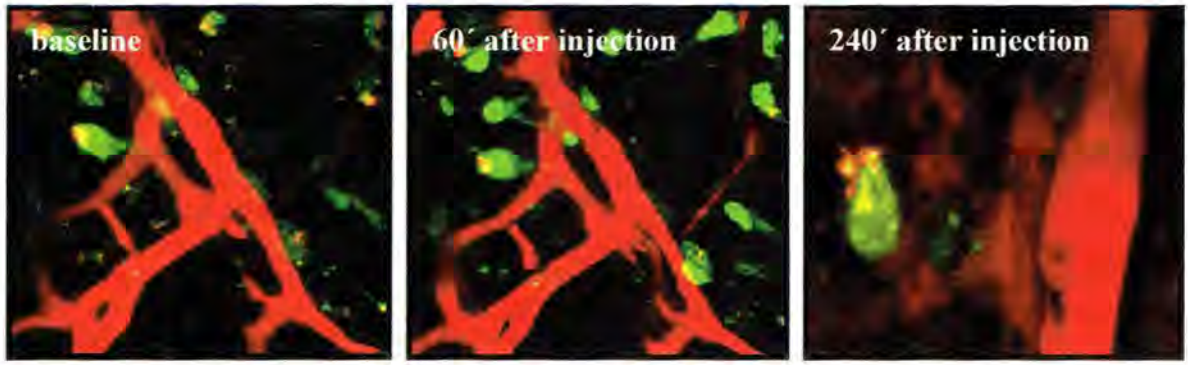


Fig. 31.2. Three-dimensional reconstructions of time-lapse laser confocal fluorescence microscopy of tumor microvasculature revealing extravasation of fluorescently labeled albumin (*red*) into the interstitial space. Tumor cells (*green*) demonstrate partial uptake of extravasated albumin

technique for the intravital microscopic assessment of tumor microcirculation. While epifluorescence techniques will remain the gold standard for the study of the dynamics of tumor perfusion and tumor microcirculation, intravital laser scanning confocal microscopy has advanced to the technique of choice when multiple cellular players are to be studied simultaneously and 3D reconstructions of the vascular compartment are of interest. Intravital laser scanning confocal microscopy may therefore lead to further insights into the complex mechanisms of tumor angiogenesis and may contribute to the design and validation of future anti-angiogenic treatments.

31.8 Role of Intravital Microscopy for Anti-angiogenic Therapy

The tumor microcirculation is of special interest in the light of evolving novel therapeutic modalities that aim at interfering with tumor angiogenesis and microcirculation, such as anti-angiogenic and anti-vascular drug therapy as well as vascular targeting strategies. For a large variety of tumors, microvessel density has been described to be an independent prognostic factor for survival (Weidner and Folkman 1996). However, simply focusing on vessel density as the sole parameter when evaluating the efficacy of anti-angiogenic and anti-vascular strategies does not fully respect the complexity of potential microcirculatory and microhemodynamic changes that may occur within the treated tumor microvascular system. For example, specific VEGFR-2 inhibition using a small molecule inhibitor has been demonstrated to result not only in a reduction in vessel density but also in a significant increase in red blood cell velocity and blood flow rate within the remaining blood vessels, partially compensating for the successful reduction of functional vascular density (Vajkoczy et al. 1999b). Thus, intravital microscopy represents the most powerful tool to analyze these specific alterations in different therapy models and may unravel unexpected results already at a pre-clinical stage, rather than at the end of potentially unsuccessful clinical testing.

In addition to assessing the consequences of anti-angiogenic/anti-vascular therapies on the tumor microcirculation alone, intravital microscopy may also provide an insight into their consequences for nutritional supply and tumor tissue oxygenation. Especially the latter has proven to be relevant in order to confirm that an individual anti-angiogenic/anti-vascular intervention does in fact result in the expected tumor hypoxia. Using intravital microscopy and the phosphorescence-quenching method in coordinated fashion, the non-invasive measurement of

intravascular as well as interstitial oxygenation can be performed in vivo (Kerger et al. 2003). Applying this versatile technique in a C6 glioma experiment, inhibition of VEGF and PDGF signaling pathways resulted in significant tumor vessel regression, leading to reduced PO2 and thus hypoxia in the tumor interstitium (Erber et al. 2004).

However, intravital microscopy not only allows one to directly visualize the effects of anti-angiogenic/anti-vascular therapies, but also helps to determine their interaction with clinically relevant combination therapies. The bioavailability of chemotherapeutic drugs critically depends on microvascular perfusion, microvascular extravasation and interstitial transport. Poor tumor perfusion remains an important obstacle to the successful delivery of chemotherapeutic agents. As a consequence, it seems contradictory, at first glance, that the efficacy of chemotherapeutic strategies may be increased by a combination with anti-angiogenic/anti-vascular therapies (Hurwitz et al. 2004). Again, intravital microscopy has recently assisted better understanding of this paradox (Vajkoczy et al. 1999b). As expected, anti-angiogenic strategies will reduce tumor vessel density and overall tumor perfusion. However, a certain amount of blood vessels will escape this intervention and will allow tumor cells to survive within their proximity (Fig. 31.3). As demonstrated by intravital microscopy these remaining blood vessels are characterized by an increased red blood cell velocity and improved blood flow rate (Fig. 31.3) (Vajkoczy et al. 1999b). As a consequence, chemotherapeutic drugs that are administered in combination with anti-angiogenic therapies will be delivered more efficiently to the viable parts of the tumor than they would when given as monotherapy.

31.9 Conclusion

Intravital microscopy represents a highly sophisticated experimental technique for the assessment of tumor microcirculation. A further refinement of this technique will help to observe biological processes taking place beyond the microvascular and cellular level. The in vivo analysis of processes on the molecular level using fluorescent reporter genes and bioluminescence will enable future experiments to shed more light on the biomolecular mechanisms underlying the regulation of angiogenesis, microcirculation and tumor growth. Additionally, the application of in vivo laser confocal fluorescence microscopy will generate new insights into the relationship and interactions of tumor cell invasion, microvasculature and extracellular matrix. Finally, intravital microscopy not only represents a versatile tool to study tumor vascular biology, but also al-

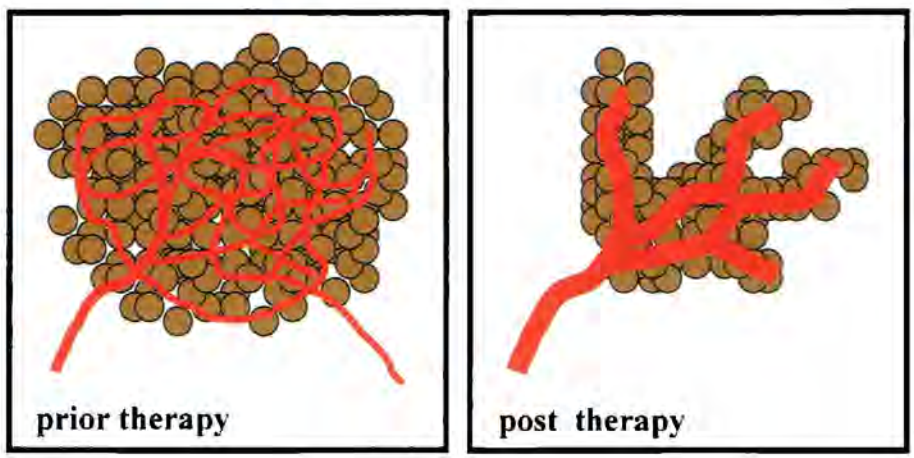


Fig. 31.3. Schematic drawings illustrating the consequences of anti-angiogenic/anti-vascular therapy on the tumor microcirculation as revealed by intravital microscopy. A reduction in tumor vessel density is accompanied by an improved blood flow rate in remaining blood vessels, with surviving tumor cells preferentially located within their proximity

lows evaluation of anti-angiogenic therapies in more detail at a pre-clinical stage, thereby providing a methodological bridge from experimental in vivo validation to successful clinical application.

Acknowledgements

The authors would like to thank Violetta Powajbo and Claudius Friedel for providing the illustrations.

References

Amos WB, White JG (2003) How the confocal laser scanning microscope entered biological research. Biol Cell 95:335–342

Baish JW, Gazit Y, Berk DA, Nozue M, Baxter LT, Jain RK (1996) Role of tumor vascular architecture in nutrient and drug delivery: an invasion percolation-based network model. Microvasc Res 51:327–346

Carmeliet P (2003) Angiogenesis in health and disease. Nat Med 9:653–660

Castellani P, Viale G, Dorcaratto A, Nicolo G, Kaczmarek J, Querze G, Zardi L (1994) The fibronectin isoform containing the ED-B oncofetal domain: a marker of angiogenesis. Int J Cancer 59:612–618

Chance B, Cohen P, Jobsis F, Schoener B (1962) Intracellular oxidation-reduction states in vivo. Science 137:499–508

Dailey ME, Waite M (1999) Confocal imaging of microglial cell dynamics in hippocampal slice cultures. Methods 18:222–230

Erber R, Thurnher A, Katsen AD, Groth G, Kerger H, Hammes HP, Menger MD, Ullrich A, Vajkoczy P (2004) Combined inhibition of VEGF and PDGF signaling enforces tumor vessel regression by interfering with pericyte-mediated endothelial cell survival mechanisms. FASEB J 18:338–340

Field SB, Needham S, Burney IA, Maxwell RJ, Coggle JE, Griffiths JR (1991) Differences in vascular response between primary and transplanted tumours. Br J Cancer 63:723–726

Fukumura D, Salehi HA, Witwer B, Tuma RF, Melder RJ, Jain RK (1995) Tumor necrosis factor alpha-induced leukocyte adhesion in normal and tumor vessels: effect of tumor type, transplantation site, and host strain. Cancer Res 55:4824–4829

Fukumura D, Xavier R, Sugiura T, Chen Y, Park EC, Lu N, Selig M, Nielsen G, Taksir T, Jain RK, Seed B (1998) Tumor induction of VEGF promoter activity in stromal cells. Cell 94:715–725

Giese A, Westphal M (1996) Glioma invasion in the central nervous system. Neurosurgery 39:235–250; discussion 250–232

Groothuis DR, Pasternak JF, Fischer JM, Blasberg RG, Bigner DD, Vick NA (1983) Regional measurements of blood flow in experimental RG-2 rat gliomas. Cancer Res 43:3362–3367

Helmlinger G, Yuan F, Dellian M, Jain RK (1997) Interstitial pH and pO2 gradients in solid tumors in vivo: high-resolution measurements reveal a lack of correlation. Nat Med 3:177–182

Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R, Kabbinavar F (2004) Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 350:2335–2342

Intaglietta M, Zweifach BW (1974) Microcirculatory basis of fluid exchange. Adv Biol Med Phys 15:111–159

Jain RK, Koenig GC, Dellian M, Fukumura D, Munn LL, Melder RJ (1996) Leukocyte-endothelial adhesion and angiogenesis in tumors. Cancer Metastasis Rev 15:195–204

Jiang F, Zhang Z, Kalkanis S, Katakowksi M, Robin AM, Zhang X, Gotlib A, Chelst I, Mikkelsen T, Chopp M (2005) A quantitative model of tumor-induced angiogenesis in the nude mouse. Neurosurgery 57:320–324

Kan Z, Ivancev K, Lunderquist A, McCuskey PA, Wright KC, Wallace S, McCuskey RS (1993) In vivo microscopy of hepatic tumors in animal models: a dynamic investigation of blood supply to hepatic metastases. Radiology 187:621–626

Kerger H, Groth G, Kalenka A, Vajkoczy P, Tsai AG, Intaglietta M (2003) pO(2) measurements by phosphorescence quenching: characteristics and applications of an automated system. Microvasc Res 65:32–38

Konerding MA, Malkusch W, Klapthor B, van Ackern C, Fait E, Hill SA, Parkins C, Chaplin DJ, Presta M, Denekamp J (1999) Evidence for characteristic vascular patterns in solid tumours: quantitative studies using corrosion casts. Br J Cancer 80:724–732

Kunkel EJ, Jung U, Ley K (1997) TNF-alpha induces selectin-mediated leukocyte rolling in mouse cremaster muscle arterioles. Am J Physiol 272:H1391–H1400

Lawrence MB, Springer TA (1991) Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. Cell 65:859–873

Lehr HA, Leunig M, Menger MD, Nolte D, Messmer K (1993) Dorsal skinfold chamber technique for intravital microscopy in nude mice. Am J Pathol 143:1055–1062

Lehr HA, Vollmar B, Vajkoczy P, Menger MD (1999) Intravital fluorescence microscopy for the study of leukocyte interaction with platelets and endothelial cells. Methods Enzymol 300:462–481

Less JR, Posner MC, Skalak TC, Wolmark N, Jain RK (1997) Geometric resistance and microvascular network architecture of human colorectal carcinoma. Microcirculation 4:25–33

Leunig M, Yuan F, Menger MD, Boucher Y, Goetz AE, Messmer K, Jain RK (1992) Angiogenesis, microvascular architecture, microhemodynamics, and interstitial fluid pressure during early growth of human adenocarcinoma LS174T in SCID mice. Cancer Res 52:6553–6560

Ley K (1994) Histamine can induce leukocyte rolling in rat mesenteric venules. Am J Physiol 267:H1017–H1023

Menger MD, Marzi I, Messmer K (1991) In vivo fluorescence microscopy for quantitative analysis of the hepatic microcirculation in hamsters and rats. Eur Surg Res 23:158–169

O'Rourke NA, Fraser SE (1990) Dynamic changes in optic fiber terminal arbors lead to retinotopic map formation: an in vivo confocal microscopic study. Neuron 5:159–171

Rosenkilde MM, Schwartz TW (2004) The chemokine system – a major regulator of angiogenesis in health and disease. APMIS 112:481–495

Saetzler RK, Jallo J, Lehr HA, Philips CM, Vasthare U, Arfors KE, Tuma RF (1997) Intravital fluorescence microscopy: impact of light-induced phototoxicity on adhesion of fluorescently labeled leukocytes. J Histochem Cytochem 45:505–513

Secomb TW, Hsu R, Dewhirst MW, Klitzman B, Gross JF (1993) Analysis of oxygen transport to tumor tissue by microvascular networks. Int J Radiat Oncol Biol Phys 25:481–489

Skobe M, Rockwell P, Goldstein N, Vosseler S, Fusenig NE (1997) Halting angiogenesis suppresses carcinoma cell invasion. Nat Med 3:1222–1227

Suematsu M, Schmid-Schonbein GW, Chavez-Chavez RH, Yee TT, Tamatani T, Miyasaka M, Delano FA, Zweifach BW (1993) In vivo visualization of oxidative changes in microvessels during neutrophil activation. Am J Physiol 264:H881–H891

Suzuki Y, Ohtani H, Mizoi T, Takeha S, Shiiba K, Matsuno S, Nagura H (1995) Cell adhesion molecule expression by vascular endothelial cells as an immune/inflammatory reaction in human colon carcinoma. Jpn J Cancer Res 86:585–593

Torres Filho IP, Intaglietta M (1993) Microvessel PO2 measurements by phosphorescence decay method. Am J Physiol 265:H1434–1438

Vajkoczy P, Menger MD, Simpson E, Messmer K (1995) Angiogenesis and vascularization of murine pancreatic islet isografts. Transplantation 60:123–127

Vajkoczy P, Schilling L, Ullrich A, Schmiedek P, Menger MD (1998) Characterization of angiogenesis and microcirculation of high-grade glioma: an intravital multifluorescence microscopic approach in the athymic nude mouse. J Cereb Blood Flow Metab 18:510–520

Vajkoczy P, Goldbrunner R, Farhadi M, Vince G, Schilling L, Tonn JC, Schmiedek P, Menger MD (1999a) Glioma cell migration is associated with glioma-induced angiogenesis in vivo. Int J Dev Neurosci 17:557–563

Vajkoczy P, Menger MD, Vollmar B, Schilling L, Schmiedek P, Hirth KP, Ullrich A, Fong TA (1999b) Inhibition of tumor growth, angiogenesis, and microcirculation by the novel Flk-1 inhibitor SU5416 as assessed by intravital multi-fluorescence videomicroscopy. Neoplasia 1:31–41

Vajkoczy P, Laschinger M, Engelhardt B (2001) Alpha4-integrin-VCAM-1 binding mediates G protein-independent capture of encephalitogenic T cell blasts to CNS white matter microvessels. J Clin Invest 108:557–565

Vajkoczy P, Blum S, Lamparter M, Mailhammer R, Erber R, Engelhardt B, Vestweber D, Hatzopoulos AK (2003) Multistep nature of microvascular recruitment of ex vivo-expanded embryonic endothelial progenitor cells during tumor angiogenesis. J Exp Med 197:1755–1765

Verheul HM, Voest EE, Schlingemann RO (2004) Are tumours angiogenesis-dependent? J Pathol 202:5–13

Vollmar B, Glasz J, Leiderer R, Post S, Menger MD (1994) Hepatic microcirculatory perfusion failure is a determinant of liver dysfunction in warm ischemia-reperfusion. Am J Pathol 145:1421–1431

Vollmar B, Burkhardt M, Minor T, Klauke H, Menger MD (1997a) High-resolution microscopic determination of hepatic NADH fluorescence for in vivo monitoring of tissue oxygenation during hemorrhagic shock and resuscitation. Microvasc Res 54:164–173

Vollmar B, Wolf B, Siegmund S, Katsen AD, Menger MD (1997b) Lymph vessel expansion and function in the development of hepatic fibrosis and cirrhosis. Am J Pathol 151:169–175

Von Andrian UH, Chambers JD, McEvoy LM, Bargatze RF, Arfors KE, Butcher EC (1991) Two-step model of leukocyte–endothelial cell interaction in inflammation: distinct roles for LECAM-1 and the leukocyte beta 2 integrins in vivo. Proc Natl Acad Sci USA 88:7538–7542

Wallen P, Carlsson K, Liljeborg A, Grillner S (1988) Three-dimensional reconstruction of neurons in the lamprey spinal cord in whole-mount, using a confocal laser scanning microscope. J Neurosci Methods 24:91–100

Weidner N, Folkman J (1996) Tumoral vascularity as a prognostic factor in cancer. Important Adv Oncol:167–190

Wright SJ, Walker JS, Schatten H, Simerly C, McCarthy JJ, Schatten G (1989) Confocal fluorescence microscopy with the tandem scanning light microscope. J Cell Sci 94:617–624

Yuan F, Salehi HA, Boucher Y, Vasthare US, Tuma RF, Jain RK (1994) Vascular permeability and microcirculation of gliomas and mammary carcinomas transplanted in rat and mouse cranial windows. Cancer Res 54:4564–4568

Cellular Actions of Angiogenesis Inhibitors on Blood Vessels

32

PETER BALUK, BEVERLY L. FALCÓN, HIROYA HASHIZUME, BARBARA SENNINO, DONALD M. MCDONALD

Abstract

Blood vessels of tumors have multiple cellular abnormalities that impair blood flow and drug delivery but provide targets for novel therapeutics. These abnormalities involve all components of the vessel wall, including endothelial cells, pericytes, and vascular basement membrane. Endothelial cells of tumor vessels have abnormal gene expression, are dynamic, and undergo sprouting, proliferation, and regression. The endothelial cells have a defective barrier function, are leaky, and may depend on vascular endothelial growth factor (VEGF) for survival. Pericytes of tumor vessels lack the normally intimate association with endothelial cells, and the surrounding basement membrane has redundant loose layers that reflect continual remodeling of the vasculature. Studies of the cellular actions of VEGF inhibitors on mouse models of spontaneous and implanted tumors have shown rapid, robust changes in tumor vessels. Strikingly, in some tumors, within 24 h, endothelial fenestrations disappear, vascular sprouting is suppressed, blood flow ceases, and patency is lost in many vessels. As many as 80% of tumor vessels may regress within 7 days. Similarly, normal blood vessels with abundant endothelial fenestrations, as in the gastrointestinal tract, kidney, and endocrine organs, may be sensitive to VEGF inhibitors. Surviving tumor vessels acquire more normal features, including uniform caliber, diminished branching, reduced VEGFR-2 expression, and tighter pericyte coverage. Empty sleeves of vascular basement membrane, left behind after endothelial cells degenerate, provide a record of pretreatment vessel number and a scaffold for vascular regrowth after cessation of therapy. Normalization of surviving tumor vessels may improve local hemodynamics and perivascular delivery of oxygen, nutrients, and therapeutics, despite reduced angiogenesis and tumor vascularity.

CONTENTS

Introduction

Angiogenesis inhibitors as anti-cancer drugs have been widely examined in animal studies, and are now in clinical use. Yet their biology is still being elucidated, and more work is needed to understand their effects on the structure and function of the component cells that constitute the microvasculature in tumors and in normal tissues (Folkman 2003a, 2003b; Carmeliet 2005). One recent realization is that although tumor blood vessels are abnormal, and in many ways unique, they share some features with quiescent blood vessels and some with blood vessels in other angiogenic contexts. Much effort has been devoted to characterize precisely the differences between tumor blood vessels and normal blood vessels, with the goal of developing 'magic bullet' therapeutics that can selectively target tumor vessels. The original concept of blocking the growth of tumor blood vessels with inhibitors of angiogenesis has recently been broadened by the recognition that these agents do more than inhibit angiogenesis. Indeed, these agents can destroy some existing tumor vessels and normalize the surviving tumor vessels (Jain 2001, 2005; Inai et al. 2004). Existing tumor vessels can also be destroyed by vascular disrupting agents (Tozer et al. 2005; Siemann et al. 2005; Gaya and Rustin 2005). Despite convincing evidence that tumor growth is angiogenesis-dependent, and that attacking the blood supply may stop tumor growth or induce tumor regression, knowledge of the abnormalities of tumor blood vessels is still incomplete. The goal of this chapter is to review the cellular abnormalities of tumor blood vessels and their relation to the actions of angiogenesis inhibitors.

Structure of Normal Blood Vessels

32.2.1 Hierarchy of Microvascular Segments

Normal blood vessels have a highly orderly architecture with distinct vascular segments composed of endothelial cells, mural cells (pericytes or smooth muscle cells), and basement membrane. Each organ has a stereotyped hierarchical arrangement of arteries, arterioles, capillaries, postcapillary venules, collecting venules, and veins (Fig. 32.1A). The overall architecture and the specialized cellular properties of each vascular segment are so well matched to the needs of the organ that the identity of the organ can be deduced from the architecture of the vasculature

alone. Individual segments of the microvasculature are specialized for functional needs: arterioles and precapillary sphincters control vascular caliber and thereby resistance and blood flow, capillaries are specialized for exchange of nutrients, while postcapillary venules are the predominant sites for plasma extravasation and leukocyte emigration during inflammation (Michel and Curry 1999).

32.2.2 Endothelial Cells

Endothelial cells of normal arterioles, capillaries, and venules each have a characteristic size, shape and phenotype adapted to their function. They form a monolayer with well-joined cell borders (Hashizume et al. 2000) (Fig. 32.1B). Endothelial cell division occurs rarely in normal blood vessels of most adult tissues and organs. However, there are notable exceptions: normal physiological cycles of growth and regression occur in the female reproductive organs (Augustin 2005) and during hair growth (Augustin 2005; Yano et al. 2003), and in pathological events such as wound healing. Consequently, such dynamic blood vessels may be susceptible to angiogenesis inhibitors (McCarty and Ellis 2002).

32.2.3 Pericytes

Pericytes are mural cells in the microvasculature that are embedded within the vascular basement membrane. Like smooth muscle cells, pericytes are of mesenchymal origin. While they share some features with smooth muscle cells, pericytes have a greater structural diversity (Sims 2000; Allt and Lawrenson 2001). Pericytes typically have a cell body with cytoplasmic processes with intimate connections with the abluminal endothelial cell surface (Fig. 32.1C). Pericytes on capillaries are long and slender and oriented along the length of the vessel but cover little of its surface area, while those on venules have multiple branching processes and cover more of the vessel surface (Fig. 32.1A,D). Pericytes communicate with endothelial cells both through direct cell contacts and through paracrine signaling (Hirschi and d'Amore 1996). In addition, the cytoplasmic processes from one pericyte can contact multiple endothelial cells and even multiple capillaries. This feature may integrate signals along the length of the vessel. Pericytes have diverse functions (Sims 2000; Allt and Lawrenson 2001; Betsholtz et al. 2005), including stabilization of endothelial cells; regulation of vascular caliber; control of endothelial permeability, sprouting, proliferation, survival, migration, and maturation (Hirschi and d'Amore 1996; Gerhardt and Betsholtz 2003). Pericytes may also influence the extravasation of tumor cells (Xian et al. 2006). Recent studies have highlighted the importance of the interactions between pericytes and endothelial cells and have shown that disruption of these intimate interactions leads to severe impairments of the vasculature (Betsholtz et al. 2005; Armulik et al. 2005).

32.2.4 Basement Membrane

The abluminal or basal surface of endothelial cells is covered by a thin sleeve of basement membrane (Fig. 32.1E), which reflects the polarity of endothelial cells. However, the vascular basement membrane is unusually rich in type IV collagen and differs in molecular composition from that of epithelial cells and most epithelial-derived tumor cells (Kalluri 2003). Vascular basement membrane of normal blood vessels observed by transmission electron microscopy is a uniform, well-defined layer coating the entire endothelial surface. Even in angiogenic blood vessels of growing muscle or mesentery, the basement membrane is intact on remodeling endothelial cells and is only absent by ultrastructural criteria at the extreme tips of filopodia (Stingl and Rhodin 1994; Rhodin and Fujita 1989). Both pericytes and endothelial cells contribute to the synthesis of the basement membrane. The vascular basement membrane is a storage site for growth factors and some of its components are a source of angiogenesis inhibitors formed by proteolytic cleavage (Kalluri 2003; Sund et al. 2004).

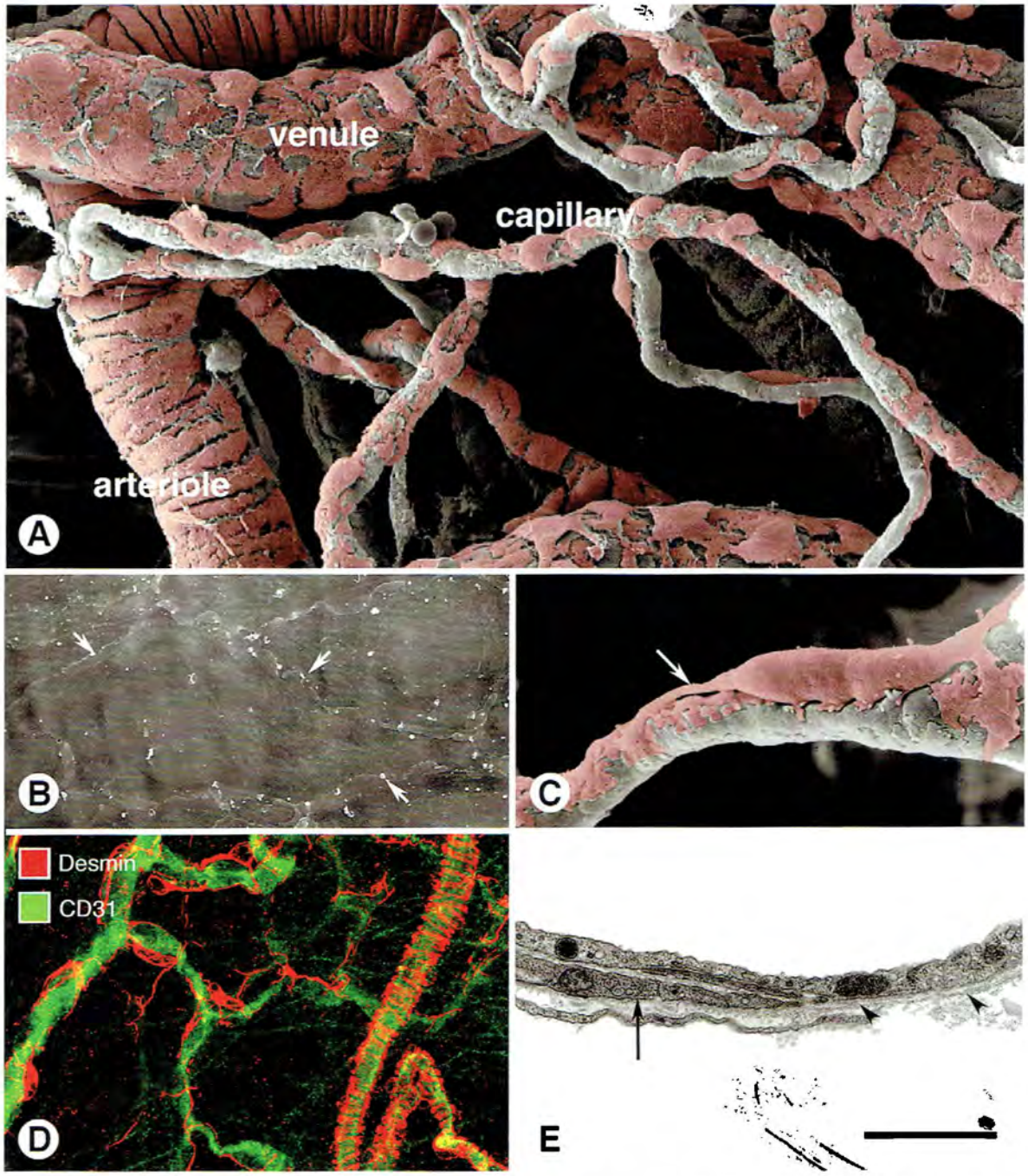


Fig. 32.1A–E. Structure of normal blood vessels. **A** Scanning electron micrograph showing hierarchy of normal blood vessels in rat dorsal skin organized as a well-ordered network of characteristic arterioles, capillaries and venules. Connective tissue elements have been removed by hydrolytic digestion to reveal smooth muscle cells and pericytes (*pink*) and endothelial cells. **B** Scanning electron micrograph of smooth luminal surface and borders (*arrows*) of normal endothelial cells of venule. **C** Pericyte (*pink*) with long process (*arrow*) on capillary. **D** Confocal image of normal blood vessels in mouse trachea. Periendothelial cells (smooth muscle cells and pericytes, red) are closely associated with endothelial cells (*green*) in capillaries and arterioles. **E** Transmission electron micrograph of normal venule. Pericyte (*arrow*) and basement membrane (*arrowheads*) are closely associated with an endothelial cell (from Baluk et al. 2004). *Scale bar*: A 20 μm; B, C 10 μm; D 70 μm; E 3 μm

Abnormalities of Tumor Blood Vessels

32.3.1 Irregular Shape and Abnormal Hierarchical Arrangement

Tumor blood vessels have structural and functional abnormalities in all of their components (Baluk et al. 2005). Architecturally, tumor vessels are irregularly shaped, tortuous, and lack the normal hierarchy of arterioles, capillaries, and venules. By implication, proper control of vasomotion, nutrient exchange, and extravasation of fluid and cells is also disrupted. Nevertheless, each type of tumor appears to have its own characteristic vascular abnormalities in terms of vessel number, branching, and size (Konerding et al. 1995; Tozer et al. 2005). A well-documented feature of many subcutaneously implanted tumors is a high density of vessels at the rapidly growing rim, and fewer vessels in the core (Dvorak et al. 1988). Regions of necrosis that have no vessels may be scattered in the tumor interior. In some implanted tumors, tumor cells grow as cylinders approximately 100–150 μm in radius around blood vessels (Hashizume et al. 2000), beyond which the tumor is necrotic. Spontaneous tumors tend to have more uniformly distributed vessels than implanted tumors, less necrosis, and their vessels have some features of those in their tissue of origin. For example, blood vessels in pancreatic islet tumors in RIP-Tag2 transgenic mice have endothelial fenestrations like the capillaries of normal pancreatic islets (Hashizume et al. 2000).

32.3.2 Sprouting

Tumor blood vessels are dynamic. They undergo continual growth, remodeling, or regression, and endothelial sprouts are a common feature in tumors (Fig. 32.2A,B). Endothelial sprouts have been carefully studied in growing blood vessels in the developing mesentery and retina (Rhodin and Fujita 1989; Gerhardt et al. 2003), where they consist of two types of specialized endothelial cells. The endothelial cells at the migrating tip of the sprout have long motile cellular projections termed filopodia. Filopodia are believed to detect gradients of growth factors such as vascular endothelial growth factor (VEGF) in a manner resembling axonal growth cones. They appear to sense the local gradient of growth factor and determine the direction of growth, whereas the endothelial stalk cells located just behind the tip undergo cell division and form a lumen (Gerhardt et al. 2003). Both processes are regulated by the distribution of VEGF and its receptors, with tip cell migration depending on the local gradient of VEGF, and proliferation of endothelial stalk cells being regulated by the concentration of VEGF. The precise cellular architecture of vascular sprouts may differ in tumors where filopodia form not only at the tips of growing vessels but also along the surface of tumor vessels (Fig. 32.2B). Imbalances between the formation of filopodia on tip cells and division of stalk cells and the distribution of VEGF and its receptors may contribute to the abnormal vascular patterns in tumors (Gerhardt and Betsholtz 2005).

32.3.3 Defective Blood Flow, Barrier Function, and Plasma Leakage

Leakiness is one of the abnormalities of tumor vessels that influences not only angiogenesis, tumor growth, and metastasis, but also drug delivery, and presents novel targets for anti-cancer therapy. The endothelial cells of tumor vessels form a defective barrier because of widened intercellular gaps, holes, and pores and multiple layers of disorganized, loosely connected, branched, overlapping, or sprouting endothelial cells (Hashizume et al. 2000) (Fig. 32.2A–C). Tumor blood vessels are an order of magnitude more leaky than normal blood vessels: the pore cutoff size is measured in hundreds, rather than in tens, of nanometers (Hobbs et al. 1998). The magnitude of leakage, which varies greatly among

different tumor types, is governed by hydrostatic driving forces as well as vascular surface area and permeability. VEGF-dependent ovarian tumors can produce huge amounts of ascites in mice and in humans (Hu et al. 2005), although this may not be due exclusively to leaky tumor vessels. The extravasation of proteins from tumor vessels is spatially and temporally heterogeneous and is actually less than might be predicted from the pore size (Hobbs et al. 1998). An abnormally small hydrostatic gradient across the vessel wall results from high interstitial pressure within tumors and reduces the driving force for convective movement. The high interstitial pressure in tumors is a consequence of leakage from the blood vessels coupled with an impaired fluid clearance through lymphatic vessels, and perhaps other factors (McDonald and Baluk 2002). One consequence of leakage from tumor vessels is the extravasation of fibrinogen and its conversion to deposits of fibrin in the tumor stroma (Brown et al. 1988; Nakahara et al. 2006). Blood vessels in tumors may also be prone to hemorrhage.

The leakiness of tumor vessels shows promise in clinical practice as a surrogate marker for estimating the aggressiveness or grade of tumors by using non-invasive approaches including dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI). When available for clinical use, macromolecular tracers such as albumin–gadolinium complexes or super-paramagnetic iron oxide particles will make DCE-MRI even more powerful (Daldrup-Link et al. 2006).

Endothelial cells of blood vessels in many types of tumor, like those of normal endocrine organs, gastrointestinal mucosa, choroid plexus, and choriocapillaris, have regularly arranged fenestrae, approximately 100 nm in diameter, usually covered by a thin diaphragm. Such fenestrations are involved in fluid and perhaps solute transport and are now believed to be dynamic rather than static structures. Capillaries of kidney glomeruli have abundant fenestrations that lack diaphragms. VEGF appears to be responsible for the induction and/or maintenance of endothelial fenestrations in normal blood vessels and in vessels at sites of pathology, including tumors (Roberts and Palade 1995, 1997; Esser et al. 1998). Inhibition of VEGF signaling results in the reduction or disappearance of endothelial fenestrations (Inai et al. 2004; Kamba et al. 2006).

32.3.4 Differential Gene Expression

Given the importance of angiogenesis for tumor growth, one strategy in the development of angiogenesis inhibitors has been the search for molecules that are differentially or exclusively expressed in angiogenic endothelial cells. Such molecules could be used to target tumor blood vessels, alone or conjugated to toxins, antibodies, or inhibitory molecules. Although theoretically attractive, a limitation of such an approach in practice is that many genes upregulated in tumor endothelial cells are also expressed in endothelial cells of normal organs or sites of angiogenesis in healing wounds or female reproductive organs. Molecules that are exclusively expressed in tumor blood vessels have proven difficult to find.

Several strategies have been used to hunt for specialized tumor vessel phenotypes. In one approach, clues from biochemical studies or in vitro models of angiogenesis have been extended to tumors grown in situ. These studies have elucidated specific cell-surface receptors, integrins, and cell-junctional molecules as candidate targets for angiogenesis inhibitors and as diagnostic markers for novel imaging techniques. Receptors for VEGF are abundant on angiogenic endothelial cells and may partially account for the action of certain receptor tyrosine kinase (RTK) inhibitors on tumor vessels. Several integrins, including $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha 4\beta 1$, and $\alpha 5\beta 1$, are overexpressed in tumor blood vessels (Eliceiri and Cheresh 2000; Jin and Varner 2004; Hwang and Varner 2004; Stupack and Cheresh 2004; Parsons-Wingerter et al. 2005; Magnussen et al. 2005; Garmy-Susini et al. 2005) (Fig. 32.2D). The role of αv integrins in angiogenesis is somewhat paradoxical, since mice deficient in $\beta 3$ or $\beta 5$ integrin exhibit extensive angiogenesis (Hynes 2002). Nevertheless, antibody and peptide inhibitors of integrins are currently in clinical trials for cancer (Jin and Varner 2004;

Hwang and Varner 2004). VEGF overexpression appears to weaken or open cell junctions in tumor vessels, which have been suggested to contribute to vessel leakiness and tumor metastasis (Corada et al. 2002; Weis and Cheresh 2005). Cell-junctional molecules are other potential targets for anti-angiogenic drugs, and antibodies to VE-cadherin and JAM-C inhibit angiogenesis and tumor growth (Corada et al. 2002, May et al. 2005; Lamagna et al. 2005; Cavallaro et al. 2005; Vincent et al. 2005).

Systematic approaches have identified proteins overexpressed on tumor vasculature. In vivo phage display methodology has led to the concept of specific molecular "vascular ZIP codes" (Pasqualini et al. 2002) and the identification, for example, of aminopeptidases A and N (CD13) as potential targets on tumor blood vessels (Pasqualini et al. 2000; Marchio et al. 2004; Hajitou et al. 2006). Serial analysis of gene expression (SAGE) has led to the identification of 46 novel human "tumor endothelial markers" (TEM) and corresponding mouse homologues (St. Croix et al. 2000; Nanda and St. Croix 2004). Some of these markers expressed on the endothelial cell surface may be useful for targeting angiogenesis inhibitors (Nanda and St. Croix 2004).

32.3.5 Pericyte Abnormalities

Some early studies reported that pericytes were sparse or absent from tumor vessels, but more recent studies using multiple immunohistochemical markers have shown that pericytes are present but abnormal (Morikawa et al. 2002). Most pericytes in tumors lack the normally intimate association with endothelial cells. Cytoplasmic processes of pericytes may extend into the tumor parenchyma (Fig. 32.2F). Pericytes may even be more abundant in tumors than in normal organs, but may be difficult to detect because of abnormalities in marker expression (Morikawa et al. 2002; Bergers and Song 2005; Abramsson et al. 2003). Despite their abnormalities, pericytes may play an important role in vessel stability in tumors (Pietras et al. 2001; Bergers et al. 2003; Uehara et al. 2003; Erber et al. 2004).

32.3.6 Defective Basement Membrane

The basement membrane of tumor vessels is defective in both composition and structure, with multiple layers only loosely associated with endothelial cells and pericytes, and broad extensions projecting away from the vessel (Baluk et al. 2003) (Fig. 32.2E). Angiogenesis inhibitors can stop the growth of tumor vessels and prune some existing vessels, but the loss of endothelial cells does not necessarily result in corresponding loss of surrounding basement membrane. Empty sleeves of basement membrane can provide a scaffold for rapid regrowth of tumors vessels after treatment ends (Mancuso et al. 2006). Rapid vascular regrowth reflects the potent drive for angiogenesis in tumors and illustrates how the bizarre microenvironment in tumors provides challenges for complete and irreversible destruction of tumor vessels.

Cellular Effects of Angiogenesis Inhibitors on Tumor Blood Vessels

The mechanism of action of anti-angiogenic agents is still not well understood at the cellular level. In the past their effects were assessed mainly by measuring tumor growth. More recently, some progress has been made by examining the effects of angiogenesis inhibitors on tumor blood vessels in animal models and in cancer patients. It has often been difficult to distinguish cause and effect and to identify meaningful readouts (Tozer 2003). Additionally, as many of the approaches are technically challenging, much effort has been devoted to finding suitable surrogate markers of anti-angiogenic effects. In view of the expanding range of angiogenesis inhibitors, different agents are unlikely to have the same cellular actions. Also, angiogenesis inhibitors have multiple effects that do not all have the same therapeutic relevance. VEGF inhibitors may influence

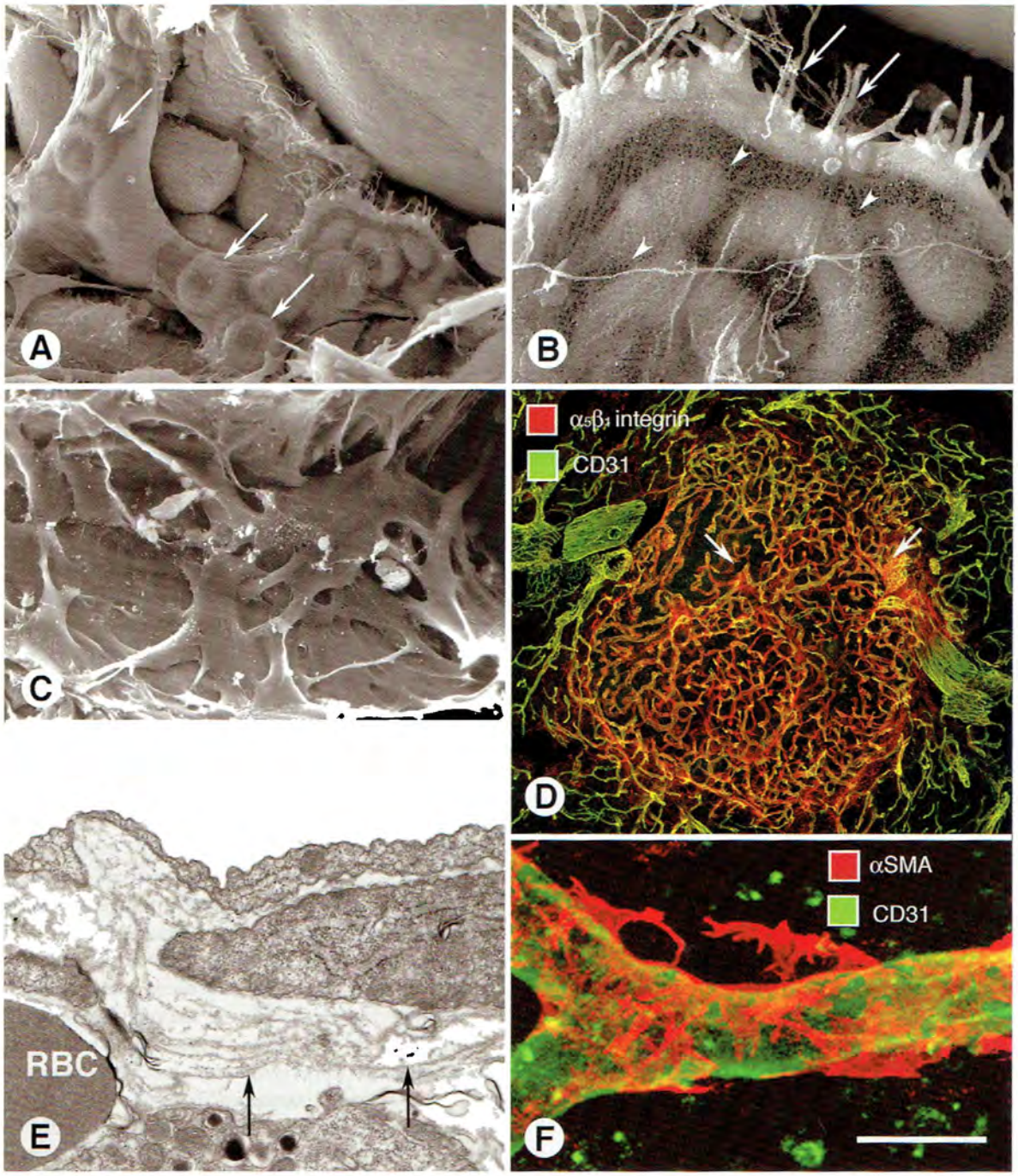


Fig. 32.2A–F. Structural abnormalities of tumor blood vessels. **A** Scanning electron micrograph of a blood vessel in a RIP-Tag2 tumor made visible by hydrolytic removal of connective tissue matrix. Intravascular erythrocytes are visible through the thin blood vessel wall (*arrows*). Cellular processes from a pericyte are loosely associated with the vessel. **B** Enlargement shows endothelial cell filopodia (*arrows*) and fenestrations (*arrowheads*). **C** Scanning electron micrograph of luminal aspect of a defective endothelial cell lining of blood vessel in a MCa-IV tumor (from Hashizume et al. 2000). **D** Overexpression of α5β1 integrin on blood vessels in tumor (*arrows*) in RIP-Tag2 mouse (from Magnussen et al. 2005). **E** Transmission electron micrograph of blood vessel wall in RIP-Tag2 tumor showing multiple layers of basement membrane (*arrows*) and extravasated erythrocyte (*RBC*) (from Baluk et al. 2003). **F** Confocal microscopic image of abnormal pericytes (*red*) loosely associated with endothelial cells (*green*) of blood vessel in a MCa-IV tumor (from Morikawa et al. 2002). *Scale bar*: A 12 μm, B 5 μm, C 40 μm, D 500 μm, E 2 μm, F 20 μm

endothelial cell survival, migration, growth, plasma leakage, blood flow and recruitment of leukocytes and stem cells. Proximal events such as vasodilatation may influence distal events such as plasma leakage.

The actions of angiogenesis inhibitors fall into three categories: (1) inhibition of the growth of new blood vessels from existing vessels; (2) regression of tumor vessels; and (3) 'normalization' of the phenotype of blood vessels that survive the treatment. The factors that determine which of these effects are most important are as yet poorly defined.

32.4.1 Inhibition of Blood Vessel Growth in Tumors

32.4.1.1 Inhibition of Pro-Angiogenic Signaling

By definition, angiogenesis inhibitors block endothelial cell sprouting and proliferation. The best understood of these inhibitors block the action of VEGF by absorbing the ligand by anti-VEGF antibodies, soluble receptors, or oligonucleotide aptamers or by inhibiting VEGF receptors by antibodies or small-molecule inhibitors of tyrosine kinase phosphorylation. Inhibition of pro-angiogenic signaling shifts the balance in favor of endothelial cell apoptosis and regression.

32.4.1.2 Endothelial Cell Proliferation

The vascularity of tumors is controlled by the balance of angiogenesis inducers and inhibitors (Iruela-Arispe and Dvorak 1997). Multiple angiogenic factors work together in a coordinated manner to induce endothelial cell growth and the formation of functional vessels. Many types of tumor cells secrete factors that drive endothelial cell proliferation; pericytes and stromal cells also make a contribution. One approach to inhibiting endothelial cell division is to block the signaling pathways of mitogens such as VEGF. Another approach is to stabilize or depolymerize microtubules essential for formation of mitotic spindles in dividing cells (Jordan and Wilson 2004). Actively proliferating cells in which nuclear division is arrested eventually undergo apoptosis. The synthetic taxanes paclitaxel and docetaxel and the naturally occurring *Vinca* alkaloids vinblastine and vincristine are examples of agents that block microtubule dynamics and thereby act as anti-mitotic agents. Other anti-cancer drugs such as combretastatin appear to exert at least part of their effect by causing vascular collapse by rapidly depolymerizing microtubules in endothelial cells (Jordan and Wilson 2004) and by interfering with their signaling via endothelial-specific VE-cadherin junctional molecules (Vincent et al. 2005). Anti-mitotic drugs can apparently disrupt microtubule dynamics at lower doses in endothelial cells than in tumor cells, and can thus manifest a selective anti-angiogenic effect (Pasquier et al. 2005).

32.4.1.3 Reduction of Vascular Sprouting

As local VEGF gradients and concentrations influence the activities of both the tip and stalk cells of endothelial sprouts (Gerhardt and Betsholtz 2003), it is perhaps not surprising that many agents that interfere with VEGF signaling, e.g. by absorbing VEGF or inhibiting VEGF receptor phosphorylation, not only inhibit the formation of new endothelial sprouts but also cause the regression of existing sprouts (Inai et al. 2004).

32.4.1.4 Blocking Changes in Extracellular Matrix

The integrity of tumor blood vessels is influenced by the extracellular matrix and other components of the tumor stroma (de Wever and Mareel 2003). Broad-spectrum matrix metalloproteinase inhibitors, although effective in reducing blood vessel growth in various animal tumor models, have generally been disappointing in clinical trials (Rundhaug 2005). This may be partially explained by matrix metalloproteinases having both pro-and anti-angiogenic actions (Rundhaug 2005).

Targeting the interface of blood vessels and the surrounding matrix by inhibiting binding of matrix ligands to endothelial integrins may prove more effective (Ruegg et al. 2004, Akalu et al. 2005; Serini et al. 2006). Inhibition of N-cadherin-mediated junctions between endothelial cells and pericytes or between adjacent tumor cells is another approach (Erez et al. 2004). Macrophages and other inflammatory cells in the tumor stroma produce growth factors, cytokines, chemokines, and enzymes that regulate angiogenesis, invasion, and tumor growth and/or metastasis (Balkwill and Coussens 2004; Lewis and Pollard 2006). Fibroblasts in tumors are not only sources of pro- and anti-angiogenic growth factors and cytokines, but also contribute to the composition of the extracellular matrix, and can reportedly transform into pericytes or myofibroblasts (Kalluri and Zeisberg 2006).

32.4.2 Regression of Tumor Blood Vessels

32.4.2.1 Cessation of Blood Flow, Endothelial Cell Apoptosis, and Reduction in Vascular Density

Angiogenesis inhibitors were originally believed to be able to shut down blood flow in tumors. The assumption was made that cessation of blood flow would lead to endothelial cell death by apoptosis or necrosis, vessel regression, and subsequent death of tumor cells. Remarkably, vascular disrupting agents such as combretastatin and other microtubule-disrupting agents shut down blood flow in certain tumors within minutes (Tozer et al. 2005; Thorpe 2004). Blood flow in tumors has also been stopped by inducing intravascular coagulation by directing tissue factor to antigenic epitopes selectively expressed on tumor blood vessels (Huang et al. 1997). Growth factors such as VEGF not only induce the growth of new blood vessels, but also regulate endothelial cell survival in existing vessels (Folkman 2003c; Sakamaki 2004). Removal or blockade of these survival molecules by inhibitors of VEGF or bFGF, or with endogenous inhibitors thrombospondin-1, endostatin, angiostatin, and tumstatin, decreases the patency of tumor vessels and increases apoptosis of endothelial cells (Fig. 32.3A–F) (Inai et al. 2004; Sund et al. 2005).

Inhibition of VEGF signaling can decrease the expression of endothelial cell markers in tumor vessels (Fig. 32.3G–I) and decrease the vascular area density (Fig. 32.4). Cytotoxic chemotherapy can also cause endothelial apoptosis that precedes death of tumor cells (Folkman 2003c). The direct pro-apoptotic effects of cytotoxic chemotherapeutic drugs on proliferating vascular endothelium can contribute to their anti-cancer action. The elimination of a population of tumor vessels is one of the earliest detectable actions of many angiogenesis inhibitors in vivo and can precede any noticeable changes in tumor cells themselves. However, caution is needed in interpretation of changes in vascular density alone, as the vascularity of a tumor can increase, decrease, or even stay the same during treatment with an angiogenesis inhibitor (Hlatky et al. 2002).

32.4.2.2 Regression of Pericytes and Dual Targeting Therapies

Until recently, most anti-angiogenic therapies sought to target endothelial cells exclusively. Since the recognition that pericytes are present on tumor blood vessels, several studies have shown that pericytes are an important complementary target to endothelial cells for anti-angiogenic therapy (Bergers and Song 2005; Bergers et al. 2003). Numerous studies targeting pericytes directly or by inhibitingthe pericyte-dependent platelet-derived growth factor (PDGF) signaling pathway have shown reductions in angiogenesis and tumor growth (Hwang et al. 2003; Roberts et al. 2005; Ozerdem 2006). In addition, several studies have shown greater regression of tumor cells when both endothelial cells (VEGF inhibition) and pericytes (PDGF inhibition) are targeted than when either cell type is targeted alone (Bergers et al. 2003; Erber et al. 2004).

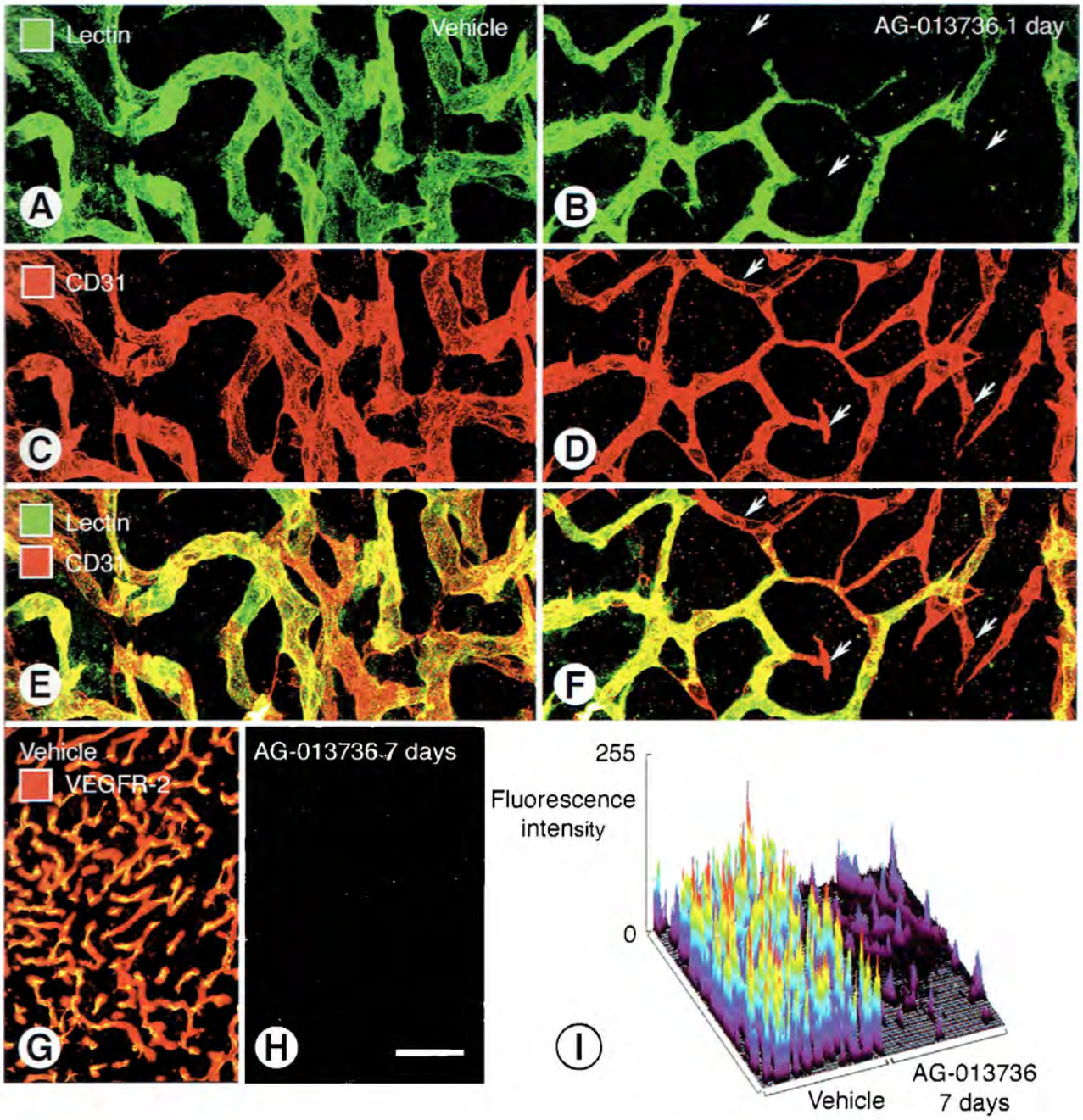


Fig. 32.3A–I. Regression of tumor vessels after inhibition of VEGF signaling. Most blood vessels in untreated RIP-Tag2 tumor (A, C, E) have a patent lumen as shown by presence of *Lycopersicon esculentum* lectin (*green*) circulating in the bloodstream. Endothelial cells are marked by CD31 immunoreactivity (*red*). In contrast, only 1 day after inhibition of VEGF signaling (B, D, F), many vessels are narrowed and lack blood flow, as shown by absence of lectin staining (*arrows*). G, I Down-regulation of VEGFR-2. Brightness of VEGFR-2 immunoreactivity is reduced in blood vessels of RIP-Tag2 tumor after treatment with VEGF inhibitor. G Vehicle-treated tumor; H tumor treated with AG-013736 for 7 days. I Surface plot of VEGFR-2 fluorescence intensity corresponding to panels G and H. Decreased vessel number and receptor expression in treated tumor are illustrated by fewer and lower peaks (from Inai et al. 2004). *Scale bar*: A–F 25 μm; G, H 80 μm

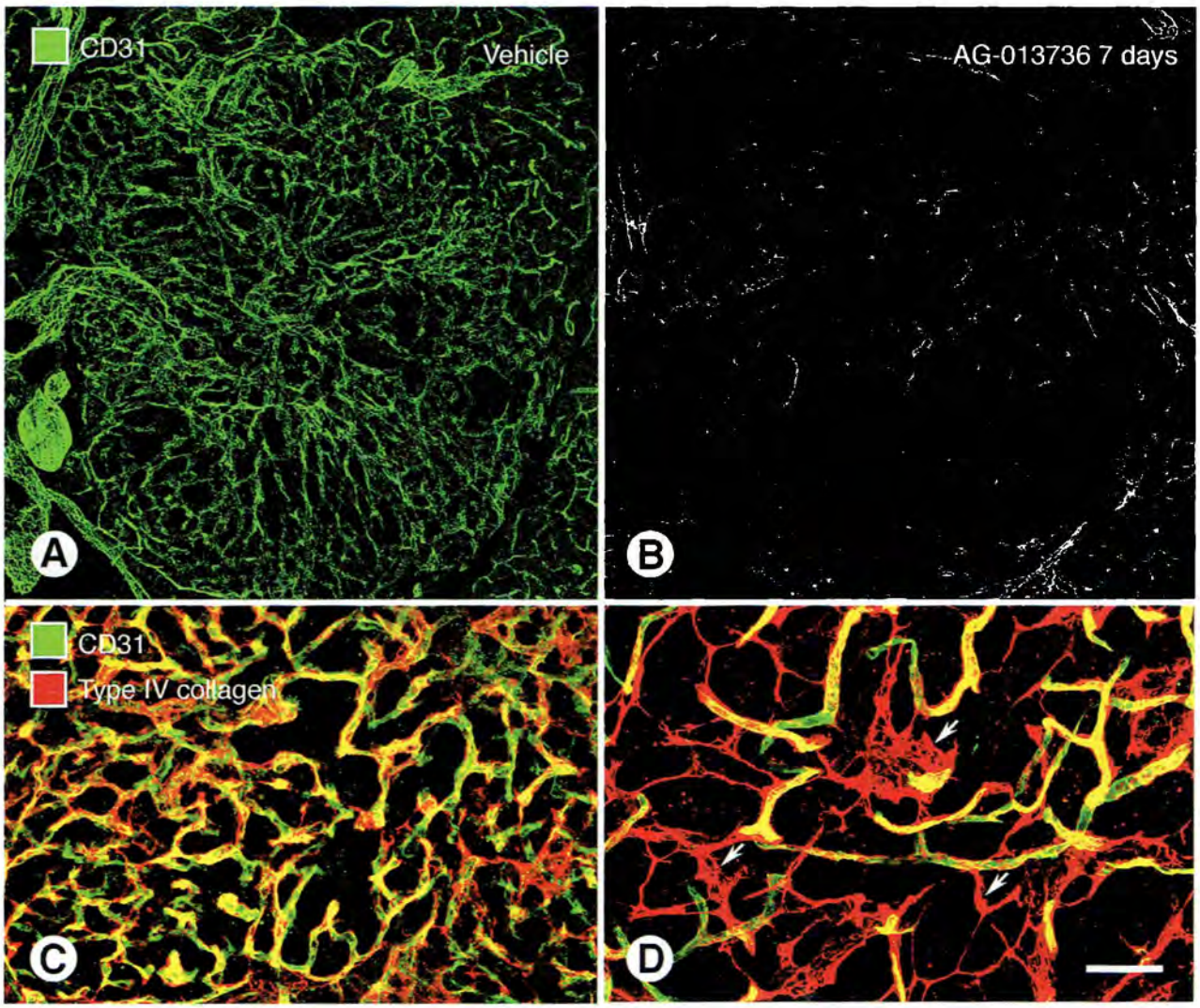


Fig. 32.4A–D. Regression of tumor blood vessels after treatment with inhibitor of VEGF receptors, AG-013736. A, B Confocal microscopic images showing reduction of overall vascular density in confocal microscopic image of RIP-Tag2 tumor after treatment with VEGF receptor inhibitor for 7 days. A Vehicle-treated tumor; B Inhibitor-treated tumor. Endothelial cells stained for CD31 (*green*). C, D Basement membrane sleeves remain as historical record of eliminated blood vessels. RIP-Tag2 tumor stained for endothelial cell marker CD31 (*green*) and type IV collagen of basement membrane (*red*). C In vehicle-treated tumor, CD31 and type IV collagen immunoreactivities are colocalized in most blood vessels. D In contrast, in tumor treated with VEGF receptor inhibitor for 7 days, blood vessel density is markedly reduced and many narrow empty basement membrane sleeves (*red, arrows*) remain (from Inai et al. 2004). *Scale bar*: A, B 130μm; C, D 50 μm

32.4.3 Normalization of Surviving Tumor Blood Vessels

32.4.3.1 Normalization of Endothelial Cells of Tumor Blood Vessels

The concept of "normalization" of tumor blood vessels by anti-angiogenic drugs was introduced by Rakesh Jain as an alternative hypothesis to the view that such drugs acted solely by inducing hypoxia (Jain 2001). It was proposed that such agents could transiently "normalize" the abnormal structure and function of tumor vessels so they provide improved blood flow and more efficient local delivery of oxygen and therapeutic agents to tumor cells, thereby increasing their sensitivity to radiation and chemotherapy (Jain 2005). Inhibition of growth factor signaling may have additional consequences, suggested by the finding that angiogenic factors are protective against radiation damage (Okunieff et al. 1998). Anti-angiogenic drugs can also normalize the overall architecture, diameter, pericyte coverage, basement membrane, and permeability of tumor vessels (Jain 2005) (Fig. 32.5).

The population of blood vessels surviving anti-angiogenic treatment resembles normal vessels more than conventional tumor vessels. They are straighter, more uniform in caliber, and less branched than tumor vessels (Fig. 32.5A,B). Normalization of tumor blood vessels does not simply reflect pruning of abnormal vessels to reveal a few previously hidden relatively normal vessels. The surviving vessels have features different from any vessels present before treatment.

Normalization of tumor vessels may prove beneficial for anti-cancer therapy. Blockade of VEGFR-2 decreases leakiness, increases blood flow, and improves the delivery of macromolecular therapeutics by restoring the gradient in driving force between blood vessel lumen and interstitium (Tong et al. 2004). Other anti-angiogenic drugs such as TNP-470 and angiostatin also appear to reduce the permeability of tumor blood vessels, and this action may contribute to their anti-tumor activity (Satchi-Fainaro et al. 2005).

After inhibition of VEGF signaling, loss of endothelial fenestrations (Fig. 32.5C,D) (Inai et al. 2004) precedes the loss of the blood vessels and could be a useful surrogate marker of their sensitivity to VEGF inhibitors. Surviving blood vessels of RIP-Tag2 tumors treated with anti-VEGF agents have reduced expression of VEGFR-2 and VEGFR-3, in addition to a more normal caliber (Inai et al. 2004). The normalization of endothelial cell surface receptors is not an isolated change related to VEGF biology, as other endothelial cell surface proteins, e.g. $\alpha 5\beta 1$ integrin, that are overexpressed on tumor vessels also decrease to more normal values (Yao et al. 2006).

32.4.3.2 Normalization of Pericytes, Basement Membrane and Extracellular Matrix

Treatment with VEGF inhibitors causes pericytes to become closely associated with surviving tumor vessels in Lewis lung carcinomas, RIP-Tag2 tumors (Inai et al. 2004) (Fig. 32.5E,F), and other tumor models (Tong et al. 2004; Willett et al. 2004). Further work is needed to determine how much of the increase in pericyte coverage is due to the preferential pruning of pericyte-poor vessels (Benjamin et al. 1999) or to the recruitment of pericytes to tumor vessels by upregulation of PDGF or angiopoietin-1 (Winkler et al. 2004).

Inhibition of the factors leading to an abnormal tumor stroma may permit a swing in the balance of proteolytic and anti-proteolytic processes, such that the extracellular matrix becomes more normal in structure and function, having reduced matrix proteases (Miller et al. 2005; Vosseler et al. 2005) and fewer immune cells (Balkwill and Coussens 2004; van Kempen et al. 2006).

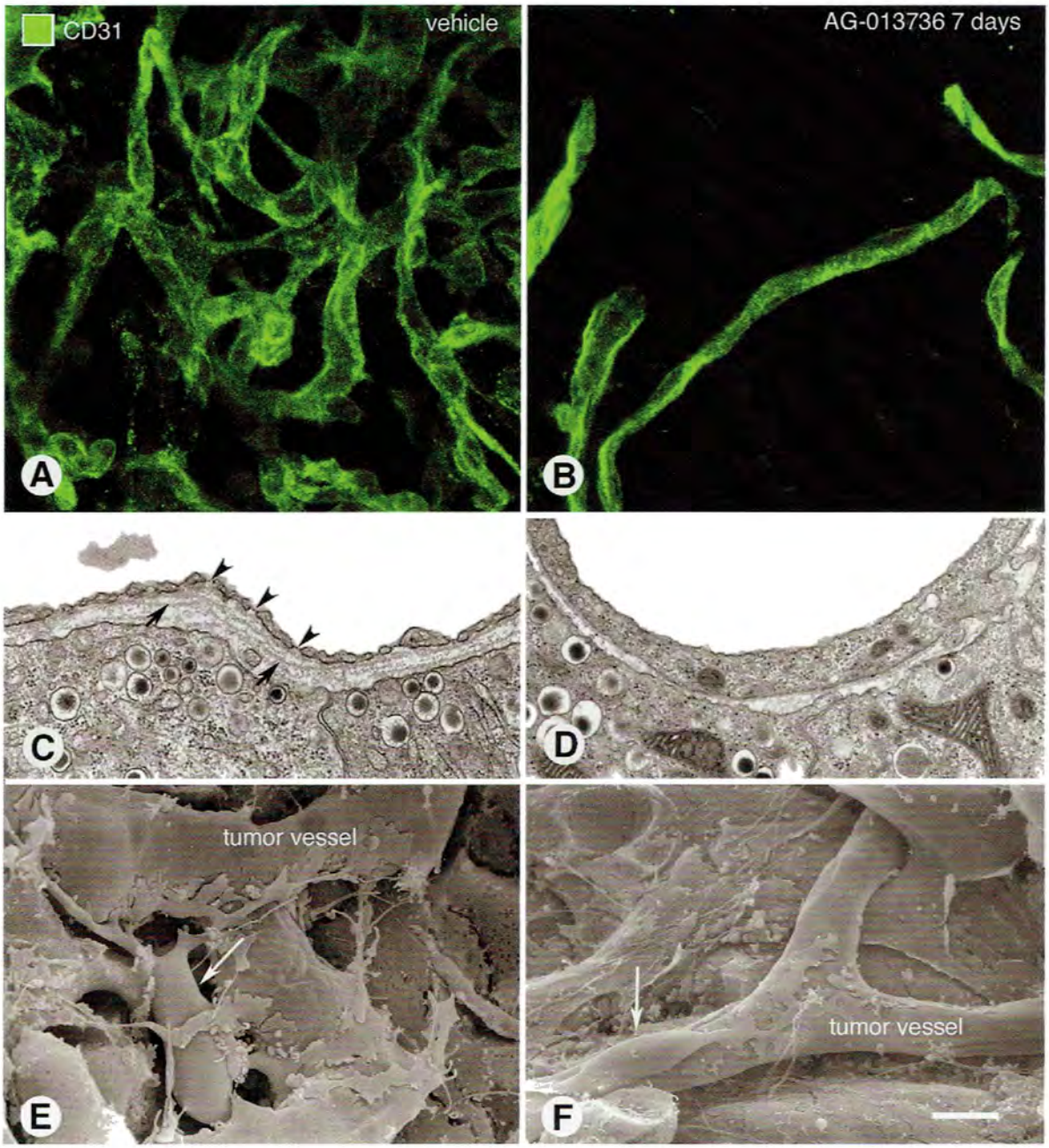


Fig. 32.5A–F. Normalization of vascular features in RIP-Tag2 tumor after treatment with VEGF receptor inhibitor AG-013736 for 7 days. A, B Confocal images showing normalization of vessel branching and caliber. **A** In vehicle-treated tumor, vessels branch and interconnect in a complicated manner and vary in diameter. **B** In AG-013736 treated tumor, vessels are far fewer in number, have much less branching and have a regular caliber. Endothelial cells stained for CD31 (*green*). **C, D** Transmission electron micrographs showing normalization of endothelial cell. **C** In untreated tumor, endothelium is thin and fenestrated (*arrowheads*) and basement membrane has multiple layers (*arrows*). **D** In treated tumor, endothelium is thicker and lacks fenestrations. Basement membrane consists of a single layer (from Inai et al. 2004). **E, F** Scanning electron micrographs showing normalization of pericyte–endothelial relationships (from Baluk et al. 2005). **E** In untreated tumor, pericyte cell body (*arrow*) and processes are only loosely associated with endothelial cells. **F** Pericyte (*arrow*) is more intimately associated with endothelium in treated tumor. *Scale bar*: **A, B** 20 μm; **C, D** 1 μm; E 10 μm; F 8 μm

Effects of VEGF Inhibitors on Normal Blood Vessels

Angiogenesis and vascular regression occur not only in tumors, but also during the physiological processes of female reproductive and hair growth cycles (Fraser 2006; Hoffman 2005) and in pathological processes of wound healing (Dvorak 2005), rheumatoid arthritis (Clavel et al. 2003), asthma and other chronic inflammatory conditions of the respiratory tract (McDonald 2001; Tang et al. 2006), and macular degeneration in the eye (Witmer et al. 2003).

Angiogenesis inhibitors can be a two-edged sword. Although they inhibit angiogenesis in disease, they can also inhibit desirable forms of angiogenesis (McCarty and Ellis 2002). In general, the underlying nature of these various forms of angiogenesis and their sensitivity to angiogenic inhibitors are poorly understood.

Furthermore, VEGF inhibitors can modify the baseline phenotype and even cause regression of certain normal adult blood vessels, where there is no evidence of active vessel growth. Examples of such effects range from relatively mild to severe: a change of vascular phenotype from a fenestrated endothelium to a non-fenestrated endothelium (Kamba et al. 2006), or complete cessation of blood flow and elimination of blood vessels (Baffert et al. 2006). Different vascular beds have varying sensitivity to elimination of selected blood vessels by VEGF inhibitors; endocrine organs with fenestrated capillaries are among the most sensitive, while most vessels in brain appear to be unaffected (Fig. 32.6A–H). The effects of VEGF inhibitors on normal vessels resemble those on tumor vessels. Fibrin accumulates within vessels that have no blood flow (Fig. 32.6I–K). Such effects of VEGF inhibitors on sensitive mature normal vessels may be best understood in the context of the relative importance of VEGF biology in particular organs, such as local concentration of VEGF and distribution and phosphorylation state of VEGF receptors (Maharaj et al. 2006).

Conventional isoforms of VEGF may not tell the whole story. For example, an endogenous splice variant, VEGF165b, has been reported to be anti-angiogenic (Woolard et al. 2004). Furthermore, blood vessels in different organs may be dependent on other growth factors. Endocrine gland VEGF (EG-VEGF), although unrelated to other members of the VEGF family, despite its name, is expressed selectively in endocrine glands, where it appears to work in concert with conventional VEGF to induce fenestrations (LeCouter et al. 2001).

The large, numerous fenestrations without diaphragms in kidney glomerular capillaries are also highly dependent on VEGF, and neutralization of VEGF or inhibition of VEGF signaling results in disappearance of glomerular fenestrations and proteinuria within hours (Kamba et al. 2006; Sugimoto et al. 2003) (Fig. 32.6L,M). Other physiological consequences of cellular changes induced by angiogenesis inhibitors in normal blood vessels are just beginning to be investigated systematically (Kamba et al. 2006). At the very least, there is a need for better understanding of the differences between blood vessels in tumors and normal organs in their dose–response characteristics to VEGF inhibitors (Betsholtz and Armulik 2006).

Summary

Tumor blood vessels have multiple abnormalities. They are irregularly shaped, tortuous, and lack the hierarchical arrangement of normal arterioles, capillaries, and venules. Their endothelial cells are poorly connected by intercellular junctions, pericytes are loosely attached, and the vascular basement membrane has multiple redundant layers. Wall defects make tumor vessels leaky to plasma proteins and prone to hemorrhage. Tumor vessels are dynamic and continually undergo proliferation, remodeling, and regression. Sprouting occurs not only at the tips of tumor vessels but also in a disorganized manner along their length. Endothelial cells of many tumor vessels require VEGF or other growth factors for survival, which makes them sensitive to

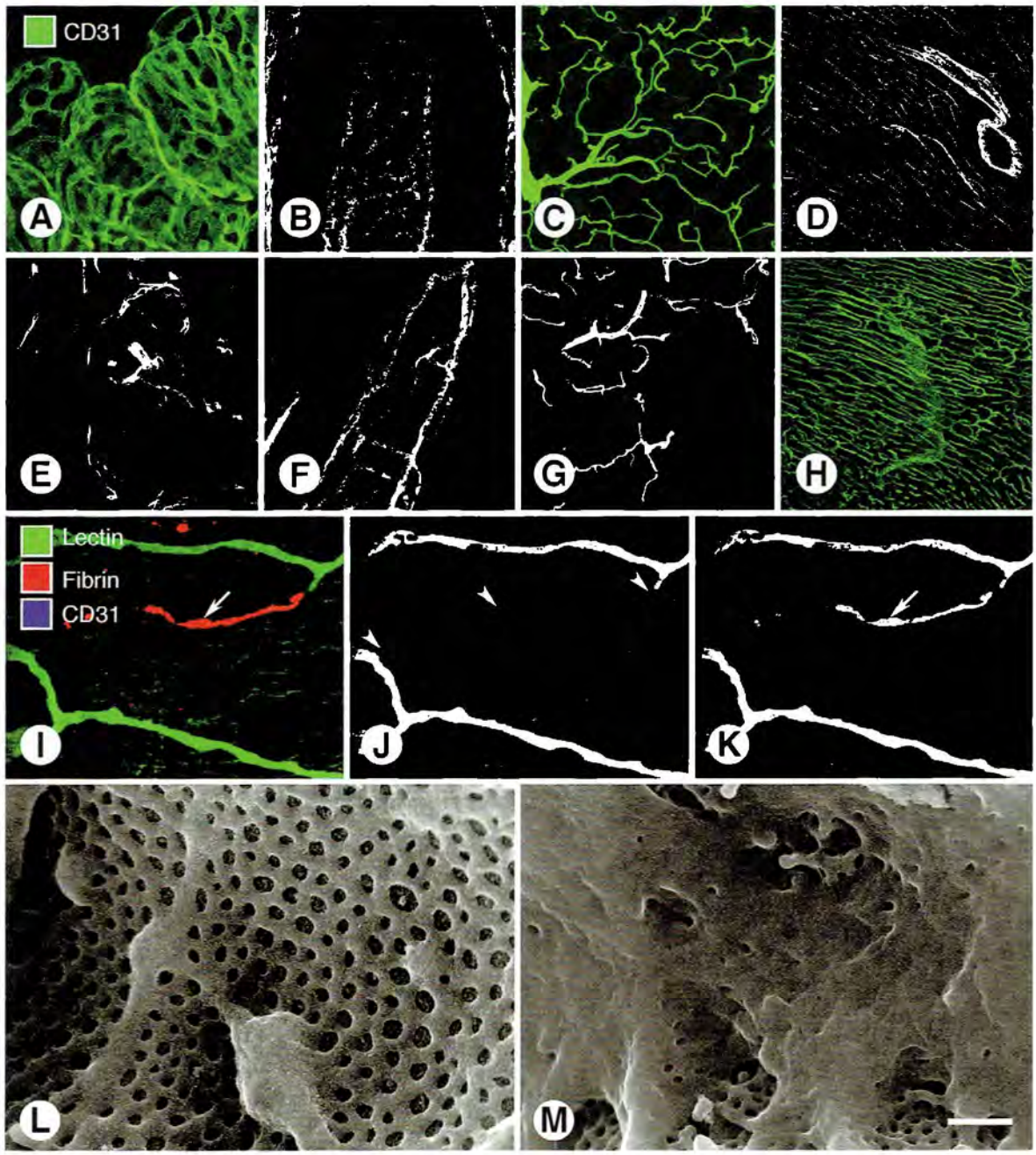


Fig. 32.6A–M. Effects of VEGF inhibitors on normal blood vessels. A–H Reduction of VEGF-dependent capillaries in organs of adult mice 14 days after inhibition of VEGF signaling by systemic treatment with adenoviral delivery of soluble VEGFR-1 (from Kamba et al. 2006). I–K Intravascular fibrin deposition in non-patent region (*arrow*) of tracheal capillary 1 day after treatment with VEGF inhibitor AG-013736 (from Baffert et al. 2006). I Fibrin deposit in capillary that has no blood flow (*arrow*), as show by lack of lectin staining. J Same capillary segment has non-uniform CD31 staining (*arrowheads*) suggestive of early endothelial regression. K Fibrin deposit coincides with region of interrupted CD31 immunoreactivity (*arrow*). L, M Reduction of endothelial fenestrations in kidney glomerular capillaries 14 days after systemic treatment with adenovirally delivered soluble VEGFR-1 (Ad-sVEGFR-1). L Control virus-treated mouse. M Ad-sVEGFR-1-treated mouse. (from Kamba et al. 2006). *Scale bar*: A, B, E, F 50 μm; C, D, G, H 100 μm; I–K 40 μm; L, M 0.3 μm

inhibitors of VEGF signaling. Endothelial cells of normal capillaries with endothelial fenestrations, which are abundant in endocrine glands, gastrointestinal tract, and kidney, may also have VEGF as a survival factor. Inhibition of VEGF signaling in tumor vessels can stop the growth of new vessels, cause regression of existing vessels, and normalize surviving vessels. Loss of the vascular lumen and cessation of blood flow are followed by endothelial cell apoptosis and regression. Loss of endothelial cells may not be accompanied by loss of pericytes and basement membrane, which can thereby provide a scaffold for vessel regrowth after anti-VEGF therapy is withdrawn.

Acknowledgements

Supported in part by the US National Institutes of Health grants HL-24136 and HL-59157 from the National, Heart, Lung, and Blood Institute and CA-082923 from the National Cancer Institute, and funding from AngelWorks Foundation to D.McD. and a fellowship from the California Tobacco-Related Disease Research Program (14FT-0152) to B.F. We thank Amy Haskell for skillful transmission electron microscopy and former members of the McDonald laboratory Fabienne Baffert, Tetsuo Inai, Tomomi Kamba, Ian Kasman, Anette Magnussen and Patricia Parsons-Wingerter for confocal microscopic images.

References

Abramsson A, Lindblom P, Betsholtz C (2003) Endothelial and nonendothelial sources of PDGF-B regulate pericyte recruitment and influence vascular pattern formation in tumors. J Clin Invest 112:1142–1151

Akalu A, Cretu A, Brooks PC (2005) Targeting integrins for the control of tumour angiogenesis. Exp Opin Invest Drugs 14:1475–1486

Allt G, Lawrenson JG (2001) Pericytes: cell biology and pathology. Cells Tissues Org 169:1–11

Armulik A, Abramsson A, Betsholtz C (2005) Endothelial/pericyte interactions. Circ Res 97:512–523

Augustin HG (2005) Angiogenesis in the female reproductive system. Exs 94:35–52

Baffert F, Le T, Sennino B, Thurston G, Kuo CJ, Hu-Lowe D, McDonald DM (2006) Cellular changes in normal blood capillaries undergoing regression after inhibition of VEGF signaling. Am J Physiol Heart Circ Physiol 290:H547–H559

Balkwill F, Coussens LM (2004) Cancer: an inflammatory link. Nature 431:405–406

Baluk P, Morikawa S, Haskell A, Mancuso M, McDonald DM (2003) Abnormalities of basement membrane on blood vessels and endothelial sprouts in tumors. Am J Pathol 163:1801–1815

Baluk P, Lee CG, Link H, Ator E, Haskell A, Elias JA, McDonald DM (2004) Regulated angiogenesis and vascular regression in mice overexpressing vascular endothelial growth factor in airways. Am J Pathol 165:1071–1085

Baluk P, Hashizume H, McDonald DM (2005) Cellular abnormalities of blood vessels as targets in cancer. Curr Opin Genet Dev 15:102–111

Benjamin LE, Golijanin D, Itin A, Pode D, Keshet E (1999) Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. J Clin Invest 103:159–165

Bergers G, Song S (2005) The role of pericytes in blood-vessel formation and maintenance. Neuro-oncol 7:452–464

Bergers G, Song S, Meyer-Morse N, Bergsland E, Hanahan D (2003) Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. J Clin Invest 111:1287–1295

Betsholtz C, Armulik A (2006) Homeostatic functions of vascular endothelial growth factor in adult microvasculature. Am J Physiol Heart Circ Physiol 290:H509–H511

Betsholtz C, Lindblom P, Gerhardt H (2005) Role of pericytes in vascular morphogenesis. EXS 94:115–125

Brown LF, van de Water L, Harvey VS, Dvorak HF (1988) Fibrinogen influx and accumulation of cross-linked fibrin in healing wounds and in tumor stroma. Am J Pathol 130:455–465

Carmeliet P (2005) Angiogenesis in life, disease and medicine. Nature 438:932–936

Cavallaro U, Liebner S, Dejana E (2005) Endothelial cadherins and tumor angiogenesis. Exp Cell Res 312:659–667

Clavel G, Bessis N, Boissier MC (2003) Recent data on the role for angiogenesis in rheumatoid arthritis. Joint Bone Spine 70:321–326

Corada M, Zanetta L, Orsenigo F, Breviario F, Lampugnani MG, Bernasconi S, Liao F, Hicklin DJ, Bohlen P, Dejana E (2002) A monoclonal antibody to vascular endothelial-cadherin inhibits tumor angiogenesis without side effects on endothelial permeability. Blood 100:905–911

Daldrup-Link HE, Simon GH, Brasch RC (2006) Imaging of tumor angiogenesis: current approaches and future prospects. Curr Pharm Des 12:2661–2672

De Wever O, Mareel M (2003) Role of tissue stroma in cancer cell invasion. J Pathol 200:429–447

Dvorak HF (2005) Angiogenesis: update 2005. J Thromb Haemost 3:1835–1842

Dvorak HF, Nagy JA, Dvorak JT, Dvorak AM (1988) Identifica-

tion and characterization of the blood vessels of solid tumors that are leaky to circulating macromolecules. Am J Pathol 133:95–109

Eliceiri BP, Cheresh DA (2000) Role of alpha v integrins during angiogenesis. Cancer J 6 [Suppl 3]:S245–249

Erber R, Thurnher A, Katsen AD, Groth G, Kerger H, Hammes HP, Menger MD, Ullrich A, Vajkoczy P (2004) Combined inhibition of VEGF and PDGF signaling enforces tumor vessel regression by interfering with pericyte-mediated endothelial cell survival mechanisms. FASEB J 18:338–340

Erez N, Zamir E, Gour BJ, Blaschuk OW, Geiger B (2004) Induction of apoptosis in cultured endothelial cells by a cadherin antagonist peptide: involvement of fibroblast growth factor receptor-mediated signalling. Exp Cell Res 294:366–378

Esser S, Wolburg K, Wolburg H, Breier G, Kurzchalia T, Risau W (1998) Vascular endothelial growth factor induces endothelial fenestrations in vitro. J Cell Biol 140:947–959

Folkman J (2003a) Angiogenesis inhibitors: a new class of drugs. Cancer Biol Ther 2:S127–S133

Folkman J (2003b) Fundamental concepts of the angiogenic process. Curr Mol Med 3:643–651

Folkman J (2003c) Angiogenesis and apoptosis. Semin Cancer Biol 13:159–167

Fraser HM (2006) Regulation of the ovarian follicular vasculature. Reprod Biol Endocrinol 4:18

Garmy-Susini B, Jin H, Zhu Y, Sung RJ, Hwang R, Varner J (2005) Integrin alpha4beta1-VCAM-1-mediated adhesion between endothelial and mural cells is required for blood vessel maturation. J Clin Invest 115:1542–1551

Gaya AM, Rustin GJ (2005) Vascular disrupting agents: a new class of drug in cancer therapy. Clin Oncol (R Coll Radiol) 17:277–290

Gerhardt H, Betsholtz C (2003) Endothelial-pericyte interactions in angiogenesis. Cell Tissue Res 314:15–23

Gerhardt H, Betsholtz C (2005) How do endothelial cells orientate? EXS 94:3–15

Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, Jeltsch M, Mitchell C, Alitalo K, Shima D, Betsholtz C (2003) VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. J Cell Biol 161:1163–1177

Hajitou A, Pasqualini R, Arap W (2006) Vascular targeting: recent advances and therapeutic perspectives. Trends Cardiovasc Med 16:80–88

Hashizume H, Baluk P, Morikawa S, McLean JW, Thurston G, Roberge S, Jain RK, McDonald DM (2000) Openings between defective endothelial cells explain tumor vessel leakiness. Am J Pathol 156:1363–1380

Hirschi KK, d'Amore PA (1996) Pericytes in the microvasculature. Cardiovasc Res 32:687–698

Hlatky L, Hahnfeldt P, Folkman J (2002) Clinical application of antiangiogenic therapy: microvessel density, what it does and doesn't tell us. J Natl Cancer Inst 94:883–893

Hobbs SK, Monsky WL, Yuan F, Roberts WG, Griffith L, Torchilin VP, Jain RK (1998) Regulation of transport pathways in tumor vessels: role of tumor type and microenvironment. Proc Natl Acad Sci USA 95:4607–4612

Hoffman RM (2005) In vivo cell biology of cancer cells visualized with fluorescent proteins. Curr Top Dev Biol 70:121–144

Hu L, Hofmann J, Holash J, Yancopoulos GD, Sood AK, Jaffe RB (2005) Vascular endothelial growth factor trap combined with paclitaxel strikingly inhibits tumor and ascites, prolonging survival in a human ovarian cancer model. Clin Cancer Res 11:6966–6971

Huang X, Molema G, King S, Watkins L, Edgington TS, Thorpe PE (1997) Tumor infarction in mice by antibody-directed targeting of tissue factor to tumor vasculature. Science 275:547–550

Hwang R, Varner J (2004) The role of integrins in tumor angiogenesis. Hematol Oncol Clin North Am 18:991–1006

Hwang RF, Yokoi K, Bucana CD, Tsan R, Killion JJ, Evans DB, Fidler IJ (2003) Inhibition of platelet-derived growth factor receptor phosphorylation by STI571 (Gleevec) reduces growth and metastasis of human pancreatic carcinoma in an orthotopic nude mouse model. Clin Cancer Res 9:6534–6544

Hynes RO (2002) A reevaluation of integrins as regulators of angiogenesis. Nat Med 8:918–921

Inai T, Mancuso M, Hashizume H, Baffert F, Haskell A, Baluk P, Hu-Lowe DD, Shalinsky DR, Thurston G, Yancopoulos GD, McDonald DM (2004) Inhibition of vascular endothelial growth factor (VEGF) signaling in cancer causes loss of endothelial fenestrations, regression of tumor vessels, and appearance of basement membrane ghosts. Am J Pathol 165:35–52

Iruela-Arispe ML, Dvorak HF (1997) Angiogenesis: a dynamic balance of stimulators and inhibitors. Thromb Haemost 78:672–677

Jain RK (2001) Normalizing tumor vasculature with anti-angiogenic therapy: a new paradigm for combination therapy. Nat Med 7:987–989

Jain RK (2005) Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science 307:58–62

Jin H, Varner J (2004) Integrins: roles in cancer development and as treatment targets. Br J Cancer 90:561–565

Jordan MA, Wilson L (2004) Microtubules as a target for anticancer drugs. Nat Rev Cancer 4:253–265

Kalluri R (2003) Basement membranes: structure, assembly and role in tumour angiogenesis. Nat Rev Cancer 3:422–433

Kalluri R, Zeisberg M (2006) Fibroblasts in cancer. Nat Rev Cancer 6:392–401

Kamba T, Tam BY, Hashizume H, Haskell A, Sennino B, Mancuso MR, Norberg SM, O'Brien S M, Davis RB, Gowen LC, Anderson KD, Thurston G, Joho S, Springer ML, Kuo CJ, McDonald DM (2006) VEGF-dependent plasticity of fenestrated capillaries in the normal adult microvasculature. Am J Physiol Heart Circ Physiol 290:H560–H576

Konerding MA, Miodonski AJ, Lametschwandtner A (1995) Microvascular corrosion casting in the study of tumor vascularity: a review. Scanning Microsc 9:1233–1243; discussion 1243–1234

Lamagna C, Hodivala-Dilke KM, Imhof BA, Aurrand-Lions M (2005) Antibody against junctional adhesion molecule-C inhibits angiogenesis and tumor growth. Cancer Res 65:5703–5710

LeCouter J, Kowalski J, Foster J, Hass P, Zhang Z, Dillard-Telm L, Frantz G, Rangell L, DeGuzman L, Keller GA, Peale F, Gurney A, Hillan KJ, Ferrara N (2001) Identification of an angiogenic mitogen selective for endocrine gland endothelium. Nature 412:877–884

Lewis CE, Pollard JW (2006) Distinct role of macrophages in different tumor microenvironments. Cancer Res 66:605–612

Magnussen A, Kasman IM, Norberg S, Baluk P, Murray R, McDonald DM (2005) Rapid access of antibodies to alpha-5beta1 integrin overexpressed on the luminal surface of tumor blood vessels. Cancer Res 65:2712–2721

Maharaj AS, Saint-Geniez M, Maldonado AE, d'Amore PA (2006) Vascular endothelial growth factor localization in the adult. Am J Pathol 168:639–648

Mancuso M, Davis R, Norberg S, O'Brien S, Sennino B, Nakahara T, Yao V, Inai T, Brooks P, Freimark B, Shalinsky D, Hu-Lowe D, McDonald D (2006) Rapid vascular regrowth in tumors after reversal of VEGF inhibition. J Clin Invest 116:2610–2621

Marchio S, Lahdenranta J, Schlingemann RO, Valdembri D, Wesseling P, Arap MA, Hajitou A, Ozawa MG, Trepel M, Giordano RJ, Nanus DM, Dijkman HB, Oosterwijk E, Sidman RL, Cooper MD, Bussolino F, Pasqualini R, Arap W (2004) Aminopeptidase A is a functional target in angiogenic blood vessels. Cancer Cell 5:151–162

May C, Doody JF, Abdullah R, Balderes P, Xu X, Chen CP, Zhu Z, Shapiro L, Kussie P, Hicklin DJ, Liao F, Bohlen P (2005) Identification of a transiently exposed VE-cadherin epitope that allows for specific targeting of an antibody to the tumor neovasculature. Blood 105:4337–4344

McCarty ME, Ellis LM (2002) Mechanisms of anti-angiogenic tyrosine kinase inhibition on wound healing - the obvious and not so obvious. Cancer Biol Ther 1:127–129

McDonald DM (2001) Angiogenesis and remodeling of airway vasculature in chronic inflammation. Am J Respir Crit Care Med 164:S39–S45

McDonald DM, Baluk P (2002) Significance of blood vessel leakiness in cancer. Cancer Res 62:5381–5385

Michel CC, Curry FE (1999) Microvascular permeability. Physiol Rev 79:703–761

Miller DW, Vosseler S, Mirancea N, Hicklin DJ, Bohlen P, Volcker HE, Holz FG, Fusenig NE (2005) Rapid vessel regression, protease inhibition, and stromal normalization upon short-term vascular endothelial growth factor receptor 2 inhibition in skin carcinoma heterotransplants. Am J Pathol 167:1389–1403

Morikawa S, Baluk P, Kaidoh T, Haskell A, Jain RK, McDonald DM (2002) Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. Am J Pathol 160:985–1000

Nakahara T, Norberg SM, Shalinsky DR, Hu-Lowe DD, McDonald DM (2006) Effect of inhibition of vascular endothelial growth factor signaling on distribution of extravasated antibodies in tumors. Cancer Res 66:1434–1445

Nanda A, St. Croix B (2004) Tumor endothelial markers: new targets for cancer therapy. Curr Opin Oncol 16:44–49

Okunieff P, Mester M, Wang J, Maddox T, Gong X, Tang D, Coffee M, Ding I (1998) In vivo radioprotective effects of angiogenic growth factors on the small bowel of C3H mice. Radiat Res 150:204–211

Ozerdem U (2006) Targeting of pericytes diminishes neovascularization and lymphangiogenesis in prostate cancer. Prostate 66:294–304

Parsons-Wingerter P, Kasman IM, Norberg S, Magnussen A, Zanivan S, Rissone A, Baluk P, Favre CJ, Jeffry U, Murray R, McDonald DM (2005) Uniform overexpression and rapid accessibility of alpha5beta1 integrin on blood vessels in tumors. Am J Pathol 167:193–211

Pasqualini R, Koivunen E, Kain R, Lahdenranta J, Sakamoto M, Stryhn A, Ashmun RA, Shapiro LH, Arap W, Ruoslahti E (2000) Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis. Cancer Res 60:722–727

Pasqualini R, Arap W, McDonald DM (2002) Probing the structural and molecular diversity of tumor vasculature. Trends Mol Med 8:563–571

Pasquier E, Honore S, Pourroy B, Jordan MA, Lehmann M, Briand C, Braguer D (2005) Antiangiogenic concentrations of paclitaxel induce an increase in microtubule dynamics in endothelial cells but not in cancer cells. Cancer Res 65:2433–2440

Pietras K, Ostman A, Sjoquist M, Buchdunger E, Reed RK, Heldin CH, Rubin K (2001) Inhibition of platelet-derived growth factor receptors reduces interstitial hypertension and increases transcapillary transport in tumors. Cancer Res 61:2929–2934

Rhodin JA, Fujita H (1989) Capillary growth in the mesentery of normal young rats. Intravital video and electron microscope analyses. J Submicrosc Cytol Pathol 21:1–34

Roberts WG, Palade GE (1995) Increased microvascular permeability and endothelial fenestration induced by vascular endothelial growth factor. J Cell Sci 108:2369–2379

Roberts WG, Palade GE (1997) Neovasculature induced by vascular endothelial growth factor is fenestrated. Cancer Res 57:765–772

Roberts WG, Whalen PM, Soderstrom E, Moraski G, Lyssikatos JP, Wang HF, Cooper B, Baker DA, Savage D, Dalvie D, Atherton JA, Ralston S, Szewc R, Kath JC, Lin J, Soderstrom C, Tkalcevic G, Cohen BD, Pollack V, Barth W, Hungerford W, Ung E (2005) Antiangiogenic and antitumor activity of a selective PDGFR tyrosine kinase inhibitor, CP-673,451. Cancer Res 65:957–966

Ruegg C, Dormond O, Mariotti A (2004) Endothelial cell integrins and COX-2: mediators and therapeutic targets of tumor angiogenesis. Biochim Biophys Acta 1654:51–67

Rundhaug JE (2005) Matrix metalloproteinases and angiogenesis. J Cell Mol Med 9:267–285

Sakamaki K (2004) Regulation of endothelial cell death and its role in angiogenesis and vascular regression. Curr Neurovasc Res 1:305–315

Satchi-Fainaro R, Mamluk R, Wang L, Short SM, Nagy JA, Feng D, Dvorak AM, Dvorak HF, Puder M, Mukhopadhyay D, Folkman J (2005) Inhibition of vessel permeability by TNP-470 and its polymer conjugate, caplostatin. Cancer Cell 7:251–261

Serini G, Valdembri D, Bussolino F (2006) Integrins and angiogenesis: a sticky business. Exp Cell Res 312:651–658

Siemann DW, Bibby MC, Dark GG, Dicker AP, Eskens FA, Horsman MR, Marme D, Lorusso PM (2005) Differentiation and definition of vascular-targeted therapies. Clin Cancer Res 11:416–420

Sims DE (2000) Diversity within pericytes. Clin Exp Pharmacol Physiol 27:842–846

St. Croix B, Rago C, Velculescu V, Traverso G, Romans KE, Montgomery E, Lal A, Riggins GJ, Lengauer C, Vogelstein B, Kinzler KW (2000) Genes expressed in human tumor endothelium. Science 289:1197–1202

Stingl J, Rhodin JA (1994) Early postnatal growth of skeletal muscle blood vessels of the rat. Cell Tissue Res 275:419–434

Stupack DG, Cheresh DA (2004) Integrins and angiogenesis. Curr Top Dev Biol 64:207–238

Sugimoto H, Hamano Y, Charytan D, Cosgrove D, Kieran M, Sudhakar A, Kalluri R (2003) Neutralization of circulating vascular endothelial growth factor (VEGF) by anti-VEGF antibodies and soluble VEGF receptor 1 (sFlt-1) induces proteinuria. J Biol Chem 278:12605–12608

Sund M, Hamano Y, Sugimoto H, Sudhakar A, Soubasakos M, Yerramalla U, Benjamin LE, Lawler J, Kieran M, Shah A, Kalluri R (2005) Function of endogenous inhibitors of angiogenesis as endothelium-specific tumor suppressors. Proc Natl Acad Sci USA 102:2934–2939

Sund M, Xie L, Kalluri R (2004) The contribution of vascular basement membranes and extracellular matrix to the mechanics of tumor angiogenesis. APMIS 112:450–462

Tang ML, Wilson JW, Stewart AG, Royce SG (2006) Airway remodelling in asthma: current understanding and implications for future therapies. Pharmacol Ther 112:474–488

Thorpe PE (2004) Vascular targeting agents as cancer therapeutics. Clin Cancer Res 10:415–427

Tong RT, Boucher Y, Kozin SV, Winkler F, Hicklin DJ, Jain RK (2004) Vascular normalization by vascular endothelial growth factor receptor 2 blockade induces a pressure gradient across the vasculature and improves drug penetration in tumors. Cancer Res 64:3731–3736

Tozer GM (2003) Measuring tumour vascular response to antivascular and antiangiogenic drugs. Br J Radiol 76:S23–S35

Tozer GM, Kanthou C, Baguley BC (2005a) Disrupting tumour blood vessels. Nat Rev Cancer 5:423–435

Tozer GM, Ameer-Beg SM, Baker J, Barber PR, Hill SA, Hodgkiss RJ, Locke R, Prise VE, Wilson I, Vojnovic B (2005b) Intravital imaging of tumour vascular networks using multi-photon fluorescence microscopy. Adv Drug Deliv Rev 57:135–152

Uehara H, Kim SJ, Karashima T, Shepherd DL, Fan D, Tsan R, Killion JJ, Logothetis C, Mathew P, Fidler IJ (2003) Effects of blocking platelet-derived growth factor-receptor signaling in a mouse model of experimental prostate cancer bone metastases. J Natl Cancer Inst 95:458–470

Van Kempen LC, de Visser KE, Coussens LM (2006) Inflammation, proteases and cancer. Eur J Cancer 42:728–734

Vincent L, Kermani P, Young LM, Cheng J, Zhang F, Shido K, Lam G, Bompais-Vincent H, Zhu Z, Hicklin DJ, Bohlen P, Chaplin DJ, May C, Rafii S (2005) Combretastatin A4 phosphate induces rapid regression of tumor neovessels and growth through interference with vascular endothelial-cadherin signaling. J Clin Invest 115:2992–3006

Vosseler S, Mirancea N, Bohlen P, Mueller MM, Fusenig NE (2005) Angiogenesis inhibition by vascular endothelial growth factor receptor-2 blockade reduces stromal matrix metalloproteinase expression, normalizes stromal tissue, and reverts epithelial tumor phenotype in surface heterotransplants. Cancer Res 65:1294–1305

Weis SM, Cheresh DA (2005) Pathophysiological consequences of VEGF-induced vascular permeability. Nature 437:497–504

Willett CG, Boucher Y, di Tomaso E, Duda DG, Munn LL, Tong RT, Chung DC, Sahani DV, Kalva SP, Kozin SV, Mino M, Cohen KS, Scadden DT, Hartford AC, Fischman AJ, Clark JW, Ryan DP, Zhu AX, Blaszkowsky LS, Chen HX, Shellito PC, Lauwers GY, Jain RK (2004) Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. Nat Med 10:145–147

Winkler F, Kozin SV, Tong RT, Chae SS, Booth MF, Garkavtsev I, Xu L, Hicklin DJ, Fukumura D, di Tomaso E, Munn LL, Jain RK (2004) Kinetics of vascular normalization by VEGFR2 blockade governs brain tumor response to radiation: role of oxygenation, angiopoietin-1, and matrix metalloproteinases. Cancer Cell 6:553–563

Witmer AN, Vrensen GF, Van Noorden CJ, Schlingemann RO (2003) Vascular endothelial growth factors and angiogenesis in eye disease. Prog Retin Eye Res 22:1–29

Woolard J, Wang WY, Bevan HS, Qiu Y, Morbidelli L, Pritchard-Jones RO, Cui TG, Sugiono M, Waine E, Perrin R, Foster R, Digby-Bell J, Shields JD, Whittles CE, Mushens RE, Gillatt DA, Ziche M, Harper SJ, Bates DO (2004) VEGF165b, an inhibitory vascular endothelial growth factor splice variant: mechanism of action, in vivo effect on angiogenesis and endogenous protein expression. Cancer Res 64:7822–7835

Xian X, Hakansson J, Stahlberg A, Lindblom P, Betsholtz C, Gerhardt H, Semb H (2006) Pericytes limit tumor cell metastasis. J Clin Invest 116:642–651

Yano K, Brown LF, Lawler J, Miyakawa T, Detmar M (2003) Thrombospondin-1 plays a critical role in the induction of hair follicle involution and vascular regression during the catagen phase. J Invest Dermatol 120:14–19

Yao VJ, Ozawa MG, Varner AS, Kasman IM, Chanthery YH, Pasqualini R, Arap W, McDonald DM (2006) Antiangiogenic therapy decreases integrin expression in normalized tumor blood vessels. Cancer Res 66:2639–2649

Antiangiogenic Therapy for Normalization of Tumor Vasculature and Microenvironment

33

RAKESH K. JAIN and DAN G. DUDA

Abstract

Surpassing several setbacks, the clinical development of antiangiogenic agents has accelerated remarkably over the past 3–4 years. As a result, there are currently three direct blockers of the VEGF pathway approved for use in cancer, and two others for age-related wet macular degeneration. Other agents that block the VEGF pathway are currently in advanced stages of clinical development and have shown promising results. With these exciting developments come critical questions regarding the use of these new molecularly targeted agents, alone or in combination with standard cytotoxic or targeted agents.

CONTENTS

33.1 Introduction

New blood vessel formation (angiogenesis) is a condition for continued growth and metastatic spread of tumors. Over the past three decades, basic researchers have explored the targeting of antiangiogenic pathways as a therapeutic approach to cancer. Unfortunately, the initial clinical trials for antiangiogenic agents yielded disappointing results. This was in contrast to the very promising results of antiangiogenic drugs in preclinical models of cancer (Garber

2002). Recently, however, two approaches blocking the vascular endothelial growth factor (VEGF) pathway have yielded survival benefit in cancer patients in randomized phase III trials. In one approach, the addition of bevacizumab, a VEGF-specific antibody, to standard chemotherapy improved overall survival in colorectal and lung cancer patients and progression-free survival in breast cancer patients. In the second approach, multitargeted tyrosine kinase inhibitors (TKIs) that block VEGF receptor and other kinases in both endothelial and cancer cells demonstrated survival benefit in gastrointestinal stromal tumor and renal cell carcinoma patients. By contrast, adding bevacizumab to chemotherapy failed to increase survival in patients with previously treated and refractory metastatic breast cancer. Furthermore, addition of vatalanib, a kinase inhibitor developed as a VEGF receptor-selective agent, to chemotherapy did not show a similar benefit in metastatic colorectal cancer patients. These contrasting responses raise critical questions about how these agents work and how to combine them optimally. We discuss here the current understanding of one of the many potential mechanisms of action of antiangiogenic agents – normalization of tumor vasculature and microenvironment for improved delivery and efficacy of therapeutics.

Use of Antiangiogenic Agents for Cancer Therapy

Solid tumors account for more than 85% of cancer mortality. Tumor angiogenesis is a rational target for therapy given the dependence of solid tumor growth and metastasis on blood vessels (Carmeliet 2005; Ferrara et al. 2004; Folkman 1971; Jain 2005a; Kerbel and Folkman 2002). Strategies have been pursued to inhibit neovascularization, or destroy existing tumor vessels. These include direct targeting of endothelial cells, supporting perivascular cells, or indirect targeting by inhibition of pro-angiogenic growth factors released by cancer cells or certain stromal cells. Unlike multiple preclinical studies in mice, no randomized phase III trial has demonstrated a survival benefit with currently available targeted (e.g., anti-VGEF) antiangiogenic agents used as monotherapy. However, the addition of a VEGF-specific antibody, bevacizumab (Avastin®, Genentech) to current cytotoxic regimens led to improved outcomes in previously untreated colorectal, breast and lung cancer and in previously treated colorectal cancer patients (Hurwitz et al. 2004; Sandler et al. 2006).

In contrast, adding bevacizumab to cytotoxic therapy did not enhance survival in previously treated breast cancer patients (Miller et al. 2005). Moreover, replacing bevacizumab with vatalanib, a potent VEGF receptor TKI, in the combined regimen did not show similar efficacy in chemo-naïve or previously treated colorectal cancer patients (Jain et al 2006). Nevertheless, monotherapy using agents with a broader spectrum of inhibitory effect on growth factor pathways (i.e., targeting both endothelial and cancer cells) has resulted in significant antitumor activity against renal cell cancer and increased survival in gastrointestinal stromal tumors (GIST) (Demetri et al. 2006; Escudier et al. 2007; Motzer et al. 2007). Finally, several agents that indirectly inhibit angiogenesis, such as the epidermal growth factor receptor (EGFR/HER)-specific antibodies or thalidomide, have yielded increased survival in clinical trials and are approved by the Food and Drug Administration (FDA) in the United States and elsewhere.

These contrasting results raise important questions about the use of antiangiogenic agents. Why has anti-VEGF monotherapy not been shown to produce increased survival in randomized trials? How can tumor vessel destruction by combined anti-VEGF treatment – instead of compromising the delivery and efficacy of cytotoxic treatment– prolong survival in previously treated colorectal cancer patients and in chemotherapy-naïve colorectal, lung and breast cancer patients? Why are agents that target both endothelial and cancer cells effective as monotherapy? In this chapter, we will summarize the knowledge gained from preclinical and clinical studies of antiangiogenic agents for cancer therapy with emphasis on vascular normalization.

Tumor Vascular Normalization: A Mechanism of Action of Antiangiogenic Agents

The widely held view is that antiangiogenic therapies should destroy the tumor vasculature, thereby depriving the tumor of oxygen and nutrients. The failure of bevacizumab to increase survival in heavily treated breast cancer patients was initially explained by, first, the highly refractory and advanced nature of the tumors in the patients enrolled and second, the increased expression of other angiogenic factors during breast cancer progression caused by chemotherapy, which rendered VEGF less critical for continued tumor growth (Miller et al. 2005). These hypotheses were partially supported by the success of a subsequent trial of bevacizumab combined with a different chemotherapeutic agent in treatment-naïve advanced breast cancer patients (Jain et al. 2006). Yet the hypotheses seem to be contradicted by the efficacy of bevacizumab with chemotherapy in heavily treated colorectal cancer patients. Vatalanib does not confer the same survival advantage as bevacizumab in colorectal cancer patients when combined with chemotherapy (Jain et al. 2006). These contrasting results raise questions about the mechanisms of action of these agents alone and in combination. Vascular normalization is a mechanism of action that can reconcile the differing outcomes in clinical trials.

33.3.1 Normalization of the Vasculature and Microenvironment by Antiangiogenic Agents in Tumors

More than a decade ago, Teicher (1996) proposed that combining antiangiogenic therapy with cytotoxic treatments will have synergistic effects because it allows targeting of both the malignant cell compartment and the vascular stroma. However, the destruction of tumor vasculature by antiangiogenic agents should antagonize chemo- and radiotherapy by impeding the delivery of therapeutics and oxygen, respectively. Indeed, a number of preclinical studies have demonstrated such antagonism (Jain 2005a,b). At the same time, such combinations have been successful in a number of preclinical and clinical studies. To resolve this paradox, we proposed in 2001 that antiangiogenic agents can transiently "normalize" tumor vasculature (Jain 2001). Normalization of tumor vasculature can be defined as the structural and functional changes that allow more efficient delivery of drugs and oxygen, ultimately leading to improved outcomes (Jain 2001). A better understanding of the molecular and cellular underpinnings of vascular normalization may ultimately lead to more effective therapies not only for cancer but also for diseases with abnormal vasculature (e.g., macular degeneration, unstable plaques), as well as regenerative medicine, in which the goal is to create and maintain a functionally normal vasculature.

33.3.2 Why Normalize the Tumor Vasculature?

Tumor vasculature is structurally and functionally abnormal. Blood vessels are leaky, tortuous, dilated, and saccular and have a haphazard pattern of interconnection (Fig. 33.1). The endothelial cells lining these vessels have aberrant morphology, pericytes (cells that provide support for the endothelial cells) are loosely attached or absent, and the basement membrane is often abnormal – unusually thick at times, entirely absent at others (Table 33.1). These structural abnormalities contribute to spatial and temporal heterogeneity in tumor blood flow. In addition, solid pressure generated by proliferating cancer cells compresses intratumor blood and lymphatic vessels, which further impairs not only the blood flow but also the lymphatic flow (Padera et al. 2004). Collectively, thesevascular abnormalities lead to an abnormal tumor microenvironment characterized by interstitial hypertension (elevated hydrostatic pressure out-

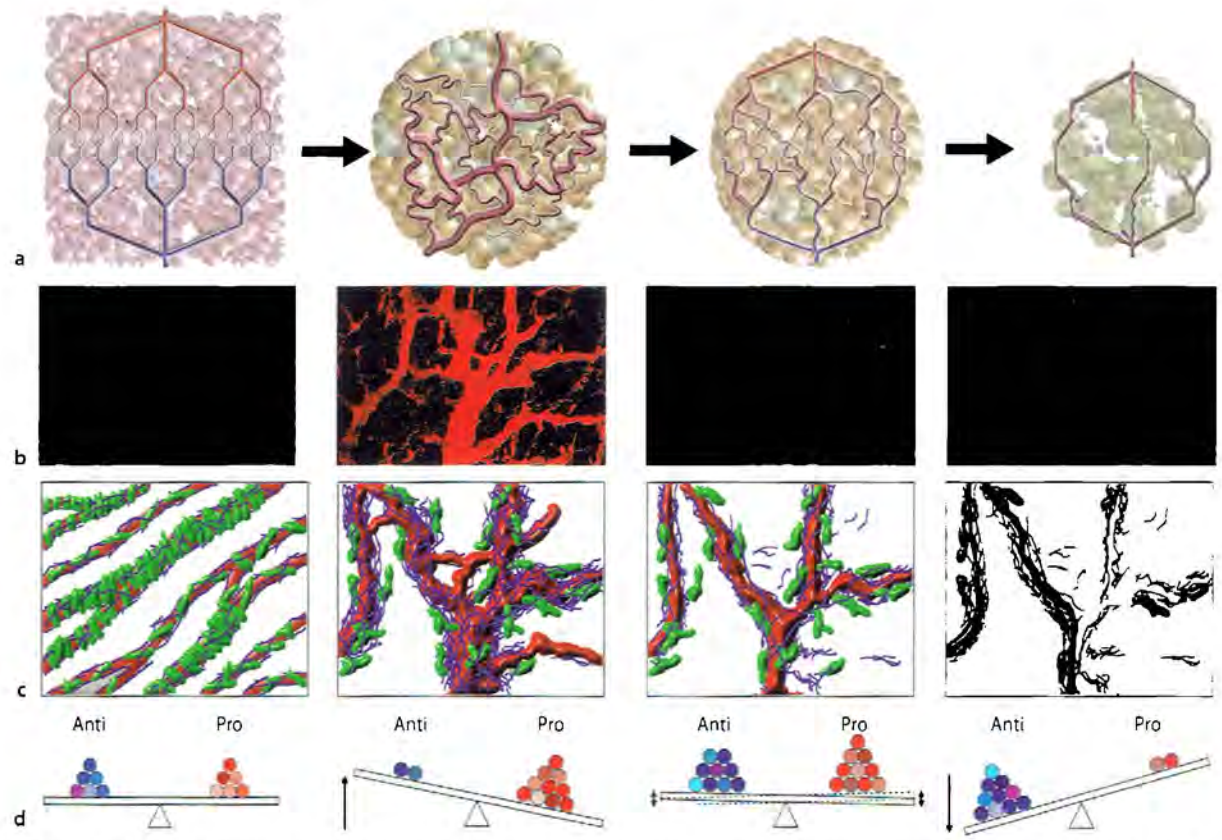


Fig. 33.1a–d. Proposed role of vessel normalization in the response of tumors to antiangiogenic therapy. a Tumor vasculature is structurally and functionally abnormal. It is proposed that antiangiogenic therapies initially improve both the structure and the function of tumor vessels. However, sustained or aggressive antiangiogenic regimens may eventually prune away these vessels, resulting in a vasculature that is both resistant to further treatment and inadequate for the delivery of drugs or oxygen. b Dynamics of vascular normalization induced by VEGF-R2 blockade. On the left is a two-photon image showing normal blood vessels in skeletal muscle; subsequent images show human colon carcinoma vasculature in mice at day 0, day 3, and day 5 after administration of VEGF-R2-specific antibody. c Diagram depicting the concomitant changes in pericyte (*red*) and basement membrane (*blue*) coverage during vascular normalization. d These phenotypic changes in the vasculature may reflect changes in the balance of pro- and antiangiogenic factors in the tissue. Figure obtained with permission from (Jain 2005a)

side the blood vessels), hypoxia, and acidosis. Impaired blood supply and interstitial hypertension interfere with the delivery of therapeutics to solid tumors. Hypoxia renders tumor cells resistant to both radiation and several cytotoxic drugs. Independent of these effects, hypoxia also induces genetic instability and selects for more malignant cells with increased metastatic potential (Bottaro and Liotta 2003). Hypoxia and low pH also compromise the cytotoxic functions of immune cells that infiltrate a tumor. Unfortunately, cancer cells are able to survive in this abnormal microenvironment. In essence, the abnormal vasculature of tumors and the resulting abnormal microenvironment together pose a formidable barrier to the delivery and efficacy of cancer therapy. This

Table 33.1. Differences between normal vasculature and tumor vasculature

Normal vasculature	Tumor vasculature
Organized	Disorganized
Evenly distributed	Unevenly distributed
Uniformly shaped	Twisted
Non-permeable	Leaky
Vascular pressure is greater than interstitial pressure	Vascular pressure is similar to tumor interstitial pressure
Properly matured	Immature
Supporting cells present (e.g., pericytes)	Supporting cells absent
Appropriate membrane protein expression	Inappropriate membrane protein expression
Independent of cell survival factors	Dependent on cell survival factors (e.g., VEGF)

suggests that if we knew how to correct the structure and function of tumor vessels, we would have a chance to normalize the tumor microenvironment and ultimately to improve cancer treatment. The fortified tumor vasculature may also inhibit the shedding of cancer cells into the circulation – a prerequisite for metastasis. In the past, higher doses of drugs and hyperbaric oxygenation have been used to increase the tumor concentrations of drugs and oxygen, respectively. These strategies have not shown much success in the clinic, however. One reason for this failure is that tumor vessels have large holes in their walls (Hobbs et al. 1998). As stated earlier, this leakiness leads to interstitial hypertension as well as spatially and temporally nonuniform blood flow. If the delivery system is flawed, it does not matter how much material is pumped into it. The drugs and oxygen will become concentrated in regions that already have enough and will still not reach the inaccessible regions (Jain 1999). However, if we fix the delivery system, more cells are likely to encounter an effective concentration of drugs and oxygen. This is the rationale for developing therapies that normalize the tumor vasculature. These therapies do not merely increase the delivery of drugs and oxygen but distribute these molecules to a larger fraction of the tumor cells by fixing the delivery system.

33.3.3 How Should One Normalize the Tumor Vasculature?

In normal tissues, the collective action of angiogenic stimulators (e.g., VEGF) is counterbalanced by the collective action of angiogenic inhibitors such as thrombospondin-1 (Fig. 33.1). This balance tips in favor of the stimulators in both pathological and physiological angiogenesis (Jain 2005a). However, in pathological angiogenesis, the imbalance persists. Therefore, restoring the balance may render the tumor vasculature close to normal. On the other hand, tipping this balance in favor of inhibitors may lead to vascular regression and, ultimately, to tumor regression. If we had antiangiogenic agents that completely destroyed tumor vessels without harming normal vessels, we would not need to add cytotoxic therapy. Unfortunately, such agents are not currently available. It is conceivable that increased doses of currently available antiangiogenic agents could produce complete tumor regression, but such doses are likely to adversely affect the vasculature of normal tissues, including the cardiovascular, endocrine, and nervous systems. Indeed, antiangiogenic therapy with agents such as bevacizumab is associated with an increased risk of arterial thromboembolic events, and such adverse

effects could be more pronounced with increased doses (Jain et al. 2006). Furthermore, excessive vascular regression may be counterproductive because it compromises the delivery of drugs and oxygen (Figs. 33.1, 33.2). Indeed, suboptimal doses or scheduling of antiangiogenic agents might lower tumor oxygenation and drug delivery and, thus, antagonize rather than augment the response to radiotherapy or chemotherapy (Fenton et al. 2004; Ma et al. 2001; Murata et al. 1997). This need for a delicate balance between normalization and excessive vascular regression emphasizes the requirement for careful selection of the dose and administration schedule for antiangiogenic agents.

33.3.4 Can Blocking VEGF Signaling Normalize Tumor Vessels? How About Multitargeted Antiangiogenic Agents or Indirect Inhibitors of Angiogenesis?

VEGF is overexpressed in the majority of solid tumors. Thus, if one were to judiciously down-regulate VEGF signaling in tumors, then the vasculature might revert back to a more "normal" state. Indeed, blockade of VEGF signaling passively prunes the immature and leaky vessels of transplanted tumors in mice and actively remodels the remaining vasculature

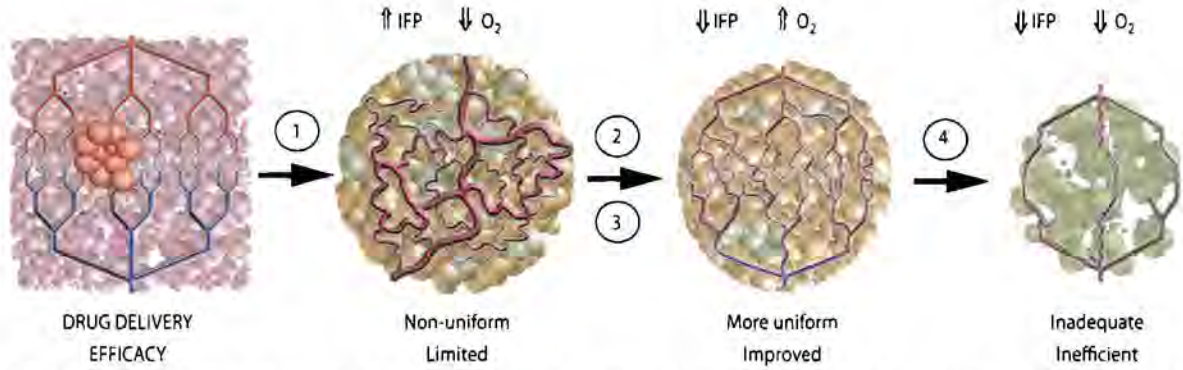


Fig. 33.2. Potential mechanisms of action of bevacizumab on tumor vasculature. Owing to high levels of proangiogenic molecules produced locally, such as VEGF, tumors make the transition from in situ carcinoma to frank carcinoma (*1*). At this stage, tumors become hypervascular, but the vessels are leaky and the blood flow is spatially and temporally heterogeneous. This leads to increased interstitial fluid pressure (*IFP*) and focal hypoxia, creating barriers to delivery and efficacy of therapeutics. The proposed mechanism of action of the VEGF-specific antibody bevacizumab is twofold: inhibition of new vessel formation and killing of immature tumor vessels (*2*), and transient normalization of the remaining vasculature by decrease in macromolecular permeability (and thus the IFP) and hypoxia, and improvement of blood perfusion (*3*). Another effect of bevacizumab may be the direct killing of cancer cells in subsets of tumors in which the cells express VEGF receptors. Regardless of the mechanisms involved, monotherapy with bevacizumab is not curative because it cannot kill all cancer cells, and in the longer term leads to a vasculature that is inefficient for drug delivery (*4*), and to tumor relapse using alternative pathways for neovascularization. Combinations of bevacizumab with chemotherapeutics have therefore been pursued in phase III trials and have led to a survival benefit in patients with chemosensitive tumors, showing the synergistic effect of the two treatment modalities. Synergy may have been achieved as a result of increased cell killing following tumor vascular normalization: the lowered IFP leads to improved delivery of chemotherapeutics and molecularly targeted agents; the improved oxygenation sensitizes cancer cells to cytotoxic therapeutics and reduces the selection of more malignant phenotype; and, finally, increased cellular proliferation around normalized vessels might increase the cytotoxicity of chemotherapeutics. Normalization of the vasculature might also benefit the direct killing of cancer cells by bevacizumab, in synergy with the chemotherapeutics. Of interest, cytotoxic therapeutics may kill proliferating endothelial cells, and thus may also normalize the tumor vasculature and improve drug delivery to tumors. Figure adapted with permission from (Jain et al. 2006)

so that it more closely resembles the normal vasculature (Fig. 33.1). This normalized vasculature is characterized by less leaky, less dilated, and less tortuous vessels with a more normal basement membrane and greater coverage by pericytes. These morphological changes are accompanied by functional changes – decreased interstitial fluid pressure, increased tumor oxygenation, and improved penetration of drugs in these tumors (Fig. 33.2 and Table 33.2) (Jain 2005a). Normalization of tumor vasculature was reported by other groups and our own during treatment of experimental tumors with FDA-approved drugs such as thalidomide and trastuzumab (Izumi et al. 2002; Segers et al. 2006) as well as with antiangiogenic agents currently under clinical development (Table 33.3).

33.3.5 What About Human Tumors?

Thousands of patients worldwide have received anti-VEGF therapy. The effect of VEGF blockade on human tumors was recently studied in rectal carcinoma patients receiving an antibody to VEGF, bevacizumab, together with radiation and chemotherapy (Willett et al. 2004, 2005). The results in patients mirrored those seen in transplanted tumors in mice: Two weeks after a single injection of bevacizumab alone, the global (mean) blood flow of tumors, as measured by contrast-enhanced computed tomography (CT), decreased by 30–50% in six consecutive patients. Tumor microvascular density, vascular volume, and interstitial fluid pressure were all reduced by VEGF blockade. Surprisingly, however, there was no concurrent decrease in the uptake of radioactive tracers in tumors, which suggests that vessels in the residual "normalized" tumor vasculature were more efficient in delivering these agents to tumor parenchyma than they were prior to bevacizumab treatment. Similar reductions in blood flow, as measured by magnetic resonance imaging (MRI), had been noted previously in patients treated daily with small-molecule inhibitors of VEGF-R tyrosine kinase activity [vatalanib/PTK787 and SU6668 (Morgan et al. 2003; Xiong et al. 2004)]. Interestingly, however, positron emission

Table 33.2 Tumor vascular normalization by anti-VEGF-R2 antibody DC101 in animals or anti-VEGF antibody bevacizumab in patients: comparison of preclinical data from transplanted tumors in mice with clinical data from rectal carcinoma patients

Properties	Preclinical data (Tong et al. 2004; Winkler et al. 2004)			Clinical data (Willett et al. 2004, 2005)		
	Control	Treatment	Change	Control	Treatment	Change
Blood volume	19.3±2.2	5.4±1.0	−72%	6.8±2.1	5.0±0.9	−26%
Vascular density	52.1±4.6	41.9±3.0	−19%	13.0±3.2	6.9±1.8	−47%
Permeability (BSA)	7.3±0.8	2.8±0.8	−62%	N/A	N/A	N/A
PS product (small molecules)	N/A	N/A	N/A	14±2	12.9±3.1	−7.9%
Interstitial fluid pressure	6.1±1.0	3.1±0.5	−49%	14.0±1.2	4.0±1.5	−71%
Perivascular cell coverage	0.67±0.04	0.81±0.04	+21%	9.9±3.8	17.8±1.5	+80%
Angiopoietin-2 level	10.4±1.3	2.2±0.5	−79%	0.046±0.002	0.020±0.001	−57%
Tumor apoptosis	0.86±0.24	2.50±0.31	+190%	1.7±0.2	3.6±0.7	+112%
Plasma VEGF level	N/D	182.5±135.8		22.5±8.3	272±22.5	+1109%

N/A, not applicable; N/D, not detectable

Table 33.3. Independent reports consistent with the induction of tumor vascular normalization by antiangiogenic agents

Antiangiogenic agent	Target/ action	Other therapeutics	Tumor model	Results	Reference
Anti-hVEGF mAb (A4.6.1)	Blocks human VEGF	CPT-11	Mice implanted with human colon adenocarcinoma	Decreased vascular density Increased intratumoral CPT-11 concentration Increased tumor perfusion	Wildiers et al. (2003)
Thalidomide	Inhibits bFGF and VEGF expression	X-ray	Mice implanted with fibrosarcoma	Induced tumor re-oxygenation Lowered IFP Increased perfusion Vascular remodeling Radiosensitization occurred in a narrow time window	Ansiaux et al. (2005)
Bevacizumab	Blocks human VEGF	Two different immunotoxins (SS1P and HA22)	Mice implanted with human mesothelioma and Burkitt's lymphoma	Combination treatment provided additive anti-tumor activity	Bang et al. (2005)
Soluble TGFbeta receptor type II	Inhibits TGF-beta signaling	Doxorubicin	Mice implanted with anaplastic thyroid carcinoma cells	Lowered IFP	Salnikov et al. (2005)
AG013736, VEGF-Trap	VEGF, PIGF	N/A	Mice with islet-cell tumors or implanted with lung carcinoma	Decreased vascular density Decreased endothelial fenestrations Improved perivascular cell coverage	Inai et al. (2004)
DC101	Blocks murine VEGF-R2	N/A	Mice implanted with squamous cell carcinoma	Decreased vascular density Improved perivascular cell coverage Improved basement membrane coverage with down-regulation of MMP9 and MMP13	Vosseler et al. (2005)
SU11657	Blocks VEGF-R1, VEGF-R2, PDGF-R, c-kit, flt3	X-ray pemetrexed	Mice implanted with human epidermoid carcinoma	Lowered IFP Increased αSMA-cell coverage Increased the anti-tumoral efficacy of doxorubicin Reduced intratumoral edema Direct radiosensitization of endothelial cells	Huber et al. (2005)
DC101	Blocks murine VEGF-R2	N/A	Mice implanted with squamous cell carcinoma	Decreased vascular density Less aggressive phenotype Normalized basement membrane	Miller et al. (2005)
Thalidomide	Inhibits bFGF and VEGF expression	cyclophosphamide	Mice implanted with liver tumors	Increased pO2 Increased retention time and anti-tumor efficacy of cyclophosphamide	Segers et al. (2006)

tomography (PET) analysis of patients treated with endostatin, an endogenous inhibitor of angiogenesis, revealed a biphasic response – an increase in tumor blood flow at lower doses and a decrease at intermediate doses (Herbst et al. 2002). With newer, improved imaging techniques, we may be able to measure the spatial and temporal changes in blood flow and other physiological parameters with higher resolution, and definitively establish the effects of antiangiogenic treatment on vascular function in human tumors growing at different sites.

33.3.6 Is There an Optimal Time or Drug Dose for Normalization?

Optimal scheduling of antiangiogenic therapy with chemotherapy and/or radiation therapy requires knowledge of the time window during which the vessels initially become normalized, as well as an understanding of how long they remain in that state. Recent studies, in which human tumors growing in mice were treated with an antibody to VEGF receptor-2, have identified such a "normalization window," that is, a period during which the addition of radiation therapy yields the best therapeutic outcome (Fig. 33.3) (Winkler et al. 2004). This window was short-lived (about 6 days) and was characterized by an increase in tumor oxygenation, which enhances radiation therapy by increasing the concentration of reactive oxygen species created by the radiation. During the normalization window, but not before or after it, VEGF-R2 blockade was found to increase pericyte coverage of vessels in a human brain tumor grown in mice. Vessel normalization was accompanied by upregulation of angiopoietin 1 and activation of matrix metalloproteinases (MMPs). The prevailing hypothesis is that VEGF blockade passively prunes nascent vessels that are not covered with pericytes. In contrast, this study found that pericyte coverage increased prior to vascular pruning. Improved understanding of the molecular mechanisms of vessel normalization may suggest new strategies for extending the normalization window to provide ample time for cytotoxic therapy. The dose of antiangiogenic agents also determines the efficacy of combination therapy. Although it is tempting to increase the dose of antiangiogenic agents or to use a more potent angiogenic blocker, as one would for chemotherapeutic agents, doing so might lead to normal-tissue toxicity and compromise the tumor vessels to the point that drug delivery is impaired. Indeed, renal cell carcinoma patients on a high dose of bevacizumab (10 mg per kg of body weight every 2 weeks) were more likely to develop hypertension and proteinuria than those on a low dose, although the sample size was too small for comparison of the rates of serious adverse events (Yang et al. 2003). Even the low dose of bevacizumab (5 mg/kg) given in combination with chemotherapy has contributed to an increased risk of cardiovascular problems, including death, in some cancer patients (Ratner 2004). Although no dose comparison has yet been made in large clinical trials, it is conceivable that such serious adverse events might increase with higher doses. In studies of mice, more potent blockers of VEGF signaling have induced regression of normal tracheal and thyroid vessels (Baffert et al. 2004).

33.3.7 Is Tumor Growth Accelerated During Vascular Normalization?

One would expect that the improved delivery of oxygen and nutrients during vascular normalization would enhance tumor growth. However, both preclinical and clinical studies to date show that, despite normalization of the vasculature, tumor growth is not accelerated during antiangiogenic monotherapy. There are several possible explanations for this apparent paradox: (1) It is important to remember that vascular normalization occurs in the context of antiangiogenic treatment and that the main effect of this treatment is a reduction in the number of blood vessels (vessel density), which should lead to tumor regression. Moreover, tumors are highly heterogeneous. Not all regions are equally vascularized, some tumor vessels are more mature than others, and the balance of pro- and antiangiogenic molecules differs from region to region and

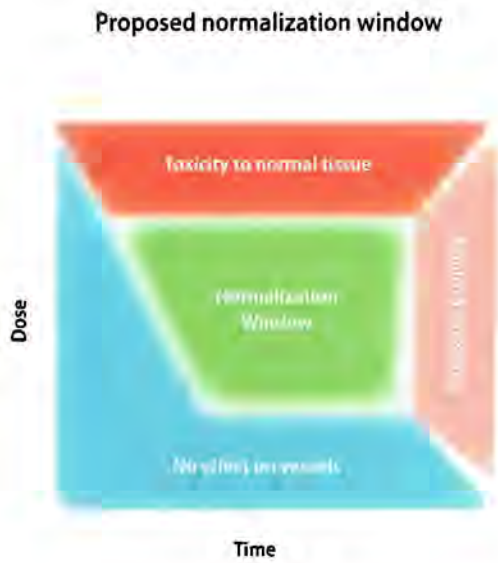


Fig. 33.3. Proposed effect of drug dose and schedule on tumor vascular normalization. The efficacy of cancer therapies that combine antiangiogenic and cytotoxic drugs depends on the dose and delivery schedule of each drug. The vascular normalization model posits that a well-designed strategy should passively prune away immature, dysfunctional vessels and actively fortify those remaining, while incurring minimal damage to normal tissue vasculature. During this "normalization" window (*green*), cancer cells may be more vulnerable to traditional cytotoxic therapies and to novel targeted therapies. The degree of normalization will be spatially and temporally dependent in a tumor. Vascular normalization will occur only in regions of the tumor where the imbalance of pro- and antiangiogenic molecules has been corrected. Figure obtained with permission from (Jain 2005a)

from one moment to the next. Hence, it may be that the effects of vessel normalization in some regions of the tumor are swamped by simultaneous vessel regression in other regions. In addition, the inability of tumors to grow new vessels during antiangiogenic therapy limits the ability of this transient increase in vascular efficiency to expand the tumor mass. If it were easy to achieve complete tumor regression with antiangiogenic monotherapy, vascular normalization would be of marginal importance, because it is expected to affect only a subset of cells and to do so only temporarily. Unfortunately, some tumor cells are able to survive antiangiogenic monotherapy, and these cells must be targeted with combined therapy. (2) The transient normalization of tumor vessels produces a temporary increase in oxygen and nutrient delivery to the cancer cells that surround these "normalized" vessels. This might be expected to enhance the proliferation of these cells and hence to accelerate tumor growth. Indeed, we have shown that bevacizumab monotherapy can increase cancer-cell proliferation in some rectal cancer patients (Willett et al. 2005). These proliferating cells are likely to be more sensitive to cytotoxic therapy. The increase in proliferation, however, occurred in the context of a significantly increased apoptosis of cancer cells. Of interest, Gullino (1982) found no correlation between tumor growth rate in vivo and blood flow rate, vascular volume, or use of oxygen or glucose. (3) It is widely assumed that hypoxia leads to the death of cells. Therefore, alleviation of hypoxia during transient normalization of tumor vasculature should accelerate tumor growth. However, a growing body of evidence indicates that hypoxia may in fact promote cancer progression (Nelson et al. 2004). These two competing effects of antiangiogenic therapy may cancel each other out. (4) Finally, in some tumors, cancer cells depend on the same angiogenic growth factors (e.g., VEGF) for their survival as do the endothelial cells. In these tumors, antiangiogenic agents may kill both cancer cells and endothelial cells and will likely induce tumor regression – similar to hormone withdrawal from a hormone-dependent tumor (Jain et al. 1998) – despite vessel normalization. For all these reasons, any acceleration in tumor growth during transient normalization is presumably masked by indirect and direct killing of cancer cells by antiangiogenic agents. Thus, it is not surprising that tumor regression is slow and/or modest after antiangiogenic monotherapy despite a significant decrease in microvascular density (Willett et al. 2004).

33.3.8 Vascular Normalization: Clinical Relevance for Cancer Therapy

Emerging preclinical and clinical data from our laboratory and others support the concept of tumor vascular normalization by antiangiogenic therapy (Ansiaux et al. 2005; Batchelor et al. 2007; Huber et al. 2005; Inai et al. 2004; Salnikov et al. 2005; Tong et al. 2004; Vosseler et al. 2005; Wildiers et al. 2003; Willett et al. 2004, 2005; Winkler et al. 2004). Vascular normalization could explain why bevacizumab was efficacious in combination with chemotherapy, despite its limited efficacy as monotherapy (Jain 2005a, 2005b). The process suggests that bevacizumab increased survival rates in chemotherapy-naive metastatic breast cancer patients by improving the delivery of chemotherapeutics to chemoresponsive tumors, while such an increase was not seen in chemotherapy-refractory metastatic breast cancer patients, in whom improved delivery of chemotherapeutics might have less of an effect. Vascular stabilization during VEGF blockade potentially also decreased the shedding of metastatic cancer cells from the primary tumors. The alleviation of hypoxia by bevacizumab might make the tumors more chemosensitive and less metastatic. Finally, if during vascular normalization the improved tumor microenvironment led to increased proliferation of the surviving cancer cells, this might have rendered them more sensitive to cytotoxic agents (Jain 2005a, 2005b; Jain et al. 2006; Willett et al. 2005).

Then why did VEGF blockade by a multitargeted agent (vatalanib) not show a clear benefit with FOLFOX4 regimen in metastatic colorectal cancer patients? Besides the simple explanation that it is not as effective an agent at administered doses, vatalanib has a considerably shorter half-life (~6 h) than bevacizumab (~20 days), and the phase III trials for vatalanib used a single daily dose of the drug. Contradicting these data, however, is the fact that pharmacokinetic data suggest that an active dose of vatalanib is maintained in the blood circulation for 24 h, and that it has a rapid and pronounced anti-vascular effect (Morgan et al. 2003). Another explanation could be the off-target effects (i.e. other than on the VEGF receptor kinases). For example, vatalanib might target PDGFR-β on perivascular cells. This action was shown in mice to be beneficial for vascular targeting, since the PDGF-B–PDGFR-β axis is known to control vascular stabilization/maturation by recruitment of supporting perivascular cells. Blocking PDGFR-β, however, may interfere with vascular normalization, by blocking perivascular cell recruitment and excessive vessel pruning, and thus prevent the synergistic effect of combined therapy (Jain 2003). Thus, the clinical benefit of targeting perivascular cells in addition to endothelial cells with multitargeted TKIs in the context of chemotherapy remains unclear. How anti-VEGF therapy affects the recruitment or response of cells of the immune system in cancer patients is not known.

Given these facts, why did other multitargeted TKIs, with broader inhibitory spectra and longer half-lives, prove efficacious in other tumor types? Our hypothesis is that broad-spectrum multitargeted TKIs (i.e. those that simultaneously target multiple receptor or soluble kinases such as c-Kit, Raf, FLT3, PGDFR-α, etc.) mimic the synergistic effect offered by vascular normalization for combinations of anti-VEGF antibody and chemotherapy more effectively than the combinations of multitargeted VEGF receptor kinases–selective TKIs used with chemotherapeutics. This concept is yet to be proven in the clinic, but is strongly supported by the PFS gain produced by sorafenib (Nexavar®, Bayer Pharmaceuticals) in renal cell carcinoma and by sunitinib (Sutent®, Pfizer) in imatinib-resistant GIST patients.

Collectively, these considerations imply that, if we are to optimally use single-targeted or multitargeted anti-VEGF agents, treatment schemes must be tailored for each agent. For example, it is not yet clear whether the addition to chemotherapy of existing multitargeted TKIs– which selectively target VEGF receptor kinases– to chemotherapy will impact outcome (e.g. vatalanib or semaxinib) to an extent comparable with the responses seen for the combination of bevacizumab – which specifically

targets VEGF – with chemotherapy. In choosing a multitargeted agent, the ability to define its spectrum and to match it at the molecular level with the disease will be desirable.

Some agents that target cancer cells directly may indirectly block angiogenesis. In a preclinical model of HER2-overexpressing human breast cancer, trastuzumab (Herceptin®; a HER2-specific antibody) decreased the expression of several angiogenic factors (including VEGF by cancer cells), while increasing the expression of the endogenous angiogenesis inhibitor thrombospondin 1, and induced changes (i.e. reduction in tumor vascular permeability, vessel diameter and vascular volume, but not vessel length) consistent with vascular normalization (Izumi et al. 2002). In this tumor model, however, trastuzumab actually increased VEGF expression in stromal cells. Thus, efficacious targeting of endothelial and cancer cells might be achieved by regimens that combine tumor-cell targeting by anti- EGFR-specific agents with direct anti-VEGF agents (such as bevacizumab). Alternatively, monotherapy regimens might consist of multitargeted TKIs that concomitantly target the EGFR/HER2 on cancer cells in addition to VEGF receptor kinases. Preliminary data from the combination of cetuximab and bevacizumab, either alone or in combination with the chemotherapeutic agent irinotecan, for patients with irinotecan refractory colorectal cancer, suggest that these combinations are feasible and have potentially promising response rates. Additional trials combining trastuzumab, cetuximab or erlotinib with bevacizumab, as well as trials of Zactima (ZD6474; AstraZeneca Pharmaceuticals, Cheshire, UK), which is a multitargeted TKI (selective for VEGFR, EFGR and RET), have reached phase II and/or III (in thyroid, breast, colorectal, lung, pancreatic and head-and-neck cancer patients), and the results will have important implications for the therapy of HER2-positive or EGFR-positive cancers (Herbst et al. 2005).

Future Directions for Anti-Cancer Therapy with Antiangiogenic Agents

The approval of the first antiangiogenic agent for clinical use in patients with colorectal carcinoma has taught us many lessons, the most important of which is that these agents must be used in combination with agents that target cancer cells to have an appreciable impact on patient survival (Jain et al. 2006). Increasing the dose of antiangiogenic agent may harm normal tissues and destroy too much of the tumor vasculature, leading to hypoxia and poor drug delivery in the tumor and to toxicity in normal tissues. However, optimal doses and schedules of these reagents tailored to the angiogenic profile of tumors can normalize tumor vasculature and microenvironment without harming normal tissue. At least three major challenges must be met before therapies based on this vascular normalization model can be successfully translated to the clinic. The first challenge is to determine which other direct or indirect antiangiogenic therapies lead to vascular normalization. In principle, any therapy that restores the balance between pro- and antiangiogenic molecules should induce normalization. Indeed, withdrawing hormones from a hormone-dependent tumor lowers VEGF levels and leads to vascular normalization (Jain et al. 1998). Recently, metronomic therapy – a drug delivery method in which low doses of chemotherapeutic agents are given at frequent intervals – has also been shown to increase the expression of thrombospondin-1, which is a potent endogenous angiogenesis inhibitor (Kerbel and Kamen 2004). Conceivably, this therapy might also induce normalization and improve oxygenation and drug penetration into tumors. Whether various synthetic kinase inhibitors (e.g., Novartis PTK787, Bayer 43–9006, Pfizer SU11248, and AstraZeneca AZD2171), endogenous inhibitors (e.g., angiostatin, endostatin, and tumstatin), antivasocrine agents (i.e., razoxane), conventional che-

motherapeutic agents (e.g., Taxol), and vascular targeting agents do the same remains to be seen. Some of these agents may be effective because they target both stromal and cancer cells. To date, most clinical trials are designed primarily to measure changes in the size of the tumor and may therefore not shed light on changes in the vascular biology of tumors. Clinical studies, such as the rectal carcinoma study described earlier, and other ongoing translational clinical trials should help bridge the gaps in this aspect of our knowledge. The second challenge is to identify suitable surrogate markers of changes in the structure and function of the tumor vasculature and to develop imaging technology that will help to identify the timing of the normalization window during antiangiogenesis therapy. Measurement of blood-vessel density requires tissue biopsy and provides little information on vessel function. Although imaging techniques are expensive and far from optimal, they can provide serial measures of vascular permeability, vascular volume, blood perfusion, and uptake of some drugs and can therefore be used to monitor the window of normalization in patients. The number of circulating mature endothelial cells and their less differentiated progenitors does decrease after VEGF blockade (Duda et al. 2006; Willett et al. 2004, 2005), but whether this decline coincides with the normalization window is not known. During the course of therapy, serial blood measurements of molecules involved in vessel maturation have the potential to identify surrogate markers. PET with 18-fluoromisonidazole and MRI can provide some indication of tumor oxygenation and might be useful for tracking the normalization window. Finally, the measurement of the interstitial fluid pressure is minimally invasive, inexpensive, and easy to implement for anatomically accessible tumors. Hence, this parameter could be used in the interim as a useful indicator of vessel function until novel noninvasive methods are developed. The third challenge is to fill gaps in our understanding of the molecular and cellular mechanisms of the vascular normalization process (Jain 2005a). With rapid advances in genomic and proteomic technology and access to tumor tissues during the course of therapy, we can begin to monitor tumor response to antiangiogenic therapies at the molecular level.

Summary

Tumors require blood vessels for growth and for local and distant invasion. Therefore, many new cancer therapies are directed against the tumor vasculature. The widely held view is that these antiangiogenic therapies should destroy the tumor vasculature, thereby depriving the tumor of oxygen and nutrients. Here, we discussed an alternative mechanism– that certain antiangiogenic agents can also transiently "normalize" the abnormal structure and function of tumor vasculature to make it more efficient for oxygen and drug delivery. Drugs that induce vascular normalization can alleviate hypoxia and increase the efficacy of conventional therapies if both are carefully scheduled.

The recent successes of the antiangiogenic agents have raised great hope and have taught us important lessons about the significance of the target, timing and dosage of each agent (Jain et al. 2006). More antiangiogenic agents are now expected to make a difference in cancer patients with a wide variety of tumor types. With the advent of specific and potent new agents – approved or in the process of being approved – oncologists have a variety of direct and indirect antiangiogenic agents to choose from when designing therapy protocols. Whether the regimens used in the successful trials are optimal, however, and whether antiangiogenic agents will work in patients outside the rigorous inclusion criteria used for those trials remains to be determined. Establishing the most advantageous combinations will require a better understanding of the mechanisms of action of each antiangiogenic agent and the sensitivity of each tumor type, as well as development of robust biomarkers and imaging techniques to guide patient selection and protocol design. A better understanding of the molecular and cellular underpinnings of vascular normalization may ultimately lead to more effective therapies not only for cancer but also for diseases with abnormal vasculature, as well as regenerative medicine, in which the goal is to create and maintain a functionally normal vasculature.

Acknowledgements

The studies described in this chapter were supported by National Cancer Institute grants PO1 CA80124 and R01 CA115767 to Rakesh K. Jain, and R21 CA099237 to Christopher G. Willett, and by a National Foundation for Cancer Research grant and a Goldhirsh Foundation grant to Rakesh K. Jain. Dan G. Duda's research is supported by an AACR-Genentech BioOncology Award. This chapter is adapted from two recent review articles (Jain 2005a; Jain et al. 2006). The authors thank the American Association for the Advancement of Science and the Nature Publishing Group for permission to use material from these articles.

References

Ansiaux R, Baudelet C, Jordan BF, Beghein N, Sonveaux P, De Wever J, Martinive P, Gregoire V, Feron O, Gallez B (2005) Thalidomide radiosensitizes tumors through early changes in the tumor microenvironment. Clin Cancer Res 11:743

Batchelor TT, Sorensen AG, di Tomaso E, Zhang WT, Duda DG, Cohen KS, Kozak KR, Cahill DP, Chen PJ, Zhu M, Ancukiewicz M, Mrugala MM, Plotkin S, Drappatz J, Louis DN, Ivy P, Scadden DT, Benner T, Loeffler JS, Wen PY, Jain RK (2007) AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor, normalizes tumor vasculature and alleviates edema in glioblastoma patients. Cancer Cell 11:83

Baffert F, Thurston G, Rochon-Duck M, Le T, Brekken R, McDonald DM (2004) Age-related changes in vascular endothelial growth factor dependency and angiopoietin-1-induced plasticity of adult blood vessels. Circ Res 94:984

Bang S, Hassan R, Pastan I (2005) Additive anti-tumor effect of Avastin and Immunotoxin combination therapy on tumor bearing mouse models. Proceedings of the 96th Annual Meeting, American Association for Cancer Research, abstract no. 4989

Bottaro DP, Liotta LA (2003) Cancer: out of air is not out of action. Nature 423:593

Carmeliet P (2005) Angiogenesis in life, disease and medicine. Nature 438:932

Demetri GD, van Oosterom AT, Garrett CR, Blackstein ME, Shah MH, Verweij J, McArthur G, Judson IR, Heinrich MC, Morgan JA, Desai J, Fletcher CD, George S, Bello CL, Huang X, Baum CM, Casali PG (2006) Efficacy and safety of sunitinib in patients with advanced gastrointestinal stromal tumour after failure of imatinib: a randomised controlled trial. Lancet 368:1329

Duda DG, Cohen KS, di Tomaso E, Au P, Klein RJ, Scadden DT, Willett CG, Jain RK (2006) Differential CD146 expression on circulating versus tissue endothelial cells in rectal cancer patients: implications for circulating endothelial and progenitor cells as biomarkers for antiangiogenic therapy. J Clin Oncol 24:1449

Escudier B, Eisen T, Stadler WM, Szczylik C, Oudard S, Siebels M, Negrier S, Chevreau C, Solska E, Desai AA, Rolland F, Demkow T, Hutson TE, Gore M, Freeman S, Schwartz B, Shan M, Simantov R, Bukowski RM; TARGET Study Group (2007) Sorafenib in advanced clear-cell renal-cell carcinoma. N Engl J Med 356:125

Fenton BM, Paoni SF, Ding I (2004) Effect of VEGF receptor-2 antibody on vascular function and oxygenation in spontaneous and transplanted tumors. Radiother Oncol 72:221

Ferrara N, Hillan KJ, Gerber HP, Novotny W (2004) Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. Nat Rev Drug Discov 3:391

Folkman J (1971) Tumor angiogenesis: therapeutic implications. N Engl J Med 285:1182

Garber K (2002) Angiogenesis inhibitors suffer new setback. Nat Biotechnol 20:1067

Gullino PM (1982) In: Gautherie M, Albert E (eds) Biomedical thermology. Liss, New York, pp 1–20

Herbst RS, Johnson DH, Mininberg E, Carbone DP, Henderson T, Kim ES, Blumenschein G Jr, Lee JJ, Liu DD, Truong MT, Hong WK, Tran H, Tsao A, Xie D, Ramies DA, Mass R, Seshagiri S, Eberhard DA, Kelley SK, Sandler A (2005) Phase I/II trial evaluating the anti-vascular endothelial growth factor monoclonal antibody bevacizumab in combination with the HER-1/epidermal growth factor receptor tyrosine kinase inhibitor erlotinib for patients with recurrent non-small-cell lung cancer. J Clin Oncol 23:2544

Herbst RS, Mullani NA, Davis DW, Hess KR, McConkey DJ, Charnsangavej C, O'Reilly MS, Kim HW, Baker C, Roach J, Ellis LM, Rashid A, Pluda J, Bucana C, Madden TL, Tran HT, Abbruzzese JL (2002) Development of biologic markers of response and assessment of antiangiogenic activity in a clinical trial of human recombinant endostatin. J Clin Oncol 20:3804

Huber PE, Bischof M, Jenne J, Heiland S, Peschke P, Saffrich R, Grone HJ, Debus J, Lipson KE, Abdollahi A (2005) Trimodal cancer treatment: beneficial effects of combined antiangiogenesis, radiation, and chemotherapy. Cancer Res 65:3643

Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R, Kabbinavar F (2004) Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 350:2335

Inai T, Mancuso M, Hashizume H, Baffert F, Haskell A, Baluk P, Hu-Lowe DD, Shalinsky DR, Thurston G, Yancopoulos GD, McDonald DM (2004) Inhibition of vascular

endothelial growth factor (VEGF) signaling in cancer causes loss of endothelial fenestrations, regression of tumor vessels, and appearance of basement membrane ghosts. Am J Pathol 165:35

Izumi Y, Xu L, di Tomaso E, Fukumura D, Jain RK (2002) Tumour biology: herceptin acts as an anti-angiogenic cocktail. Nature 416:279

Jain RK (1999) Understanding barriers to drug delivery: high resolution in vivo imaging is key. Clin Cancer Res 5:1605

Jain RK (2001) Normalizing tumor vasculature with anti-angiogenic therapy: a new paradigm for combination therapy. Nat Med 7:987

Jain RK (2003) Molecular regulation of vessel maturation. Nat Med 9:685

Jain RK (2005a) Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science 307:58

Jain RK (2005b) Antiangiogenic therapy for cancer: current and emerging concepts. Oncology (Williston Park) 19 [Suppl]:7

Jain RK, Safabakhsh N, Sckell A, Chen Y, Jiang P, Benjamin L, Yuan F, Keshet E (1998) Endothelial cell death, angiogenesis, and microvascular function after castration in an androgen-dependent tumor: role of vascular endothelial growth factor. Proc Natl Acad Sci USA 95:10820

Jain RK, Duda DG, Clark JW, Loeffler JS (2006) Lessons from phase III clinical trials on anti-VEGF therapy for cancer. Nat Clin Pract Oncol 3:24

Kerbel R, Folkman J (2002) Clinical translation of angiogenesis inhibitors. Nat Rev Cancer 2:727

Kerbel RS, Kamen BA (2004) The anti-angiogenic basis of metronomic chemotherapy. Nat Rev Cancer 4:423

Ma J, Pulfer S, Li S, Chu J, Reed K, Gallo JM (2001) Pharmacodynamic-mediated reduction of temozolomide tumor concentrations by the angiogenesis inhibitor TNP-470. Cancer Res 61:5491

Miller KD, Chap LI, Holmes FA, Cobleigh MA, Marcom PK, Fehrenbacher L, Dickler M, Overmoyer BA, Reimann JD, Sing AP, Langmuir V, Rugo HS (2005) Randomized phase III trial of capecitabine compared with bevacizumab plus capecitabine in patients with previously treated metastatic breast cancer. J Clin Oncol 23:792

Morgan B, Thomas AL, Drevs J, Hennig J, Buchert M, Jivan A, Horsfield MA, Mross K, Ball HA, Lee L, Mietlowski W, Fuxuis S, Unger C, O'Byrne K, Henry A, Cherryman GR, Laurent D, Dugan M, Marme D, Steward WP (2003) Dynamic contrast-enhanced magnetic resonance imaging as a biomarker for the pharmacological response of PTK787/ZK 222584, an inhibitor of the vascular endothelial growth factor receptor tyrosine kinases, in patients with advanced colorectal cancer and liver metastases: results from two phase I studies. J Clin Oncol 21:3955

Motzer RJ, Hutson TE, Tomczak P, Michaelson MD, Bukowski RM, Rixe O, Oudard S, Negrier S, Szczylik C, Kim ST, Chen I, Bycott PW, Baum CM, Figlin RA (2007) Sunitinib versus interferon alfa in metastatic renal-cell carcinoma. N Engl J Med 356:115

Murata R, Nishimura Y, Hiraoka M (1997) An antiangiogenic agent (TNP-470) inhibited reoxygenation during fractionated radiotherapy of murine mammary carcinoma. Int J Radiat Oncol Biol Phys 37:1107

Nelson DA, Tan TT, Rabson AB, Anderson D, Degenhardt K, White E (2004) Hypoxia and defective apoptosis drive genomic instability and tumorigenesis. Genes Dev 18:2095

Padera TP, Stoll BR, Tooredman JB, Capen D, di Tomaso E, and Jain RK (2004) Pathology: cancer cells compress intratumour vessels. Nature 427:695

Ratner M (2004) Genentech discloses safety concerns over Avastin. Nat Biotechnol 22:1198

Salnikov AV, Roswall P, Sundberg C, Gardner H, Heldin NE, Rubin K (2005) Inhibition of TGF-beta modulates macrophages and vessel maturation in parallel to a lowering of interstitial fluid pressure in experimental carcinoma. Lab Invest 85:512

Sandler A, Gray R, Perry MC, Brahmer J, Schiller JH, Dowlati A, Lilenbaum R, Johnson DH (2006) Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. N Engl J Med 355:2542

Segers J, Fazio VD, Ansiaux R, Martinive P, Feron O, Wallemacq P, Gallez B (2006) Potentiation of cyclophosphamide chemotherapy using the anti-angiogenic drug thalidomide: importance of optimal scheduling to exploit the 'normalization' window of the tumor vasculature. Cancer Lett 244:129–135

Teicher BA (1996) A systems approach to cancer therapy. (Antioncogenics + standard cytotoxics–>mechanism(s) of interaction). Cancer Metastasis Rev 15:247

Tong RT, Boucher Y, Kozin SV, Winkler F, Hicklin DJ, Jain RK (2004) Vascular normalization by vascular endothelial growth factor receptor 2 blockade induces a pressure gradient across the vasculature and improves drug penetration in tumors. Cancer Res 64:3731

Vosseler S, Mirancea N, Bohlen P, Mueller MM, Fusenig NE (2005) Angiogenesis inhibition by vascular endothelial growth factor receptor-2 blockade reduces stromal matrix metalloproteinase expression, normalizes stromal tissue, and reverts epithelial tumor phenotype in surface heterotransplants. Cancer Res 65:1294

Wildiers H, Guetens G, de Boeck G, Verbeken E, Landuyt B, Landuyt W, de Bruijn EA, van Oosterom AT (2003) Effect of antivascular endothelial growth factor treatment on the intratumoral uptake of CPT-11. Br J Cancer 88:1979

Willett CG, Boucher Y, di Tomaso E, Duda DG, Munn LL, Tong RT, Chung DC, Sahani DV, Kalva SP, Kozin SV, Mino M, Cohen KS, Scadden DT, Hartford AC, Fischman AJ, Clark JW, Ryan DP, Zhu AX, Blaszkowsky LS, Chen HX, Shellito PC, Lauwers GY, Jain RK (2004) Direct evidence that the VEGF-specific antibody bevacizumab has anti-

vascular effects in human rectal cancer. Nat Med 10:145

Willett CG, Boucher Y, Duda DG, di Tomaso E, Munn LL, Tong RT, Kozin SV, Petit L, Jain RK, Chung DC, Sahani DV, Kalva SP, Cohen KS, Scadden DT, Fischman AJ, Clark JW, Ryan DP, Zhu AX, Blaszkowsky LS, Shellito PC, Mino-Kenudson M, Lauwers GY (2005) Surrogate markers for antiangiogenic therapy and dose-limiting toxicities for bevacizumab with radiation and chemotherapy: continued experience of a phase I trial in rectal cancer patients. J Clin Oncol 23:8136

Winkler F, Kozin SV, Tong R, Chae S, Booth MF, Garkavtsev I, Xu L, Hicklin DJ, Fukumura D, di Tomaso E, Munn LL, Jain RK (2004) Kinetics of vascular normalization by VEGFR2 blockade governs brain tumor response to radiation: role of oxygenation, angiopoietin-1 and matrix metalloproteinases. Cancer Cell 6:553

Xiong HQ, Herbst R, Faria SC, Scholz C, Davis D, Jackson EF, Madden T, McConkey D, Hicks M, Hess K, Charnsangavej CA, Abbruzzese JL (2004) A phase I surrogate endpoint study of SU6668 in patients with solid tumors. Invest New Drugs 22:459

Yang JC, Haworth L, Sherry RM, Hwu P, Schwartzentruber DJ, Topalian SL, Steinberg SM, Chen HX, Rosenberg SA (2003) A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. N Engl J Med 349:427

Metronomic Antiangiogenic Chemotherapy: Questions and Answers

34

Robert S. Kerbel, Urban Emmenegger, Shan Man, Raquel Munoz, Francesco Bertolini, Yuval Shaked

Abstract

"Metronomic" (antiangiogenic) chemotherapy refers to the close, regular administration of low doses (non-toxic) of conventional chemotherapy drugs, in the absence of any prolonged drug-free break periods, over long periods of time, even several years. Unlike "dose-dense" and intensive chemotherapy it is minimally toxic and thus does not usually require supportive care drugs. The preclinical antitumor effects of certain metronomic chemotherapy regimens can be surprisingly good, especially when used in combination with concurrent administration of a targeted biologic antiangiogenic agent. It is thought that the antitumor basis of metronomic chemotherapy is mainly via antiangiogenic mechanisms as a result of the local targeting of dividing endothelial cells in the growing tumor neovasculature, and also the systemic targeting of bone marrow-derived circulating endothelial progenitor cells (CEPs). Maximum tolerated dose (MTD) chemotherapy may, in some circumstances, also target CEPs but a hemopoiesis-like proangiogenic acute CEP "rebound" can occur immediately afterwards which is hypothesized to nullify this potential antiangiogenic effect. Shortening or eliminating the drug-free break periods compromises this robust repair process. This CEP rebound phenomenon may also help explain the ability of certain antiangiogenic drugs such as bevacizumab (Avastin®) to enhance the efficacy of some conventional chemotherapy regimens, i.e., by preventing the systemic CEP rebound. Several phase II metronomic chemotherapy clinical trials, some randomized, have been completed, most using daily low-dose (e.g. 50 mg) oral cyclophosphamide, in conjunction with a targeted biologic agent such as bevacizumab or letrozole for treatment of either advanced or early stage breast cancer, or celecoxib for advanced non-Hodgkin's lymphoma, with encouraging results, despite the obvious drawback of the empiricism associated with metronomic dosing. However, advances are being made, both preclinically and clinically, in the discovery of surrogate markers to monitor biologic activity of metronomic chemotherapy and help determine the optimal biologic dose. These markers include circulating apoptotic endothelial cells and CEPs.

CONTENTS

What Is Metronomic Antiangiogenic Chemotherapy?

"Metronomic" chemotherapy is a term that was coined in 2000 to describe a dosing and administration schedule for conventional chemotherapy drugs which is thought to induce antitumor effects indirectly, primarily by antiangiogenic mechanisms, rather than by direct targeting of the tumor cell population (Hanahan et al. 2000). The term refers to the close, regular administration of a chemotherapy drug in the absence of any prolonged drug-free break periods, over long periods of time, using relatively low, non-toxic doses of drug (Browder et al. 2000; Kerbel and Kamen 2004; Klement et al. 2000). The original basis for exploiting chemotherapy drugs as antiangiogenics was based on the hypothesis that dividing endothelial cells present in the growing neovasculature of tumors should be susceptible to the cytotoxic action of such drugs, like any other normal dividing cell population (Kerbel 1991). As such, it was hypothesized by Kerbel that it should be possible to induce responses in tumors even when the tumor cell population is resistant to a given chemotherapy drug, by virtue of targeting the drug-sensitive dividing host endothelial cell population in the tumor's growing neovasculature. Indeed, there is an extensive body of literature dating back to the mid-1980s showing that a wide spectrum of chemotherapeutics belonging to virtually every class of such drugs can cause antiangiogenic effects in a variety of assays (Miller et al. 2001). However, Folkman's laboratory reported in 2000 that the antiangiogenic effects of chemotherapy, using a common alkylating agent – cyclophosphamide (CTX) – were largely lost if the drug was administered in a conventional, pulsatile fashion using maximum tolerated doses (MTDs) separated by long (2-week) drug-free break periods between successive courses of CTX chemotherapy (Browder et al. 2000). Evidence was obtained to show direct endothelial cell apoptosis in the tumor vasculature shortly after drug administration, but this damage inflicted on the tumor vasculature was apparently rapidly repaired during the subsequent drug-free break periods, which are necessary to allow the host (in this case, tumor-bearing mice) to recover from the harmful side effects of chemotherapy such as myelosuppression. Therefore, it was reasoned that if the drug was administered in a condensed

schedule, e.g. weekly rather than in cycles every with 3 week breaks, this would compromise the repair process involved in replacing damaged or killed vascular endothelial cells (Browder et al. 2000). Browder et al. designated this form of chemotherapy "antiangiogenic chemotherapy" and the major hallmarks of this method of chemotherapy drug administration are its prolonged nature and the absence of any long drug-free break periods, and hence the need for much lower individual doses of drug. In addition, it was reported by Browder et al. that a variety of transplantable mouse tumors that had been previously selected for acquired resistance in vivo to CTX by using conventional MTD dosing and scheduling of CTX could be induced to respond once again to the same drug simply by switching to the weekly lower-dose metronomic protocol (Browder et al. 2000). In other words, a state of acquired resistance could be reversed simply by altering the dosing and administration schedule of the drug, which resulted in a different cellular target. There are some precedents for this in the clinic, e.g. ovarian or breast cancer patients responding to a weekly taxane regimen after seemingly becoming resistant to a taxane regimen administered at the MTD once every 3 weeks (Kerbel and Kamen 2004).

Results similar to those of Browder et al. were reported by Klement and colleagues using vinblastine to treat large established human tumor neuroblastoma xenografts, where the drug was administered every 3 days over long periods without any prolonged drug-free breaks (Klement et al. 2000). In addition, Klement and colleagues reported that combining this method of administering vinblastine with concurrent administration of a targeted antiangiogenic drug, e.g. antibodies to vascular endothelial growth factor receptor-2 (VEGFR-2) also administered every 3 days, resulted in remarkably effective tumor responses, which included complete and sustained regressions of large established tumors, without any evidence of tumor relapse over the 7-month long period of therapy (Klement et al. 2000). Effectively, the mice were cured with little evidence of any serious toxic side effects (see Fig. 34.1). The rationale for this particular treatment combination was based on the hypothesis that the endothelial cell targeting effects of a metronomic chemotherapy regimen might be compromised by locally high levels of endothelial cell survival factors, especially VEGF, and if so, neutralizing the pro-survival function of VEGF at the same time as administration of metronomic chemotherapy would significantly improve the effects of the latter (Klement et al. 2000).

The term "metronomic chemotherapy" was coined by Hanahan and colleagues in an editorial commentary summarizing the results and significance of the two studies by Browder et al. and Klement et al. (Hanahan et al. 2000). It is meant to convey the idea of regular beats of a metronome over long periods of time, where each "beat" is administration of a chemotherapy drug. Because of the increased frequency of drug administration, comparatively low doses of chemotherapy drug are required for each administration. In some cases, the cumulative doses of the drug are less than, or equivalent to, the respective chemotherapy administered in a conventional MTD fashion. As a result, the acute toxicities associated with metronomic chemotherapy regimens are often minimal and, as a result, do not require supportive care drugs. This is in marked contrast to "dose-dense" chemotherapy, where a drug is also administered using a more frequent schedule, but in fairly high doses such that toxicities remain significant, thus requiring the use of supportive care drugs (Citron 2004). Indeed, it is the use of recombinant G-CSF to accelerate recovery from myelosuppression to 2 weeks from 3 weeks that makes dose-dense chemotherapy possible (Kim and Tannock 2005).

What Is the Basis for the Endothelial Cell Repair Process During the Drug-free Break Period Between Cycles of MTD Chemotherapy?

Originally, the target responsible for mediating the antiangiogenic effects of chemotherapy was assumed to be the differentiated dividing vascular endothelial cell present in the growing tumor neo-

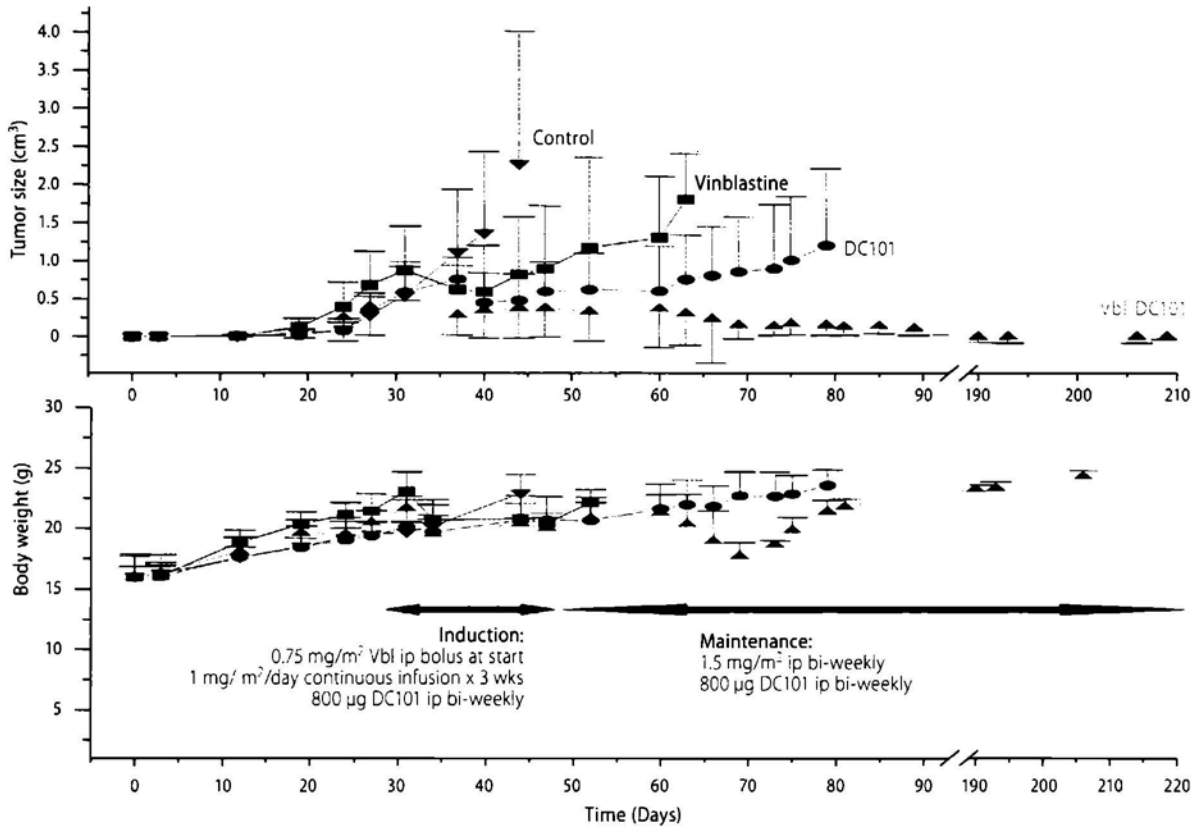


Fig. 34.1. Effects of extended metronomic low-dose vinblastine treatment combined with DC101, an anti-VEGFR-2 neutralizing antibody, on the growth of large subcutaneously transplanted human (SK-NMC NB) neuroblastoma xenografts in immune deficient mice (*upper panel*). The long-term vinblastine metronomic/maintenance therapy was initiated after an induction/up-front 3-week treatment of a cumulatively higher dose of vinblastine, using an infusion pump. Note the 7-month-long treatment in the combination group, which resulted in complete and sustained tumor regressions in the absence of significant toxicity as assessed by body weight change (*lower panel*). The drugs were administered intraperitoneally every 3 days with no long break periods. This result and others (Browder et al. 2000; Pietras and Hanahan 2005) have significantly contributed to the initiation of phase II clinical trials evaluating similar therapeutic strategies, e.g. low-dose metronomic CTX and letrozole for the adjuvant treatment of breast cancer (Bottini et al. 2006). Taken from Klement et al. (2000) with permission from the publishers

vasculature, albeit in low percentages (Browder et al. 2000; Klement et al. 2000), as discussed above. However, it is now known that new endothelial cells during the process of angiogenesis can be derived not only from the aforementioned local (tumor) process, but also systemically, as a result of the mobilization of cells from the bone marrow compartment, which then enter the peripheral blood circulation and home to sites of angiogenesis, where a proportion of such cells incorporate into the lumina of growing vessels and differentiate into endothelial cells (Asahara et al. 1997). Such cells are referred to as "circulating endothelial progenitor cells" (CEPs) (Asahara et al. 1997; Shaked et al. 2005a). A few years ago it was reported that shortly after administration of an intensive week long MTD course of CTX, the levels of CEPs were substantially reduced, but that this was followed by an abrupt and marked

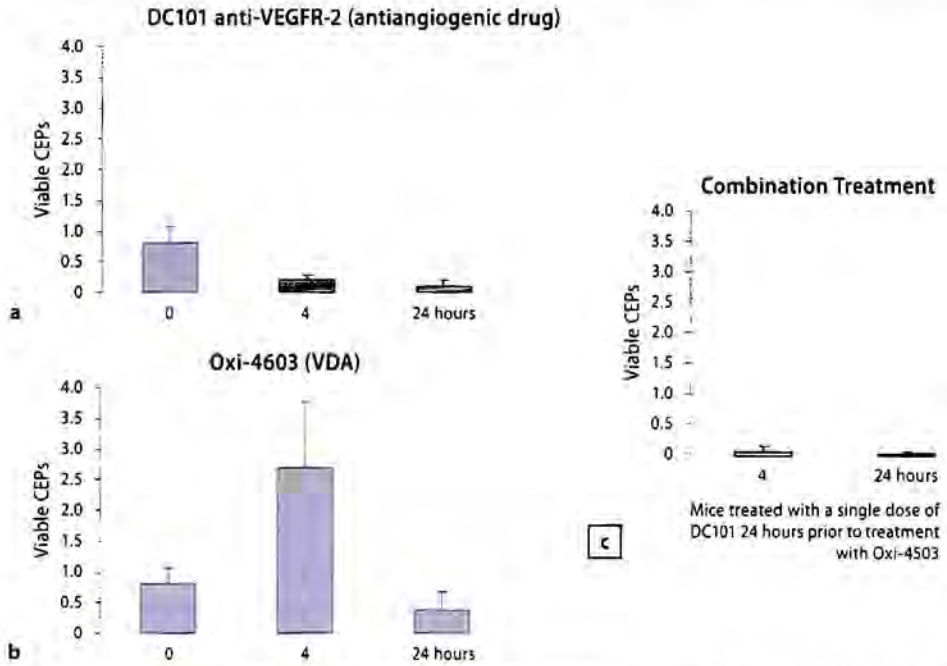


Fig. 34.2a–c. Effects of VDA and/or antiangiogenic drug (DC101) treatment on levels of viable CEPs in peripheral blood, as assessed by four-color flow cytometry. a The anti-VEGFR-2 antibody DC101 causes a decrease in CEPs, as described previously (Shaked et al. 2005a), observed at both 4 h and 24 h after a single i.p. injection of 800 μg/mouse. b In contrast, the VDA known as Oxi-4503 causes a rapid increase in levels of CEPs observed at 4 h after i.p. injection of the drug, following which levels return to near normal at 24 h. c The VDA-induced "flare" observed at 4 h post treatment can be completely blocked by DC101 given 24 h before Oxi-4503. Taken from Shaked et al. (2006) with permission of the publishers

rebound of such cells, similar to the process of hemopoiesis (Bertolini et al. 2003). The rapid mobilization of CEPs following their initial decline could conceivably account for a significant portion of the repair process during the extended drug-free break periods following MTD chemotherapy, assuming that such mobilized cells actually home to sites of tumor angiogenesis/damaged blood vessels and contribute to new blood vessel formation and vessel repair. Recently, it was reported that exactly such a scenario appears to be the case after administration of so-called cytotoxic "vascular disrupting agents" (VDAs) such as combretastatin, a microtubule inhibitor, or a second-generation derivative of this drug called Oxi-4503 (Shaked et al. 2006). Thus, VDA administration can cause a rapid and marked increase in the peripheral blood levels of CEPs (see Fig. 34.2), which then home to the viable tissue that typically remains at the tumor rim (Shaked et al. 2006) that characteristically remain after VDA treatment, surrounding a large area of intra-tumoral necrosis (Tozer et al. 2005). These bone marrow-derived cells are then retained at the tumor periphery, as shown in Fig. 34.3 using the example of mice that had been previously lethally irradiated and reconstituted with syngeneic GFP-positive bone marrow cells, where they contribute to tumor regrowth (repopulation), at least in part by stimulating tumor angiogenesis (Shaked et al. 2006). If a similar scenario holds for chemotherapy drugs administered at the MTD, this could obviously account for the rapid repair that was first noted by Browder and colleagues to the tumor vasculature after MTD CTX treatment (Browder et al. 2000).

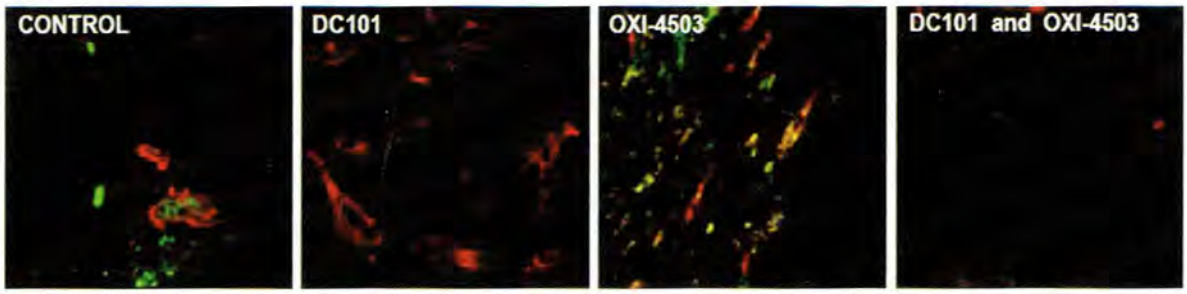


Fig. 34.3. Acute homing of bone marrow-derived circulating cells (including CEPs) to tumors shortly after treatment of tumor-bearing mice with a single injection of a VDA (Oxi-4503). Lewis lung carcinomas were grown in syngeneic C57BI6 mice that had previously been lethally irradiated and reconstituted with GFP-positive bone marrow cells. Note low levels of GFP-positive cells in tumors from untreated mice. However, 72 h after Oxi-4503 treatment a pronounced GFP signal is evident in the tumor, indicating a massive homing of such cells to treated tumors, a process which can be prevented by prior treatment with DC101, the anti-VEGFR-2 antibody. The CEP homing phenomenon was shown to contribute to tumor angiogenesis and tumor growth at the viable tumor rim which characteristically remains after VDA treatment. Taken from Shaked et al. (2006) with permission of the authors

34.3 If Shortening the Breaks Compromises Endothelial Cell Repair Mechanisms, Should This Not Also Apply to Other Types of Normal Drug-sensitive Cycling Cells?

This particular question represents a major paradox regarding the mechanistic basis of metronomic chemotherapy, as one might expect metronomic chemotherapy would compromise repair of other tissues/cells damaged or killed by the therapy. It has been noted by a number of investigators, both clinically (Colleoni et al. 2002) and preclinically (Emmenegger et al. 2004), that metronomic administration of chemotherapy drugs such as CTX, or CTX plus methotrexate (MTX), is not associated with significant high-grade common toxicities such as myelosuppression. How does one explain maximizing endothelial cell damage by more frequently administered chemotherapy, while not at the same time increasing the severity of other side effects such as toxicity to bone marrow progenitors, leading to an increase in the severity of neutropenia? There are several possible explanations to this question. The first is that for unknown reasons dividing differentiated endothelial progenitor cells, and possibly bone marrow-derived CEPs could be exquisitely sensitive to very low doses of chemotherapy which are not toxic to other types of normal cycling cells that are usually sensitive to higher doses of chemotherapy. There is some limited evidence in support of this possibility. For example, several groups have reported that extremely low concentrations of chemotherapy drugs in vitro can cause anti-endothelial effects, including apoptosis, at concentrations which no longer have such effects on any other types of normal or malignant cell populations tested (Bocci et al. 2002; Vacca et al. 1999; Wang et al. 2003). In other words, at extremely low doses of chemotherapy, either in vitro or in vivo, there might be a marked and selective sensitivity of vascular endothelial cells. If so, this conceivably might also apply to bone marrow-derived CEPs (Shaked et al. 2005c). Indeed, metronomic chemotherapy using CTX administered at an approximate dose of 20 mg/kg/day through the drinking water is not associated with any myelosuppression, i.e., neutropenia (Emmenegger et al. 2004), but causes a marked decline in CEPs (Shaked et al. 2005c), as shown in Fig. 34.4. A second explanation is that the effects of low-dose metronomic chemotherapy are mediated by an indirect, secondary mechanism that is highly specific for activated vascular endothelial cells and/or CEPs. Again, there

is some limited evidence for this hypothesis. Bocci and colleagues first reported that administration of low-dose CTX can result in a systemic induction of the well-known endogenous angiogenesis inhibitor, thrombospondin-1 (TSP-1). Thus, the effects of metronomic CTX on tumors were largely lost when tumors were grown and treated in TSP-1-deficient mice (Bocci et al. 2003). Several other groups have now also reported that metronomic chemotherapy can result by unknown mechanisms in an increase in the tissue expression of TSP-1, including in the tumor cells and tumor stroma, as well as increased circulating levels of TSP-1 in peripheral blood (Damber et al. 2006; Hamano et al. 2004). The key point here is that TSP-1 would not be expected to affect bone marrow progenitors, hair follicle cells, epithelial cells lining the gut, etc., so that cells/tissues that are normally sensitive to the toxic effects of MTD chemotherapy would not be strongly affected, if at all, by low-dose metronomic chemotherapy if a TSP-1-mediated mechanism was involved in targeting endothelial cells or CEPs.

What Are the Advantages of Metronomic Chemotherapy Regimens?

Given the aforementioned discussion, it is apparent that one significant advantage of most metronomic chemotherapy regimens would be the absence of high-grade adverse events normally associated with conventional MTD chemotherapy dosing, such as nausea, vomiting, myelosuppression, mucositis, and hair loss (Emmenegger et al. 2004). This is not to say that toxicity would be absent, but it would be minimal, and this obviously represents a significant potential advantage, especially with respect to treatment of children and the elderly. A second advantage, at least potentially, is convenience when using orally bioavailable chemotherapy drugs such as CTX (Colleoni et al. 2002), etoposide (Kieran et al. 2005), MTX (Colleoni et al. 2002), capecitabine, and tegafur-uracil (UFT) (Kato et al. 2004). Such drugs can be administered at fixed or varying doses

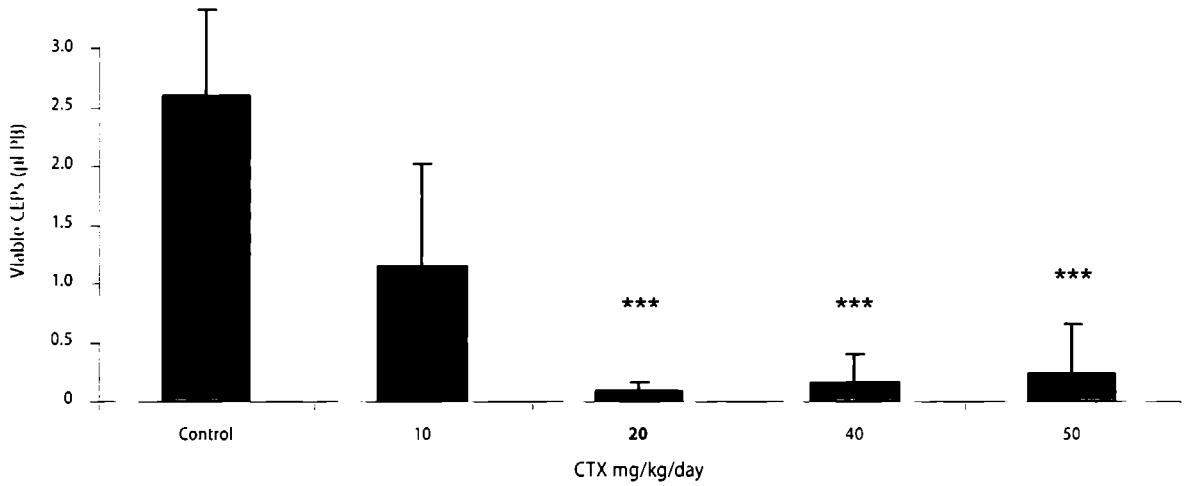


Fig. 34.4. Effect of different doses of CTX administered daily through the drinking water on levels of viable CEPs. An estimated dose of 20 mg/kg/day caused maximal decline in levels of viable CEPs, and this figure was not significantly changed by increasing the dose to 40 or 50 mg/kg/day. However, the latter higher doses were found to be toxic to mice (Shaked et al. 2005c). Parallel experiments involving 1 week's treatment of tumor-bearing mice with these different doses of CTX showed that the OBD, taking toxicity into account, was 20 mg/kg/day – which matches the CEP results shown above. In addition, a dose of 10 mg/kg/day does not cause an increase in the levels of apoptotic circulating endothelial cells, whereas doses of 20 mg/kg/day or above do so (Mancuso et al. 2006). Taken from Shaked et al. (2005c) with permission of the publishers

on an out-patient or at-home basis. This particular advantage, however, must be balanced with the known disadvantages of oral drugs, such as patient compliance and inconsistent pharmacokinetics due to variable drug absorption. A third advantage relates to reduced costs (Bocci et al. 2004b) when using inexpensive off-patent drugs such as CTX or MTX. This represents a potentially significant benefit, especially given the rapidly increasing costs of new anticancer drugs, the extent of which is placing enormous and growing burdens on health care systems (Schrag 2004). A fourth advantage is the ability to combine targeted biologic agents that are relatively non-toxic in a chronic fashion with metronomic chemotherapy. Such chronic combination treatments would not be possible when using only toxic MTD chemotherapy regimens.

In Addition to Combination with Targeted Antiangiogenic Drugs, What Other Combinations Can Be Used with Metronomic Chemotherapy?

While most published preclinical studies have shown the benefit of combining a targeted antiangiogenic drug with a particular metronomic chemotherapy regimen, other promising drug combinations have been reported. For example, immunotherapeutic tumor vaccines can be combined with metronomic chemotherapy to enhance the overall effects of either form of therapy (Hermans et al. 2003). This is particularly interesting since one of the supposed disadvantages of using immunotherapy approaches for cancer treatment is the inability to combine such approaches with potentially immunosuppressive MTD chemotherapy regimens. However, in the case of metronomic chemotherapy, at least with some drugs such as CTX, it has been shown that not only is this form of chemotherapy non-immunosuppressive, but it actually can stimulate the immune system (Loeffler et al. 2005). Indeed, it has been known for many years that administration of single low doses of CTX to mice or rats can deplete the host of regulatory immunosuppressive T-cells and, as a result, amplify the effects of cytotoxic T-cells (Ghiringhelli et al. 2004). Thus, low-dose metronomic chemotherapy may be particularly ideal as a combination treatment with tumor vaccines (Hermans et al. 2003). In addition to tumor vaccines it has also been reported that other agents such as COX-2 inhibitors (Buckstein et al. 2006; Coras et al. 2004; Hafner et al. 2005; Reichle et al. 2004), letrozole, an aromatase inhibitor (Bottini et al. 2006), trastuzumab (Herceptin®) (du Manoir et al. 2006), and dexamethasone (Glode et al. 2003) can each be combined with metronomic chemotherapy regimens involving daily low-dose oral CTX. Some of these studies are preclinical in nature, while others are clinical. It may be that the enhanced antitumor effects of combining a drug such as trastuzumab or celecoxib with metronomic chemotherapy is due to the antiangiogenic effects of the aforementioned biologic agents (Buckstein et al. 2006; du Manoir et al. 2006). Indeed, part of the rationale for testing trastuzumab with metronomic CTX was based on previous studies showing that trastuzumab could have antiangiogenic effects that contribute to its overall antitumor efficacy (Viloria-Petit et al. 1997).

How Does One Determine the Optimal Biologic Dose for Metronomic Chemotherapy?

This particular question highlights the current major disadvantage of daily oral metronomic chemotherapy (Lam et al. 2006). Clearly, it is a reasonably straightforward proposition to determine the MTD of a given chemotherapy drug. It is a different matter to determine the optimal biologic (low) dose (OBD) for a drug administered in a metronomic fashion (Lam et al. 2006). This problem is, of course, not restricted to metronomic chemotherapy. It is well known that many of the new anticancer agents do not necessarily have the dose-limiting toxicities which are normally used to define an MTD. In addition,

even when a particular drug has a definable MTD, the OBD may be less than the MTD (Cristofanilli et al. 2002). This is thought to be the case with many of the targeted antiangiogenic drugs that are currently commercially available, or still under development (Cristofanilli et al. 2002). However, some progress has been made recently with respect to defining surrogate pharmacodynamic biomarkers which may be useful for monitoring the biologic activity of antiangiogenic drugs and metronomic chemotherapy, including determining the OBD (Bocci et al. 2004a; Shaked et al. 2005a, 2005c). Preclinically, it has been reported that the OBDs of a number of different metronomic chemotherapy regimens can be determined by using CEPs as a surrogate biomarker (Shaked et al. 2005c). This is based on prior findings, in mice, that enumeration of peripheral blood CEPs can be used as a readout for angiogenesis and therefore to determine the OBDs of targeted antiangiogenic agents such as anti-VEGFR-2 antibodies (Shaked et al. 2005a). To date, the OBDs for seven different chemotherapy drugs have been determined for mice using CEPs. These drugs are CTX (Shaked et al. 2005c), cisplatinum (Shaked et al. 2005c), vinblastine (Shaked et al. 2005c), vinorelbine (Shaked et al. 2005c), paclitaxel (Shaked et al. 2005c), abraxane (Ng et al. 2006), and UFT (Munoz et al. 2006). Whether endothelial progenitor cells can be used successfully in a similar fashion in humans has not yet been shown, and may be difficult given the low numbers of endothelial progenitor cells in humans compared to mice. Instead, a more practical cellular surrogate marker may be apoptotic circulating endothelial cells (CECs). Recently, Mancuso et al. have shown that apoptotic CECs measured after 2 months of metronomic CTX/MTX chemotherapy treatment of metastatic breast cancer patients has potential as a surrogate marker to monitor biologic activity of metronomic chemotherapy, and quite possibly, to predict future clinical benefit, such as progression-free survival and overall survival (Fig. 34.5) (Mancuso et al. 2006). Based on preclinical studies, the presumed source in humans of these apoptotic CECs is the tumor vasculature. Thus, administra-

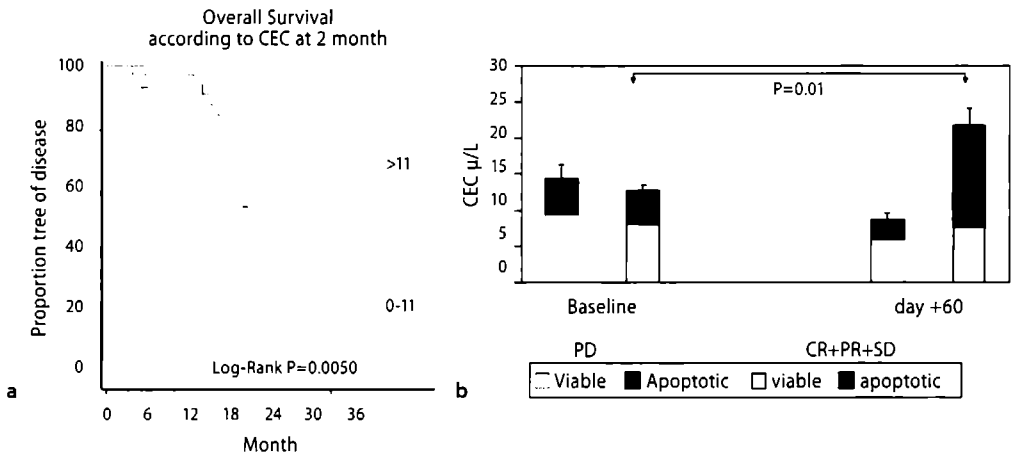


Fig. 34.5a,b. Increases in levels of apoptotic CECs may be a surrogate marker indicative of the antiangiogenic activity of metronomic chemotherapy in patients, as well as predicting clinical benefit. a Results of a phase II clinical trial involving low-dose oral metronomic CTX and MTX chemotherapy (combined with thalidomide) showed that an increase in the levels of apoptotic CECs, as detected by flow cytometry, after 2 months of treatment indicated a clinical benefit when the levels of apoptotic CECs exceeded greater than 11/µl of blood. Clinical benefit was also defined as either complete response (*CR*), partial response (*PR*), or stable disease (*SD*), as opposed to progressive disease (*PD*). b Patients experiencing CR, PR, or SD showed an elevated level of apoptotic CECs after 60 days of metronomic chemotherapy treatment, compared to baseline values observed before treatment was initiated. Taken from Mancuso et al. (2006) with permission of the publishers

tion of metronomic CTX to normal mice does not result in a detectable increase in the levels of apoptotic CECs, whereas such increases are noted when using biologically active doses of the same drug in tumor-bearing mice (Mancuso et al. 2006). Clearly, validation of such markers in humans will be an important step towards improving the likelihood of achieving significant clinical benefits using metronomic chemotherapy protocols in patients. In this regard, the benefits that have been noted so far in empirical metronomic chemotherapy trials (summarized below) are particularly encouraging.

Is It Useful to Combine Cyclic MTD Chemotherapy with Metronomic Low-Dose Chemotherapy?

In one of the original metronomic chemotherapy studies (Klement et al. 2000), the once every 3 day low-dose vinblastine administration schedule was actually preceded by a 3-week treatment regimen where the drug was given at a cumulative higher dose, using a continuous infusion pump, to treat large established neuroblastoma xenografts. The initial upfront higher cumulative dose regimen was used to bring about a rapid tumor response, i.e., some degree of tumor shrinkage, which would then be followed immediately by the long-term 'maintenance' metronomic chemotherapy regimen using the same drug. This protocol highlighted the possibility of using conventional chemotherapy dosing in sequence with metronomic chemotherapy. Indeed, a preclinical study by Pietras and Hanahan clearly showed the significant benefits that can be derived by a short upfront course of MTD CTX therapy, immediately followed by long-term daily low-dose metronomic CTX, where the drug was administered daily through the drinking water (Pietras and Hanahan 2005), as first reported by Man et al. (2002). A variation of the aforementioned two studies was reported by Shaked and colleagues using three different transplantable tumor models, in which bolus dose (BD) intraperitoneal injection of approximately one third of the MTD CTX was administered every 3 or 6 weeks, along with daily low-dose oral CTX administered through the drinking water at the optimal biologic (metronomic) dose, as shown in Fig. 34.6. This combination of BD plus low-dose metronomic chemotherapy was found to significantly improve the effects of the low-dose metronomic chemotherapy regimen used alone, and in some cases was associated with surprisingly effective long-term antitumor effects (Shaked et al. 2005b). Thus, conventional MTD-type chemotherapy and less toxic low-dose metronomic chemotherapy should not be thought of in terms of "either/or" but rather as possibly complementary ways of giving the same drug to enhance overall antitumor efficacy. Clinically, there are protocols being tested that are somewhat similar in some respects, e.g. daily low-dose oral CTX with weekly vinblastine (Young et al. 2006) or weekly platinum and daily oral etoposide (Correale et al. 2006).

Do the Early Clinical Trial Results of Metronomic Chemotherapy Support the Preclinical Studies of This Chemotherapy Treatment Concept?

As with any new anticancer treatment concept initially demonstrated in preclinical models, especially using tumor-bearing mice, there is always the concern that the encouraging preclinical results will not be observed in patients. However, early indications suggest that metronomic chemotherapy may become a clinically validated concept, though formal confirmation of this awaits initiation and completion of larger randomized phase III clinical trials. In the meantime, there have been a number of both non-randomized and randomized phase II clinical trials involving a number of chemotherapy drugs, especially oral low-dose CTX, sometimes combined with oral low-dose MTX, where these two drugs are used together, or in combination with a targeted antiangiogenic drug such as bevacizumab, celecoxib, or letrozole, with promising results. These trials

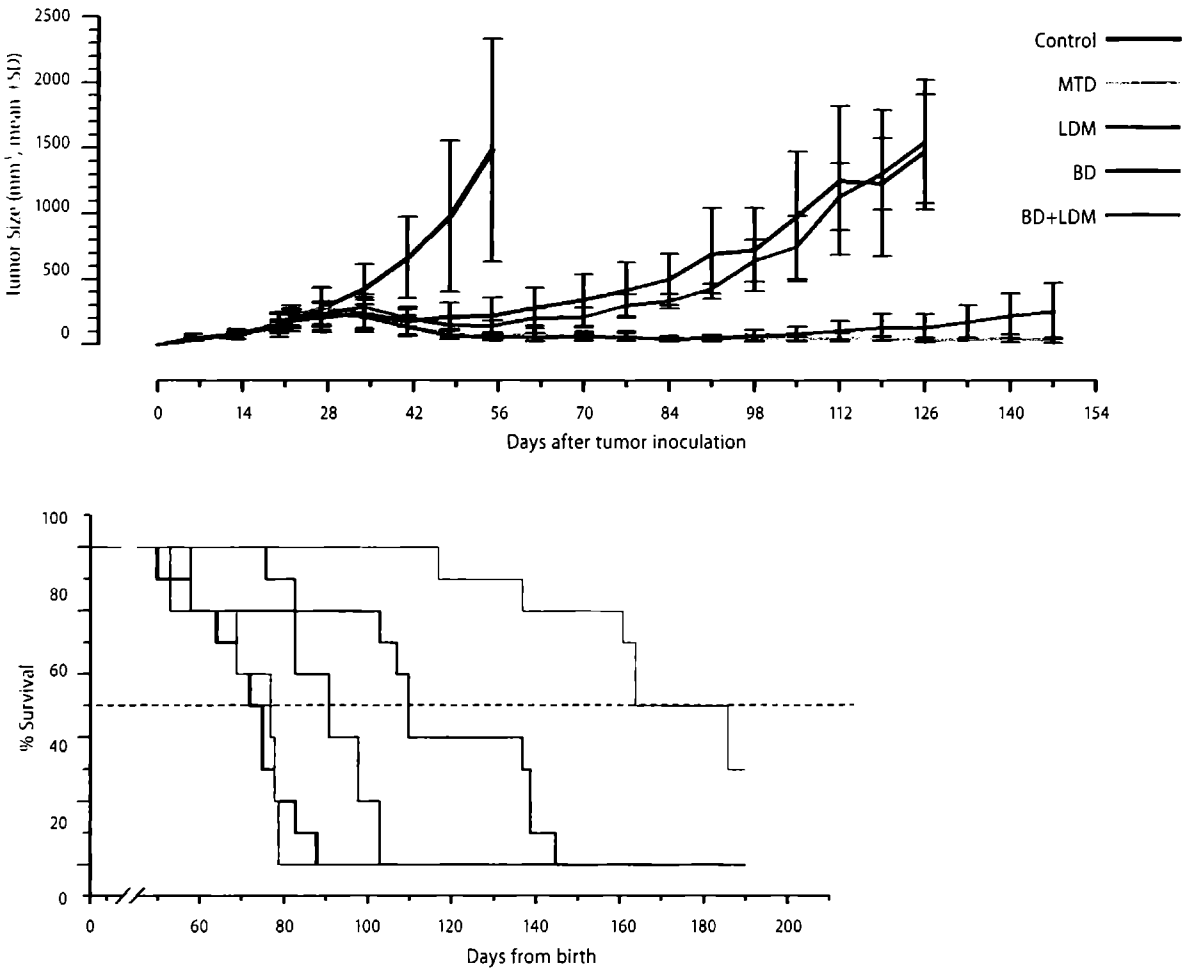


Fig. 34.6a,b. Extended survival and increased antitumor efficacy in mice treated with intermittent bolus dose (BD) CTX plus low-dose metronomic (*LDM*) CTX ("fast" plus "slow" metronomic chemotherapy). Human PC3 prostate cancer cells (a) or Friend erythroleukemia (b) induced mice were treated with CTX in various regimens, including MTD – total of 450 mg/kg CTX administered intraperitoneally over a 6-day cycle consisting of three injections of 150 mg/kg each every other day; LDM – approximately 20 mg/kg/day of the same drug administered through the drinking water; BD – a single injection of 150 mg/kg of CTX administered every 3 or 6 weeks; or a combination of BD plus LDM. Mice were monitored regularly for tumor volume changes (a) or survival (b). Taken from Shaked et al. (2005b) with permission of the publishers

include a non-randomized trial of relapsed, refractory non-Hodgkin's lymphoma using daily low-dose CTX in combination with celecoxib (Buckstein et al. 2006); a randomized trial of recurrent epithelial ovarian cancer using daily low-dose CTX in combination with biweekly bevacizumab (Garcia et al. 2005, 2007); a randomized phase II trial of daily low-dose CTX and low-dose MTX administered 2 days a week in combination with bevacizumab for the treatment of metastatic breast cancer (cited in Singletary 2007); a randomized phase II trial of daily low-dose cyclophosphamide and letrozole for metastatic breast cancer in elderly patients (Bottini et al. 2006); a non-randomized trial in pediatric

cancer patients using a 3-week alternating sequence of daily low-dose CTX and then daily low-dose etoposide, in conjunction with daily administration of thalidomide and celecoxib (Kieran et al. 2005); and a non-randomized trial involving different malignancies involving daily low-dose CTX and weekly vinblastine, along with concurrent daily rofecoxib (Young et al. 2006). All of these trials have been associated with minimal or modest toxicity along with putative clinical benefit in terms of time to progression, response rate, or progression-free survival and sometimes overall survival. The emphasis on CTX, and in some cases, MTX along with CTX, stems from a pivotal non-randomized phase II trial involving 66 women with metastatic breast cancer who were treated with daily low-dose CTX (50 mg orally) and MTX (orally) 2 days a week for a total dose of 10 mg a week by Colleoni et al. (2002). Based on a number of preclinical studies, especially Klement et al. (2000), it was decided to use this empirical but all-oral convenient metronomic chemotherapy protocol in combination with targeted biologic agents such as bevacizumab. In addition, the results of other chemotherapy drugs in clinical trials are being re-examined retrospectively from the point of view of the metronomic chemotherapy concept. For example, over a decade ago a clinical trial was initiated using the 5-fluorouracil (5-FU) oral prodrug known as UFT for the treatment of early stage, resected non-small cell lung cancer (NSCLC) where the drug was orally administered by tablet at low, non-toxic doses every day for 2 years (Kato et al. 2004). UFT, or its metabolites, has been shown to have antiangiogenic effects, especially when administered by continuous infusion at lower doses, as opposed to intermittent bolus injections (Yonekura et al. 1999). UFT generates three different metabolites – 5-FU, gamma butyrolactone (GBL), and gamma hydroxybutyrate (GHB) – all of which have been shown to induce antiangiogenic effects in vivo (Yonekura et al. 1999).

In addition, results of smaller pilot studies have been reported using low-dose CTX and dexamethasone for advanced prostate cancer (Glode et al. 2003) and rofecoxib with trofosfamide for advanced melanoma and soft tissue sarcoma (Reichle et al. 2004), among others.

What About Combination Metronomic Chemotherapy Using Two Different Chemotherapy Drugs?

An interesting question is whether two different chemotherapy drugs, administered as a 'doublet' regimen using metronomic dosing and schedules, would improve upon the effects of either drug used alone. There would seem to be a compelling rationale for testing such drug combinations, as it is well known that combinations of two chemotherapy drugs administered in an MTD fashion are often more effective than either drug alone, e.g. a platinum drug in combination with a taxane ("PC") for the treatment of NSCLC; the combination of 5-FU, leucovorin and irinotecan, or oxaliplatin, for the treatment of colorectal cancer; and CTX and adriamycin (AC) for breast cancer. To this end, Munoz and colleagues investigated the effects of a combination of daily low-dose CTX administered through the drinking water on a non-stop basis combined with UFT administered by gavage, also on a daily non-stop basis, where both drugs were dosed at the OBD, using CEPs as a surrogate biomarker to determine the OBD (Munoz et al. 2006). This combination chemotherapy treatment was tested in a newly developed model of advanced, high-volume (terminal) visceral human metastatic breast cancer in immunodeficient mice. Indeed, evidence emerged that the combination of metronomic CTX and UFT could cause remarkable long-term antitumor effects, if not cure, and moreover do so in the absence of any significant discernable toxicity (Munoz et al. 2006), as shown in Fig. 34.7. Other doublet combinations of metronomic chemotherapy are currently being evaluated in different models of advanced metastatic cancer, including malignant melanoma.

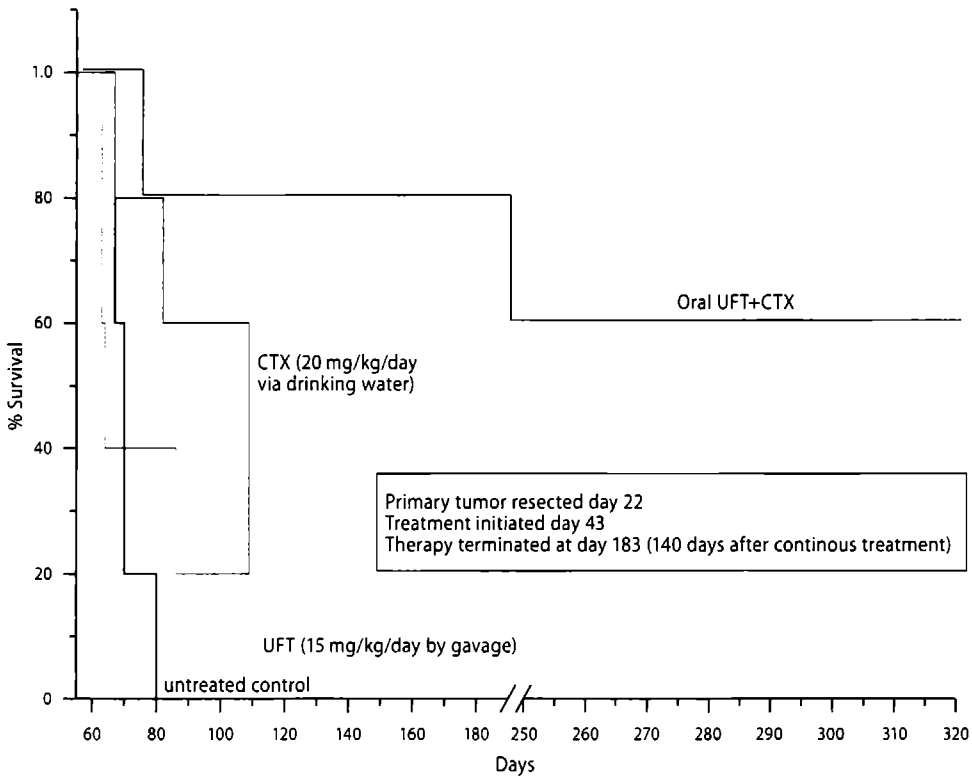


Fig. 34.7. Effects of combination 'doublet' oral low-dose metronomic chemotherapy on survival of mice with advanced, high-volume visceral metastatic breast cancer. A new model of advanced metastatic breast cancer was developed using the MDA-MB-231 human breast cancer cell line, as described by Munoz et al (2006), which involves a combination of orthotopic transplantation, surgical removal of primary tumors, and establishment of cell lines from subsequently forming metastases found in the lungs. One such variant is called LM2-4. This variant was orthotopically transplanted into SCID mice and the tumor removed approximately a month later, and then treatment was initiated approximately 1 month after surgery when extensive microscopic metastases were established in several different organ sites, such as the lungs, liver, and lymph nodes. Treatment consisted of daily oral low-dose CTX and/or daily oral low-dose UFT administered by gavage at the indicated OBDs, as determined by using CEP as a surrogate biomarker of tumor angiogenesis. Therapy was maintained continuously for 140 days. The combination treatment caused a remarkable antitumor effect and extension of survival. Taken from Munoz et al. (2006) with permission of the publishers

Metronomic Chemotherapy: The Way(s) Ahead

The first clinical reports of metronomic chemotherapy are sufficiently encouraging to warrant further clinical trial testing in the context of larger randomized phase II or phase III trials. For example, in a randomized phase II trial of metronomic daily low-dose (50 mg) oral CTX combined with daily oral letrozole involving 57 early-stage breast cancer patients per arm, those receiving the combination treatment had an exceptionally high response rate of 88.1% while the letrozole arm had a response rate of 72%; toxicities were very mild in both arms (Bottini et al. 2006). This might be viewed as remarkable given the empirical nature of the CTX dosing. Clearly we need to learn more about the best chemotherapy drugs for

metronomic chemotherapy (Drevs et al. 2004), the best chemotherapy drug combinations, the optimal dose for such drugs, and the best biologic agents to be used in combination with metronomic chemotherapy regimens. Also important will be choices with respect to the types or stages of cancer most suitable for metronomic chemotherapy treatment regimens. Preclinical metronomic chemotherapy studies have been very helpful in guiding the design of clinical trials. Some clinical trials are now providing directions for new preclinical studies that could improve the results of future clinical trials. For example, the results of Mancuso et al. showing the potential of apoptotic CECs as a surrogate marker for metronomic chemotherapy biologic activity in breast cancer patients (Mancuso et al. 2006) need to be confirmed and extended in preclinical models to determine whether such cells might be used successfully to determine the OBD for metronomic chemotherapy in patients. In addition, some molecular markers circulating in blood, such as soluble erbB2/Her2 or EGFR, may have promise as predictive markers (in breast cancer patients) treated with metronomic chemotherapy (Sandri et al. 2007).

References

Asahara T et al (1997) Isolation of putative progenitor endothelial cells for angiogenesis. Science 275:964–967

Bertolini F et al (2003) Maximum tolerable dose and low-dose metronomic chemotherapy have opposite effects on the mobilization and viability of circulating endothelial progenitor cells. Cancer Res 63:4342–4346

Bocci G et al (2002) Protracted low-dose effects on human endothelial cell proliferation and survival in vitro reveal a selective antiangiogenic window for various chemotherapeutic drugs. Cancer Res 62:6938–6943

Bocci G et al (2003) Thrombospondin-1, a mediator of the antiangiogenic effects of low-dose metronomic chemotherapy. Proc Natl Acad Sci USA 100:12917–12922

Bocci G et al (2004a) Increased plasma VEGF as a surrogate marker for optimal therapeutic dosing of VEGFR-2 monoclonal antibodies. Cancer Res 64:6616–6625

Bocci G et al (2004b) Cyclophosphamide–methotrexate "metronomic" chemotherapy for the palliative treatment of metastatic breast cancer. A comparative pharmacoeconomic evaluation. Ann Oncol 16:1243–1252

Bottini A et al (2006) Randomized phase II trial of letrozole and letrozole plus low-dose metronomic oral cyclophosphamide as primary systemic treatment in elderly breast cancer patients. J Clin Oncol 24:3623–3628

Browder T et al (2000) Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. Cancer Res 60:1878–1886

Buckstein R et al (2006) High-dose celecoxib and metronomic 'low-dose' cyclophosphamide is an effective and safe therapy in patients with relapsed and refractory aggressive histology non-Hodgkin's lymphoma. Clin Cancer Res 12:5190–5198

Citron ML (2004) Dose density in adjuvant chemotherapy for breast cancer. Cancer Invest 22:555–568

Colleoni M et al (2002) Low dose oral methotrexate and cyclophosphamide in metastatic breast cancer: antitumor activity and correlation with vascular endothelial growth factor levels. Ann Oncol 13:73–80

Coras B et al (2004) Antiangiogenic therapy with pioglitazone, rofecoxib, and trofosfamide in a patient with endemic Kaposi sarcoma. Arch Dermatol 140:1504–1507

Correale P et al (2006) A novel metronomic chemotherapy regimen of weekly platinum and daily oral etoposide in high-risk non-small cell lung cancer patients. Oncol Rep 16:133–140

Cristofanilli M et al (2002) Angiogenesis modulation in cancer research: novel clinical approaches. Nat Rev Drug Discov 1:415–426

Damber JE et al (2006) The anti-tumour effect of low-dose continuous chemotherapy may partly be mediated by thrombospondin. Cancer Chemother Pharmacol 58:354–360

Drevs J et al (2004) Antiangiogenic potency of various chemotherapeutic drugs for metronomic chemotherapy. Anticancer Res 24:1759–1763

Du Manoir JM et al (2006) Strategies for delaying or treating in vivo acquired resistance to trastuzumab (Herceptin®) in human breast cancer xenografts. Clin Cancer Res 12:904–916

Emmenegger U et al (2004) A comparative analysis of low dose metronomic cyclophosphamide reveals absent or low grade toxicity on tissues highly sensitive to the toxic effects of maximum tolerated dose regimens. Cancer Res 64:3994–4000

Garcia AA et al (2005) Interim report of a phase II clinical trial of bevacizumab (Bev) and low dose metronomic oral cyclophosphamide (mCTX) in recurrent ovarian (OC) and primary peritoneal carcinoma: a California Cancer Consortium Trial. Proc Am Soc Clin Oncol abstract # 5000

Garcia AA et al (2007) Phase II clinical trial of bevacizumab and low dose metronomic oral cyclophosphamide in recurrent ovarian cancer. A study of the California, Chicago and Princess Margaret Hospital Phase II Consortia. J Clin Oncol (submitted)

Ghiringhelli F et al (2004) CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. Eur J Immunol 34:336–344

Glode LM et al (2003) Metronomic therapy with cyclophosphamide and dexamethasone for prostate cancer. Cancer 98:1643–1648

Hafner C et al (2005) New indications for established drugs: combined tumor-stroma-targeted cancer therapy with PPARgamma agonists, COX-2 inhibitors, mTOR antagonists and metronomic chemotherapy. Curr Cancer Drug Targets 5:393–419

Hamano Y et al (2004) Thrombospondin-1 associated with tumor microenvironment contributes to low-dose cyclophosphamide-mediated endothelial cell apoptosis and tumor growth suppression. Cancer Res 64:1570–1574

Hanahan D et al (2000) Less is more, regularly: metronomic dosing of cytotoxic drugs can target tumor angiogenesis in mice. J Clin Invest 105:1045–1047

Hermans IF et al (2003) Synergistic effect of metronomic dosing of cyclophosphamide combined with specific antitumor immunotherapy in a murine melanoma model. Cancer Res 63:8408–8413

Kato H et al (2004) A randomized trial of adjuvant chemotherapy with uracil-tegafur for adenocarcinoma of the lung. N Engl J Med 350:1713–1721

Kerbel RS (1991) Inhibition of tumor angiogenesis as a strategy to circumvent acquired resistance to anti-cancer therapeutic agents. BioEssays 13:31–36

Kerbel RS, Kamen BA (2004) Antiangiogenic basis of low-dose metronomic chemotherapy. Nat Rev Cancer 4:423–436

Kieran MW et al (2005) A feasibility trial of antiangiogenic (metronomic) chemotherapy in pediatric patients with recurrent or progressive cancer. J Pediatr Hematol Oncol 27:573–581

Kim JJ, Tannock IF (2005) Repopulation of cancer cells during therapy: an important cause of treatment failure. Nat Rev Cancer 5:516–525

Klement G et al (2000) Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. J Clin Invest 105: R15–R24

Lam T et al (2006) From total empiricism to a rational design of metronomic chemotherapy phase I dosing trials. Anticancer Drugs 17:113–121

Loeffler M et al (2005) Immunostimulatory effects of low-dose cyclophosphamide are controlled by inducible nitric oxide synthase. Cancer Res 65:5027–5030

Man S et al (2002) Antitumor and anti-angiogenic effects in mice of low-dose (metronomic) cyclophosphamide administered continuously through the drinking water. Cancer Res 62:2731–2735

Mancuso P et al (2006) Circulating endothelial cell kinetics and viability predict survival in breast cancer patients receiving metronomic chemotherapy. Blood 108:452–459

Miller KD et al (2001) Redefining the target: chemotherapeutics as antiangiogenics. J Clin Oncol 19:1195–1206

Munoz R et al (2006) Highly efficacious non-toxic treatment for advanced metastatic breast cancer using combination UFT-cyclophosphamide metronomic chemotherapy. Cancer Res 66:3386–3391

Ng SSW et al (2006) Influence of formulation vehicle on metronomic taxane chemotherapy: nanoparticle albumin-bound versus cremophor EL-based paclitaxel. Clin Cancer Res 12:4331–4338

Pietras K, Hanahan D (2005) A multitargeted, metronomic, and maximum-tolerated dose „chemo-switch" regimen is antiangiogenic, producing objective responses and survival benefit in a mouse model of cancer. J Clin Oncol 23:939–952

Reichle A et al (2004) Pioglitazone and rofecoxib combined with angiostatically scheduled trofosfamide in the treatment of far-advanced melanoma and soft tissue sarcoma. Cancer 101:2247–2256

Sandri MT et al (2007) Serum EGFR and serum HER-2/neu are useful predictive and prognostic markers in metastatic breast cancer patients treated with metronomic chemotherapy cancer.Schrag D (2004) The price tag on progress – chemotherapy for colorectal cancer. N Engl J Med 351:317–319

Shaked Y et al (2005a) Genetic heterogeneity of the vasculogenic phenotype parallels angiogenesis: implications for cellular surrogate marker analysis of antiangiogenesis. Cancer Cell 7:101–111

Shaked Y et al (2005b) Low-dose metronomic combined with intermittent bolus-dose cyclophosphamide is an effective long-term chemotherapy treatment strategy. Cancer Res 65:7045–7051

Shaked Y et al (2005c) The optimal biological dose of metronomic chemotherapy regimens is associated with maximum antiangiogenic activity. Blood 106:3058–3061

Shaked Y et al (2006) Therapy-induced acute recruitment of circulating endothelial progenitor cells to tumors. Science 313:1785–1787

Singletary SE (2007) Multidisciplinary frontiers in breast cancer management: a surgeon's perspective. Cancer 109:1019–1029

Tozer GM et al (2005) Disrupting tumour blood vessels. Nat Rev Cancer 5:423–435

Vacca A et al (1999) Antiangiogenesis is produced by nontoxic doses of vinblastine. Blood 94:4143–4155

Viloria-Petit AM et al (1997) Neutralizing antibodies against EGF and ErbB-2/neu receptor tyrosine kinases down-regulate VEGF production by tumor cells in vitro and in vivo: angiogenic implications for signal transduction therapy of solid tumors. Am J Pathol 151:1523–1530

Wang J et al (2003) Paclitaxel at ultra low concentrations inhibits angiogenesis without affecting cellular microtubule assembly. Anticancer Drugs 14:13–19

Yonekura K et al (1999) UFT and its metabolites inhibit the angiogenesis induced by murine renal cell carcinoma, as determined by a dorsal air sac assay in mice. Clin Cancer Res 5:2185–2191

Young SD et al (2006) Phase II clinical trial results involving treatment with low-dose daily oral cyclophosphamide, weekly vinblastine, and rofecoxib in patients with advanced solid tumors. Clin Cancer Res 12:3092–3098

Anti-Angiogenic Tumor Therapy in Clinical Studies

The Development of Avastin

35

Robert Mass

Abstract

Bevacizumab (Avastin; Genentech, Inc., South San Francisco, CA, USA) is a humanized monoclonal antibody that specifically targets and neutralizes vascular endothelial growth factor (VEGF-A), an essential endothelial cell mitogen and survival factor. As the first anti-angiogenic therapy developed and approved for human cancer, it represents the culmination of many years of biologic and human research in many laboratories and clinics around the world. This chapter will review (1) the development of bevacizumab beginning with the cloning of human VEGF which allowed for the generation of murine-derived, human-specific anti-VEGF monoclonal antibodies; (2) the process by which a single clone, A4.6.1, was identified and selected for clinical development; (3) the process of "humanizing" the murine antibody to form bevacizumab to enable human testing; (4) the clinical development program from phase I through phase III clinical experiments; (5) the future areas for clinical evaluation of bevacizumab.respect to differential efficacy and adverse effect profiles.

CONTENTS

35.1 Introduction

The concept of targeting tumor vasculature as a therapeutic strategy in human cancer dates to an observation made in 1939 by Gordon Ide and colleagues that rapid growth of transplanted tumors was often preceded by an intense local increase in vascular density (Ide et al. 1939). Based on their

experiments, which involved the use of a transparent chamber to observe the growth of transplanted rabbit carcinomas, they postulated the following: "one is almost forced to the conclusion that there is, associated with the viable growing tumor, some blood vessel growth stimulating factor". Later work, reported in 1945 by Algire and Chalkley at the National Cancer Institute, refined this technique such that it could be applied to mice and vessel growth could be quantitated directly by enumerating vessel counts (Algire et al. 1945). Their conclusions were similar: "the rapid growth of tumor explants is dependent on the development of a rich vascular supply". In 1968, Greenblatt and Ehrmann demonstrated that this process, termed angiogenesis, was mediated by diffusible factors released by tumor cells (Greenblatt and Shubik 1968; Ehrmann and Knoth 1968).

However, it was the landmark treatise by Folkman in 1971 that accelerated the field of "anti-angiogenesis" as a therapeutic option to treat cancer (Folkman 1971). His group had recently reported the isolation of a serum protein termed tumor angiogenesis factor (TAF), which they showed to be an endothelial cell mitogen (Folkman et al. 1971). He postulated at that time that blockade of this factor might arrest cancers at a tiny diameter of a few millimeters. TAF was ultimately shown to be vascular endothelial growth factor (VEGF), one of the most potent of a family of endothelial cell-specific mitogens. VEGF is a highly conserved, homodimeric, heparin-binding glycoprotein that exists in several isoforms. VEGF mediates its effects by interacting with the membrane-bound tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR, flk-1), activating specific downstream survival and proliferation pathways (Ferrara and Davis-Smyth 1997). VEGF is considered essential for normal developmental vasculogenesis, and there is substantial evidence implicating VEGF as a critical factor in tumor angiogenesis. Transformed cell lines commonly express increased levels of both VEGF mRNA and protein. Transfection of Chinese hamster ovary cells with expression vectors encoding VEGF allows these cells to form tumors in nude mice (Ferrara et al. 1993). Increased VEGF expression has been described in most human tumors and in many instances is correlated with an adverse prognosis (increased risk of tumor recurrence and metastasis and decreased survival) (Takahashi et al. 1995, 1997; Radinsky and Ellis 1996; Tokunaga et al. 1998).

The biology of VEGF and the target receptors involved in signaling are described in detail in other chapters of this text. This chapter will review the pre-clinical and clinical development of bevacizumab, the first therapeutic anti-angiogenic drug developed for human cancer.

Creating a Therapeutic Antibody

The cloning of human VEGF by Napoleone Ferrara in 1989 created the opportunity to envision strategies to antagonize the growth factor and potentially inhibit angiogenesis in humans (Leung et al. 1989). Engineering a highly specific, neutralizing, humanized antibody directed against VEGF represented one potential therapeutic strategy to pursue. Mice were first immunized with recombinant human VEGF165 and a library of murine anti-human VEGF antibodies was created (Kim et al. 1992). Four antibodies, A3.13.1, A4.6.1, B4.3.1 and B2.6.2, were selected for further characterization based on their high-affinity binding to VEGF with dissociation constants (K_d) ranging from 2.2×10^{-9} to 4.4×10^{-10}. Epitope mapping indicated that A4.6.1 had the favorable characteristic of recognizing a continuous as opposed to a discontinuous epitope that was essential for angiogenesis, and it also recognized all four isoforms of VEGF ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$) created by alternative splicing. Based on these results, A4.6.1 was selected for further pre-clinical testing as a potential anti-tumor agent.

In 1993, a landmark series of experiments was reported by Kim and colleagues (Kim et al. 1993). They studied the effect of A4.6.1 on in vivo and in vitro growth characteristics of three immortalized human malignant cell lines: A673 rhabdomyosar-

coma, G55 glioblastoma multiforme, and SK-LMS-1 leiomyosarcoma. Each of these lines were known to express VEGF mRNA and were tumorigenic when grown as xenografts in immunodeficient mice. As seen in Fig. 35.1, A4.6.1 exerted a dose-dependent inhibition of tumor growth in both A673 and G55 tumors. Histologic examination of the tumors revealed a significant reduction in vascular density within the A4.6.1-treated tumors. In order to separate paracrine and autocrine mechanisms, Kim also reported the activity of both recombinant VEGF and A4.6.1 on the same cell lines grown in agar and plastic. As seen in Fig. 35.2, neither the growth factor itself nor the high-affinity anti-VEGF antibody had any growth effects on isolated tumor cells. These observations confirmed findings in earlier studies

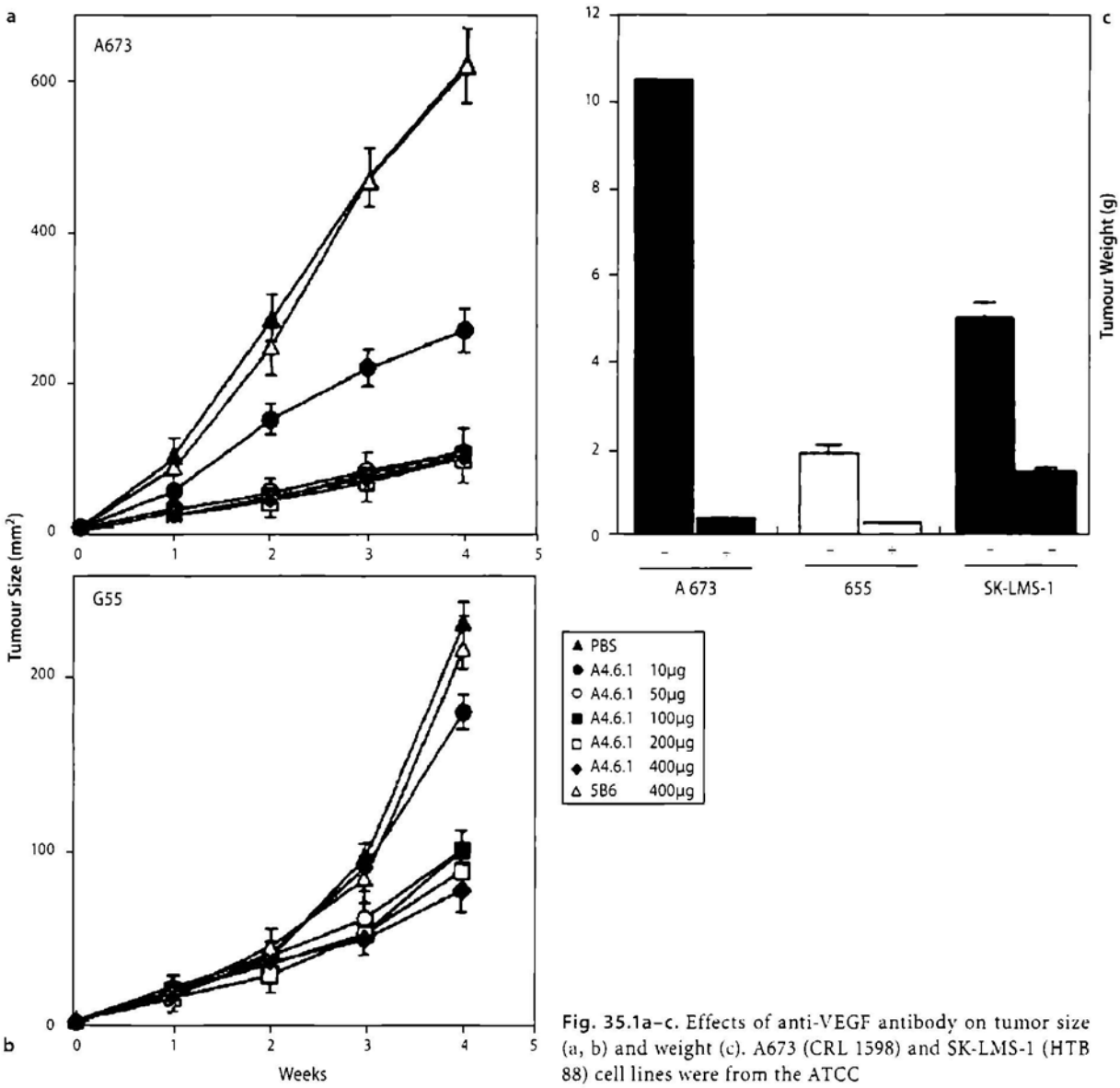


Fig. 35.1a–c. Effects of anti-VEGF antibody on tumor size (a, b) and weight (c). A673 (CRL 1598) and SK-LMS-1 (HTB 88) cell lines were from the ATCC

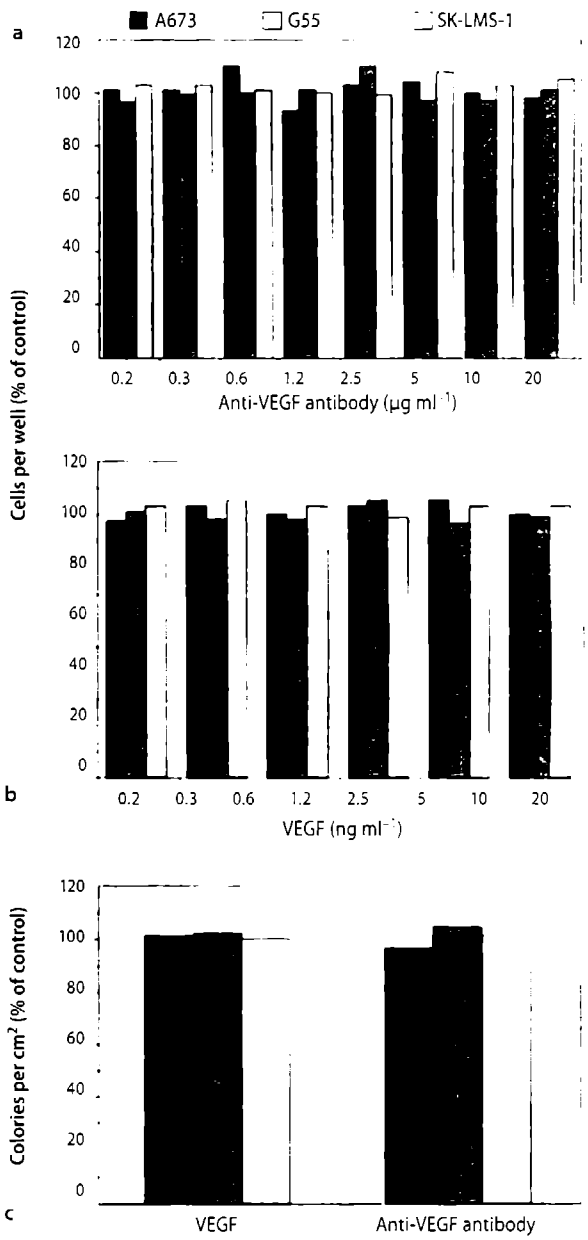


Fig. 35.2a–c. Effects of VEGF or anti-VEGF antibody on the growth of tumor cell lines in plastic or in soft agar

suggesting that VEGF is an endothelial cell-specific mitogen and survival factor and blocking VEGF with a neutralizing antibody exerts anti-tumor activity indirectly through effects on tumor endothelial cells rather than a direct effect on tumor cells. These experiments represented the first direct evidence that blocking angiogenesis could induce an anti-tumor response in human cancer.

Similar results were reported by Warren using two different colon cancer cell lines, HM7 and LS-LiM6 (Warren et al. 1995). In addition to subcutaneous xenograft experiments, they also described a model of hepatic metastases where a fixed quantity of HM7 cells was inoculated into the splenic pulp of immunodeficient mice, reliably producing large hepatic metastases within 28 days. Figure 35.3 shows the results of treatment with A4.6.1 versus control antibody starting 1 day after the tumor cell inoculation. The observations of Kim and Warren provided strong pre-clinical evidence that A4.6.1 might have anti-tumor activity in human use.

The next step in the creation of bevacizumab involved humanization of A4.6.1 (Presta 1997). Although the potential therapeutic use of monoclonal antibodies had been recognized for decades, early clinical experiments with murine-derived antibodies were limited by the rapid and uniform development of neutralizing human anti-murine antibodies (HAMAs) (Miller et al. 1983). Chronic human administration would require some modification to traditionally generated murine antibodies in order to reduce or eliminate immunogenicity. The first approach to this problem involved the development of chimeric antibodies, constructed by combining mouse variable and human constant domains by linking the genes encoding for each domain and then expressing the recombinant antibody. Although successful in some instances, chimeric antibodies retain approximately 30% murine elements and are frequently immunogenic (Neuberger et al. 1985). Subsequently, techniques were developed to graft only the specific complementary-determining regions (CDRs), the hypervariable loops responsible for antigen binding, of a murine antibody directly into a human antibody (Jones et al. 1986; Riechman et al. 1988). These initial humaniza-

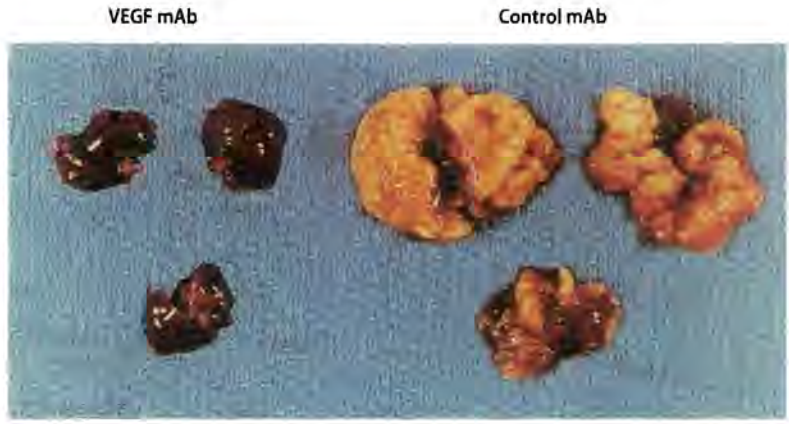


Fig. 35.3. Neutralizing antibody to human VEGF inhibits growth of experimental hepatic metastases in the athymic mouse. One day after splenic-portal tumor cell inoculation (2 million HM7 cells), twice-weekly antibody injections were begun, and animals were killed after 4 weeks. Livers of representative animals are shown

tion methodologies were cumbersome and time-consuming in identifying specific clones retaining the critical high-affinity binding characteristics of the parental murine antibody. However, advances in recombinant technology led to sophisticated methods of site-specific mutagenesis that allow for the rapid development and selection of humanized antibody constructs with optimal antigen binding characteristics (Carter et al. 1992).

To create bevacizumab, previously identified murine CDRs from the murine parental antibody A4.6.1 were grafted onto the V_H and V_L regions of a recombinant human IgG1 framework. In addition to the CDRs, eight additional critical human to murine substitutions were identified via site-specific mutagenesis and affinity-binding experiments: seven involving the heavy chain and one involving the light chain. Figure 35.4 shows the specific amino acid sequences of the V_H and V_L domains of A4.6.1, the human IgG_1 framework, and clone $F_{(ab)}$-12, known as bevacizumab. Figure 35.5 shows a ribbon diagram of bevacizumab and the specific location of the critical, non-CDR human to murine substitutions at H49, H69, H71, H73, H76, H78, H94, and L46. It is thought that these sequences indirectly influence the conformation of the respective CDR loops and the specific binding characteristics with VEGF. By engineering this humanized variant of A4.6.1, bevacizumab retains specific high-affinity binding with VEGF with a K_d of 1.8 nM, only a twofold reduction from the parental murine antibody A4.6.1 at 0.9 nM. Bevacizumab was then evaluated in vitro in a biologic assay

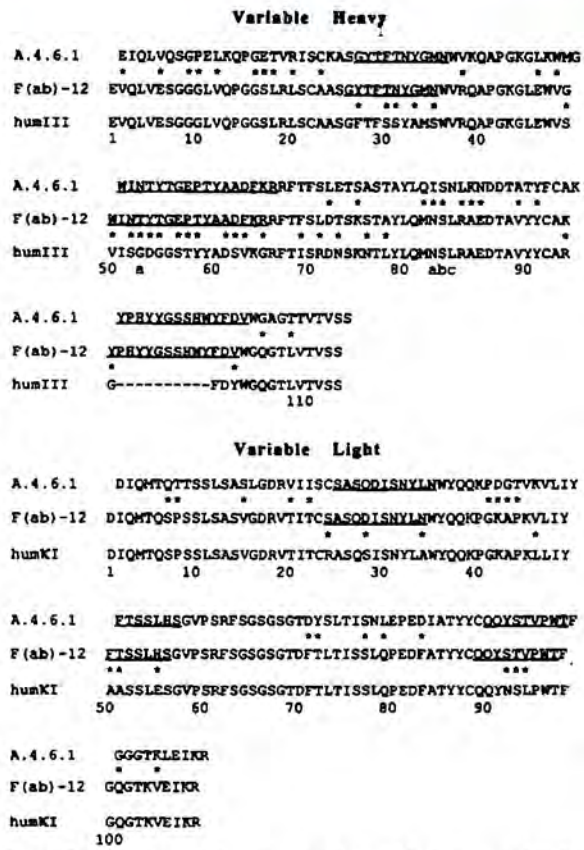


Fig. 35.4. Amino acid sequence of variable heavy and light domains of muMAbVEGF A4.6.1 humanized F(ab) with optimal VEGF binding [F(ab)-12] and human consensus frameworks (*humIII*, heavy subgroup III; *humKI*, light K subgroup I). *Asterisks*, differences between humanized F(ab)-12 and the murine MAb or between F(ab)-12 and the human framework. CDRs are *underlined*

Fig. 35.5. Ribbon diagram of the model of humanized F(abl-12) VL and VH domains. The VL domain is shown in *brown* with CDRs in tan. The side chain of residue L46 is shown in *yellow*. The VH domain is shown in *purple* with CDRs in *pink*. Side chains of VH residues changed from human to murine are shown in yellow

of VEGF-induced endothelial cell proliferation. As shown in Fig. 35.6, the inhibitory effects of bevacizumab were identical to the effect of A4.6.1. Finally, the anti-tumor activity of bevacizumab was assessed in vivo using A673 xenografts implanted subcutaneously in immunodeficient mice. Figure 35.7 shows the marked, dose-dependent effect of both antibodies in this model. After the successful engineering of a humanized antibody retaining approximately 93% human elements and 7% murine elements, bevacizumab was ready for human testing.

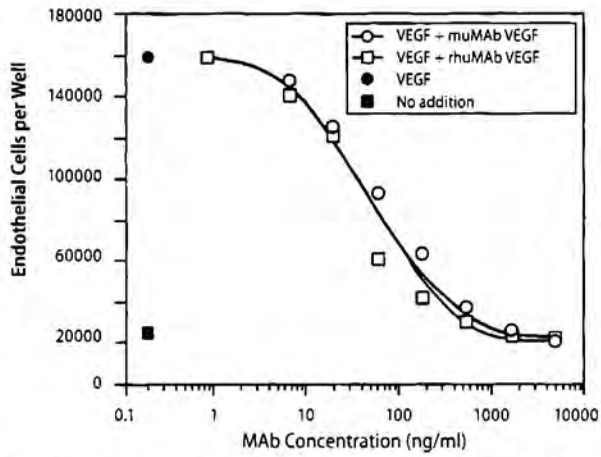


Fig. 35.6. Inhibition of VEGF-induced mitogenesis. Bovine adrenal cortex-derived capillary endothelial cells were seeded at the density of 6×10^3 cells/well in six-well plates. Either muMAb VEGF A4.6.1 or rhuMAb VEGF (IgGl) was added at the indicated concentrations. After 2-3 h, $rhVEGF_{165}$ was added at the final concentration of 3 ng/ml. After 5 or 6 days, cells were trypsinized and counted. Values shown are means of duplicate determinations. The variation from the mean did not exceed 10%

35.3 Phase I and Pharmacokinetics

The clinical development of bevacizumab was initiated in 1997, when 25 human subjects were enrolled in a phase I single-agent trial. Cohorts of five patients received a single dose of bevacizumab, followed by a 4-week wash-out period and then three weekly doses. The doses evaluated were 0.1, 0.3, 1, 3, and 10 mg/kg. Earlier pre-clinical experiments in A673 xenografts had suggested a plateau in antitumor activity at antibody concentrations exceeding 10 μg/ml. Scaling from murine and primate pharmacokinetic studies indicated that human doses between 1.0 and 10 mg/kg should achieve trough concentrations in excess of this targeted plasma level. Infusions were initially administered over 90 min and, if tolerated, subsequent infusions were admin-

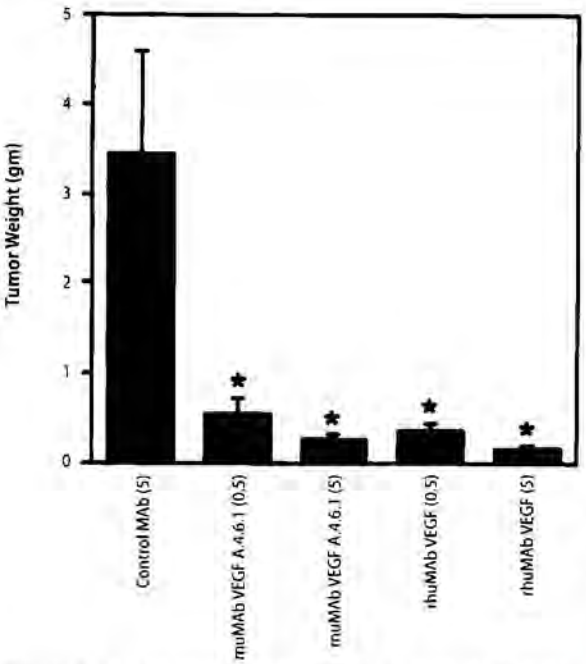


Fig. 35.7. Inhibition of tumor growth in vivo. A673 rhabdomyosarcoma cells were injected in BALB/c nude mice at the density of 2×10^6 per mouse. Starting 24 h after tumor cell inoculation, animals were injected with a control Mab, muMAb VEGF A.4.6.1, or rhuMab VEGF (IgG1) twice weekly, i.p. The dose of the control Mab was 5 mg/kg; the anti-VEGF Mabs were given at 0.5 or 5 mg/kg as indicated (n=10). Four weeks after tumor cell injection, animals were killed and tumors were removed and weighed. *Significant difference compared to the control group by ANOVA (P<0.05)

istered over 30 min. In this experiment, the drug was well tolerated and no dose-limiting toxicities were observed. The commonest adverse events included asthenia, headache, and fever. Four patients developed NCI-CTC grade 3 or 4 adverse events, including anemia (0.1 mg/kg dose), dyspnea (0.3 mg/kg dose), and two episodes of bleeding (both with the 3.0 mg/kg dose). One of these bleeding events was an intracranial hemorrhage associated with an unsuspected CNS metastasis from hepatocellular carcinoma and the other was tumor-associated bleeding from a soft tissue sarcoma involving the thigh. Both systolic and diastolic blood pressure were noted to increase in excess of 10 mm Hg at the 3 mg/kg and 10 mg/kg dose levels at some point during the treatment period. Of the 23 patients evaluable at day 70, 12 were reported to have stable disease while 11 had progressive disease. Of 7 patients with renal cell cancer, 5 had stable disease with one demonstrating a minor response.

The pharmacokinetic data from this trial are shown in Fig. 35.8. There were dose-related increases in C_{max} concentrations ranging from 2.8 μg/ml at 0.1 mg/kg to 284 μg/ml at 10 mg/kg, with corresponding increases in AUC and Cmin concentrations. Linear kinetics were observed at doses greater than 0.3 mg/kg and the half-life estimate was 15 days, consistent with that of other humanized IgG1 antibodies. Based on these PK and safety observations, the doses selected for phase II testing ranged from 3 to 10 mg/kg with a 2-week dosing interval or from 7.5 to 15 mg/kg with a 3-week interval.

35.4 Phase II Development

Two important preclinical observations helped define the approach to the phase II "proof of concept" program for bevacizumab. The first related to disease selection, while the second involved the selection of appropriate endpoints and clinical trial designs. Virtually all of the common malignant diseases had been previously been evaluated with respect to (1) the frequency of increased VEGF expression and/or other markers of angiogenesis (i.e. microvessel density) and (2) the clinical consequences of increased VEGF expression. There was a consistent association between increased VEGF expression and unfavorable clinical outcomes. However, the degree of VEGF overexpression was modest in most tumor collections reported. The one exception to this finding was the clear-cell variant of renal cell cancer. This malignancy is commonly associated with mutations in the Von Hippel-Lindau (VHL) gene leading to constitutive activation of hypoxia-inducible factor 1 alpha (HIF1α) and subsequent high-level VEGF expression. No other tumor, with the exception of

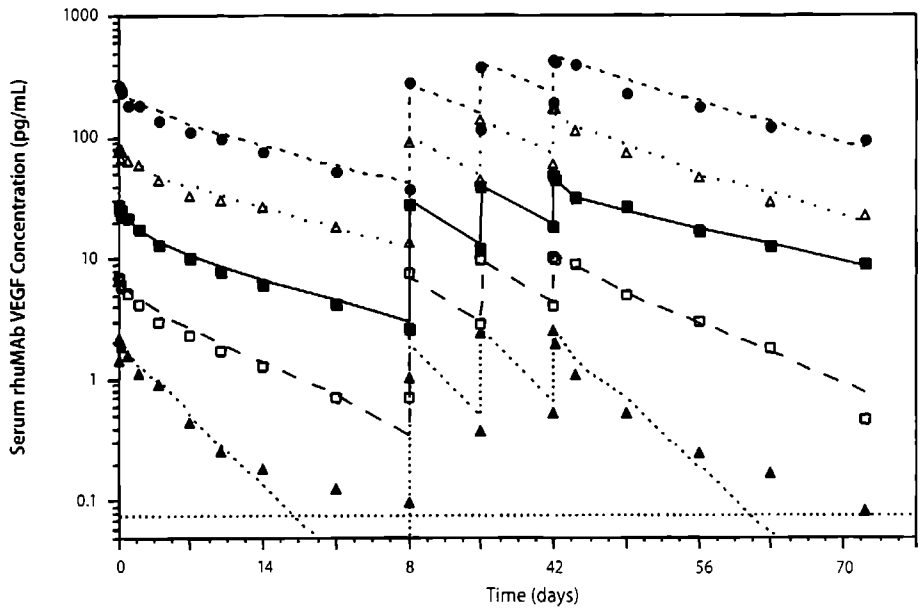


Fig. 35.8. Mean serum rhuMAb VEGF concentrations. Serum levels of rhuMAb VEGF after serial administrations (days 0, 28, 35, and 42) at doses of 0.1 (*closed triangle*), 0.3 (*open square*), 1.0 (*closed square*), 3.0 (*open triangle*), and 10.0 (*closed circle*) mg/kg. Cohorts consist of four to five patients

grade 4 glioma, expresses these very high levels of VEGF. In pre-clinical xenograft experiments, the level of VEGF expression in human tumors did not correlate with the anti-tumor activity of anti-VEGF antibody therapy. Based on these observations, five tumor types were selected for phase II testing. These were clear-cell renal cell cancer, based on the unique VEGF-driven biology, and four common solid tumors with high unmet medical need: breast, lung, colon, and prostate cancer.

Pre-clinical observations also provided important information with regard to clinical trial design and endpoint selection for the phase II program. A consistent finding in pre-clinical experiments with bevacizumab was the lack of objective tumor response or regression. In xenograft and orthotopic tumor models, bevacizumab resulted in delay of tumor growth rather than overt tumor shrinkage. This finding was consistent with the known anti-angiogenic mechanism of action and a predominant cytostatic effect. A second pre-clinical observation was that the activity of an anti-VEGF antibody seemed to be more impressive when combined with classical cytotoxic chemotherapy than when used as a single agent. These two critical observations, a primarily cytostatic mechanism of action and improved activity in combination with chemotherapy, drove the basic design of the phase II program.

Interpreting the clinical relevance of a delay in tumor growth in clinical trials, measured typically as a delay in time to tumor progression (TTP) or, more acceptably, an improvement in progression-free survival (PFS), which includes death prior to progression as well as actual disease progression within the endpoint, is fraught with risk, particularly without the utilization of an untreated control population and treatment blinding by the use of placebo. Investigators and trial subjects with life-threatening medical conditions are often reluctant to participate in randomized and placebo-controlled trials, especially those conducted as phase II experiments. Although often criticized for lacking statistical power for formal comparisons, randomized phase II designs provide a critical advantage over non-randomized designs by minimizing selection bias. Drug development in oncology is littered with failed phase III experiments that were based on non-randomized phase II experiments where the "promising" outcome resulted from inadvertently biased selection of patients with unrecognized favorable pre-treatment characteristics rather than from the action of the new agent itself. Based on these observations,

the phase II program with bevacizumab consisted of two components: three trials of single-agent bevacizumab where both response rate (RR) and PFS were evaluated, and two trials of bevacizumab in combination with standard chemotherapy. Three of the five trials included a randomized phase II, controlled design. Based on the known mechanism of action and the toxicities reported in the phase I trial, all of the phase II trials had a common list of defined eligibility exclusions that included: no recent (<28 days) surgery, no current anticoagulation, no known CNS involvement, no recent (<6 months) stroke or myocardial infarction, no significant (>grade 1) proteinuria, and no "uncontrolled" (>150/100) hypertension. In all of the clinical trials, bevacizumab was continued until disease progression or unacceptable toxicity.

Phase II Single-Agent Program

35.5.1 Breast Cancer

This clinical experiment, conducted in multiple institutions, was designed as a classic Simon two-stage trial with co-primary endpoints of RR and landmark PFS. The eligibility criteria required that patients had progressed on at least one prior chemotherapy regimen for metastatic disease, and 72% had received two regimens or more. A total of 75 patients were eventually enrolled. At the first dose level/cohort of 3 mg/kg every 2 weeks, only a single response was observed in the first 18 patients; based on the pre-specified analysis plan, a second cohort was then studied at 10 mg/kg. A total of 41 patients were enrolled in this cohort. Finally, an amendment to the protocol allowed an additional 16 patients to be enrolled at a dose level of 20 mg/kg every 2 weeks. Overall, bevacizumab was well tolerated in this study. Hypertension requiring medical therapy developed in 19% of the patients and did not appear to be dose dependent. Proteinuria of any grade was seen in 24% of the patients, and serious proteinuria (>3.5g/24 h) was noted in 4%. This trial is noteworthy in that it is the only clinical experiment where a dose level of 20 mg/kg every 2 weeks was evaluated, and 4/16 patients (25%) developed severe headache associated with nausea and vomiting.

The overall RR in this trial was 7/75 patients (9.3%), with 1 response in the 3 mg/kg cohort, 5 in the 10 mg/kg cohort, and 1 in the 20 mg/kg cohort. In addition, the rate of non-progressive disease at day 154 was 16%. Based on these results, two phase III trials of bevacizumab in metastatic breast cancer were conducted; one trial compared capecitabine alone to the combination of capecitabine plus bevacizumab in women with highly refractory disease, and the other compared paclitaxel alone to the combination of paclitaxel plus bevacizumab in newly diagnosed metastatic breast cancer (see Sect 35.7)

35.5.2 Renal Cell Cancer

This clinical experiment was conducted exclusively at the National Cancer Institute in Bethesda, MD, USA. It was designed as a double-blind, placebo-controlled randomized phase II trial comparing placebo with two bevacizumab doses: 3 mg/kg and 10 mg/kg every 2 weeks. In addition, patients in this trial received a "loading" dose of bevacizumab that was 150% of the assigned dose. Patients were required to have clear-cell histology, measurable disease, and progressive disease following interleukin (IL)-2 therapy or intolerance of IL-2. The primary endpoints were time to disease progression (TTP) and RR. A total of 116 patients were enrolled. Patient characteristics are shown in Table 35.1 and are balanced between the groups, over 90% of the patients had progressed following IL-2 therapy.

The adverse events noted in this trial were similar to those seen in phase I and included fever, hypertension, and proteinuria; the latter two were potentially dose dependent in this patient population,

with >90% having only a single kidney. Bleeding was also noted, typically epistaxis and/or hematuria of little clinical consequence.

Although initially designed to enroll 150 patients, the trial was stopped at the second, planned interim efficacy analysis when the Data Safety Monitoring Board determined that the TTP of the high-dose group had crossed a pre-specified O'Brian–Fleming boundary for efficacy. The Kaplan–Meier curves for each dose group compared to placebo are shown in Fig. 35.9 (Yang et al. 2003). The median TTP in the 10 mg/kg group was 4.8 months (vs 2.5 months, $p<0.001$) and that in the 3 mg/kg group was 3.0 months (vs 2.5 months,

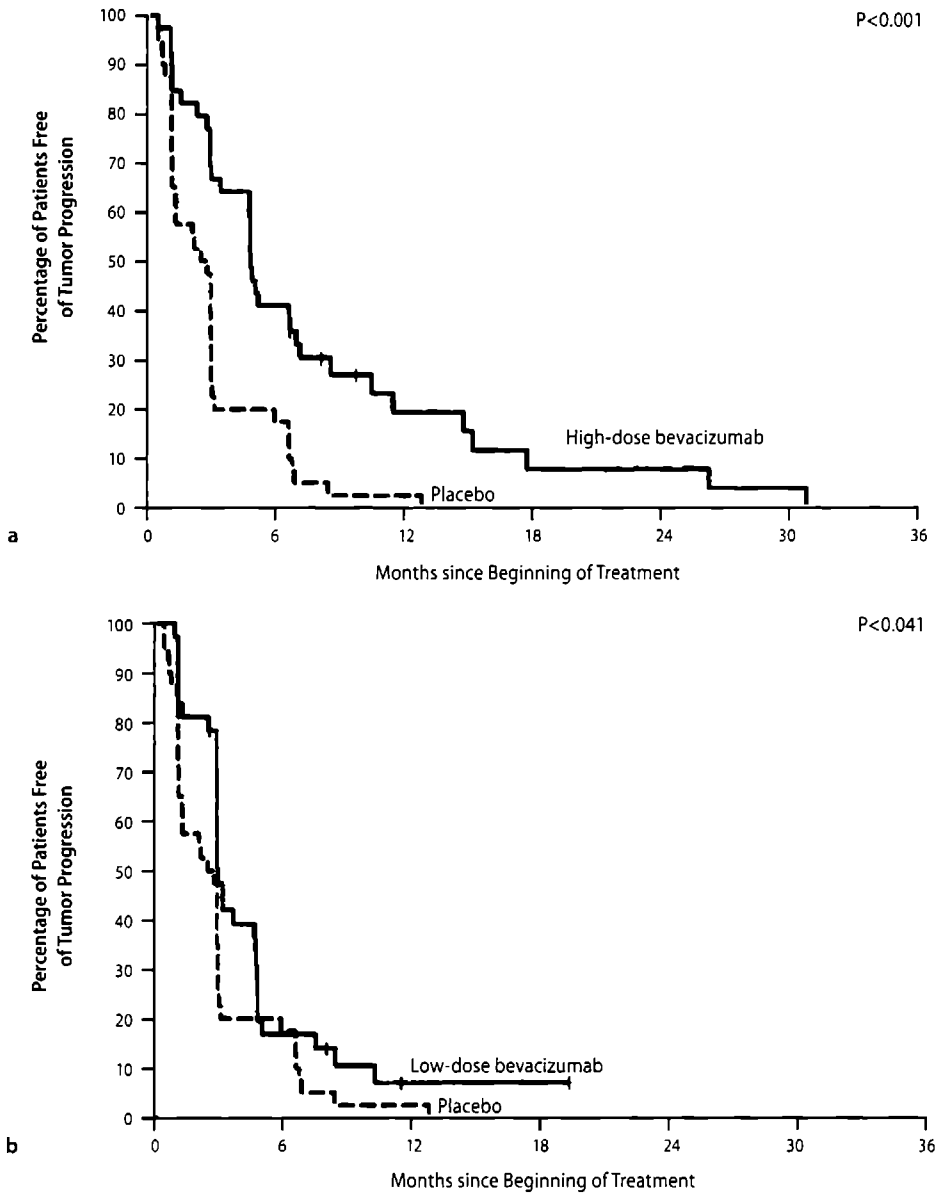


Fig. 35.9a,b. Kaplan–Meier analysis of survival-free tumor progression for patients receiving high-dose bevacizumab (a) or low-dose bevacizumab (b), compared with placebo. The high dose of bevacizumab was 10 mg per kilogram of body weight. The low dose of bevacizumab was 3 mg per kilogram. Doses were given every 2 weeks. P values were calculated using the log-rank test

p=0.041). In a pre-planned landmark analysis at 8 months from randomization the proportion of patients free from disease progression was 5% (placebo), 14% (3 mg/kg) and 30% (10 mg/kg). Only four patients, all in the 10 mg/kg dose group, had objective responses. These results were consistent with the pre-clinical experiments suggesting that objective RRs would underestimate the full clinical benefit from bevacizumab, which would be better captured via lengthening TTP. Based on these results, two phase III trials are being conducted in newly diagnosed metastatic renal cell cancer, both comparing interferon alpha alone to interferon alpha plus bevacizumab (Table 35.1).

35.5.3 Prostate Cancer

A small phase II open-label, single-agent clinical experiment was conducted exclusively at UCSF San Francisco, CA, USA with a classic Simon two-stage design. The dose selected was 10 mg/kg every 2 weeks and the primary endpoint was RR. A total of 15 men with hormone-refractory prostate cancer were enrolled. The first tumor evaluation was conducted at day 70 following six infusions of bevacizumab. Of the 14 patients who were evaluable, none had an objective response, 7 had progressive disease

Table 35.1. Characteristics of patients before treatment (p>0.05 for all comparisons)

Characteristic	High-dose bevacizumab (n=39)	Low-dose bevacizumab (n=37)	Placebo (n=40)
Median age (years)	53	54	53
Male sex (%)	74	84	68
ECOG performance status (no.)[a]			
0	30	30	31
1 or 2	9	7	9
Prior interleukin-2 therapy (no.)	37	34	37
Prior chemotherapy (no.)	10	7	8
Prior radiation therapy (no.)	8	6	12
Prior nephrectomy (no.)	35	33	38
Anemia (no.)	14	15	16
Hypercalcemia (no.)	12	18	14
Interval from diagnosis to randomization (no.)	14	13	12
1–2 years	8	6	9
>2 years	17	18	19
Liver involvement (no.)	10	10	10
Bone involvement (no.)	2	3	6

[a]ECOG denotes Eastern Cooperative Oncology Group. Higher performance status indicates greater impairment.

and 7 had stable disease. Too few patients with stable disease remained on bevacizumab to evaluate the potential for prolonged disease control. There were no new safety issues noted in this trial. Despite these results, other investigators elected to combine bevacizumab with both docetaxel and estramustine, with promising results which led to a phase III trial currently being conducted (CALGB 90401) comparing docetaxel alone to docetaxel plus bevacizumab in hormone-refractory prostate cancer.

Phase II Combination Program

35.6.1 Colon Cancer

Between June and November 1998, a total of 104 patients were enrolled from 36 centers in a three-arm, randomized controlled clinical experiment (Kabbinavar et al. 2003). Patients were required to have metastatic colon or rectal cancer with measurable disease and no prior chemotherapy for metastatic disease. The three treatment groups included standard bolus 5-fluorouracil (5FU) and leucovorin (LV) delivered weekly for 6 weeks followed by a 2-week rest (Petrelli et al. 1989), either alone (control) or with bevacizumab at a dose of 5 mg/kg or 10 mg/kg every 2 weeks. Treatment was continued until documented disease progression and, at that time, patients in the control arm were permitted to receive bevacizumab alone at 10 mg/kg every 2 weeks. The primary endpoints were TTP and RR. The patient baseline characteristics are shown in Table 35.2. There were some modest imbalances in the three groups, including gender, adjuvant therapy, and extent of known metastases which could influence the interpretation of the results given the small overall sample size.

At the time of the planned analysis, the overall RR was 17% in the control group versus 40% in the 5 mg/kg bevacizumab group (p=0.029) and 24% in the 10 mg/kg group (p=0.434). In addition, 22 of the 36 patients in the control arm crossed over to receive single-agent bevacizumab and 2 patients experienced a response with an additional 7 patients achieving stable disease. The detailed data regarding PFS are shown in Table 35.3. There were significant improvements in PFS in the 5 mg/kg group and an exploratory, pooled analysis of both dose levels. The adverse event profile, shown in Table 35.4, was similar to that seen with the single-agent trials, including mild bleeding (epistaxis) and hypertension with possible increases in the rate of both bleeding and thrombotic events. These results formed the basis for three phase III trials of bevacizumab in colorectal cancer, in combination with 5FU/LV, 5FU/LV and irinotecan, and 5FU/LV and oxaliplatin (see Sect. 35.7)

35.6.2 Non-small Cell Lung Cancer

This clinical experiment was designed to be very similar to the phase II trial in colorectal cancer. During1998, a total of 99 patients were enrolled from 12 centers in a three-arm, randomized, controlled trial comparing standard chemotherapy with carboplatin and paclitaxel (CP) alone to CP with two different dose levels of bevacizumab: 7.5 mg/kg or 15 mg/kg every 3 weeks. The chemotherapy was delivered for six cycles and the bevacizumab was given concurrently and then continued after chemotherapy every 3 weeks until disease progression. Patients were required to have previously untreated inoperable stage IIIB or stage IV non-small cell lung cancer (NSCLC). The primary endpoints were time to disease progression and RR. Again, at progression in the control group, patients were permitted to receive bevacizumab alone at the 15 mg/kg dose level. The baseline characteristics of the patients are shown in Table 35.5. The one noticeable imbalance is the relatively large number of patients (10) with squamous cell histology in the 7.5 mg/kg group.

The overall efficacy results are shown in Table 35.6 and Fig. 35.10 (Johnson et al. 2004). This

Table 35.2. Baseline patient characteristics (from Kabbinavar et al. 2003)

Characteristic	Control (n=36) No. of patients	%	Bevacizumab 5 mg/kg (n=35) No. of patients	%	10 mg/kg (n=33) No. of patients	%	Total (n=104) No. of patients	%
Sex								
Male	27	75	17	49	15	46	59	57
Female	9	25	18	51	18	54	45	43
ECOG performance status								
0	22	61	21	60	18	54	61	59
1	14	39	14	40	14	42	42	40
2	0	0	0	0	1	3	1	1
Prior cancer therapy								
Adjuvant chemotherapy	8	22	5	14	7	21	20	19
Radiation therapy	5	14	5	14	5	15	15	14
Surgery	35	97	28	80	28	85	91	88
No. of metastatic sites								
1	23	64	20	57	17	52	60	58
2	10	28	9	26	10	30	29	28
≥3	3	8	6	17	6	18	15	14
Sites of metastases								
Liver	25	69	29	83	27	82	81	78
Lung	8	22	14	40	12	36	34	33
Liver and lung	4	11	9	26	8	24	21	20
Baseline albumin <3 g/dl	2	6	6	17	5	15	13	13

trial utilized an independent, blinded radiology review facility (IRF) in addition to the investigators to assess the efficacy endpoints in the trial, and Table 35.6 includes both sets of data. There appeared to be improved clinical benefit, including response, TTP and survival, in the 15 mg/kg group compared to control, with the investigator assessment of TTP reaching statistical significance (4.2 vs 7.4 months, p=0.023); however, little or no benefit was noted in the 7.5 mg/kg group. A total of 19 patients crossed over from the control arm at progression and received single-agent bevacizumab. Although no patient had a response, five patients experienced disease stabilization and remained on drug for greater than 6 months. The adverse events noted in this trial, shown in Table 35.7, again were similar to those noted in the phase II program and included headache, hypertension, epistaxis, and thrombosis. However a new,

Table 35.3. Analysis of PFS outcomes (from Kabbinavar et al. 2003)

Assessment	Control (n=36)	Bevacizumab 5 mg/kg (n=35)	Bevacizumab 10 mg/kg (n=33)	Pooled (n=68)
No. of progressions	26	22	23	45
Patients free of progression, %	28	37	30	34
Time to progression				
Median, months	5.2	9,0	7.2	7.4
Range, months	0.2–11.0+	0.6±13.5+	0.7±12.7	0.6±12.7
Hazard ratio, unadjusted	–	0.46	0.66	0.54
95% Confidence interval	3.5–5.6	5.8–10.9	3.8–9.2	5.7–9.2
p, log-rank	–	.005	.217	.013

Table 35.4. Adverse event profile (from Kabbinavar et al. 2003)

	Control (n=35)			Bevacizumab 5 mg/kg (n=35)			Bevacizumab 10 mg/kg (n=32)		
	All events		Grade 3/4	All events		Grade 3/4	All events		Grade 3/4
	No. of patients	%	No. of patients	%	No. of patients	%	No. of patients	%	No. of patients
Any event	35	100	19	35	100	26	32	100	25
Diarrhea	29	83	13	32	91	10	24	75	10
Leukopenia	1	3	1	4	11	2	1	3	1
Stomatitis	6	17	0	8	23	0	6	19	0
Fever	4	11	0	13	37	0	11	34	1
Headache	5	14	0	11	31	0	12	38	1
Rash	7	20	0	16	46	1	11	34	0
Chills	1	3	0	5	14	0	5	16	0
Abdominal pain	19	54	1	16	46	3	15	47	4
Weight loss	8	23	0	5	14	1	3	9	0
GI hemorrhage	0	0	0	2	6	0	5	16	3
Epistaxis	4	11	0	16	46	0	17	53	0
Hypertension	1	3	0	4	11	3	9	28	8
Infection	7	20	0	14	40	0	8	25	1
Thrombotic events	3	9	1	9	26	5	4	13	2

Table 35.5. Baseline patient characteristics (from Phase II Non-small Cell Lung Cancer Trial)

Parameter	Bevacizumab			
	Control (n =32)	7.5 mg/kg (n=32)	15 mg/kg (n=35)	Total (n=99)
Sex				
Female	8	12	19	39
Male	24	20	16	60
ECOG status				
0	15	16	19	50
1	15	15	12	42
2	2	1	4	7
Duration of current cancer				
<1 year	22	24	28	74
1 year	4	2	4	10
2 years	2	2	1	5
≥3 years	4	4	4	10
Prior cancer therapy				
Any	13	10	10	33
Radiation	8	9	7	24
Other*	11	7	9	27
Histology				
Adenocarcinoma	17	20	23	60
Large-cell anaplastic	4	1	5	10
Squamous cell	7	10	3	20
Other	4	1	4	9
Cancer stage				
IIIB	6	2	7	15
IV	26	30	28	84

serious toxicity was noted: life-threatening or fatal pulmonary hemorrhage. Six of these events were reported, four resulting in death. Five of the six events occurred in the 7.5 mg/kg group, and four occurred in patients with squamous cell histology. Because of the significant imbalance, with over-representation of squamous cell cancer patients in the 7.5 mg/kg group, an exploratory analysis was conducted which excluded these patients from all three groups. This result showed a dose-dependent improvement in all three efficacy endpoints: RR of 20% vs 32% vs 50%, TTP of 4.0 months vs 6.3 vs 7.0, and survival of 12.2 months versus 14 vs 17. It was this subset analysis which led to a phase III trial comparing CP alone to CP plus bevacizumab at 15 mg/kg in non-squamous NSCLC (see Sect. 35.7).

Table 35.6. Overall efficacy results (from (from Phase II Non-small Cell Lung Cancer Trial)

		Bevacizumab	
	Control (n=32)	7.5 mg/kg (n=32)	15 mg/kg (n=34)
Response rate, %			
Investigator	18.8	28.1	31.5
IRF	31.3	21.9	40.0
TTP, months			
Investigator			
Median	4.2	4.3	7.7
Range	0–12.6*	0.2–12.9*	0.7–12.5
IRF			
Median	5.9	4.1	7.0
Range	0.2–12.6*	0.2–13.1*	0.3–13.2*
Survival, months			
Median	14.9	11.6	17.7
Range	0.2–57.0	0.2–56.8*	0.8–57.8*
p		.84	.63

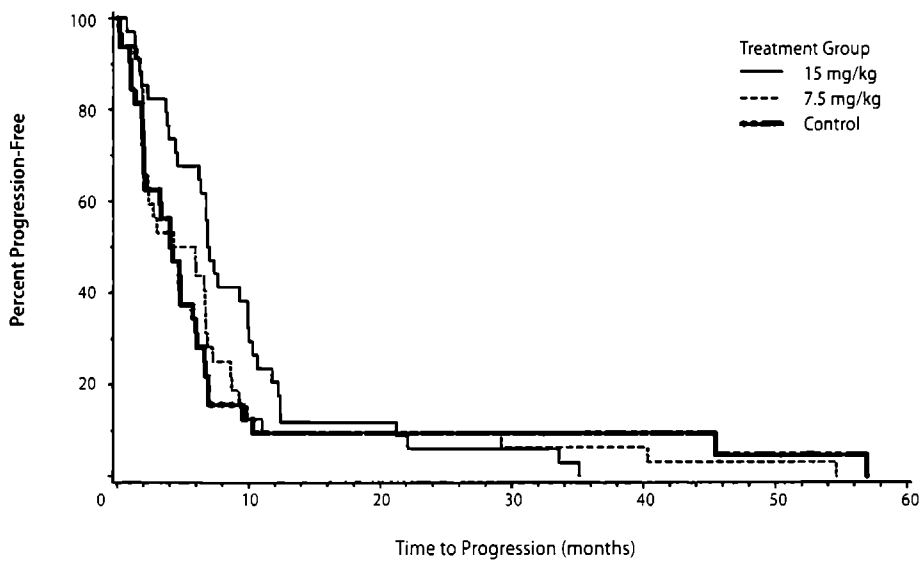


Fig. 35.10. Kaplan–Meier curve showing time to progression according to independent review facility/investigator assessment for carboplatin/paclitaxel (control), bevacizumab 7.5 mg/kg and bevacizumab 15 mg/kg arms, respectively

Table 35.7. Adverse events (from Phase II Non-small Cell Lung Cancer Trial)

	Control			7.5 mg/kg			15 mg/kg		
	All events			All events			All events		
	No. of patients	%	Grade 3/4	No. of patients	%	Grade 3/4	No. of patients	%	Grade 3/4
Chills	3	9.4	0	4	12.6	0	4	11.8	0
Diarrhea	6	18.8	0	9	28.1	3	14	41.2	1
Epistaxis	2	6.3	0	10	31.3	0	15	44.1	0
Fever	4	12.5	0	11	34.4	2	11	32.4	2
Headache	3	9.4	0	10	31.3	1	16	47.1	2
Hemorrhage	0	0	0	4	12.5	2	0	0	0
Hypertension	1	3.1	1	5	15.6	0	6	17.6	2
Hemoptysis	2	6.3	0	9	28.1	3	4	11.8	1
Infection	8	25.0	1	10	31.3	0	12	36.3	2
Leukopenia	10	31.3	7	15	46.9	10	19	55.9	13
Nausea	15	46.9	1	16	50.0	1	17	50.0	2
Neuropathy	9	28.1	0	4	12.5	0	5	14.7	1
Paresthesia	7	21.9	0	9	28.1	0	12	35.3	0
Peripheral neuritis	9	28.1	1	8	25.0	0	13	38.2	2
Rash	3	9.4	0	11	34.4	0	8	23.5	0
Stomatitis	3	9.4	0	5	15.6	0	8	23.5	0
Thrombocytopenia	5	15.6	0	2	6.3	0	7	20.6	1
Thrombotic events	3	9.4	3	4	12.5	2	6	17.6	5
Vomiting	6	18.8	1	6	18.8	1	8	23.5	1

35.6.3 Phase II Program Summary

At the completion of these phase II experiments there were some consistent conclusions. First, bevacizumab appeared to have clinical activity in human cancer. The results in renal cell cancer were particularly compelling, but all of the phase II trials, with the exception of prostate cancer, clearly indicated activity. The results tended to confirm the pre-clinical observations that RR was a poor surrogate for clinical activity and that delay in tumor progression was clearly a better endpoint for ongoing clinical experiments. The significant number of patients demonstrating prolonged stable disease during cross-over from the control groups to single-agent bevacizumab in the colon and NSCLC trials reinforces this finding. This work also defined a preliminary adverse event profile for bevacizumab

which included hypertension, proteinuria, thrombosis and bleeding with a unique finding of life-threatening and fatal pulmonary bleeding in patients with NSCLC. These adverse events would become a focus of the phase III programs.

Phase III Program

35.7.1 Dose Selection

At the completion of the phase II program questions remained regarding the optimal dose of bevacizumab to evaluate in phase III trials. No obvious dose-limiting toxicities had been observed, although doses in excess of 15 mg/kg every 3 weeks seemed unwise, given the headache syndromes observed in the single-agent phase II breast cancer program. Pharmacokinetic data had demonstrated that doses between 2.5 mg/kg/week (5 mg/kg every 2 weeks or 7.5 mg/kg every 3 weeks) and 5.0 mg/kg/week (10 mg/kg every 2 weeks or 15 mg/kg every 3 weeks) achieved steady-state trough concentrations that were in excess of the targeted optimal inhibitory concentration of 10μg/ml extrapolated from xenograft models. These concentrations of bevacizumab were approximately 3 logs higher than the concentration of circulating VEGF, and "saturation" of target was estimated to occur with bevacizumab doses of greater than 0.1–0.3 mg/kg. Despite these pharmacokinetic observations, the clinical results in three of the four dose-ranging phase II trials suggested a dose effect, including studies in breast, lung, and renal cell cancers. The one exception to this observation occurred in the colorectal study, where the results with the lower dose level appeared to be superior to those obtained at the higher dose level. Whether this observation may have been a chance event, given the small sample size of the study, remains an unanswered question. However, based on the clinical results of these phase II trials, the dose of bevacizumab selected for phase III testing was 5 mg/kg/week, with the exception of colorectal cancer, where the lower dose of 2.5 mg/kg/week was also studied.

35.7.1.1 Colorectal Cancer

The pivotal phase III trial of bevacizumab combined with chemotherapy for the initial treatment of metastatic colorectal cancer (CRC), known as study AVF2107, involved a complicated study design (Hurwitz et al. 2004). The enabling phase II experiment had been conducted with the chemotherapy regimen of bolus 5FU and leucovorin LV, one of several commonly accepted "standard" regimens at the time for treating CRC. The initial intent of the phase III experiment was simply to repeat the phase II design with sufficient power to demonstrate a statistically significant effect on patient survival. However, just as the study design was being finalized, a new chemotherapy regimen emerged for the treatment of CRC, specifically irinotecan with 5FU and LV, or IFL (Saltz et al. 2000). It was apparent that a phase III study of bevacizumab with 5FU/LV would not be acceptable for patients given the newly established standard of IFL. However, bevacizumab had never been combined with IFL, which itself had some significant toxicities, including diarrhea, myelosuppression and thromboembolic events. In order to avoid a significant delay in the phase III program, the CRC trial incorporated a novel design element that pre-specified a regimen selection process early in the conduct of the phase III trial.

The clinical experiment was initiated in September 2000 in the United States, New Zealand and Australia and completed recruitment of 923 patients by May 2002. Initially patients were randomly assigned to one of three treatment groups; the control group of IFL plus placebo, one experimental group of 5FU/LV plus bevacizumab, and a second experimental group of IFL plus bevacizumab. All treatment including chemotherapy and bevacizumab or placebo was continued until the time of disease progression. After 313 patients were enrolled, an independent data safety and monitoring board (DSMB)

conducted a formal safety interim analysis. The specific intent of that interim analysis was to determine the safety of combining bevacizumab with IFL. If the DSMB determined that the combination was safe, then the experiment would become a two-group study of IFL with or without bevacizumab. If the DSMB determined that the combination was unsafe, the experiment would become a two-group study of IFL versus 5FU/LV plus bevacizumab. In fact, the DSMB determined that the combination of bevacizumab and IFL was safe and the trial proceeded as a simple two-group comparison of IFL plus placebo or bevacizumab. The primary endpoint of the study was overall survival (OS), and it was designed to have 80% power to detect a hazard ratio of 0.75 for death with a type 1 error of less than 5%. To achieve this power approximately 385 deaths were required for the final analysis, and a total of 813 patients were randomized between the two primary comparison groups. Stratification variables included baseline performance status, site of primary disease (colon vs rectum), number of metastatic sites (one vs two or more), and study center. In addition to survival, PFS, overall RR and response duration were important secondary efficacy endpoints.

The patient characteristics are shown in Table 35.8. There was good balance for all of the stratification variables and other important demographic and prognostic characteristics.

The overall efficacy results of the study are shown in Table 35.9 and Fig. 35.11. It achieved the primary goal of improving the survival of patients with advanced CRC, with a 34% reduction in the hazard of death for the group receiving bevacizumab compared to the control IFL group. In addition there were highly significant improvements in both PFS and RR.

In addition to the overall treatment effect, the survival benefit was also analyzed in a number of pre-specified patient subsets, including age, gender, race, performance status, location of primary tumor (colon vs rectum), number of metastatic sites (1 vs >1), prior adjuvant therapy (yes vs no), tumor burden by volume (<median vs ≥ median), albumin (<median vs ≥ median), alkaline phosphatase (<median vs ≥ median), and lactate dehydrogenase

Table 35.8. Demographic and baseline patient characteristics (from Hurwitz et al. 2004)

Characteristic	IFL plus placebo (n=411)	IFL plus bevacizumab (n=402)
Sex (%)		
Male	60	59
Female	40	41
Mean age (years)	59.2	59.5
Race (%)		
White	80	79
Black	11	12
Other	9	9
Location of center (%)		
USA	99	99
Australia or New Zealand	<1	<1
ECOG performance status (%)		
0	55	58
1	44	41
2	<1	<1
Type of cancer (%)		
Colon	81	77
Rectal	19	23
No. of metastatic sites (%)		
1	39	37
>1	61	63
Prior cancer therapy (%)		
Adjuvant chemotherapy	28	24
Radiation therapy	14	15
Median duration of metastatic disease (months)	4	4

Table 35.9. Overall efficacy (from Hurwitz et al. 2004)

Endpoint	IFL plus placebo	IFL plus bevacizumab	*p* value
Median survival (mo)	15.6	20.3	<0.001
Hazard ratio for death		0.66	
One-year survival rate (%)	63.4	74.3	<0.001
Progression-free survival (months)	6.2	10.6	<0.001
Hazard ratio for progression		0.54	
Overall response rate (%)	34.8	44.8	0.004
Complete response	2.2	3.7	
Partial response	32.6	41.0	
Median duration of response (months)	7.1	10.4	0.001

(<median vs ≥ median). Interestingly, there was significant and consistent benefit in every patient subset analyzed.

Overall, the combination of IFL and bevacizumab was well tolerated and selected adverse event information is shown in Table 35.10. There were significantly higher rates of grade 3 or 4 adverse events in the combination group, primarily represented by an increase in hypertension, an adverse event that was well described in the phase II program. Grade 5 adverse events (death) and overall 60-day mortality were similar in both groups. It was interesting that the rates of thrombosis were quite high in both treatment arms, and these results underscore the importance of placebo-controlled randomized studies in assessing unbiased safety information in clinical drug development. There was also no increase in the rate of serious bleeding or proteinuria, safety events that had been noted during the open-label phase II program. One important new safety issue was noted in this study, namely gastrointestinal perforation. This event was observed in six bevacizumab-treated patients versus none in the control group. The presentations were variable and included predominantly large-bowel perforations but also gastric perforation. The underlying etiology remains unclear, but may represent a spectrum of impaired wound healing, occurring in patients with a variety of bowel pathologies (benign gastric ulceration, diverticular disease, recent bowel surgery, or malignant bowel involvement). Further study is required to better understand the potential risks of this serious complication.

One interesting exploratory analysis reported from this study involved the assessment of the treatment effect obtained from bevacizumab in patients who either did or did not obtain an objective response to their assigned therapy. Traditional cytotoxic therapies for cancer are typically assessed by their ability to shrink cancers, and the lack of an objective tumor response is usually viewed as evidence of no activity. In addition, chemotherapy is often discontinued at the time of "maximal" tumor response, the lack of further tumor shrinkage again being interpreted as a surrogate for no further activity or benefit. Because bevacizumab provided a much greater benefit on endpoints of PFS and OS as compared to RR in the phase III trial, the results implied that even patients who do not demonstrate objective tumor response might gain significant benefit from the agent. Figure 35.12 shows the results of these exploratory analyses. Although patients with objective tumor responses had longer PFS and OS, the actual treatment effect from bevacizumab as assessed by the hazard ratio for progression or death was similar in the responding and non-responding patient groups. This observation is consistent with the predominantly cytostatic effects of bevacizumab and underscores the importance of

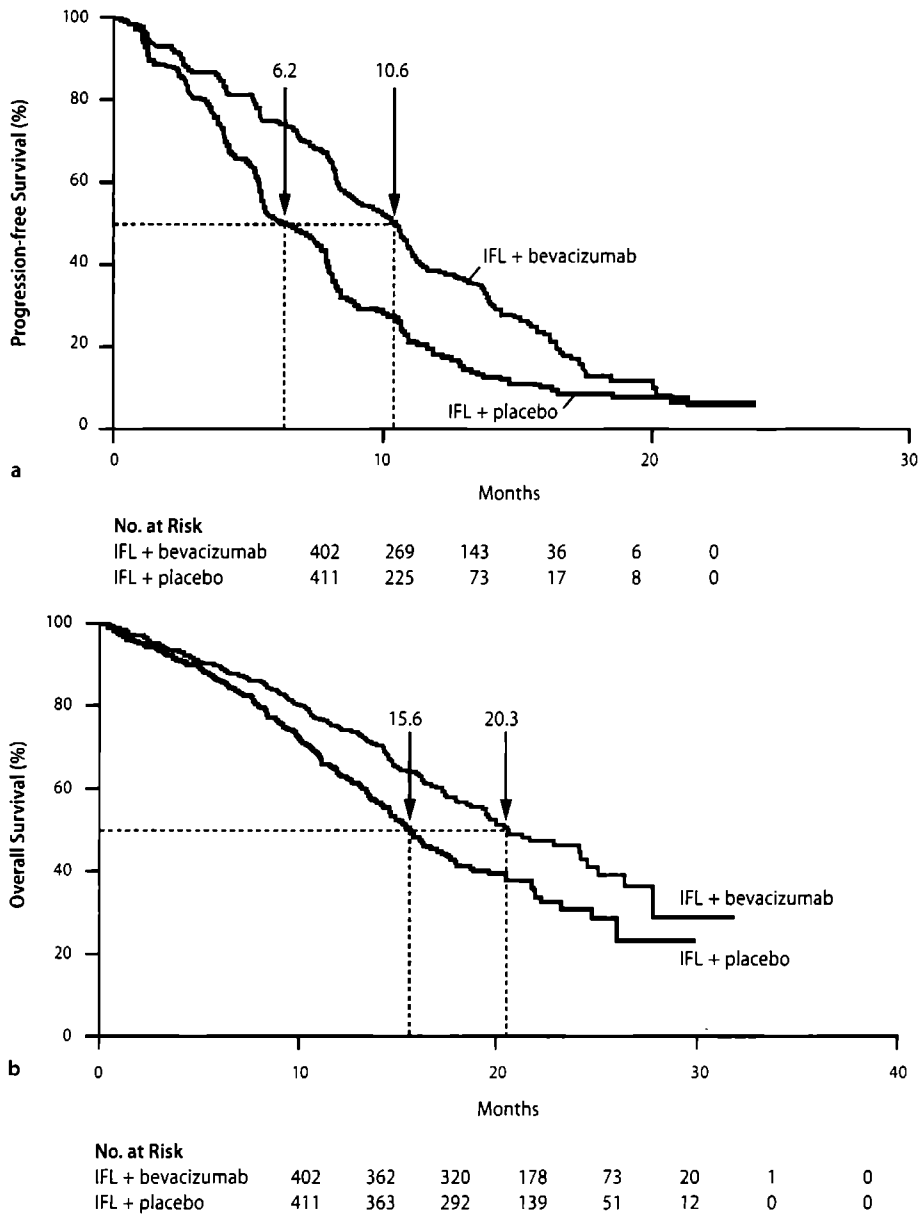


Fig. 35.11a,b. Kaplan–Meier estimates of PFS (*top*) and OS (*bottom*) (from Hurwitz et al. 2004)

differentiating this biologic agent from traditional chemotherapy when designing endpoints for clinical experiments in addition to assessing its benefit in routine clinical use.

The significance of the overall results of the study by Hurwitz and colleagues (Hurwitz et al. 2004) should not be underestimated. This represented the first definitive experiment showing clinical benefit associated with the use of a specific anti-angiogenic agent in the treatment of human cancer and essentially validated the hypothesis of Ferrara that blocking VEGF signaling with a specific humanized antibody would represent a novel therapeutic strategy in oncology.

Table 35.10. Selected adverse events (from Hurwitz et al. 2004)

Adverse event	IFL plus placebo (n=397) %	IFL plus bevacizumab (n=393)
Any grade 3 or 4 adverse event	74.0	34.9
Adverse event leading to hospitalization	39.6	44.9
Adverse event leading to discontinuation of treatment	7.1	8.4
Adverse event leading to death	2.8	2.6
Death within 60 days	4.9	1.0
Grade 3 or 4 leukopenia	31.1	37.0
Grade 3 or 4 diarrhea	24.7	32.4
Hypertension		
Any	8.2	22.4
Grade 3	2.3	11.0
Any thrombotic event	16.2	19.4
Deep thrombophlebitis	6.3	8.9
Pulmonary embolus	5.1	3.6
Grade 3 or 4 bleeding	2.5	3.1
Proteinuria		
Any	21.7	36.5
Grade 2	5.8	3.1
Grade 3	0.8	0.8
Gastrointestinal perforation	0.0	1.5

In addition to the pivotal phase III trial with IFL in CRC, a second supportive randomized phase II experiment was conducted concurrently in a group of first-line patients not felt to be candidates for IFL chemotherapy (Kabbinavar et al. 2005). Study AVF2192 required patients to have at least one of four known features that predicted either lack of benefit or increased toxicity from the addition of irinotecan to 5FU/LV based therapy: age ≥65 years, performance status of 1 or 2, serum albumin ≤3.5 g/dl, or prior radiotherapy to the abdomen or pelvis. The trial design was a straightforward comparison of 5FU/LV plus placebo versus 5FU/LV plus bevacizumab. The primary endpoint was OS, with secondary endpoints of PFS, RR, and duration of response along with safety. Between August 2000 and July 2002, 209 patients were randomized between the two treatment groups, stratified for baseline performance status (0 vs 1 or 2), site of primary disease (colon vs rectum), number of metastatic sites (1 vs >1), and study center. The study was relatively small and had 80% power to detect a hazard ratio of 0.61 for death with a type 1 error of <5%, which required approximately 133 deaths.

The baseline demographic characteristics are shown in Table 35.11 and are reasonably well bal-

Table 35.11. Selected demographic and baseline characteristics (from Kabbinavar et al. 2005)

Characteristic	5-FU/LV/ placebo (n=105)	5-FU/LV/ bevacizumab (n=104)
Sex		
Male	51%	56%
Female	49%	44%
Mean age (years)	70.7	71.3
Race		
White	85%	83%
Black	7%	10%
Other	9%	8%
ECOG performance status		
0	28%	29%
1	67%	64%
2	6%	8%
Cancer type		
Colon	80%	82%
Rectal	20%	18%
Number of metastatic sites		
1	31%	39%
>1	70%	62%
Prior cancer therapy		
Adjuvant chemotherapy	21%	19%
Radiation therapy	14%	15%
Albumin		
3.5 g/dl	51 (49%)	42 (42%)
>3.5 g/dl	53 (51%)	57 (58%)

anced between the two groups, with the exception of a lower proportion of patients with low serum albumin in the bevacizumab group.

The overall efficacy results are shown in Table 35.12 and Fig. 35.13. Although the trial did not achieve the primary endpoint of a 39% reduction in the hazard of death, the estimated improvement in median survival observed in this trial, 3.7 months, was similar to the 4.7 months survival benefit observed in the pivotal IFL trial. Study AVF2192 did demonstrate a significant improvement in PFS, with a hazard for progression of 0.50, and a borderline significant improvement in RR, 15.2% versus 26.0%. These results were particularly encouraging given the poor prognostic group of patients studied in this experiment and the lack of effective treatment options beyond 5FU/LV that had been demonstrated to date.

The incidence of selected adverse events in this experiment is shown in Table 35.13 and is consistent with the safety profile of bevacizumab that was observed in the pivotal phase III trial with IFL. The only significant adverse event that occurred at a statistically higher rate in the bevacizumab group was grade 3 hypertension (3% vs 16%). Again, gastrointestinal perforation was observed in 2 of the 100 patients randomly assigned to the bevacizumab group versus none in the placebo group, further supporting the conclusion that this event is uncommon but associated with bevacizumab use in CRC.

Another large phase III trial of bevacizumab in CRC has recently been reported which adds to the previous three randomized trials of bevacizumab in the treatment of this disease (Fig. 35.14; Giantonio et al. 2007). As previously noted, during the development of bevacizumab in CRC several improvements were reported in chemotherapeutic approaches to the disease. On the basis of a number of randomized trials it was determined that the infusional delivery of 5FU was safer and more effective than bolus schedules of the agents (Meta-analysis Group in Cancer 1998). Furthermore, two new cytotoxic agents were developed for the disease; irinotecan (CPT-11) and oxaliplatin (Saltz et al. 2000; de Gramont et al. 2000; Rothenberg et al. 2003).

With this background information available, the Eastern Cooperative Oncology Group (ECOG) in collaboration with the National Cancer Institute conducted a phase III trial comparing an infusional 5FU/LV plus oxaliplatin regimen known as FOLFOX4 with and without bevacizumab for patients with relapsed metastatic CRC who had previ-

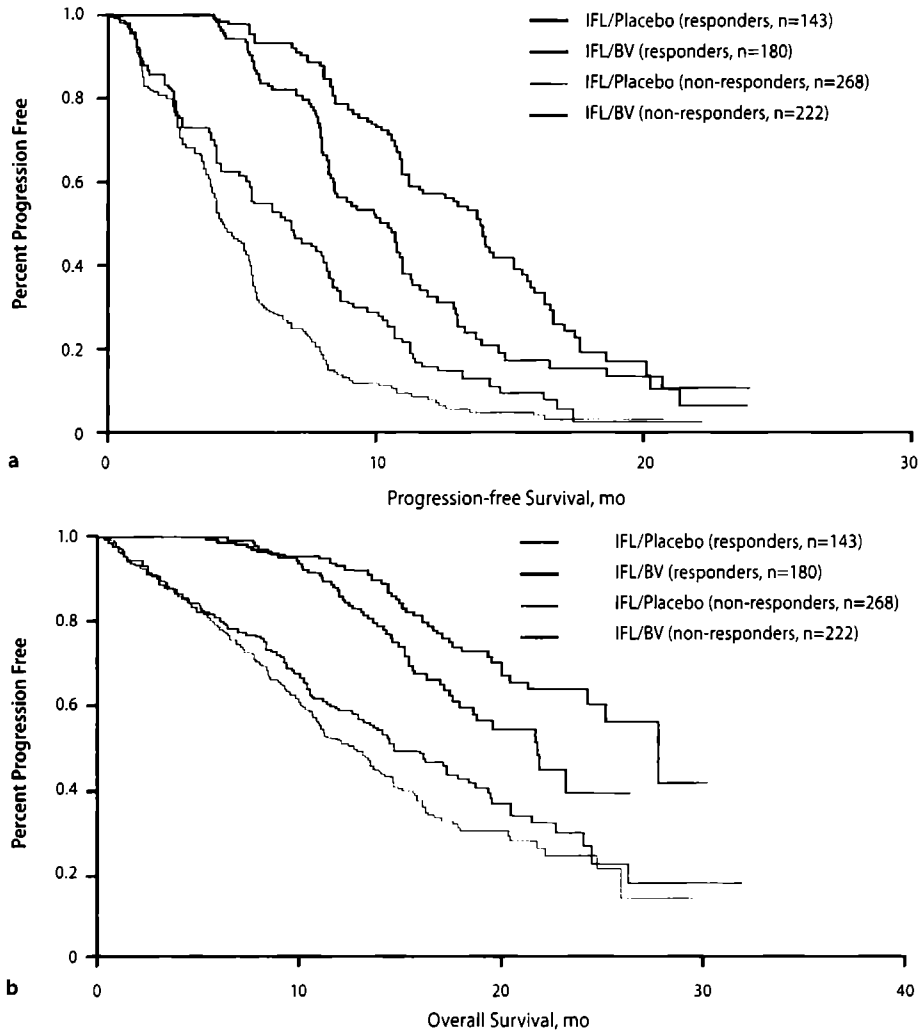


Fig. 35.12a,b. Bevacizumab treatment effect (*top*, PFS; *bottom*, OS) in responding and non-responding patient groups (from Mass et al. 2004)

BV= bevacizumab; IFL= irinotecan, 5-fluorouracil, and leucovorin

ously been treated with both 5FU/LV and irinotecan. It is important to note that the dose of bevacizumab utilized in this clinical study was 5 mg/kg/week, rather than the 2.5 mg/kg/week dose used in the pivotal phase III first-line CRC study. In addition to this primary comparison, a third treatment group received bevacizumab alone. The trial was designed to provide 90% power to detect a 50% improvement in survival (from 7 months to 10.5 months) for the primary comparison (FOLFOX vs FOLFOX plus bevacizumab) with a type 1 error of <5%. PFS, landmark 1-year survival, and RR were secondary efficacy endpoints. Between November 2001 and April 2003 a total of 820 patients known to meet the eligibility requirements were randomized utilizing two stratification variables, prior radiation therapy (yes vs no) and performance status (0 vs 1 vs 2). In February 2003, the DSMB determined during a planned interim analysis that the bevacizumab alone group appeared to have survival inferior to that in the FOLFOX groups and randomization to the bevacizumab alone group was closed. The final intent-to-

Table 35.12. Summary of efficacy analysis (from Kabbinavar et al. 2005)

Efficacy parameter	5-FU/LV/placebo (n=105)	5-FU/LV/bevacizumab (n=104)	p value
Median survival (months)	12.9	16.6	0.160
Hazard ratio		0.79	
95% CI		0.56–1.10	
Progression-free survival (months)	5.5	9.2	0.0002
Hazard ratio		0.50	
95% CI		0.34 to 0.73	
Overall response rate (%)	15.2	26.0	0.055
Complete response	0	0	
Partial response	15.2	26.0	
Duration of response (months)	6.8	9.2	0.088
Hazard ratio		0.42	
95% CI		0.15 to 1.17	

treat analysis included 291 patients in the FOLFOX group, 286 in the FOLFOX plus bevacizumab group, and 243 in the bevacizumab alone group. The baseline demographics and disease characteristics are shown in Table 35.14 and are well balanced between the groups.

The efficacy results are shown in Table 35.15 and Fig. 35.15. The trial achieved its pre-specified endpoint of improving OS, with an observed hazard ratio for death of 0.75 (p=0.001) and an absolute 13% higher rate of 1-year survival when bevacizumab was added to FOLFOX chemotherapy in relapsed CRC. In addition to the survival benefit, highly significant improvements in both PFS and RR were also observed. Although the DSMB closed recruitment to the bevacizumab alone group, the final survival analysis did not suggest inferior results when compared to FOLFOX alone (10.2 months vs 10.8 months); however, PFS and RR both appeared to be better in the FOLFOX-containing groups, although formal comparative statistics were not reported for group B vs group C.

As noted in the previous studies of bevacizumab in CRC, the drug was well tolerated in E3200, and Table 35.16 lists some selected adverse events. Hypertension was again noted to occur, although the absolute incidence was lower than that observed in the pivotal study. Vomiting and neuropathy also occurred at a higher rate when bevacizumab was added to FOLFOX and may be related to the greater number of chemotherapy cycles (10 vs 7) received by the FOLFOX plus bevacizumab group. In addition, serious bleeding, which was predominantly gastrointestinal, occurred at a higher frequency (3.4% vs 0.4%) in the FOLFOX plus bevacizumab group and, again, gastrointestinal perforation was observed in three patients in each of the bevacizumab-containing arms.

In summary, bevacizumab has been evaluated in four randomized controlled studies in CRC, two phase II and two phase III trials, utilizing three different chemotherapy backbones (5FU/LV, IFL, and FOLFOX) in both first-line and relapsed patient populations. It consistently provided statistical improvements in time to disease progression and, in the two large phase III trials, a statistical improvement in OS. Use of the agent to the time of disease progression appears to be important, with a retro-

Table 35.13. Selected adverse events

Adverse event	5-FU/LV/placebo (n=104)	5-FU/LV/bevacizumab (n=100)
Any grade 3 or 4 adverse event	71%	87%
Adverse event leading to study discontinuation	12%	10%
Adverse event leading to death	7%	4%
All-cause mortality at 60 days	14%	5%
Diarrhea, grade 3 or 4	40%	39%
Leukopenia, grade 3 or 4	7%	5%
Hypertension, any	5%	32%
Hypertension, grade 3*	3%	16%
Thrombotic events, any	18%	18%
Deep thrombophlebitis	9%	6%
Pulmonary embolus	2%	3%
Arterial thrombotic event, any	5%	10%
Bleeding, grade 3 or 4	3%	5%
Proteinuria, any	19%	38%
Grade 2	4%	7%
Grade 3	0%	1%
Gastrointestinal perforation	0%	2%

spective analysis suggesting PFS and OS improvements were independent of objective response. The doses used in these experiments varied between 2.5 and 5.0 mg/kg/week, and the optimal dose for individual patients has not been established. Overall, the drug was well tolerated, with consistent increases in hypertension requiring medical therapy and low-grade proteinuria noted in these trials. Increases in serious bleeding were reported in one of the phase III studies, and more data will be required to more fully understand the precise incidence and risk factors for this event. Gastrointestinal perforation has been consistently observed at a rate of 1–2%, and patients should be informed of this potentially life-threatening event. Based on the results of these four clinical trials, bevacizumab in combination with 5FU-based chemotherapy is now considered a standard treatment option for both first-line and relapsed metastatic CRC. Studies are ongoing to evaluate the potential role of bevacizumab in the adjuvant treatment of this common malignant disease.

35.7.1.2 Breast Cancer

Following the initial single-agent phase II study of bevacizumab in refractory breast cancer, two subsequent phase III clinical experiments of bevacizumab in combination with chemotherapy in metastatic breast cancer (MBC) have been completed and reported.

The first phase III experiment, known as study AVF2119, was conducted in a highly refractory MBC

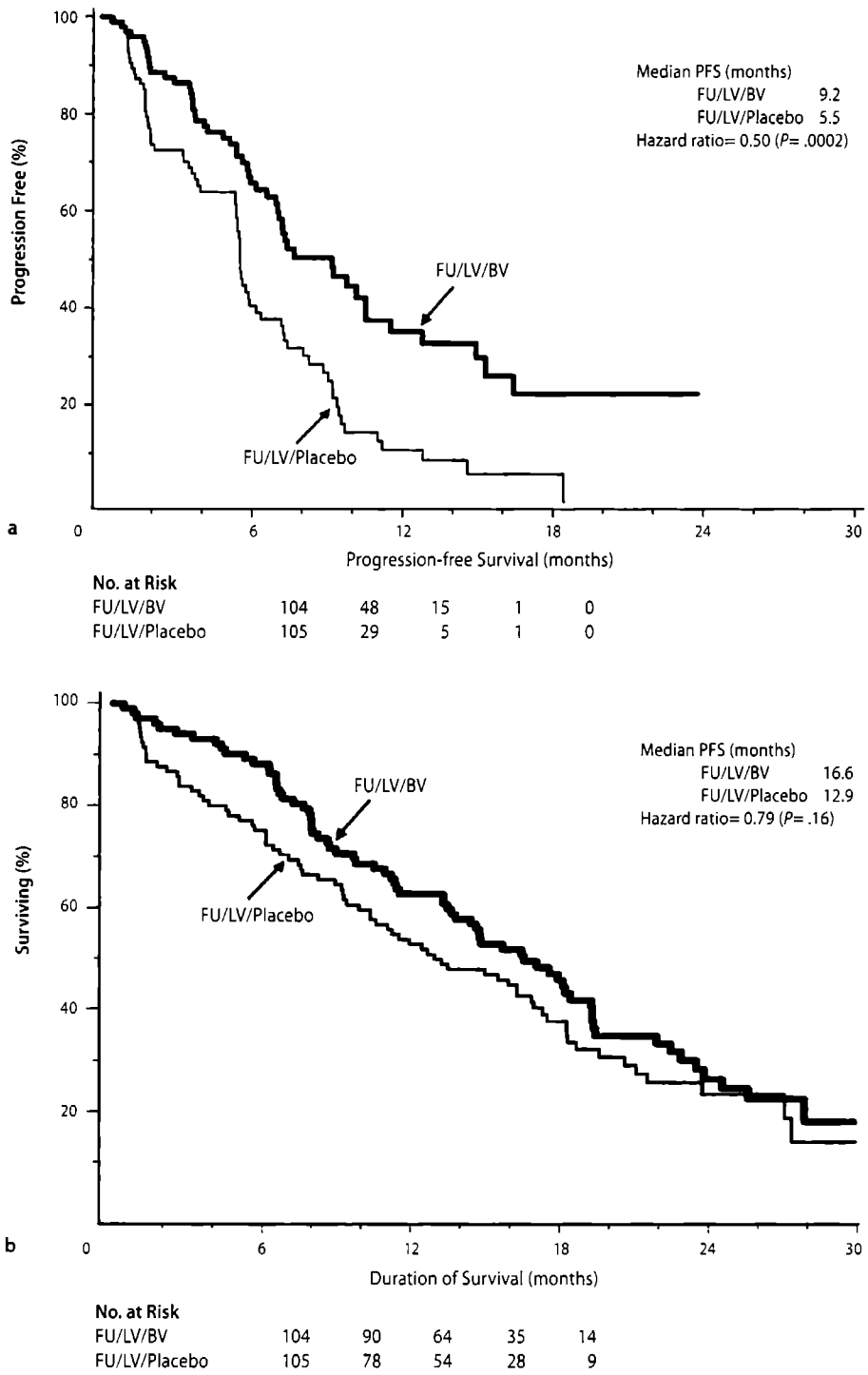


Fig. 35.13a,b. Kaplan-Meier estimates of PFS (*top*) and OS (*bottom*) (from Kabbinavar et al. 2005)

patient population (Miller et al. 2005). Patients were required to have progressive disease following therapy with both anthracyclines and taxanes. The patients without prior therapy for MBC were required to have relapsed within 12 months of completing adjuvant therapy with both anthracyclines and taxanes, a patient population with a particularly poor prognosis. Capecitabine had recently been approved for the treatment of this refractory patient population based on a non-randomized phase II experiment documenting a finite objective RR (Blum et al. 1999). It seemed reasonable to design a phase III experiment comparing capecitabine alone to capecitabine with bevacizumab. The dose of capecitabine in this study was 2500 mg/m^2 twice daily for 14 days followed by a 7-day rest period, with cycles repeated every 21 days. Bevacizumab was used at a dose of 15 mg/kg every 3 weeks (5 mg/kg/week).

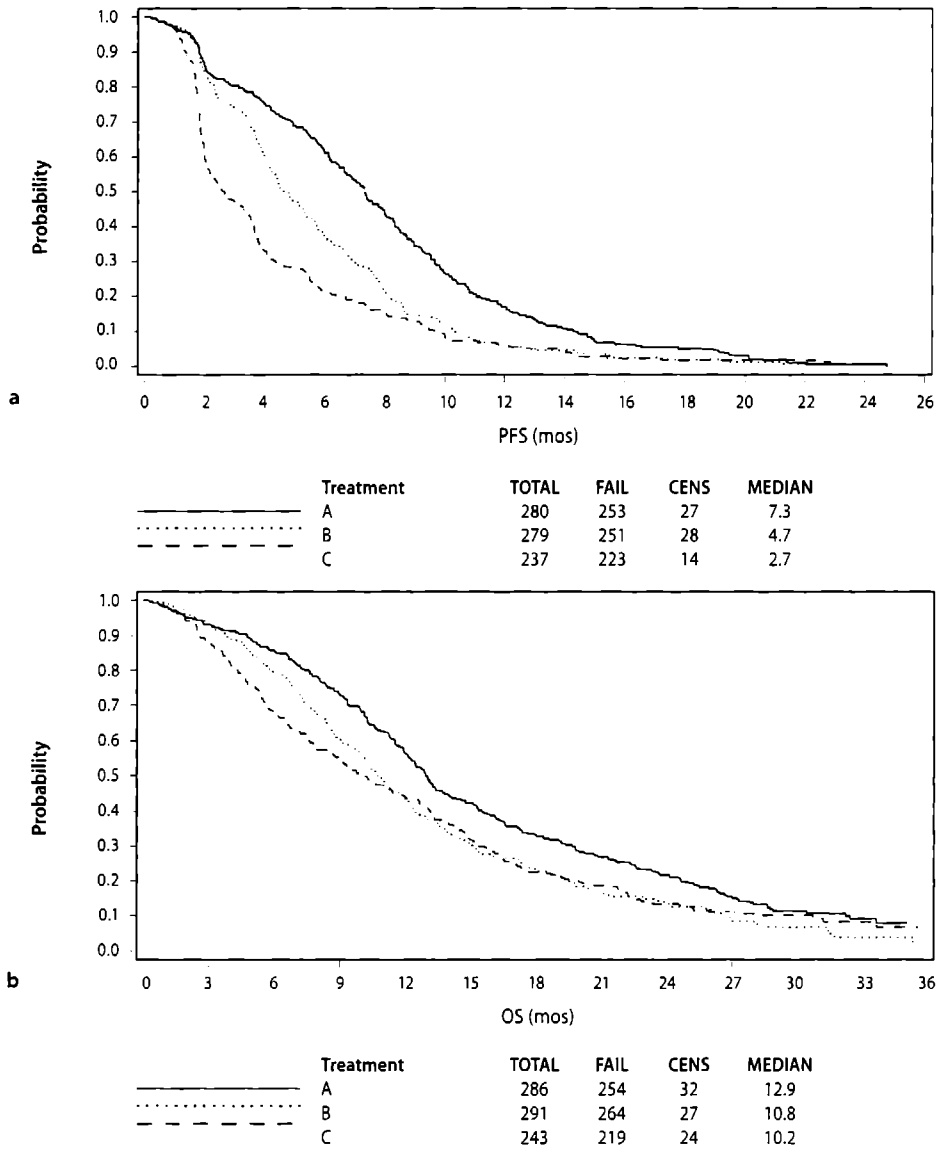


Fig. 35.14a,b. Kaplan–Meier estimates of PFS (*top*) and OS (*bottom*) (from Giantonio et al. 2007)

Table 35.14. Baseline patient characteristics: E3200 study (from Giantonio et al. 2007

	FOLFOX4 + bevacizumab (n=286)	FOLFOX4 (n=291)	Bevacizumab (n=243)
Median age (years)	62.0	60.8	59.6
Range	21–85	25–84	23–82
Sex			
Female	39.5%	39.2%	40.7%
Performance status			
0	48.9%	51.2%	48.6%
1	46.9%	43.0%	43.6%
2	4.2%	5.8%	7.8%
Prior radiation therapy	25.9%	24.7%	25.9%
Disease sites			
Liver	73.4%	75.9%	70.8%
Lung	55.5%	51.2%	59.7%

Table 35.15. Efficacy results: E3200 study (from Giantonio et al. 2007)

	FOLFOX4 + bevacizumab	FOLFOX4	Bevacizumab	p A vs B
Median survival (months)	12.9	10.8	10.2	0.0011
Hazard ratio for death	0.75			
Progression-free survival (months)	7.3	4.7	2.7	<0.0001
Hazard ratio for progression	0.61			
One-year survival (%)	56	43	44	
Overall response rate (%)	22.7	8.6	3.3	<0.0001
Complete response (%)	1.7	0.7	0	
Partial response (%)	21.0	7.9	3.3	

Between November 2000 and March 2002, 462 patients were randomized between the two treatment groups utilizing three stratification variables: performance status (0 vs 1), number of metastatic sites (0 vs ≥1), and study center. The primary endpoint of the study was PFS and the secondary efficacy endpoints included RR, duration of response, and OS. Because the trial was unblinded, the primary PFS analysis was based on a blinded, independent radiographic assessment rather than the investigator assessment of progression. The sample size was determined to provide 90% power to observe a hazard for progression of 0.67 with a type 1 error of <5% and required 265 progression events. Table 35.17 shows the baseline demographic and disease characteristics in the two treatment groups, which were well

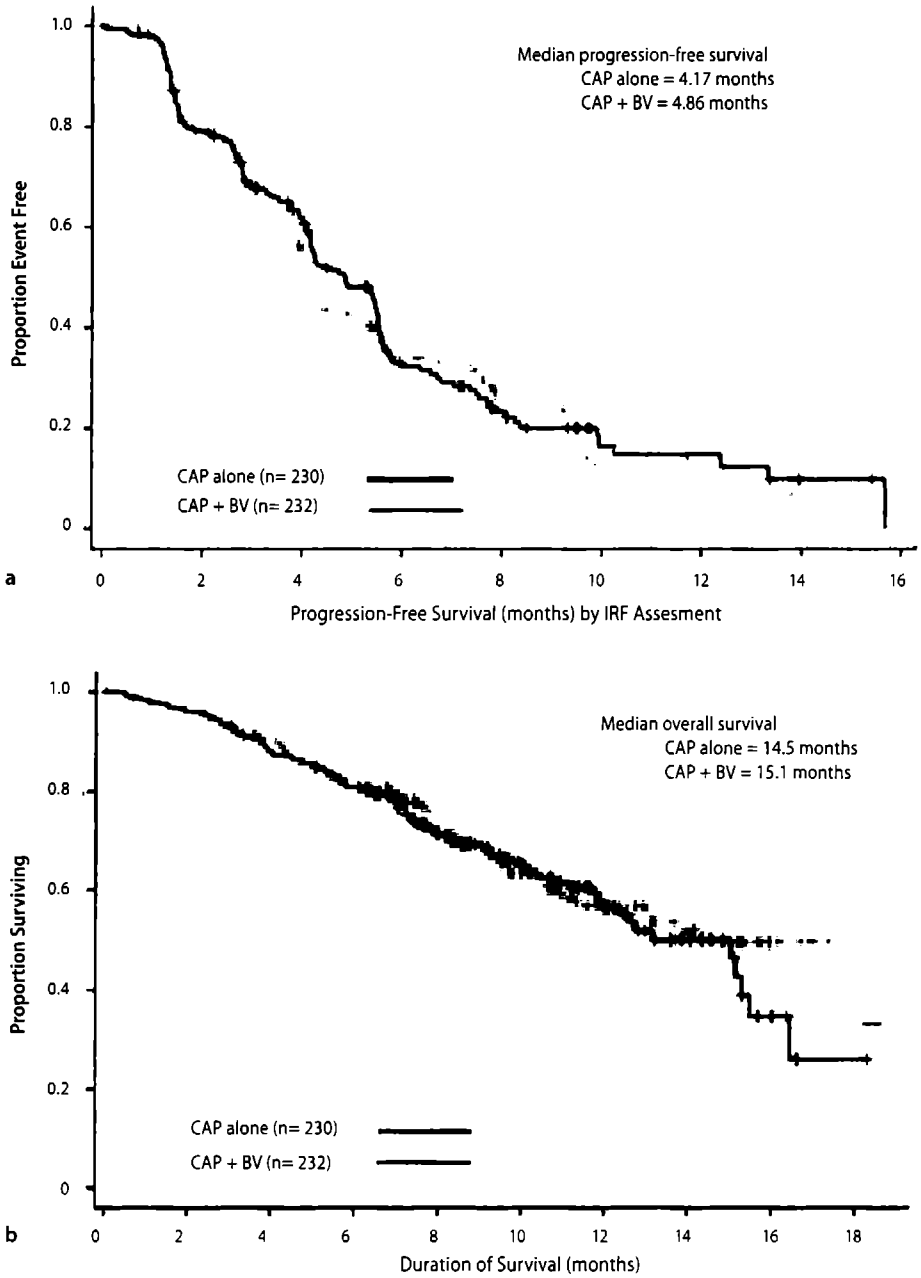


Fig. 35.15a,b. Kaplan-Meier estimates of PFS (*top*) and OS (*bottom*) (from Miller et al. 2005)

Table 35.16. Percentage of adverse events: E3200 study (from Giantonio et al. 2007)

	FOLFOX4 + bevacizumab (n=287)		FOLFOX4 (n=285)		Bevacizumab (n=234)		p A vs B
	G3	G4	G3	G4	G3	G4	
Hypertension	5.2	1.0	1.4	0.4	7.3	0	0.008
Bleeding	3.1	0.3	0.4	0	2.1	0	0.011
Vomiting	8.7	1.4	2.8	0.4	4.7	0	0.001
Proteinuria	0.7	0	0	0	0	0	0.50
Neuropathy	16	0.3	8.8	0.4	0.4	0.4	0.011
Thromboembolism	3.1	0.3	1.1	1.4	0	0.4	0.62
Cardiac ischemia	0.3	0.3	0	0.4	0	0	0.62[a]
Cerebrovascular ischemia	0.3	0	0	0	0	0	
Any adverse event	49.5	25.8	36.1	24.9	27.8	8.1	
Adverse event leading to treatment discontinuation	23.4		23.9		12.0		
Death from any cause within 60 days	5		4		6		

[a]Cardiac ischemia and cerebrovascular ischemia combined

balanced except for a slightly higher proportion of ER/PR+ patients in the capecitabine alone group.

The efficacy results showed a statistically significant improvement in RR when adding bevacizumab to capecitabine (9.1% vs 19.8%, p=0.001). However, the study failed to meet the primary endpoint of improved PFS. The median PFS values were 4.2 months in the control group and 4.9 months in the bevacizumab group, with an observed progression hazard of 0.98 (p=0.86) and there was no difference in overall survival (see Fig. 35.15). Tables 35.18 and 35.19 outline the adverse events reported, focusing on capecitabine-associated events and bevacizumab-associated events. There was no suggestion that bevacizumab worsened the common toxicities (hand–foot syndrome, stomatitis, and diarrhea) associated with capecitabine use. With regard to bevacizumab toxicities, only hypertension requiring medical therapy occurred with increased frequency (0.5 vs 17.9%). No reports of gastrointestinal perforation were observed in this study. However, an imbalance was noted in the incidence of congestive heart failure; two events were reported in the control arm versus seven in the bevacizumab arm. All patients had received prior anthracycline therapy, and this observation highlighted the possible link between prior or concurrent use of anthracyclines, which are known cardiotoxic agents, and bevacizumab-associated congestive heart failure.

While study AV2119 was ongoing, investigators in the Eastern Cooperative Oncology Group (ECOG) and the NCI were interested in evaluating bevacizumab in less refractory patients with MBC. Preclinical observations had suggested that progressive drug resistance in tumor cells in vitro is associated with up-regulation of multiple angiogenic growth factors. A logical inference from these observations was that utilizing a very specific anti-VEGF antibody like bevacizumab in highly refractory breast cancer might be less likely to demonstrate benefit than applying the therapy to less highly refractory patients. In addition, observations were emerging with regard to concepts of "metronomic" scheduling of traditional chemotherapy agents that dem-

Table 35.17. Patient characteristics: AVF2119 study (from Miller et al. 2005

	Capecitabine (n=230)	Capecitabine + bevacizumab (n=232)
Mean age (range)	52.3 years (30–77)	51.1 years (29–78)
Race		
Black	25 (10.9%)	30 (12.9%)
White	185 (80.4%)	187 (80.6%)
Other	20 (8.7%)	15 (6.5%)
ECOG PS		
0	115 (50.0%)	117 (50.4%)
1	115 (50.0%)	114 (49.1%)
2	0	1 (0.4%)
ER positive	119 (51.7%)	97 (41.8%)
PR positive	96 (41.7%)	75 (32.3%)
HER2 positive[a]	47 (20.4%)	61 (26.3%)
Median duration of metastatic disease (range)	1.3 years (0–19.3)	1.0 years (0–13.2)
Visceral disease	184 (80.0%)	180 (77.6%)
>3 sites of disease	116 (50.4%)	114 (49.1%)
Prior chemotherapy regimens for MBC		
0	37 (16.1%)	35 (15.1%)
1	98 (42.6%)	107 (46.1%)
≥2	95 (41.3%)	90 (38.8%)

[a]HER2 positive = 3+ by immunohistochemistry or amplification by FISH

onstrated anti-angiogenic properties in pre-clinical models (Kerbel and Kamen 2004). Taxanes, specifically paclitaxel, had shown promising activity when delivered on weekly as compared to 3-weekly schedules and definitive phase III trials in the neo-adjuvant and adjuvant settings have confirmed the superior activity of this metronomic scheduling (Green et al. 2005). In order to exploit the potential anti-angiogenic properties of metronomic chemotherapy together with bevacizumab, ECOG designed study E2100 (Miller et al. 2007). This trial compared paclitaxel alone given weekly at 90 mg/m^2 for 3 weeks followed by a 1 week rest with the same schedule of paclitaxel plus bevacizumab 10 mg/kg every 2 weeks. Both agents were continued until disease progression.

The key eligibility criteria for this study included locally recurrent or metastatic breast cancer with no prior chemotherapy for advanced disease. Adjuvant chemotherapy was permitted and adjuvant taxanes were allowed provided more than 12 months had elapsed between the completion of taxanes and recurrence of breast cancer. HER2+ breast cancer was permitted only if the patient had received prior therapy with trastuzumab without chemotherapy. Between December 2001 and March 2004, 722 patients were randomized, with 680 determined to meet all of the eligibility criteria, based on four stratification variables: adjuvant chemotherapy (yes vs no), disease-free interval (≤24 months vs >24 months), number of metastatic sites (<3 vs ≥3), and estrogen receptor status (ER+ vs ER– vs ER unknown). The sample size was selected to provide 85% power to detect a 33% improvement (6–8 months) in the primary endpoint of PFS with a type 1 error of <5%. The statistical plan included two pre-specified interim efficacy analyses to occur at 270 and 425 events, and the final analysis was planned after 546 progression events. Table 35.20 describes the baseline demographic information and tumor characteristics, which were equally balanced between the two groups.

In April 2005, at the first formal interim analysis for efficacy, the DSMB recommended that the trial be stopped based on crossing the pre-defined O'Brian–Fleming boundaries for efficacy. The data was originally reported at ASCO 2005 and the efficacy data shown in Figs 35.16 and 35.17 represents updated information after 484 progression or death events that was presented at the San Antonio Breast Cancer Symposium in December 2005. The objective RR was 13.8 versus 29.9% (p<0.001) for the entire eligible population of 680 patients and was 16.0 versus 37.7% (p<0.001) for the population of 498 patients

Table 35.18. Common capecitabine toxicities

	Capecitabine (n=215)			Capecitabine + bevacizumab (n=229)		
	Grade 2 (%)	Grade 3 (%)	Grade 4 (%)	Grade 2 (%)	Grade 3 (%)	Grade 4 (%)
Diarrhea	34 (15.8)	23 (10.7)	0	37 (16.2)	27 (11.8)	0
Stomatitis	11 (5.1)	0	1 (0.5)	16 (7.0)	4 (1.7)	0
Hand-foot syndrome	77 (35.8)	52 (24.2)	0	97 (42.4)	63 (27.5)	0

National Cancer Institute Common Toxicity Criteria worst grade experienced per patient

Table 35.19. Common bevacizumab toxicities

	Capecitabine (n=215)				Capecitabine + bevacizumab (n=229)			
	Grade 1 (%)	Grade 2 (%)	Grade 3 (%)	Grade 4 (%)	Grade 1 (%)	Grade 2 (%)	Grade 3 (%)	Grade 4 (%)
Hypertension	4 (1.9)	0	1 (0.5)	0	9 (3.9)	4 (1.7)	41 (17.9)	0
Proteinuria	14 (6.5)	2 (0.9)	0	0	42 (18.3)	7 (3.1)	2 (0.9)	0
Bleeding	19 (8.8)	4 (1.9)	1 (0.5)	0	60 (26.2)	5 (2.2)	1 (0.4)	0
Thrombotic event	0	4 (1.9)	5 (2.3)	3 (1.4)	1 (0.4)	3 (1.3)	9 (3.9)	4 (1.7)
Pulmonary embolism	0	0	0	3 (1.4)	0	0	0	3 (1.3)

National Cancer Institute Common Toxicity Criteria worst grade experienced per patient

Table 35.20. Demographic and disease characteristics: E2100 study (Miller et al. 2007)

	Paclitaxel (n=339)	Paclitaxel + bevacizumab (n=341)
Median age	55 (27–85)	56 (29–84)
DFI ≤24 months	42%	42%
≥3 sites	43%	43%
Adjuvant chemotherapy	64%	65%
Taxane	18%	18%
ER+	64%	59%
HER2+	4%	5%

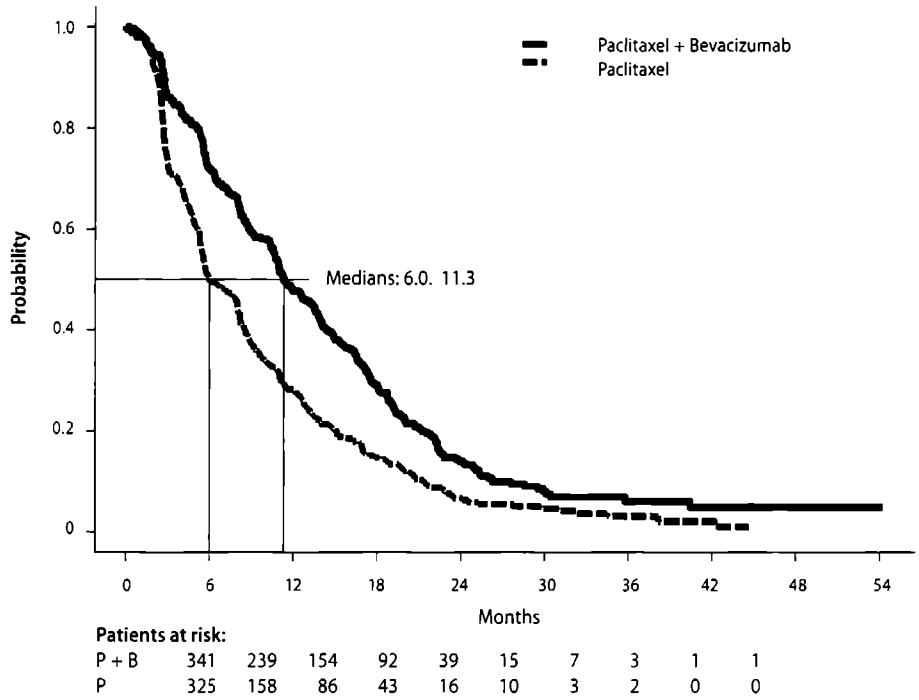


Fig. 35.16. Kaplan–Meier estimate of PFS in the E2100 study (from Miller et al. 2007)

with measurable disease at study entry. The primary efficacy endpoint, PFS, is shown in Fig. 35.16. Bevacizumab provided nearly a doubling of PFS with medians of 6.1 versus 11.4, and the hazard for progression was 0.51 ($p<0.001$).

Figure 35.17 portrays, via Forest plot, the treatment effect on PFS for a variety of clinically relevant patient subgroups. In general there was very consistent benefit noted in all of the subsets reported, although the 95% confidence intervals crossed 1.0 for the small subsets of ER+/PR– patients and age >64 years. It is important to note that the treatment effect in the ER–/PR– group was very significant, as this group essentially represents the "basal-like" or "triple negative" group of breast cancer, which is a particularly poor prognostic group with inferior benefit from cytotoxic chemotherapy. The subset of patients with prior adjuvant taxane exposure also represents a poor prognostic group that gained substantial benefit from the addition of bevacizumab to paclitaxel. Although preliminary survival data was presented suggesting a non-significant trend favoring the bevacizumab group, an insufficient number of deaths have been reported to enable the formal, specified survival analysis, which is expected to occur sometime in 2007.

Overall bevacizumab was well tolerated in this clinical study. Tables 35.21 and 35.22 describe the incidence of selected grade 3 and 4 adverse events. Hypertension was more frequent (2% vs 16%) as were bleeding (0% vs 3%) and proteinuria (0% vs 2%). Patients receiving bevacizumab also experienced more neuropathy and fatigue, perhaps related to the longer time on treatment with paclitaxel and higher cumulative exposure. Only a single episode of CHF was reported during this analysis.

In summary, bevacizumab has been evaluated in two randomized controlled studies in MBC; both were phase III trials and utilized different chemotherapy backbones (capecitabine and paclitaxel) in both first-line and refractory patient populations. The trials showed inconsistent results, with highly significant improvements in clinical benefit when used in the initial treatment of MBC while be-

Group	N	HR	95% CI	PFS (mos.) P	P + B
ER+, PR+	284	0.53	(0.40, 0.70)	8.2	14.1
ER+, PR+	108	0.86	(0.56, 1.30)	9.2	12.5
ER+, PR+	231	0.56	(0.44, 0.73)	4.5	8.5
No adjuvant chemo	228	0.74	(0.56, 0.98)	7.4	12.5
Non-taxane	328	0.59	(0.47, 0.75)	7.7	10.7
Taxane	109	0.45	(0.30, 0.69)	3	12.3
Anthracycline	271	0.56	(0.43, 0.72)	5.6	10.7
Non-anthracycline	166	0.55	(0.39, 0.78)	6.9	12.5
Age 27–49	216	0.52	(0.39, 0.70)	5.6	11.9
Age 50–64	304	0.56	(0.44, 0.72)	7.2	11.4
Age 65–85	146	0.86	(0.61, 1.22)	8	10.6
DFI 0–24 months	276	0.61	(0.47, 0.79)	5.1	10.3
DFI > 24 months	390	0.6	(0.48, 0.74)	8.2	13.8
< 3 sites	366	0.58	(0.46, 0.73)	7.8	13.2
3 or more sites	299	0.64	(0.50, 0.81)	5.6	10.7
No visceral disease	108	0.65	(0.41, 1.03)	8,5	15.5
Visceral disease	557	0.59	(0.49, 0.71)	5.9	10.9
Not only bone	605	0.58	(0.49, 0.69)	5.8	11
Bone only	60	0.64	(0.34, 1.19)	13	18.8
Measurable	487	0.56	(0.46, 0.68)	5.6	11
Non-measurable	179	0.75	(0.53, 1.05)	11.4	13.5
Overall	**666**	**0.6**	**(0.51, 0.71)**	**6**	**11.3**

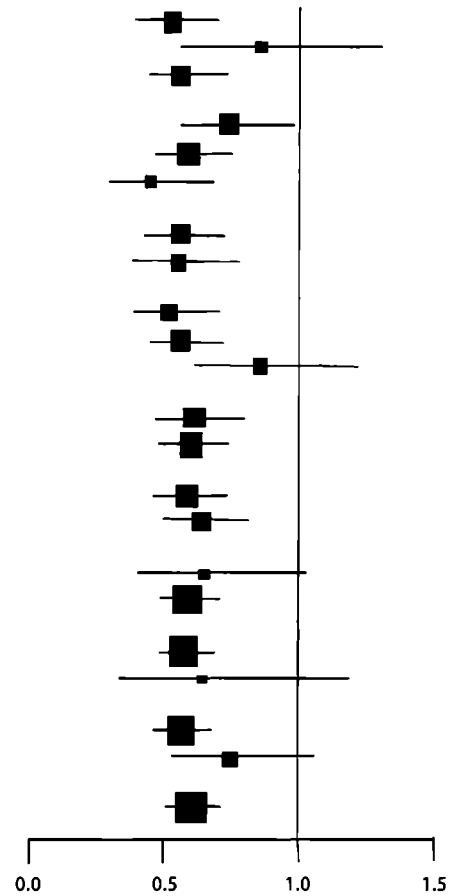


Fig. 35.17. Forest plot of PFS for selected clinical subsets in the E2100 study (from Miller et al. 2007)

vacizumab use in refractory patients provided only modest improvement in RRs with no real beneficial effect on PFS or OS. The reason for these differences is not clear. It has been hypothesized that highly refractory cancer often activates multiple angiogenic pathways such that inhibition of a single, albeit critical ligand, VEGF, may be insufficient to effectively inhibit tumor angiogenesis. It is also possible that highly refractory cancers with very short PFS times may simply be a difficult environment to effectively evaluate new therapies. It will be important to evaluate the results of several ongoing phase III clinical trials in first- and second-line MBC to ensure consistency of treatment effect in this disease. Until those results are available, the combination of weekly paclitaxel and bevacizumab should be considered one appropriate standard for the treatment of first-line MBC. Studies are ongoing to evaluate the potential role of bevacizumab in the adjuvant treatment of this common malignant disease in both HER2– and HER2+ disease.

35.7.1.3 Non-small Cell Lung Cancer

Non-small cell lung cancer represents a serious global health problem. Linked to tobacco use, NSCLC is the leading cause of cancer-related death in the US and EU and is likely to become a major health issue in the developing world over the coming

Table 35.21. Bevacizumab-associated adverse events: E2100 study (from Miller et al. 2007)

	Paclitaxel (n=332) %		Paclitaxel + bevacizumab (n=350)	
	Grade 3	Grade 4	Grade 3	Grade 4
Hypertension*	2	0	15	<1
Thromboembolism	2	2	2	0
Bleeding**	0	0	2	<1
Proteinuria***	0	0	1	1

NCI-CTC v3.0, worst per patient. *p<0.0001; **p=0.02; ***p=0.002

Table 35.22. Other adverse events: E2100 study (from Miller et al. 2007)

	Paclitaxel (n=332) %		Paclitaxel + bevacizumab (n=350)	
	Grade 3	Grade 4	Grade 3	Grade 4
Neuropathy	17	1	22	1
Fatigue*	4	<1	8	<1
Neuropenia	NR	3	NR	4
Decreased LVEF	0	0	<1	0

NCI-CTC v3.0, worst per patient. *p=0.05

decades. Modest improvements in treatment have been achieved over the past 20 years; however, no substantial progress has been made since the introduction of platin-based doublet chemotherapy more than 10 years ago. An evaluation of four modern doublet regimens revealed no substantial benefit of any one over any of the others (Schiller et al. 2002). Although the randomized phase II results with carboplatin and paclitaxel with bevacizumab were encouraging, "triplet" therapy in advanced NCSLC had never provided better outcomes than doublet therapy. This was true of three-drug combinations of cytotoxic chemotherapy as well as two-drug chemotherapy regimens with a third "biologic" agent. In addition to the skepticism around multiple failures of three-drug combinations in this disease was the significant problem of life-threatening and fatal pulmonary bleeding that was noted in the phase II experiment. Six of 66 bevacizumab-treated patients, about 9%, experienced this adverse event, and serious questions existed at the time as to whether further development of the agent in this disease was possible. However further evaluation of risk factors, including both univariate and multivariate analyses, demonstrated that the single significant risk factor for this event was predominant squamous cell histology in the tumor. Although this histologic subtype was noted in only 13 patients treated with bevacizumab in the phase II experiment, four of the six serious bleeding events occurred in this group, for an estimated risk of 31%. Only 2 of 53, or 4%, of the non-squamous NSCLC patients in this study experienced a serious pulmonary bleeding event. Why squamous cell patients appear to have an increased

Table 35.23. Baseline demographic and disease characteristics: E4599 study (from Schiller et al. 2002)

	PC (n=433)	BPC (n=417)
Gender[a]		
Male	253 (58%)	210 (50%)
Female	180 (42%)	207 (50%)
Age ≥65 years	189 (44%)	177 (42%)
Race (44 unknown)		
White	378 (91%)	352 (90%)
Black	23 (6%)	22 (6%)
Other	14 (3%)	17 (4%)
ECOG PS (6 unknown)		
0	170 (40%)	167 (40%)
1	260 (60%)	247 (60%)
Measurable disease	392 (91%)	381 (91%)
Prior weight loss ≥5%	121 (28%)	117 (28%)
Stage IIIB non-recurrent	55 (13%)	50 (12%)
Stage IV non-recurrent	337 (78%)	310 (74%)
Recurrent	40 (9%)	57 (14%)
Prior RT	37 (9%)	33 (8%)
Adeno or NOS	380 (88%)	366 (88%)
Large cell	29 (7%)	17 (4%)
BAC	11 (3%)	12 (3%)
Other histology	11 (3%)	22 (5%)
>2 sites involved	229 (53%)	216 (52%)
Pleura involved	111 (26%)	112 (27%)
Liver involved	73 (17%)	90 (22%)
Bone involved	149 (34%)	118 (28%)
Adrenal involved	72 (17%)	53 (13%)

PC, paclitaxel and carboplatin; BPC, paclitaxel and carboplatin plus bevacizumab. [a]Males: PC 58%, BPC 50%; p=0.03, Fisher's exact test

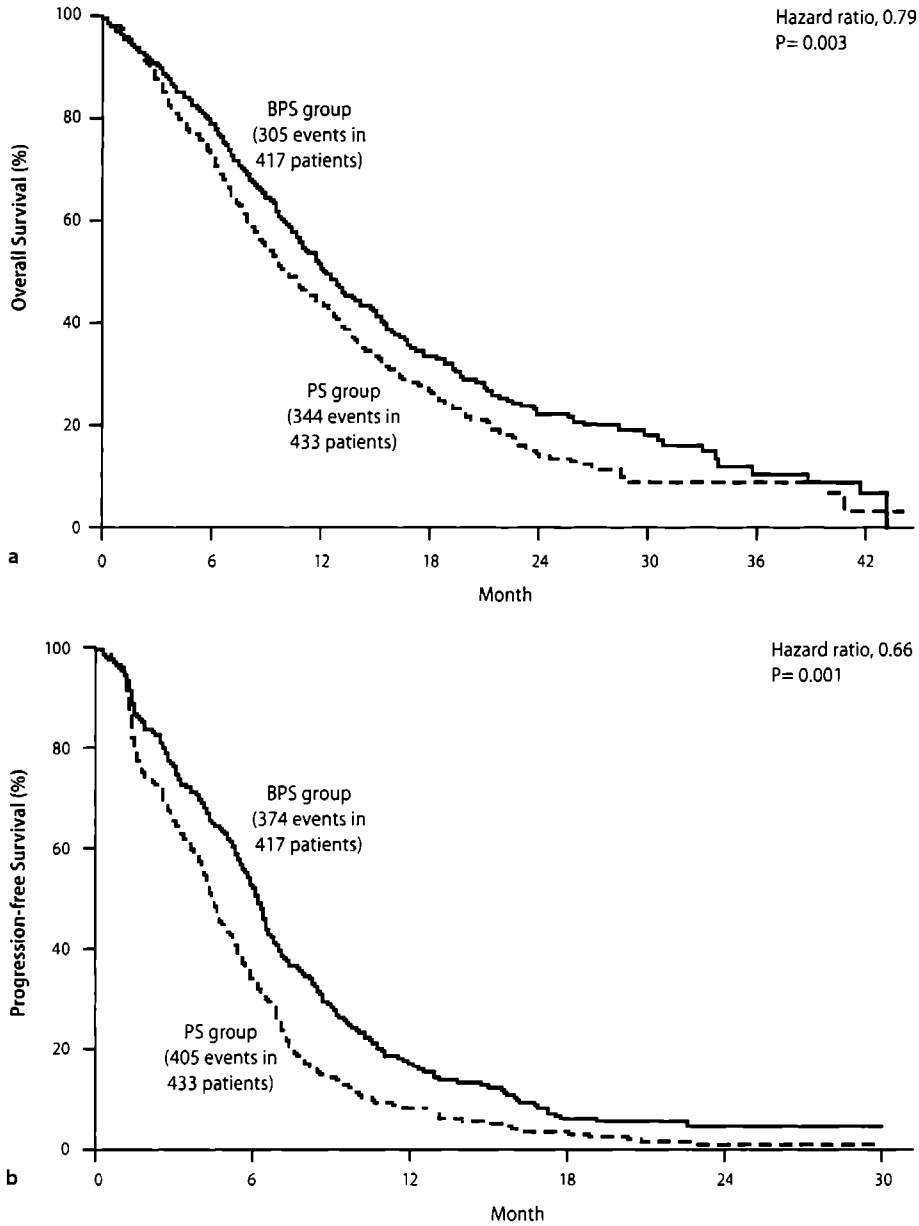


Fig. 35.18a,b. Kaplan–Meier estimates of survival (*top*) and PFS (*bottom*) in the E4599 study (from Sandler et al. 2006)

risk of pulmonary bleeding with bevacizumab therapy is not known; however, further development of the drug in NSCLC requires exclusion from subsequent studies of patients with this histology.

Once again, investigators from the ECOG and the NCI took the lead in designing a phase II/III experiment to definitively evaluate bevacizumab in NSCLC with study E4599. The key eligibility criteria for the study included non-squamous NSCLC not amenable to surgery or radiation therapy. Patients with brain metastases were also excluded, and no prior chemotherapy was permitted. The trial design compared

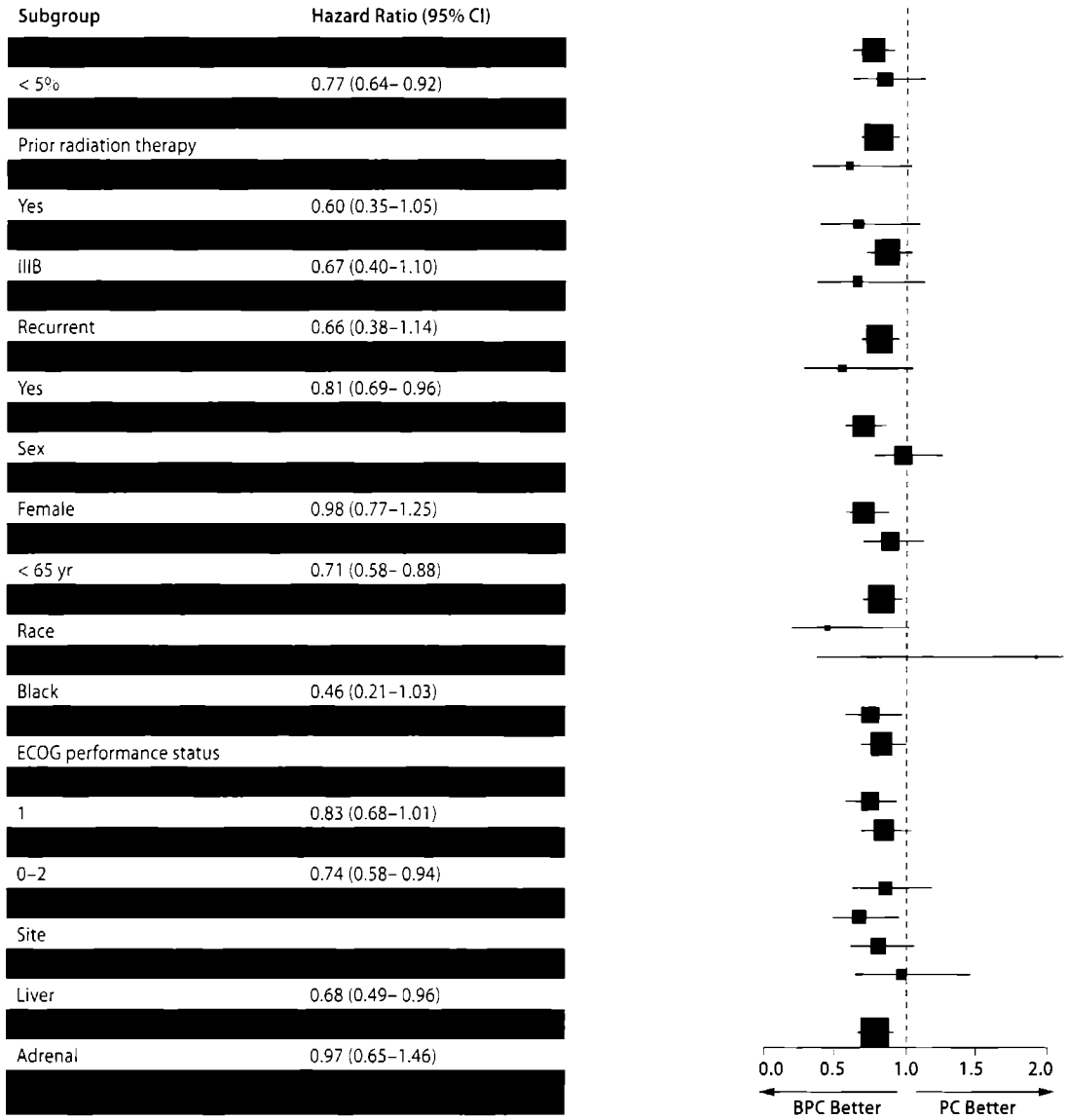


Fig. 35.19. Survival treatment hazard ratios (BPC/PC) in subgroups in the E4599 study

standard treatment with up to 6 cycles of carboplatin (AUC=6) and paclitaxel (200 mg/m^2) every 3 weeks versus the same chemotherapy plus bevacizumab 15 mg/kg every 3 weeks. At the completion of up to 6 cycles of chemotherapy, bevacizumab was continued as a single agent until the time of disease progression.

Between July 2001 and April 2004 a total of 850 eligible patients were randomized between the two groups utilizing four stratification variables; measurable vs non-measurable disease, prior radiation

therapy (yes vs no), prior weight loss (<5% vs ≥5%), and stage (IIIB vs IV). The study design was somewhat complicated in that there was a randomized phase II component embedded in the overall phase III study. The primary endpoint of the phase II component was safety, particularly the rate of pulmonary bleeding. In fact, after the first 112 patients were randomized enrollment was suspended between February 2002 and August 2002 so that 6-month safety information could be made available to the DSMB to conduct the formal interim safety analysis. Only after the DSMB concluded that the observed bleeding rates were within the acceptable limits pre-specified in the protocol did recruitment resume to the planned sample size of 842 patients. The primary endpoint for the phase III component was overall survival, with secondary endpoints of PFS and RR. The sample size provided 80% power to detect a 20% reduction in the hazard for death with a type 1 error of <5%, accounting for two planned interim efficacy analyses at 40% and 70% of the planned 650 death events required for the final analysis. Table 35.23 shows the baseline demographic and tumor characteristics in the two groups, which were balanced except for a significant difference in gender, with more males in the chemotherapy-alone group and more women in the bevacizumab group.

In March 2005, at the second planned interim efficacy analysis, the DSMB recommended that the trial be stopped because the pre-defined O'Brian-

Table 35.24. Selected adverse events (%): E4599 study

Toxicity type	Treatment group						
	PC (n=440)			BPC (n=427)			p value
	Grade 3	Grade 4	Grade 5	Grade 3	Grade 4	Grade 5[b]	
Neutropenia[a]	–	16.8	–	–	25.5	–	0.002
Thrombocytopenia[a]	–	0.2	–	–	1.6	–	0.04
Anemia[a]	-	0.9	–	-	0.0	-	NS
Febrile neutropenia	1.8	–	0.2	4.0	–	1.2	0.02
Hyponatremia	0.9	0.2	–	2.6	0.9	–	0.02
Hypertension	0.5	0.2	–	6.8	0.2	–	<0.001
Proteinuria	–	–	–	2.6	0.5	–	<0.001
Headache	0.5	–	–	3.0	–	–	0.003
Rash/desquamation	0.5	–	–	2.3	–	–	0.02
Bleeding events (all)	0.7			4.4			<0.001
CNS hemorrhage	–	–	–	–	0.7	–	
Epistaxis	0.2	–	–	0.7	–	–	
Hematemesis	–	–	–	–	–	0.5	
Hemoptysis	0.2	–	–	0.5	0.2	1.2	
Melena/GI bleeding	0.2	–	0.2	0.7	0.2	–	
Hemorrhage, other	–	–	–	0.2	0.2	–	

Unadjusted for differences in median duration of therapy between the groups. [a]Only grades 4–5 were collected for hematologic toxicities. [b]Three other grade 5 toxicities on arm BPC: cerebrovascular event, 2; pulmonary embolus, 1

Table 35.25. Causes of death: E4599 study

	PC	BPC
Total deaths	344	305
Lung cancer	309	260
Toxicity	2	14
Other medical problems	16	16
Unknown cause	17	15

One patient on the BPC arm who suffered a grade 5 event was ineligible because advanced disease was not documented and is excluded from this table (but was included in the toxicity analysis)

Fleming boundaries for efficacy had been crossed. The data were originally reported at ASCO 2005 and the efficacy data shown in Figs 35.18 and 35.19 represent updated information through October 2005 with a median follow-up duration of 19 months and a minimum of 18 months. In the 773 patients with measurable disease at study entry, bevacizumab provided a significant improvement in RR (15% vs 35%, $p<0.001$). The treatment effects on the primary endpoint of survival and the secondary endpoint of PFS are shown in Fig. 35.18. Survival was improved from 10.3 to 12.3 months with a hazard of death of 0.79 ($p=0.003$). PFS was improved from 4.5 to 6.2 months with a hazard of progression of 0.66 ($p<0.001$). This represented the first time that a third agent had provided statistical and clinical benefit to doublet chemotherapy in NSCLC and was the first large phase III trial to achieve a median survival in excess of 1 year. Figure 35.19 displays the survival results in several important clinical subsets. Although there was generally a consistent treatment effect, females did not appear to show a survival benefit with bevacizumab. The reasons for this finding are not known. The relative treatment effect on PFS was very similar between men and women, suggesting that bevacizumab has a similar benefit in both genders; however, women may have better prognostic characteristics or may benefit preferentially from second-line therapies (i.e., EGFR tyrosine kinase inhibitors). Further clinical trials are necessary to more clearly understand this observation in E4599.

Table 35.24 lists the adverse events that occurred more frequently in the bevacizumab group as compared to the control group. Bleeding was more common with bevacizumab, although the rate of pulmonary bleeding was reduced to 1.9% by selecting non-squamous cell NSCLC patients. Both GI and CNS bleeding were also increased in the bevacizumab group. The other new safety event noted in this trial was a higher rate of neutropenia. The phase III trials in breast and colon cancer did not use highly myelosuppressive regimens, as this trial did, and it appears that such regimens may be a risk factor for neutropenia. Table 35.25 lists the causes of death for study patients. Although there was a significant reduction in cancer-associated death, there was higher treatment-related mortality in the bevacizumab group, including deaths from bleeding, thrombosis, and febrile neutropenia. Typical bevacizumab-related toxicities of hypertension and proteinuria were observed.

In summary, bevacizumab has been evaluated in two randomized controlled studies in NSCLC: one phase II and one phase III trial. Both utilized the same chemotherapy backbone and both studied first-line patients. Consistent clinical benefit was seen on the endpoint of PFS, while the phase III trial also showed a statistically significant improvement in survival. It is important to note that patients with predominant squamous cell histology were excluded from the phase III study and this change in the study population brought about a significant, though incomplete, reduction in the risk of serious pulmonary bleeding. Additional studies aiming to improve the understanding of risk factors for this adverse event are in progress, as is work on potential approaches to administer bevacizumab safely to squamous cell patients. In addition, confirmatory studies in first-line metastatic disease are under way along with phase III trials of the second-line treatment of metastatic disease, together with a large international trial of bevacizumab in the adjuvant treatment of NSCLC.

Conclusions

Bevacizumab represents a prominent landmark in therapeutic oncology as the first anti-angiogenic agent to show clinical benefit in the treatment of human cancer. It has validated many years of clinical and pre-clinical observations and hypotheses from laboratories and clinics around the world and opened many new and exciting areas of study to extend and enhance the impact of inhibiting angiogenesis in cancer. In 2006, bevacizumab is being tested in more than 25 malignant conditions, including the adjuvant treatment of colon, lung, and breast cancer, where the possibility of long-term remission is being sought. Its development represents a remarkable collaboration of basic science, protein engineering, and clinical development involving both industry and the public sector that substantially accelerated definitive phase III testing and has brought new hope to thousands of cancer patients around the world.

References

Algire GH, Chalkley HW, Legallais FY, Park HD (1945) Vascular reactions of normal and malignant tissues in vivo. I. Vascular reactions of mice to wounds and to normal and neoplastic transplants. J Natl Cancer Inst 6:73–85

Blum JL, Jones SE, Buzdar AU et al (1999) Multicenter phase II study of capecitabine in paclitaxel-refractory metastatic breast cancer. J Clin Oncol 127:485–493

Carter P et al (1992) Humanization of an anti-p185HER2 antibody for human cancer therapy. PNAS 89:4285–4289

De Gramont A, Figer A, Seymour M et al (2000) Leucovorin and fluorouracil with or without oxaliplatin as first-line treatment of metastatic colorectal cancer. J Clin Oncol 18:2938–2947

Ehrmann RL, Knoth M (1968) Choriocarcinoma. Transfilter stimulation of vasoproliferation in the hamster cheek pouch. Studied by light and electron microscopy. J Natl Cancer Inst 41:1329–1341

Ferrara N, Davis-Smyth T (1997) The biology of vascular endothelial growth factor. Endocr Rev 18:4–25

Ferrara N, Winer J, Burton T, Rowland A, Siegel M, Phillips HS et al (1993) Expression of vascular endothelial growth factor does not promote transformation but confers a growth advantage in vivo to Chinese hamster ovary cells. J Clin Invest 91:160–170

Folkman J (1971) Tumor angiogenesis: therapeutic implications. N Engl J Med 285:1182–1186

Folkman J, Merler E, Abernathy C, Williams G (1971) Isolation of a tumor factor responsible for angiogenesis. J Exp Med 133:275–288 (abstract/free full text)

Giantonio BJ, Catalano PJ, Meropol NJ et al (2007) Bevacizumab in combination with oxaliplatin, fluorouracil and leucovorin (FOLFOX4) for previously treated metastatic colorectal cancer: results from the Eastern Cooperative Oncology Group Study E3200. J Clin Oncol (in press)

Green MC, Buzdar AU, Smith T et al (2005) Weekly paclitaxel improves pathologic complete remission in operable breast cancer when compared with paclitaxel once every 3 weeks. J Clin Oncol 23:5983–5992

Greenblatt M, Shubik P (1968) Tumor angiogenesis: transfilter diffusion studies in the hamster by the transparent chamber technique. J Natl Cancer Inst 41:111–124

Hurwitz H, Fehrenbacher L, Novotny W et al (2004) Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colon cancer. N Engl J Med 350:2335–2342

Ide AG, Baker NH, Warren SL (1939) Vascularization of the Brown Pearce rabbit epithelioma transplant as seen in the transparent ear chamber. Am J Roentgenol 42:891–899

Johnson DH, Fehrenbacher L, Novotny WF, Herbst RS, Nemunaitis JJ, Jablons DM, Langer CJ, DeVore RF, Gaudreault J, Damico LA, Holmgren E, Kabbinacar F (2004) Randomized phase II trial comparing bevacizumab plus carboplatin and paclitaxel with carboplatin and paclitaxel alone in previously untreated locally advanced or metastatic non-small-cell lung cancer. J Clin Oncol 22:2184–2191

Jones PT et al (1986) Replacing the complementarity-determining regions in a human antibody with those from a mouse. Nature 321:522–525

Kabbinavar F et al (2003) Phase II, randomized trial comparing bevacizumab plus fluorouracil (FU)/leucovorin (LV) with FU/LV alone in patients with metastatic colorectal cancer. J Clin Oncol 21:60–65

Kabbinavar FF, Schulz J, McCleod M et al (2005) Addition of bevacizumab to bolus fluorouracil and leucovorin in first-line metastatic colorectal cancer: results of a randomized phase II trial. J Clin Oncol 23:3697–3705

Kerbel RS, Kamen BA (2004) The anti-angiogenic basis of metronomic chemotherapy. Nat Rev Cancer 4:423–36

Kim K, La B, Houck K., Winer J, Ferrara N (1992) The vascular endothelial growth factor proteins: identification of biologically relevant regions by neutralizing monoclonal antibodies. Growth Factors 7:53–64

Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS, Ferrara N (1993) Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumor growth in vivo. Nature 362:841–844

Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 246:1306–1309

Mass RD et al (2004) Clinical benefit from bevacizumab (BV) in responding (R) and non-responding (NR) patients (pts) with metastatic colorectal cancer (mCRC). Proc Am Soc Clin Oncol 23 (abstr 3514)

Meta-analysis Group in Cancer (1998) Efficacy of intravenous continuous infusion of fluorouracil compared with bolus adminstration in advanced colorectal cancer. J Clin Oncol 16:301–308

Miller KD, Chap LI, Holmes FA et al (2005) Randomized phase III trial of capecitabine compared with bevacizumab plus capecitabine in patients with previously treated metastatic breast cancer. J Clin Oncol 23:792–799

Miller KD, Wang M, Gralow J et al (2007) **A randomized phase III trial of paclitaxel versus paclitaxel plus bevacizumab as first-line therapy for locally recurrent or metastatic breast cancer: a trial coordinated by the Eastern Cooperative Oncology Group (E2100).** J Clin Oncol (submitted for publication)

Miller RA, Oseroff AR, Stratte PT, Levy R (1983) Monoclonal antibody therapeutic trials in seven patients with T-cell lymphoma. Blood 62:988–995

Neuberger MS et al (1985) A hapten-specific chimaeric IgE antibody with human physiological effector function. Nature 314:268–270

Petrelli N, Douglas O et al (1989) Modulation of fluorouracil with leucovorin in metastatic colorectal carcinoma: a prospective randomized phase III trial. J Clin Oncol 7:1419–1426

Picus J (2003) The use of bevacizumab (B) with docetaxel (D) and estramustine (E) in hormone refractory prostate cancer (HRPC): Initial results of CALGB 90006. Proc Am Soc Clin Oncol 22 (abstr 1578)

Presta LG, Chen H, O'Connor SJ, Chisholm V, Meng YG, Krummen L, Winkler M, Ferrara N (1997) Humanization of an anti-VEGF monoclonal antibody for the therapy of solid tumors and other disorders. Cancer Res 57:4593–4599

Radinsky R, Ellis LM (1996) Molecular determinants in the biology of liver metastasis. Surg Oncol Clin North Am 5:215–229

Reese D (1999) A phase II trial of humanized monoclonal anti-vascular endothelial growth factor antibody (rhuMAb VEGF) in hormone refractory prostate cancer (HRPC). Proc Am Soc Clin Oncol 18 (abstr 1355)

Riechmann L et al (1988) Reshaping human antibodies for therapy. Nature 332:323–327

Rothenberg ML, Oza AM, Bigelow RH et al (2003) Superiority of oxaliplatin and fluorouracil-leucovorin compared with either therapy alone in patients with progressive colorectal cancer after irinotecan and fluorouracil-leucovorin: interim results of a phase III trial. J Clin Oncol 21:2059–2069

Saltz LB, Cox JV, Blancke C et al (2000) Irinotecan plus fluorourical and leucovorin for metastatic colorectal cancer. N Eng J Med 343:905–914

Sandler A. Gray R. Perry MC. Brahmer J. Schiller JH. Dowlati A. Lilenbaum R. Johnson DH (2006) Paclitaxel–carboplatin alone or with bevacizumab for non-small-cell lung cancer. New Engl J Med 355:2542–2550

Schiller J, Harrington D, Belani C et al (2002) Comparison of four chemotherapy regimens for advanced non-small cell lung cancer: ECOG 1594. N Engl J Med 346:92–98

Takahashi Y, Kitadai Y, Bucana CD, Cleary KR, Ellis LM (1995) Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. Cancer Res 55:3964–3968

Takahashi Y, Tucker SL, Kitadai Y, Koura AN, Bucana CD, Cleary KR et al (1997) Vessel counts and expression of vascular endothelial growth factor as prognostic factors in node-negative colon cancer. Arch Surg 132:541–546

Tokunaga T, Oshika Y, Abe Y, Ozeki Y, Sadahiro S, Kijima H et al (1998) Vascular endothelial growth factor (VEGF) mRNA isoform expression pattern is correlated with liver metastasis and poor prognosis in colon cancer. Br J Cancer 77:998–1002

Warren RS, Yuan H, Matli MR, Gillett NA, Ferrara N (1995) Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. J Clin Invest 95:1789–1797

Yang JC, Haworth L, Sherry RM, Hwu P, Schwartzentruber DJ, Topalian SL, Steinberg SM, Chen HX, Rosenberg SA (2003) A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. N Engl J Med 349:427–434

Clinical Development of Sorafenib (BAY 43-9006), a VEGFR and RAF Inhibitor

36

DIMITRIS VOLIOTIS and J. DUMAS

Abstract

Sorafenib is a multi-kinase inhibitor with effects on the tumor cell and tumor vasculature that inhibit proliferation, promote cell death, and disrupt neo-angiogenesis. Originally identified as a Raf kinase inhibitor, sorafenib also inhibits VEGFR-1/-2/-3; PDGF-β receptor (PDGFR-β); Fms-like tyrosine kinase-3 (FLT-3); c-Kit protein (c-Kit); and RET receptor tyrosine kinases. Sorafenib has demonstrated potent anti-tumor activity in preclinical xenograft models of different tumor types by virtue of its anti-angiogenic, anti-proliferative and pro-apoptotic effects. This orally administered drug is well tolerated and received approval for the treatment of metastatic renal cell carcinoma (RCC) primarily based on a large international trial, the Treatment Approaches in Renal Cancer Global Evaluation Trial (TARGETs), in which 903 patients were randomized to receive sorafenib or placebo. At a planned interim analysis, patients treated with sorafenib had a lower risk for death than those treated with placebo. However, improvement in survival did not meet criteria for statistical significance. In a planned interim analysis of 769 patients, sorafenib significantly prolonged median progression-free survival compared with placebo (167 days vs 84 days; hazard ratio 0.44; P<0.000001). Sorafenib has also demonstrated early signs of clinical efficacy, alone or in combination regimens, in patients with advanced hepatocellular carcinoma (HCC) and melanoma. Ongoing phase III trials are currently being conducted to further evaluate sorafenib in HCC, melanoma, and non-small cell lung cancer. This chapter summarizes the discovery, preclinical findings, and clinical development of sorafenib in RCC and its potential use in other tumor types.

CONTENTS

Introduction

The blood vessel network of the adult vasculature is formed during embryogenesis by a process termed vasculogenesis. Vasculogenesis involves the de novo formation of blood vessels from angioblasts, which are the precursors of endothelial cells (Papetti and Herman 2002). Angioblasts proliferate, coalesce, and differentiate into vascular endothelial cells to form a network of vessels called the primary capillary plexus (Papetti and Herman 2002). The primary capillary plexus is then remodeled by the sprouting and branching of new blood vessels from pre-existing vessels in a process called angiogenesis (Table 36.1).

Most angiogenesis occurs early in life to establish an adequate vasculature and thereby support growth and embryonic development; very little endothelial cell turnover occurs in adults. Angiogenesis in the adult is limited to the ovarian cycle and the wound healing process (Papetti and Herman 2002). However, dysregulated angiogenesis has been implicated in several pathological processes. Downregulation of the normal angiogenic process may be involved in ischemic heart disease, atherosclerosis, stroke, Alzheimer's disease, and chronic wounds. Uncontrolled upregulation of angiogenesis has also been associated with diabetic retinopathy, arthritis, psoriasis, and cancer.

Tumor-induced angiogenesis or neo-angiogenesis is the process by which new blood vessels develop from the endothelium of the pre-existing vasculature to supply the tumor with the necessary nutrients to facilitate tumor growth, and is a key step in malignant growth, invasion, and metastasis (Folkman 1992). Neo-angiogenesis induction is essential for the establishment of the necessary vasculature to facilitate solid tumor growth beyond 2–3 mm^3 (Wary et al. 2003). Solid tumors induce this new blood vessel growth by secreting up to 20 different growth factors and cytokines, including the pro-angiogenic factors transforming growth factor-alpha (TGF-α), vascular endothelial growth factor (VEGF), platelet-derived growth factor-beta (PDGF-β), and basic fibroblast growth factor (bFGF). The expression of these pro-angiogenic factors is driven by the lack of oxygen in the tumor tissue (hypoxia) (Wary et al. 2003).

Angiogenesis involves the destabilization of the existing microvasculature, resulting in vascular hyperpermeability and matrix remodeling, and transition of existing endothelial cells from a quiescent to a plastic proliferative phenotype (Table 36.1) (Papetti and Herman 2002). Activated endothelial cells then proliferate, migrate, and undergo tube formation (i.e. formation of new microvessels) (Papetti and Herman 2002). The final stage involves the differentiation, proliferation, and recruitment of mural cells, such as pericytes, which stabilize the newly formed vasculature (Table 36.1) (Papetti and Herman 2002). However, unlike angiogenesis under normal physiological conditions, it appears that tumor-induced angiogenesis results in new microvessels with multiple structural and functional abnormalities (Baluk et al. 2005). These microvessels vary in diameter and have a tortuous shape; they also have thin walls, which lack the usual layer of pericytes (Baluk et al. 2005).

The angiogenic effects of hypoxia-induced, tumor-derived growth factors (VEGF-A, bFGF, PDGF-β) are summarized in Table 36.1. These growth factors induce signaling via their respective receptor tyrosine kinases (RTKs), on the surface of endothelial cells and/or pericytes, followed by activation of the ubiquitous mitogen-activated protein kinase (MAPK) intracellular signal transduction pathway. This highly conserved pathway comprises Raf kinase, mitogen extracellular kinase (MEK), and extracellular signal-regulated kinase (ERK) (Kolch et al. 2002). Two of the most widely studied pro-angiogenic growth factors, VEGF and bFGF, have been shown to promote new blood vessel formation in vivo and in vitro. These factors also induce mitogenesis and enhance capillary morphogenesis in vivo (Baluk et al. 2005). The vascular abnormalities and embryonic lethality observed in VEGF and VEGF-receptor (VEGFR) knockout mice, and the in vivo regression of tumor vasculature induced by VEGF/VEGFR inhibitors, established a role for VEGF in promoting tu-

Table 36.1. The main stages of angiogenesis under normal physiological conditions

Stage of angiogenesis	Pro-angiogenic factors involved	Citation
1. Vessel destabilization	Ang2/Tie2	Phelps et al. 2006
Removal of pericytes from the vascular endothelium		
Change in endothelial cells from growth-arrested to proliferative phenotype		
2. Vessel hyperpermeability	VEGF, VE-cadherin	Yamaoka-Tojo et al. 2006
Allows local extravasation of proteases and matrix components from bloodstream		
3. Matrix remodeling	TGF-β	Wu et al. 2006
4. Endothelial cell proliferation	VEGF, FGF-2, EGF	Wu et al. 2006; Lampugnani et al. 2006: Sahni et al. 2006
5. Endothelial cell migration	α, β-integrin, VEGF, EGFR, ephrin-B2/eph-B4	Alghisi and Ruegg 2006; Kim et al. 2006; Semino et al. 2006
6. Cell–cell adhesion	VE-cadherin, ephrin-B2/eph-B4	Lampugnani et al. 2006; Esser et al. 1998; Kertesz et al. 2006
7. Tube formation	FGF, PDGF, TNF-α, ephrin-2A, ephrin-B2/eph-B4	Kertesz et al. 2006; Leibovich et al. 1987; d'Amore and Smith 1993; Ogawa et al. 2000
8. Mesenchymal cell (i.e. pericyte precursors) proliferation	PDGF	d'Amore and Smith 1993
Proliferating mesenchymal cells migrate along growing tubules		
9. Differentiation into pericytes	TGF-β	Pardali and Moustakas 2007

Ang, Angiopoietin; *eph*, ephrine; *EGFR*, epidermal growth factor receptor; *FGF*, fibroblast growth factor; *PDGF*, platelet derived growth factor; *TGF-β*, transforming growth factor-beta; *Tie*, angiopoietin receptor; *TNF-α*, tumor necrosis factor-alpha; *VE*, vascular endothelial; *VEGF*, vascular endothelial growth factor

mor survival and angiogenesis (Ferrara 2002). In particular, signaling through the endothelial cell VEGF-A/VEGFR-2 pathway has been shown to be of pivotal importance in stimulating angiogenesis (Takahashi and Shibuya 2005).

The concept that tumor growth could be impacted by inhibiting the angiogenic process was first proposed by Judah Folkman in 1971. Since then, clinical trials involving targeted anti-angiogenic agents have demonstrated clinical efficacy and good tolerability, leading to the approval of the first targeted anti-angiogenic drug, a humanized anti-VEGF monoclonal antibody, bevacizumab (Chen 2004).

The MAPK cascade has also been recognized as a key target for therapeutic intervention in oncology for over a decade. The three Raf serine/threonine kinase isoforms [A-Raf, B-Raf and Raf-1 (also called c-Raf)] are the first kinases in the MAPK cascade and regulate cellular proliferation, survival, and an-

giogenesis (Kolch et al. 2002). Wild-type Raf-1 can also prolong cell survival independent of MAPK signaling by direct interaction with anti-apoptotic and pro-apoptotic regulatory proteins (Fig. 36.1) (Kolch et al. 2002; O'Neill et al. 2004).

Because hyperactivation of the constituent kinases of the MAPK cascade – particularly the Raf serine/threonine kinase isoforms – often occurs in human solid tumors (~30%), increased signaling through this pathway may have an important role in the onset and progression of cancer (Kolch et al. 2002; O'Neill et al. 2004). Although increased activation of wild-type B-Raf kinase has not been implicated in tumorigenesis, in certain human tumor types oncogenic mutations have been identified within the kinase domain of the B-Raf gene which have resulted in constitutive kinase activation (Brose et al. 2002; Salvatore et al. 2004; Melillo et

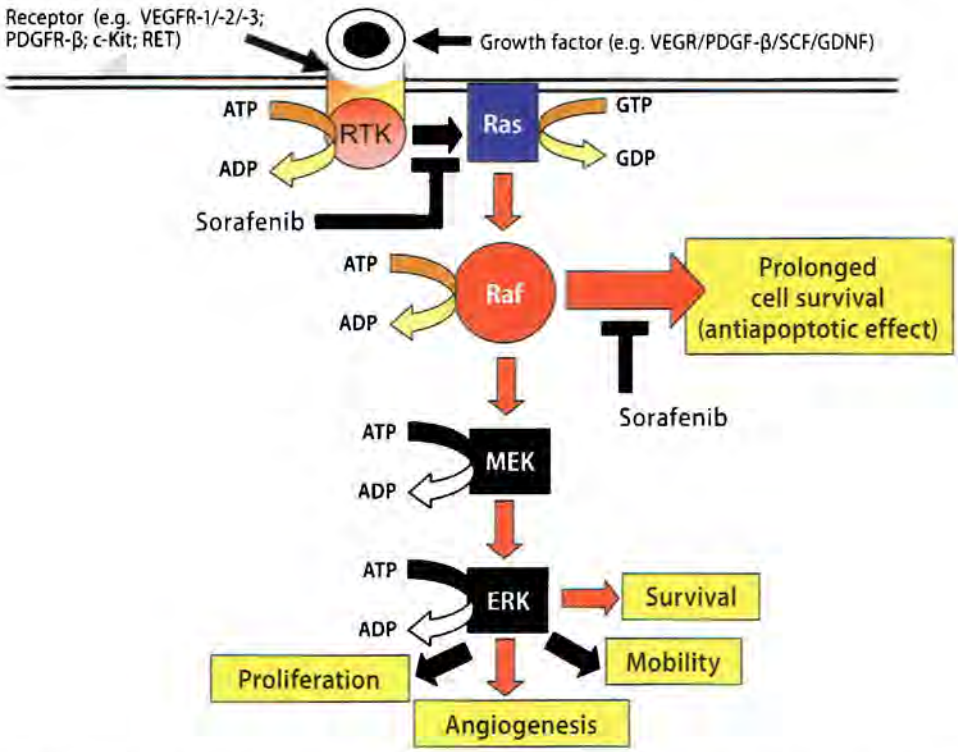


Fig. 36.1. Raf is a downstream mediator of growth-factor signaling in normal cells, and exerts its effects via MEK/ERK activation or independently of MEK/ERK activation. Dysregulated signaling through receptor tyrosine kinases and Raf is implicated in tumorigenesis. Sorafenib inhibits signaling through pro-angiogenic (VEGFR-1/-2/-3; PDGFR-β) and tumor-promoting (c-Kit; RET) receptor tyrosine kinases as well as downstream Raf serine/threonine kinases to exert an antitumor effect. *ADP*, Adenosine diphosphate; *ATP*, adenosine triphosphate; *ERK*, extracellular signal-regulated kinase; *GDNF*, glial-cell line-derived neurotrophic factor; *GDP*, guanosine diphosphate; *GTP*, guanosine triphosphate; *MEK*, mitogen extracellular kinase; *PDGF-β*, platelet-derived growth factor-beta; *PDGFR-β*, PDGF-beta receptor; Ras, rat sarcoma oncogene; RET, rearranged during transfection tyrosine kinase; *RTK*, receptor tyrosine kinase; *SCF*, stem cell factor; *c-Kit*, stem cell factor receptor kinase; *VEGF*, vascular endothelial growth factor; *VEGFR*, VEGF receptor

al. 2005). In particular, the *b-raf* V600E oncogenic mutation has been implicated in the development of melanomas and papillary thyroid carcinomas (Brose et al. 2002; Salvatore et al. 2004; Melillo et al. 2005)and is frequently detected in these tumor types. The *b-raf* V600E oncogenic mutation is frequently detected in these tumor types (melanoma 63%; papillary thyroid carcinoma 50%) (Salvatore et al. 2004; Melillo et al. 2005).

Although there is little or no evidence suggesting that Raf-1 acts as an oncogene in human cancer, uncontrolled activation of wild-type Raf-1 kinase is a common feature of human solid tumors and could arise by a number of mechanisms. Constitutively activating oncogenic mutations within the upstream Ras gene (particularly *k-ras* mutations), which occur in a wide variety of human solid tumor types (e.g. 90% of pancreatic and 45% of colon cancers), can result in activation of downstream Raf-1 (Downward 2003). In addition, the uncontrolled upregulation of upstream growth factors, or the constitutive activation of upstream RTKs by oncogenic mutations, could result in activation of downstream wild-type Raf-1 kinase. Wild-type Raf-1 activation is frequently detected in renal cell carcinoma (RCC; 50% of tumor biopsies in one study) (Oka et al. 1995) and hepatocellular cancer (HCC; 100% of biopsies in one study) (Hwang et al. 2004). Wild-type Raf 1 activation is also associated with poor prognosis in cancers of the reproductive tract, including ovarian (McPhillips et al. 2001) and androgen-independent prostate tumors (Mukherjee et al. 2005).

Targeted disruption of the Raf-1 gene by a specific antisense oligonucleotide (ISIS 5132; Isis Pharmaceuticals Inc, Carlsbad, CA) was first reported to inhibit tumor growth in animal models (i.e. xenografts in athymic mice) of human breast, lung, and ovarian cancer in 1989 (Kasid and Dritschilo 2003). This landmark study by Kasid et al. represented the first proof of concept that targeting Raf-1 is an effective anticancer strategy. ISIS 5132 is a 20-base phosphorothioate antisense oligonucleotide which hybridizes to the 3'-untranslated sequence of Raf-1 mRNA (Kasid and Dritschilo 2003). This hybridization results in the degradation of Raf-1 mRNA, thereby decreasing Raf-1 protein synthesis (Kasid and Dritschilo 2003).

In the past few years, several oral multi-kinase inhibitors have entered late-stage clinical trials for the treatment of advanced cancer. One such multi-kinase inhibitor is sorafenib (BAY 43-9006), which has been approved for the treatment of metastatic RCC – a disease characterized by its hypervascularity (Wilhelm et al. 2004). The kinase inhibition profile of sorafenib encompasses pro-angiogenic stromal RTKs, such as VEGFR-1/-2/-3 and PDGF-ô ɛceptor (PDGFR-β); RTKs directly involved in tumor progression, such as c-Kit (stem cell factor receptor kinase), FLT-3 (Fms-like tyrosine kinase-3), and RET (rearranged during transfection tyrosine kinase); and the Raf serine/threonine kinases (Wilhelm et al. 2004; Levy et al. 2006; Carlomagno et al. 2006). Sorafenib inhibits tumor growth by acting on the endothelial cell and the tumor cell to inhibit proliferation, promote cell death, and disrupt neo-angiogenesis (Wilhelm et al. 2004). This chapter summarizes the discovery of sorafenib and preclinical findings which support a rationale for sorafenib as a treatment for advanced RCC, and suggest it has potential utility in other solid tumor types, including HCC and melanoma. We will then focus on the clinical development of sorafenib for the treatment of RCC and on the continuing clinical evaluations in HCC and melanoma.

Discovery

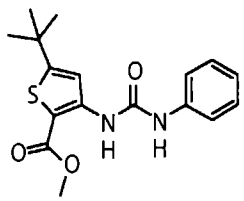
Although an inhibitor of several pro-angiogenic molecules, sorafenib was originally selected on the basis of its potent Raf kinase inhibitory activity (Lyons et al. 2001). Bayer and Onyx engaged in collaboration to discover novel therapies targeting the MAPK (i.e. Raf/MEK/ERK) pathway and initiated a high-throughput screening program in 1995 to obtain novel inhibitors of human recombinant Raf-1 kinase (Smith et al. 2001). This led to the identification of the 3-thienyl urea de-

rivative 1 (Fig. 36.2) as a lead structure. Structure-activity relationships were then explored using a combination of traditional medicinal and combinatorial chemistry approaches. This program resulted in the identification of sorafenib as a potent in vitro Raf-1 inhibitor (Fig. 36.2) (Lowinger et al. 2002). Further details of this optimization process have been published elsewhere (Lowinger et al. 2002). X-ray crystallography studies have shown that sorafenib binds to Raf kinase, stabilizing the protein in an inactive conformation (Wan et al. 2004). Extensive in vitro kinase profiling confirmed that sorafenib is a potent in vitro inhibitor of Raf-1, wild-type B-Raf, and oncogenic *b-raf* V600E serine/threonine kinases (Table 36.2) (Wilhelm et al. 2004). Sorafenib was also shown to potently inhibit several RTKs (VEGFR-1/-2/-3, PDGFR-β, c-Kit, Flt-3, and RET) in biochemical and autophosphorylation assays (Table 36.2) (Wilhelm et al. 2004; Levy et al. 2006; Carlomagno et al. 2006). With the exception of wild-type B-Raf, these molecular targets have been implicated in the etiology of several forms of human cancer. However, sorafenib did not inhibit all RTKs [notably those of the epidermal growth factor receptor (EGFR) and the insulin-like growth factor receptor (IGFR) families] and had no significant inhibitory effect on MEK-1, ERK-1, protein kinase B, protein kinase A, protein kinase Cα, or protein kinase Cγ in biochemical assays (Table 36.2) (Wilhelm et al. 2004).

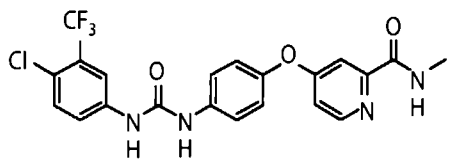
Preclinical Overview

36.3.1 Cellular Studies

Sorafenib inhibited signaling through the MAPK pathway in a wide variety of tumor cell lines harboring *k-ras* and/or *b-raf* oncogenes (Wilhelm et al. 2004; Liu et al. 2005), as evidenced by phosphorylated (i.e. activated) ERK (pERK). Furthermore, sorafenib inhibited VEGF-stimulated phosphorylation of the VEGFR-2 RTK and PDGF-β-stimulated phosphorylation of the PDGFR-β RTK in human cells (Table 36.2) (Wilhelm et al. 2004). Sorafenib has been shown to induce apoptosis in several human tumor cell lines, including ACHN renal carcinoma, MDA-MB-231 breast carcinoma, A549 non-small cell lung cancer (NSCLC), HT-29 colon carcinoma, KMCH cholangiocarcinoma, and PLC/PRF/5 and HepG2 HCC cells (Liu et al. 2005). It also induced apoptosis in human leukemia cells, including K562 (chronic myelogenous), Jurkat (acute T-cell), and MEC-2 (chronic lymphocytic) lines. Sorafenib acted by downregulating the levels of the anti-apoptotic protein myeloid cell leukemia sequence-1 in these human tumor cell lines to induce apoptosis (Yu et al. 2005; Rahmani et al. 2005). Sorafenib also induced



1
Raf-1 IC_{50} = 17μM



BAY 43-9006
Raf-1 IC_{50} = 6nM

Fig. 36.2. Chemical structure of sorafenib (BAY 43-9006)

Table 36.2. In vitro inhibitory profile of sorafenib (Wilhelm et al. 2004; Levy et al. 2006; Carlomagno et al. 2006; Salvatore et al. 2006)

Kinase target	In vitro IC50 value (nM)
Biochemical (kinase) assay	
Raf-1	6
Wild-type B-Raf	25
Oncogenic *b-raf* V600E	38
VEGFR-1	26
VEGFR-2	90
Murine VEGFR-3	20
Murine PDGFR-β	57
Flt-3	33
p38	38
c-Kit	68
FGFR-1	580
MEK-1, ERK-1, EGFR, HER2, c-met, IGFR-1, PKA, PKB, cdk1/cyclin B, pim-1, PKC-α, PKC-γ	>10,000
Cellular kinase assay	
MEK phosphorylation in MDA MB 231 cells[a]	40
ERK 1/2 phosphorylation in MDA MB 231 cells[a]	90
MEK 1/2, and p44/p42 MAPK phosphorylation in FRO cells[b]	500
RET phosphorylation in NIH3T3 fibroblasts	47
Oncogenic V804L RET human thyroid carcinoma cells[c]	110
Oncogenic V804M RET human thyroid carcinoma cells[c]	147
Oncogenic b-raf V600E in human thyroid carcinoma cells	1000
VEGFR-2 phosphorylation in NIH 3T3 fibroblasts	30
VEGF-ERK 1/2 phosphorylation in HUVEC cells[d]	60
PDGFR-β phosphorylation in HAoSMC[e]	80
VEGFR-3 phosphorylation in HEK-293 cells	100
Flt-3 phosphorylation in HEK-293 cells with human ITDs[f]	20

[a]Human breast carcinoma cells containing G463V *b-raf* and G13D k-ras oncogenes. [b]ARO and FRO human thyroid carcinoma cells. [c]Human thyroid carcinoma cell lines with RET mutations (V804L or V804M) conferring resistance to anilinoquinazolines and pyrazolopyrimidines, [d]Human umbilical vein endothelial cells (HUVEC). [e]Human aortic smooth muscle cells (HAoSMC). [f]Internal tandem duplication Flt-3 mutants found in human acute myeloid leukemia. c-Kit, stem cell factor receptor; *c-met*, hepatocyte growth factor receptor; *cdk*, cyclin-dependent kinase; *EGFR*, epidermal growth factor receptor; *ERK*, extracellular signal-regulated kinase; *FGFR*, fibroblast growth factor receptor; *Flt-3*, FMS-like tyrosine kinase; *HER2*, human epidermal growth factor receptor-2; *IFGR*, insulin-like growth factor receptor; *ITD*, internal tandem duplication; *MAPK*, mitogen-activated protein kinase; *MEK*, mitogen extracellular kinase; *PDGFR-β*, platelet-derived growth factor receptor-beta ; *pim-1*, human proviral integration site for murine leukemia virus; *PKA*, protein kinase A; *PKB*, protein kinase B; *PKC*, protein kinase C; *RET*, rearranged during transfection tyrosine kinase; *VEGFR*, vascular endothelial growth factor receptor

the apoptosis of human melanoma cell lines by a MEK/ERK-independent and caspase-independent mechanism involving the nuclear translocation of apoptosis-inducing factor (Panka et al. 2006).

36.3.2 In Vivo Studies

Mechanism of action studies were performed to demonstrate that sorafenib can target both the tumor and vascular compartments. Post-treatment tumor samples were examined by immunohistochemistry for a series of pharmacodynamic markers following daily oral dosing (30 mg/kg) for 5 days in nude mice. These markers were present either in the tumor tissue (pERK-1/2 and Ki-67), or in the tumor vasculature (CD31 and smooth muscle cell actin).

Sorafenib exerted a potent anti-angiogenic effect in virtually all models tested, as demonstrated by a significant reduction in microvessel area and microvessel density (Wilhelm et al. 2004). The growth-inhibitory effects of sorafenib in the Renca murine renal cancer model are associated with disruption of the tumor vasculature without significant down-regulation of pERK levels (Chang et al. 2005). The anti-angiogenic effects of sorafenib in these models are probably attributable to its inhibition of pro-angiogenic endothelial cell (VEGFR-2) and pericyte (PDGFR-β) RTKs.

In contrast, although sorafenib inhibited the growth of two NSCLC xenograft models (A549 and NCI-H460), it did not appear to inhibit the MAPK pathway in these experiments (Wilhelm et al. 2004). Sorafenib's inhibitory activity against human colon Colo-205 xenografts was also not associated with a detectable reduction in pERK (Wilhelm et al. 2004). However, in other human tumor xenograft models (MDA-MB-231 breast; HT-29 colon; PLC/PRF/5 HCC) (Liu et al. 2005), sorafenib's activity involved both inhibition of signaling through the MAPK pathway (i.e. antiproliferative effect) and inhibition of angiogenesis. Similar results were observed in a human melanoma xenograft model expressing *b-raf* V600E (Sharma et al. 2005).

These data suggest that sorafenib can target the tumor cell MAPK pathway in some, but not all, tumor types. In addition, sorafenib targeted the tumor vasculature in all models tested. Recent data also indicate that inhibition of Raf-1 may promote cell death in endothelial cells as a downstream event of VEGFR-2 and FGF-receptor-1 stimulation (Alavi et al. 2003).

36.3.3 Preclinical and Clinical Combination Overview

Sorafenib showed promising in vivo activity in tumor xenograft models in nude mice when dosed in combination with other chemotherapeutic agents (Carter et al. 2007). Phase I clinical trials have subsequently confirmed that sorafenib shows promise in combination with standard chemotherapeutic agents, such as oxaliplatin (Kupsch et al. 2005), 5-fluorouracil and leucovorin (Figer et al. 2004), paclitaxel/carboplatin (Flaherty et al. 2006), gemcitabine (Siu et al. 2006), taxotere (Awada et al. 2004), irinotecan (CPT-11) (Steinbild et al. 2005), and dacarbazine (DTIC) (Eisen et al. 2005). In these trials, co-administered sorafenib did not significantly increase the toxicity of the chemotherapeutic agent in patients with advanced cancer. A full discussion of the clinical trials on sorafenib in combination with chemotherapeutics is beyond the scope of this chapter; the most significant findings are described below.

Rationale for Sorafenib in RCC

Clear-cell RCC is the most prevalent and aggressive histological type of renal cancer, and is associated with loss of function of the von Hippel–Lindau (VHL) tumor suppressor gene (Brauch et al. 2000). Loss of VHL function results in overexpression of hypoxia-inducible factors (HIF)-1 and -2, and subsequent overexpression of pro-angiogenic (e.g. VEGF) and autocrine growth factors (e.g. TGF-α) (Leung

and Ohh 2002; Gunaratnam et al. 2003). Clear-cell RCC is a hypervascularized solid tumor also associated with increased Raf-1 activity (Oka et al. 1995). Results of preclinical studies in a murine model of RCC, in which mice lack a functional VHL gene, have shown that TGF-α is overproduced and binds the EGFR of tumor cells to initiate an autocrine growth loop that involves HIF-2 upregulation, increased VEGF production, and tumor angiogenesis (Fig. 36.3). Furthermore, downregulation of HIF-2 or EGFR inhibition prevents tumor growth in this model of RCC (Gunaratnam et al. 2003; Rini 2005; Smith et al. 2005; de Paulsen et al. 2001).

The signal transduction pathway activated by VEGF is a clear target of sorafenib, via inhibition of VEGFR-2 and/or Raf-1. This fact alone provides a strong rationale for the evaluation of sorafenib in advanced RCC (Fig. 36.3). Additional effects of sorafenib either directly on Raf-1, or on TGF-α signaling via Raf-1, can also be postulated but have not yet been clearly established in vivo.

36.5 Clinical Development of Sorafenib in RCC

Sorafenib was generally well tolerated in phase I trials investigating various oral dosing schedules in patients with several forms of advanced cancer. These trials helped determine the recommended dose of sorafenib for phase II and III trials [i.e. 400 mg con-

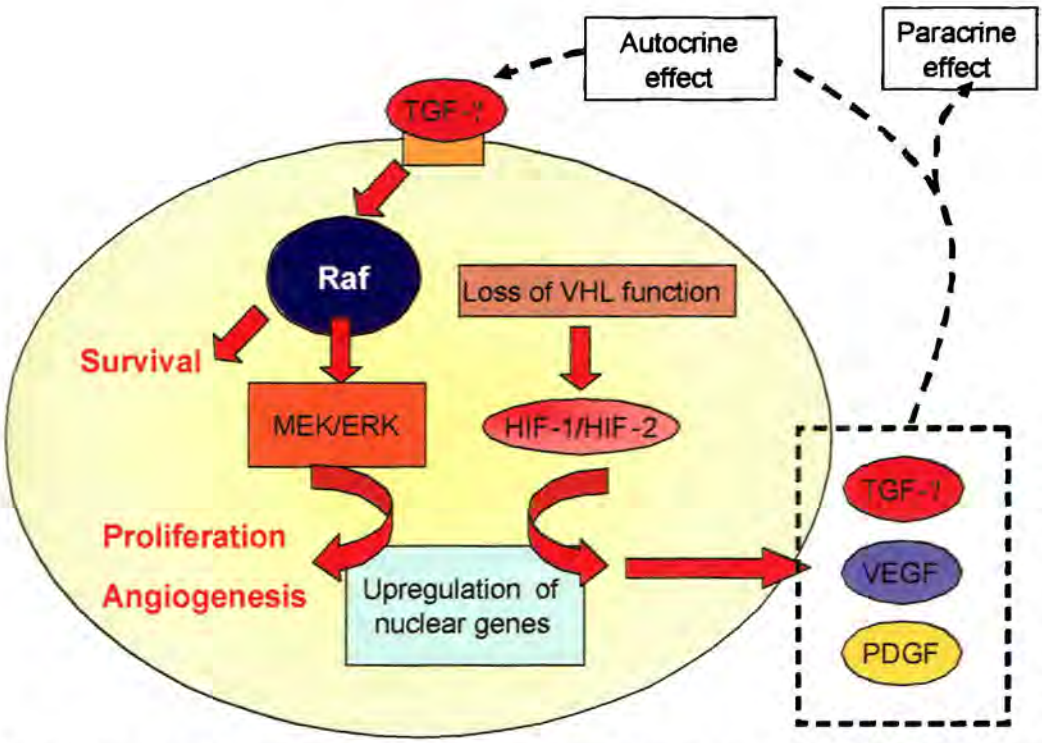


Fig. 36.3. Key pathways involving Raf-1 that are activated in solid tumors (e.g. renal cell carcinoma) by hypoxia-inducible growth factors in the tumor cell, endothelial cells, and pericytes. *ERK*, Extracellular signal-regulated kinase; *HIF*, hypoxia-inducible factor; *MEK*, mitogen extracellular kinase; *PDGF*, platelet-derived growth factor; *TGF-α*, transforming growth factor-alpha; *VEGF*, vascular endothelial growth factor; *VHL*, von Hippel-Lindau gene

tinuous twice daily (b.i.d.)]. Dose-limiting toxicities at continuous doses higher than 400 mg b.i.d. were diarrhea, fatigue, and skin rash (Awada et al. 2005; Clark et al. 2005; Moore et al. 2005; Strumberg et al. 2005, 2006). The phase I trials included 11 RCC patients who were evaluable for response (Awada et al. 2005; Clark et al. 2005; Moore et al. 2005; Strumberg et al. 2005). One RCC patient had a partial response (PR) on a 600 mg b.i.d. regimen that was sustained for 104 days (Awada et al. 2005), while two other RCC patients who received sorafenib had stable disease (SD), which lasted for ≥2 years (Clark et al. 2005; Strumberg et al. 2005). Subsequently, an analysis of a cohort of 202 patients participating in a large phase II randomized discontinuation trial (RDT) showed that sorafenib 400 mg b.i.d. significantly prolonged median progression-free survival (PFS) compared with placebo (24 weeks vs 6 weeks; P=0.0087) in patients with advanced RCC (Fig. 36.4) (Ratain et al. 2006).

The anti-angiogenic effects of sorafenib on the t mor vasculature and the relationship between the anti-angiogenic effects and outcome were investigat in 17 patients with RCC who participated in the pha II RDT using dynamic contrast-enhanced magnet resonance imaging (O'Dwyer et al. 2005). This tecl nique quantifies changes in tumor blood flow, perm ability, and vascular surface area over time (Padhar and Husband 2001). Sorafenib treatment significantl decreased the gadolinium exchange constant (K_{trans} – a measure of the extent of neovascularization – be tween the serum and tumor in these 17 patients; a observation consistent with an anti-angiogenic effec (O'Dwyer et al. 2005). The decline in K_{trans} was as sociated with subsequent clinical response and PF (O'Dwyer et al. 2005). Baseline K_{trans} was also significantly associated with PFS (O'Dwyer et al. 2005). Therefore, elevated Ktrans may be a marker of poor prognosis in RCC.

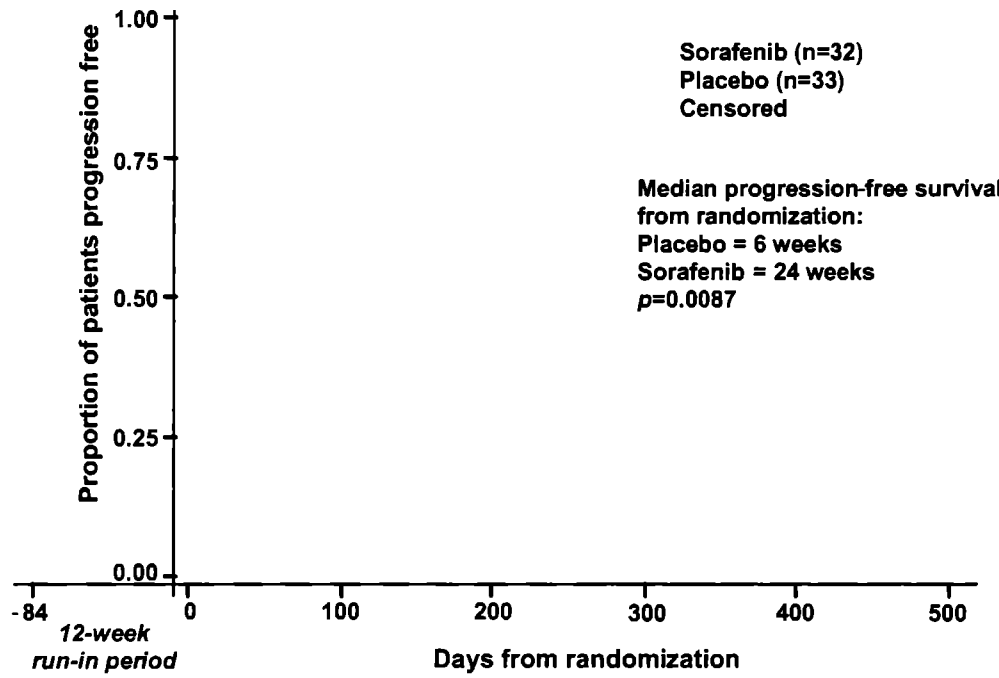


Fig. 36.4. Randomized discontinuation trial. Kaplan–Meier plot of investigator-assessed progression-free survival from week 12 randomization for patients randomized to placebo (n=33) or to sorafenib (n=32) (Ratain et al. 2006). This figure is reprinted with permission from the American Society of Clinical Oncology from Ratain et al. (2006)

Common adverse events included skin rash/desquamation, hand–foot skin reaction (HFSR), and fatigue (Ratain et al. 2006). A subsequent phase III trial was performed to confirm the observed antitumor efficacy of sorafenib in patients with advanced RCC, and the results of this trial formed the basis of its recent approval by the FDA for this indication.

36.5.1 Phase III Sorafenib RCC Trial

The phase III Treatment Approaches in Renal Cancer Global Evaluation Trial (TARGETs) was the largest randomized trial conducted in RCC to date. It was conducted in 117 centers in 19 countries in patients with progressive, treatment-refractory, metastatic clear-cell RCC (Escudier et al. 2005). The study design, including inclusion and exclusion criteria, has been reported previously (Escudier et al. 2005).

36.5.1.1 TARGETs Endpoints

The primary efficacy endpoint was overall survival. Secondary endpoints included investigator-reviewed PFS, independentlyreviewed PFS, and overall response rate, as assessed using the Response Evaluation Criteria In Solid Tumors (RECIST) (Escudier et al. 2005).

36.5.1.2 TARGETs Efficacy

In total, 903 patients were randomized to receive treatment in TARGETs (Eisen et al. 2006). In a planned interim overall survival analysis based on 220 deaths, patients treated with sorafenib had a 39% improvement in median overall survival relative to those receiving placebo (hazard ratio 0.72; $P<0.018$) (Eisen et al. 2006). However, this survival improvement did not meet prespecified criteria for statistical significance (Bayer 2006). A final survival analysis will be undertaken after 540 deaths.

Sorafenib significantly prolonged median PFS versus placebo [24 weeks (167 days) vs 12 weeks (84 days); hazard ratio 0.44; $P<0.000001$] in a planned interim analysis of 769 patients assessed by independent radiologic review (Fig. 36.5) (Escudier et al. 2005; Bayer 2006). This PFS benefit was independent of age (above or below 65 years), prior cytokine (interleukin-2 or interferon) therapy, baseline Eastern Cooperative Oncology Group (ECOG) performance status, Memorial Sloan-Kettering Cancer Center risk group, and time since diagnosis (Bayer 2006). At 3 months post-randomization, 75% of patients on sorafenib were progression-free compared with 43% on placebo (Escudier et al. 2005).

The anti-angiogenic effects of sorafenib were evaluated in a subset of 27 patients in this phase III trial using Doppler ultrasonography with perfusion software (vascular recognition imaging, Toshiba) and contrast agent injection (Lamuraglia et al. 2005). With this technique, a decrease in contrast uptake reflects reduced blood flow consistent with an anti-angiogenic effect. Good responders to sorafenib, defined as patients who experienced a decrease in contrast uptake of 10% and a stable or decreased tumor volume, had a longer PFS (53 vs 30 weeks) and overall survival (57 vs 33 weeks) than poor responders (Lamuraglia et al. 2005).

36.5.1.3 TARGETs Safety

Sorafenib was generally well tolerated in TARGETs (Escudier et al. 2005), with dermatologic symptoms, such as rash or HFSR, among the most common adverse events (Bayer 2006). These dermatologic symptoms usually appeared within 6 weeks of initiating treatment and were generally mild or moderate (grade 1–2) in severity (Bayer 2006). These dermatologic toxicities were easily managed by topical treatments, dose interruptions, or dose reductions. Only 3 of 451 patients who received sorafenib discontinued permanently due to HFSR (Bayer 2006). Treatment-emergent hypertension, typically occurring early in the course of therapy, was more common with sorafenib (16.9%) than placebo (1.8%). However, the hypertension was usually mild to moder-

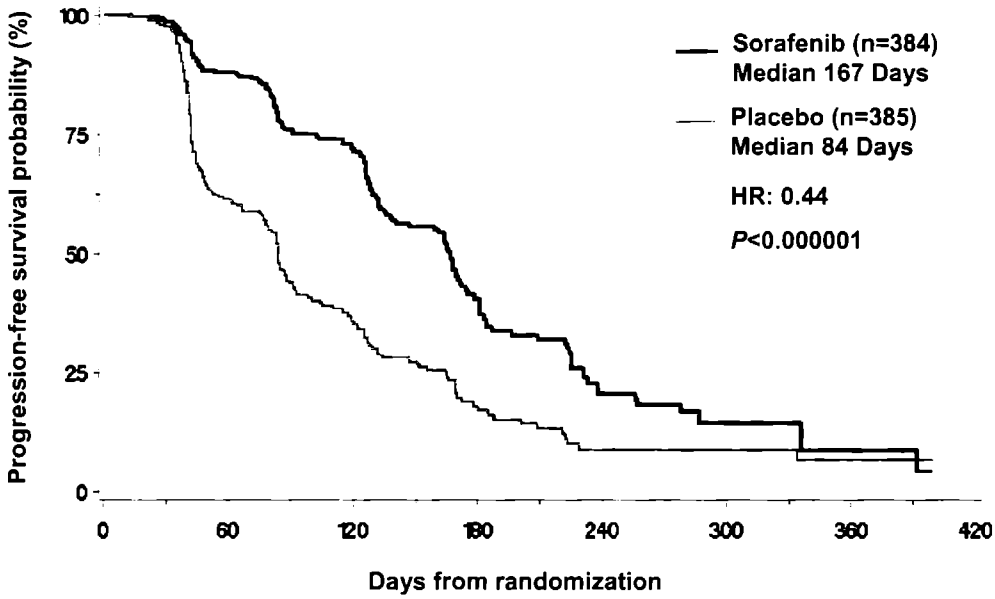


Fig. 36.5. Phase III TARGETs: Kaplan–Meier plot showing that sorafenib significantly prolonged progression-free survival compared with placebo in patients with treatment-refractory, metastatic clear-cell renal cell carcinoma (Bayer 2006)

ate (grade 1 or 2) and easily managed by standard antihypertensive medications (Bayer 2006). Hypertension resulted in only one discontinuation out of 451 patients who received sorafenib in TARGETs (Bayer 2006). The incidence of bleeding of any cause was also higher with sorafenib treatment than placebo (15.3% vs 8.2%). Although cardiac ischemia was rarely reported, it was also more common with sorafenib (2.9%) than placebo (0.4%).

Drug-related adverse events that were more common with sorafenib treatment than placebo included rash/desquamation (31% vs 11%), diarrhea (30% vs 7%), HFSR (26% vs 5%), and hypertension (8% vs <1%). However, the incidence of fatigue was similar between sorafenib-treated (18%) and placebo (14%) patients (Escudier et al. 2005). No significant biochemical toxicities were associated with sorafenib in this interim phase III trial analysis (Escudier et al. 2005).

36.5.1.4 TARGETs Quality of Life

The impact of sorafenib on health-related quality of life (HRQOL) and on kidney cancer-related symptoms was also evaluated in TARGETs by comparison with placebo. The impact of sorafenib treatment on HRQOL was measured by the Functional Assessment of Cancer Therapy-General (FACT-G) questionnaire, and symptoms were measured by the FACT-Kidney Cancer Symptom Index (FKSI) questionnaire. In the FKSI questionnaire, patients used a Likert scale (0–4) to respond to each of 15 items. The results showed that sorafenib did not adversely affect HRQOL and had a positive impact on kidney cancer-related symptoms, including cough, fevers, worry that the condition would worsen, shortness of breath, and ability to enjoy life (Dhanda et al. 2006).

Clinical Trials of Sorafenib in Other Tumor Types

36.6.1 Hepatocellular Carcinoma

Hepatocellular carcinoma is a highly vascularized tumor that expresses high levels of VEGF (Park et al. 2000; Yamaguchi et al. 1998). Therefore, there is a good rationale for investigating the benefits of sorafenib in this tumor type. The primary risk factor for HCC is cirrhosis, particularly cirrhosis attributable to hepatitis B (HBV) and hepatitis C (HCV) viral infections – the two most important etiological factors for HCC (Yamaguchi et al. 1998; Cougot et al. 2005). HBV and HCV account for >70% of HCC cases worldwide (Cougot et al. 2005). When integrated into the DNA of their human host, HBV and HCV encode proteins that hyperactivate Raf kinase, one of sorafenib's molecular targets. Furthermore, Raf-1 has been reported to be overactivated in 100% of HCC biopsies (30/30) in a recent study (Hwang et al. 2004). Early evidence of the potential for combining sorafenib with other agents in the treatment of HCC was provided by a phase I trial of sorafenib monotherapy, a phase I trial of sorafenib combined with doxorubicin, and a phase II trial of single-agent sorafenib. In the phase I sorafenib monotherapy trial in patients with advanced refractory solid tumors, one patient with HCC had a PR (Strumberg et al. 2005). In the phase I combination therapy trial, four of 16 patients with SD had HCC (Richly et al. 2004a). In addition, the phase II trial showed that sorafenib monotherapy had antitumor activity in HCC patients, as assessed by modified World Health Organization response criteria (Abou-Alfa et al. 2006). In this trial, sorafenib induced a PR in three patients (2.2%), a minor response (MR) in eight patients (5.8%), and SD (lasting ≥16 weeks) in a further 46 patients (33.6%). Therefore, sorafenib had a 41.6% clinical benefit (CR+PR+MR+SD). Investigator-assessed median time to progression (TTP) was 4.2 months, and median overall survival was 9.2 months. Interestingly, despite appearing to increase in size (i.e. showing progressive disease by standard response criteria), the tumors of many patients receiving sorafenib in the trial showed extensive areas of necrosis. Furthermore, patients with tumors with high levels of pERK staining (i.e. high levels of signaling through Raf and MEK) at baseline had a significantly longer TTP in this trial. However, only 18 of the 33 patients evaluated had high levels of pERK staining at baseline (Abou-Alfa et al. 2006), suggesting that the proportion of patients with HCC whose tumors have high levels of signaling through the MAPK pathway may be lower than previously reported in the literature.

The results of an extended phase I trial of sorafenib and doxorubicin in 18 patients with advanced HCC showed that the safety profile of sorafenib and doxorubicin in combination was similar to that expected of either agent alone (Richly et al. 2004b). Of 13 evaluable patients, the best response observed was SD for at least 6 months in four patients (30%) and at least 3 months in seven patients (54%). The high proportion of patients included with previous systemic therapy (35%) could explain the low objective response rate observed in this trial. Because systemic chemotherapy has been shown to be ineffective in HCC (Bruix et al. 2001), it is possible that the disease stabilizations observed in this trial could be due to potentiation of doxorubicin's cytotoxic effects by sorafenib.

An ongoing phase III trial is evaluating single-agent sorafenib in 560 patients with advanced HCC. Eligible patients will have a baseline ECOG performance status of 0–2, adequate liver function (i.e. Child–Pugh status A), and a life expectancy of at least 12 weeks. In this trial, patients will be randomized to receive either sorafenib 400 mg b.i.d. or placebo. Clinical efficacy will be assessed using overall survival and time to symptomatic progression as primary endpoints. Secondary endpoints include TTP, overall disease control rate (i.e. clinical benefit, as previously defined), and quality of life.

36.6.2 Melanoma

Increased signaling through the Raf/MEK/ERK pathway, as a result of autocrine stimulation by bFGF and hepatocyte growth factor, is implicated in melanocytic tumorigenesis (tumor growth, invasion, and metastasis) (Satyamoorthy et al. 2003). Furthermore, the activity of ERK, which is downstream of Raf, has been shown to increase from early- to advanced-stage melanoma (Satyamoorthy et al. 2003). This increased ERK activity may be the consequence of activating *b-raf* mutations, which are present in up to 80% of human melanomas (Davies et al. 2002; Chang et al. 2004; Garnett and Marais 2004). The most prevalent oncogenic *b-raf* mutation is the *b-raf* V600E mutation (previously known as V599E), which is present in 63% of melanomas (Brose et al. 2002).

The increased apoptosis observed in human melanoma cell lines when *b-raf* expression is downregulated using RNA interference supports a role for oncogenic *b-raf* driven MEK/ERK overactivation in maintaining the transformed phenotype of malignant melanoma cells (Hingorani et al. 2003; Karasarides et al. 2004). This observation suggests that *b-raf* is the target of choice for the treatment of melanoma. There is also evidence of elevated expression of several angiogenic factors, including VEGF, bFGF, and interleukin-8, in primary cutaneous melanomas (Streit and Detmar 2003). The overproduction of VEGF165 and its association with VEGFR expression favors cell growth and survival of melanoma cells through MAPK and phosphatidyl inositol-3-kinase (PI3K) signaling pathways. These data support the involvement in melanoma growth and survival of a VEGF-dependent internal autocrine loop mechanism, at least in vitro (Graells et al. 2004). Therefore, there is a solid rationale for the use of sorafenib in the treatment of melanoma through inhibition of the VEGFR and Raf kinase isoforms.

The effects of the combination of paclitaxel, carboplatin, and sorafenib have been investigated in an open-label phase I trial in patients with progressive stage IV melanoma who had received multiple prior chemotherapy regimens (Flaherty et al. 2004). Most patients (60%) had received at least one prior therapy and had American Joint Committee on Cancer M1c stage disease (68%). Interestingly, the preliminary results showed a high rate of PR (31%), ongoing 3–16 months at the time of analysis, and 54% SD, but the antitumor activity appeared to be independent of tumor *b-raf* status. These results were encouraging and supported further evaluation of this combination in patients with melanoma.

Ongoing phase III trials are investigating the combination of sorafenib and repeated cycles of paclitaxel/carboplatin in patients with unresectable stage III or IV melanoma. Sorafenib has also been evaluated in combination with repeated cycles of DTIC in a single-center, open-label, phase I dose-escalation trial that enrolled patients with metastatic melanoma (Eisen et al. 2005). Among 11 evaluable patients, two had PRs, two had tumor shrinkage that did not reach a PR, and one had SD after cycle 2. This combination is also undergoing further evaluation in clinical trials, including a phase II randomized, placebo-controlled trial in patients with unresectable stage III or IV melanoma.

36.7 Summary

Sorafenib is a novel, multi-kinase inhibitor which targets the tumor cell and the tumor vasculature. This orally administered drug is well tolerated and shows promise against notoriously chemoresistant solid tumors. Based on its ability to significantly prolong PFS versus placebo, rather than its response rate, sorafenib was the first targeted agent to be approved for the treatment of patients with advanced refractory RCC. It has now been approved for this indication in the US, Switzerland, Mexico, Chile, Brazil, Korea, and Argentina. Sorafenib has also recently received marketing approval from the European Commission for the treatment of patients with advanced RCC who have failed prior interferon or interleukin-2 therapy or are ineligible for cytokine

therapy. Sorafenib also shows early signs of clinical efficacy, either alone or in combination regimens, in patients with advanced HCC and melanoma. Ongoing phase III clinical trials are further evaluating sorafenib combinations in HCC, melanoma, and NSCLC. Ongoing adjuvant trials will ultimately determine whether treatment with sorafenib post nephrectomy improves survival in patients with RCC. Finally, sorafenib is being compared head-to-head against interferon-α in previously untreated patients with RCC.

References

Abou-Alfa GK et al (2006) Phase II study of sorafenib in patients with advanced hepatocellular carcinoma. J Clin Oncol 24:4293–4300

Alavi A, Hood JD, Frausto R, Stupack DG, Cheresh DA (2003) Role of Raf in vascular protection from distinct apoptotic stimuli. Science 301:94–96

Alghisi GC, Ruegg C (2006) Vascular integrins in tumor angiogenesis: mediators and therapeutic targets. Endothelium 13:113–135

Awada A et al (2004) A phase I study of BAY 43-9006, a novel Raf kinase and VEGFR inhibitor, in combination with taxotere in patients with advanced solid tumors. Eur J Cancer [Suppl] 2:114

Awada A et al (2005) Phase I safety and pharmacokinetics of BAY 43-9006 administered for 21 days on/7 days off in patients with advanced, refractory solid tumours. Br J Cancer 92:1855–1861

Baluk P, Hashizume H, McDonald DM (2005) Cellular abnormalities of blood vessels as targets in cancer. Curr Opin Genet Dev 15:102–111

Bayer (2006) Nexavar full prescribing information. http://www.univgraph.com/bayer/inserts/nexavar.pdf

Brauch H et al (2000) VHL alterations in human clear cell renal cell carcinoma: association with advanced tumor stage and a novel hot spot mutation. Cancer Res 60:1942–1948

Brose MS et al (2002) BRAF and RAS mutations in human lung cancer and melanoma. Cancer Res 62:6997–7000

Bruix J et al (2001) Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. J Hepatol 35:421–430

Carlomagno F et al (2006) BAY 43-9006 inhibition of oncogenic RET mutants. J Natl Cancer Inst 98:326–334

Carter CA et al (2007) Sorafenib is efficacious and tolerated in combination with cytotoxic or cytostatic agents in preclinical models of human non-small cell lung carcinoma. Cancer Chemother Pharmacol 59:183–195

Chang DZ et al (2004) Clinical significance of BRAF mutations in metastatic melanoma. J Transl Med 2:46

Chang YS et al (2005) BAY 43-9006 (Sorafenib) inhibits ectopic and orthotopic growth of a murine model of renal adenocarcinoma (Renca) predominantly through inhibition of tumor angiogenesis. Clin Cancer Res 46:5831

Chen HX (2004) Expanding the clinical development of bevacizumab. Oncologist 9 [Suppl 1]:27–35

Clark JW, Eder JP, Ryan D, Lathia C, Lenz H-J (2005) Safety and pharmacokinetics of the dual action Raf kinase and vascular endothelial growth factor receptor inhibitor, BAY 43-9006, in patients with advanced, refractory solid tumors. Clin Cancer Res 11:5472–5480

Cougot D, Neuveut C, Buendia MA (2005) HBV induced carcinogenesis. J Clin Virol 34 [Suppl 1]:S75–S78

D'Amore PA, Smith SR (1993) Growth factor effects on cells of the vascular wall: a survey. Growth Factors 8:61–75

Davies H et al (2002) Mutations of the BRAF gene in human cancer. Nature 417:949–954

De Paulsen N et al (2001) Role of transforming growth factor-alpha in von Hippel-Lindau (VHL)(-/-) clear cell renal carcinoma cell proliferation: a possible mechanism coupling VHL tumor suppressor inactivation and tumorigenesis. Proc Natl Acad Sci USA 98:1387–1392

Dhanda R et al (2006) A comparison of quality of life and symptoms in kidney cancer patients receiving sorafenib versus placebo. J Clin Oncol 24:225s

Downward J (2003) Targeting RAS signalling pathways in cancer therapy. Nat Rev Cancer 3:11–22

Eisen T et al (2005) Phase I trial of BAY 43-9006 (sorafenib) combined with dacarbazine (DTIC) in metastatic melanoma patients. J Clin Oncol 23:7508

Eisen T et al (2006) Randomized phase III trial of sorafenib in advanced renal cell carcinoma (RCC): impact of crossover on survival. J Clin Oncol 24:4524

Escudier B et al (2005) Randomized phase III trial of the multi-kinase inhibitor sorafenib (BAY 43-9006) in patients with advanced renal cell carcinoma (RCC). Eur J Cancer [Suppl] 3:226

Esser S, Lampugnani MG, Corada M, Dejana E, Risau W (1998) Vascular endothelial growth factor induces VE-cadherin tyrosine phosphorylation in endothelial cells. J Cell Sci 111:1853–1865

Ferrara N (2002) Role of vascular endothelial growth factor in physiologic and pathologic angiogenesis: therapeutic implications. Semin Oncol 29:10–14

Figer A et al (2004) Phase I trial of BAY 43-9006 in combination with 5-fluorouracil (5-FU) and leucovorin (LCV) in patients with advanced refractory solid tumors. Ann Oncol 15:87

Flaherty KT et al (2004) Phase I/II trial of BAY 43-9006, carboplatin (C) and paclitaxel (P) demonstrates preliminary antitumor activity in the expansion cohort of patients with metastatic melanoma. J Clin Oncol 22:7507

Flaherty KT et al (2006) Sorafenib combined with carboplatin and paclitaxel for metastatic melanoma: PFS and response versus B-Raf status. Proceedings of the 4th International Symposium on Targeted Anticancer Therapies (TAT), Amsterdam, The Netherlands, http://www.nddo.org/files/pdf/TAT_symp06_proceedings.pdf

Folkman J (1992) The role of angiogenesis in tumor growth. Semin Cancer Biol 3:65–71

Folkman J (1971) Tumor angiogenesis: therapeutic implications. N Engl J Med 285:1182–1186

Garnett MJ, Marais R (2004) Guilty as charged: B-RAF is a human oncogene. Cancer Cell 6:313–319

Graells J et al (2004) Overproduction of VEGF concomitantly expressed with its receptors promotes growth and survival of melanoma cells through MAPK and PI3 K signaling. J Invest Dermatol 123:1151–1161

Gunaratnam L et al (2003) Hypoxia inducible factor activates the transforming growth factor-alpha/epidermal growth factor receptor growth stimulatory pathway in VHL(-/-) renal cell carcinoma cells. J Biol Chem 278:44966–44974

Hellstrom M, Kalen M, Lindahl P, Abramsson A, Betsholtz C (1999) Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. Development 126:3047–3055

Hingorani SR, Jacobetz MA, Robertson GP, Herlyn M, Tuveson DA (2003) Suppression of BRAF (V599E) in human melanoma abrogates transformation. Cancer Res 63:5198–5202

Hwang YH et al (2004) Over-expression of c-raf-1 proto-oncogene in liver cirrhosis and hepatocellular carcinoma. Hepatol Res 29:113–121

Karasarides M et al (2004) B-RAF is a therapeutic target in melanoma. Oncogene 23:6292–6298

Kasid U, Dritschilo A (2003) RAF antisense oligonucleotide as a tumor radiosensitizer. Oncogene 22:5876–5884

Kertesz N et al (2006) The soluble extracellular domain of EphB4 (sEphB4) antagonizes EphB4-EphrinB2 interaction, modulates angiogenesis, and inhibits tumor growth. Blood 107:2330–2338

Kim CW, Son KN, Choi SY, Kim J (2006) Human lactoferrin upregulates expression of KDR/Flk-1 and stimulates VEGF-A-mediated endothelial cell proliferation and migration. FEBS Lett 580:4332–4336

Kolch W, Kotwaliwale A, Vass K, Janosch P (2002) The role of Raf kinases in malignant transformation. Exp Rev Mol Med 2002:1–18

Kupsch P et al (2005) Results of a phase I trial of sorafenib (BAY 43-9006) in combination with oxaliplatin in patients with refractory solid tumors, including colorectal cancer. Clin Colorectal Cancer 5:188–196

Lampugnani MG, Orsenigo F, Gagliani MC, Tacchetti C, Dejana E (2006) Vascular endothelial cadherin controls VEGFR-2 internalization and signaling from intracellular compartments. J Cell Biol 174:593–604

Lamuraglia M et al (2005) Doppler ultrasonography with perfusion software and contrast agent injection as a to for early evaluation of metastatic renal cancers treate with the Raf kinase and VEGFR inhibitor: a prospectiv study. J Clin Oncol 23:209s

Leibovich SJ et al (1987) Macrophage-induced angiogenesi is mediated by tumour necrosis factor-alpha. Natur 329:630–632

Leung SK, Ohh M (2002) Playing tag with HIF: the VHL story J Biomed Biotechnol 2:131–135

Levy AP et al (2006) Analysis of transcription and proteir expression changes in the 786-O human renal cell carcinoma tumor xenograft model in response to treatment with the multi-kinase inhibitor sorafenib (BAY 43-9006). Proc Am Assoc Cancer Res 47:213–214

Liu L et al (2005) Sorafenib (BAY 43-9006) inhibits the Raf/MEK/ERK pathway in hepatocellular carcinoma (HCC) cells and produces robust efficacy againts PLC/PRF/5 HCC tumors in mice. Poster presentation at AACR-NCI-EORTC. Philadelphia, PA

Lowinger TB, Riedl B, Dumas J, Smith RA (2002) Design and discovery of small molecules targeting raf-1 kinase. Curr Pharm Des 8:2269–2278

Lyons JF, Wilhelm S, Hibner B, Bollag G (2001) Discovery of a novel Raf kinase inhibitor. Endocr Relat Cancer 8:219–225

McPhillips F et al (2001) Association of c-Raf expression with survival and its targeting with antisense oligonucleotides in ovarian cancer. Br J Cancer 85:1753–1758

Melillo RM et al (2005) The RET/PTC-RAS-BRAF linear signaling cascade mediates the motile and mitogenic phenotype of thyroid cancer cells. J Clin Invest 115:1068–1081

Moore M et al (2005) Phase I study to determine the safety and pharmacokinetics of the novel Raf kinase and VEGFR inhibitor BAY 43-9006, administered for 28 days on/7 days off in patients with advanced, refractory solid tumors. Ann Oncol 16:1688–1694

Mukherjee R, Bartlett JM, Krishna NS, Underwood MA, Edwards J (2005) Raf-1 expression may influence progression to androgen insensitive prostate cancer. Prostate 64:101–107

O'Dwyer PJ, Rosen M, Gallagher M, Schwartz B, Flaherty KT (2005) Pharmacodynamic study of BAY 43-9006 in patients with metastatic renal cell carcinoma. J Clin Oncol 23:193s

Ogawa K et al (2000) The ephrin-A1 ligand and its receptor, EphA2, are expressed during tumor neovascularization. Oncogene 19:6043–6052

Oka H et al (1995) Constitutive activation of mitogen-activated protein (MAP) kinases in human renal cell carcinoma. Cancer Res 55:4182–4187

O'Neill E, Rushworth L, Baccarini M, Kolch W (2004) Role of the kinase MST2 in suppression of apoptosis by the proto-oncogene product Raf-1. Science 306:2267–2270

Padhani AR, Husband JE (2001) Dynamic contrast-enhanced MRI studies in oncology with an emphasis on

quantification, validation and human studies. Clin Radiol 56:607–620
Panka DJ, Wang W, Atkins MB, Mier JW (2006) The Raf inhibitor BAY 43-9006 (Sorafenib) induces caspase-independent apoptosis in melanoma cells. Cancer Res 66:1611–1619
Papetti M, Herman IM (2002) Mechanisms of normal and tumor-derived angiogenesis. Am J Physiol Cell Physiol 282:C947–C970
Pardali K, Moustakas A (2007) Actions of TGF-beta as tumor suppressor and pro-metastatic factor in human cancer. Biochim Biophys Acta 1775:21–62
Park YN, Kim YB, Yang KM, Park C (2000) Increased expression of vascular endothelial growth factor and angiogenesis in the early stage of multistep hepatocarcinogenesis. Arch Pathol Lab Med 124:1061–1065
Phelps ED, Updike DL, Bullen EC, Grammas P, Howard EW (2006) Transcriptional and posttranscriptional regulation of angiopoietin-2 expression mediated by IGF and PDGF in vascular smooth muscle cells. Am J Physiol Cell Physiol 290:C352–C361
Rahmani M, Davis EM, Bauer C, Dent P, Grant S (2005) Apoptosis induced by the kinase inhibitor BAY 43-9006 in human leukemia cells involves downregulation of Mcl-1 through inhibition of translation. J Biol Chem 280:35217–35227
Ratain MJ et al (2006) Phase II placebo-controlled randomized discontinuation trial of sorafenib in patients with metastatic renal cell carcinoma. J Clin Oncol 24:2505–2512
Richly H et al (2004a) Results of a phase I trial of BAY 43-9006 in combination with doxorubicin in patients with refractory solid tumors. J Clin Oncol 23:207
Richly H et al (2004b) Results of a phase I trial of BAY 43-9006 in combination with doxorubicin in patients with primary hepatic cancer. Ann Oncol 15:104
Rini BI (2005) VEGF-targeted therapy in metastatic renal cell carcinoma. Oncologist 10:191–197
Sahni A, Khorana AA, Baggs RB, Peng H, Francis CW (2006) FGF-2 binding to fibrin(ogen) is required for augmented angiogenesis. Blood 107:126–131
Salvatore G et al (2004) Analysis of BRAF point mutation and RET/PTC rearrangement refines the fine-needle aspiration diagnosis of papillary thyroid carcinoma. J Clin Endocrinol Metab 89:5175–5180
Salvatore G et al (2006) B-RAF is a therapeutic target in aggressive thyroid carcinoma. Clin Cancer Res 12:1623–1629
Satyamoorthy K et al (2003) Constitutive mitogen-activated protein kinase activation in melanoma is mediated by both BRAF mutations and autocrine growth factor stimulation. Cancer Res 63:756–759
Semino CE, Kamm RD, Lauffenburger DA (2006) Autocrine EGF receptor activation mediates endothelial cell migration and vascular morphogenesis induced by VEGF under interstitial flow. Exp Cell Res 312:289–298
Sharma A et al (2005) Mutant V599E B-Raf regulates growth and vascular development of malignant melanoma tumors. Cancer Res 65:2412–2421
Siu LL et al (2006) Phase I/II trial of sorafenib and gemcitabine in advanced solid tumors and in advanced pancreatic cancer. Clin Cancer Res 12:144–151
Smith K et al (2005) Silencing of epidermal growth factor receptor suppresses hypoxia-inducible factor-2-driven VHL-/- renal cancer. Cancer Res 65:5221–5230
Smith RA et al (2001) Discovery of heterocyclic ureas as a new class of raf kinase inhibitors: identification of a second generation lead by a combinatorial chemistry approach. Bioorg Med Chem Lett 11:2775–2778
Steinbild S et al (2005) Phase I study of BAY 43-9006 (sorafenib), a Raf kinase and VEGFR inhibitor, combined with irinotecan (CPT-11) in advanced solid tumors. J Clin Oncol 23:3115
Streit M, Detmar M (2003) Angiogenesis, lymphangiogenesis, and melanoma metastasis. Oncogene 22:3172–3179
Strumberg D et al (2005) Phase I clinical and pharmacokinetic study of the novel Raf kinase and vascular endothelial growth factor receptor inhibitor BAY 43-9006 in patients with advanced refractory solid tumors. J Clin Oncol 23:965–972
Strumberg D et al (2006) Pooled safety analysis of BAY 43-9006 (sorafenib) monotherapy in patients with advanced solid tumours: Is rash associated with treatment outcome? Eur J Cancer 42:548–556
Takahashi H, Shibuya M (2005) The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions. Clin Sci (Lond) 109:227–241
Wan PT et al (2004) Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. Cell 116:855–867
Wary KK, Thakker GD, Humtsoe JO, Yang J (2003) Analysis of VEGF-responsive genes involved in the activation of endothelial cells. Mol Cancer 2:25
Wilhelm SM et al (2004) BAY 43-9006 exhibits broad spectrum oral anti-tumor activity and targets the Raf/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. Cancer Res 64:7099–7109
Wu X, Ma J, Han JD, Wang N, Chen YG (2006) Distinct regulation of gene expression in human endothelial cells by TGF-beta and its receptors. Microvasc Res 71:12–19
Yamaguchi R et al (1998) Expression of vascular endothelial growth factor in human hepatocellular carcinoma. Hepatology 28:68–77
Yamaoka-Tojo M et al (2006) IQGAP1 mediates VE-cadherin-based cell-cell contacts and VEGF signaling at adherence junctions linked to angiogenesis. Arterioscler Thromb Vasc Biol 26:1991–1997
Yu C et al (2005) The role of Mcl-1 downregulation in the proapoptotic activity of the multikinase inhibitor BAY 43-9006. Oncogene 24:6861–6869

Clinical Development of the VEGFR Signalling Inhibitor AZD2171

37

Joachim Drevs and Clemens Unger

Abstract

New blood vessel formation is fundamental to tumour growth and spread. The key signalling molecules involved in the promotion of tumour angiogenesis are the vascular endothelial growth factors (VEGF), which exert their effects by binding to tyrosine kinase receptors: VEGFR-1, VEGFR-2 and VEGFR-3. VEGFR activation results in a range of downstream processes involved in angiogenesis, neovascular survival and induction of vascular permeability. Targeting of VEGF signalling is therefore a rational and potentially valuable therapeutic strategy. AZD2171 is an oral, highly potent and selective VEGF signalling inhibitor of all three VEGFRs. Preclinical studies of AZD2171 have demonstrated selective inhibition of VEGF-stimulated human umbilical vein endothelial cell proliferation in vitro, as well as dose-dependent inhibition of tumour growth in a broad range of established human tumour xenografts. These results are consistent with inhibition of VEGF signalling and an anti-angiogenic mode of action rather than a direct antiproliferative effect on tumour cells. The encouraging data obtained from preclinical studies have led to the clinical evaluation of AZD2171 as a single agent and in combination with other anticancer treatments. The phase I programme has demonstrated that AZD2171 is generally well tolerated at doses up to 45 mg/day, with a manageable adverse event profile. Pharmacokinetic data support a once-daily oral dosing schedule. Preliminary efficacy data demonstrate that AZD2171 has encouraging antitumour activity in a range of tumour types, including renal, glioblastoma multiforme, lung and colorectal cancers. These data warrant further clinical evaluation of AZD2171, and recruitment to a series of phase II, phase II/III and phase III trials is ongoing.

CONTENTS

Introduction

New blood vessel formation (angiogenesis) is fundamental to tumour growth and spread (Ferrara et al. 2003). Vascular endothelial growth factor (VEGF) has a key, rate-limiting role in promoting tumour angiogenesis and exerts its effects by binding to one of three tyrosine kinase receptors: VEGF receptor-1 (VEGFR-1), VEGFR-2 and VEGFR-3. VEGFR-1 (ligands include VEGF-A, -B and placental growth factor, PlGF) and VEGFR-2 (ligands include VEGF-A, -C and -D) are predominantly expressed on vascular endothelial cells, and activation of VEGFR-2 appears to be both necessary and sufficient to mediate VEGF-dependent angiogenesis (Meyer et al. 1999) and induction of vascular permeability (Gille et al. 2001). Although the exact contribution of VEGFR-1 signalling to angiogenesis is unclear, it has been shown to co-operate directly with VEGFR-2 via heterodimerization and can bind two additional VEGF homologues, VEGF-B and PlGF (Autiero et al. 2003). VEGFR-3, largely restricted to lymphatic endothelial cells, binds the VEGF homologues VEGF-C and VEGF-D and may play an important role in the regulation of lymphangiogenesis (Jussila and Alitalo 2002; Cao 2005).

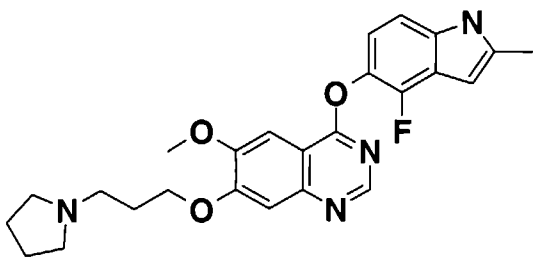


Fig. 37.1.
Structure of AZD2171

Inhibiting tumour angiogenesis by targeting VEGF signalling is therefore a rational and potentially valuable therapeutic strategy. Indeed, recent studies with the anti-VEGF-A monoclonal antibody bevacizumab in combination with certain chemotherapy regimens have demonstrated clinically relevant improvements in survival in colorectal (Hurwitz et al. 2004), lung (Sandler et al. 2005) and breast cancer (Miller et al. 2005). Antitumour activity has also been observed with small molecules such as sorafenib (Escudier et al. 2005) and sunitinib (Motzer et al. 2005), both of which possess activity against multiple kinases, including VEGFR tyrosine kinases.

AZD2171 (Fig. 37.1) is an oral, highly potent inhibitor of VEGF signalling that selectively inhibits all known VEGFR tyrosine kinase activity (VEGFR-1, -2 and -3; Fig. 37.2). This chapter reviews the encouraging results obtained to date with AZD2171 in a range of clinical studies.

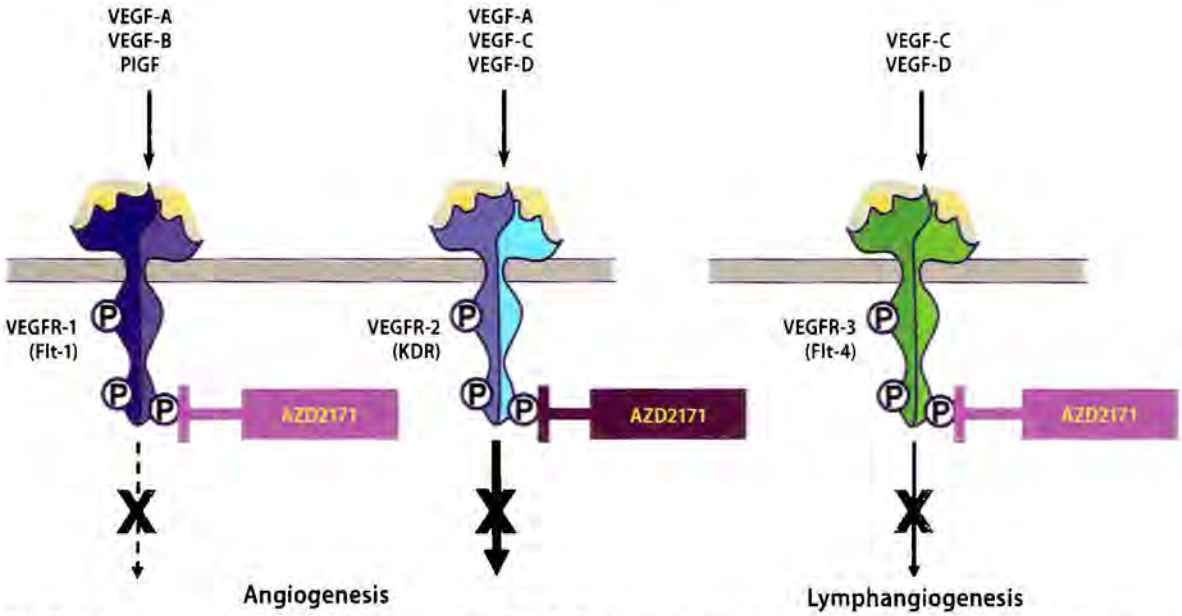


Fig. 37.2. Inhibition of VEGFR activation by AZD2171. *PlGF*, placental growth factor; *VEGF*, vascular endothelial growth factor; *VEGFR*, VEGF receptor

37.2 Preclinical Development of AZD2171

37.2.1 In Vitro Potency and Selectivity

The effects of AZD2171 on receptor kinase activity were assessed in a recombinant enzyme assay versus isolated kinases (Wedge et al. 2005). AZD2171 was shown to be a highly potent inhibitor of VEGFR-2 tyrosine kinase activity (IC_{50}<1 nM). It also demonstrated potent activity versus VEGFR-1 and VEGFR-3 (IC_{50}=5 nM and ≤3 nM, respectively) and additional activity versus c-Kit (IC_{50}=2 nM). AZD2171 showed excellent selectivity versus a range of additional tyrosine and serine/threonine kinases, including Flt-3 (IC_{50}<1000 nM) and epidermal growth factor receptor (EGFR; IC_{50}<1000 nM).

The ability of AZD2171 to inhibit growth factor-stimulated receptor phosphorylation was determined in a range of cell lines (Wedge et al. 2005). AZD2171 produced a dose-dependent inhibition of VEGF-induced VEGFR-2 phosphorylation in human umbilical vein endothelial cells (HUVECs), with an IC_{50} value of 0.5 nM. Furthermore, this effect was also associated with inhibition of MAP kinase phosphorylation, a downstream marker of VEGF signalling. Compared with inhibition of VEGFR-2 phosphorylation in HUVECs, a 2 times higher concentration of AZD2171 was required to inhibit phosphorylation of c-Kit receptors in NCI-H5262 cells. In contrast, 10–16 times higher concentrations of AZD2171 were required to produce similar inhibition of platelet-derived growth factor (PDGF)-induced phosphorylation of PDGFR-α and -β in MG63 cells. Consistent with inhibition of VEGFR-2 tyrosine kinase activity, AZD2171 was also shown to be a potent and selective inhibitor of VEGF-stimulated HUVEC proliferation (IC_{50}=0.4 nM). Approximately 275- and 1250-fold higher concentrations were required to inhibit proliferation induced by basic fibroblast growth factor (IC_{50}=110 nM) or EGF (IC_{50}=500 nM). The effect of AZD2171 on PDGF-AA/PDGFR-α-dependent cellular proliferation was examined in MG63 cells, with

an antiproliferative effect only occurring at AZD2171 concentrations that were 100 times greater than those required for comparable inhibition of VEGF-driven HUVEC proliferation (Wedge et al. 2005). These data suggest that AZD2171 can selectively inhibit VEGFR-dependent proliferation but appreciable functional selectivity is evident against other targets, including EGFR, FGFR and PDGFR-α.

37.2.2 In Vivo Inhibition of VEGF Signalling and Angiogenesis

The in vivo activity of AZD2171 was investigated in a model of vascular sprouting (Wedge et al. 2005). In nude mice implanted with a VEGF-containing Matrigel plug, once-daily oral administration of both low (1.5 mg/kg/day) and high (6 mg/kg/day) doses of AZD2171 for 7 days completely abolished VEGF-induced vessel formation. These results demonstrate that AZD2171 is a potent inhibitor of VEGF-induced angiogenesis (Wedge et al. 2005).

The VEGF signalling pathway is one of several involved in mammalian blood pressure homeostasis through its vasodilatory properties. By removing an important contributing factor to overall vasodilatory tone, agents that inhibit VEGF signalling may, indirectly, cause vasoconstriction. Preclinical studies have demonstrated that hypertension may occur following treatment with AZD2171; however, normotension may be successfully maintained using antihypertensive agents (Curwen et al. 2005).

37.2.3 In Vivo Inhibition of Tumour Growth

AZD2171 has demonstrated antitumour efficacy in a number of in vivo preclinical studies, including xenograft, orthotopic, metastatic and spontaneous models of human cancer (Wedge et al. 2005; Chap. 23 of this book).

Once-daily oral administration of AZD2171 (1.5–6 mg/kg/day) produced dose-dependent inhibition of tumour growth in a range of histologically distinct human tumour xenografts (lung, colon, breast, prostate and ovarian). In each of the tumour models, treatment with the highest dose investigated (6 mg/kg/day) resulted in >90% inhibition of tumour volume compared with time-matched controls. For example, Fig. 37.3 shows the effect of AZD2171 on growth of MDA-MB-231 human breast tumour xenografts.

The effects of AZD2171 on both tumour volume and tumour vascular density were examined in Calu-6 human lung tumour xenografts (Wedge et al. 2005). Chronic treatment with AZD2171 (6 mg/kg/day) for 21 days inhibited tumour growth by 68% compared with controls, and there was a corresponding reduction in tumour vessel density of ≥70%. Acute treatment showed a 47% reduction in vessel number after only three doses of AZD2171. These findings indicate that AZD2171 can cause vascular regression in tumours and this is likely to be due to a direct action of AZD2171 on tumour endothelium, derived from potent inhibition of VEGF signalling and neovascular survival.

AZD2171 has also been shown to decrease primary tumour growth, metastasis and microvessel density in an orthotopic model of murine renal cell carcinoma (Drevs et al. 2004). This model develops primary tumours within 1 week and generally metastasizes to the lung and abdominal lymph nodes within 10 days. Treatment with AZD2171 (6.3 mg/kg/day p.o. for 7 days), which commenced 8 days after tumour implantation, resulted in a significant reduction in primary tumour volume (by 50%) and vessel density (by 45%), as well as a 54% reduction in lung metastases (Fig. 37.4).

AZD2171 has also been used to investigate the potential role of VEGF signalling in early-stage tumour development using a mouse model of spontaneous multiple intestinal neoplasia (Min) (Goodlad et al. 2005). Administration of AZD2171 (5 mg/kg/day p.o.) to 6-week old Min mice for 28 days significantly reduced polyp number in the small bowel by 46% and in the colon by 62% (both $P<0.05$).

An orthotopic human lung adenocarcinoma mouse model was used to study the effects of AZD2171 (6 mg/kg/day p.o.) in combination with gefitinib (25 mg/kg/day p.o.) or paclitaxel (150 μg/

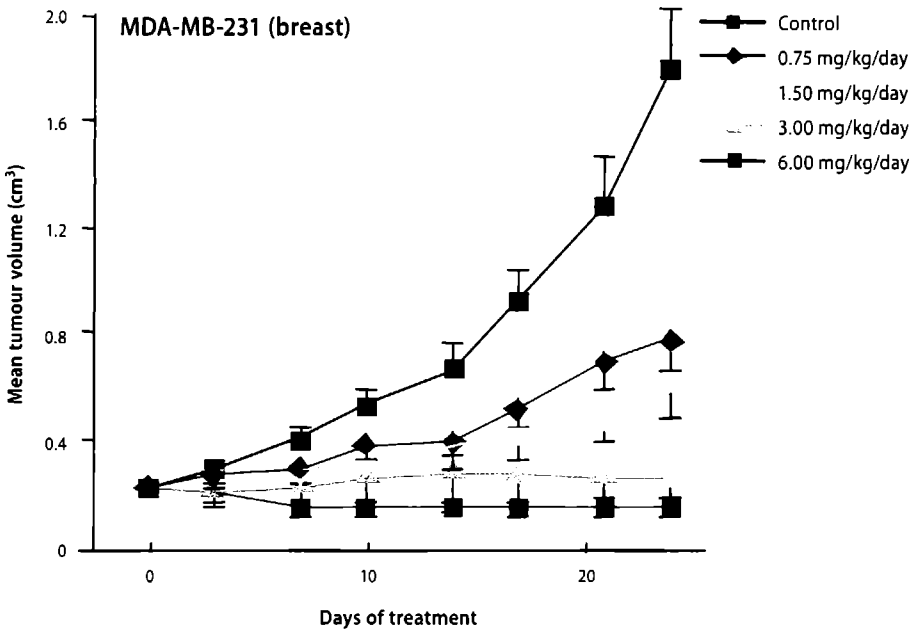


Fig. 37.3. Effect of AZD2171 on growth of MDA-MB-231 human breast tumour xenografts

weekly i.p.) (Wu et al. 2005). AZD2171 showed monotherapy efficacy in this model, and combination treatments suppressed tumour growth, lymphatic and chest wall metastasis, and pleural effusion to a greater extent than either agent alone.

Taking all these findings together, AZD2171 has shown antitumour activity in a range of preclinical in vivo models consistent with inhibition of VEGF signalling and an anti-angiogenic mode of action rather than a direct antiproliferative effect on tumour cells.

Clinical Development

The encouraging data obtained from preclinical studies have led to the clinical evaluation of AZD2171 in patients with cancer. The clinical development programme includes a comprehensive series of phase I clinical trials to investigate AZD2171 as a single agent and in combination with certain other anticancer treatments (Table 37.1).

37.3.1 Phase I Dose-finding Study

This single-centre, two-part open study was the first phase I clinical evaluation of AZD2171 (Drevs et al. 2007). The primary objective was to assess the safety and tolerability of ascending doses of AZD2171 in patients with advanced solid tumours refractory to standard treatments, or for whom no standard treatment existed. Secondary objectives included assessments of pharmacokinetics, pharmacodynamics and efficacy.

A total of 83 patients entered the study and received treatment with AZD2171. All patients in part A (n=36) had metastatic liver disease, whereas patients with (n=36) or without (n=11) liver lesions were recruited to part B. The primary tumour types for part A were colorectal (n=7); breast (n=4); renal (n=3); skin/soft tissue (n=3); lung (n=1); prostate (n=1); small bowel (n=1); liver (n=1); other (n=7). In part A, patients received a single oral dose of AZD2171 (0.5, 1, 2.5, 5, 10, 20, 30, 45 or 60 mg) followed by a 2- to 7-day observation period. At the end of this period, patients received once-daily treatment

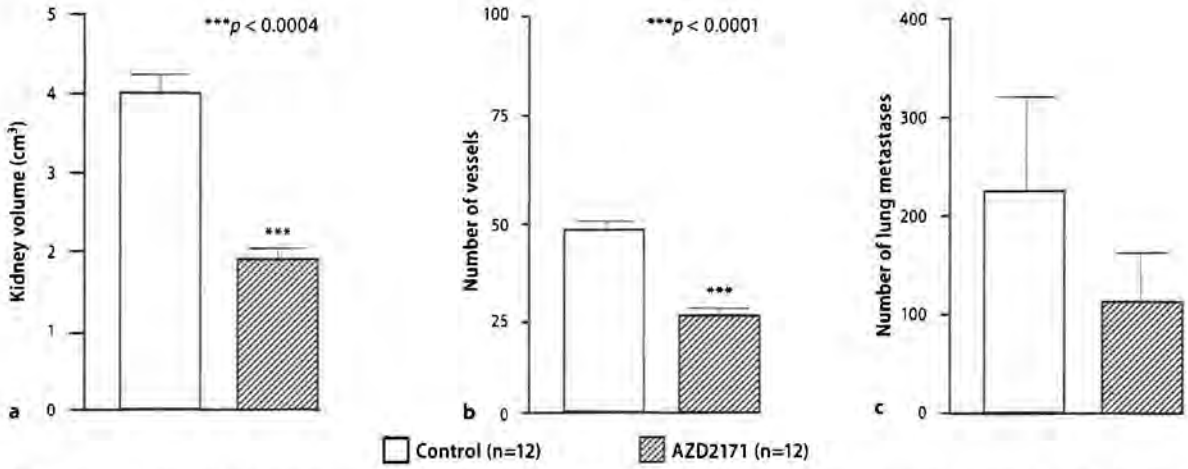


Fig. 37.4a–c. AZD2171 (6.3 mg/kg/day) inhibits a primary tumour growth, b tumour vessel density and c lung metastases in an orthotopic model of renal cell carcinoma (Drevs et al. 2004)

until tumour progression or uncontrollable toxicity was observed. Part B was a cohort expansion phase in which patients were randomized to receive oral AZD2171 once daily at one of three potentially active and well-tolerated doses from part A (20, 30 and 45 mg). In both parts, patients received AZD2171 until tumour progression or uncontrollable toxicity was observed.

Serial blood samples were collected for pharmacokinetic assessments following single and multiple doses. Pharmacodynamic parameters included soluble markers of angiogenesis measured in serum and plasma samples, iAUC60 and K^{trans} [indicators of tumour blood flow and permeability, assessed using dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) of liver lesions] (Strecker et al. 2003). A solitary measurable target lesion, with other lesions classified and followed as non-target lesions, was evaluated according to RECIST (Therasse et al. 2000) before the start of the study and every 28 days following the start of daily AZD2171 dosing.

AZD2171 was generally well tolerated at doses of ≤45 mg/day. The most frequently occurring adverse events, irrespective of causality, were fatigue (n=47), diarrhoea (n=39), nausea (n=34), dysphonia (n=30), and hypertension (n=29). Among the adverse events reported, dysphonia, hypertension and diarrhoea were considered by the investigator to be mostly drug related. The overall incidence of drug-related grade 3 or 4 adverse events was low, and none were recorded at doses ≤10 mg (Table 37.2). As predicted from the preclinical data, the most common dose-limiting toxicity was hypertension (n=7; reported only at doses ≥20 mg). Importantly, most cases of hypertension were manageable by standard antihypertensive therapy, and a hypertension management protocol has been developed for use in future clinical studies. This outcome is consistent with the results from a preclinical study of AZD2171 in conscious rats, which showed that hypertension resulting from inhibition of VEGF signalling could be reversed with an appropriate antihypertensive agent (Curwen et al. 2005). Hypertension has also been observed with clinical evaluation of small-molecule VEGF tyrosine kinase inhibitors other than AZD2171 (Drevs et al. 2002; Raymond et al. 2003; Veronese et al. 2004), as well as the anti-VEGF-A monoclonal antibody bevacizumab (Hurwitz et al. 2004; Johnson et al. 2004).

Table 37.1. Summary of phase I clinical trials for AZD2171

Study (code)	Patients (n)[a]	Status[b] and key findings[a]
Monotherapy		
AZD2171 dose-finding study in patients with advanced solid malignant tumours (2171IL/0001) (Drevs et al. 2006)	83	Completed: once-daily, oral AZD2171 at doses ≤45 mg/day was generally well tolerated and was associated with encouraging antitumour activity. Orally bioavailable; $t_{1/2}$ ~20 h
AZD2171 in patients with advanced prostate adenocarcinoma (2171IL/0003) (Ryan et al. 2005)	24	Completed: AZD2171 was considered to be well tolerated at doses ≤20 mg/day
Combination therapy		
AZD2171 in combination with carboplatin and paclitaxel in patients with advanced NSCLC (2171IL/0009) (Laurie et al. 2006)	20	Completed: adverse events were manageable and predictable, with encouraging evidence of antitumour activity with AZD2171 (30–45 mg) in combination with carboplatin and paclitaxel
AZD2171 in combination with gefitinib in patients with advanced cancer (2171IL/0004) (van Cruijsen et al. 2006)	70	Ongoing: no unexpected toxicities associated with AZD2171 (20–45 mg) in combination with gefitinib (250 or 500 mg); preliminary response data are encouraging
AZD2171 in combination with selected chemotherapy regimens (including mFOLFOX6, irinotecan, docetaxel and pemetrexed) in patients with advanced solid tumours (2171IL/0008) (LoRusso et al. 2006)	46	Ongoing: combination regimens have been well tolerated, with no unexpected toxicities, and have shown preliminary evidence of antitumour activity
AZD2171 in combination with selected chemotherapy regimens (including mFOLFOX6, irinotecan, docetaxel and pemetrexed) in patients with advanced solid tumours (2171IL/0008) (LoRusso et al. 2006)	46	Ongoing: combination regimens have been well tolerated, with no unexpected toxicities, and have shown preliminary evidence of antitumour activity

mFOLFOX6, fluorouracil, leucovorin and oxaliplatin, [a]As reported in the referenced publication, [b]Status as of May 2006

Following a single oral dose of AZD2171, maximal plasma concentration was measured at between 1 h and 8 h post dosing (overall median 3 h). The plasma concentration declined in an apparently biexponential manner, with a terminal half-life ranging from 12.4 h to 35.7 h (overall arithmetic mean 22±6.5 h). Following multiple once-daily dosing for 28 days, the maximal plasma concentration was measured at between 1 h and 6.4 h (overall median 2.1 h; Fig. 37.5). Steady-state plasma concentrations were attained after approximately 7 days. In addition, steady-state plasma concentrations were predicted by the single-dose pharmacokinetics, with the grand arithmetic mean temporal change parameter value being 0.988. This supports no auto-induction or auto-inhibition of AZD2171 metabolism following multiple doses. Following multiple oral doses of AZD2171 20 mg, the unbound $C_{ss,min}$ was almost 5 times greater than the measured IC_{50} for inhibition of HUVEC proliferation. This suggests that under steady-state conditions, AZD2171 at doses of 20 mg will produce plasma concentrations sufficient for inhibition of target enzymes throughout the entire 24-h dosing interval. This is supportive of the once-daily oral dosing of AZD2171.

Table 37.2. Grade 3 or 4 drug-related[a] adverse events occurring in more than one patient

		Dose of AZD2171 (mg)				
MedDRA-preferred term	CTC grade[b]	20 (n=19)	30 (n=21)	45 (n=19)	60 (n=8)	All (n=83)
Hypertension	3	4	3	4	2	13
Hypertensive crisis	4	2	0	1	0	3
GGT increased	3	0	3	0	0	3
PPE syndrome	3	0	0	2	1	3
Diarrhoea	3	0	1	1	0	2

Adverse events occurring at both grades 3 and 4 in the same patient are only counted once. *MedDRA*, Medical Dictionary for Regulatory Activities; *CTC*, Common Terminology Criteria; *GGT*, gamma-glutamyl transferase; *PPE* syndrome, palmar-plantar erythrodysaesthesia syndrome (hand-foot syndrome). [a]The investigator considered there was reasonable possibility that the event may have been caused by the study treatment or procedure. [b]No drug-related CTC grade 3 or 4 adverse events were reported at doses ≤10 mg

The following DCE-MRI assessments were used to measure the vascular response following AZD2171 treatment. The initial area under the curve (defined over 60 s; $iAUC_{60}$) parameter was based on the change in contrast agent concentration. Additionally, pharmacokinetic modelling was used to calculate the volume transfer constant (K^{trans}) of the contrast agent from the plasma to the extracellular extravascular space. Both $iAUC_{60}$ and K^{trans} represent composite measurements of vascular flow, volume and permeability (Parker et al. 1997; Tofts et al. 1999; Evelhoch 1999; Medved et al. 2004).

The specimen DCE-MRI images in Fig. 37.6 show that AZD2171 modulates tumour blood flow and vascular permeability. Following 28 days of multiple dosing with AZD2171 (0.5–60 mg) in parts A and B, multiple dose pharmacokinetic parameters (AUC_{ss}, $C_{max,ss}$ and $C_{min,ss}$) appeared to be a major determinant (R^2=0.33–0.49) of the decrease in tumour vascular permeability and blood flow as assessed by percentage change in $iAUC_{60}$ (Fig. 37.7). The relationship between each pharmacokinetic parameter and changes in $iAUC_{60}$ was significant (P<0.01). Findings from the statistical analysis of K^{trans} were

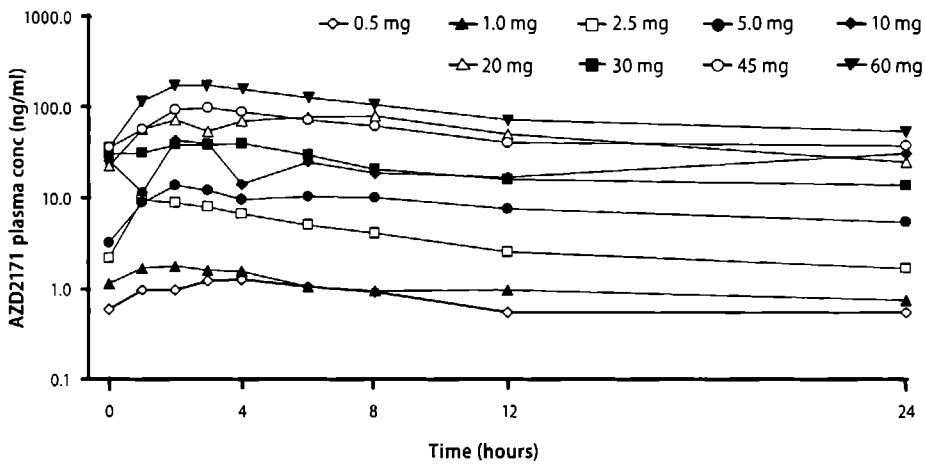
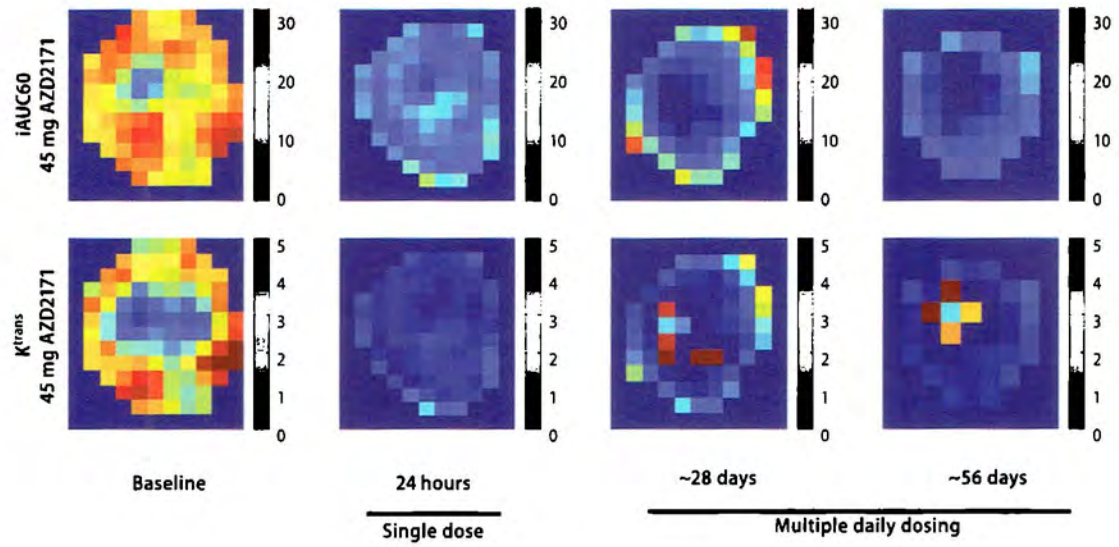


Fig. 37.5. AZD2171 plasma concentration–time profile after multiple oral dosing in part A. Data are shown as the geometric mean for each dose level (Drevs et al. 2007)



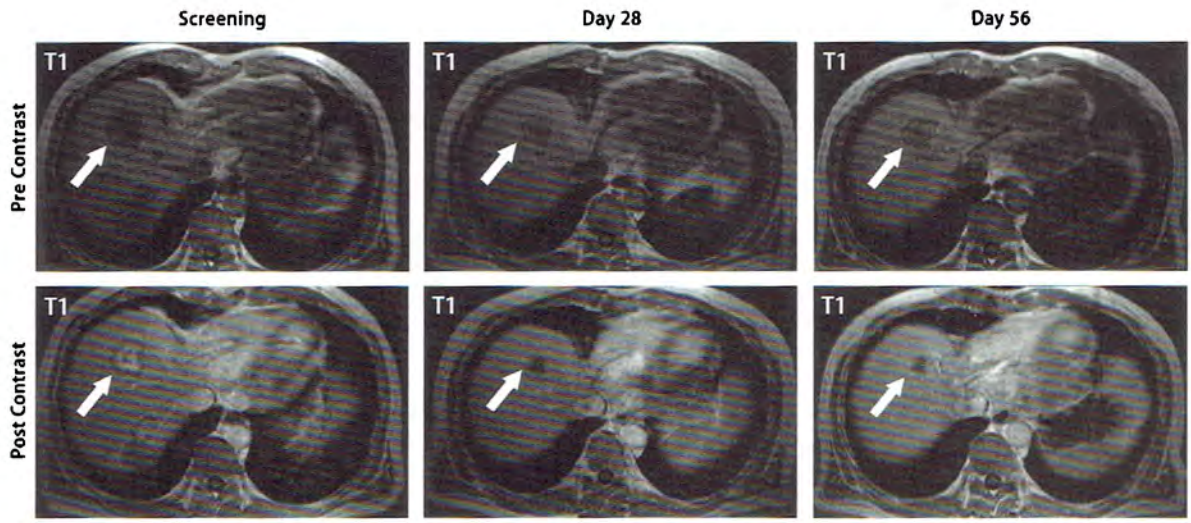


Fig. 37.6. *Top*: Example of DCE-MRI parametric images of tumour $iAUC_{60}$ and K^{trans} after single (24 h, day 2) and multiple daily dosing (28 and 56 days) with AZD2171. *Bottom*: Corresponding T1-weighted pre- and post-contrast MRI scans. This patient with prostate cancer experienced a best overall response of partial response and had overall tumour reductions in $iAUC_{60}$ (68% reduction at 24 h, 73% at 28 days and 84% at 56 days) and K^{trans} (67% reduction at 24 h, 83% at 28 days and 95% at 56 days) (Drevs et al. 2007)

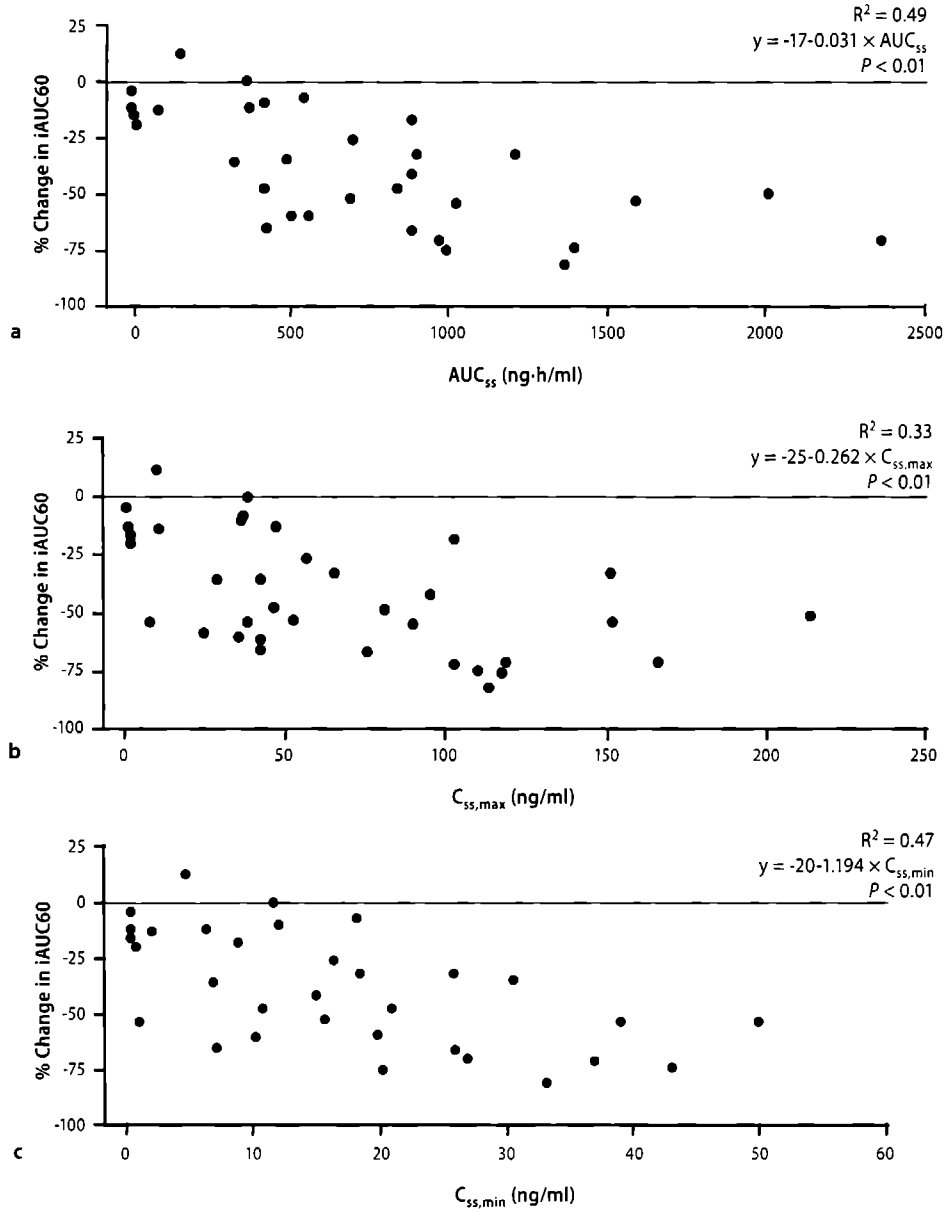


Fig. 37.7a–c. Relationship between change from baseline $iAUC_{60}$ at day 28 with a AUC_{ss}, b $C_{ss,max}$ and c $C_{ss,min}$ (Drevs et al. 2007)

consistent with those for $iAUC_{60}$. However, when the dose range was restricted to the doses explored in the randomized part B (20, 30 and 45 mg), the pharmacokinetic parameters were not a major determinant (R^2 range 0.12–0.35; data not shown). Statistical analyses revealed significant average reductions from baseline in $iAUC_{60}$ for all three doses in part B (Fig. 37.8), although there was no evidence of a dose effect when the dose range was restricted to these doses.

Once-daily dosing with AZD2171 was also associated with time- and dose-dependent reductions in levels of soluble VEGFR-2. Conversely, VEGF levels showed an apparent increase at all doses with no

evidence of a dose relationship. The decreased levels of VEGFR-2 and increased levels of VEGF may be related to the highly potent anti-angiogenic activity of AZD2171.

Tumour response evaluations confirmed partial responses in two patients, one each with prostate (45 mg) and renal (60 mg) cancer as the primary tumour type. A further 22 patients had stable disease, including two unconfirmed partial response (20 mg) and seven confirmed minor responses (20 mg; 10–30% reduction). The minor responses were observed in patients with the following primary tumour types: breast (n=2), colorectal, lung, head and neck, liver and skin/soft tissue (n=1 each). The smallest post-dose measurements of the target lesion size for each patient during the study suggested dose-related decreases in tumour size (Fig. 37.9).

In summary, the results of this phase I dose-finding study demonstrated that once-daily oral administration of AZD2171 at doses of ≤45 mg were generally well tolerated in patients with advanced cancer. In addition, the pharmacokinetic profile supports a once-daily dosing regimen. AZD2171 demonstrated exposure-related reductions in tumour blood flow and vascular permeability, time- and dose-related decreases in soluble VEGFR-2 levels and increased VEGF levels, all of which are consistent with inhibition of VEGF signalling. The encouraging preliminary efficacy data, including the dose-related decreases in tumour size, suggest that AZD2171 has clinical activity in a range of tumour types, including those refractory to previous treatment.

37.3.2 Other Phase I Studies

37.3.2.1 AZD2171 Monotherapy in Prostate Cancer

In this multicentre trial, patients with advanced, hormone-refractory prostate adenocarcinoma received once-daily oral doses of AZD2171 until a withdrawal criterion was met (Ryan et al. 2005). The primary objective was to assess the safety and tolerability of AZD2171. Secondary objectives included determination of the pharmacokinetic profile and preliminary evaluation of efficacy.

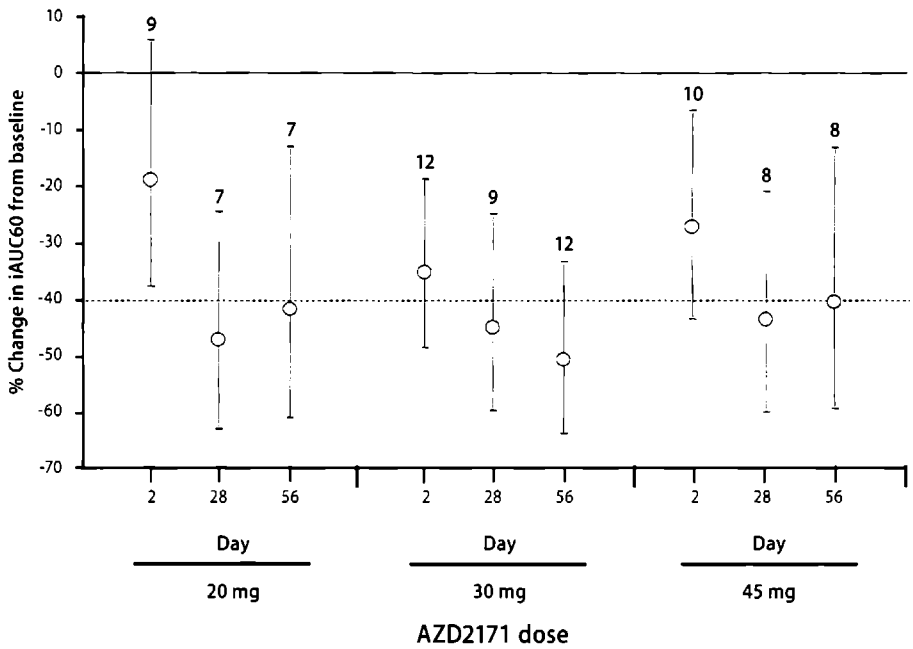


Fig. 37.8. Estimated percentage change from baseline in $iAUC_{60}$ (± 90% confidence interval) after 1 day, 28 days and 56 days (20, 30 and 45 mg). Changes at doses ≤10 mg were not significant. No significant differences were detected between the doses. The number of patients in each group is shown above the data points (Drevs et al. 2007)

Twenty-four patients received treatment with AZD2171 (1, 2.5, 5, 10, 20 or 30 mg), which was considered to be generally well tolerated at doses ≤20 mg. Adverse events were generally mild at doses ≤10 mg, with only fatigue, anorexia and nausea reported in more than one patient. Among the 10 patients in the 20 mg cohort, grade 3 adverse events considered by the investigator to be possibly drug related were hypertension (n=1), myalgia (n=1) and transient ischaemic attack (n=1). Accrual to the 30 mg cohort was discontinued per protocol after two patients experienced a total of three suspected dose-limiting toxicities (n=1 each for fatigue, muscle weakness and hypertension, all grade 3).

Plasma concentrations at all dose levels reached their maximal value at 2–8 h post dosing with an overall median value of 2 h. Steady-state plasma concentrations were attained after approximately 7 days of repeated once daily dosing. The observed accumulation ratio supports an effective half-life of approximately 25 h. Following multiple oral doses of 20 mg, the unbound $C_{ss,min}$ is 5 times above the HUVEC proliferation IC_{50}. These data are consistent with the pharmacokinetic profile determined in the initial dose-finding study and provide further evidence supporting the once-daily oral dosing regimen of AZD2171 (Ryan et al. 2005).

In this refractory population, one patient receiving 20 mg had a prostate-specific antigen (PSA) decline of 30%. A 30-day follow-up showed a second patient in this cohort experienced a >50% decrease in PSA and computed tomography revealed complete resolution of retroperitoneal adenopathy. This patient has remained progression-free for >6 months after stopping treatment and has not required any further antitumour therapy. The final results from this study are expected to be available late 2006.

37.3.2.2 AZD2171 with Carboplatin and Paclitaxel in NSCLC

This recently completed phase I study recruited 20 patients with stage IIIB/IV non-small-cell lung cancer (NSCLC) who had not received prior chemotherapy for metastatic disease and had no significant haemoptysis or bleeding (Laurie et al. 2006). The primary objective was to determine the recommended dose of AZD2171 to be given with standard doses of carboplatin and paclitaxel in phase II studies. Secondary objectives included evaluations of tolerability, safety, pharmacokinetics and antitumour activity.

Patients received once-daily oral doses of AZD2171 30 mg (n=9) or 45 mg (n=11) with carboplatin (tar-

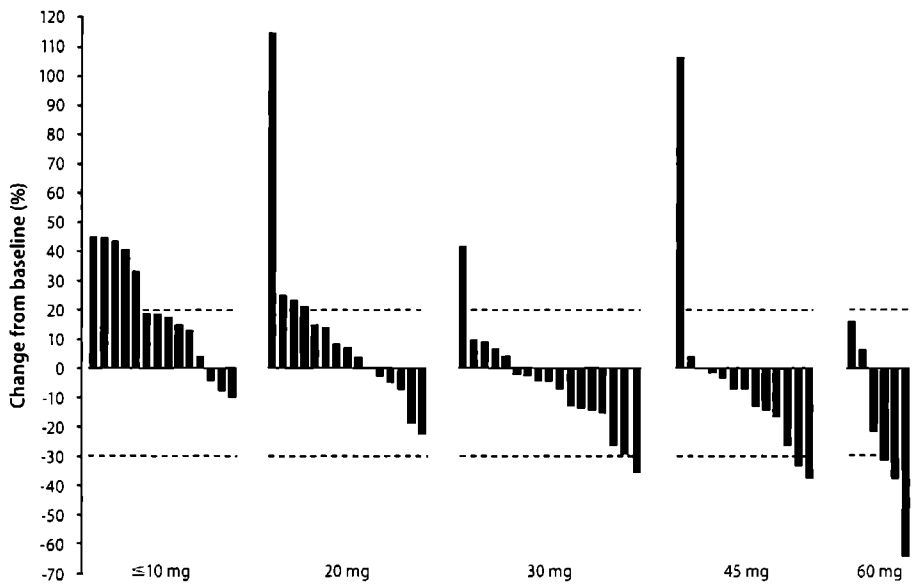


Fig. 37.9. Smallest post-dose measurement of target lesion size from baseline. Data not available for 18 patients: 16 withdrew prior to scanning due to adverse events (n=11), progressive disease (n=4) or unwillingness to continue study (n=1); no scan data available for 2 patients (Drevs et al. 2007)

get AUC=6 mg/ml·min) and paclitaxel (200 mg/m^2). Adverse events appeared to be manageable. Dose-limiting toxicity was observed in one patient from each cohort: grade 3 increased alanine aminotransferase (30 mg) and grade 3 febrile neutropenia with grade 3 mucositis (45 mg). Six patients experienced hypertension (grade 2), which prompted the use of a standardized algorithm for management of this adverse event.

There was encouraging preliminary evidence of antitumour activity with this combination regimen. Among the 15 patients evaluable for tumour response, six showed a partial response, eight had stable disease (many with evidence of tumour shrinkage) and one patient had progressive disease.

37.3.2.3 AZD2171 with Selected Chemotherapy Regimens in Advanced Cancer

This phase I trial in patients with heavily pretreated solid tumours was designed to evaluate the safety and tolerability of escalating doses of AZD2171 (20, 30 and 45 mg) in combination with selected chemotherapy regimens, including mFOLFOX6 (oxaliplatin 85 mg/m^2, 5-FU 400 mg/m^2, leucovorin 400 mg/m^2 every 2 weeks; arm 1); irinotecan 300 mg/m^2 every 3 weeks (arm 2); docetaxel 75 mg/m^2 every 3 weeks (arm 3) and pemetrexed 500 mg/m^2 every 3 weeks (arm 4) (Lo-Russo et al. 2006). Secondary objectives include evaluation of any potential pharmacokinetic interaction and clinical efficacy.

Forty-six patients had been recruited to this ongoing study by January 2006. Dose-limiting toxicities (grade 3 unless stated) considered to be related to combination therapies included: arm 1 – diarrhoea and fatigue; arm 2 – neutropenia (grade 3 or 4) and thrombocytopenia (grade 3 or 4); arm 3 – fatigue, neutropenia (grade 3 or 4), abdominal pain, hand–foot syndrome, leucopoenia and small intestinal obstruction; arm 4 – fatigue and hypertension.

AZD2171 did not appear to alter the pharmacokinetic profiles of any chemotherapy regimen tested, and the steady-state pharmacokinetic values of AZD2171 are similar to those observed from monotherapy studies. Preliminary response data have been encouraging in this heavily pretreated population, with three partial tumour responses and a further eight patients experiencing stable disease.

37.3.2.4 AZD2171 with Gefitinib in Advanced Cancer

The primary aim of this phase I trial is to assess the safety and tolerability of AZD2171 in combination with gefitinib (IRESSA®), an inhibitor of EGFR tyrosine kinase (van Cruijsen et al. 2006). As of September 2005, 70 patients with advanced solid tumours refractory to standard therapy had received once-daily oral doses of AZD2171 (20, 25, 30 or 45 mg) in combination with gefitinib (250 or 500 mg).

Table 37.3. Summary of key phase II and phase II/II clinical trials

Study	Tumour type	Primary objectives
Phase II		
AZD2171+FOLFOX vs bevacizumab+FOLFOX	Second-line metastatic CRC	Assessment of PFS
Phase II/III		
Paclitaxel and carboplatin ± AZD2171	First-line NSCLC	Assessment of PFS and overall survival. Determination of pharmacogenomics and pharmacodynamic aspects of these regimens.

CRC, colorectal cancer; *FOLFOX*, fluorouracil, leucovorin and oxaliplatin; *NSCLC*, non-small cell lung cancer; *PFS*, progression-free survival;

There have been no unexpected toxicities associated with combination treatment. The most commonly reported grade 3 or 4 adverse events were diarrhoea, anorexia, hypertension and fatigue.

The steady-state pharmacokinetic profile for AZD2171 30 mg and gefitinib 250 mg is consistent with that seen previously for either agent alone. Partial tumour responses have been confirmed in two patients, one each with mesothelioma and renal cancer as the primary tumour type; following surgery, the patient with renal cancer is currently free from disease. Stable disease was reported for a further 28 patients.

37.3.3 Summary of Clinical Development Programme

The phase I programme has demonstrated that once-daily, oral administration of AZD2171 is generally well tolerated, with a manageable adverse event profile. Moreover, the encouraging preliminary evidence of efficacy in a range of tumour types warrants further clinical evaluation, and recruitment to a series of randomized, double-blind phase II and phase II/III trials is currently under way (Table 37.3).

Conclusions and Future Directions

AZD2171 is one of the most potent inhibitors of VEGFR-2 tyrosine kinase activity in development. Preclinical studies have demonstrated that AZD2171 inhibits VEGF-dependent signalling, angiogenesis and neovascular survival. AZD2171 is also a potent inhibitor of VEGFR-1 and -3 tyrosine kinases, and shows selectivity for VEGFRs versus a range of other kinases. Consistent with an anti-angiogenic effect, once-daily treatment with AZD2171 produced dose-dependent inhibition of tumour growth in a broad range of established human tumour xenografts.

A series of phase I studies have been conducted to investigate AZD2171 in patients with cancer, both as monotherapy and in combination with certain other anticancer strategies. These investigations have shown AZD2171 to be generally well tolerated, with side effects that are tolerable and manageable. Currently available pharmacokinetic data are supportive of a once-daily oral dosing schedule for AZD2171. Furthermore, preliminary efficacy data demonstrate that AZD2171 has potential antitumour activity in multiple tumour types. Recruitment to a number of clinical trials has been initiated to further determine the activity of AZD2171 in a wide range of tumours.

Acknowledgements

IRESSA® is a registered trade mark of the AstraZeneca group of companies. is We thank Dr Jen Jackson, who provided medical writing support funded by AstraZeneca.

References

Autiero M, Luttun A, Tjwa M, Carmeliet P (2003) Placental growth factor and its receptor, vascular endothelial growth factor receptor-1: novel targets for stimulation of ischemic tissue revascularization and inhibition of angiogenic and inflammatory disorders. J Thromb Haemost 1:1356–1370

Cao Y (2005) Opinion: emerging mechanisms of tumour lymphangiogenesis and lymphatic metastasis. Nat Rev Cancer 5:735–743

Curwen JO, Musgrove HL, Graham GA, Kendrew J, Richmond GHP, Ogilvie DJ, Wedge SR (2005) Management of hypertension induced by AZD2171, an oral, highly potent and reversible VEGFR signaling inhibitor, in rat. Clin Cancer Res 11: abstract B9

Drevs J, Muller-Driver R, Wittig C, Fuxius S, Esser N, Hugenschmidt H, Konerding MA, Allegrini PR, Wood J, Hennig J, Unger C, Marme D (2002) PTK787/ZK 222584, a specific vascular endothelial growth factor-receptor tyrosine kinase inhibitor, affects the anatomy of the tumor vascular bed and the functional vascular properties as detected by dynamic enhanced magnetic resonance imaging. Cancer Res 62:4015–4022

Drevs J, Esser N, Wedge SR, Ryan AJ, Oglivie DJ, Marme D (2004) Effect of AZD2171, a highly potent VEGF recep-

tor tyrosine kinase inhibitor, on primary tumor growth, metastasis and vessel density in murine renal cell carcinoma. Proc Am Assoc Cancer Res 45:1051

Drevs J, Siegert P, Medinger M, Mross K, Strecker R, Zirrgiebel U, Harder J, Blum H, Robertson J, Jürgensmeier JM, Puchalski TA, Young H, Saunders O, Unger C (2007) Phase I clinical study of AZD2171, an oral, highly potent VEGF signaling inhibitor, in patients with advanced solid tumors. J Clin Oncol (in press)

Escudier B, Szczylik C, Eisen T, Stadler WM, Schwartz B, Shan M, Bukowski RM (2005) Randomized Phase III trial of the Raf kinase and VEGFR inhibitor sorafenib (BAY 43-9006) in patients with advanced renal cell carcinoma (RCC). Proc Am Soc Clin Oncol abstract 4510

Evelhoch JL (1999) Key factors in the acquisition of contrast kinetic data for oncology. J Magn Reson Imaging 10:254–259

Ferrara N, Gerber HP, LeCouter J (2003) The biology of VEGF and its receptors. Nat Med 9:669–676

Gille H, Kowalski J, Li B, LeCouter J, Moffat B, Zioncheck TF, Pelletier N, Ferrara N (2001) Analysis of biological effects and signaling properties of Flt-1 (VEGFR-1) and KDR (VEGFR-2). A reassessment using novel receptor-specific vascular endothelial growth factor mutants. J Biol Chem 276:3222–3230

Goodlad RA, Watkins AJ, Poulsom R, Ryan A, Wedge SR, Pyrah IT, Mandir N, Alferez DG, Wilkinson RW (2005) AZD2171, an orally active inhibitor of VEGFR tyrosine kinase activity, reduces polyp burden in a mouse model of intestinal cancer. Proc Am Assoc Cancer Res 46: abstract 5841

Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R, Kabbinavar F (2004) Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 350:2335–2342

Johnson DH, Fehrenbacher L, Novotny WF, Herbst RS, Nemunaitis JJ, Jablons DM, Langer CJ, DeVore RF, III, Gaudreault J, Damico LA, Holmgren E, Kabbinavar F (2004) Randomized phase II trial comparing bevacizumab plus carboplatin and paclitaxel with carboplatin and paclitaxel alone in previously untreated locally advanced or metastatic non-small-cell lung cancer. J Clin Oncol 22:2184–2191

Jussila L, Alitalo K (2002) Vascular growth factors and lymphangiogenesis. Physiol Rev 82:673–700

Laurie SA, Arnold A, Gauthier I, Chen E, Goss G, Ellis P, Shepherd F, Matthews S, Robertson J, Seymour L (2006) Final results of a phase I study of daily oral AZD2171, an inhibitor of the vascular endothelial growth factor receptors (VEGFR), in combination with carboplatin (C) + paclitaxel (T) in patients with advanced non-small cell lung cancer (NSCLC): a study of the National Cancer Institute of Canada Clinical Trials Group. Proc Am Soc Clin Oncol abstract 3054

LoRusso P, Heath E, Malburg L, Pilat M, DeLuca P, Wozniak A, Gadgeel S, Puchalski T, Ewesuedo R (2006) Phase I multi-cohort study evaluating the safety and tolerability of AZD2171 in combination with various anticancer regimens. Ann Oncol 17 [Suppl 9]:ix136, abstract 405P

Medved M, Karczmar G, Yang C, Dignam J, Gajewski TF, Kindler H, Vokes E, MacEneany P, Mitchell MT, Stadler WM (2004) Semiquantitative analysis of dynamic contrast enhanced MRI in cancer patients: variability and changes in tumor tissue over time. J Magn Reson Imaging 20:122–128

Meyer M, Clauss M, Lepple-Wienhues A, Waltenberger J, Augustin HG, Ziche M, Lanz C, Buttner M, Rziha HJ, Dehio C (1999) A novel vascular endothelial growth factor encoded by Orf virus, VEGF-E, mediates angiogenesis via signalling through VEGFR-2 (KDR) but not VEGFR-1 (Flt-1) receptor tyrosine kinases. EMBO J 18:363–374

Miller KD, Wang M, Gralow J, Dickler M, Cobleigh MA, Perez EA, Shenkler TN, Davidson NE (2005) A randomized Phase III trial of paclitaxel versus paclitaxel plus bevacizumab as first-line therapy for locally recurrent or metastatic breast cancer. ASCO oral presentation (no abstract available)

Motzer RJ, Rini BI, Michaelson MD, Redman BG, Hudes GR, Wilding G, Bukowski RM, George DJ, Kim ST, Baum CM, the SU11248 Study Group (2005) Phase 2 trials of SU11248 show antitumor activity in second-line therapy for patients with metastatic renal cell carcinoma (RCC). Proc Am Soc Clin Oncol abstract 4508

Parker GJ, Suckling J, Tanner SF, Padhani AR, Revell PB, Husband JE, Leach MO (1997) Probing tumor microvascularity by measurement, analysis and display of contrast agent uptake kinetics. J Magn Reson Imaging 7:564–574

Raymond E, Faivre S, Vera K, Delbaldo C, Robert C, Spatz A, Lozahic S, Bello C, Deprimo S, Brega N, Scigala P, Armand J-P (2003) Final results of a phase I and pharmacokinetic study of SU11248, a novel multi-target tyrosine kinase inhibitor, in patients with advanced cancers. Proc Am Soc Clin Oncol abstract 769

Ryan C, Stadler WM, Roth B, Puchalski T, Morris C, Small E (2005) Phase I evaluation of AZD2171, a highly potent VEGFR tyrosine kinase inhibitor, in patients with advanced prostate adenocarcinoma. Clin Cancer Res 11: abstract A4

Sandler AB, Gray R, Brahmer J, Dowlati A, Schiller JH, Perry MC, Johnson DH (2005) Randomized phase II/III trial of paclitaxel (P) plus carboplatin (C) with or without bevacizumab (NSC # 704865) in patients with advanced non-squamous non-small cell lung cancer (NSCLC): An Eastern Cooperative Oncology Group (ECOG) Trial - E4599. Proc Am Soc Clin Oncol abstract LBA4

Strecker R, Scheffler K, Buchert M, Mross K, Drevs J, Hennig J (2003) DCE-MRI in clinical trials: data acquisition techniques and analysis methods. Int J Clin Pharmacol Ther 41:603–605

Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, van Glabbeke M, van Oosterom AT, Christian MC, Gwyther SG (2000) New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 92:205–216

Tofts PS, Brix G, Buckley DL, Evelhoch JL, Henderson E, Knopp MV, Larsson HB, Lee TY, Mayr NA, Parker GJ, Port RE, Taylor J, Weisskoff RM (1999) Estimating kinetic parameters from dynamic contrast-enhanced T(1)-weighted MRI of a diffusable tracer: standardized quantities and symbols. J Magn Reson Imaging 10:223–232

Van Cruijsen H, Voest EE, van Herpen CML, Hoekman K, Witteveen PO, Tjin-A-Ton ML, Punt CJ, Puchalski T, Milenkova T, Giaccone G (2006) Phase I evaluation of AZD2171, a highly potent, selective VEGFR signaling inhibitor, in combination with gefitinib, in patients with advanced tumors. Proc Am Soc Clin Oncol abstract 3017

Veronese ML, Flaherty KT, Townsend R, Osenkis A, Zimmer R, Rosen M, Gallagher M, O'Dwyer PJ (2004) Pharmacodynamic study of the raf kinase inhibitor BAY 43-9006: mechanisms of hypertension. J Clin Oncol 22 [Suppl]: abstract 2035

Wedge SR, Kendrew J, Hennequin LF, Valentine PJ, Barry ST, Brave SR, Smith NR, James NH, Dukes M, Curwen JO, Chester R, Jackson JA, Boffey SJ, Kilburn LL, Barnett S, Richmond GH, Wadsworth PF, Walker M, Bigley AL, Taylor ST, Cooper L, Beck S, Jürgensmeier JM, Ogilvie DJ (2005) AZD2171: a highly potent, orally bioavailable, vascular endothelial growth factor receptor-2 tyrosine kinase inhibitor for the treatment of cancer. Cancer Res 65:4389–4400

Wu W, Fujitaka K, Mandal J, Imagumbai T, Ryan A, Jürgensmeier JM, Fidler IJ, O'Reilly MS, Herbst RS (2005) AZD2171, an oral, highly potent VEGFR signaling inhibitor, in combination with gefitinib or paclitaxel: results of a study in an orthotopic human lung adenocarcinoma model. Clin Cancer Res 11: abstract B7

Clinical Development of Sunitinib Malate

38

Isan Chen, Carlo Bello, and Zuleima Aguilar

Abstract

Sunitinib malate (SUTENT®, SU11248, Pfizer Inc.) is an oral multitargeted receptor tyrosine kinase (RTK) inhibitor with antiangiogenic and antitumor activity. RTKs are transmembrane proteins, the dysregulated activity of which has been linked to the growth and metastatic spread of human cancers. Sunitinib has shown efficacy in phase II and phase III trials of metastatic renal cell carcinoma (RCC) and gastrointestinal stromal tumors (GIST). The phase III study in imatinib-resistant or -intolerant GIST patients was unblinded early when the planned interim analysis revealed a significant time to progression advantage for sunitinib compared with placebo (27.3 weeks vs 6.4 weeks; $P<0.0001$). In the recently reported phase III comparison of first-line sunitinib and interferon alfa (IFN-α) in metastatic RCC patients, treatment with sunitinib also resulted in a significantly longer progression-free survival than IFN-α (11 vs 5 months; $P<0.001$). Sunitinib is well tolerated and has a consistent, predictable safety profile. The most common treatment-related adverse events reported in the clinical program have been fatigue, diarrhea, nausea, vomiting, and anorexia. Fatigue was not commonly associated with treatment discontinuation. Gastrointestinal toxicities were generally manageable. Encouraging results from single-agent studies in other tumor types such as metastatic breast cancer and non-small cell lung cancer have led to planned and ongoing investigations of this agent in first- and second-line combination studies in these and other common solid tumor types.

CONTENTS

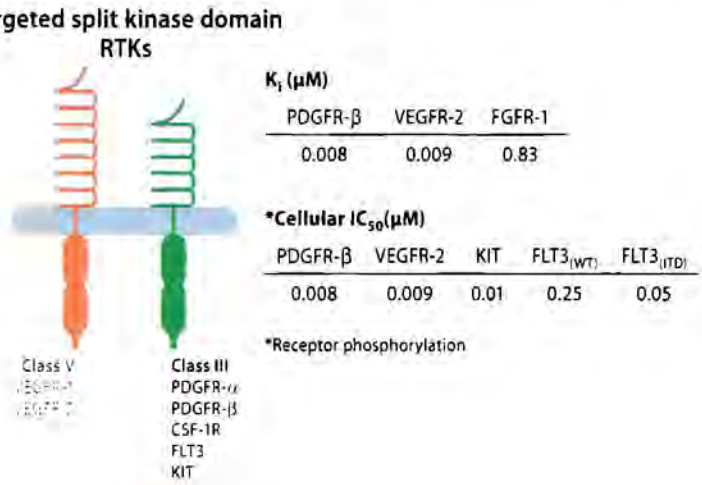
Introduction

Sunitinib malate (SUTENT®, SU11248, Pfizer Inc.) is an oral multitargeted tyrosine kinase inhibitor with antiangiogenic and antitumor activity in clinical development for a variety of advanced solid malignancies. It emerged from a drug discovery program that sought to identify small molecules with suitable pharmacologic properties capable of inhibiting the activity of selected receptor tyrosine kinases (RTKs), the dysregulated activity of which has been linked to various human cancers.

RTKs are transmembrane proteins containing an extracellular ligand-binding domain and an intracellular catalytic domain. Ligand-bound, or activated, RTKs use ATP to catalyze the phosphorylation of tyrosine residues located in the intracellular domain of the receptor, setting off a signaling cascade within the cell. Many of the processes involved in tumor growth, progression, and metastasis are mediated by signaling molecules acting downstream from activated RTKs (Hanahan and Weinberg 2000). In addition to their direct role in tumor cell growth and survival, several RTKs, most notably the vascular endothelial cell receptors (VEGFR-1, -2, and -3) and platelet-derived growth factor receptors (PDGFR-α, -β), are implicated in tumor-dependent angiogenesis (Hicklin and Ellis 2005; Saharinen and Alitalo 2003; Yu et al. 2003). These RTKs are expressed not only on tumor cells, but also on cells in the tumor stroma and supportive environment, including vascular endothelial cells, pericytes and fibroblasts (Saharinen and Alitalo 2003), and tumor development depends upon complex interactions between these different cell types.

Therapeutic approaches using RTK inhibition have involved strategies to target either a single pathway or simultaneous inhibition of multiple pathways. The involvement of multiple cellular abnormalities in tumor development suggests that a multitargeted approach, including inhibition of RTKs (or their associated ligands) on both tumor and extratumor cells, may be associated with greater clinical benefit than use of agents targeting only a single pathway (Hanahan and Weinberg 2000). For example, the pivotal study by Yang et al suggests that targeting the VEGFR pathway with an anti-VEGF antibody may be of use in renal cell carcinoma (RCC) (Yang et al. 2003). However, the investigators concluded that, given the modest clinical activity exhibited by high-dose bevacizumab (10 mg/kg every other week) in patients with metastatic RCC and the known involvement of other proteins in promoting angiogenesis, future treatments should target more than VEGF alone. There exists, for instance, strong evidence that PDGFRs are critical in recruitment of pericytes to nascent vessels during neoangiogenesis, resulting in formation of more mature and stable tumor vasculature (Pietras et al. 2003; Bergers and Song 2005), making it an appropriate additional target. In preclinical experiments, dual inhibition of VEGFR and PDGFR has produced greater antitumor and antiangiogenic effects than inhibition of either receptor type alone (Bergers et al. 2003; Erber et al. 2004; Potapova et al. 2006).

Sunitinib is a potent and selective inhibitor of split kinase domain RTKs (Fig. 38.1), including VEGFR, PDGFR, stem cell factor receptor (KIT), the RTK encoded by the ret proto-oncogene (RET), the receptor for M-CSF (CSF-1R), and Fms-like tyrosine kinase-3 receptor (FLT3), and has shown potent antitumor activity in a range of mouse tumor models (Abrams et al. 2003a, 2003b; Mendel et al. 2003; O'Farrell et al. 2003; Schueneman et al. 2003; Sun et al. 2003). Initial characterization of sunitinib in a series of biochemical assays demonstrated it to be an ATP-competitive inhibitor of the catalytic activity of VEGFR-1, -2, and -3 (K_i=2, 9, and 17 nM, respectively) and PDGFR-β (K_i=8 nM). Potent inhibition of target RTK phosphorylation or ligand-stimulated RTK-target-dependent cell proliferation by sunitinib (and its active N-desethyl metabolite, SU12662) in cells was demonstrated utilizing cell lines that either normally express or were engineered to express *VEGFR-2*, *PDGFR-β*, *KIT*, *FLT3-ITD*, or a mutant form of *RET* associated with endocrine tumors (Mendel et al. 2003; Pfizer, data on file). Phosphorylation of each of these RTKs was inhibited at a comparable potency range (0.008–0.05 μM), indicating that pharmacologically relevant inhibitory activity against each of these



K_i (μM)

PDGFR-β	VEGFR-2	FGFR-1
0.008	0.009	0.83

*Cellular IC_{50}(μM)

PDGFR-β	VEGFR-2	KIT	$FLT3_{(WT)}$	$FLT3_{(ITD)}$
0.008	0.009	0.01	0.25	0.05

*Receptor phosphorylation

Fig. 38.1. Sunitinib exhibits potent and selective RTK inhibition (Mendel et al. 2003; Pfizer, data on file)

RTKs could be attained at a given dose in vivo (Mendel et al. 2003; Pfizer, data on file).

Based on the favorable preclinical properties of sunitinib, clinical studies in healthy volunteers began in December 2000 and in cancer patients in April 2001. Sunitinib is currently being investigated in several cancer indications in phase II and III trials and has received multinational approval for the treatment of advanced RCC and of imatinib-resistant or -intolerant gastrointestinal stromal tumors (GIST).

38.2 Molecular Formula, Structure, and Formulation

Sunitinib is a small molecule with the molecular formula $C_{22}H_{27}FN_4O_2$. The free base has a molecular weight of 398.48 and the L-malate salt, the form used in clinical trials (Fig. 38.2), has a molecular weight of 532.56. The chemical name of the L-malate salt is (Z)-*N*-[2-(diethylamino)ethyl]-5-[(5-fluoro-2-oxo-1,2-dihydro-3*H*-indol-3-ylidene)methyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxamide (S)-2-hydroxysuccinate.

Sunitinib is an oral drug dispensed as hard gelatin capsules containing the free-base equivalent of 12.5, 25, and 50 mg of sunitinib L-malate.

38.3 Clinical Development

The clinical pharmacokinetics, safety and efficacy of sunitinib have been studied in key phase I, II, and III clinical trials, and as of November 2006 approximately 17,000 patients with advanced malignancies, distributed equally across clinical trials and marketing experience, had been treated with sunitinib.

38.3.1 Clinical Pharmacokinetics

The results of both single-dose and multiple-dose studies have demonstrated the favorable pharmacokinetic properties of sunitinib in humans. Pharmacokinetic studies show that sunitinib is well absorbed and that its bioavailability is unaffected by food (Bello et al. 2006a). After absorption, sunitinib is converted to its active N-desethyl metabolite, SU12662 (Sakamoto 2004; Bello et al. 2006a). Because SU12662 has a similar inhibitory profile to sunitinib in preclinical assays, the combination of sunitinib plus SU12662 represents the total active drug in plasma.

Plasma levels of sunitinib peak between 6 h and 12 h after a single oral dose, and pharmacokinet-

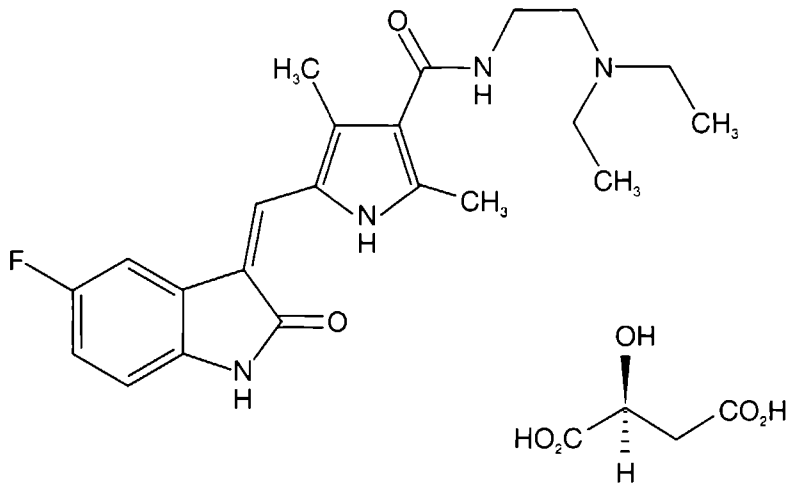


Fig. 38.2. The structural formula of sunitinib L-malate salt

ics are linear at doses of 50–100 mg/day (Sakamoto 2004). With repeated daily administration, the AUC_{0-24} for sunitinib increases 3- to 4-fold, while the AUC_{0-24} for SU12662 increases 7- to 10-fold, compared to day 1; steady-state concentrations are typically achieved within 10–14 days. There is no additional drug accumulation upon repeated dosing (Pfizer, data on file). No significant differences in pharmacokinetics are observed comparing either repeated dosing with single dosing, or healthy volunteers with cancer patients. In addition, the pharmacokinetic profile of sunitinib and SU12662 was not shown to be altered in subjects with mild or moderate hepatic impairment as compared to subjects with normal liver function (Bello et al. 2006b). A similar starting dose of sunitinib is therefore recommended in patients with mild or moderate hepatic impairment. Sunitinib and SU12662 have prolonged terminal half-lives of approximately 40–60 h and 80–110 h, respectively (Pfizer, data on file).

Both sunitinib and SU12662 are metabolized predominantly by cytochrome P450 3A4 (CYP3A4) enzyme and elimination is primarily via the feces (61% of administered dose) and secondarily in urine (16%) (Pfizer, data on file). SU12662 comprises 23–37% of the total exposure.

Concurrent administration of sunitinib with the potent CYP3A4 inhibitor ketoconazole resulted in 49% and 51% increases in the combined (sunitinib+SU12662) C_{max} and $AUC_{0-\infty}$ values, respectively, compared with sunitinib alone (Washington et al. 2003). Results from a study of sunitinib administered with the potent CYP3A4 inducer rifampin showed reductions of 23% and 46% in the combined C_{max} and AUC, respectively, compared with sunitinib alone (Bello et al. 2005). These data indicate that dose adjustments for sunitinib should be considered when co-administered with CYP3A4 inhibitors and inducers. Sunitinib and its metabolite appear to have minimal potential to inhibit or induce CYP3A4-mediated metabolism.

38.3.2 Phase I Dose-finding Studies

Phase I dose-escalation studies of patients with solid tumors were designed to determine the primary dose-limiting toxicity (DLT) and maximum tolerated dose (MTD) for sunitinib (Rosen et al. 2003; Faivre et al. 2006; Demetri et al. 2003). Studies were also designed to determine a safe and effective treatment schedule for repeated dosing. Patients in the multiple-dose studies received daily oral doses of 25–150 mg, according to one of three dosing schedules: the 4/2 schedule, consisting of 4 weeks on treat-

ment followed by 2 weeks off; the 2/2 schedule, consisting of 2 weeks on treatment followed by 2 weeks off; and the 2/1 schedule, with 2 weeks on treatment followed by 1 week off.

The primary DLT in the phase I studies was fatigue/asthenia, which generally occurred 1–2 weeks after the start of therapy and was readily reversible during the portion of the cycle when sunitinib was not administered (Rosen et al. 2003; Faivre et al. 2006; Demetri et al. 2003). Additional adverse events in phase I trials included nausea, vomiting, diarrhea, neutropenia, thrombocytopenia, and skin/hair discoloration. The frequency and severity of adverse events were generally correlated with doses higher than 50 mg/day and were generally manageable and reversible.

Consequently, the MTD of sunitinib was defined as 50 mg daily in phase I clinical studies in patients with advanced solid tumors (Rosen et al. 2003; Faivre et al. 2006; Demetri et al. 2003). Patients receiving this dose achieved combined trough plasma concentrations of sunitinib plus SU12662 of >50 ng/ml. Since sunitinib and SU12662 are 95% and 90% plasma protein bound, respectively, >50 ng/ml, in humans, is the concentration predicted from preclinical data to inhibit receptor phosphorylation and result in tumor regression. As the 4/2 schedule provided the longest drug exposure, compared to the 2/2 and 2/1 schedules, it was selected for phase II and III studies. A continuous dosing schedule of sunitinib at 37.5 mg/day is being investigated.

Clinically relevant antitumor activity was demonstrated in patients with solid tumors in phase I studies (Raymond et al. 2003; Rosen et al. 2003; Pfizer, data on file). Confirmed partial responses were achieved by 19 patients treated at the 50 mg or 75 mg dose level with either the 4/2 schedule or the 2/2 schedule in phase I studies, which included patients with the following tumor types: GIST, RCC, neuroendocrine tumor (NET), sarcoma, thyroid carcinoma, non-small-cell lung carcinoma (NSCLC), and malignant melanoma. These early findings suggested that sunitinib has applicability to a broad range of human malignancies, as expected from its mechanism of action.

38.3.3 Rationale to Target GIST and RCC

Sunitinib showed efficacy in all the diverse, preclinical models in which it was evaluated as a single agent or in combination, and regression occurred in multiple tumor types. As discussed above, phase I studies in cancer patients were likewise encouraging, with clinical responses demonstrated in advanced stages of several different tumor types.

Malignant GIST is a sarcoma comprised of tumors of mesenchymal origin that occur primarily in the gastrointestinal (GI) tract and abdomen. While GIST is a rare neoplasm, estimated to represent <1% of all GI tumors, it is the most common mesenchymal malignancy of the GI tract. Before the advent of molecularly targeted therapies, nearly half of GIST patients presented with metastatic disease, with a median survival of 19 months (DeMatteo et al. 2000).

The vast majority (>95%) of malignant GISTs express the CD117 (KIT) tyrosine kinase receptor, with activating *KIT* gene mutations present in approximately 85% of GIST patients (Corless et al. 2004; Hirota et al. 1998), resulting in constitutive activation of the KIT protein, proliferation of GIST cells, and inhibition of apoptosis. Approximately 35% of GISTs lacking detectable *KIT* mutations harbor an alternative oncogenic signal consisting of intragenic activating *PDGFRA* mutations (Heinrich et al. 2003a, 2003b; Hirota et al. 1998).

Before tyrosine kinase inhibitors became available, there were no effective systemic therapies for advanced GIST. However, trials with the selective KIT tyrosine kinase inhibitor imatinib mesylate have shown that inhibition of the uncontrolled kinase function of KIT is an effective anticancer therapy for the majority of patients with malignant GIST (Benjamin et al. 2003; Verweij et al. 2004). In spite of these results, approximately 12–14% of GIST patients have primary resistance to imatinib (Demetri et al. 2002; van Glabbeke et al. 2005), another 8% discontinue imatinib therapy due to intolerance (Gleevec (imatinib mesylate) prescribing information 2005), and >40% of initially responsive GIST patients develop

secondary imatinib resistance after a median of 25 months of treatment (van Glabbeke et al. 2005). Therefore, GIST patients intolerant or resistant to imatinib represent an unmet medical need. Because KIT is one of the primary targets of sunitinib and early evidence of efficacy was observed in GIST patients in phase I trials, imatinib-resistant GIST was an indication pursued for further clinical development of sunitinib.

RTK activity appears to play a prominent role in the malignant transformation, growth (L11), and metastasis of RCC, often through the involvement of the von Hippel-Lindau gene (*VHL*), which is believed to be inactivated in as many as 80% of sporadic clear-cell RCCs by deletion, mutation (L15), or methylation (Gnarra et al. 1994; Herman et al. 1994). This tumor suppressor gene encodes a protein that is involved in regulating the transcription of VEGF, PDGF-β (L18), and a number of other hypoxia-inducible proteins. *VHL* inactivation leads to overexpression of these agonists of VEGFR and PDGFR, amongst others, which may promote tumor angiogenesis, tumor growth (L22), and metastasis. VEGFR and PDGFR are primary targets of sunitinib, and early evidence of activity in RCC patients was observed in phase I studies of sunitinib, encouraging further investigation of this agent in RCC.

In conclusion, the rationale for pursuing further studies in GIST and RCC with sunitinib was based on its mechanism of action, the role of RTKs targeted by sunitinib in GIST and RCC, and on the objective tumor responses seen in GIST and RCC patients in early phase I studies with sunitinib.

38.3.4 Phase I/II Study of Patients with Imatinib-resistant GIST

An open-label, non-randomized, dose-escalating phase I/II study of sunitinib in patients with metastatic or unresectable GIST was designed to establish one or more recommended dosing schedules for phase II development (Maki et al. 2005; Morgan et al. 2005). All patients enrolled in this study had failed treatment with imatinib mesylate therapy because of either resistance (primary resistance, or progression after initial response; 96% of patients) or intolerance (4% of patients); 66% were male and 94% had an Eastern Cooperative Group (ECOG) performance status of 0 or 1. Of the 97 patients enrolled (phase I and II), 55 were treated on a 4/2 schedule and 42 on either a 2/2 schedule or a 2/1 schedule, at daily doses ranging from 25 mg to 75 mg. As discussed above in Sect. 38.3.2, the recommended sunitinib dose for phase II studies was identified as 50 mg once daily, administered using the 4/2 schedule.

Eight patients (8%) experienced a partial response, 36 patients (37%) stable disease for at least 6 months, and 32 patients (33%) stable disease for 6 weeks to 6 months; the remaining 21 patients (22%) experienced disease progression or stable disease for less than 6 weeks (Maki et al. 2005). Median time to tumor progression was 7.8 months (95% CI 5.1–10.6), and median overall survival was 19.8 months (95% CI 13.6–25.8) (Maki et al. 2005). Thirty-two patients with a partial response or stable disease for more than 6 months entered a continuation study, of whom 15 remained on study without progressive disease after a median follow-up of more than 1.5 years (Maki et al. 2005).

38.3.5 Phase III Study of Patients with Imatinib-resistant or -intolerant GIST

The encouraging phase I/II GIST study results were subsequently confirmed in a double-blind, placebo-controlled, multicenter phase III trial (Demetri et al. 2006). A total of 312 GIST patients with documented imatinib resistance or intolerance were randomized 2:1 to receive sunitinib 50 mg daily (n=207) or placebo (n=105), administered using the 4/2 schedule.

The trial was unblinded early when the planned interim analysis, conducted after the first 149 cases of RECIST-defined disease progression or death, revealed a significantly longer time to tumor progression in the sunitinib group than in the placebo group. Median time to tumor progression was more than four times as long with sunitinib as with placebo treatment (27.3 weeks vs 6.4 weeks; hazard ratio 0.33; P<0.0001) (Fig. 38.3). Sunitinib treatment also significantly improved overall survival (hazard

ratio 0.49; P=0.007), although median overall survival had not been reached in either group at the time of the interim analysis (Fig. 38.4). The survival benefit of sunitinib treatment may be underestimated in a future analysis due to the early unblinding and crossing over of the placebo patients to active treatment.

At the time of the interim analysis, 7% of patients had achieved a partial response, 58% exhibited stable disease, and 19% progressive disease in the sunitinib group, compared with rates of 0%, 48%, and 37%, respectively, in the placebo group. The confirmed objective response rate was significantly higher in the sunitinib group than in the placebo group (7% vs 0%; P=0.006). Of the 59 patients in the placebo group who crossed over to sunitinib after disease progression, 6 patients (10%) subsequently achieved a partial response.

This is the first phase III trial to demonstrate efficacy of a tyrosine kinase inhibitor in cancer patients refractory to or intolerant of another targeted agent. Sunitinib significantly improved time to progression and overall survival, as well as other measures of tumor response, in patients with imatinib-resistant or imatinib-intolerant GIST. In addition, sunitinib was reasonably well tolerated compared with placebo (Table 38.1).

In addition to the phase I–III trials described above, sunitinib has also been studied in a 'treatment-use' trial, in which the drug was made available to GIST patients ineligible for sunitinib clinical trials because of pre-specified entry criteria, or because there were no available GIST trials prior to regulatory approval in a particular country (Dileo et al. 2006). Consistent with phase I–III data, preliminary results from this treatment-use study indicate that sunitinib is associated with acceptable tolerability and significant efficacy in patients with imatinib-resistant or -intolerant GIST.

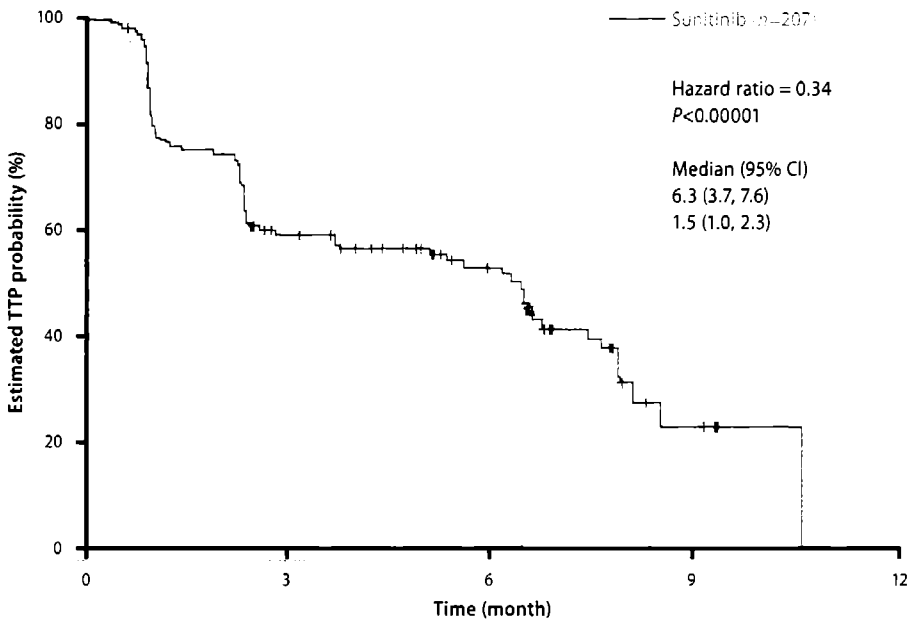


Fig. 38.3. Kaplan–Meier estimates for time to tumor progression in patients with malignant GIST treated with sunitinib or placebo (reproduced with permission from Demetri et al. 2006)

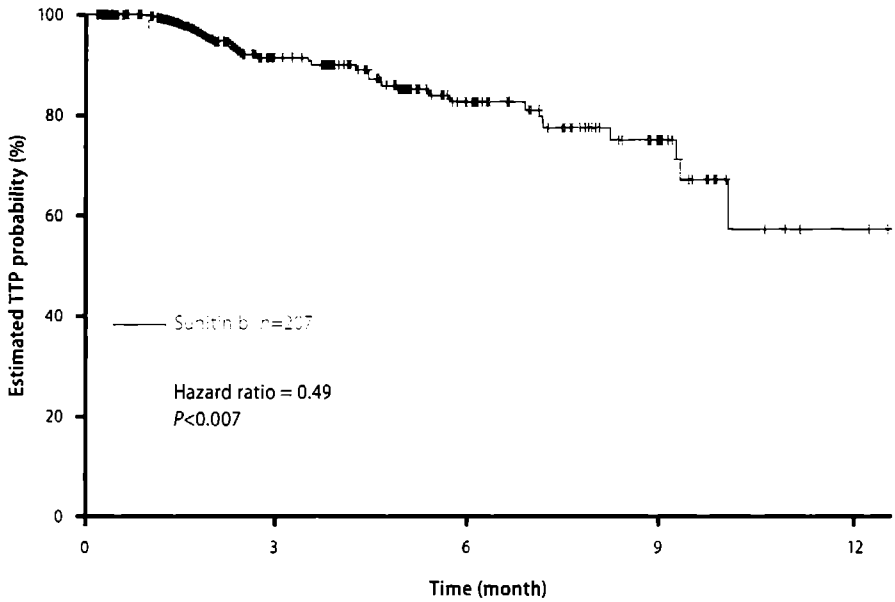


Fig. 38.4. Kaplan–Meier estimates for overall survival of patients with malignant GIST treated with sunitinib or placebo (reproduced with permission from Demetri et al. 2006)

38.3.6 Sunitinib GIST Antitumor Activity and Kinase Mutation Status

As discussed above, most malignant GISTs have activating mutations in the *KIT* RTK, while among those lacking detectable *KIT* mutations, approximately 35% (or about 5% of all malignant GISTs) are associated with mutations in *PDGFRA*, the gene encoding PDGFR-α (Heinrich et al. 2003a, 2003b; Hirota et al. 1998). Previous studies have shown that the presence and type of primary *KIT* or *PDGFRA* mutation influences the clinical activity of imatinib in GIST (Debiec-Rychter et al. 2004; Heinrich et al. 2003a), while secondary *KIT* point mutations are commonly associated with acquired imatinib resistance (Antonescu et al. 2005; Debiec-Rychter et al. 2005).

The relationship between tumor kinase genotype and sunitinib activity was examined in patients with metastatic GIST entered in the phase I/II study described above via analysis of tumor specimens for primary and secondary mutations of *KIT* and *PDGFRA* (Maki et al. 2005). Median time to tumor progression was significantly longer in patients with primary *KIT* exon 9 mutations or wild-type status versus primary *KIT* exon 11 mutations (14.3, 13.8, and 5.1 months, respectively; $P \leq 0.01$). Median overall survival was also significantly longer with primary *KIT* exon 9 mutation or wild-type versus primary *KIT* exon 11 mutation ($P \leq 0.01$). Of note, these are results from imatinib-resistant or -intolerant GIST patients participating in this study and cannot yet be extrapolated to treatment-naïve patients.

For the enrolled population in the studies described above, sunitinib appeared to be more active against GISTs harboring *KIT* exon-9 than *KIT* exon-11 mutations, while in the treatment-naïve GIST patients, imatinib seems to exhibit greater activity in GIST associated with primary *KIT* exon-11 mutations (Debiec-Rychter et al. 2004; Heinrich et al. 2003a). However, because more than half of the patients in the sunitinib mutational analysis

Table 38.1. Treatment-related adverse events and laboratory abnormalities that occurred with at least a 5% greater frequency with sunitinib than with placebo in the per-protocol population of a phase III GIST trial (adapted with permission from Demetri et al., 2006)

	Sunitinib (n=202)			Placebo (n=102)		
	Grade 1/2	Grade 3	Grade 4	Grade 1/2	Grade 3	Grade 4
Treatment-related adverse events, no. (%)						
Fatigue	58 (29)	10 (5)	0	20 (20)	2 (2)	0
Diarrhea	52 (26)	7 (3)	0	8 (8)	0	0
Skin discoloration	50 (25)	0	0	6 (6)	0	0
Nausea	47 (23)	1 (1)	0	10 (10)	1 (1)	0
Anorexia	38 (19)	0	0	5 (5)	1 (1)	0
Dysgeusia	36 (18)	0	0	2 (2)	0	0
Stomatitis	30 (15)	1 (1)	0	2 (2)	0	0
Vomiting	30 (15)	1 (1)	0	5 (5)	1 (1)	0
Hand–foot syndrome	19 (9)	9 (4)	0	2 (2)	0	0
Rash	24 (12)	2 (1)	0	5 (5)	0	0
Asthenia	18 (9)	6 (3)	0	2 (2)	2 (2)	0
Mucosal inflammation	24 (12)	0	0	0	0	0
Dyspepsia	22 (11)	1 (1)	0	1 (1)	0	0
Hypertension	15 (8)	6 (3)	0	4 (4)	0	0
Epistaxis	14 (7)	0	0	0	0	0
Hair-color changes	14 (7)	0	0	2 (2)	0	0
Dry mouth	13 (6)	0	0	1 (1)	0	0
Glossodynia	11 (6)	0	0	0	0	0
Laboratory abnormalities, no. (%)						
Anemia[a]	117 (58)	7 (4)	0	59 (58)	2 (2)	0
Leukopenia	104 (52)	7 (4)	0	5 (5)	0	0
Neutropenia	86 (43)	17 (8)	3 (2)	4 (4)	0	0
Lymphopenia	80 (40)	18 (9)	1 (1)	31 (30)	2 (2)	1 (1)
Thrombocytopenia	72 (36)	8 (4)	1 (1)	4 (4)	0	0

Severity of adverse events was rated by investigators using the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0. [a]Anemia was included because of its frequency and clinical relevance in GIST although the difference between treatment groups was less than 5%.

with primary *KIT* exon-11 mutations developed secondary *KIT* mutations during prior treatment with imatinib, the true effect of primary *KIT* genotype on sunitinib activity can only be assessed in a study of sunitinib as first-line treatment in imatinib-naïve GIST patients. Future sunitinib studies are planned to answer these questions.

38.3.7 Phase II Studies of Patients with Metastatic RCC

Two independent, open-label, consecutive phase II studies have investigated the efficacy and safety of sunitinib as second-line treatment in patients with metastatic RCC (Motzer et al. 2006a, 2006b). In both studies, patients received repeated 6-week cycles of treatment, each comprising sunitinib 50 mg/day administered using the 4/2 schedule. All patients had failed one prior cytokine-based therapy. The primary endpoint for each study was objective response rate; secondary endpoints included time to progression in the first study, and progression-free survival in the second study.

Results from the first phase II study show that the majority of patients had a reduction in measurable disease (Fig. 38.5) (Motzer et al. 2006a). Of the 63 patients treated with sunitinib, 25 (40%) achieved a partial response, while a further 17 patients (27%) had stable disease lasting 3 months. [By comparison, only 4 (6%) of the 63 patients entered in this study had achieved a complete or partial response to prior cytokine therapy.] Twenty-four of the patients with a partial response had clear-cell histology, and one had a papillary-cell type; in the study population overall, 55 patients (87%) had clear-cell histology. At the time of analysis, several of the 8 responding patients who remained on sunitinib treatment were progression-free for over 24 months from the start of treatment. Median time to tumor progression for all patients was 8.7 months and median time to overall survival was 16.4 months.

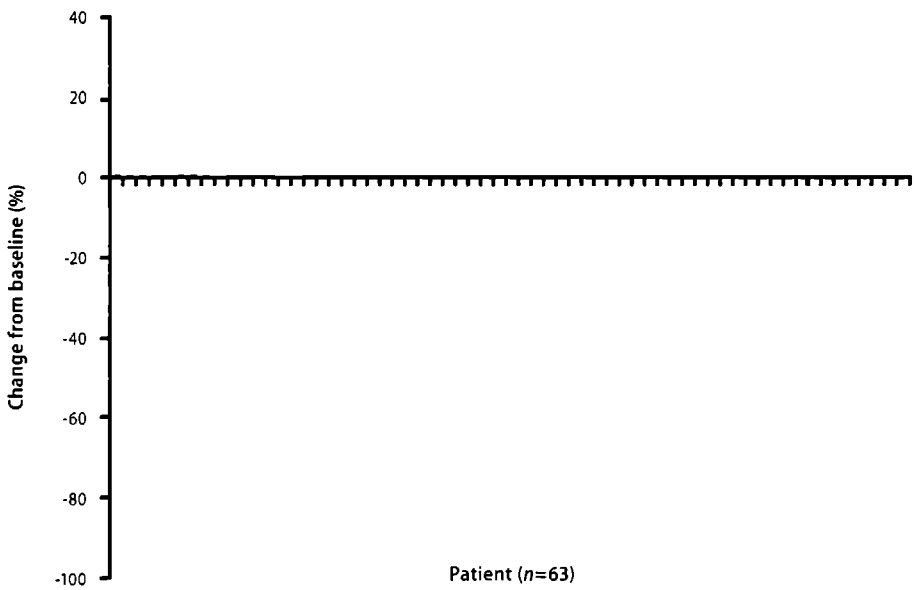


Fig. 38.5. Maximal percentage of tumor reduction for target lesions in each patient with metastatic RCC treated with sunitinib (reproduced with permission from Motzer et al. 2006a)

A second, larger phase II trial of 106 patients was designed to confirm these initial results (Motzer et al. 2006b). All patients entered in this study were required to have had prior nephrectomy, a component of clear-cell histology and documented evidence of disease progression following prior cytokine therapy. According to the investigator assessment, the overall objective response rate to sunitinib in this study was 44%: one patient (1%) achieved a complete response and 45 patients (43%) a partial response. An additional 23 patients (22%) had stable disease of at least 3 months duration. As in the first trial, most patients with stable disease had some decrease in tumor size, albeit insufficient to be categorized as a partial response by the RECIST criteria. At the time of analysis, 34 of the 46 responding patients remained progression-free, including one patient with a complete response for over 10 months. Median progression-free survival was 8.1 months. At the time of analysis, the median overall survival had not been reached and the 6-month survival rate was 79%. In a pooled analysis of the phase II studies in which demographics and efficacy data were combined, an objective response rate of 42% was reported and associated with a median progression-free survival of 8.2 months (Motzer et al. 2006b).

Patients with progressive metastatic RCC after cytokine therapy are generally managed by supportive care. In clinical trials of other agents targeting the VEGFR pathway, the median time to progression for such patients is approximately 2–5 months (Escudier et al. 2005; Yang et al. 2003). The efficacy demonstrated by sunitinib in these two phase II studies compares very favorably with that of other agents used for the treatment of RCC. The cytokines interferon-α (IFN-α) and interleukin-2 (IL-2) produce responses in approximately 5–20% of patients, with a median survival of approximately 13–15 months when used in first-line settings (Law et al. 1995; Motzer et al. 2004; Rohrmann et al. 2005; Vogelzang et al. 1993). Figure 38.6 shows an example of tumor response achievable with sunitinib in a patient with local recurrence of RCC.

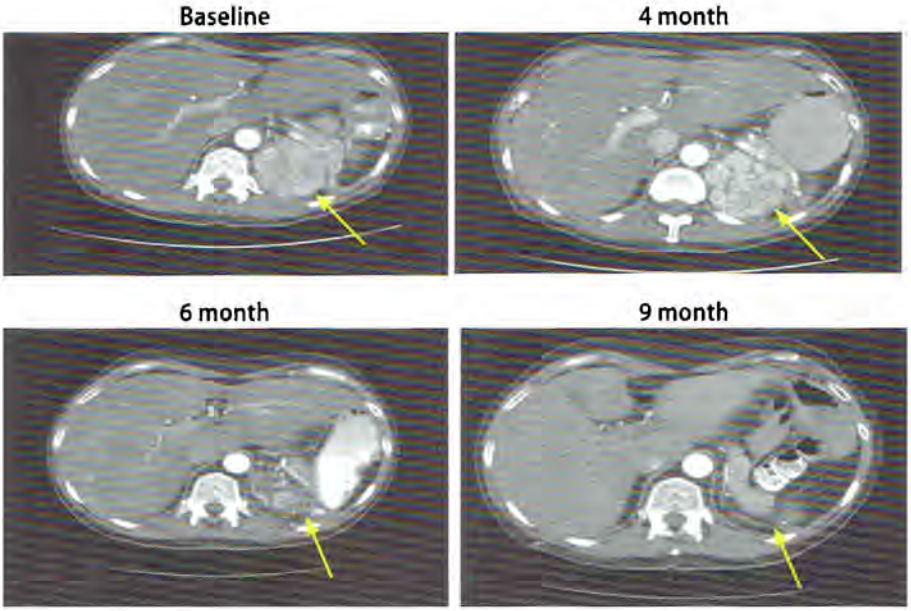


Fig. 38.6. CT scans showing tumor response to sunitinib treatment in a patient with local recurrence of RCC (scans courtesy of M. Dror Michaelson, MD, PhD, Massachusetts General Hospital Cancer Center, Boston, MA)

38.3.8 Phase III Study of Patients with Metastatic RCC

The high objective response rate of sunitinib as second-line therapy for metastatic RCC is more than double that reported with cytokines as first-line treatment (Rohrmann et al. 2005). The efficacy results of these two phase II studies in cytokine-failure patients provided the clinical rationale to assess the role of sunitinib as first-line treatment in metastatic RCC patients. Data from a prospectively planned interim analysis of an international, multicenter, randomized phase III trial in which sunitinib was compared with IFN-α as first-line therapy for metastatic RCC were reported by Motzer et al (2007). Treatment with sunitinib was found to be well tolerated and resulted in significantly longer progression-free survival (Fig. 38.7) and a higher objective response rate than IFN-α as assessed by blinded central review (11 vs 5 months and 31% vs 6%, respectively; $P<0.001$).

Most general adverse events of all grades occurred more frequently in the sunitinib group than in the IFN-α group (Table 38.2). However, the proportion of patients with grade 3 or 4 adverse events was relatively low in both groups and, of the two most common treatment-related adverse events, the proportion with grade 3 or 4 fatigue was significantly higher in the IFN-α group ($P<0.05$), whereas grade 3 or 4 diarrhea was significantly more frequent in the sunitinib group ($P<0.05$). In addition, fewer patients on sunitinib than on IFN-α discontinued treatment because of adverse events (8% vs 13%; $P=0.05$). Patients receiving sunitinib also reported a significantly better quality of life than patients receiving IFN-α ($P<0.001$).

In addition to the phase I–III trials described above, sunitinib has also been studied in a treatment-use trial, in which the drug was made available to metastatic RCC patients ineligible for sunitinib clinical trials, as described above for GIST patients (Bukowski et al. 2006). In line with phase I–III results, preliminary data from this study suggest that sunitinib has acceptable tolerability in this patient population.

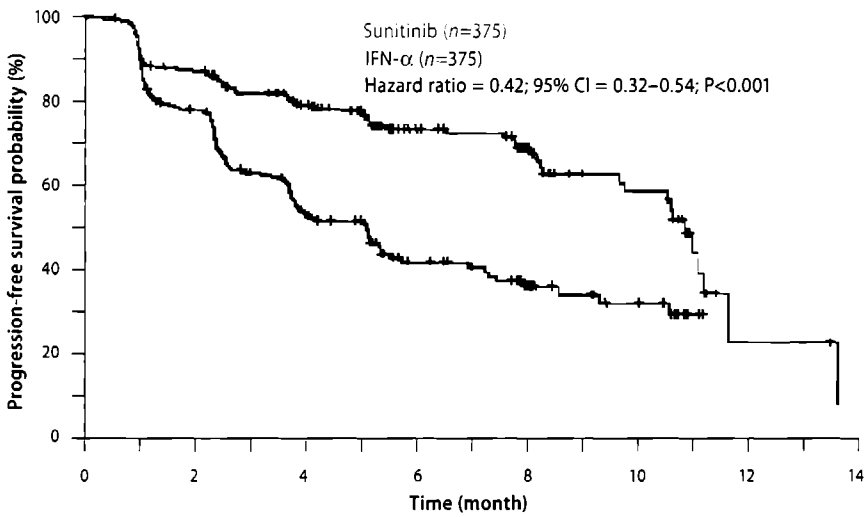


Fig. 38.7. Kaplan–Meier estimates of progression-free survival (as assessed by blinded central review) for patients with metastatic RCC treated with sunitinib or IFN-α (reproduced with permission from Motzer et al. 2007)

Table 38.2. Treatment-related adverse events and selected laboratory abnormalities with sunitinib versus IFN-α in a phase III metastatic RCC trial (adapted with permission from Motzer et al. 2007)

	Sunitinib (n=375)		IFN-α (n=360)	
	All grades (%)	Grade 3/4 (%)	All grades (%)	Grade 3/4 (%)
Adverse event				
Diarrhea[a]	53	5/0	12	0/0
Fatigue[a]	51	7/0	51	11/1
Nause[a]	44	3/0	33	1/0
Stomatitis	25	1/0	2	1/0
Vomiting[a]	24	4/0	10	1/0
Hypertension[a]	24	8/0	1	1/0
Hand–foot syndrome[a]	20	5/0	1	0/0
Mucosal inflammation	20	2/0	1	1/0
Rash	19	1/1	6	1/0
Asthenia	17	4/0	20	4/0
Dry skin	16	1/0	5	0/0
Skin discoloration	16	0/0	0	0/0
Changes in hair color	14	0/0	1	0/0
Epistaxis	12	1/0	1	0/0
Pain in limb	11	1/0	3	0/0
Headache	11	1/0	14	0/0
Dry mouth	11	0/0	6	1/0
Decline in ejection fraction	10	2/0	3	1/0
Pyrexia	7	1/0	34	0/0
Chills	6	1/0	29	0/0
Myalgia	5	1/0	16	1/0
Influenza-like illness	1	0/0	7	1/0
Laboratory abnormality				
Leukopenia[a]	78	5/0	56	2/0
Neutropenia[a]	72	11/1	46	7/0
Anemia	71	3/1	64	4/1
Increased creatinine	66	1/0	49	1/0
Thrombocytopenia[a]	65	8/0	21	0/0
Lymphopenia[a]	60	12/0	63	22/0
Increased lipase[a]	52	13/3	42	5/1

	Sunitinib (n=375)		IFN-α (n=360)	
	All grades (%)	Grade 3/4 (%)	All grades (%)	Grade 3/4 (%)
Increased aspartate aminotransferase	52	2/0	34	2/0
Increased alanine aminotransferase	46	2/1	39	2/0
Increased alkaline phosphatase	42	2/0	35	2/0
Increased uric acid	41	0/12	31	0/8
Hypophosphatemia	36	4/1	32	6/0
Increased amylase[a]	32	4/1	28	2/1
Increased total bilirubin	19	1/0	2	0/0

Listed are treatment-related adverse events of interest and those occurring in at least 10% of patients in the sunitinib group. All severity was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events, version 3.0. [a]The difference between the sunitinib group and the IFN-α group was significant (P<0.05) with the use of Fisher's exact test applied to the sum of grade 3 and 4 adverse events. The significance of the difference between treatment groups for "all grades" of adverse events is not shown.

38.3.9 Phase II Study of Patients with Metastatic Breast Cancer

The rationale for investigating sunitinib in breast cancer is supported by data implicating the VEGFR signaling pathway in breast cancer angiogenesis (Price et al. 2001) and by the association between overexpression of PDGFR-α and breast cancer progression (Carvalho et al. 2005). Consequently, an open-label, multicenter phase II study was conducted to investigate the efficacy of sunitinib in breast cancer (Miller et al. 2005). The study population comprised patients with metastatic breast cancer (mBC) who had been previously treated with anthracyclines and taxanes and who were resistant to at least one of these therapies. No more than two prior regimens for mBC were allowed. All patients received sunitinib 50 mg daily on the 4/2 schedule.

In this study, 7 of the 64 refractory mBC patients (11%) enrolled achieved a partial response, and a further 3 patients exhibited stable disease for 6 months, resulting in an overall clinical benefit rate of 16%. Responses were observed in patients with visceral as well as with superficial tumor involvement at the time of enrolment. Median time to progression was 10 weeks.

38.3.10 Phase II Study of Patients with Previously Treated Advanced NSCLC

In NSCLC, elevated expression of VEGF is a strong prognostic indicator, predicting increased tumor angiogenesis, early postoperative relapse and decreased survival (Yuan et al. 2001). Increased expression of PDGF has also been associated with poor prognosis in NSCLC (Koukourakis et al. 1997). A phase II trial evaluated the single-agent activity of sunitinib (50 mg/day on the 4/2 schedule) (Socinski et al. 2006). At the time of analysis, six confirmed partial responses were reported among 63 treated patients (9.5%; 95% CI 3.6–19.6). Stable disease was observed in an additional 12 patients (19.0%). The study was extended to explore a continuous dosing schedule of 37.5 mg/day.

38.3.11 Phase II Study of Patients with Unresectable NET

Neuroendocrine tumors are highly vascular, and elevated levels of VEGF and VEGFR have been found in both carcinoid and pancreatic endocrine tumors

(Christofori et al. 1995; Terris et al. 1998). NET is also known to express other growth factors and their receptors, including PDGFR-α and -β, which, like VEGFR, are targeted by sunitinib (Chaudhry et al. 1993). In phase I studies, patients with NET achieved a partial response to sunitinib (Faivre et al. 2006).

The efficacy of sunitinib in the treatment of patients with NET was investigated in a multicenter, phase II study (Kulke et al. 2005). A total of 109 patients with advanced, unresectable NET were recruited; 102 patients were evaluable, comprising 41 with carcinoid and 61 with pancreatic islet cell tumors. Patients received 50 mg sunitinib daily on the 4/2 schedule. Eight patients (13%) with pancreatic islet cell tumors achieved a partial response to sunitinib, compared with only one patient (2%) with carcinoid. Notably, the majority of patients experienced stable disease, including 38 patients (93%) with carcinoid and 46 patients (75%) with pancreatic islet cell tumor. Median time to tumor progression was 42 weeks and 33 weeks in the carcinoid and pancreatic islet tumor groups, respectively. Sunitinib is the first RTK inhibitor to show clinical activity in patients with NET.

Summary of Clinical Safety

The safety profile of sunitinib was broadly consistent across all patients with solid malignant tumors treated in all clinical studies as of July 2005 (450 patients). The most commonly reported treatment-related adverse events were fatigue (60.0%), diarrhea (50%), nausea (44%), vomiting (33%), and anorexia (31%) (Pfizer, data on file). Although fatigue was the most common DLT reported in early clinical pharmacology studies, it was not commonly associated with treatment discontinuation in phase II and III studies. Gastrointestinal toxicities were generally manageable with interventions such as dose reduction, temporary dosing delay, or symptomatic therapy. Occasional decreases in left ventricular ejection fraction (LVEF) were observed after systematic evaluation, but in only one patient was it associated with clinical signs or symptoms of congestive heart failure. Less than 10% of patients discontinued sunitinib treatment due to adverse events. Adverse events were generally mild to moderate in intensity.

There were a total of four deaths in the phase III study of GIST and one in the larger, pivotal phase II study of RCC. Of the four deaths in the phase III GIST study, two occurred in patients randomized to sunitinib and two in patients randomized to placebo.

The most commonly reported treatment-related events were hypertension (17%) and hemorrhagic events (including tumor bleeding; 14%). No patient experienced grade 4 or 5 hypertension or discontinued treatment for reasons related to this event. Epistaxis was the most frequently reported treatment-related hemorrhagic event, but the events rarely were considered serious. Two patients stopped treatment with sunitinib following hemorrhage and epistaxis.

Although transient increases in lipase levels have been observed in patients treated with sunitinib, these do not appear to correlate with clinical signs or symptoms of pancreatitis over the course of prolonged treatment. Pancreatitis was reported in less than 1% of patients. Grade 3 and 4 neutropenia were experienced by 13.1% and 0.9% of patients, respectively; grade 3 and 4 lymphopenia by 14.9% and 2.1% of patients; grade 3 and 4 thrombocytopenia by 4% and 0.5% of patients; and grade 3 and 4 anemia by 6.3% and 0.7% of patients. One patient developed febrile neutropenia; none developed neutropenic sepsis.

Overall, sunitinib has a favorable safety profile, with manageable toxicities that rarely result in discontinuation of patients from treatment. The safety profile of sunitinib should facilitate its combination with other agents, and trials testing the safety of sunitinib combined with a range of chemotherapeutic and biological agents are ongoing.

Future Plans

As outlined above, sunitinib has shown significant efficacy with acceptable tolerability across several studies, including two phase III studies of GIST and RCC, respectively, and phase II studies of mBC, NSCLC and NET. Several trials are either under way or will soon begin recruitment to further investigate its effectiveness in these cancers and others, such as colorectal cancer, pediatric cancers, prostate cancer, gastric cancer, and other solid tumors. In addition, these studies will explore alternative treatment regimens, including continuous dosing of sunitinib and combinations with commonly used chemotherapeutic regimens. The multitargeted mechanism of action of sunitinib, its favorable safety profile, and its broad clinical activity warrant further investigation of this agent in first-line, adjuvant and neoadjuvant settings.

References

Abrams TJ, Lee LB, Murray LJ, Pryer NK, Cherrington JM (2003a) SU11248 inhibits KIT and platelet-derived growth factor receptor beta in preclinical models of human small cell lung cancer. Mol Cancer Ther 2:471–478

Abrams TJ, Murray LJ, Pesenti E et al (2003b) Preclinical evaluation of the tyrosine kinase inhibitor SU11248 as a single agent and in combination with "standard of care" therapeutic agents for the treatment of breast cancer. Mol Cancer Ther 2:1011–1021

Antonescu CR, Besmer P, Guo T et al (2005) Acquired resistance to imatinib in gastrointestinal stromal tumour occurs through secondary gene mutation. Clin Cancer Res 11:4182–4190

Bello C, Houk B, Sherman L, et al (2005) Effect of rifampin on the pharmacokinetics of SU11248 in healthy volunteers. J Clin Oncol, Proc Am Soc Clin Oncol 23, abstract 3078

Bello C, Sherman L, Zhou J et al (2006a) Effect of food on the pharmacokinetics of sunitinib malate (SU11248), a multitargeted receptor tyrosine kinase inhibitor: results from a phase I study in healthy subjects. Anticancer Drugs 3:353–358

Bello C, Garrett M, Smeraglia J et al (2006b) Pharmacokinetics (PK) of sunitinib malate (SU11248) in subjects with hepatic impairment (poster presentation). Ann Oncol 17: ix148 (abstract 451P)

Benjamin RS, Rankin C, Fletcher C et al (2003) Phase III dose-randomized study of imatinib mesylate (Gleevec, STI571) for GIST: Intergroup S0033 early results. (Oral presentation, abstract 3271). American Society of Clinical Oncology 39th Annual Meeting, Chicago, Illinois, USA, 31 May–3 June 2003. Available at: www.asco.org

Bergers G, Song S (2005) The role of pericytes in blood-vessel formation and maintenance. Neuro-oncol 7:452–464

Bergers G, Song S, Meyer-Morse N et al (2003) Benefits of targeting both pericytes and endothelial cells in the tumour vasculature with kinase inhibitors. J Clin Invest 111:1287–1295

Bukowski RM, Szyzylik C, Porta C et al (2006) Preliminary results of an expanded-access trial of sunitinib malate (SU11248) for the treatment of patients with refractory metastatic renal cell carcinoma (mRCC) (Oral presentation). Ann Oncol 17: ix144 (abstract 437O)

Carvalho I, Milanezi F, Martins A, Reis RM, Schmitt F (2005) Overexpression of platelet-derived growth factor receptor alpha in breast cancer is associated with tumour progression. Breast Cancer Res 7:R788–R795

Chaudhry A, Funa K, Öberg K et al (1993) Expression of growth factor peptides and their receptors in neuroendocrine tumours of the digestive system. Acta Oncol 32:107–114

Christofori G, Naik P, Hanahan D (1995) Vascular endothelial growth factor and its receptors, flt-1 and flk-1, are expressed in normal pancreatic islets and throughout islet cell tumorigenesis. Mol Endocrinol 9:1760–1770

Corless CL, Fletcher JA, Heinrich MC (2004) Biology of gastrointestinal stromal tumors. J Clin Oncol 22:3813–3825

Debiec-Rychter M, Dumez H, Judson I et al (2004) Use of c-KIT/PDGFRA mutational analysis to predict the clinical response to imatinib in patients with advanced gastrointestinal stromal tumours entered on phase I and II studies of the EORTC Soft Tissue and Bone Sarcoma Group. Eur J Cancer 40:689–695

Debiec-Rychter M, Cools J, Dumez H et al (2005) Mechanisms of resistance to imatinib mesylate in gastrointestinal stromal tumors and activity of the PKC412 inhibitor against imatinib-resistant mutants. Gastroenterology 128:270–279

DeMatteo RP, Lewis JJ, Leung D, Mudan SS, Woodruff JM, Brennan MF (2000) Two hundred gastrointestinal stromal tumours: recurrence patterns and prognostic factors for survival. Ann Surg 231:51–58

Demetri GD, Von Mehren M, Blanke CD et al (2002) Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumours. N Engl J Med 347:472–480

Demetri DG, George S, Heinrich MC et al (2003) Clinical activity and tolerability of the multi-targeted tyrosine kinase inhibitor SU11248 in patients with metastatic gastrointestinal stromal tumor refractory to imatinib mesylate (Oral presentation, abstract 3273). American Society

of Clinical Oncology 39th Annual Meeting, Chicago, IL, USA, 31 May–3 June 2003. Available at: www.asco.org

Demetri DG, van Oosterom AT, Garrett CR et al (2006) Efficacy and safety of sunitinib in patients with advanced gastrointestinal stromal tumour after failure of imatinib: a randomized controlled trial. Lancet 368:1329–1338

Dileo P, Morgan JA, Garrett CR et al (2006) Updated results from a "treatment-use" trial of sunitinib in advanced gastrointestinal stromal tumor (GIST). Ann Oncol 17:ix162 (abstract 507PD)

Erber R, Thurnher A, Katsen AD et al (2004) Combined inhibition of VEGF and PDGF signalling enforces tumour vessel regression by interfering with pericyte-mediated endothelial cell survival mechanisms. FASEB J 18:338–340

Escudier B, Szczylik C, Eisen T et al (2005) Randomized phase III trial of sorafenib (BAY 43-9006) – an oral multikinase inhibitor – in patients with advanced RCC. (Oral presentation, abstract 4510). American Society of Clinical Oncology 41st Annual Meeting, Orlando, Florida, USA, 13–17 May 2005. Available at: www.asco.org

Faivre S, Delbaldo C, Vera K et al (2006) Safety, pharmacokinetic, and antitumor activity of SU11248, a novel oral multitarget tyrosine kinase inhibitor, in patients with cancer. J Clin Oncol 24:25–35

Gleevec (imatinib mesylate) prescribing information (2005) Novartis Pharmaceuticals Corporation, East Hanover, NJ, USA

Gnarra JR, Tory K, Weng Y et al (1994) Mutations of the VHL tumour suppressor gene in renal carcinoma. Nat Genet 7:85–90

Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100:57–70

Heinrich MC, Corless CL, Demetri GD et al (2003a) Kinase mutations and imatinib response in patients with metastatic gastrointestinal stromal tumor. J Clin Oncol 21:4342–4349

Heinrich MC, Corless CL, Duensing A et al (2003b) PDGFRA activating mutations in gastrointestinal stromal tumours. Science 299:708–710

Herman JG, Latif F, Weng Y et al (1994) Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. Proc Natl Acad Sci USA 91:9700–9704

Hicklin DJ, Ellis LM (2005) Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. J Clin Oncol 23:1011–1027

Hirota S, Isozki K, Moriyama Y et al (1998) Gain-of-function mutations of c-kit in human gastrointestinal stromal tumours. Science 279:577–580

Koukourakis MI, Giatromanolaki A, O'Byrne KJ et al (1997) Platelet-derived endothelial cell growth factor expression correlates with tumour angiogenesis and prognosis in non-small-cell lung cancer. Br J Cancer 75:477–481

Kulke M, Lenz HJ, Meropol NJ et al (2005) A phase 2 study to evaluate the efficacy and safety of SU11248 in patients (pts) with unresectable neuroendocrine tumors (NETs). (Oral presentation, abstract 4008). American Society of Clinical Oncology 41st Annual Meeting, Orlando, Florida, USA, 13–17 May 2005. Available at: www.asco.org

Law TM, Motzer RJ, Mazumdar M et al (1995) Phase III randomized trial of interleukin-2 with or without lymphokine-activated killer cells in the treatment of patients with advanced renal cell carcinoma. Cancer 76:824–832

Maki RG, Fletcher JA, Heinrich MC et al (2005) SU11248 in patients with imatinib-resistant GIST: results from a continuation trial (Oral presentation). American Society of Clinical Oncology 41st Annual Meeting, Orlando, Florida, USA, 13–17 May 2005. Available at: www.asco.org

Mendel DB, Laird AD, Xin X et al (2003) In vivo antitumor activity of SU11248, a novel tyrosine kinase inhibitor targeting vascular endothelial growth factor and platelet-derived growth factor receptors: determination of a pharmacokinetic/pharmacodynamic relationship. Clin Cancer Res 9:327–337

Miller K, Burstein HJ, Elias AD et al (2005) Phase II study of SU11248, a multitargeted receptor tyrosine kinase inhibitor (TKI), in patients (pts) with previously treated metastatic breast cancer (MBC). (Poster; abstract 1066.) San Antonio Breast Cancer Symposium 28th Annual Meeting, San Antonio, Texas, USA, 8–11 December 2005. Available at: www.sabcs.org

Morgan JA, Demetri GD, Fletcher JA et al (2005) Patients with imatinib mesylate-resistant GIST exhibit durable responses to sunitinib malate (SU11248). Eur J Cancer [Suppl] 3:421 (Abstract 1456)

Motzer RJ, Bacik J, Mazumdar M (2004) Prognostic factors for survival of patients with stage IV renal cell carcinoma: Memorial Sloan-Kettering Cancer Center experience. Clin Cancer Res 10:6302S–633S

Motzer R, Michaelson M, Redman BG et al (2006a) Activity of SU11248, a multitargeted inhibitor of vascular endothelial growth factor receptor and platelet-derived growth factor receptor, in patients with metastatic renal cell carcinoma. J Clin Oncol 24:16–24

Motzer RJ, Rini BI, Bukowski RM et al (2006b) Sunitinib in patients with metastatic renal cell carcinoma. JAMA 295:2516–2524

Motzer RJ, Hutson TE, Tomczak P et al (2007) Sunitinib versus interferon alfa in metastatic renal-cell carcinoma. N Engl J Med 356:115–124

O'Farrell AM, Abrams TJ, Yuen HA et al (2003) SU11248 is a novel FLT3 tyrosine kinase inhibitor with potent activity in vitro and in vivo. Blood 101:3597–3605

Pietras K, Sjoblom T, Rubin K, Heldin CH, Ostman A (2003) PDGF receptors as cancer drug targets. Cancer Cell 3:439–443

Potapova O, Laird AD, Nannini MA et al (2006) Contribution of individual targets to the antitumor efficacy of the multitargeted receptor tyrosine kinase inhibitor SU11248. Mol Cancer Ther 5:1280–1289

Price DJ, Miralem T, Jiang S, Steinberg R, Avraham H (2001) Role of vascular endothelial growth factor in the stimu-

lation of cellular invasion and signaling of breast cancer cells. Cell Growth Differ 12:129–135

Raymond E, Faivre S, Vera K et al for the SU11248 working group (2003) Final results of a phase I and pharmacokinetic study of SU11248, a novel multi-target tyrosine kinase inhibitor, in patients with advanced cancers. (Oral presentation, abstract 769.) American Society of Clinical Oncology 39th Annual Meeting, Chicago, Illinois, USA, 31 May-3 June 2003. Available at: www.asco.org

Rohrmann K, Staehler M, Haseke N, Bachmann A, Stief CG, Siebels M (2005) Immunotherapy in metastatic renal cell carcinoma. World J Urol 23:196–201

Rosen L, Mulay M, Long J et al (2003) Phase I trial of SU011248, a novel tyrosine kinase inhibitor in advanced solid tumors. (Oral presentation, abstract 765). American Society of Clinical Oncology 39th Annual Meeting, Chicago, Illinois, USA, 31 May-3 June 2003. Available at: www.asco.org

Saharinen P, Alitalo K (2003) Double target for tumor mass destruction. J Clin Invest 111:1277–1280

Sakamoto KM (2004) SU-11248 Sugen. Curr Opin Invest Drugs 5:1329–1339

Schueneman AJ, Himmelfarb E, Geng L et al (2003) SU11248 maintenance therapy prevents tumor regrowth after fractionated irradiation of murine tumor models. Cancer Res 63:4009–4016

Socinski MA, Novello S, Sanchez JM et al (2006) Efficacy and safety of sunitinib in previously treated, advanced non-small cell lung cancer (NSCLC): preliminary results of a multicenter phase II trial. Proc Am Soc Clin Oncol 24:143s (abstract 7001)

Sun L, Liang C, Shirazian S et al (2003) Discovery of 5-[5-fluoro-2-oxo-1,2- dihydroindol-(3Z)-ylidenemethyl]-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylaminoethyl)amide, a novel tyrosine kinase inhibitor targeting vascular endothelial and platelet-derived growth factor receptor tyrosine kinase. J Med Chem 46:1116–1119

Terris B, Scoazec JY, Rubbia L et al (1998) Expression of vascular endothelial growth factor in digestive neuroendocrine tumours. Histopathology 32:133–138

Van Glabbeke M, Verweij J, Casali PG et al (2005) Initial and late resistance to imatinib in advanced gastrointestinal stromal tumors are predicted by different prognostic factors: a European Organisation for Research and Treatment of Cancer-Italian Sarcoma Group-Australasian Gastrointestinal Trials Group study. J Clin Oncol 23:5795–5804

Verweij J, Casali PG, Zalcberg J et al for the EORTC Soft Tissue and Bone Sarcoma Group, the Italian Sarcoma Group, and the Australasian Gastrointestinal Trials Group (2004) Progression-free survival in gastrointestinal stromal tumours with high-dose imatinib: randomised trial. Lancet 364:1127–1134

Vogelzang NJ, Lipton A, Figlin RA (1993) Subcutaneous interleukin-2 plus interferon alfa-2a in metastatic renal cancer: an outpatient multicenter trial. J Clin Oncol 11:1809–1816

Washington C, Eli M, Bello C, et al (2003) The effect of ketoconazole (KETO), a potent CYP3A4 inhibitor, on SU011248 pharmacokinetics (PK) in caucasian and Asian healthy subjects. Proc Am Soc Clin Oncol 22, abstract 553

Yang JC, Haworth L, Sherry RM et al (2003) A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. N Engl J Med 349:427–434

Yu J, Ustach C, Kim HR (2003) Platelet-derived growth factor signaling and human cancer. J Biochem Mol Biol 36:49–59

Yuan A, Yu CJ, Kuo SH et al (2001) Vascular endothelial growth factor 189 mRNA isoform expression specifically correlates with tumor angiogenesis, patient survival, and postoperative relapse in non-small-cell lung cancer. J Clin Oncol 19:432–441

The EGF(R) and VEGF(R) Pathways as Combined Targets for Anti-Angiogenesis Trials in Cancer Therapy

39

KLAAS HOEKMAN, HESTER VAN CRUIJSEN, GIUSEPPE GIACCONE

Abstract

In tumor biology both the EGF(R) and the VEGF(R) pathway are constitutively activated due to genetic abnormalities and ongoing tumor-associated hypoxia. In addition, both pathways can be activated by anticancer therapies such as chemotherapy and radiotherapy, which contributes to the resistance to these treatments. Moreover, VEGF modulates EGFR signaling and EGF induces VEGF activity. Therefore, both pathways are logical targets for therapy, and because of their parallel and reciprocal activation, dual inhibition of the EGF(R) and VEGF(R) signaling makes sense. In this chapter we discuss the possibilities for integrated anti-angiogenesis therapy directed at these two biological systems.

CONTENTS

39.1 Introduction

Tumor progression depends on tumor-specific genetic abnormalities and neoangiogenesis. A multitude of inhibitors and stimulators are involved in tumor-induced angiogenesis. The key initiator of angiogenesis is vascular endothelial growth factor (VEGF), which induces proliferation, migration of endothelial cells, and permeability and vasorelaxation in intact vessel walls (Carmeliet 2005; Ferrara 2005). Furthermore, VEGF is a survival factor for endothelial cells. Four subtypes of VEGF have been described (VEGF-A, -B, -C and -D), and several isoforms are known for VEGF-A, with different solubility and activity, either in the direct vicinity of the tumor or at a distance via the circulation. Four VEGF receptors (R) have been identified that bind these ligands. Interaction between VEGF-A

and VEGFR-2 greatly dominates the tumor-associated neovascularization, while interaction between VEGF-C/-D and VEGFR-3 preferentially stimulates lymphangiogenesis. The presence of VEGF and VEGFRs in tumor tissues has a prognostic impact on survival of cancer patients (Ferrara 2005). So far, the VEGF(R) pathway has been the most frequent and successful target for anti-angiogenesis therapy.

There is growing evidence that modulation of the epidermal growth factor receptor (EGFR) pathway also significantly affects tumor-induced angiogenesis (van Cruijsen et al. 2005). EGFR, a tyrosine kinase receptor of the ErbB family, is highly expressed in most human solid tumors and high expression has been associated with poor prognosis. Tumoral activation of EGFR signaling includes production of EGFR ligands by tumor cells or environmental stromal cells, high expression of EGFR associated with an enhanced sensitivity to EGF-like ligands, and constitutively active EGF-receptor mutations. EGF–EGFR signaling in endothelial cells appears to occur directly, through EGF receptors on tumor endothelial cells, and indirectly, via an effect on the production of angiogenesis stimulators by tumor cells. This makes both pathways potential targets for a combined therapy with anti-EGFR and anti-VEGF(R) drugs.

39.2 The Activity of EGF(R) and VEGF(R) in Cancer Biology

The EGF(R) and VEGF(R) pathways are often active in tumor biology (Ciardiello and de Vita 2005; Carmeliet 2005). This may be a result of direct genetic alterations of the targets (e.g. EGFR gene mutations or amplification) or an indirect effect of mutations in certain oncogenes or suppressor genes (Rak et al. 2000). Furthermore, both pathways can be activated by stress factors. Hypoxia, a well-known stress inducer, is induced by the enhanced tumor cell proliferation that precedes tumor neovascularization, and it activates the EGFR pathway in tumors (Laderoute et al. 1992; Bos et al. 2005; Gunaratnam et al. 2003; Swinson et al. 2004; O'Reilly et al. 2006). Hypoxia-induced stabilization of hypoxia-inducible factor (HIF-1/-2α) is known to be a strong stimulator of VEGF production by tumor cells (Carmeliet 2005), while the expression of VEGFR-1 and VEGF-2 in (tumor-associated) endothelial cells is upregulated by hypoxia (Waltenberger et al. 1996).

Anti-cancer therapy (irradiation, chemotherapy or growth factor deprivation) also causes intra-tumoral stress. It has been demonstrated that irradiation enhances both EGFR and VEGF expression (Frederick et al. 2006) in tumor cells and in endothelial cells (Bozec et al. 2005). Chemotherapy also upregulates the EGFR expression (Frassoldati et al. 1997; Sumitomo et al. 2004; Qiu et al. 2005) and may stimulate directly (Wild et al. 2004) or indirectly, via chemo-enhanced cyclooxygenase (cox)-2 expression in tumor cells (Watwe et al. 2005), the production of VEGF in tumoral tissue. Upregulation of both pathways may be involved in resistance to radiotherapy and chemotherapy (Chakravarti et al. 2002).

Altogether, this information indicates that both the EGFR and the VEGFR pathway are upregulated constitutively and transiently, due to additional stress factors, in tumor tissues. The interesting results obtained in clinical trials directed at both pathways suggest that dual inhibition may be better than inhibition of only one target.

39.3 EGF(R) Signaling and Angiogenesis: Direct Effects on Endothelial Cells

Two types of endothelial cells are used for in vitro studies of angiogenesis: human microvascular endothelial cells (HMVECs), which respond to EGF-like ligands and VEGF, and human umbilical vein endothelial cells (HUVECs). HMVECs express EGFR/ErbB1, and stimulation with EGF-like ligands induced tube formation while

treatment with gefitinib, an EGFR tyrosine kinase (TK) inhibitor, inhibited EGF-induced migration and tube formation of these cells. HUVECs express ErbB2, ErbB3 and ErbB4, but not EGFR. Stimulation of HUVECs with recombinant neuregulin (ligand for ErbB3 and ErbB4) induced cell proliferation, while stimulation with betacellulin (ligand for EGFR and ErbB4) resulted in dimerization and subsequent phosphorylation of ErbB2 and ErbB4, and proliferation, migration and tube formation of HUVECs in collagen gels. Recently, Amin et al. (2006) showed that tumor endothelial cells (isolated from melanoma, liposarcoma and breast cancer xenografts, and stained in situ) express EGFR, ErbB2 and ErbB4. The functionality of these receptors was shown by treating these cells with EGF ligands and EGFR inhibitors. Various types of normal endothelial cells expressed ErbB2, ErbB3 and ErbB4. The presence of EGFR in tumor endothelial cells was confirmed in a pancreatic cancer model (Yokoi et al. 2005). This finding suggests that tumor endothelial cells might be sensitive to EGFR-TK inhibitors (Amin et al. 2006). A recent study by Semino and colleagues suggested that VEGF might induce this switch in tumor endothelial EGFR status. They treated HUVECs with VEGF and showed that migration and morphogenesis of these cells was mediated by autocrine activation of EGFR (Semino et al. 2006).

Mature endothelial vessels are covered by smooth muscle cells (SMCs/pericytes). VEGF and angiopoietin-1 stimulate the production of heparin-binding EGF (HB-EGF, an ErbB1 and ErbB4 ligand), a potent inducer of angiogenesis (Ongusaha et al. 2004), by endothelial cells (Arkonac et al. 1998), which caused migration of SMCs (ErbB1 and ErbB2 positive) (Iivanainen et al. 2003). These cells, on the other hand, produce VEGF when stimulated by (tumoral) EGF (Sini et al. 2005). Only mature vessels effectively transport erythrocytes, and cooperation of VEGF and EGF appears to be engaged in the generation of these vessels.

EGF(R) Signaling and Angiogenesis: Indirect Effects on Endothelial Cells

Ligands of EGFR have no direct effect on HUVECs. An indirect effect of EGF on HUVECs was demonstrated by Hirata et al. (2002), who showed that HUVECs when cultured alone did not migrate in the presence of EGF, but did migrate when these cells were co-cultured with A431 cells, which express high levels of EGFR. EGF enhanced the migration of HUVECs, while gefitinib and SU5416, a selective TK inhibitor of VEGFR-2, blocked the migration of HUVECs. This finding was explained by the fact that A431 cells produced VEGF and IL-8 in response to EGF (Hirata et al. 2002). The production of angiogenic molecules by tumor cells stimulated with EGF-like ligands has been confirmed in many other studies. EGF stimulation of glioma, bladder cancer and gastric tumor cells consistently increased the synthesis of VEGF by these cells (Goldman et al. 1993; Perrotte et al. 1999; Akagi et al. 2003). In gastric and pancreatic cell lines EGF induced enhanced production of neuropilin-1, a co-receptor of VEGFR-2, increasing the affinity of VEGF to VEGFR-2 (Parikh et al. 2003).

Other ErbB receptors than EGFR, just like other ligands of the ErbB family than EGF, also play a role in angiogenesis. For example, stimulation of breast and lung cancer cells that had a constitutive or engineered overexpression of ErbB2 with heregulin-β, which stimulates ErbB3 and ErbB4 heterodimerization with ErbB2, induced the secretion of VEGF, which was absent in normal mammary and bronchial cells (Iivanainen et al. 2003; Akagi et al. 2003). Furthermore, overexpression of ErbB2 per se in human tumor cells appears to increase angiogenesis and expression of VEGF (Iivanainen et al. 2003; Perrotte et al. 1999; Laughner et al. 2001). Finally, transformation of rat fibroblasts with an ErbB2 mutant resulted in increased VEGF production (Petit et al. 1997).

Inhibition of EGFR signaling using antibodies or specific TK inhibitors confirmed the involvement of EGFR in tumor-induced angiogenesis (Petit et al. 1997; Perrotte et al. 1999; Bruns et al. 2000; Ciardiello et al. 2001; Kedar et al. 2002). Cetuximab, a monoclonal antibody to EGFR, reduced the production of VEGF and IL-8 by human transitional, pancreatic, colon and epidermoid cancer cells in vitro (Petit et al. 1997; Perrotte et al. 1999; Bruns et al. 2000; Ciardiello et al. 2001), whereas antibodies directed against ErbB2 downregulated VEGF production in an ErbB2-positive breast cancer cell line (Petit et al. 1997). Gefitinib decreased the VEGF and bFGF production in vitro in various cancer cell lines (Ciardiello et al. 2001).

In summary, we may conclude that activity of EGFR signaling in tumors is associated with the production of multiple angiogenic molecules, while inhibition of this pathway reduces their angiogenic profile. In addition, we may conclude that the effect of EGF-like ligands on tumoral endothelial cells can be direct as well as indirect.

EGFR Signaling and Angiogenesis: In Vivo Studies

Several ErbB ligands stimulate angiogenesis in preclinical models. This has been shown in a mouse cornea model (Bruns et al. 2000), in a chorioallantoic membrane assay (Goldman et al. 1993) and in bladder cancer xenografts (Ciardiello et al. 2001).

Furthermore, EGFR inhibitors inhibit tumoral angiogenesis in preclinical models. Xenografts of pancreaticcarcinomashowedadecreasedexpression of VEGF and IL-8, a decrease in microvessel density (MVD) and endothelial cell apoptosis after several weeks of treatment with either cetuximab or PKI 166 (an EGFR-TK inhibitor) (Ciardiello et al. 2001). Cetuximab suppressed VEGF in xenografts of A431 and transitional cancer cells (Petit et al. 1997; Perrotte et al. 1999). The decrease in VEGF, IL-8 and bFGF, assessed by immunohistochemistry (IHC), preceded the involution of tumor blood vessels. Treatment of squamous cell cancer xenografts with cetuximab inhibited the growth and local invasion of these cells and their associated endothelial cells (Huang et al. 2002). Treatment with either cetuximab or gefitinib resulted in a decrease of VEGF, bFGF and TGF-ô expression in colon cancer xenografts and a reduction in MVD (Ciardiello et al. 2001). Finally, treatment with PKI 166 of human renal cell cancer xenografts in nude mice downregulated expression of VEGF, IL-8 and bFGF and decreased MVD (Kedar et al. 2002). These studies show that activation of EGF–EGFR signaling induces tumor-associated angiogenesis and that inhibition of this effect may contribute to the anticancer effects of EGFR inhibitors. This idea has recently been indirectly confirmed by Chung et al. who showed that patients with EGFR-negative tumors responded to cetuximab, an effect which might be explained by the direct effect of cetuximab on the tumoral endothelial cell compartment (Chung et al. 2005).

Resistance to Anti-EGFR and Anti-VEGFR Therapy

Specific mutations in the ATP-binding site of the EGFR and the status of specific downstream signaling molecules (Akt, MAPK) are predictive for a clinical response to EGFR TK inhibitors (Janne et al. 2004; Miller et al. 2005). Acquired resistance to EGFR inhibitors occurs in clinical studies and can be induced in preclinical models by continuous treatment with anti-EGFR agents (Viloria-Petit et al. 2001; Ciardiello et al. 2004).This reduced sensitivity to anti-EGFR therapy could not be explained by changes in EGFR status in these two studies, since total protein and phosphorylation of EGFR did not significantly change during treatment. The mechanisms of acquired resistance to EGFR inhibitors have recently been reviewed (Viloria-Petit and Kerbel 2004; Camp et al. 2005). Genetic altera-

tion or overactivity of oncogenic signaling pathways independent of EGFR, but also mutations in the EGFR gene, are responsible for resistance to EGFR inhibitors (Kobayashi et al. 2005). Upregulation of VEGF and other angiogenic molecules by tumor cells is another mechanism. Chronic treatment of mice bearing A431 tumor xenografts with cetuximab induced resistance, which was associated with retained high EGFR expression and unaltered sensitivity to cetuximab when these tumor cells were harvested and cultured in vitro. Their increased growth potential in vivo could be explained by an increased production of VEGF, resulting in increased angiogenic potential (Viloria-Petit et al. 2001). Ciardiello and colleagues induced resistance to cetuximab and gefitinib by continuous treatment of colon cancer xenografts in athymic mice (Ciardiello et al. 2004). These resistant colon cancer cells showed a five- to tenfold increase in activated MAPK and expression of cox-2 and VEGF, compared with parental colon cancer cells. Perez-Soler found that acquired resistance of squamous cancer cell lines to erlotinib was associated with increased activity of AKT and overexpression of fibroblast growth factor receptor (FGFR), platelet-derived growth factor receptor (PDGFR) and fibronectin (Perez-Soler 2004). All these molecules are involved in angiogenesis. Finally, resistance to trastuzumab, the antibody against ErbB2, was also found to be associated with elevated levels of VEGF and could be treated with bevacizumab in a breast cancer model (du Manoir et al. 2006).

In a recent study, it was shown that an antibody against VEGFR-2 inhibited the growth of intracerebral glioblastoma cells, but caused increased tumor cell invasion along the preexisting vasculature, which could be blocked by cetuximab (Lamszus et al. 2005). Together, these data suggest that VEGF may be involved in the resistance to anti-EGFR treatment, while EGF may play a role in the resistance to anti-VEGF(R) therapies.

Combined Targeting of EGFR and VEGF(R) in Preclinical and Clinical Studies

It is appealing to combine anti-EGFR drugs with anti-angiogenic interventions. This has been investigated preclinically using agents with dual inhibition of both EGFR and VEGFR (e.g. ZD6474 and AEE788), and with combinations of EGFR and VEGF(R) inhibitors.

ZD6474, a TK inhibitor of VEGFR and EGFR (Heymach 2005), has been shown to induce substantial anti-tumor effects in preclinical models studying gastric cancer (McCarthy et al. 2004; Arao et al. 2006), hepatocellular carcinoma (Giannelli et al. 2006), central nervous tumors (Sandstrom et al. 2004; Rich et al. 2005), small cell lung cancer (Yano et al. 2005), renal cell cancer (Drevs et al. 2004) and prostate cancer (Checkley et al. 2003). The anti-tumor effects were accompanied by increased apoptosis of tumor cells and associated endothelial cells in these studies. ZD6474 induced an immediate reduction of vascular permeability in prostate xenografts (Checkley et al. 2003). ZD6474 was still effective in colon cancer cell xenografts resistant to cetuximab and gefitinib, and continuous treatment with ZD6474 monotherapy resulted in efficient growth inhibition for up to 150 days in this model (Ciardiello et al. 2004).

AEE788, a TK inhibitor of ErbB1 and ErbB2 and VEGFR-1 and -2, demonstrated anti-proliferative activity against a range of EGFR-overexpressing tumor cell lines and EGF- and VEGF-stimulated HUVECs. In preclinical models featuring anaplastic or follicular thyroid carcinoma (Kim et al. 2005; Younes et al. 2005), squamous cancer (Yigitbasi et al. 2004; Park et al. 2005), ovarian cancer (Thaker et al. 2005), pancreatic cancer (Yokoi et al. 2005) and prostate cancer (Yazici et al. 2005) this agent had substantial anti-tumor activity, accompanied by apoptosis of tumor-associated endothelial cells and decreased microvessel density.

Addition of cetuximab to an anti-VEGFR antibody, DC101, resulted in further reduction in tumor growth, tumor vascularity and formation of ascites, and increase in apoptosis of both tumor cells and endothelial cells, in a colon cancer model (Shaheen et al. 2001). In this model cetuximab augmented significantly the anti-angiogenic effects of DC101. In an orthotopic gastric cancer mice model the combination of these two agents was superior in tumor growth inhibition compared with either agent alone (Jung et al. 2002). Cetuximab plus an antisense VEGF oligonucleotide in mice with human colon cancer also induced a prolonged inhibition of tumor growth and reduction in MVD (Ciardiello et al. 2000). Moreover, Hidalgo reported additional antitumor effects when erlotinib was combined with the anti-VEGF antibody bevacizumab in a variety of preclinical models (Hidalgo 2003).

Together, these preclinical investigations indicate that therapy combining anti-EGFR with anti-VEGF(R) could be of interest for cancer patients.

Several clinical studies combining anti-EGFR compounds with angiogenesis inhibitors are under way. Results of small phase I/II trials combining erlotinib with bevacizumab have recently been presented. Herbst et al. showed that in patients with recurrent non-small cell lung cancer (NSCLC) this combination resulted in 20% partial responses (PR) and 65% stable disease (SD) (Herbst et al. 2005). These results appear better than those obtained with erlotinib alone in unselected patients (Shepherd et al. 2005). The results of a study for patients with renal cell cancer were of special interest. The combination resulted in 25% PR and 61% SD (Hainsworth et al. 2005), while EGFR inhibitors were not effective (Dawson et al. 2004) and bevacizumab alone induced only 10% PR in this disease (Yang 2004). However, a phase II trial with ZD6474 for 46 heavily pretreated breast cancer patients showed no activity (Miller et al. 2005). The toxicity in these studies was limited to grade 2. Rash, diarrhea, hypertension and fatigue were the most common side effects, which seldom necessitated discontinuation of treatment. A great number of trials based on this concept are running at this moment. So far we may conclude that preliminary data support the preclinical evidence that combination of anti-EGFR and anti-VEGF(R) therapy is promising. A next step will be combination of antibodies and TK inhibitors with the same target (Matar et al. 2004), and the use of drugs (alone or in combination) with a broader target profile: for instance plus bFGF(R) or PDGF(R). Combination of drugs targeting both EGFR and VEGF(R) with chemotherapy (Yigitbasi et al. 2004; Yokoi et al. 2005) or radiotherapy (Williams et al. 2004; Damiano et al. 2005; Frederick et al. 2006) has been shown to be successful in preclinical settings. In the ATLAS trial patients with locally advanced or metastatic NSCLC will be treated with chemotherapy plus bevacizumab and, after progression of disease, by bevacizumab followed by erlotinib or by the combination of these drugs. In the Betalung trial patients with advanced or metastatic NSCLC previously treated with chemotherapy will receive erlotinib or erlotinib with bevacizumab, while in another trial such patients will be randomized between docetaxel + pemetrexed with or without bevacizumab and erlotinib + bevacizumab. The outcome of these trials will certainly have great impact on the oncology practice of the future.

Conclusion

Strong preclinical evidence points to important crosstalk between activated EGFR and VEGF(R) pathways in tumor biology. Both pathways are upregulated in tumors due to the genetic profile, hypoxia and stress induced by anti-cancer strategies. Moreover, both families of proteins stimulate the production of each other. Inhibition of one or both pathways is consequential for the activity of the other. In preclinical studies, resistance to anti-EGFR therapy could be circumvented and survival could be improved with anti-angiogenic therapy. Therefore, the combination of inhibitors of both pathways is logical and may be more effective than blocking one or the other. The first clinical experiences encourage further clinical exploration of this combination.

References

Akagi M, Kawaguchi M, Liu W, McCarty MF et al (2003) Induction of neuropilin-1 and vascular endothelial growth factor by epidermal growth factor in human gastric cancer cells. Br J Cancer 88:796–802

Amin DN, Hida K, Bielenberg DR, Klagsbrun M (2006) Tumor endothelial cells express epidermal growth factor receptor (EGFR) but not ErbB3 and are responsive to EGF and to EGFR kinase inhibitors. Cancer Res 66:2173–2180

Arao T, Yanagihara K, Takigahira M, Takeda M et al (2006) ZD6474 inhibits tumor growth and intraperitoneal dissimination in a highly metastatic orthotopic gastric cancer model. Int J Cancer 118:483–489

Arkonac BM, Foster LC, Sibinga NE, Patterson C et al (1998) Vascular endothelial growth factor induces heparin-binding epidermal growth factor-like growth factor in vascular endothelial cells. J Biol Chem 20:4400–4405

Baker CH, Kedar D, McCarthy MF, Tsan R et al (2002) Blockade of epidermal growth factor receptor signaling on tumor cells and tumor-associated cells endothelial cells for therapy of human carcinomas. Am J Pathol 161:929–938

Bos R, van Diest PJ, de Jong JS, van der Groep P et al (2005) Hypoxia-inducible factor-1alpha is associated with angiogenesis, and expression of bFGF, PDGF-BB, and EGFR in invasive breast cancer. Histopathology 46:31–36

Bozec A, Formento P, Ciccolini J, Fanciullino R et al (2005) Response of endothelial cells to a dual tyrosine kinase receptor inhibition combined with irradiation. Mol Cancer Ther 4:1962–1971

Bruns CJ, Harbison MT, Davis DW, Portera CA et al (2000) Epidermal growth factor receptor blockade with C225 plus gemcitabine results in regression of human pancreatic carcinoma growing orthotopically in nude mice by antiangiogenic mechanisms. Clin Cancer Res 6:1936–1948

Camp ER, Summy J, Bauer TW, Liu W et al (2005) Molecular mechanisms of resistance to therapies targeting the epidermal growth factor. Clin Cancer Res 11:397–405

Carmeliet P (2005) VEGF as a key mediator of angiogenesis in cancer. Oncology 69 [Suppl 3]:4–10

Chakravarti A, Chakladar A, Delaney MA, Latham DE, Loeffler JS (2002) The epidermal growth factor receptor pathway mediates resistance to sequential administration of radiation and chemotherapy in primary glioblastoma cells in a RAS-dependent manner. Cancer Res 62:4307–4315

Checkley D, Tessier JJ, Kendrew J, Waterton JC, Wedge SR (2003) Use of dynamic contrast-enhanced MRI to evaluate acute treatment with ZD6474, a VEGF signalling inhibitor, in PC-3 prostate tumours. Br J Cancer 89:1889–1895

Chung KY, Shia J, Kemeny NE, Shah M et al (2005) Cetuximab shows activity in colorectal cancer patients with tumors that do not express the epidermal growth factor receptor by immunohistochemistry. J Clin Oncol 23:1803–1810

Ciardiello F, de Vita F (2005) Epidermal growth factor receptor (EGFR) inhibitors in cancer therapy. Prog Drug Res 63:93–114

Ciardiello F, Bianco R, Damiano V, Fontanini G et al (2000) Antiangiogenic and antitumor activity of anti-epidermal growth factor receptor C225 monoclonal antibody in combination with vascular endothelial growth factor antisense oligonucleotide in human GEO colon cancer cells. Clin Cancer Res 6:3739–3747

Ciardiello F, Caputo R, Bianco R, Damiano V et al (2001) Inhibition of growth factor production and angiogenesis in human cancer cells by ZD1839 (Iressa), a selective epidermal growth factor receptor tyrosine kinase inhibitor. Clin Cancer Res 7:1459–1465

Ciardiello F, Bianco R, Caputo R, Caputo R et al (2004) Antitumor activity of ZD6474, a vascular endothelial growth factor receptor tyrosine kinase inhibitor, in human cancer cells with acquired resistance to antiepidermal growth factor receptor therapy. Clin Cancer Res 10:784–193

Damiano V, Melisi D, Bianco C, Raben D et al (2005) Cooperative antitumor effect of multitargeted kinase inhibitor ZD6474 and ionizing radiation in glioblastoma. Clin Cancer Res 11:5639–5644

Dawson NA, Guo C, Zak R, Dorsey B et al (2004) A phase II trial of gefitinib (Iressa, ZD1839) in stage IV and recurrent renal cell carcinoma. Clin Cancer Res 10:7812–7819

Drevs J, Konerding MA, Wolloscheck T, Wedge SR et al (2004) The VEGF receptor kinase inhibitor, ZD6474, inhibits angiogenesis and affects microvascular architecture within an orthotopically implanted renal cell carcinoma. Angiogenesis 7:347–354

Du Manoir JM, Francia G, Man S, Mossoba M et al (2006) Strategies for delaying or treating in vivo acquired resistance to trastuzumab in human breast cancer xenografts. Clin Cancer Res 12:904–916

Ferrara N (2005) VEGF as therapeutic target in cancer. Oncology 69 [Suppl 3]:11–16

Frassoldati A, Adami F, Banzi C, Criscuolo M et al (1997) Changes of biological features in breast cancer cells determined by primary chemotherapy. Breast Cancer Res Treatment 44:185–192

Frederick B, Gustafson D, Bianco C, Ciardiello F et al (2006) ZD6474, an inhibitor of VEGFR and EGFR tyrosine kinase activity in combination with radiotherapy. Int J Rad Oncol Biol Phys 64:33–37

Gianelli G, Azzariti A, Sgarra C, Porcelli L et al (2006) ZD6474 inhibits proliferation and invasion of human hepatocellular carcinoma cells. Biochem Pharmacol 71:479–485

Goldman CK, Kim J, Wong WL, King V et al (1993) Epidermal growth factor stimulates vascular endothelial growth factor production by human malignant glioma cells: a model of glioblastoma multiforme pathophysiology. Mol Biol Cell 4:121–133

Gunaratnam L, Morley M, Franovic A, de Paulsen N et al (2003) Hypoxia-inducible factor activates the transforming growth factor-alpha/ epidermal growth factor receptor growth stimulatory pathway in VHL(-/-) renal cell carcinoma cells. J Biol Chem 278:44966–44974

Hainsworth JD, Sosman JA, Spigel DR, Schwert RC et al

(2005) Treatment of metastatic renal carcinoma with a combination of bevacizumab and erlotinib. J Clin Oncol 23:7889–7896

Herbst RS, Johnson DH, Mininberg E, Carbone DP et al (2005) Phase I/II trial evaluating the anti-vascular endothelial growth factor monoclonal antibody bevacizumab in combination with the HER-1/epidermal growth factor receptor tyrosine kinase inhibitor erlotinib for patients with recurrent non-small-cell lung cancer. J Clin Oncol 23:2544–2555

Heymach JV (2005) ZD6474: clinical experience to date. Br J Cancer 92:S14–S20

Hidalgo M (2003) Erlotinib: preclinical investigations. Oncology 17:11–16

Hirata A, Ogawa S, Kometani T, Kuwano T et al (2002) ZD1839 (Iressa) induces antiangiogenic effects through inhibition of epidermal growth factor receptor tyrosine kinase. Cancer Res 62:2554–2560

Huang SM, Li J, Harari PM (2002) Molecular inhibition of angiogenesis and metastatic potential in human squamous cell carcinomas after epidermal growth factor receptor blockade. Mol Cancer Ther 1:507–514

Iivanainen E, Nelimarkka L, Elenius V, Heikkinen SM et al (2003) Angiopoietin-regulated recruitment of vascular smooth muscle cells by endothelial-derived heparin binding EGF-like growth factor. FASEB J 17:1609–1621

Janne PA, Gurubhagavatula S, Yeap BY, Lucca J et al (2004) Outcomes of patients with advanced non-small cell lung cancer treated with gefitinib (ZD1839, "Iressa") on an expanded access study. Lung Cancer 44:221–230

Jung YD, Mansfield PF, Akagi M, Takeda A et al (2002) Effects of combination anti-vascular endothelial growth factor receptor and anti-epidermal growth factor receptor therapies on the growth of gastric cancer in a nude mouse model. Eur J Cancer 38:1133–1140

Kedar D, Baker CH, Killion JJ, Dinney CP, Fidler IJ (2002) Blockade of the epidermal growth factor receptor signaling inhibits angiogenesis leading to regression of human renal cell carcinoma growing orthotopically in nude mice. Clin Cancer Res 8:592–600

Kim S, Schiff BA, Yigitbasi OG, Doan D et al (2005) Targeted molecular therapy of anaplastic thyroid carcinoma with AEE788. Mol Cancer Ther 4:632–640

Kobayashi S, Boggon TJ, Dayaram T, Janne PA et al (2005) EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med 352:786–792

Laderoute KR, Grant TD, Murphy BJ, Sutherland RM (1992) Enhanced epidermal growth factor receptor synthesis in human squamous carcinoma cells exposed to low levels of oxygen. Int J Cancer 52:428–432

Lamszus K, Brockmann MA, Eckerich C, Bohlen P et al (2005) Inhibition of glioblastoma angiogenesis and invasion by combined treatments directed against vascular endothelial growth factor receptor-2, epidermal growth factor receptor, and vascular endothelial-cadherin. Clin Cancer Res 11:4934–4940

Laughner E, Taghavi P, Chiles K, Mahon PC, Semenza GL (2001) Her2 (neu) signaling increases the rate of hypoxia-inducible factor 1 alpha (HIF-1α) synthesis: novel mechanism for HIF-mediated vascular endothelial growth factor expression. Mol Cell Biol 21:3995–4004

Matar P, Rojo F, Cassia R, Moreno-Bueono G et al (2004) Combined epidermal growth factor receptor targeting with the tyrosine kinase inhibitor gefinitib (ZD1939) and the monoclonal antibody cetuximab (IMC-C225): superiority over single-agent receptor targeting. Clin Cancer Res 10:6487–6501

McCarty MF, Wey J, Stoeltzing O, Liu W et al (2004) ZD6474, a vascular endothelial growth factor receptor tyrosine kinase inhibitor with additional activity against epidermal growth factor receptor tyrosine kinase, inhibits orthotopic growth and angiogenesis of gastric cancer. Mol Cancer Ther 3:1041–1048

Miller KD, Trigo JM, Wheeler C, Barge A et al (2005) A multicenter phase II trial of ZD6474, a vascular endothelial growth factor receptor-2 and epidermal growth factor tyrosine kinase inhibitor, in patients with previously treated metastatic breast cancer. Clin Cancer Res 11:3369–3376

Ongusaha PP, Kwak JC, Zwible AJ, Macip S et al (2004) HB-EGF is a potent inducer of tumor growth and angiogenesis. Cancer Res 64:5283–5290

O'Reilly SM, Leonard MO, Kieran N et al (2006) Hypoxia induces epithelial amphiregulin gene expression in a CREB-dependent manner. Am J Physiol Cell Physiol 290: C592–C600

Parikh AA, Liu WB, Fan F, Stoeltzing O et al (2003) Expression and regulation of the novel vascular endothelial growth factor receptor neuropilin-1 by epidermal growth factor in human pancreatic carcinoma. Cancer 98:720–729

Park YW, Younes MN, Jasser SA, Yigitbasi OG et al (2005) AEE788, a dual tyrosine kinase receptor inhibitor, induces endothelial cell apoptosis in human cutaneous squamous cell carcinoma xenografts in nude mice. Clin Cancer Res 11:1963–1973

Perez-Soler R (2004) Phase II clinical trial data with the epidermal growth factor receptor tyrosine kinase inhibitor erlotinib (Osi-774) in non-small-cell lung cancer. Clin Lung Cancer 6:S20–S23

Perez-Soler R, Piperdi B, Haigentz M, Ling YH (2004) Determinants of sensitivity to the EGFR TK inhibitor erlotinib (E) in a panel of NSCLC cell lines. Proc Am Soc Clin Oncol 7026

Perrotte P, Matsumoto T, Inoue K, Kuniyasu H et al (1999) Anti-epidermal growth factor receptor antibody C225 inhibits angiogenesis in human transitional cell carcinoma growing orthotopically in nude mice. Clin Cancer Res 5:257–265

Petit AM, Rak J, Hung MC, Rockwell P et al (1997) Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells in vitro

and in vivo: angiogenic implications for signal transduction therapy of solid tumors. Am J Pathol 151:1523–1530

Qiu L, Di W, Jiang Q, Scheffler E et al (2005) Targeted inhibition of transient activation of the EGFR-mediated cell survival pathway enhances paclitaxel-induced ovarian cancer cell death. Int J Oncol 27:1441–1448

Rak J, Yu JL, Klement G, Kerbel RS (2000) Oncogenes and angiogenesis: signaling three-dimensional tumor growth. J Invest Dermatol Proc 5:24–33

Rich JN, Sathornsumetee S, Keir ST, Kieran MW et al (2005) ZD6474, a novel tyrosine kinase inhibitor of vascular endothelial growth factor receptor and epidermal growth factor receptor, inhibits tumor growth of multiple nervous system tumors. Clin Cancer Res 11:8145–8157

Sandstrom M, Johansson M, Andersson U, Bergh A et al (2004) The tyrosine kinase inhibitor ZD6474 inhibits tumour growth in an intracerebral rat glioma model. Br J Cancer 91:1174–1180

Semino CE, Kamm RD, Lauffenburger DA (2006) Autocrine EGF receptor activation mediates endothelial cell migration and vascular morphogenesis induced by VEGF under interstitial flow. Exp Cell Res 312:289–298

Shaheen RM, Ahmad SA, Liu W, Reinmuth N et al (2001) Inhibited growth of colon cancer carcinomatosis by antibodies to vascular endothelial and epidermal growth factor receptors. Br J Cancer 85:584–589

Shepherd FA, Rodrigues Pereira J, Ciuleanu T, Ciuleanu T et al (2005) Erlotinib in previously treated non-small-cell lung cancer. N Engl J Med 353:123–132

Sini P, Wyder L, Schnell C, Littlewood A et al (2005) The antitumor and antiangiogenic activity of vascular endothelial growth factor receptor inhibition is potentiated by ErbB1 blockade. Clin Cancer Res 11:4521–4532

Sumitomo M, Asano T, Asakuma J, Asano T et al (2004) ZD1839 modulates paclitaxel response in renal cell cancer by blocking paclitaxel-induced activation of the epidermal growth factor receptor-extracellular signal-regulated kinase pathway. Clin Cancer Res 10:794–801

Swinson DE, Jones JL, Cox G, Richardson D et al (2004) Hypoxia-inducible factor-1 alpha in non small cell lung cancer: relation to growth factor, protease and apoptosis pathway. Int J Cancer 111:43–50

Thaker PH, Yazici S, Nilsson MB, Yokoi K et al (2005) Antivascular therapy for orthotopic human ovarian carcinoma through blockade of the vascular endothelial growth factor and epidermal growth factor receptors. Clin Cancer Res 11:4923–4933

Van Cruijsen H, Giaccone G, Hoekman K (2005) Epidermal growth factor receptor and angiogenesis: opportunities for combined anticancer strategies. Int J Cancer 118:883–888

Viloria-Petit AM, Kerbel RS (2004) Acquired resistance to EGF inhibitors: mechanisms and prevention strategies. Int J Radiat Oncol Biol Phys 58:914–926

Viloria-Petit A, Crombet T, Jothy S, Hicklin D et al (2001) Acquired resistance to the antitumor effect of epidermal growth factor receptor-blocking antibodies in vivo: a role for altered tumor angiogenesis. Cancer Res 61:5090–101

Wedge SR, Kendrew J, Hennequin LF, Valentine PJ et al (2005) AZD2171: a highly potent, orally bioavailable, vascular endothelial growth factor receptor-2 tyrosine kinase inhibitor for the treatment of cancer. Cancer Res 65:4389–4400

Waltenberger J, Mayr U, Pentz S, Hombach V (1996) Functional upregulation of the vascular endothelial growth factor receptor KDR by hypoxia. Circulation 94:1647–1654

Watwe V, Javle M, Lawrence D, Groth J et al (2005) Cyclooxygenase-2 (cox-2) levels before and after chemotherapy: a study in rectal cancer. Am J Clin Oncol 28:560–564

Wild R, Dings RP, Subramanian I, Ramakrishnan S (2004) Carboplatin selectively induces the VEGF stress response in endothelial cells: potentiation of antitumor activity by combination treatment with antibody to VEGF. Int J Cancer 110:343–351

Williams KJ, Telfer BA, Brave S, Kendrew J et al (2004) ZD6474, a potent inhibitor of vascular endothelial growth factor signaling, combined with radiotherapy: schedule-dependent enhancement of antitumor activity. Clin Cancer Res 10:8587–8593

Yang JC (2004) Bevacizumab for patients with metastatic renal cancer: an update. Clin Cancer Res 10:6367S–6370S

Yano S, Muguruma H, Matsumori Y, Goto H et al (2005) Antitumor vascular strategy for controlling experimental metastatic spread of human small-cell lung cancer cells with ZD6474 in natural killer cell-depleted severe combined immunodeficient mice. Clin Cancer Res 11:8729–8798

Yazici S, Kim SJ, Busby JE, He J et al (2005) Dual inhibition of the epidermal growth factor and vascular endothelial growth factor phosphorylation for antivascular therapy of human prostate cancer in the prostate of nude mice. Prostate 65:203–215

Yigitbasi OG, Younes MN, Doan D, Jasser SA et al (2004) Tumor cell and endothelial cell therapy of oral cancer by dual tyrosine kinase receptor blockade. Cancer Res 64:7977–7984

Yokoi K, Sasaki T, Bucana CD, Fan D et al (2005) Simultaneous inhibition of EGFR, VEGFR, and platelet-derived growth factor receptor signaling combined with gemcitabine produces therapy of human pancreatic carcinoma and prolongs survival in an orthotopic nude mouse model. Cancer Res 65:10371–10380

Younes MN, Yigitbasi OG, Park YW, Kim SJ et al (2005) Antivascular therapy of human follicular thyroid cancer experimental bone metastasis by blockade of epidermal growth factor receptor and vascular growth factor receptor phosphorylation. Cancer Res 65:4716–4727

Imaging the Effect of Anti-Angiogenic Tumor Therapy in Clinical Studies

40

Bruno Morgan and Mark A. Horsfield

Abstract

Although tumor size measurements taken from CT scans are useful for monitoring treatment, this approach has several weaknesses due to heterogeneity of response, inter-observer variability, potentially long time frames to gain data, and lack of specificity to the mechanism of response. This requires increasingly long-term measures to assess efficacy that may offer little advantage over clinical assessment measures. Increasing knowledge of molecular medicine and its application to the study of cancer has dramatically changed treatment approaches, and there are many potential cancer treatments in the "pipeline" of the major pharmaceutical companies and research institutes. Clinical studies are particularly important, since animal studies do not directly translate to humans, and most pre-clinical studies are carried out in "non-wild-type" tumors with different locations, maturity and stages of development. This is particularly true for treatments based on tumor angiogenesis. The questions in early clinical trials have changed from toxicity and pharmacokinetics to include whether the agent is having its intended effect in vivo. There is, therefore, an increasing need to study biological processes in vivo at both the pre-clinical and clinical stages of pharmacological development and application. Imaging studies sensitive to these biological processes have been used extensively in the pre-clinical setting, but translation of this work from bench to bedside is more difficult. This article discusses the development of such imaging and attempts to address the questions being asked of imaging departments by oncologists and pharmaceutical companies.

CONTENTS

Introduction

Although medical imaging for cancer has changed greatly over the past three decades, there is still an emphasis on a surgical approach to disease, concentrating on the localization, size, shape and appearance of tumor lesions. Advanced scanning equipment, using X-ray computed tomography, ultrasound, magnetic resonance and positron-emitting agents, is largely used to assess new cancer patients accurately prior to surgical treatment of their tumors. For patients with advanced cancer on systemic therapy, imaging is used mainly to document increases or decreases in tumor size. Pressure for change has been limited by the lack of innovative pharmacological approaches to cancer therapy, with surgical resection still giving the best chance of cure in most solid tumors (Bailar and Gornik 1997).

Although, as might be expected, tumor size measurements taken from X-ray computed tomography scans have a correlation with clinical outcome in chemotherapy (Buyse et al. 2000), and are useful for monitoring treatment where rapid size changes are expected, this approach has several weaknesses. In lung cancer the tumor shrinkage (response rate) shows some correlation with survival but not as much as might be hoped (Shanafelt et al. 2004). Further, for gastric cancer it has been demonstrated that it is probably the duration of the stable disease phase, and not tumor shrinkage, that has the major effect in prolonging survival of patients (Takahashi et al. 2000). This requires increasingly long-term measures to assess efficacy that may offer little advantage over assessment of clinical measures of progression-free survival or quality of life. A second weakness is that inter- and intraobserver variation in assessment of response rates, using cross-sectional imaging, is poor and requires a 30% reduction in size of tumors to reliably gauge the response to treatment (Therasse et al. 2000). Thus, to assess the efficacy of a cancer treatment properly, long-term markers of efficacy are currently required, requiring large-scale expensive studies.

Increasing knowledge of molecular medicine and its application to the study of cancer has dramatically changed treatment approaches, and there are many potential cancer treatments in the "pipeline" of the major pharmaceutical companies and research institutes. Clinical studies are particularly important, since animal studies do not directly translate to humans, and most pre-clinical studies are carried out in "non-wild-type" tumors varying in location, maturity and stage of development.

This is particularly true for the introduction of targeted agents that inhibit specific tumor pathways, such as treatments based on tumor angiogenesis. Traditionally, the purpose of a phase I clinical study is to establish pharmacokinetics and potential toxicity in humans. However, with so many potential therapeutic options being developed, the questions in early trials have changed to: does the agent has an effect via the intended mechanism (e.g., changes in vascularity) in vivo; does it affect the intended receptor in vivo; and is it getting to the right place in sufficient quantities and times in the studied dose regime (Jain 1994)? These are important for early clinical studies in advanced cancer, since it may be difficult to detect clinical efficacy by traditional methods such as survival or a reduction in tumor size. However, trials do not solely need to confirm that new agents are acting in their intended way, but also to sieve through large numbers of agents for potential efficacy in the shortest time possible. There is, therefore, an increasing need to study biological processes in vivo at both the pre-clinical and clinical stages of pharmacological development and application.

There are two potential roles for imaging: first as a "biomarker", and second as a "surrogate clinical endpoint" for the activity of the drug. A biological marker (biomarker) is defined as an objective measurement indicating a pharmacological response to a therapeutic intervention. A surrogate clinical endpoint is a biomarker that is intended to substitute for a clinical endpoint; a characteristic or variable that reflects the patient's well-being (Atkinson et al. 2001). Surrogate endpoints potentially provide a quicker trial result, since "true" endpoints include parameters such as 5-year survival. They also help to avoid clouding of the results by factors incidental to the treated disease. Another advantage is that fewer patients are required, because each patient can act as their own control.

However, a balance is needed between the use of specific biomarkers and true clinical endpoints. On the one hand, it is useful to know whether treatments are promising in early therapeutic trials, even if advanced tumors are not the ultimate target, since this avoids rejecting a potentially useful therapy. On the other hand, in combination treatment, anti-angiogenic treatment may be expected to reduce vasculature, which may compromise the effectiveness of radiotherapy or chemotherapy. In this case, biomarkers showing specific drug activity are less helpful, and 'true' indirect clinical endpoints, such as tumor size response or delayed time to progression, are required (Hurwitz et al. 2005).

This has lead to the search for new surrogate endpoints as markers of efficacy for angiogenesis inhibitors. Angiogenesis is a local tissue phenomenon, so attempts to measure surrogate markers in the blood, while promising, may be non-specific (Drevs et al. 2005). Biopsies with histopathological staining are invasive, may not be representative of the whole tumor, and only give information at a single time point (Kerbel 2000). Several imaging modalities potentially offer a non-invasive in vivo test, with the ability both to measure tumors spatially and to map their changes over time. Functional and molecular information is increasingly being emphasized, and the in vivo characterization and measurement of biological processes at the cellular and molecular level is often described as 'molecular imaging' (Weissleder and Mahmood 2001). Measuring vascular or other physiological changes can be described as 'functional' imaging. Such molecular or functional imaging could very quickly show whether the drug is working at a mechanistic/molecular level. Imaging is already changing the study of drug development in the pre-clinical phase, but is just starting to have an impact in the clinic.

Imaging studies investigating the molecular basis of cancer have been used extensively in the pre-clinical setting (Evelhoch et al. 2000), but translation of this work from bench to bedside is more difficult, since radiology departments are often focused on high-turnover clinical service work, with little money or poor organizational systems for research. Furthermore, using complex imaging in clinical trials to study biological processes asks more time and greater commitment from the patient.

Possible Imaging Targets

The "switch" to angiogenesis involves many processes and factors which may be potential treatment targets. These range from genetic alterations, to the consequences of angiogenic factors, such as vascular endothelial growth factor (VEGF), causing changes in vascular permeability, proliferation and maturity. Vascular changes may result in increased circulation or, more commonly, the presence of inadequate vascular networks and lymphatics, leading to hypoxia and high interstitial pressure (Carmeliet and Jain 2000).

There are, therefore, several aspects of the angiogenic process that are amenable to imaging. First are the direct processes that may be manifested by over-expression of cell surface markers, falling into the realms of 'molecular imaging'. These processes may also lead to changes in circulating angiogenic factors (Drevs et al. 2005). Less directly, there will be changes in the vasculature itself, including the vascular permeability to macromolecules (and therefore contrast agents), the perfusion of the tissue,

and the maturity of the vessels in the tumor which may change in response to pharmacologic manipulation. Ultimately, failure of tissue perfusion will lead to cell death by necrosis and apoptosis, which suggests further potential imaging targets including hypoxia and apoptosis markers as well as tumor volume. Anti-angiogenic treatment may therefore have a range of effects on the vascular characteristics of a tumor but, ultimately, all should be amenable to imaging of some kind.

There are three major mechanisms by which pharmacological targeting of tumor blood vessels could be achieved: true angiogenesis inhibition, vascular targeting (directly destroying blood vessels) and non-selective anti-angiogenic effects such as have been proposed for some chemotherapeutic agents at low dose (Miller et al. 2001). Thus, any successful cancer treatment may affect vasculature and therefore alter imaging-derived vascular parameters. Furthermore, any imaging test that has been shown to correlate with a successful clinical outcome may be useful in clinical studies of anti-angiogenesis agents.

The vascularity of a tumor can be measured in terms of the blood volume (the volume of the intravascular space compared with the volume of the tumor) and perfusion (rate of blood flow into the tumor per unit volume of tumor). A further aspect is the permeability of the vasculature, which determines the ease with which substances can pass from the intravascular to the extravascular, extracellular (interstitial) space. The permeability varies according to the molecular weight (size) of the substance, and angiogenesis promoters such as VEGF have been shown to increase permeability to macromolecules (Dvorak 2002). There is debate about the exact mechanism of changes in macromolecular permeability that may impact on interpretation of imaging results. One possibility is that macromolecules extravasate predominantly by an opening of the junctions between adjacent endothelial cells (McDonald et al. 1999). However, Dvorak and colleagues argue that, although it is likely that very small hydrophilic molecules up to 3 nm in diameter pass through intact interendothelial cell junctions, in response to VEGF-A macromolecules up to 50–70 nm in diameter cross the endothelium predominantly by means of a transendothelial cell pathway that involves vesiculovacuolar organelles (Dvorak 2002). Thus, there are potentially two distinct mechanisms for the leakage of macromolecules up to 3 nm in diameter.

Phase I trial design is made difficult by the lack of toxicity of these drugs, such that toxicity-based selection of dose for further development may not be the best way forward. Although conventional imaging techniques are still needed, since tumor size monitoring will remain an important response variable (Carmeliet and Jain 2000), methods to demonstrate biological activity before reaching maximum tolerated dose, or even to show an optimal dose well below the maximum tolerated dose, would greatly enhance the utility of such studies. There is currently no proven method of imaging the angiogenic process. The reasons for this are easy to grasp: angiogenesis is a complex process involving many steps, defying a simple single-method approach. Furthermore, if the individual molecular processes are to be studied, the method has to be sensitive to microscopic changes or nanomolar concentrations of naturally occurring substances or deliverable imaging contrast agents.

It should be noted that in a dose-escalating trial, even if a potential biomarker shows a correlation between dose and efficacy, this may represent purely a side effect or even toxicity of the drug, and does not imply cause and effect. Although many imaging tests are in the early stages of validation as biomarkers, there are, as yet, no studies validating "functional imaging" as surrogate endpoint of clinical efficacy for anti-angiogenic treatments.

Imaging Modalities

Imaging can be performed by a variety of modalities, including X-ray computed tomography (CT), magnetic resonance imaging (MRI), radioisotope imaging (single photon emission computed tomog-

raphy, SPECT, and positron emission computed tomography, PET), ultrasound and optical imaging. One fundamental parameter of all imaging modalities is their resolution, which impacts on the ability to separate tissues that are different either by structure or function in a spatial and temporal manner. Resolution is related to the ratio of signal to noise, or the ratio of the information returned from the tissue to the random variation in that information due to measurement imperfections. All in vivo imaging techniques have their own strengths and weaknesses due to the different types of information returned, and therefore have varying limits of spatial and temporal resolution. In some cases, the imaging modality alone provides information that is relevant for studying angiogenesis, e.g. Doppler ultrasound, which provides blood flow measures, or MRI using diffusion or spectroscopy techniques. However, with other techniques, a contrast agent (or probe, possibly radioactively labeled) is required. In these cases, it is the attributes of the agent that largely dictate what information can be gained. The imaging test determines at what concentration, speed and spatial resolution the agent can be studied.

When a contrast agent is used, it may provide direct information related to a specific aspect of angiogenesis, or indirect (downstream) information related to a consequence of successful therapy. Most tests performed without the use of contrast media or probes are generally indirect. The multiple potential targets and treatment strategies suggest that the most useful approach may be an indirect measure of angiogenesis. The most common current indirect indicators of angiogenesis are changes in metabolism and vascularity. These represent the expected downstream consequences of depriving the tumor of blood supply, and are particularly useful if there is uncertainty about the exact nature of the mechanisms of action of the drug, and the need to test drugs with different mechanisms in combination.

This chapter reviews these imaging methods and concentrates on the modalities that are available to most cancer centers, including dynamic contrast-enhanced MRI and FDG-PET scanning.

MRI with Contrast Agents

Studies of the vasculature with MRI normally include rapid injection of a contrast agent, often referred to as dynamic contrast-enhanced (DCE)-MRI. Consequent changes in the image brightness are then used to detect and characterize lesions. DCE-MRI is already being proposed for routine clinical application in MR mammography.

The MR image is created from the nuclei of hydrogen atoms (protons), which are present mainly in water. Application of both a large static magnetic field and a series of radio-frequency pulses causes "excitation" of the protons and generates the signal. The image (a spatial location map of the proton signal) is created by applying magnetic field gradients in different directions. Although the signal intensity is largely dependent on the water concentration (or proton density), the image can be made sensitive to two different ways in which the signal changes or 'relaxes': the time constants that govern these two relaxation processes are called the T_1 and T_2 relaxation times. When the image is sensitized to one of the relaxation processes, this is called T_1-weighted or T_2-weighted imaging.

Contrast media are available that can cause both T_1 and T_2 to change, with a consequent change in signal intensity. The presence of contrast agent is indicated by either signal hyperintensity (with T_1-weighted imaging), or hypointensity (with T_2-weighted imaging). In normal clinical use in the brain, just a single image is acquired some time (typically 5 min) after contrast injection to show the distribution of the agent and to confirm opening of the blood–brain barrier. Outside the brain, however, where even in healthy tissue the contrast agent leaks from the vasculature, measuring the time course of the signal change in the tissue can be much more revealing.

Using contrast media of varying molecular weights and magnetic properties, MRI can be used to measure blood volume, perfusion and blood vessel permeability (Su et al. 1998). High-molecular-weight contrast agents will stay within the intravascular space, and

by collecting MR images continuously as the contrast is injected, both blood volume and perfusion can be estimated. Very small molecules, such as water, will leak rapidly into the interstitial space, again providing a guide to perfusion in the dynamic phase and the size of the interstitial space. Agents of low to intermediate molecular weight, however, are neither freely diffusible nor do they remain purely in the blood pool, and the degree of signal change will be related to both flow and permeability parameters. Starting before the injection of contrast, DCE-MR images are acquired as the contrast first 'washes' into the tissue, and may continue as it begins to wash out, with the plasma concentration diminishing as contrast disperses and is cleared via the kidneys. Temporal analysis of the enhancement pattern for intermediate size agents can help elucidate the separate components of blood volume, perfusion and permeability, but for low-molecular-weight compounds such as the standard gadolinium (Gd) chelates available for use in humans, the enhancement pattern seen often results from an inseparable combination of flow, blood volume and permeability (Tofts et al. 1999). As well as perfusion and permeability, the enhancement profile depends on the volume of tissue to which the contrast agent has access – the extravascular, extracellular space: the higher its volume fraction, the slower the contrast agent will equilibrate between the blood and the tissue.

Although non-specific, all these factors are related to angiogenesis (Carmeliet and Jain 2000), and microvascular density, malignancy, and prognosis have all been correlated with enhancement parameters (Buadu et al. 1996; Hawighorst et al. 1998). Correlations are not reliable, however, probably due to the variable effects of malignancy on vascularity and vascular permeability (Hulka et al. 1997). Several studies have shown that successful therapies may result in changes in parameters derived from DCE-MRI data in animals and humans, which may prove a more accurate and earlier indication of response than standard clinical and imaging parameters (Pham et al. 1998).

The signal intensity from T_2-weighted MRI depends on inherent tissue properties and requires MRI sequences that are insensitive to local magnetic field inhomogeneity (spin-echo sequences). A similar type of scan (T_2'-weighted imaging) is sensitized to any local magnetic field inhomogeneity and shows a reduction in signal intensity in regions of poor field uniformity. Standard gadolinium chelates in high concentration cause shortening of the T_2^* relaxation time. Such concentrations are found in the vascular tree after bolus injection, causing a decrease in observed signal intensity. This T_2^* effect reduces dramatically as leakage into the extravascular space occurs. Therefore, gadolinium chelates are sometimes considered as extravascular agents with T_1-weighted imaging and as intravascular agents for T_2^*-weighted imaging.

Gadolinium chelates are used routinely in clinical practice with T_2^*-weighted imaging for cerebral perfusion studies, where the blood–brain barrier prevents leakage into the extravascular space. In tumors, breakdown of the blood–brain barrier, which is essential for standard contrast enhancement, makes the T_2^* effect less consistent and more difficult to interpret quantitatively.

Other contrast agents are now becoming available for use in humans, and these are based around iron oxide particles in a dextran coating, giving a strong T_2' change even at low concentrations. These agents, called super-paramagnetic iron oxide particles (SPIOs) or ultra-small super-paramagnetic iron oxide particles (USPIOs), may have potential as blood pool markers when used in conjunction with a dynamic MRI scan. They are already proving useful in the clinic as specific lymph node markers. In a trial of 80 patients with pre-surgical, clinical stage T1, T2, or T3 prostate cancer, high-resolution MRI with highly lymphotropic super-paramagnetic nanoparticles allowed the detection of small and otherwise undetectable lymph-node metastases (Harisinghani et al. 2003).

These developments, and the fact that contrast-enhanced MRI is often performed routinely in cancer patients, has led to increased interest in the use of this imaging modality to study the effects of treatment. This has met with varying degrees of success, with some clinical studies showing that DCE-MRI with standard gadolinium chelates (using a variety of imaging and analysis methods) can successfully be used to assess different types of therapy.

40.4.1 DCE-MRI Sequence

Ideally, DCE-MRI data acquisition should have high spatial resolution and allow mapping of enhancement parameters for the tumor in a pixel-by-pixel manner. This is important for gaining prognostic information about a tumor where small areas of increased tumor activity could otherwise be missed. This approach typically involves image acquisition times of between 6 s and 30 s for each image in the dynamic series. This may not be practical for many phase I studies, as a large proportion of metastatic disease treated by chemotherapy is in parts of the body that cannot be easily immobilized, such as liver and lung. In these areas, pixel-by-pixel data analysis is complicated by the need for sophisticated registration of the tumor in consecutive images. Long imaging times also involve multiple breath-holds, which may be difficult for a patient with advanced cancer. Good temporal resolution is needed to capture the dynamics of contrast uptake fully, but also with increased scan times there is the risk of patient movement during the acquisition, which can result in artifacts and unusable images. The choice of pulse sequence is therefore a compromise depending on multiple factors, including the expected rate of tumor contrast uptake and the tumor location, since anatomical areas that are easily immobilized (such the head, limbs or breast) are more suitable for high-resolution imaging.

40.4.2 Quantification of DCE-MRI

There are many ways of quantifying the "enhancement" of tumors after injection of a contrast agent, including semi-quantitative analysis such as measuring peak enhancement or the maximum upslope of the enhancement curve. However, the shape of the tissue enhancement curve depends on different physiological parameters at different times. If the peak enhancement occurs after, say, 1 min, it will depend mainly on the perfusion and permeability of the vasculature whilst if it occurs after 10 min, it may depend more on the volume of the extravascular, extracellular space. Maximum slope can also be a problem, since in rapidly enhancing tumors it may be difficult to measure because of the coarse sampling of the enhancement curve, while in heterogeneous tumors there will be more than one component to the initial slope. Perhaps the simplest semi-quantitative approach is to evaluate the area under the enhancement curve out to a certain fixed time after contrast injection. This measurement has no direct physiologic meaning, but is a robust measure that depends on the vascularity of the tumor in its broadest sense. This is often termed the initial area under the enhancement curve (IAUC).

All such properties of the enhancement curve are, however, dependent on the concentration of contrast agent in the artery that feeds the tumor (the arterial input function, AIF): the total amount injected, the rate of injection, and the dispersion of the bolus between injection and delivery to the tumor all affect the enhancement. Hence, it is usual to "normalize" the enhancement curve to take the AIF into account by, for example, dividing the IAUC by the AUC for a feeding artery out to the same time point. In fact, any robust analysis method must be able to take the AIF into account, although in follow up studies, where relative changes from pre-treatment values are important, it may possible to assume that the AIF is consistent from one scan to the next.

A more sophisticated quantitative approach attempts to quantify the perfusion/permeability in terms of a non-specific leakage rate constant K^{trans} and the extravascular, extracellular space volume fraction ve (Tofts et al. 1999). These methods make a few assumptions. First is that the imaged tissue comprises three compartments: the vascular space, the extracellular, extravascular space and the intracellular space. Since contrast agent enters neither red blood cells nor tissue cells in normal tissue, the method models movement of contrast agent between the plasma and the extracellular, extravascular space. It is also assumed that there is no active transport mechanism and therefore the flux depends only on the concentration gradient and the vascular permeability (Kety 1951). A

slightly more sophisticated model also estimates the vascular volume fraction, which is otherwise assumed to be negligible. The imaging community is working to develop uniformity of imaging and analysis protocols, but this will remain difficult until successful treatments are available by which to assess the utility of different methods in various tumors and organ systems.

The image acquisition technique and analysis method used depend on the tumor site and tumor heterogeneity. There is no doubt that calculating enhancement parameters for all pixels in a tumor increases the information available, but this approach is difficult where there is motion in the tissue (such as in lung), or where the tumor changes size or shape on treatment. In these, cases averaging parameters over the whole tumor may be more useful.

The lack of agreement about data acquisition and analysis methods makes comparison of results among groups difficult. It is possible that different methods will lend themselves to different agents with different mechanisms of action, and too much standardization may stifle development. Furthermore, in all models, basic assumptions are made about the distribution of the contrast agent or isotope, which may not prove true in tumors.

40.4.3 Clinical Trials Using DCE-MRI

In phase I studies, conducted in Leicester (UK) and Freiburg (Germany), we have shown that DCE-MRI can provide useful information in the clinical study of an angiogenesis inhibitor (Morgan et al. 2003; Thomas et al. 2005). The agent (PTK787/ZK222584) is a small-molecule inhibitor of VEGF tyrosine kinases. In a sub-cohort of 25 patients with liver metastases from colorectal carcinoma, we showed significant reductions in enhancement rates as early as day 2 after start of treatment, which may persist for months. These changes correlated significantly with increasing dose and plasma levels. Furthermore, the degree of enhancement reduction correlated well with changes in tumor size and clinical response. Similar changes have been seen in glioblastoma multiforme with the same agent (Yung et al. 2003). These studies have helped in the selection of the dose and tumor types to be used in phase II and III studies. Similar trials using an anti-VEGF antibody HuMV833 and the small-molecule tyrosine kinase inhibitor AG-013736 show a similar magnitude of changes in contrast enhancement parameters (Jayson et al. 2002; Liu et al. 2005).

Although there is a clear relationship between the measured enhancement parameters and treatment with the drug, the exact mechanism is not clear. The mechanism therefore has to be inferred, based on our knowledge of pre-clinical data and knowledge of the various factors that can affect the imaging test. In pre-clinical studies with other anti-VEGF agents, changes in endothelial cell survival and vessel density may not occur for some days after initiation of therapy (Bruns et al. 2000). However, some studies show more rapid reductions in the density of immature vessels (Benjamin et al. 1999). Ktrans reflects not only tumor vascularity and blood flow, but also vascular permeability. The effects observed may therefore be due to acute changes in permeability, vascularity or even other factors such as the action of VEGF on nitric oxide production (He et al. 1999). It seems likely, however, that specific targeting of immature vessels contributes to rapid enhancement changes and the "normalization" of the vasculature as proposed by Jain (2005).

Similar results have been obtained with the vascular targeting agent combretastatin A4 phosphate (CA4P) (Galbraith et al. 2003; Stevenson et al. 2003). DCE-MRI studies were performed to examine changes in parameters related to blood flow and vascular permeability (K^{trans}) and the IAUC during a 24-h period after treatment with CA4P. Eighteen patients in a phase I trial received escalating doses, and significant reductions in tumor K^{trans} after treatment were seen. A similar study of 16 patients treated with 5,6-dimethylxanthenone-4-acetic acid (DMXAA), an agent that causes vascular shutdown in pre-clinical models, in a dose-escalating trial (Galbraith et al. 2002b) showed that 9 of 16 patients had significant reductions in IAUC 24 h after treatment.

Effects on blood flow have also been observed with classical chemotherapy agents. DCE-MRI measurements using Ktrans have been made in 16 patients receiving preoperative chemo-radiotherapy (George et al. 2001). However in a separate, similar trial from the same centre, taxane-based chemotherapy showed no effect (Lankester et al. 2003).

It is important to know whether chemotherapy regimes affect vascularity as seen on DCE-MRI, not only to judge DCE-MRI results from chemotherapy in combination with anti-angiogenesis agents, but also to assess possible anti-angiogenic effects of low-dose metronomic chemotherapy. Initial DCE-MRI results may also predict response to treatment, generally with increased enhancement parameters predicting a good response (George et al. 2001; DeVries et al. 2001). This may be related to many factors, including tumor oxygenation, access of chemotherapy, and potential correlation with angiogenesis.

Cerebral contrast-enhanced T_2^*-weighted imaging also proved useful in the brain in 24 patients undergoing treatment with carboplatin and thalidomide for malignant gliomas. Cerebral blood volume (CBV) maps created for the tumors before and after treatment showed marked reduction in patients treated with thalidomide and carboplatin in comparison to carboplatin alone. These changes correlated with efficacy after 1 year (Cha et al. 2000).

Other Imaging Methods Measuring Blood Flow

40.5.1 MRI Measures of Tumor Blood Flow Without Contrast Media

For some time, MRI angiography methods have been available that can measure flow velocity in large blood vessels, but these are of no use in the tumor microvasculature. A newer technique, MRI arterial spin labeling (ASL), is a perfusion imaging technique that involves exciting protons in blood (spin tagging) in a well-defined vessel that feeds the organ, and then recording the consequent signal change in that organ/tumor. This effectively measures tissue perfusion using arterial water as the probe. ASL has shown correlation with contrast-enhanced methods of cerebral blood flow in brain tumors (Warmuth et al. 2003). Although this technique has the advantage of using no exogenous contrast agent, allowing multiple studies to be performed sequentially, the signal to noise ratio (SNR) is considerably poorer than with contrast-enhanced methods. The available SNR has been improved with the introduction of clinical MRI scanners operating at a higher magnetic field of 3 tesla (compared with 1.5 tesla in more common usage) (Wang et al. 2005). In investigational studies, the ability to perform multiple sequential measurements on the same day may allow assessment of the perfusion changes over time, particularly in combination with pharmacokinetic measurements.

40.5.2 Computed Tomography

Computed tomography has the advantage of being widely available and, like T_1-weighted MRI, shows enhancement of the image with increasing concentration of contrast agent, although the volumes of contrast agent used are much higher with CT. Iodinated contrast agents in CT show similar distribution to MRI media, i.e. they are extravascular, extracellular agents with no specific uptake mechanism. However, the mechanism of contrast in CT is different: the image brightness depends on the degree of attenuation of the X-ray beam, and is related to the density of the tissue or contrast agent. With iodinated compounds, the degree of enhancement (i.e. increase in attenuation) is proportional to the concentration of iodine, making quantification straightforward, particularly when measuring arterial contrast enhancement during bolus injections. As for DCE-MRI, "functional" contrast-enhanced CT techniques have shown increases in tissue perfusion that may reflect malignancy and stage (Miles 1999). CT is faster and easier to perform than MRI,

with fewer potential artifacts and a higher spatial resolution (typically around 0.5 mm). Clinical MRI has a resolution in the order of 1–2 mm, although pre-clinical MRI can achieve resolutions of a few tens of micrometers. This makes CT a more robust technique that potentially allows automated analysis. Indeed, in one example, a retrospective trial was possible in 130 patients with primary lung carcinoma showing correlations with VEGF expression and microvascular density based on relatively straightforward acquisition parameters (Tateishi et al. 2002).

CT has the weakness of potentially poor anatomical coverage, which is being solved to some extent by multislice spiral technology. Generally, CT contrast media are safe, but have a worse side-effect profile than standard MRI contrast agents. In particular, the "hot flush" that many patients experience can make reliable multiple breath-holding protocols for dynamic enhancement studies problematic.

The chief weakness of CT compared to MRI, and MRI compared to nuclear medicine, is the low sensitivity for detecting current clinical contrast agents or labeled probe. While CT contrast agents are often used in the millimolar concentration range, and MRI agents range from millimolar to micromolar, nuclear medicine agents are down to true "trace" picomolar concentrations. One must therefore be careful not to overwhelm the system under investigation, and the development of new, more targeted CT agents is difficult due to potential toxicity.

Theoretically, since standard CT and MRI contrast agents have similar pharmacokinetics, any DCE-MRI findings should be translatable to CT. This was demonstrated in a study of the VEGF-specific antibody bevacizumab in human rectal cancer, where a rapid antivascular effect was shown by DCE-CT (Willett et al. 2004).

Despite the ease of using CT in clinical applications, there is concern about the associated radiation exposure. The increased risks of radiation from a CT scan may seem trivial for many cancer patients; however, there are now strict regulations in Europe concerning techniques used for research that involve radiation, but are not of direct benefit to the patient. Currently, tumor perfusion studies are not of proven benefit to the patient, so these regulations make MRI easier to organize in clinical trials, unless CT studies can be linked with standard clinical CT protocols.

40.5.3 Radionuclide Imaging

Single photon emission computed tomography (SPECT) and positron emission tomography (PET) utilize compounds labeled with radioisotopes as molecular probes. Both techniques have considerably poorer spatial resolution than CT and MRI, but much better sensitivity to low concentrations of tracer. While SPECT uses gamma ray emitters, PET uses positron emitters, with the annihilation of the emitted positron and an electron producing two photons (gamma rays of 512 keV energy) that travel in almost exactly opposite directions. Detection of both these photons allows the location of the original positron emission to be determined to within a few millimeters. PET has advantages over SPECT: it has better spatial resolution, greater sensitivity to radiopharmaceuticals, easier quantification of tissue radiopharmaceutical concentration, and biologically important radiopharmaceuticals are easier to manufacture (Alavi et al. 2004). Since most SPECT applications (such as labeled annexin to image apoptosis) can be adapted to PET imaging techniques, this section will concentrate on PET applications.

A wide variety of simple positron-emitting atoms can be created, such as isotopes of oxygen, nitrogen, carbon and fluorine (chemically similar to hydrogen) and numerous others, without changing their chemical and biological properties. Since very low concentrations of probe are required, pharmacological effect is not usually a concern. Unfortunately, PET is not widely available, since, in addition to the scanner, on-site (or nearby) radiochemistry facilities and a cyclotron are needed to generate the short-lived isotopes, making it an expensive technique. Quantification of the tracer concentration is difficult, due to variable attenuation of photons from the deep struc-

tures, which can make follow-up studies difficult (Pomper 2001).

Despite the expense, PET imaging in oncology is becoming standard in some areas. Its main applications use the probe 18-fluorodeoxyglucose (^{18}FDG) as an indirect marker of metabolically active cancer cells. This is a glucose analogue, which is transported into cells and undergoes hexokinase-mediated phosphorylation. The end product, FDG-6-PO4, is not a significant substrate for subsequent reactions and is retained in the cell in proportion to the rate of glycolysis. Increased metabolism is a biomarker for the presence of a tumor, since many tumors have high levels of glucose utilization via glycolysis rather than oxidative metabolism (Warburg effect). The relative specificity for FDG uptake by tumors has lead to PET becoming a standard tool in the staging of lung cancer, particularly in combination with CT scanning, which improves spatial localization. Fig. 40.1 shows CT/PET images in a case of non-small-cell lung cancer, with no evidence of distant spread.

^{18}FDG-PET is often used during treatment of tumors such as lymphoma, and early work has shown changes related to prognosis after one cycle of chemotherapy (Kostakoglu et al. 2002). Dramatic responses have been seen in patients with advanced gastrointestinal stromal tumors within days of the first dose of the signal transduction inhibitor imatinib (Gleevec) (Joensuu et al. 2001).

Using radiotracers, such as $H_2{}^{15}O$, ^{11}CO or $C^{15}O$, and dynamic phase (monitoring the concentration over time) ^{18}FDG-PET, blood flow and blood volume estimations can be made. Water provides perfusion information and CO, which binds to hemoglobin, gives blood volume information. In order to calculate flow, tissue and arterial tracer concentration measurements need to be made. The methods (and the problems encountered) are similar to those described for quantifying DCE-MRI enhancement and

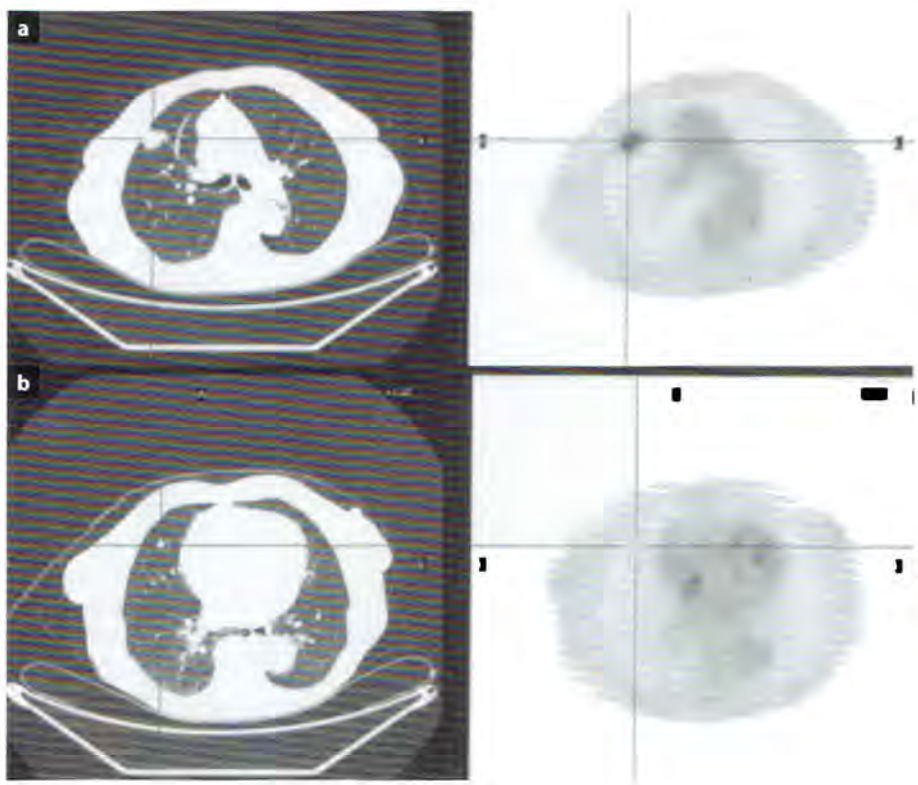


Fig. 40.1a,b. Combined CT/PET study showing axial CT images with the corresponding FDG-PET images registered to the same slice position. a A malignant lung lesion with high ^{18}FDG uptake on the PET image. b An incidental benign granuloma which does not show high uptake

are largely based on the principles of Fick and Kety (Kety 1951; Anderson and Price 2002). Measurement of arterial tracer concentration is difficult for both PET and DCE-MRI. Unlike DCE-MRI, however, where arterial tracer concentrations can be estimated based on injection rate, injected volume and patient body weight, radionuclide studies require that the tracer activity is measured during the scan, since it changes over time due to rapid radioactive decay. Measurement of arterial tracer activity can be determined directly by arterial sampling, or by measuring the signal from a region of interest over the left ventricle or large arteries such as the aorta. PET perfusion studies are complex and may take as long as 3 h. The half-life of non-^{18}FDG tracers is short: for ^{15}O it is 123 s, requiring an on-site cyclotron facility. Like CT and MRI, the image is made of voxels – cuboid volumes each of which has signal intensity – and smaller voxels imply better spatial resolution. Partial volume effects occur when voxels are too large to capture the details of signal intensities that change over short distances. These partial volume effects may be significant if the tumor size is in the order of (or less than) the resolution of the scanner (~2 cm). Partial volume effects result in a phenomenon called "spill-over" or "spill-in" of signal counts from surrounding structures with high blood flow, such as the heart and aorta, or within areas of relatively high flow, such as liver (Laking and Price 2003).

As for MRI, PET has been used to measure the effects of combretastatin A4 phosphate on tumor and normal tissue perfusion and blood volume in humans. Significant dose-dependent reductions were seen in tumor perfusion and tumor blood volume within 30 min after dosing, although by 24 h there was evidence of tumor vascular recovery (Anderson et al. 2003). Interestingly, the twofold decrease seen in humans was not nearly as dramatic as the eightfold reduction in tumor perfusion seen in rats at 1 h or the 100-fold decrease at 6 h. This again emphasizes the need to confirm pre-clinical findings in the clinic.

Herbst et al. (2002) imaged primary and metastatic lesions serially using $H_2{}^{15}$O-PET and ^{18}FDG-PET to assess changes in tumor blood flow and metabolism during treatment with human recombinant endostatin. They showed measurable effects on tumor blood flow and metabolism even in the absence of demonstrable anticancer effects. The data suggest that there is a complex, possibly nonlinear, relationship between tumor blood flow, tumor metabolism, and endostatin dose. In a study of 35 patients with locally advanced breast cancer, ^{18}FDG and $H_2{}^{15}$O-PET imaging before and after 2 months of chemotherapy were used to assess metabolism and perfusion. Although both resistant and responsive tumors had an average decline in metabolic rate over the course of chemotherapy, resistant tumors had an average increase in blood flow. Patients whose tumors failed to show a decline in blood flow after 2 months of therapy had poorer disease-free and overall survival (Mankoff et al. 2003). These studies clearly show that measured perfusion is not necessarily coupled with metabolism or response.

40.5.4 Ultrasound

Ultrasound imaging is inexpensive, quick to perform and a mainstay in obstetrics and the diagnosis of disease. Ultrasound uses pulses of high-frequency sound waves (usually between 3 MHz and 20 MHz) that are transmitted into the body and reflected by the different structures. These echoes are detected by a piezoelectric crystal, which turns the reflected sound waves into a voltage. The resolution of traditional ultrasound depends on the frequency used, with higher frequency giving better resolution but poorer depth penetration, a problem for high-resolution clinical imaging. High-frequency ultrasound may be useful in accessible human tumors such as ocular melanoma and skin tumors. The imaging of deep structures is also compromised by poor accessibility to certain anatomical regions (for example, those that are behind bone) and operator dependence. Blood flow can be measured by using the Doppler shift in the echo frequencies caused by movement of the blood. Using pulsed Doppler, this information can be displayed as a waveform of vascular flow velocity at a certain position, while color

Doppler gives an image of mean blood flow velocities (Fig. 40.2). Power Doppler, on the other hand, shows a map of blood flow amplitude, which is useful for assessing flow in small vessels. Ultrasound therefore has the potential to provide effective, low-cost, sequential monitoring of vascular changes associated with malignant tumors and their response to treatment.

Non-invasive monitoring of anti-angiogenic therapy has been performed by serial power Doppler and color Doppler ultrasound imaging of pre-clinical tumors, showing reduction in vascularity with treatment by anti-vascular and anti-VEGF therapies (Drevs et al. 2000). Color flow Doppler has also been used to characterize superficial solid tumors in patients. In a study of 67 patients with melanomas before surgical excision, high-frequency sonography and color Doppler sonography parameters correlated with tumor aggressiveness (Lassau et al. 2002). In a further study, tumor vascularity index was evaluated with power Doppler US in 44 patients with advanced hepatocellular carcinoma treated with 200–300 mg/day thalidomide. The pre-treatment vascularity index was significantly higher in responders than in non-responders (Hsu et al. 2005).

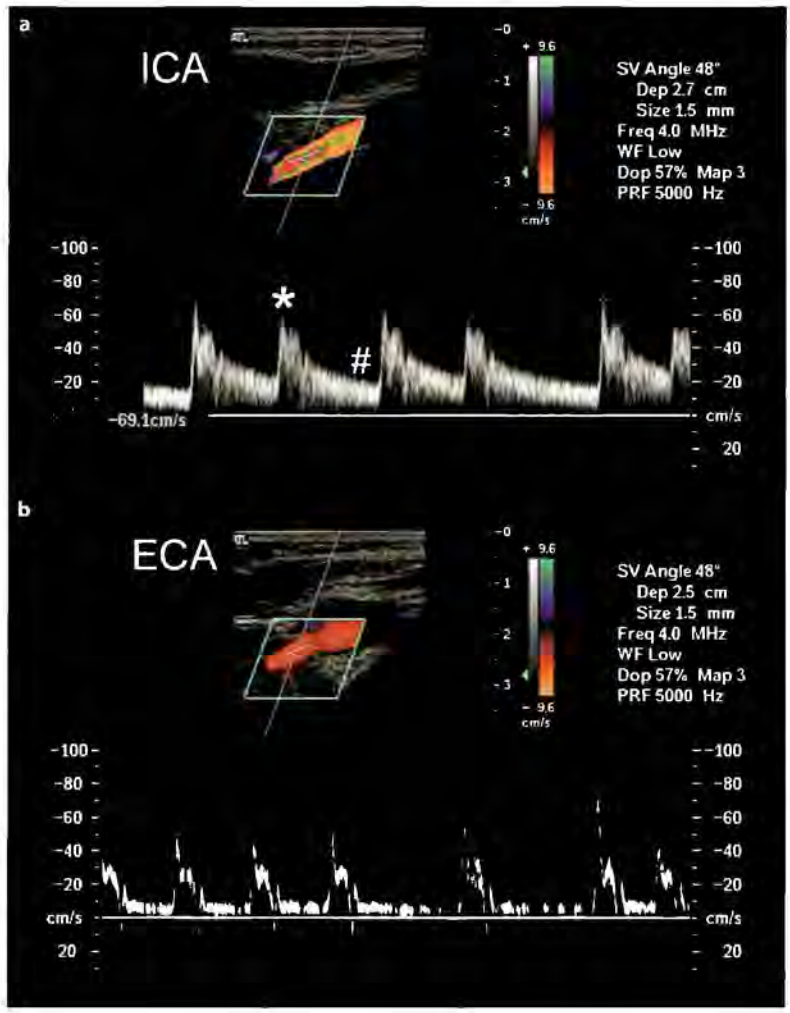


Fig. 40.2a,b. Color and pulsed Doppler images, used to analyze the internal (a) and external (b) carotid arteries (*ICA*, *ECA*). The color Doppler mode allows identification of the vessel flow, while pulsed Doppler allows waveform analysis. One parameter, the resistive index, can be calculated by comparing the systolic peak flow (*) with the end-diastolic flow (=). The end-diastolic flow in the ICA is higher than in the ECA due to lower vascular resistance. Changes in vascular resistance may be of use in monitoring treatment

The development of ultrasound contrast agents relies on one of the main disadvantages of ultrasound: the fact that ultrasound waves do not travel well through air. Any air/soft tissue interface causes strong echogenicity, and deeper structures cannot be seen. Ultrasound cannot therefore be used to 'see' through lung, and gas-filled bowel loops can prevent successful abdominal imaging. Ultrasound contrast media use "microbubbles" of air surrounded by a polymer shell, which are intensely echogenic. This improves the image of any vascular structure and enhances Doppler studies, allowing smaller vessel sizes down to 40 μm to be discriminated. Increasing the energy of the ultrasound pulse, or selecting a particular 'harmonic' frequency, can also destroy these microbubbles. This allows imaging the reappearance of the microbubbles, reflecting flow into the imaged area, and quantification of perfusion (Lassau et al. 2001).

Correlations between ultrasound-derived enhancement parameters and microvascular density have been demonstrated in animals (Lassau et al. 2001). Contrast-enhanced power Doppler ultrasonography has also been used to determine the angiogenic status of 21 patients with renal cell carcinoma. The color pixel ratios of selected images were calculated as the ratio of the number of pixels showing power Doppler signals to the total number of pixels within the lesion. A significant correlation was found between color pixel ratio and microvascular density (Kabakci et al. 2005). In 15 patients, follow-up examinations after stereotactic, single-dose radiotherapy were performed using contrast-enhanced ultrasound, showing a significant reduction of the arterial vascularization in treated tumors ($p<0.05$) (Krix et al. 2005).

Discussion of Blood Flow Imaging

The results described above have caused great excitement in the field of drug development, because they offer the hope not only of establishing a "proof of concept" of drug activity with relatively few patients, but also of aiding dose selection for phase II trials without relying on dose-limiting toxicity. There is a problem, however, in that although a positive result is reassuring, many promising agents have not revealed positive results using PET and MRI. Also, initial encouraging findings using these tests are no guarantee of later success. Positive results in combination therapy in the presence of toxicity may give encouragement for other regimes to be explored. In evaluating all biological agents, it must be recognized that they affect not only their primary target but also the activity of other kinases, some known and some possibly unknown. The exact mechanism of enhancement reduction is also unclear and may be different for different agents and at different times. A positive result from an indirect test, therefore, may not relate to the expected activity of the agent. Further, there is a danger that efficacious treatments could be dismissed because DCE-MRI with standard contrast media is insensitive to their mode of action, or their onset of action is too slow. The development of new contrast media and isotope probes will considerably aid understanding of the mechanisms of enhancement reductions. Furthermore, the facts that endostatin has produced measurable effects on tumor blood flow using PET but not MRI (Thomas et al. 2003) in the absence of tumor regression, and that, as previously stated, measures of metabolism do not always couple with measures of perfusion provide evidence that different tumor imaging methods may be required as endpoints in different situations.

There is also no consensus about how MRI or PET scans should be performed and how the data should be analyzed. Although there is a wealth of experience in animal models, these sometimes do not help in the planning of human trials, since different tumor types are often studied, with imaging protocols that are not feasible for clinical trials due to potentially toxic, unlicensed agents or clinically impractical imaging protocols. Translational imaging studies comparing effects in animals and humans using similar regimes do, however, provide information helpful in planning and interpreting clinical studies (Galbraith et al. 2003; Lee et al. 2006). Reproducibility studies

are required to judge the numbers required to obtain significant results in trials but also, possibly more importantly, to judge the significance of changes in the individual patient. Reproducibility varies depending on the methods employed, but often shows a coefficient of variation of approximately 14–20%. This implies that such studies should be sensitive to treatment changes of approximately 15% if cohorts of 10 patients are used. The intra-patient repeatability, which is an indicator of the significance on an individual patient's response, is generally higher, in the order of 30–40% (Galbraith et al. 2002a; Evelhoch et al. 2004). Technique refinement should improve these values in future.

In DCE-MRI and DCE-PET studies, care must be taken not to interpret 'reductions in enhancement' as an indication that the drug will be delivered less effectively to the tissues. In both PET and MRI, a reduction in enhancement may simply relate to a delay in achieving maximum tissue concentrations of the tracer, due to reducing the perfusion of a tumor or vascular permeability. The potential maximum concentration of the tracer in the extravascular space may never be achieved due to limited clinical imaging times, ranging from 5 min to 20 min, and the fact that the tracer concentration declines, either by renal excretion or owing to the short half-life of isotopes, during this time. This is important since, during treatment, "steady-state" plasma levels of a pharmaceutical compound should be achieved, and delays in achieving peak tissue concentration, even of several hours, should not be significant. Also, it has been suggested that blocking VEGF signaling "normalizes" the tumor vasculature by selective destruction of immature blood vessels. A further treatment effect is lowering the interstitial fluid pressure, creating a hydrostatic pressure gradient across the vascular wall. This induced pressure gradient may actually lead to better delivery of molecules into tumors. Thus, anti-VEGF therapy may paradoxically improve the access of therapeutic agents to cancer cells (Tong et al. 2004). This has been suggested as a possible mechanism for the successful combination of bevacizumab with chemotherapy in advanced colorectal cancer (Hurwitz et al. 2005). There may be a graded effect of the individual action of the agent, where the initial effect is to reduce interstitial pressure resulting in an overall increase in chemotherapy delivery, leading eventually to a more pronounced anti-vascular effect, where necrosis actually reduces the effect of chemotherapy. Currently most DCE-MRI results are reported as a Ktrans value, or possibly IAUC. For agents planned to be used with chemotherapy, however, the V_e value (volume of the extracellular, extravascular space accessible to contrast), which gives an indication of possible overall chemotherapy delivery, may also be important.

Indirect Tests Not Measuring Blood Flow

40.7.1 MR Spectroscopy

By altering the way in which the signal from hydrogen is measured, the slightly different resonance frequency of some common metabolites allows their concentration to be estimated (^{1}H-MR spectroscopy). With more specialist MRI equipment, metabolites containing phosphorus, such as adenosine triphosphate, can also be measured (^{31}P-MR spectroscopy).

Measuring the levels of different molecules in vivo has considerable appeal, although progress has been slow due to the poor sensitivity of the technique and, therefore, the limited range of molecules that can be studied. Considerable improvement in clinical results has been possible with use of increasing magnetic field strength in commercially available MRI scanners. Elevated choline levels are detectable by ^{1}H-MRS in cancer and correlate with malignancy and cell proliferation in brain tumors (Fig. 40.3). This can help diagnose malignancy and has particular clinical value in distinguishing radiation necrosis from recurrent tumor in the brain.

Although not specifically related to angiogenesis, any technique that shows a measurable difference between benign and malignant tissue could be

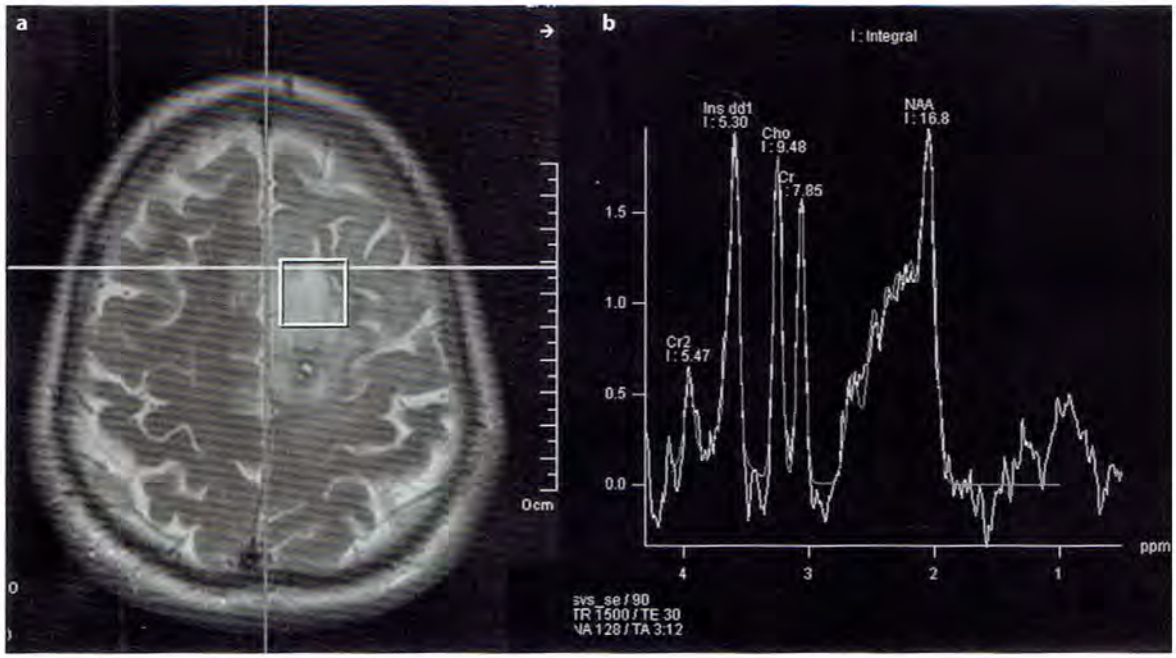


Fig. 40.3. a A *T2-weighted* MR image of a brain tumor after radiotherapy. The *white box* represents a region of interest ove the brain lesion for study by 1H-MR spectroscopy. b The spectrum obtained, showing high choline (*Cho*) level in compariso to creatine (*Cr*). The choline level can potentially be used to monitor tumors during treatment

adapted as a potential test for response to treatment. Garwood and colleagues have shown that ^{1}H-MRS can demonstrate changes as early as 1 day after the commencement of neo-adjuvant breast cancer therapy, which are correlated with response after 6 weeks (Meisamy et al. 2004).

40.7.2 Diffusion-weighted MRI

By using magnetic field gradients, the MRI signal can be made sensitive to water motion at the microscopic level, with the image brightness decreasing with increased water mobility. Since water is impeded in its random motion by contact with structures such as cell membranes, the motion is quantified as an "apparent diffusion coefficient" (ADC), in acknowledgment of the fact that the water diffusivity is measured by MRI to be lower than it really is, as a result of these interactions. Images that are sensitized in this way are used extensively in the clinical investigation of stroke, being extremely sensitive to th changes in water mobility that occur in acute ischemia because of cytotoxic edema due to membran pump failure. A high ADC value suggestive of necrosis has been shown to be a marker of poor respons of colorectal tumors to chemotherapy (Dzik-Juras 2004). There is also increasing interest in this technique as a method for monitoring necrosis cause by apoptosis, which is a demonstrated result of anti angiogenesis treatment, and relevant changes hav been shown in animals with the anti-vascular agen combretastatin A4 phosphate (Thoeny et al. 2005).

40.7.3 BOLD or T_2^* Imaging

A less direct method of assessing vasculatur uses intrinsic blood oxygenation level-dependen (BOLD) contrast MRI. The BOLD technique use

the principle that a heavily T_2^*-weighted image can depict changes in blood oxygenation because of the different magnetic properties of oxy- and deoxyhemoglobin. Signal intensities can be affected by vasodilatation by pharmacologic agents, or even by mental activity (functional brain imaging). Significant T_2^* decrease has been demonstrated in rat GH3 prolactinomas after administration of the vascular-targeting agent, ZD6126. These occur within 35 min, consistent with an ischemic insult induced by vascular collapse (Robinson et al. 2005).

T_2^*-weighted imaging may also be useful for mapping vascular maturity, since immature vessels do not have smooth muscle activity and do not respond to vasodilators. BOLD contrast MRI has been used for mapping vascular maturation using the response of mature vessels to hypercapnia (inhalation of air vs air and 5% CO_2) and the response of all vessels to hyperoxia [air and 5% CO_2 vs oxygen and 5% CO_2 (carbogen)]. This may help predict response to anti-vascular therapy and can be used in the clinic, although breathing carbogen can be unpleasant (Taylor et al. 2001).

40.7.4 Radionuclide Imaging

Although ^{18}FDG-PET scans are those most commonly used in clinical oncology, there is increasing use of other agents with indirect mechanisms of measurement but acting as more specific indicators. Agents are available that are sensitive to programmed cell death (apoptosis), due to affinity to phosphatidyl serine, which is externalized on the cell wall early in the apoptosis pathway (^{99m}Tc-labeled annexin) and proliferation by 18F-fluorothymidine (^{18}FLT-PET). These agents can also show some perfusion information in the dynamic phase. Detection of programmed cell death (apoptosis) by imaging is potentially interesting for assessing malignant and benign disorders, since apoptosis mediates tumor cell and angiogenic vascular endothelial cell regression.

Hypoxia in tumor tissue is also an important prognostic indicator of response to either chemotherapy or radiation therapy. Therefore, detection of hypoxia in advance of such interventions is of importance in optimizing the use and outcome of different therapeutic modalities. Furthermore, many anti-angiogenic therapies alter oxygen levels in tumors. Misonidazole molecules bind in inverse proportion to oxygen levels, and [^{18}F]-fluoromisonidazole (^{18}FMISO), or more recently copper-60 diacetyl-bis(N-methyl-thiosemicarbazone) (^{60}Cu-ATSM), can be used to study hypoxia and changes in oxygen status. ^{18}FMISO has been used to quantify hypoxia in the rat glioma by PET and may provide functional information about the results of anti-angiogenic therapy (Rasey et al. 2000). In 14 patients with biopsy-proved cervical cancer, ^{60}Cu-ATSM-PET, before initiation of radiotherapy and chemotherapy, showed that the frequency of loco-regional nodal metastasis was greater in hypoxic tumors (Dehdashti et al. 2003).

^{18}FLT acts as a marker for proliferation and has the potential to be used as a specific agent for assessing disease activity in various stages of different malignancies.

40.8 Specific Angiogenesis Imaging in Development

As well as observing downstream effects of successful treatment, whether specific to angiogenesis (blood flow) or simply related to successful treatment at a cellular level, there is interest in imaging specifically to document the effect of treatments on their intended site of action. This section will rely mainly on pre-clinical in vivo data to speculate about what may be achieved in human trials in the future. Imaging will almost certainly rely on contrast media or other probes which, to be successful in humans, will need to be imaged at very low concentrations. Because of this, PET imaging is at the forefront. Due to the ability to label molecules with isotopes such as oxygen and carbon for PET imaging, it is possible to label just about any specific marker. PET studies

also have the potential advantage that the treatment agent can be directly labeled. This allows direct imaging of drug delivery by 'micro-dosing', and chemotherapeutic agents, such as ^{18}F-fluorouracil, have been synthesized to assess their pharmacokinetics and metabolism. The concentration of ^{18}F-fluorouracil in metastatic colorectal cancer has been correlated with patient survival (Moehler et al. 1998). Labeled VEGF and other mediators of angiogenesis can also be used to predict response to anti-VEGF treatment (Collingridge et al. 2002). Direct labeling of the actual therapeutic agent can provide crucial information for trial design and optimal dosing. In a study of 20 patients with progressive solid tumors treated with various doses of the anti-VEGF antibody HuMV833, the agent was labeled with iodine-124. PET showed that antibody distribution and clearance were markedly heterogeneous between and within patients and between and within individual tumors (Jayson et al. 2002). This suggests future trial designs for this type of agent that use defined tumor types and potentially intra-patient dose escalation.

Molecular imaging by MRI has been thoroughly reviewed elsewhere (Weissleder and Mahmood 2001). The main problem is developing a contrast agent that can be 'seen' by MRI at nanomolar concentrations and that can be linked to specific probes. What works in animals may not be helpful in humans due to long development times and potential toxicity. Pre-clinical imaging with MRI scanners with much smaller access bores allows much higher magnet strengths to be achieved (typically six times that of a standard clinical scanner), giving greater sensitivity to low concentrations, or better spatial resolution in the range of 10–100 μm rather than millimeters. Nanoparticles composed of a perfluorocarbon emulsion coated with a layer of lipid have been developed. (Lanza et al. 2002). Linked to the lipid layer of each nanoparticle are up to 90,000 molecules of gadolinium-DTPA, enough to enable detection at low concentrations. Into the lipid outer layer, hundreds of homing molecules can be added, such as antibodies, peptides, or peptidomimetics. By targeting the protein alpha v beta 3-integrin, it is possible to detect the immature blood vessels that characterize angiogenesis in vivo in pre-clinical models (Sipkins et al. 1998).

An exciting property of MRI contrast media is that they are not imaged directly but by their effect on surrounding water. This means they have the potential to be activated by chemical reactions in the body, an effect that has been used in imaging gene expression in vivo in pre-clinical models. Where a gene transfer is attempted by a vector, a technique that may be used to modify angiogenesis in the future, transduction efficiency of the vector can be tested by the inclusion of a marker enzyme with the vector. The marker enzyme's effect could be to activate the MRI contrast agent. Such systems and further different approaches have been designed in pre-clinical models (Bremer and Weissleder 2001).

The high sensitivity of ultrasound to microbubble contrast means that high-frequency ultrasound systems can be designed to be sensitive to a single microbubble. As well as microbubbles of air, perfluorocarbon nanospheres, similar to those used in MRI, have been developed. Vectorization of these contrast agents, in particular with a specific alpha v beta 3-integrin monoclonal antibody, directed at endothelium in tumor vessels, has already been accomplished in pre-clinical models (Ellegala et al. 2003).

Optical imaging is based on the use of molecules that may affect or emit radiation in the visible or near-visible spectrum in a variety of ways, including scattering, absorption, and fluorescence. These "chromophores" or "fluorophores" may be intrinsic to the tissue, or may be administered (Weissleder and Ntziachristos 2003). Optical imaging is mainly limited to research, but with endoscopic imaging technology, fluorescent and bioluminescent probes could be seen in clinically relevant sites in humans. The inability of light to pass from deep tissues is the biggest problem, but optical tomography, using intrinsic hemoglobin concentration (Pogue et al. 2001), has successfully been used to detect breast lesions in a clinical setting.

Although these techniques do not immediately lend themselves to human studies, the approach may be useful in the long term. Whether they are translatable to humans remains to be seen, and depends on the toxicity of the agents and the ability to achieve satisfactory imaging resolution and SNR.

Conclusion

This is a continually evolving field, and it is difficult to know what the future will bring. What is clear is that imaging tests are available that can give useful information to aid development of anti-angiogenesis strategies in humans. The good news is that when changes are seen in the clinic, they are almost always rapid and there are few cases where imaging 'too early' has failed to see a response. However, what is now becoming clear is that the effects of angiogenesis inhibitors on tumor blood flow characteristics may not be consistent throughout the treatment cycle. In early therapy there may be a paradoxical increase in tumor perfusion due to constriction of inappropriately dilated vessels. This can then lead to reduced perfusion and necrosis as treatment continues, making dose selection and dose timing more complex. The correct dose for efficacy as a single agent may induce necrosis rapidly and therefore be higher than the ideal dose in combination with chemotherapy. Furthermore, intermittent pulsed therapy, by the process of selectively attacking immature vessels, may paradoxically improve vessel growth in the long term.

Many studies either show or suggest a relationship between dose and response or efficacy, although human trials are always confounded by heterogeneity of patients and tumor types. It is clear that positive results may not always correlate with each other or with clinical outcome, and there should not be overreliance on the accuracy of any one technique.

However, when imaging is "successful", there is a danger of attaching too much weight to cases where imaging is positive, ignoring tumor types where there is no imaging response. This is particularly true in comparisons of MRI enhancement changes in angiogenesis inhibition. It is reasonable to assume that treatment will have a greater (or more rapid) effect on metastatic lesions, with high proportions of immature, strongly angiogenic blood vessels, than a primary tumor. This, however, may divert attention from a more subtle but clinically significant response in the primary tumor with its larger proportion of mature vessels and better perfusion. Variations in the effect of angiogenesis treatments may also be due to differing levels of natural anti-angiogenic agents. In one case, removal of a primary colorectal tumor resulted in an increase in metabolic activity in its liver metastasis, with a concomitant drop in levels of angiostatin and endostatin in urine and plasma, respectively (Peeters et al. 2005). The level of these factors may well affect the degree of response to be expected from biomarker studies.

Many advances in the past have been made because of the observation that something works, without prior discovery of the mechanism. Now, drugs are being designed to have an effect on specific mechanisms. Unfortunately, understanding of these mechanisms is incomplete, and designing drugs to work perfectly in the test tube is no guarantee of clinical success. Furthermore, lack of understanding of the specific mechanism is no guarantee that it will not work for other reasons. Whilst it will always be important to increase understanding of mechanisms of action for both imaging and treatment, a more pragmatic approach is needed in the interim. Whether an imaging test is valuable depends on whether it can be established as a surrogate endpoint or biomarker for the desired effect, and therefore answer key questions for drug development rather than simply providing interesting data (Collins 2003). These questions include:

- Did imaging help to assess whether the mechanistic goals were achieved?
- Did imaging assist dose selection for phase II?
- Did imaging provide assistance for schedule selection for phase II?
- Can imaging select subpopulations with potentially greater response?

This approach has been used in the summary of a recent encouraging publication of the efficacy of a novel oral angiogenesis inhibitor AG-013736 (Liu et al. 2005).

To achieve further progress, a huge multidisciplinary effort is required to achieve validation and standardization of imaging methodology and to draw up guidelines to ensure consistent and standardized reporting on findings (Leach et al. 2005).

Comparison studies to determine which imaging methods work best (alone or in combination) should be instituted. The pharmaceutical companies could play a key role in developing advanced contrast agents whose main clinical role may be in the assessment of novel anti-cancer agents. Pharmaceutical companies must also take a translational "bench-to-bedside" approach to imaging: pre-clinical development of angiogenesis inhibitors should include developing imaging approaches suitable for use in subsequent clinical trials.

With further cooperation and progress, these imaging techniques, as surrogate endpoints for efficacy of biological agents, may become as commonplace as CT scans for drug development, and may even become standard imaging tests for all oncology patients.

References

Alavi A, Kung JW, Zhuang HM (2004) Implications of PET based molecular imaging on the current and future practice of medicine. Semin Nucl Med 34:56–69

Anderson H, Price P (2002) Clinical measurement of blood flow in tumours using positron emission tomography: a review. Nucl Med Commun 23:131–138

Anderson HL, Yap JT, Miller MP, Robbins A, Jones T, Price PM (2003) Assessment of pharmacodynamic vascular response in a phase I trial of combretastatin A4 phosphate. J Clin Oncol 21:2823–2830

Atkinson AJ, Colburn WA, DeGruttola VG, Demets DL, Downing GJ, Hoth DF, Oates JA, Peck CC, Schooley RT, Spilker BA, Woodcock J, Zeger SL (2001) Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharmacol Ther 69:89–95

Bailar JC, Gornik HL (1997) Cancer undefeated. N Engl J Med 336:1569–1574

Benjamin LE, Golijanin D, Itin A, Pode D, Keshet E (1999) Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. J Clin Invest 103:159–165

Bremer C, Weissleder R (2001) Molecular imaging – in vivo imaging of gene expression: MR and optical technologies. Acad Radiol 8:15–23

Bruns CJ, Liu WB, Davis DW, Shaheen RM, McConkey DJ, Wilson MR, Bucana CD, Hicklin DJ, Ellis LM (2000) Vascular endothelial growth factor is an in vivo survival factor for tumor endothelium in a murine model of colorectal carcinoma liver metastases. Cancer 89:488–499

Buadu LD, Murakami J, Murayama S, Hashiguchi N, Sakai S, Masuda K, Toyoshima S, Kuroki S, Ohno S (1996) Breast lesions: correlation of contrast medium enhancement patterns on MR images with histopathologic findings and tumor angiogenesis. Radiology 200:639–649

Buyse M, Thirion P, Carlson RW, Burzykowski T, Molenberghs G, Piedbois P (2000) Relation between tumour response to first-line chemotherapy and survival in advanced colorectal cancer: a meta-analysis. Lancet 356:373–378

Carmeliet P, Jain RK (2000) Angiogenesis in cancer and other diseases. Nature 407:249–257

Cha S, Knopp EA, Johnson G, Litt A, Glass J, Gruber ML, Lu S, Zagzag D (2000) Dynamic contrast-enhanced T2*-weighted MR imaging of recurrent malignant gliomas treated with thalidomide and carboplatin. Am J Neuroradiol 21:881–890

Collingridge DR, Carroll VA, Glaser M, Aboagye EO, Osman S, Hutchinson OC, Barthel H, Luthra SK, Brady F, Bicknell R, Price P, Harris AL (2002) The development of [I-124]iodinated-VG76e: A novel tracer for imaging vascular endothelial growth factor in vivo using positron emission tomography. Cancer Res 62:5912–5919

Collins JM (2003) Functional imaging in phase I studies: decorations or decision making? J Clin Oncol 21:2807–2809

Dehdashti F, Grigsby PW, Mintun MA, Lewis JS, Siegel BA, Welch MJ (2003) Assessing tumor hypoxia in cervical cancer by positron emission tomography with 60Cu-ATSM: relationship to therapeutic response – a preliminary report. Int J Radiat Oncol Biol Phys 55:1233–1238

DeVries AF, Griebel J, Kremser C, Judmaier W, Gneiting T, Kreczy A, Ofner D, Pfeiffer KP, Brix G, Lukas P (2001) Tumor microcirculation evaluated by dynamic magnetic resonance imaging predicts therapy outcome for primary rectal carcinoma. Cancer Res 61:2513–2516

Drevs J, Hofmann I, Hugenschmidt H, Wittig C, Madjar H, Muller M, Wood J, Martiny-Baron G, Unger C, Marme D (2000) Effects of PTK787/ZK 222584, a specific inhibitor of vascular endothelial growth factor receptor tyrosine kinases, on primary tumor, metastasis, vessel density, and flood flow in a murine renal cell carcinoma model. Cancer Res 60:4819–4824

Drevs J, Zirrgiebel U, Schmidt-Gersbach CIM, Mross K, Medinger M, Lee L, Pinheiro J, Wood J, Thomas AL, Unger C, Henry A, Steward WP, Laurent D, Lebwohl D, Dugan M, Marme D (2005) Soluble markers for the assessment of biological activity with PTK787/ZK 222584 (PTK/ZK), a vascular endothelial growth factor receptor (VEGFR) tyrosine kinase inhibitor in patients with advanced colorectal cancer from two phase I trials. Ann Oncol 16:558–565

Dvorak HF (2002) Vascular permeability factor/vascular

endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. J Clin Oncol 20:4368–4380

Dzik-Jurasz A (2004) The development and application of functional nuclear magnetic resonance to in vivo therapeutic anticancer research: 2002 Sir Godfrey Hounsfield lecture delivered at the President's Day, Manchester. Br J Radiol 77:296–307

Ellegala DB, Poi HL, Carpenter JE, Klibanov AL, Kaul S, Shaffrey ME, Sklenar J, Lindner JR (2003) Imaging tumor angiogenesis with contrast ultrasound and microbubbles targeted to alpha(v)beta(3). Circulation 108:336–341

Evelhoch JL, Gillies RJ, Karczmar GS, Koutcher JA, Maxwell RJ, Nalcioglu O, Raghunand N, Ronen SM, Ross BD, Swartz HM (2000) Applications of magnetic resonance in model systems: cancer therapeutics. Neoplasia 2:152–165

Evelhoch JL, LoRusso PM, He ZQ, DelProposto Z, Polin L, Corbett TH, Langmuir P, Wheeler C, Stone A, Leadbetter J, Ryan AJ, Blakey DC, Waterton JC (2004) Magnetic resonance imaging measurements of the response of murine and human tumors to the vascular-targeting agent ZD6126. Clin Cancer Res 10:3650–3657

Galbraith SM, Lodge MA, Taylor NJ, Rustin GJS, Bentzen S, Stirling JJ, Padhani AR (2002a) Reproducibility of dynamic contrast-enhanced MRI in human muscle and tumours: comparison of quantitative and semi-quantitative analysis. NMR Biomed 15:132–142

Galbraith SM, Rustin GJS, Lodge MA, Taylor NJ, Stirling JJ, Jameson M, Thompson P, Hough D, Gumbrell L, Padhani AR (2002b) Effects of 5,6-dimethylxanthenone-4-acetic acid on human tumor microcirculation assessed by dynamic contrast-enhanced magnetic resonance imaging. J Clin Oncol 20:3826–3840

Galbraith SM, Maxwell RJ, Lodge MA, Tozer GM, Wilson J, Taylor NJ, Stirling JJ, Sena L, Padhani AR, Rustin GJS (2003) Combretastatin A4 phosphate has tumor antivascular activity in rat and man as demonstrated by dynamic magnetic resonance imaging. J Clin Oncol 21:2831–2842

George ML, Dzik-Jurasz ASK, Padhani AR, Brown G, Tait DM, Eccles SA, Swift RI (2001) Non-invasive methods of assessing angiogenesis and their value in predicting response to treatment in colorectal cancer. Br J Surg 88:1628–1636

Harisinghani MG, Barentsz J, Hahn PF, Deserno WM, Tabatabaei S, van de Kaa CH, de la Rosette J, Weissleder R (2003) Noninvasive detection of clinically occult lymph-node metastases in prostate cancer. N Engl J Med 348:2491–2495

Hawighorst H, Weikel W, Knapstein PG, Knopp MV, Zuna I, Schonberg SO, Vaupel P, van Kaick G (1998) Angiogenic activity of cervical carcinoma: assessment by functional magnetic resonance imaging-based parameters and a histomorphological approach in correlation with disease outcome. Clin Cancer Res 4:2305–2312

He H, Venema VJ, Guo XL, Venema RC, Marrero MB, Caldwell RB (1999) Vascular endothelial growth factor signals endothelial cell production of nitric oxide and prostacyclin through Flk-1/KDR activation of c-Src. J Biol Chem 274:25130–25135

Herbst RS, Mullani NA, Davis DW, Hess KR, McConkey DJ, Charnsangavej C, O'Reilly MS, Kim HW, Baker C, Roach J, Ellis LM, Rashid A, Pluda J, Bucana C, Madden TL, Tran HT, Abbruzzese JL (2002) Development of biologic markers of response and assessment of antiangiogenic activity in a clinical trial of human recombinant endostatin. J Clin Oncol 20:3804–3814

Hsu C, Chen CN, Chen LT, Wu CY, Hsieh FJ, Cheng AL (2005) Effect of thalidomide in hepatocellular carcinoma: assessment with power Doppler US and analysis of circulating angiogenic factors. Radiology 235:509–516

Hulka CA, Edmister WB, Smith BL, Tan LJ, Sgroi DC, Campbell T, Kopans DB, Weisskoff RM (1997) Dynamic echoplanar imaging of the breast: experience in diagnosing breast carcinoma and correlation with tumor angiogenesis. Radiology 205:837–842

Hurwitz HI, Fehrenbacher L, Hainsworth JD, Heim W, Berlin J, Holmgren E, Hambleton J, Novotny WF, Kabbinavar F (2005) Bevacizumab in combination with fluorouracil and leucovorin: an active regimen for first-line metastatic colorectal cancer. J Clin Oncol 23:3502–3508

Jain RK (1994) Barriers to drug-delivery in solid tumors. Sci Am 271:58–65

Jain RK (2005) Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science 307:58–62

Jayson GC, Zweit J, Jackson A, Mulatero C, Julyan P, Ranson M, Broughton L, Wagstaff J, Hakannson L, Groenewegen G, Bailey J, Smith N, Hastings D, Lawrance J, Haroon H, Ward T, Mcgown AT, Tang M, Levitt D, Marreaud S, Lehmann FF, Herold M, Zwierzina H (2002) Molecular imaging and biological evaluation of HuMV833 anti-VEGF antibody: implications for trial design of antiangiogenic antibodies. J Natl Cancer Inst 94:1484–1493

Joensuu H, Roberts PJ, Sarlomo-Rikala M, Andersson LC, Tervahartiala P, Tuveson D, Silberman SL, Capdeville R, Dimitrijevic S, Druker B, Demetri GD (2001) Effect of the tyrosine kinase inhibitor STI571 in a patient with a metastatic gastrointestinal stromal tumor. N Engl J Med 344:1052–1056

Kabakci N, Igci E, Secil M, Yorukoglu K, Mungan U, Celebi H, Kirkali Z (2005) Echo contrast-enhanced power Doppler ultrasonography for assessment of angiogenesis in renal cell carcinoma. J Ultrasound Med 24:747–753

Kerbel RS (2000) Tumor angiogenesis: past, present and the near future. Carcinogenesis 21:505–515

Kety SS (1951) The theory and applications of the exchange of inert gas at the lungs and tissues. Pharmacol Rev 3:1–41

Kostakoglu L, Coleman M, Leonard JP, Kuji I, Zoe H, Goldsmith SJ (2002) PET predicts prognosis after 1 cycle of chemotherapy in aggressive lymphoma and Hodgkin's disease. J Nucl Med 43:1018–1027

Krix M, Plathow C, Essig M, Herfarth K, Debus J, Kauczor HU, Delorme S (2005) Monitoring of liver metastases after stereotactic radiotherapy using low-MI contrast-enhanced ultrasound - initial results. Eur Radiol 15:677–684

Laking GR, Price PM (2003) Positron emission tomographic imaging of angiogenesis and vascular function. Br J Radiol 76:S50–S59

Lankester KJ, Taylor NJ, Stirling JJ, Boxall J, Leach MO, Padhani AR et al (2003) Conventional cytotoxic chemotherapy agents do not have acute antivascular effects, as measured by dynamic contrast enhanced MRI (DCE-MRI). Proc Am Soc Clin Oncol 22:588

Lanza GM, Abendschein DR, Yu X, Winter PM, Karukstis KK, Scott MJ, Fuhrhop RW, Scherrer DE, Wickline SA (2002) Molecular imaging and targeted drug delivery with a novel, ligand-directed paramagnetic nanoparticle technology. Acad Radiol 9:S330–S331

Lassau N, Koscielny S, Opolon P, de Baere T, Peronneau P, Leclere J, Roche A (2001) Evaluation of contrast-enhanced color Doppler ultrasound for the quantification of angiogenesis in vivo. Invest Radiol 36:50–55

Lassau N, Koscielny S, Avril MF, Margulis A, Duvillard P, de Baere T, Roche A, Leclere J (2002) Prognostic value of angiogenesis evaluated with high-frequency and color Doppler sonography for preoperative assessment of melanomas. Am J Roentgenol 178:1547–1551

Leach MO, Brindle KM, Evelhoch JL, Griffiths JR, Horsman MR, Jackson A, Jayson GC, Judson IR, Knopp MV, Maxwell RJ, McIntyre D, Padhani AR, Price P, Rathbone R, Rustin GJ, Tofts PS, Tozer GM, Vennart W, Waterton JC, Williams SR, Workmanw P (2005) The assessment of antiangiogenic and antivascular therapies in early-stage clinical trials using magnetic resonance imaging: issues and recommendations. Br J Cancer 92:1599–1610

Lee L, Sharma S, Morgan B, Allegrini P, Schnell C, Brueggen J, Cozens R, Horsfield M, Guenther C, Steward W, Drevs J, Lebwohl D, Wood J, McSheehy P (2006) Biomarkers for assessment of pharmacologic activity for a vascular endothelial growth factor (VEGF) receptor inhibitor, PTK787/ZK 222584 (PTK/ZK): translation of biological activity in a mouse melanoma metastasis model to phase I studies in patients with advanced colorectal cancer with liver metastases. Cancer Chemother Pharmacol 57:761–771

Liu G, Rugo HS, Wilding G, McShane TM, Evelhoch JL, Ng C, Jackson E, Kelcz F, Yeh BM, Lee FT Jr, Charnsangavej C, Park JW, Ashton EA, Steinfeldt HM, Pithavala YK, Reich SD, Herbst RS (2005) Dynamic contrast-enhanced magnetic resonance imaging as a pharmacodynamic measure of response after acute dosing of AG-013736, an oral angiogenesis inhibitor, in patients with advanced solid tumors: results from a Phase I study. J Clin Oncol 23:5464–5473

Mankoff DA, Dunnwald LK, Gralow JR, Ellis GK, Schubert EK, Tseng J, Lawton TJ, Linden HM, Livingston RB (2003) Changes in blood flow and metabolism in locally advanced breast cancer treated with neoadjuvant chemotherapy. J Nucl Med 44:1806–1814

McDonald DM, Thurston G, Baluk P (1999) Endothelial gaps as sites for plasma leakage in inflammation. Microcirculation 6:7–22

Meisamy S, Bolan PJ, Baker EH, Bliss RL, Gulbahce E, Everson LI, Nelson MT, Emory TH, Tuttle TM, Yee D, Garwood M (2004) Neoadjuvant chemotherapy of locally advanced breast cancer: predicting response with in vivo H-1 MR spectroscopy - a pilot study. Radiology 233:424–431

Miles KA (1999) Tumour angiogenesis and its relation to contrast enhancement on computed tomography: a review. Eur J Radiol 30:198–205

Miller KD, Sweeney CJ, Sledge GW (2001) Redefining the target: chemotherapeutics as antiangiogenics. J Clin Oncol 19:1195–1206

Moehler M, Mitrakopoulou-Strauss A, Gutzler F, Raeth U, Strauss LG, Stremmel W (1998) F-18-labeled fluorouracil positron emission tomography and the prognoses of colorectal carcinoma patients with metastases to the liver treated with 5-fluorouracil. Cancer 83:245–253

Morgan B, Thomas AL, Drevs J, Hennig J, Buchert M, Jivan A, Horsfield MA, Mross K, Ball HA, Lee L, Mietlowski W, Fuxius S, Unger C, O'Byrne K, Henry A, Cherryman GR, Laurent D, Dugan M, Marme D, Steward WP (2003) Dynamic contrast-enhanced magnetic resonance imaging as a biomarker for the pharmacological response of PTK787/ZK 222584, an inhibitor of the vascular endothelial growth factor receptor tyrosine kinases, in patients with advanced colorectal cancer and liver metastases: results from two phase I studies. J Clin Oncol 21:3955–3964

Peeters CFJM, de Geus LF, Westphal JR, de Waal RMW, Ruiter DJ, Wobbes T, Oyen WJG, Ruers TJ (2005) Decrease in circulating anti-angiogenic factors (angiostatin and endostatin) after surgical removal of primary colorectal carcinoma coincides with increased metabolic activity of liver metastases. Surgery 137:246–249

Pham CD, Roberts TPL, van Bruggen N, Melnyk O, Mann J, Ferrara N, Cohen RL, Brasch RC (1998) Magnetic resonance imaging detects suppression of tumor vascular permeability after administration of antibody to vascular endothelial growth factor. Cancer Invest 16:225–230

Pogue BW, Poplack SP, McBride TO, Wells WA, Osterman KS, Osterberg UL, Paulsen KD (2001) Quantitative hemoglobin tomography with diffuse near-infrared spectroscopy: pilot results in the breast. Radiology 218:261–266

Pomper MG (2001) Functional and metabolic imaging. In: DeVita VT, Hellman S, Rosenberg SA (eds) Cancer, principles and practice of oncology. Lippincott Williams and Wilkins, Philadelphia

Rasey JS, Casciari JJ, Hofstrand PD, Muzi M, Graham MM, Chin LK (2000) Determining hypoxic fraction in a rat glioma by uptake of radiolabeled fluoromisonidazole. Radiat Res 153:84–92

Robinson SP, Kalber TL, Howe FA, McIntyre DJO, Griffiths

JR, Blakey DC, Whittaker L, Ryan AJ, Watertont JC (2005) Acute tumor response to ZD6126 assessed by intrinsic susceptibility magnetic resonance imaging. Neoplasia 7:466–474

Shanafelt TD, Loprinzi C, Marks R, Novotny P, Sloan J (2004) Are chemotherapy response rates related to treatment-induced survival prolongations in patients with advanced cancer? J Clin Oncol 22:1966–1974

Sipkins DA, Cheresh DA, Kazemi MR, Nevin LM, Bednarski MD, Li KCP (1998) Detection of tumor angiogenesis in vivo by alpha(v)beta(3)-targeted magnetic resonance imaging. Nat Med 4:623–626

Stevenson JP, Rosen M, Sun WJ, Gallagher M, Haller DG, Vaughn D, Giantonio B, Zimmer R, Petros WP, Stratford M, Chaplin D, Young SL, Schnall M, O'Dwyer PJ (2003) Phase I trial of the antivascular agent combretastatin A4 phosphate on a 5-day schedule to patients with cancer: magnetic resonance imaging evidence for altered tumor blood flow. J Clin Oncol 21:4428–4438

Su MY, Muhler A, Lao XY, Nalcioglu O (1998) Tumor characterization with dynamic contrast-enhanced MRI using MR contrast agents of various molecular weights. Magn Reson Med 39:259–269

Takahashi Y, Mai M, Taguchi T, Urushizaki I, Nishioka K (2000) Prolonged stable disease effects survival in patients with solid gastric tumor: analysis of phase II studies of doxifluridine. Int J Oncol 17:285–289

Tateishi U, Kusumoto M, Nishihara H, Nagashima K, Morikawa T, Moriyama N (2002) Contrast-enhanced dynamic computed tomography for the evaluation of tumor angiogenesis in patients with lung carcinoma. Cancer 95:835–842

Taylor NJ, Baddeley H, Goodchild KA, Powell MEB, Thoumine M, Culver LA, Stirling JJ, Saunders MI, Hoskin PJ, Phillips H, Padhani AR, Griffiths JR (2001) BOLD MRI of human tumor oxygenation during carbogen breathing. J Magn Reson Imaging 14:156–163

Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, van Glabbeke M, van Oosterom AT, Christian MC, Gwyther SG (2000) New guidelines to evaluate the response to treatment in solid tumors. J Natl Cancer Inst 92:205–216

Thoeny HC, De Keyzer F, Chen F, Vandecaveye V, Verbeken EK, Ahmed BS, Sun XH, Ni YC, Bosmans H, Hermans R, van Oosterom A, Marchal G, Landuyt W (2005) Diffusion-weighted magnetic resonance imaging allows noninvasive in vivo monitoring of the effects of combretastatin A-4 phosphate after repeated administration. Neoplasia 7:779–787

Thomas AL, Morgan B, Horsfield MA, Higginson A, Kay A, Lee L, Masson E, Puccio-Pick M, Laurent D, Steward WP (2005) Phase I study of the safety, tolerability, pharmacokinetics, and pharmacodynamics of PTK787/ZK 222584 administered twice daily in patients with advanced cancer. J Clin Oncol 23:4162–4171

Thomas JP, Arzoomanian RZ, Alberti D, Marnocha R, Lee F, Friedl A, Tutsch K, Dresen A, Geiger P, Pluda J, Fogler W, Schiller JH, Wilding G (2003) Phase I pharmacokinetic and pharmacodynamic study of recombinant human endostatin in patients with advanced solid tumors. J Clin Oncol 21:223–231

Tofts PS, Brix G, Buckley DL, Evelhoch JL, Henderson E, Knopp M, Larsson HBW, Lee TY, Mayr NA, Parker GJM, Port RE, Taylor J, Weisskoff RM (1999) Estimating kinetic parameters from dynamic contrast-enhanced T-1-weighted MRI of a diffusable tracer: standardized quantities and symbols. J Magn Reson Imaging 10:223–232

Tong RT, Boucher Y, Kozin SV, Winkler F, Hicklin DJ, Jain RK (2004) Vascular normalization by vascular endothelial growth factor receptor 2 blockade induces a pressure gradient across the vasculature and improves drug penetration in tumors. Cancer Res 64:3731–3736

Wang B, Gao ZQ, Yan X (2005) Correlative study of angiogenesis and dynamic contrast-enhanced magnetic resonance imaging features of hepatocellular carcinoma. Acta Radiol 46:353–358

Warmuth C, Gunther M, Zimmer C (2003) Quantification of blood flow in brain tumors: comparison of arterial spin labeling and dynamic susceptibility-weighted contrast-enhanced MR imaging. Radiology 228:523–532

Weissleder R, Mahmood U (2001) Molecular imaging. Radiology 219:316–333

Weissleder R, Ntziachristos V (2003) Shedding light onto live molecular targets. Nat Med 9:123–128

Willett CG, Boucher Y, di Tomaso E, Duda DG, Munn LL, Tong RT, Chung DC, Sahani DV, Kalva SP, Kozin SV, Mino M, Cohen KS, Scadden DT, Hartford AC, Fischman AJ, Clark JW, Ryan DP, Zhu AX, Blaszkowsky LS, Chen HX, Shellito PC, Lauwers GY, Jain RK (2004) Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. Nat Med 10:649

Yung WKA, Friedman H, Conrad C, Reardon D, Provenzale J, Jackson E (2003) A phase I trial of single-agent PTK 787/ZK 222584 (PTK/ZK), an oral VEGFR tyrosine kinase inhibitor, in patients with recurrent glioblastoma multiforme. Proc Am Soc Clin Oncol 22:395

Vandetanib (ZACTIMA™; ZD6474): Preclinical and Clinical Development

41

ALAN BARGE and ANDERSON J. RYAN

Abstract

Vandetanib (ZACTIMA™; ZD6474) is a once-daily, orally available agent with potential for use in a number of solid tumour types. Vandetanib targets key signalling pathways in cancer by inhibiting vascular endothelial growth factor receptor (VEGFR)-dependent tumour angiogenesis and epidermal growth factor receptor (EGFR)-dependent tumour cell growth and survival. Vandetanib also inhibits RET (rearranged during transfection) kinase activity, which is involved in the development of several human diseases, including medullary and papillary carcinomas of the thyroid. Preclinical studies of vandetanib have demonstrated potent inhibition of VEGF- and EGF-stimulated human umbilical vein endothelial cell proliferation in vitro, as well as dose-dependent inhibition of tumour growth in a histologically diverse range of human tumour xenografts. Phase I studies showed vandetanib to be generally well tolerated at doses up to 300 mg per day, with a pharmacokinetic profile that supports once-daily oral administration. Common adverse events included rash, diarrhea and asymptomatic QTc prolongation, all of which were controlled by standard management. Phase II evaluation of vandetanib in patients with advanced, refractory NSCLC (non-small-cell lung cancer) has demonstrated improvements in progression-free survival, both as monotherapy (versus gefitinib) and in combination with docetaxel (versus docetaxel alone). These positive outcomes have led to the initiation of phase III trials of vandetanib in a broad population of patients with advanced NSCLC. Clinical development is also ongoing in other tumour types, and encouraging evidence of antitumour activity has been reported in patients with metastatic hereditary medullary thyroid cancer.

CONTENTS

Introduction

Vandetanib (ZACTIMA™; ZD6474) is a once-daily, orally available, small molecule with potential for use in a number of solid tumour types. It was selected for further development after demonstrating nanomolar inhibition of vascular endothelial growth factor receptor-2 (VEGFR-2) tyrosine kinase activity in vitro, combined with oral bioavailability and sustained plasma drug levels in experimental models, supporting once-daily oral dosing in vivo (Hennequin et al. 2002). VEGF/VEGFR-2 signalling in tumour vascular endothelial cells is now recognized as a pivotal and rate-limiting step in tumour angiogenesis, and much research has focussed on generating novel agents that inhibit this pathway. Vandetanib has a unique activity profile that distinguishes it from other molecular-targeted agents (Table 41.1). In addition to its anti-VEGF signalling, vandetanib is also a potent inhibitor of the intrinsic tyrosine kinase activity associated with epidermal growth factor receptor (EGFR) and the oncoprotein RET (rearranged during transfection). Aberrant EGFR signalling is a hallmark of many solid tumours, leading to increased tumour cell proliferation, survival and invasiveness (Harari 2004). Similarly, constitutively active RET oncoproteins are involved in the development of several human neuroendocrine diseases, including medullary and papillary carcinomas of the thyroid (Ichihara et al. 2004). Consequently, vandetanib offers the possibility of inhibiting key signalling pathways in tumour growth by (1) targeting tumour growth indirectly, via inhibition of VEGF-dependent tumour angiogenesis and VEGF-dependent endothelial cell survival, and (2) targeting tumour growth directly, via inhibition of EGFR- and RET-dependent tumour cell proliferation and survival (Fig. 41.1). This chapter summarizes the promising activity of vandetanib seen in preclinical and clinical studies to date, and considers its future use in the treatment of patients with cancer.

Table 41.1. Vandetanib kinase selectivity (adapted from Wedge et al. 2002)

Kinase	IC_{50} (μM)
VEGFR-2 (KDR)	0.04
VEGFR-3 (Flt-4)	0.11
RET	0.13
EGFR	0.50
VEGFR-1 (Flt-1)	>1
PDGFR-β	>1
Tie-2	>1
FGFR1	>1
MEK	>10
CDK2	>10
c-Kit	>20
erbB2	>20
FAK	>20
PDK1	>20
AKT	>100
IGF-1R	>200

Preclinical Development of Vandetanib

41.2.1 In Vitro

Vandetanib is a novel anilinoquinazoline (Fig. 41.2) that acts as a selective and reversible inhibitor of ATP binding to VEGFR-2 and -3, EGFR and RET tyrosine kinases (Table 41.1). In recombinant enzyme assays vandetanib has been shown to be a potent inhibitor of VEGFR-2 tyrosine kinase (IC_{50}=40 nM), with additional activity against VEGFR-3 (IC_{50}=110 nM), RET (IC_{50}=130 nM) and EGFR (IC_{50}=500 nM) tyrosine kinases. The excellent selectivity of vandetanib for these kinases was demonstrated by a relative lack of inhibitory effect on structurally related receptor tyrosine kinases, such as PDGFR-β, c-Kit and erbB2, as well as kinases from other families.

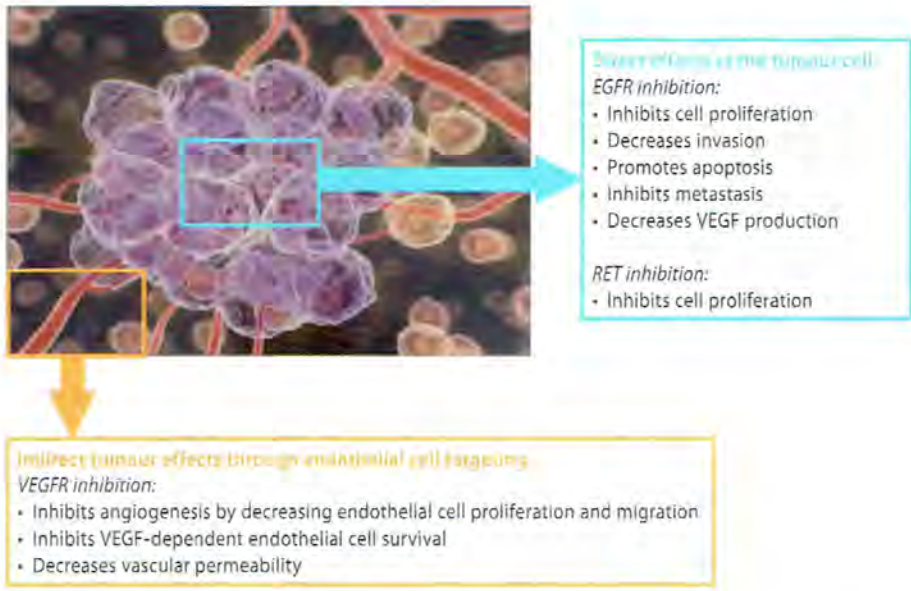
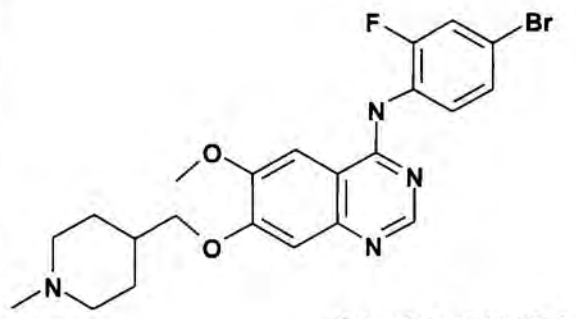


Fig. 41.1. Vandetanib's mechanism of action

The in vitro effects of vandetanib have also been investigated in growth factor-stimulated human umbilical vein endothelial cells (HUVECs). Consistent with its selectivity profile versus isolated enzymes, vandetanib was a potent inhibitor of VEGF-stimulated HUVEC proliferation (IC_{50}=60 nM). Vandetanib also inhibited EGF-stimulated HUVEC proliferation (IC_{50}=170 nM), but much higher concentrations were required to inhibit proliferation induced by basic fibroblast growth factor (IC_{50}=800 nM) or serum (basal; IC_{50}>3000 nM).

In addition to its antiproliferative effect on endothelial cell cultures, vandetanib has also demonstrated direct inhibition of tumour cell proliferation/survival in various human cancer cell lines that express functional EGFR, but not VEGFR-2 (Fig. 41.3) (Ciardiello et al. 2003; Arao et al. 2004a). The direct inhibitory effect of vandetanib on tumour cell growth in vitro in these studies is likely to be a consequence of its anti-EGFR activity. Indeed, Arao et al observed a strong correlation between the IC_{50} values for inhibition of tumour cell growth in vitro for vandetanib and gefitinib (Iressa™), a highly selective inhibitor of EGFR signalling (Arao et al. 2004a). In the same study, PC-9 cells were shown to be hypersensitive to the growth-inhibitory effects of vandetanib (IC_{50}=90 nM). The PC-9 cell line is derived from a Japanese female patient with adenocarcinoma of the lung and contains a 15-base-pair deletion mutation in the EGFR gene. This mutation leads to expression of EGFR with constitutively active EGFR tyrosine kinase that is more sensitive to inhibition by vandetanib and gefitinib than wild-type EGFR (Taguchi et al. 2004; Arao et al. 2004a). In summary, vandetanib can inhibit EGFR-dependent tumour cell growth and is more effective against tumour cell lines that are highly dependent on EGFR signalling for growth and survival, such as certain cell lines harbouring activating mutations of the *EGFR* gene.



Molecular weight 475 Da

Fig. 41.2. Structure of vandetanib

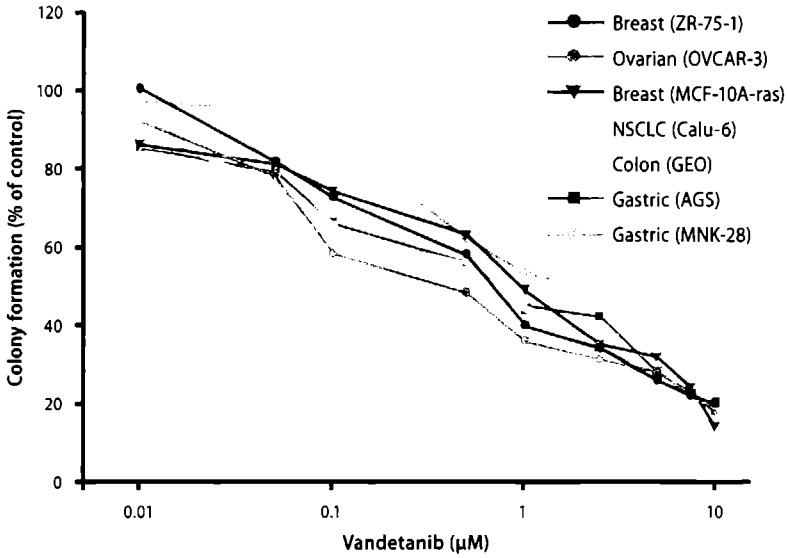


Fig. 41.3. Vandetanib is a direct inhibitor of tumour cell growth (Ciardiello et al. 2003)

Vandetanib is also a potent inhibitor of RET tyrosine kinase activity in cells (IC_{50}=100 nM), and has demonstrated selective inhibition of RET-dependent thyroid tumour cell growth both in vitro and in vivo (Fig. 41.4) (Carlomagno et al. 2002, 2004). Furthermore, point-mutation studies showed vandetanib to be an effective inhibitor of the tyrosine kinase activity of both wild-type RET receptor and RET receptors harbouring mutations commonly associated with certain forms of hereditary and sporadic thyroid carcinoma (Carlomagno et al. 2004).

41.2.2 In Vivo

41.2.2.1 Inhibition of VEGF Signalling, Angiogenesis and Vascular Permeability

The ability of vandetanib to inhibit VEGF-dependent signalling and angiogenesis in vivo was confirmed using two pharmacodynamic endpoints. In anaesthetized rats, vandetanib reversed VEGF-induced hypotension by 63% (P<0.001), but had no significant effect on bFGF-induced hypotension (Wedge et al. 2002). During bone growth, the progressive ossification of cartilage cells within the growth plate zone is dependent upon VEGF-regulated angiogenic invasion of cartilage (Horner et al. 1999). Consistent with inhibition of VEGF-dependent physiological angiogenesis, once-daily oral administration of vandetanib inhibited ossification and induced dose-dependent hypertrophy in femoro-tibial epiphyseal growth plates of young growing rats (Wedge et al. 2002).

A comprehensive series of preclinical studies has demonstrated that vandetanib inhibits tumour angiogenesis in vivo. Once-daily oral dosing with vandetanib produced significant, dose-dependent inhibition of tumour-induced blood vessel formation following intradermal transplantation of A549 human non-small-cell lung cancer (NSCLC) cells in mice (Wedge et al. 2002). In orthotopic models of human gastric (McCarty et al. 2004) and pancreatic (Bruns et al. 2003) cancer, vandetanib treatment resulted in a significant decrease in tumour microvessel density, although this was not seen in an orthotopic model of glioma (Sandstrom et al. 2004). A notable finding in the gastric cancer model was that after ad-

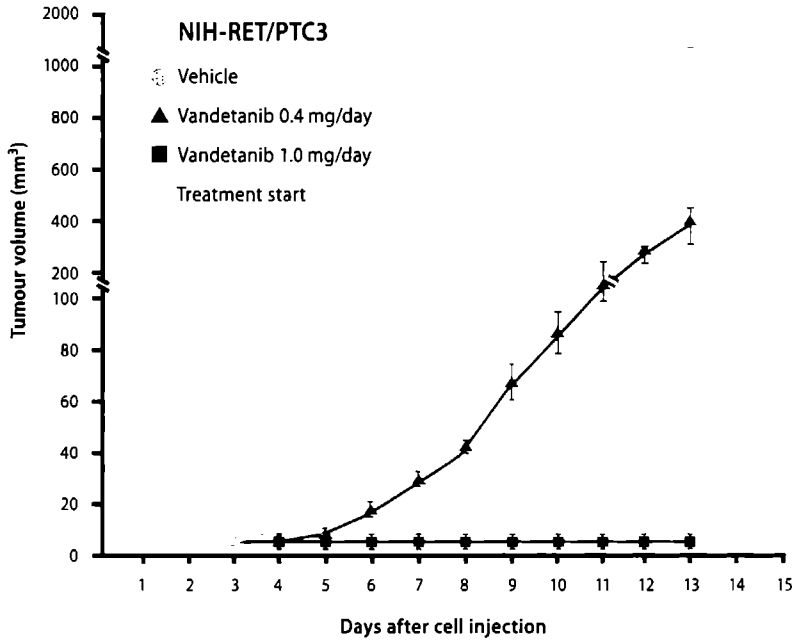


Fig. 41.4. Vandetanib: inhibition of RET-dependent thyroid tumour growth in vivo (Carlomagno et al. 2002)

ministration of vandetanib, the remaining microvessels showed a threefold increase in the percentage of pericyte coverage, despite a marked decrease in the overall number of tumour endothelial cells (McCarty et al. 2004). This may be due to selective effects of vandetanib on tumour blood vessels that are not stabilized by pericyte coverage. These immature vessels are characteristic of tumour vasculature and have been reported to be highly dependent on VEGF-induced survival signalling (Gerber et al. 1998).

Changes in haemodynamic parameters in vivo can be measured using dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI). Since VEGF is known to enhance vascular permeability (Dvorak et al. 1995), several clinical trials of VEGF signalling inhibitors have used DCE-MRI to explore the effects of drug treatment on tumour vascular permeability and blood flow (Morgan et al. 2003; Willett et al. 2004). The acute haemodynamic effects of vandetanib treatment have been assessed using this technique in preclinical models of both human prostate (Checkley et al. 2003) and colon (Bradley et al. 2004) cancer. In both studies, DCE-MRI analysis showed a dose-dependent reduction in tumour uptake of the contrast agent 24 hours after initiation of vandetanib treatment. These results are consistent with vandetanib inhibition of VEGF-induced hyperpermeability of the tumour vasculature.

41.2.2.2 Inhibition of Tumour Growth and Metastasis

The antitumour activity of vandetanib has been demonstrated in a range of in vivo preclinical studies, including xenograft, orthotopic and metastatic models (Table 41.2). Among the models that are sensitive to the effects of vandetanib are those with intrinsic or acquired resistance to inhibitors of EGFR activity. Furthermore, the results of a number of studies suggest that combining vandetanib with radiation therapy or certain cytotoxic chemotherapy may confer additional antitumour efficacy.

Xenograft Models

In a range of histologically diverse (lung, prostate, breast, ovarian, colon or vulval) xenograft models, chronic once-daily oral administration of

Table 41.2. Overview of vandetanib evaluation in preclinical models of cancer

Tumour type	Cancer model					Combination therapy	
	Xenograft	Orthograft	Metastatic	Spontaneous/ carcinogen-induced	In vitro	Radiation	Chemo-therapy
Lung	Wedge et al. 2002; Hoang et al. 2004; Taguchi et al. 2004; Williams et al. 2004	Wu et al. 2004a,b; Shibuya et al. 2005; Shintani et al. 2005	Matsumori et al. 2003; Wu et al. 2004a; Shintani et al. 2005	–	Helfrich et al. 2004; Briggs et al. 2005	Williams et al. 2003; Hoang et al. 2004; Shibuya et al. 2005	Wu et al. 2004b
RET-activated	Carlomagno et al. 2002	–	–	–	–	–	–
Breast	Wedge et al. 2002; Miller et al. 2003	–	–	Heffelfinger et al. 2004	–	–	–
Colon	Wedge et al. 2002; Ciardiello et al. 2004; Siemann and Shi 2004	–	–	Goodlad et al. 2004	Azzariti et al. 2003; Troiani et al. 2005	Siemann and Shi 2004	Azzariti et al. 2003; Troiani et al. 2005
EGFR-inhibitor resistant	Ciardiello et al. 2004; Taguchi et al. 2004	–	–	–	Helfrich et al. 2004; Briggs et al. 2005	–	–
Endothelial cells	–	–	–	–	Bradshaw and Gustafson 2004	–	Bradshaw and Gustafson 2004
Gastric	–	McCarty et al. 2004; Arao et al. 2004b	–	–	–	–	–
CNS	Damiano et al. 2005; Rich et al. 2005	Leenders et al. 2004; Sandstrom et al. 2004	Leenders et al. 2004	–	–	Damiano et al. 2005	–
Head and neck	Gustafson et al. 2004	–	–	–	–	Gustafson et al. 2004	–
Ovarian	Wedge et al. 2002	–	–	–	–	–	–
Pancreatic	–	Bruns et al. 2003	Bruns et al. 2003	–	–	–	–
Prostate	Wedge et al. 2002; Nicholson et al. 2004	–	–	–	–	–	–
Renal	–	Drevs et al. 2004	–	–	–	–	–
Vulval	Wedge et al. 2002	–	–	–	–	–	–

vandetanib at doses of 12.5–100 mg/kg resulted in significant, dose-dependent inhibition of tumour growth (Fig. 41.5) (Wedge et al. 2002). Other human xenograft models in which vandetanib also significantly inhibited tumour growth included a colon cancer model with acquired resistance to inhibitors of EGFR signalling (Ciardiello et al. 2004), and several models of CNS tumour types (Rich et al. 2005). This broad-spectrum activity of vandetanib is consistent with inhibition of VEGF signalling, and therefore an indirect (that is, antiangiogenic) effect on tumour cell growth. However, depending on the tumour type, a direct antiproliferative effect on the tumour cells through inhibition of EGFR and/or RET tyrosine kinase may also contribute to the antitumour activity of vandetanib. A striking example of an anti-EGFR mechanism was demonstrated in a study using xenografts derived from the PC-9 NSCLC cell line, which is known to be highly dependent on EGFR signalling for proliferation and survival. Vandetanib (12.5–50 mg/kg/day) resulted in marked dose-dependent regression of established PC-9 xenograft tumours, suggesting that the anti-EGFR effects of vandetanib can be an important contributing factor to its antitumour activity (Taguchi et al. 2004). Similarly, vandetanib has demonstrated significant inhibition of RET-dependent tumour growth in vivo (Carlomagno et al. 2002).

Orthotopic Models

Vandetanib has also been shown to inhibit tumour growth in orthotopic models. Because this type of model involves implantation at the anatomical site analogous to the tumour origin in man, it can be considered to mimic more closely than

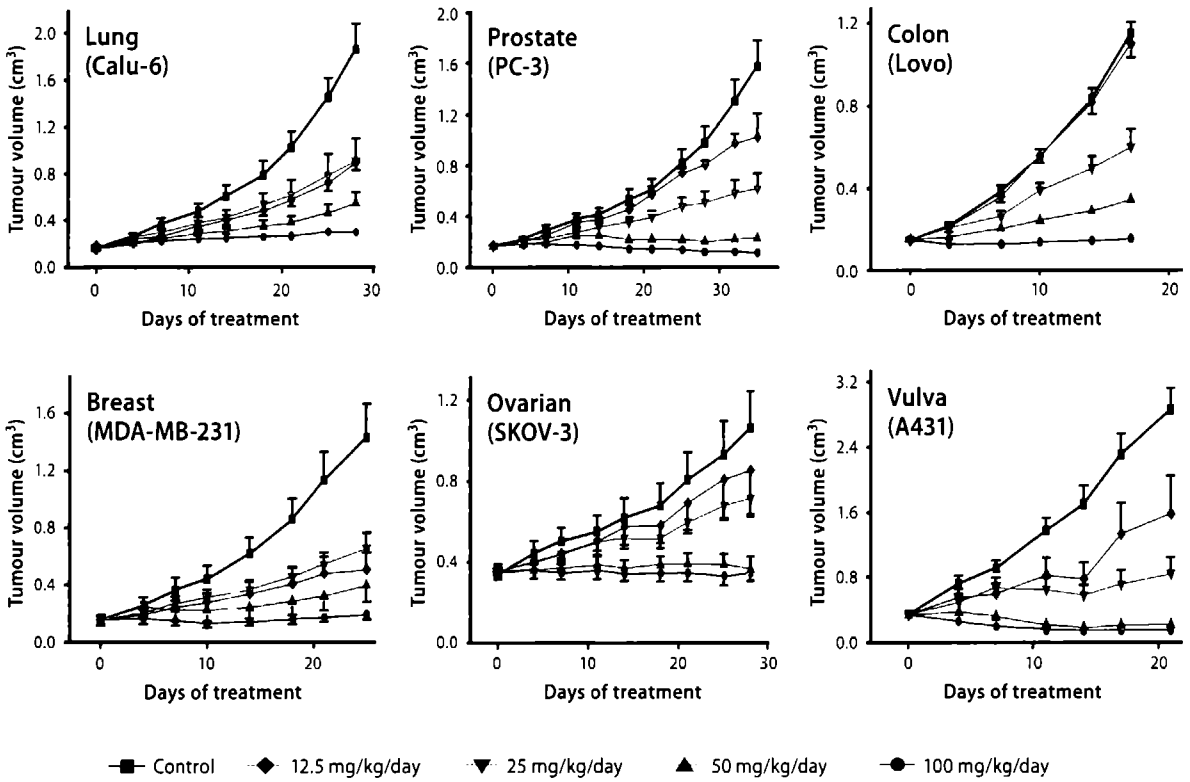


Fig. 41.5. Vandetanib demonstrates antitumour activity in a range of human tumour xenografts. Adapted from Wedge et al. (2002)

subcutaneous xenografts the patterns of growth and metastasis observed in the natural tumour (Taghian and Suit 1999). Marked inhibition of tumour growth, reduced tumour cell proliferation and increased tumour cell apoptosis were observed in orthotopic models of glioma (Sandstrom et al. 2004), and gastric (McCarty et al. 2004) and pancreatic (Bruns et al. 2003) cancer after once-daily oral administration of vandetanib. In an orthotopic model of NSCLC, almost complete suppression of tumour growth was observed in animals receiving daily oral dosing with vandetanib (25 or 50 mg/kg) (Wu et al. 2004a).

Metastatic Models

Metastasis is the leading cause of death in patients with cancer, and the growth of metastases (and primary tumours) is dependent upon the formation of new blood vessels (Folkman 2002). Consistent with an antiangiogenic mode of action, vandetanib has demonstrated effective inhibition of metastatic growth and spread in several preclinical models. In a metastatic model of human SCLC, the administration of vandetanib caused a significant reduction in the frequency of large (>3 mm) metastatic colonies in the liver, lymph nodes and bone (Yano et al. 2005). Vandetanib has also been shown to inhibit metastatic growth in experimental models of liver metastasis (Varghese et al. 2003) and NSCLC (Matsumori et al. 2003; Wu et al. 2004a). It is interesting to note that the metastatic lung cancer models PE14PE6 and H226 were sensitive to inhibition by vandetanib, but refractory to the selective EGFR inhibitor gefitinib (Matsumori et al. 2003). These results suggest that the antimetastatic effects of vandetanib were mediated via inhibition of VEGF-dependent angiogenesis in the host endothelial cells, and also suggest that vandetanib may provide some benefit in metastatic NSCLC that is resistant to EGFR tyrosine kinase inhibitors. In a metastatic model of human pancreatic cancer, the numbers of lung and lymph node metastases were reduced following treatment with vandetanib (Bruns et al. 2003). Given that previous studies have shown that lymph node metastases in this pancreatic model are not sensitive to inhibitors of VEGFR-2 (Bruns et al. 2002) or EGFR activity (Bruns et al. 2000), it may be that the reduction in lymphatic metastasis observed with vandetanib is a consequence of its activity versus both VEGFR-2 and EGFR. Alternatively, vandetanib may have the potential to inhibit lymphatic metastasis through inhibition of VEGFR-3 tyrosine kinase activity. Collectively, these results demonstrate that vandetanib has the potential to inhibit metastasis by preventing primary tumour dissemination as well as by inhibiting the growth of any secondary tumours that have become established.

Early-stage Disease Models

Vandetanib has also been used to investigate the potential role of angiogenesis in early tumourigenesis. In multiple intestinal neoplasia (Min) mice, which develop spontaneous, early, intestinal adenomas, administration of vandetanib (50 mg/kg/day) to 6-week-old mice significantly reduced polyp growth and number (Wilkinson et al. 2004). Similar results were obtained in a 7,12-dimethylbenz[a]anthracene (DMBA) model of breast cancer (Heffelfinger et al. 2004). Initiation of vandetanib treatment 1 week or 6 weeks after DMBA exposure inhibited the formation of mammary tumours by >95%. These data indicate that VEGF-dependent angiogenesis is a necessary step for progression from a premalignant to a malignant phenotype, and that vandetanib may offer a strategy for treatment of early-stage disease.

Combination Therapy

Several preclinical studies have demonstrated that combining vandetanib with certain other anticancer strategies can provide additional efficacy (Table 41.2). Vandetanib has been shown to significantly enhance the effects of radiation therapy in models of human lung cancer (Hoang et al. 2004; Williams et al. 2004; Shibuya et al. 2005), colon cancer (Siemann and Shi 2004) and glioblastoma (Damiano et al. 2005). The impact of scheduling was examined in Calu-6 lung xenografts, and it was shown that the efficacy of radiation therapy was enhanced with either concurrent or sequential administration of vandetanib, but that giving vandetanib after radiation therapy was more

effective than concurrent treatment (Fig. 41.6) (Williams et al. 2004). These data suggest that in the Calu-6 xenograft model, achieving an optimal response to vandetanib in combination with radiation therapy may depend upon the timing of its administration.

Vandetanib has also been investigated in combination with certain conventional cytotoxic chemotherapy. In a xenograft model of colon cancer, combining vandetanib (100 or 150 mg/kg) with paclitaxel (20 mg/kg) produced a significantly greater inhibition of established tumour growth compared with either agent alone (Ciardiello et al. 2004). In another study, vandetanib was investigated in combination with SC-236, a selective inhibitor of cyclooxygenase-2 (COX-2). COX-2 is an inducible enzyme that is overexpressed in several tumour types and may be involved in promoting angiogenesis and tumour growth (Masferrer et al. 2000; Masunaga et al. 2000). The effect of simultaneous blockade of VEGFR, EGFR and COX-2 signalling pathways was examined using vandetanib and SC-236 in colon and lung cancer xenografts (Tuccillo et al. 2005). Combination treatment with vandetanib and SC-236 produced sustained inhibition of tumour growth that was greater than the effects of either agent alone.

Clinical Development of Vandetanib

41.3.1 Phase I Evaluation

Vandetanib has been evaluated in two phase I studies of patients with malignant solid tumours refractory to standard therapy or for whom no therapy exists. One study was conducted at five sites in the USA and Australia (n=77; Holden et al. 2005), and a second study was conducted in Japan (n=18; Minami et al. 2003). The primary objective of both studies was to evaluate the safety and tolerability of ascending doses of vandetanib (USA/Australia, 50–600 mg; Japan, 100–400 mg). Secondary objectives included preliminary assessment of vandetanib antitumour activity and assessment of the pharmacokinetic profile of vandetanib. The most common tumour type in the USA/Australian study was colorectal cancer (n=23). Most of the patients recruited to the Japanese study had either NSCLC (n=9) or colorectal cancer (n=4).

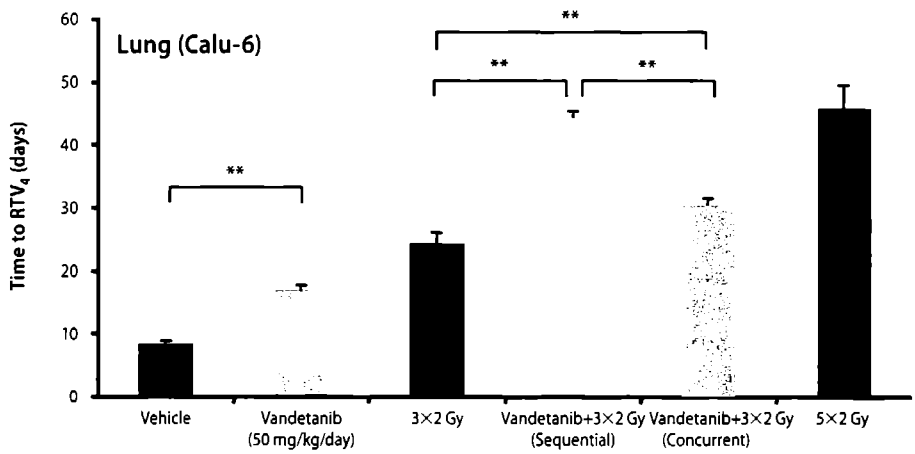


Fig. 41.6. Antitumour activity of vandetanib in combination with radiation therapy (Williams et al. 2004)

41.3.1.1 Safety and Tolerability

Adverse events were generally mild (Table 41.3) and vandetanib was generally well tolerated at daily oral doses up to and including 300 mg. In the USA/Australian study, the most common treatment-related adverse events were diarrhoea and rash, which appeared to be dose related and could be managed by dose reduction, treatment withdrawal or supportive care. Asymptomatic QTc prolongation was also noted in seven patients, but was not associated with any clinical sequelae. In the Japanese study, the most common treatment-related adverse events were rash, asymptomatic QTc prolongation, diarrhoea and proteinuria. Asymptomatic QTc prolongation was reported at all doses studied, with no clear evidence of dose dependency. All adverse events in the Japanese study were manageable with dose interruption and/or reduction.

41.3.1.2 Efficacy

In the USA/Australian study, there were no objective tumour responses and stable disease was reported in 31 patients (40%). In the Japanese study, a partial tumour response was observed in four of the nine patients with refractory NSCLC. These responses were maintained (range 90–438 days) despite subsequent reductions in daily dose.

41.3.1.3 Pharmacokinetics

In both phase I studies, plasma concentrations of vandetanib increased linearly with dose. Vandetanib was extensively distributed, with a long half-life that was supportive of once-daily oral dosing. The half-life of vandetanib was estimated to be greater than 120 h in the Western study (dose range 50–600 mg) and between 90 h and 115 h in the Japanese study (dose range 100–400 mg). No differences in the pharmacokinetic profile of vandetanib were observed between Japanese and USA/Australian patients (Fig. 41.7). The plasma levels of vandetanib attained in these patients are consistent with achieving pharmacologically relevant inhibition of VEGFR-2, EGFR and RET signalling.

41.3.1.4 Metabolism and Pharmacodynamics

The effect of food on the intrasubject variability of vandetanib pharmacokinetics was examined in healthy subjects (Smith et al. 2005). There was no clinically significant effect of food on the absorption characteristics of vandetanib, and it was concluded that vandetanib could be taken with or without food. Because the CYP3A4 isoenzyme is likely to contribute to the metabolism of vandetanib, a study was conducted in healthy subjects to assess the effect

Table 41.3. Common adverse events[a] in phase I studies of vandetanib

USA/Australian study		Japanese study	
Adverse event	n=77 (grade 3/4)	Adverse event	n=18 (grade 3)
Rash	45 (4)	Rash	14 (0)
Diarrhoea	27 (4)	QTc prolongation[b]	11 (0)
Nausea	15 (0)	Diarrhoea	10 (1)
Fatigue	14 (1)	Proteinuria	10 (0)
Hypertension	14 (4)	Hypertension	7 (4)
Anorexia	10 (0)		

[a]Irrespective of causality. [b]Asymptomatic and manageable with dose interruption and reduction

of itraconazole, a potent inhibitor of CYP3A4, on the bioavailability of vandetanib (Smith et al. 2006). The results indicated that concomitant use of vandetanib with CYP3A4 inhibitors will not result in a clinically meaningful pharmacokinetic interaction. In a similar population, the potential for a pharmacodynamic interaction between vandetanib and ondansetron was investigated. Ondansetron, a 5-HT^3 antagonist known to prolong the QTc interval, is widely used for chemotherapy-induced emesis. Co-administration of vandetanib and ondansetron did not result in greater than additive effects on QTc prolongation, and any potential cardiovascular effects are unlikely to be exacerbated with their concomitant use (Hammett et al. 2005).

41.3.2 Phase II/III Evaluation

41.3.2.1 Non-Small-Cell Lung Cancer

The preliminary evidence of efficacy observed in patients with NSCLC during phase I evaluation led to the initiation of a series of two-part, randomized, double-blind, multicentre phase II trials to investigate the efficacy of vandetanib in patients with advanced NSCLC. In all three studies, patients with squamous cell histology were eligible to enter, and brain metastases were permitted if treated at least 4 weeks before entry and clinically stable without steroid treatment for 1 week.

Vandetanib Versus Gefitinib

Vandetanib monotherapy was compared with gefitinib in patients with locally advanced or metastatic (stage IIIB/IV) NSCLC after failure of first-line ± second-line chemotherapy, either of which could be platinum-based, because of toxicity or tumour progression (Natale et al. 2006). Patients received once-daily oral doses of vandetanib 300 mg (n=83) or gefitinib 250 mg (n=85) until disease progression or toxicity (part A), with an option to switch to the alternative treatment after a washout period of 4 weeks (part B; Fig. 41.8). The dual primary objectives in part A were assessments of progression-free survival (PFS) and safety/tolerability. The study met its primary efficacy objective, with vandetanib demonstrating a significant prolongation of PFS versus gefitinib: median PFS was 11.0 weeks for vandetanib and 8.1 weeks for gefitinib [hazard ratio 0.69 (95% CI 0.50–0.96); P=0.025]. The randomized phase of the study was designed to have a >75% power to detect a 33% prolongation of PFS at a significance

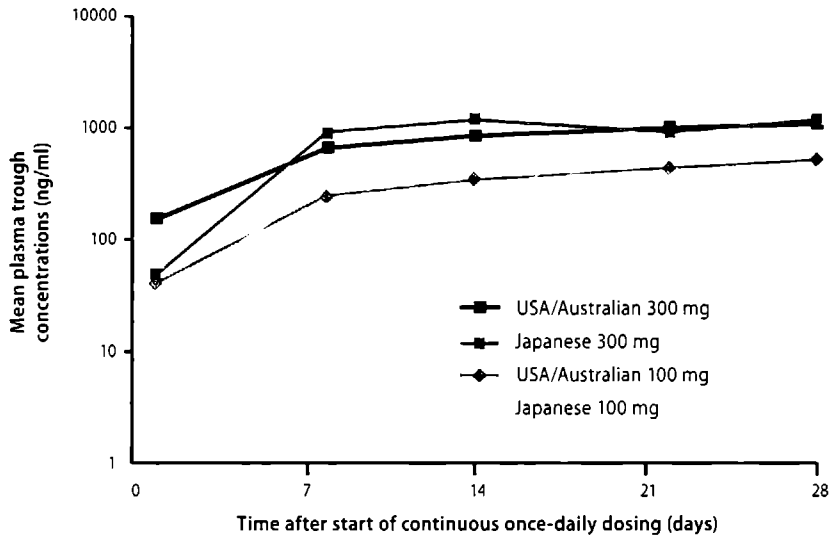


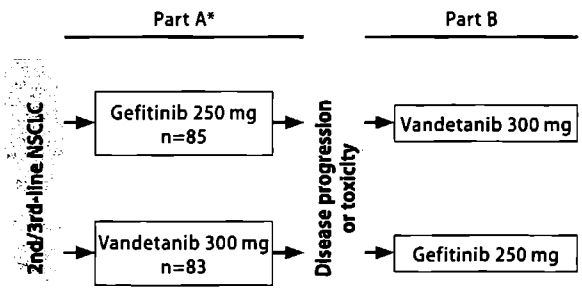
Fig. 41.7. Pharmacokinetic profile of vandetanib in USA/Australian and Japanese patients

level of $P<0.2$. A significance level of 0.2 (rather than 0.05) was used since the trial was designed to assess whether vandetanib shows sufficient promise to warrant further investigation. The adverse-event profile of vandetanib was similar to that seen in previous trials, and included diarrhoea, rash and asymptomatic QTc prolongation. There was no significant difference in overall survival between patients initially randomized to either vandetanib or gefitinib (median survival 6.1 and 7.4 months, respectively). The switch-over design of the study potentially confounds interpretation of this secondary endpoint.

Vandetanib with Docetaxel Versus Docetaxel Alone

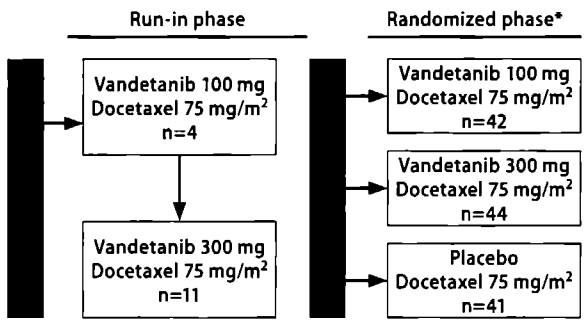
The efficacy of vandetanib plus docetaxel (a standard therapy for the treatment of second-line NSCLC) has been compared with docetaxel alone in patients with locally advanced or metastatic (stage IIIB/IV) NSCLC after failure of first-line platinum-based chemotherapy (Fig. 41.9). An initial open-label, run-in phase evaluated once-daily doses of vandetanib (100 or 300 mg) with docetaxel (75 mg/m^2 i.v. infusion every 21 days) for any potential acute toxicities and pharmacokinetic interaction (Heymach et al. 2004). The results of the run-in phase confirmed that the combination regimen was generally well tolerated and not associated with clinically significant changes in exposure to either drug. A partial tumour response is shown in Fig. 41.10.

Following the run-in phase, a different population of patients was recruited into the double-blind, randomized phase to receive vandetanib 100 mg plus docetaxel (n=42), vandetanib 300 mg plus docetaxel (n=44), or docetaxel alone (n=41) (Heymach et al. 2006). The primary objective of the randomized phase was to determine whether once-daily oral vandetanib (100 or 300 mg) plus docetaxel prolonged PFS versus docetaxel alone. The randomized phase of the study was designed to have a >75% power to detect a 50% prolongation of PFS at a significance level of $P<0.2$. A significance level of 0.2 (rather than 0.05) was used since the trial was designed to assess whether vandetanib shows sufficient promise to warrant further investigation. Combined use of vandetanib and docetaxel prolonged PFS compared with docetaxel alone (Table 41.4). Vandetanib 100 mg plus docetaxel demonstrated a significant improvement in PFS compared to docetaxel alone. Vandetanib 300 mg demonstrated a numerical advantage in PFS compared to docetaxel alone but the difference did not reach statistical significance. Overall survival data were immature at the time of PFS analysis. The adverse event profile was consistent with that reported in previous studies. Common adverse events included diarrhoea, rash and asymptomatic QTc prolongation, all responding



* Study designed to have a >75% power to detect a 33% prolongation of progression-free survival at a significant level of $P<0.2$

Fig. 41.8. Vandetanib versus gefitinib: a two-part, randomized, double-blind, multicentre phase II study



* Study designed to have a >75% power to detect a 50% prolongation of progression-free survival at a significant level of $P<0.2$

Fig. 41.9. Vandetanib plus docetaxel versus docetaxel: a two-part, randomized, double-blind, multicentre phase II

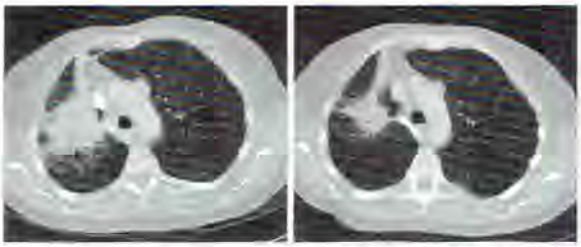


Fig. 41.10. CT evaluation of a tumour response seen in patient after receiving vandetanib (300 mg/day) and two 21-day cycles of docetaxel (75 mg/m^2)

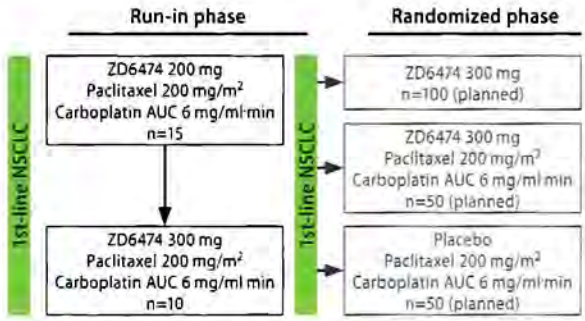


Fig. 41.11. Vandetanib with carboplatin and paclitaxel as first-line treatment for NSCLC: a two-part, randomized, double-blind, multicentre phase II study

to dose interruption and/or reduction or standard management. No fatal episodes of haemoptysis or any CNS haemorrhage adverse events were reported in patients receiving vandetanib. The encouraging efficacy results obtained with vandetanib 100 mg plus docetaxel supported the decision to initiate phase III evaluation of this combination (versus docetaxel alone) in second-line NSCLC.

Vandetanib with Carboplatin and Paclitaxel

The efficacy of vandetanib alone and in combination with carboplatin and paclitaxel as first-line therapy is being assessed in an ongoing study of patients with locally advanced (stage IIIB) or metastatic (stage IV) NSCLC (Fig. 41.11) (Johnson et al. 2005). A 21-day safety run-in phase was conducted initially to establish the appropriate dose of vandetanib to be administered in combination with carboplatin and paclitaxel: subjects received daily oral doses of vandetanib (200 mg or 300 mg) in combination with paclitaxel (200 mg/m^2 i.v.) and carboplatin, which was dosed to achieve a target AUCss of 6 mg/ml·min. Combination treatment was generally well tolerated, without evidence of mutually additive toxicity, and did not appear to result in changes in exposure to vandetanib. Based on the results of the run-in phase, patients recruited into the double-blind, randomized component of the study received vandetanib 300 mg alone, carboplatin and paclitaxel alone, or vandetanib 300 mg in combination with carboplatin and paclitaxel.

41.3.2.2 Medullary Thyroid Cancer

The clinical activity of once-daily oral vandetanib (300 mg/day) is currently being evaluated in an ongoing, open-label, single arm, phase II study in patients with unresectable, measurable, locally advanced or metastatic hereditary medullary thyroid cancer (Wells et al. 2006). As of November 2005, 16 patients had received treatment with vandetanib (300 mg/day). Encouragingly, the majority of patients have shown marked reductions in circulating levels of calcitonin and objective tumour responses have been reported in 3 of 15 evaluable patients.

41.3.2.3 Other Tumour Types

To date, the safety and clinical potential of vandetanib monotherapy has also been evaluated in phase II trials of patients with advanced breast cancer and multiple myeloma. In the breast cancer study, 46 patients with heavily pretreated metastatic breast cancer received once-daily vandetanib therapy at a dose of 100 mg or 300 mg (Miller et al. 2004). vandetanib was generally well tolerated, with an adverse event profile similar to that seen in other studies. No objective responses were seen, suggesting that vandetanib may have limited activity as monotherapy in patients with refractory metastatic breast cancer. Vandetanib monotherapy has also been investigated

Table 41.4. Progression-free survival with vandetanib plus docetaxel versus docetaxel alone in previously treated NSCLC

	Docetaxel (n=41)	Vandetanib 100 mg + docetaxel (n=42)	Vandetanib 300 mg + docetaxel (n=44)
Hazard ratio versus docetaxel alone	–	0.64	0.83
95% CI	–	0.38, 1.05	0.50, 1.36
P value	–	0.074	0.461
Median progression-free survival (weeks)	12	19	17

in 18 patients with relapsed pretreated multiple myeloma. Treatment with vandetanib 100 mg p.o. daily demonstrated that treatment was generally well tolerated in this population, but no tumour responses were observed (Kovacs et al. 2006).

An ongoing SCLC study of vandetanib 300 mg versus placebo is recruiting patients who have previously experienced a complete or partial response to induction chemotherapy with or without radiation therapy. In addition to assessing the effect of vandetanib maintenance therapy on disease progression, any correlation between outcome and tumour VEGFR expression and microvascular density will be investigated.

41.3.2.4 Summary of Phase II/III Evaluation

As part of an ongoing clinical development programme, the efficacy of vandetanib, alone and in combination with standard chemotherapy regimens, continues to be investigated in a range of tumour types. Key phase II studies and phase III studies are summarized in Table 41.5.

Conclusions and Future Perspectives

Vandetanib is a once-daily oral agent that selectively targets key signalling pathways in cancer by inhibiting VEGFR-dependent tumour angiogenesis, and EGFR- and RET-dependent tumour cell proliferation and survival. The relative contribution of VEGFR, EGFR and RET tyrosine kinase inhibition to the in vivo antitumour activity of vandetanib remains to be determined, but direct antitumour effects may be particularly important in cancers that are highly dependent on EGFR or RET tyrosine kinase activity for continued proliferation and survival. Ligand-dependent activation of EGFR in cancer cells results in the increased expression and secretion of VEGF and other proangiogenic factors (Goldman et al. 1993; Gille et al. 1997). Conversely, inhibition of EGFR-dependent signalling in tumour cells can lead to a reduction in VEGF production and hence, inhibition of angiogenesis (Ciardiello et al. 2001). Furthermore, VEGF overexpression has been shown to occur in human tumour cells following efficient blockade of EGFR activity (Viloria-Petit et al. 2001; Ciardiello et al. 2004). This 'compensatory' upregulation of VEGF may be an important mechanism in tumours that have acquired resistance to inhibitors of EGFR activity, and available data suggest that vandetanib may be an effective treatment in this disease setting (Ciardiello et al. 2004).

In addition to its nanomolar inhibition of VEGFR-2 in vitro, vandetanib is also a potent inhibitor of VEGFR-3 tyrosine kinase activity in isolated enzyme assays. Expression of VEGFR-3 in vivo is considered to be limited to lymphatic endothelial cells, but there is increasing evidence from experimental models that VEGFR-3 and its ligands VEGF-C and VEGF-D may be involved in regulating lymphangiogenesis (Jussila and Alitalo 2002) and promoting lymph node metastasis (Schietroma et al. 2003; Kurahara et al. 2004; Zeng et al. 2004; Suzuki et al. 2005). Determining the relevance of VEGFR-3 tyro-

sine kinase inhibition to the pharmacological profile of vandetanib will require additional in vitro and in vivo studies.

In preclinical studies, vandetanib has demonstrated potent inhibition of VEGF-induced endothelial cell proliferation and tumour-induced angiogenesis, and a direct antiproliferative effect on tumour cells through inhibition of EGFR and/or RET signalling. Once-daily oral dosing with vandetanib had significant antitumour effects in a histologically diverse range of xenograft, orthotopic and metastatic tumour models, and also enhanced the efficacy of radiation therapy and certain cytotoxic chemotherapy.

Phase I and phase II studies in a broad population of patients with cancer show vandetanib to be gener-

Table 41.5. Overview of key phase II and phase III trials of vandetanib

Tumour type	Vandetanib dose (mg/day)	Status[a]
Phase II		
Second-/third-line NSCLC: vandetanib versus gefitinib (6474IL0003)	300	Complete – vandetanib demonstrated a significant improvement in progression-free survival versus gefitinib
Breast cancer: two vandetanib dose cohorts (6474IL0002)	100 or 300	Complete – vandetanib therapy generally well tolerated. No clinical benefit determined
Multiple myeloma: single arm (6474IL0004)	100	Complete – vandetanib therapy generally well tolerated. No clinical benefit determined
Medullary thyroid cancer: single arm (6474IL0008)	300	Ongoing – preliminary data show sustained reduction in plasma levels of the tumour marker calcitonin
SCLC: vandetanib versus placebo (6474IL0005)	300	Ongoing
Glioma: single arm (phase I/II)	Dose escalation	Recruiting
First-line NSCLC: vandetanib versus carboplatin + paclitaxel versus combination (6474IL0007)	300	Completed recruitment
Second-line NSCLC: vandetanib + docetaxel versus docetaxel (6474IL0006)	100 or 300	Complete – vandetanib 100 mg plus docetaxel demonstrated a significant improvement in progression-free survival versus docetaxel alone
Phase III		
Second-line NSCLC: vandetanib + docetaxel versus docetaxel (6474IL0032)	100	Recruiting
Refractory NSCLC: vandetanib versus placebo in patients previously treated with anti-EGFR therapy (6474IL0044)	300	Open
Refractory NSCLC: vandetanib versus erlotinib (6474IL0057)	300	Open

[a]As of June 2006

ally well tolerated, with a pharmacokinetic profile supportive of once-daily oral dosing. Vandetanib met its primary endpoint in two phase II studies in patients with recurrent NSCLC, demonstrating improved PFS as monotherapy versus gefitinib and in combination with docetaxel versus docetaxel alone. A phase III, randomized, double-blind, multi-centre study to assess the efficacy of vandetanib plus docetaxel second-line NSCLC has been initiated, and the clinical development plan for vandetanib also includes investigations in other NSCLC settings as well as other tumour types, including medullary thyroid cancer. Indeed, based on the preliminary evidence of activity in medullary thyroid cancer, the US Food and Drug Administration has granted vandetanib orphan drug designation for the treatment of patients with follicular, medullary, anaplastic, and locally advanced and metastatic papillary thyroid cancer. Similarly, the European Medicines Agency (EMEA) has recently confirmed vandetanib orphan drug designation for the treatment of patients with medullary thyroid cancer in the European Union.

In conclusion, vandetanib has potential for use in a number of solid tumour types. Early clinical evaluation of vandetanib has demonstrated a promising efficacy and safety profile, both as a single agent and in combination with other anticancer strategies. The results of preclinical, translational and clinical investigations currently in progress are keenly anticipated.

Acknowledgements

ACTIMA and RESSA are registered trademarks of the AstraZeneca group of companies. We thank Dr John Matthew, from Mudskipper Bioscience, who provided medical writing support funded by AstraZeneca.

References

Arao T, Fukumoto H, Takeda M, Tamura T, Saijo N, Nishio K (2004a) Small in-frame deletion in the epidermal growth factor receptor as a target for ZD6474. Cancer Res 64:9101–9104

Arao T, Yanagihara K, Takigahira M, Fukumoto F, Koizumi F, Nishio K (2004b) Antitumor effect of ZD6474 in a VEGF-producing primary gastric cancer model. Proc Am Assoc Cancer Res 45:1047

Azzariti A, Porcelli L, Paradiso A (2003) In vitro activity of ZD6474 in combination with 7-ethyl-10-hydroxy-camptothecin (SN-38) on colon cancer cell line growth and survival. Clin Cancer Res 9:6148S

Bradley DP, Tessier JT, Checkley DR, Kuribayashi H, Wedge SR (2004) The VEGF signaling inhibitors ZD6474 and AZD2171 compromise hemodynamic parameters in an SW620 human colon tumor model: an analysis using perfusion-permeability dynamic contrast-enhanced magnetic resonance imaging (pp-DCE-MRI). Proc Am Assoc Cancer Res 45:1051

Bradshaw EL, Gustafson DL (2004) Inhibitory growth effect of ZD6474 alone and in combination with low-dose cytotoxic chemotherapy in mouse endothelial cells. Proc Am Assoc Cancer Res 45:882

Briggs A, Molofsky D, Wang A, Engelman J, Beaudry P, Prox D, Tracy S, Ryan A, Folkman J, Janne P, Johnson BE, Heymach JV (2005) Sensitivity of NSCLC cell lines bearing wild-type and mutated EGFR to the VEGFR/EGFR inhibitor ZD6474. Proc Am Assoc Cancer Res 46: abstr 5833

Bruns CJ, Solorzano CC, Harbison MT, Ozawa S, Tsan R, Fan D, Abbruzzese J, Traxler P, Buchdunger E, Radinsky R, Fidler IJ (2000) Blockade of the epidermal growth factor receptor signaling by a novel tyrosine kinase inhibitor leads to apoptosis of endothelial cells and therapy of human pancreatic carcinoma. Cancer Res 60:2926–2935

Bruns CJ, Shrader M, Harbison MT, Portera C, Solorzano CC, Jauch KW, Hicklin DJ, Radinsky R, Ellis LM (2002) Effect of the vascular endothelial growth factor receptor-2 antibody DC101 plus gemcitabine on growth, metastasis and angiogenesis of human pancreatic cancer growing orthotopically in nude mice. Int J Cancer 102:101–108

Bruns CJ, Kohl G, Guba M, Yezhelyev M, Wiegand U, Ryan A, Barge A, Geissler E, Jauch K-W (2003) Anti-angiogenic and anti-tumor activity of a novel VEGFR-2 tyrosine kinase inhibitor ZD6474 in a metastatic human pancreatic tumor model. Proc Am Assoc Cancer Res (2nd Ed) 44:604

Carlomagno F, Vitagliano D, Guida T, Ciardiello F, Tortora G, Vecchio G, Ryan AJ, Fontanini G, Fusco A, Santoro M (2002) ZD6474, an orally available inhibitor of KDR tyrosine kinase activity, efficiently blocks oncogenic RET kinases. Cancer Res 62:7284–7290

Carlomagno F, Guida T, Anaganti S, Vecchio G, Fusco A, Ryan AJ, Billaud M, Santoro M (2004) Disease associated mutations at valine 804 in the RET receptor tyrosine kinase confer resistance to selective kinase inhibitors. Oncogene 23:6056–6063

Checkley D, Tessier JJ, Kendrew J, Waterton JC, Wedge SR (2003) Use of dynamic contrast-enhanced MRI to evaluate acute treatment with ZD6474, a VEGF signalling inhibitor, in PC-3 prostate tumours. Br J Cancer 89:1889–1895

Ciardiello F, Caputo R, Bianco R, Damiano V, Fontanini G, Cuccato S, de Placido S, Bianco AR, Tortora G (2001) Inhibition of growth factor production and angiogenesis in human cancer cells by ZD1839 (Iressa), a selective epidermal growth factor receptor tyrosine kinase inhibitor. Clin Cancer Res 7:1459–1465

Ciardiello F, Caputo R, Damiano V, Caputo R, Troiani T, Vitagliano D, Carlomagno F, Veneziani BM, Fontanini G, Bianco AR, Tortora G (2003) Antitumor effects of ZD6474, a small molecule vascular endothelial growth factor receptor tyrosine kinase inhibitor, with additional activity against epidermal growth factor receptor tyrosine kinase. Clin Cancer Res 9:1546–1556

Ciardiello F, Bianco R, Caputo R, Caputo R, Damiano V, Troiani T, Melisi D, de Vita F, de Placido S, Bianco AR, Tortora G (2004) Antitumor activity of ZD6474, a vascular endothelial growth factor receptor tyrosine kinase inhibitor, in human cancer cells with acquired resistance to antiepidermal growth factor receptor therapy. Clin Cancer Res 10:784–793

Damiano V, Melisi D, Bianco C, Raben D, Caputo R, Fontanini G, Bianco R, Ryan A, Bianco AR, de Placido S, Ciardiello F, Tortora G (2005) Cooperative antitumor effect of multitargeted kinase inhibitor ZD6474 and ionizing radiation in glioblastoma. Clin Cancer Res 11:5639–5644

Drevs J, Konerding MA, Wolloscheck T, Wedge SR, Ryan AJ, Ogilvie DJ, Esser N (2004) The VEGF receptor tyrosine kinase inhibitor, ZD6474, inhibits angiogenesis and affects microvascular architecture within an orthotopically implanted renal cell carcinoma. Angiogenesis 7:347–354

Dvorak HF, Brown LF, Detmar M, Dvorak AM (1995) Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. Am J Pathol 146:1029–1039

Folkman J (2002) Role of angiogenesis in tumor growth and metastasis. Semin Oncol 29:15–18

Gerber HP, McMurtrey A, Kowalski J, Yan M, Keyt BA, Dixit V, Ferrara N (1998) Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. J Biol Chem 273:30336–30343

Gille J, Swerlick RA, Caughman SW (1997) Transforming growth factor-alpha-induced transcriptional activation of the vascular permeability factor (VPF/VEGF) gene requires AP-2-dependent DNA binding and transactivation. EMBO J 16:750–759

Goldman CK, Kim J, Wong WL, King V, Brock T, Gillespie GY (1993) Epidermal growth factor stimulates vascular endothelial growth factor production by human malignant glioma cells: a model of glioblastoma multiforme pathophysiology. Mol Biol Cell 4:121–133

Goodlad RA, Ryan A, Watson J, Wedge SR, Pyrah IT, Mandir N, Wilkinson RW (2004) Antitumor therapy with VEGF receptor tyrosine kinase inhibitor ZD6474 in a mouse model of intestinal cancer. EJC [Suppl] 2:51

Gustafson DL, Merz AL, Zirrolli JA, Connaghan-Jones KD, Raben D (2004) Impact of scheduling on combined ZD6474 and radiotherapy in head and neck tumor xenografts. EJC [Suppl] 2:45

Hammett T, Oliver S, Ghahramani P, Kennedy S, Fisher I, Gilmore E, Duvauchelle T, Smith R (2005) The pharmacodynamic effect on cardiac repolarization of combination single dose ZD6474 and ondansetron in healthy subjects. Proc Am Soc Clin Oncol abstr 3197

Harari PM (2004) Epidermal growth factor receptor inhibition strategies in oncology. Endocr Relat Cancer 11:689–708

Heffelfinger SC, Yan M, Gear RB, Schneider J, LaDow K, Warshawsky D (2004) Inhibition of VEGFR2 prevents DMBA-induced mammary tumor formation. Lab Invest 84:989–998

Helfrich B, Troiani T, Raben D, Frederick B, Gustafson D, Merz A, Weed S, Bunn P (2004) Anticancer effects of ZD6474 in gefitinib (Iressa™) resistant lung cancer cell lines in vitro. EJC [Suppl] 2:51

Hennequin LF, Stokes ES, Thomas AP, Johnstone C, Ple PA, Ogilvie DJ, Dukes M, Wedge SR, Kendrew J, Curwen JO (2002) Novel 4-anilinoquinazolines with C-7 basic side chains: design and structure activity relationship of a series of potent, orally active, VEGF receptor tyrosine kinase inhibitors. J Med Chem 45:1300–1312

Heymach JV, Dong R-P, Dimery I, Wheeler C, Fidias P, Lu C, Johnson B, Herbst R (2004) ZD6474, a novel antiangiogenic agent, in combination with docetaxel in patients with NSCLC: results of the run-in phase of a two-part, randomized phase II study. Proc Am Soc Clin Oncol abstr 3051

Heymach JV, Johnson BE, Prager D, Csada E, Roubec J, Pešek M, Špásová I, Hou J, Kennedy S, Herbst RS (2006) A phase II trial of ZD6474 plus docetaxel in patients with previously treated NSCLC: follow-up results. Proc Am Soc Clin Oncol abstr 7016

Hoang T, Huang SM, Armstrong EA, Harari PM (2004) Augmentation of radiation response with the VEGFR-tyrosine kinase inhibitor ZD6474. Proc Am Assoc Cancer Res 45:955

Holden SN, Eckhardt SG, Basser R, de Boer R, Rischin D, Green M, Rosenthal MA, Wheeler C, Barge A, Hurwitz HI (2005) Clinical evaluation of ZD6474, an orally active inhibitor of VEGF and EGF receptor signaling, in patients with solid, malignant tumors. Ann Oncol 16:1391–1397

Horner A, Bishop NJ, Bord S, Beeton C, Kelsall AW, Coleman N, Compston JE (1999) Immunolocalisation of vascular endothelial growth factor (VEGF) in human neonatal growth plate cartilage. J Anat 194:519–524

Ichihara M, Murakumo Y, Takahashi M (2004) RET and neuroendocrine tumors. Cancer Lett 204:197–211

Johnson BE, Ma P, West H, Kerr R, Prager D, Sandler A, Herbst RS, Stewart DJ, Dimery I, Heymach JV (2005) Preliminary Phase II safety evaluation of ZD6474, in combination with carboplatin and paclitaxel, as first-line treatment in patients with NSCLC. Proc Am Soc Clin Oncol abstr 7102

Jussila L, Alitalo K (2002) Vascular growth factors and lymphangiogenesis. Physiol Rev 82:673–700

Kovacs MJ, Reece DE, Marcellus D, Meyer RM, Mathews S, Dong RP, Eisenhauer E (2006) A phase II study of ZD6474 (Zactima™), a selective inhibitor of VEGFR and EGFR tyrosine kinase in patients with relapsed multiple myeloma-NCIC CTG IND.145. Invest New Drugs 24:529–535

Kurahara H, Takao S, Maemura K, Shinchi H, Natsugoe S, Aikou T (2004) Impact of vascular endothelial growth factor-C and -D expression in human pancreatic cancer: its relationship to lymph node metastasis. Clin Cancer Res 10:8413–8420

Leenders WP, Kusters B, Verrijp K, Maass C, Wesseling P, Heerschap A, Ruiter D, Ryan A, de Waal R (2004) Antiangiogenic therapy of cerebral melanoma metastases results in sustained tumor progression via vessel co-option. Clin Cancer Res 10:6222–6230

Masferrer JL, Leahy KM, Koki AT, Zweifel BS, Settle SL, Woerner BM, Edwards DA, Flickinger AG, Moore RJ, Seibert K (2000) Antiangiogenic and antitumor activities of cyclooxygenase-2 inhibitors. Cancer Res 60:1306–1311

Masunaga R, Kohno H, Dhar DK, Ohno S, Shibakita M, Kinugasa S, Yoshimura H, Tachibana M, Kubota H, Nagasue N (2000) Cyclooxygenase-2 expression correlates with tumor neovascularization and prognosis in human colorectal carcinoma patients. Clin Cancer Res 6:4064–4068

Matsumori Y, Goto H, Nakataki E, Kanematsu T, Yano S, Wedge S, Ryan A, Sone S (2003) ZD6474, an inhibitor of vascular endothelial growth factor receptor tyrosine kinase activity, inhibits growth of human non-small cell lung cancer metastases that are resistant to treatment with ZD1839. Proc Am Assoc Cancer Res (2nd Ed) 44:9

McCarty MF, Wey J, Stoeltzing O, Liu W, Fan F, Bucana C, Mansfield PF, Ryan AJ, Ellis LM (2004) ZD6474, a vascular endothelial growth factor receptor tyrosine kinase inhibitor with additional activity against epidermal growth factor receptor tyrosine kinase, inhibits orthotopic growth and angiogenesis of gastric cancer. Mol Cancer Ther 3:1041–1048

Miller KD, Hutchins G, Miller M, Badve S, Sledge GW (2003) ZD6474-mediated vascular changes in a breast cancer xenograft model illustrated by positron emission tomography. Clin Cancer Res 9:6139S

Miller KD, Trigo JM, Stone A, Wheeler C, Barge A, Sledge GW, Baselga J (2004) A phase II trial of ZD6474, a vascular endothelial growth factor receptor-2 (VEGFR-2) and epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, in patients with previously treated metastatic breast cancer (MBC). Breast Cancer Res Treat 88 [Suppl]: abstr 6060

Minami H, Ebi H, Tahara M, Sasaki Y, Yamamoto N, Yamada Y, Tamura T, Saijo N (2003) A Phase I study of an oral VEGF receptor tyrosine kinase inhibitor, ZD6474, in Japanese patients with solid tumors. Proc Am Soc Clin Oncol 194

Morgan B, Thomas AL, Drevs J, Hennig J, Buchert M, Jivan A, Horsfield KM, Ball HA, Lee L, Mietlowski W, Fuxius S, Unger C, O'Byrne KJ, Henry A, Cherryman GR, Leurent D, Dugan M, Marme D, Steward WP (2003) Dynamic contrast-enhanced magnetic resonance imaging as a biomarker for the pharmacological response of PTK787/ZK222584, an inhibitor of the vascular endothelial growth factor receptor tyrokine kinases, in patients with advanced colorectal cancer and liver metastases: results from two Phase I studies. J Clin Oncol 21:3955–3964

Natale RB, Bodkin D, Govindan R, Sleckman B, Rizvi N, Capo A, Germonpré P, Stockman P, Kennedy S, Ranson M (2006) ZD6474 versus gefitinib in patients with advanced NSCLC: final results from a two-part, double-blind, randomized Phase II trial. Proc Am Soc Clin Oncol abstr 7000

Nicholson B, Gulding K, Conaway M, Wedge SR, Theodorescu D (2004) Combination antiangiogenic and androgen deprivation therapy for prostate cancer: a promising therapeutic approach. Clin Cancer Res 10:8728–8734

Rich JN, Sathornsumetee S, Keir ST, Kieran MW, Laforme A, Kaipainen A, McLendon RE, Graner MW, Rasheed BK, Wang L, Reardon DA, Ryan AJ, Wheeler C, Dimery I, Bigner DD, Friedman HS (2005) ZD6474, a novel tyrosine kinase inhibitor of vascular endothelial growth factor receptor and epidermal growth factor receptor, inhibits tumor growth of multiple nervous system tumors. Clin Cancer Res 11:8145–8157

Sandstrom M, Johansson M, Andersson U, Bergh A, Bergenheim AT, Henriksson R (2004) The tyrosine kinase inhibitor ZD6474 inhibits tumour growth in an intracerebral rat glioma model. Br J Cancer 91:1174–1180

Schietroma C, Cianfarani F, Lacal PM, Odorisio T, Orecchia A, Kanitakis J, D'Atri S, Failla CM, Zambruno G (2003) Vascular endothelial growth factor-C expression correlates with lymph node localization of human melanoma metastases. Cancer 98:789–797

Shibuya K, Komaki R, Shintani T, Wu W, Itasaka S, Isobe T, Ryan A, Herbst RS, O'Reilly MS (2005) Combined blockade of VEGFR and EGFR with ZD6474 enhances the antitumor and antivascular effects of radiation therapy in an orthotopic mouse model of human lung cancer. Proc Am Assoc Cancer Res 46: abstr 5828

Shintani T, Lewis VO, Komaki R, Wu W, Ryan A, Herbst RS, O'Reilly MS (2005) ZD6474 inhibits human lung cancer bone metastases in a murine model by targeting both the tumor and its vasculature. Proc Am Assoc Cancer Res 46: abstr 5844

Siemann D, Shi W (2004) The VEGFR2 tyrosine kinase inhibitor, ZD6474, enhances the antitumor effect of radiation. EJC [Suppl] 2:49

Smith R, Kennedy S, Robertson J, Sandall D, Oliver R (2005) The effect of food on the intra-subject variability of the pharmacokinetics of ZD6474, a novel antiangiogenic agent, in healthy subjects. Proc Am Soc Clin Oncol abstr 3167

Smith R, Oliver S, Ghahramani P, Kennedy S, Gilmore E, Duvauchelle T, Hammett T (2006) The effect of the CYP3A4 inhibitor, itraconazole, on the pharmacokinetics of ZD6474 in healthy subjects. Proc Am Soc Clin Oncol abstr 3124

Suzuki K, Morita T, Tokue A (2005) Vascular endothelial growth factor-C (VEGF-C) expression predicts lymph node metastasis of transitional cell carcinoma of the bladder. Int J Urol 12:152–158

Taghian AG, Suit HD (1999) Animal systems for translational research in radiation oncology. Acta Oncol 38:829–838

Taguchi F, Koh Y, Koizumi F, Tamura T, Saijo N, Nishio K (2004) Anticancer effects of ZD6474, a VEGF receptor tyrosine kinase inhibitor, in gefitinib ("Iressa")-sensitive and resistant xenograft models. Cancer Sci 95:984–989

Troiani T, Lockerbie O, Morrow M, Eckhardt SG (2005) Sequence-dependent sensitivity of human colon cancer cells to oxaliplatin and ZD6474, a selective inhibitor of the EGFR/VEGFR tyrosine kinases. Proc Am Assoc Cancer Res 46: abstr 2171

Tuccillo C, Romano M, Troiani T, Martinelli E, Morgillo F, De Vita F, Bianco R, Fontanini G, Bianco RA, Tortora G, Ciardiello F (2005) Antitumor activity of ZD6474, a vascular endothelial growth factor-2 and epidermal growth factor receptor small molecule tyrosine kinase inhibitor, in combination with SC-236, a cyclooxygenase-2 inhibitor. Clin Cancer Res 11:1268–1276

Varghese HJ, MacKenzie LT, Groom AC, Ellis CG, Ryan A, MacDonald IC, Chambers AF (2003) The vascular-targeting agent, ZD6126, and the antiangiogenic agent, ZD6474, inhibit liver metastases via differential effects on metastatic vasculature. Clin Cancer Res 9:6141S

Viloria-Petit A, Crombet T, Jothy S, Hicklin D, Bohlen P, Schlaeppi JM, Rak J, Kerbel RS (2001) Acquired resistance to the antitumor effect of epidermal growth factor receptor-blocking antibodies in vivo: a role for altered tumor angiogenesis. Cancer Res 61:5090–5101

Wedge SR, Ogilvie DJ, Dukes M, Kendrew J, Chester R, Jackson JA, Boffey SJ, Valentine PJ, Curwen JO, Musgrove HL, Graham GA, Hughes GD, Thomas AP, Stokes ES, Curry B, Richmond GH, Wadsworth PF, Bigley AL, Hennequin LF (2002) ZD6474 inhibits vascular endothelial growth factor signaling, angiogenesis, and tumor growth following oral administration. Cancer Res 62:4645–4655

Wells S, You YN, Lakhani V, Hou J, Langmuir P, Headley D, Skinner M, Morse M, Burch W, Schlumberger M (2006) A Phase II trial of ZD6474 in patients with hereditary metastatic medullary thyroid cancer. Proc Am Soc Clin Oncol abstr 5533

Wilkinson RW, Ryan AJ, Wedge SR, Pyrah IT, Mandir N, Goodlad RA (2004) The VEGF receptor tyrosine kinase inhibitor ZD6474 significantly reduces intestinal tumor burden in the Min mouse. Proc Am Assoc Cancer Res 45:1075

Willett CG, Boucher Y, di Tomaso E, Duda DG, Munn LL, Tong RT, Chung DC, Sahani DV, Kalva SP, Kozin SV, Mino M, Cohen KS, Scadden DT, Hartford AC, Fischman AJ, Clark JW, Ryan DP, Zhu AX, Blaszkowsky LS, Chen HX, Shellito PC, Lauwers GY, Jain RK (2004) Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. Nat Med 10:145–147

Williams KJ, Telfer BA, Stratford IJ, Wedge SR (2003) Schedule-dependent effects of ZD6474, a potent inhibitor of VEGF signaling, combined with radiotherapy in a lung tumor xenograft model. Clin Cancer Res 9:6141S

Williams KJ, Telfer BA, Brave S, Kendrew J, Whittaker L, Stratford IJ, Wedge SR (2004) ZD6474, a potent inhibitor of vascular endothelial growth factor signaling, combined with radiotherapy: schedule-dependent enhancement of antitumor activity. Clin Cancer Res 10:8587–8593

Wu W, Isobe T, Itasaka S, Shintani T, Langley RR, Onn A, Hansen JC, O'Reilly MS, Herbst RS (2004a) ZD6474, a small molecule targeting VEGF and EGF receptor signaling, inhibits lung angiogenesis and metastasis and improves survival in an orthotopic model of non-small cell lung cancer. Proc Am Assoc Cancer Res 45:1051

Wu W, Onn A, Shintani T, O'Reilly MS, Herbst RS (2004b) ZD6474 enhances the anticancer efficacy of paclitaxel in human lung cancer models. Angiogenesis 7 [Suppl 1]:25

Yano S, Muguruma H, Matsumori Y, Goto H, Nakataki E, Edakuni N, Tomimoto H, Kakiuchi S, Yamamoto A, Uehara H, Ryan A, Sone S (2005) Antitumor vascular strategy for controlling experimental metastatic spread of human small-cell lung cancer cells with ZD6474 in natural killer cell-depleted severe combined immunodeficient mice. Clin Cancer Res 11:8789–8798

Zeng Y, Opeskin K, Baldwin ME, Horvath LG, Achen MG, Stacker SA, Sutherland RL, Williams ED (2004) Expression of vascular endothelial growth factor receptor-3 by lymphatic endothelial cells is associated with lymph node metastasis in prostate cancer. Clin Cancer Res 10:5137–5144

Integrins: Targets for Anti-Angiogenic Therapy

42

OLIVER KISKER

Abstract

Angiogenesis, the process responsible for the formation of new blood vessels, is an integral part of both normal development and tumor growth and metastasis. The switch of endothelial cells from a quiescent phenotype to a pro-angiogenic phenotype requires the upregulation of endogenous angiogenic factors such as the growth factors FGF2 and VEGF and the downregulation of endogenous inhibitors of angiogenesis such as endostatin and tumstatin. Analysis of the co-ordination between growth factors and components of the extracellular matrix in the regulation of angiogenesis has highlighted the role of the integrins (e.g. αvβ3, αvβ5 or α5β1) in the process. This has been demonstrated by the anti-angiogenic effects of monoclonal antibody and small-peptide αv integrin antagonists in particular, in preclinical studies. This in turn has led to the development of αv integrin antagonists as new targeted anti-cancer therapies and their investigation in clinical trials.

CONTENTS

42.1 Introduction

It is now accepted that tumor angiogenesis is essential for the growth and metastasis of solid tumors (Kerbel and Folkman 2002). Initiation of angiogenesis facilitates the rapid growth of the primary tumor and increases the chance that tumor cells will enter

the circulation and begin the process of forming metastases at different sites. Thus, angiogenesis is a critical step in ongoing tumor progression.

The process of angiogenesis involves multiple factors and cell types and is reliant on signaling molecules and receptors, across a variety of cell types, working in concert (Carmeliet 2003; Folkman 1992; Kerbel and Folkman 2002; Marmé 2003). Tumor cells begin the process of angiogenesis early in the process of tumor formation. The "angiogenic switch" (Folkman and Hanahan 1991; Hanahan and Folkman 1996), which converts quiescent endothelial cells to an active pro-angiogenic phenotype, is associated with the oncogene-driven tumor expression (Rak et al. 2000) of pro-angiogenic proteins such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF2), interleukin-8 (IL-8), platelet-derived growth factor (PDGF), placenta-like growth factor (PLGF), transforming growth factor-β (TGF-β), and platelet-derived endothelial growth factor (PD-EGF) (Kerbel and Folkman 2002). In addition, tumor hypoxia can activate tumor hypoxia-inducible factor 1-α (HIF-1α), which induces the upregulation of several angiogenic factors (Maxwell et al. 1997). Fibroblasts in or near the tumor are also responsible for the secretion of pro-angiogenic factors such as FGF2 and VEGF.

However, angiogenesis depends not only on growth factors and their receptors but also on endothelial cell interactions with the extracellular matrix (ECM). Adhesion to the ECM is primarily mediated by integrins, and evidence from various experimental systems demonstrates the importance of integrin-mediated cell adhesion in coordinating the growth factor signaling events responsible for cell proliferation and migration (Stupack and Cheresh 2002). Integrins interact directly with growth factor receptors to create integrin–growth factor receptor complexes which are responsible for downstream signaling.

VEGF is the most extensively studied of the angiogenic growth factors, and cross-talk between VEGF, its receptors and αvβ3 integrins is known to co-ordinate certain of the key angiogenic processes (De et al. 2005). The α1, α2 and β3 integrin subunits are known to modulate VEGF/VEGFR signaling (Hong et al. 2004; Reynolds et al. 2004), while VEGF is known to control the functional activity of αvβ3 integrins (De et al. 2005). The integrin αvβ3 has been proposed as 'the gatekeeper' of VEGF-mediated processes (De et al. 2005) and has been shown to regulate the production of VEGF in tumor cells that express it (De et al. 2005). Furthermore, it is becoming increasingly clear that integrins (possibly all integrins) can act to some degree as bidirectional signaling receptors inducing changes in protein activity or gene expression in response to ligand binding, with the concomitant modulation of cell adhesion (Hynes 2002). Indeed, integrins act as signal transduction receptors that are just as important to the cells as traditional growth factor receptors.

42.2 Integrins

Integrins are a family of heterodimeric, transmembrane glycoproteins comprising at least 18 α subunits and 8 β subunits (Brakebusch et al. 2002; Giancotti and Ruoslahti 1999). Different combinations of these α and β subunits dimerize non-covalently to form approximately 24 different receptors with distinct specificities in terms of their ECM ligands and distinct functions (Hynes 2002). Of the wide spectrum of integrin subunit combinations that are expressed on the surfaces of activated endothelial cells and tumor cells, the integrin αvβ3 has been identified as a receptor for a wide variety of ECM ligands expressing the RGD (arginine–lysine–aspartame) tripeptide motif, namely vitronectin, fibronectin, fibrinogen, thrombospondin, proteolyzed collagen, von Willebrand factor and osteopontin (Eliceiri and Cheresh 1999, 2000). Integrins are recognized as important transmembrane cell surface receptors for the transduction of positional cues from the ECM to the intracellular signaling pathways. These signals include elevation of intracellular pH and calcium levels, inositol lipid synthesis, and phosphorylation of a number of non-receptor tyrosine kinases such as focal adhesion kinase (FAK) and src

kinases, as well as adapter proteins shc, p130 CAS and Crk 11. These events in turn trigger downstream signaling (Fig. 42.1). Interestingly, many of the signaling pathways and effectors activated by integrin binding are also activated following growth factor stimulation, suggesting that there may be some synergy between the responses. Antagonists of the integrins αvβ3 and αvβ5 and others such as α5β1 are known to block both growth factor- and tumor-induced angiogenesis in animal models, inducing tumor regression (Brooks et al. 1994a,b; Kim et al. 2000a; Kumar et al. 2000). In addition, there is considerable preclinical evidence that endogenous components of the ECM such as endostatin (O'Reilly et al. 1997; Sudhakar et al. 2003), angiostatin (O'Reilly et al. 1994), thrombospondin (Good et al. 1990), canstatin (Kamphaus et al. 2000; Petitclerc et al. 2000), arresten (Colorado et al. 2000) and tumstatin (Maeshima et al. 2000, 2002; Petitclerc et al. 2000; Sudhakar et al. 2003) can suppress integrin-mediated angiogenesis. Members of the integrin class of cell adhesion molecules therefore appear to play a key role in endothelial cell survival and migration in relation to vascular development (Table 42.1).

42.3 Integrins and Angiogenesis

Integrins are involved in the cell–cell and cell–ECM interactions that take place during the growth of new blood vessels (Buerkle et al. 2002; Eliceiri and Cheresh 1999). To date, 9 of the 24 known integrin heterodimers have been implicated in angiogenesis (Serini et al. 2006). These are α1β1, α2β1, α3β1, α4β1, α5β1, αvβ1, αvβ3, αvβ5, αvβ8 and α6β4 (Serini et al. 2006). Historically, most of the data have pointed to the role of αv integrins in angiogenesis, in particular αvβ3, which, as stated previously, is capable of interacting with a variety of ECM proteins, and αvβ5, a receptor for vitronectin only, as major players in angiogenesis. The integrin αvβ3, which is not normally expressed on epithelial cells and only minimally on intestinal, vascular and uterine smooth muscle cells, has been shown to be expressed on vascular cells during angiogenesis and vascular remodeling. It has also been shown to be significantly upregulated during tumor-induced angiogenesis on the chick chorioallantoic membrane (CAM), on vascular cells within human

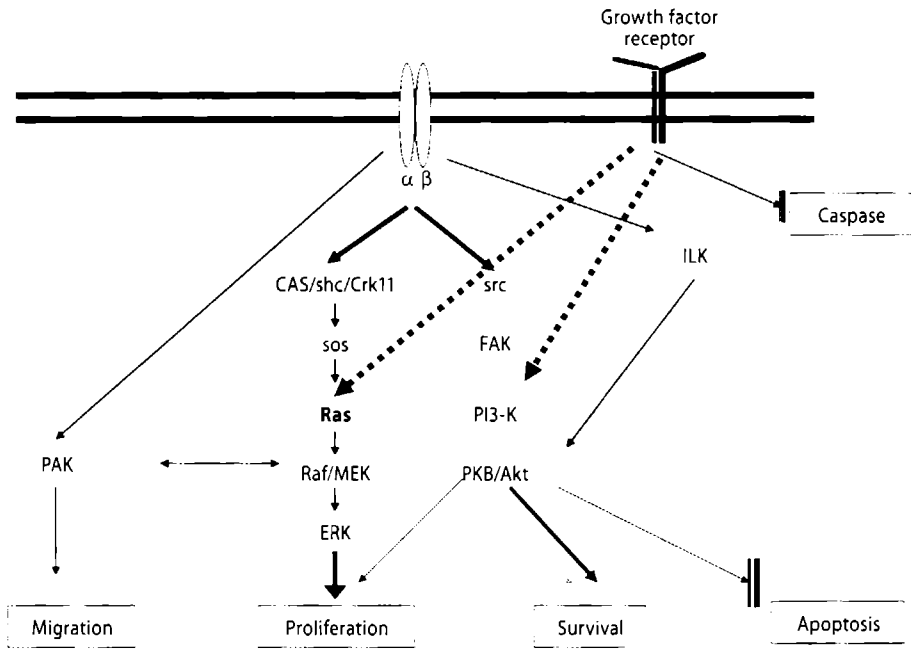


Fig. 42.1. Integrin-mediated angiogenesis pathways

Table 42.1. Summary of integrin-mediated growth factor responses in model systems

Growth factor	Integrin	Response	Reference(s)
PDGF	αvβ3	Proliferation, migration	Schneller et al. 1997
FGF2	αvβ3, α5β1	Angiogenesis, migration	Friedlander et al. 1996; Kim et al. 2000b
VEGF	αvβ5	Angiogenesis	Friedlander et al. 1995
VEGF	αvβ5, αvβ3, β1	Adhesion migration	Byzova et al. 2000b
VEGF	α2β1; α1β1, αvβ3	Angiogenesis, migration	Senger et al. 1996, 1997

tumors and in response to specific growth factors, namely FGF2 and tumor necrosis factor-α (TNF-α) (Brooks et al. 1994b; Kumar et al. 2000). The angiogenic growth factors FGF2 and VEGF have been shown to induce angiogenesis through two distinct signaling pathways which are inhibited by antagonists of αvβ3 and αvβ5 integrin respectively (Friedlander et al. 1995). These pathways can be further distinguished by their sensitivity to an inhibitor of protein kinase C (PKC) which blocks αvβ5- but not αvβ3-mediated angiogenesis (Friedlander et al. 1995). These data are consistent with the observations that αvβ5-mediated cell motility is dependent on a PKC-dependent signaling pathway (Eliceiri and Cheresh 1999; Eliceiri et al. 1999; Klemke et al. 1994). Also, inhibition of src kinase or PKC specifically disrupts VEGF-induced angiogenesis but not FGF2-induced angiogenesis. Furthermore, the αvβ3 and αvβ5 integrins contribute differently to sustained Ras-extracellular signal-related kinase (Ras-ERK) signaling in blood vessels (Hood et al. 2003), accounting for specific but distinct vascular responses during two pathways of angiogenesis. The growth factor receptors or integrins recruit adaptor proteins and nuclear exchange factors that convert Ras to a GTP-bound form, leading to recruitment of c-Raf to the cytoplasmic membrane, where it is activated. More specifically, FGF2-induced phosphorylation of c-Raf on serine 338 by p21-activated kinase (PAK) leads to c-Raf translocation to the mitochondria and endothelial cell survival in the presence of apoptotic stress, while VEGF-induced Src kinase-mediated phosphorylation of c-Raf on tyrosines 340/341 leads to endothelial cell survival in response to receptor-mediated apoptosis (Alavi et al. 2003).

However, although inhibitors of αvβ5 do not affect FGF2-induced angiogenesis, inhibitors of αvβ3 can inhibit up to 50% of VEGF-induced angiogenic activity, which is consistent with the observation, in vitro, that VEGF can promote αvβ3- and αvβ5- and β1-mediated endothelial cell adhesion and migration (Byzova et al. 2000b; Senger et al. 1997). The integrin α5β1 and its ligand fibronectin have been shown to be overexpressed on the blood vessels of human and mouse tumors (Kim et al. 2000a), while integrin-linked kinase (ILK), which is associated with the integrin β1, has also been shown to have a specific role in integrin–ECM interactions and endothelial cell survival (Friedrich et al. 2004), as has the integrin heterodimer α4β1 (Garmy-Susini et al. 2005). In turn, the integrins α1β1 and α2β1 have been shown in model systems in vitro and in vivo to be involved in VEGF-induced signaling, cell migration and tumor angiogenesis (Senger et al. 2002). Thus, angiogenesis is regulated by signals derived from both growth factor receptors and ECM molecules and there is considerable evidence of cross-talk between integrins and growth factor receptors.

42.4 Integrin–Growth Factor Receptor and Integrin–Integrin Cross-Talk

The integrin αvβ3 co-immunoprecipitates with both VEGFR-2 and the PDGF receptor (PDGFR)

(Borges et al. 2000; Byzova et al. 2000a). Several recent studies suggest that homodimers and heterodimers of VEGFR-1 and VEGFR-2 can propagate distinct signals in cultured porcine and human endothelial cells (Huang et al. 2001; Kanno et al. 2000; Rahimi et al. 2000), and have shown VEGFR-2 activation to play a positive role in angiogenesis by promoting endothelial cell proliferation in vitro in porcine endothelial cells (Rahimi et al. 2000). Conversely, VEGFR-1 activation antagonizes the VEGFR-2 responses, inhibiting endothelial cell proliferation.

Although αv integrins clearly play a critical role in angiogenesis, recent studies of integrin–growth factor receptor cross-talk provide an insight into the roles of other integrins, and their receptors and ligands, in the process. As stated previously, α1β1, α2β1 integrins are involved in VEGF signaling. Antagonists of α1β1 and α2β1 integrins are known to block VEGF-induced angiogenesis (Senger et al. 1997), while antagonists of αvβ3 or α5β1 block FGF2- but not VEGF-induced angiogenesis, suggesting that the integrins αvβ3 and α5β1 regulate similar angiogenesis pathways (Kim et al. 2000b). However, in an example of integrin–integrin cross-talk, α5β1 ligation potentiates αvβ3-mediated migration but not αvβ3-mediated cell adhesion (Kim et al. 2000b). There is also evidence of β3 and α5β1 integrin ligation during cell adhesion (Eliceiri and Cheresh 2001), while recently β1 integrins have been shown to promote random migration, whereas β3 integrins have been shown to promote persistent migration in the same epithelial cell background (Danen et al. 2005).

However, although integrins and growth factor receptors promote coordinated signaling activities, the mechanisms appear to be manifold (Fig. 42.1), and are as yet unclear. One can propose that pro-angiogenic growth factors stimulate the expression of the αv and the other integrins involved in angiogenesis, causing cells to invade the surrounding ECM and proliferate. When the ligation of these pro-angiogenic integrins is blocked by integrin antagonists, the proliferating vascular endothelial cells undergo apoptosis, accompanied by an increase in p53 activity.

Integrins as Targets for Cancer Therapy

The integrins, as outlined above, are involved in a plethora of interactions with growth factors, growth factor receptors and other molecules such as the matrix metalloproteinases MMP2 and MMP9 (Boger et al. 2001; Hamano et al. 2003; Kessler et al. 2002; Silletti et al. 2001), responsible for the regulation of key angiogenic signaling pathways (Fig. 42.1). Modulation of the balance between pro- and anti-angiogenic factors holds great promise in the treatment of cancer, and antibody and small-peptide inhibitors of the αv integrins have been shown to inhibit endothelial cell adhesion and migration, induce endothelial cell apoptosis and in some cases induce tumor regression in preclinical models (Brooks et al. 1994b, 1995; Carron et al. 1998; Kerr et al. 2000, 2002; MacDonald et al. 2001; Stromblad and Cheresh 1996).

In addition, anti-angiogenic therapy offers several potential advantages over standard cytotoxic chemotherapy as an approach to cancer treatment, due in large part to the relative genetic stability of the target endothelial cells compared with the tumor cells. Also, because most of the adult vasculature is relatively quiescent compared to tumor vasculature, the potential for adverse events with anti-angiogenic therapy is significantly reduced. Finally, the very specific, targeted nature of anti-angiogenic therapy means that there is no mechanistic overlap with the standard therapeutic approaches used for cancer. Consequently, anti-angiogenic strategies may be very effective when used in combination with cytotoxic chemotherapy, radiotherapy, hormonal therapy and even immunotherapy. In addition, high levels of integrin αvβ3 expression are associated with several invasive tumor types such as glioma, prostate, melanoma and breast cancer (Brooks et al. 1994a; Hood and Cheresh 2002; Seftor 1998). Thus, any agents that can block the integrins, on activated tumor endothelial cells or on integrin-expressing tumor cells, are candidate therapeutic agents.

Inhibitors of Integrin-mediated Angiogenesis as Potential Therapeutic Agents

In tumor-initiated angiogenesis, one of the first steps of tumor development is usually the secretion by the tumor of pro-angiogenic factors, such as FGF2 and VEGF, the pro-angiogenic effects of which are, to a large degree, αv integrin-dependent.

42.6.1 Importance of αv Integrins in Angiogenesis: Preclinical Studies of Anti-angiogenic Agents

Some of the agents used to define some of the pathways of angiogenesis outlined above have themselves the potential to become anti-tumor agents, as evidenced by the data from model systems (Table 42.2). The chick CAM model is classically used to study the stimulation and inhibition of angiogenesis. In the CAM system an anti-αvβ3 monoclonal antibody (MAb), LM609, blocked angiogenesis induced by FGF2, tumor necrosis factor-α, and human melanoma fragments, but had no effect on pre-existing vessels (Table 42.2). In support of this observation, Brooks et al., using the same LM609 MAb, showed αvβ3 integrin to be expressed on blood vessels in human wound granulation tissue, but not in normal skin (Brooks et al. 1994a). αvβ3 integrin expression increased fourfold during FGF2-induced angiogenesis in the chick CAM system. However, anti-αvβ5 and anti-β1 antibodies failed to block FGF2-induced angiogenesis in the same system (Brooks et al. 1994a, 1994b), suggesting that these integrins are not induced by FGF2.

The above findings were supported by Friedlander et al. (1995), who showed the anti-αvβ3 MAb LM609 and an anti-αvβ5 MAb (P1F6) to block FGF2- and VEGF-induced ocular angiogenesis in the rabbit, respectively (Table 42.2). To confirm these results, the experiment was repeated in the chick CAM system using FGF2, VEGF and the additional pro-angiogenic agents TNF-α, transforming growth factor-α (TGF-α) and the tumor promoter phorbol myristate acetate, and showed that angiogenesis induced by FGF2 or TNF-α was dependent on αvβ3 integrin, whilst angiogenesis induced by VEGF, TGF-α or a phorbol ester tumor promoter was dependent on the integrin αvβ5. A cyclic peptide (RGDfV/EMD 66203) specific for both integrins, as predicted, blocked both pathways and inhibited angiogenesis induced by both FGF2 and VEGF. Furthermore, Brooks et al. (1994b) had previously shown the same cyclic peptide (RGDfV/EMD 66203) to block angiogenesis, which led to the regression of human tumors transplanted on to the chick CAM, as a consequence of the selective peptide-induced apoptosis of the proliferating endothelial cells. The pre-existing quiescent vessels were unaffected. The cyclic peptide RGDfV/EMD 66203 was also shown to inhibit retinal neovasculature in a mouse model of hypoxia-induced neovascularization (Hammes et al. 1996) These studies were the first to show the potential of integrins as therapeutic targets and integrin antagonists as potential therapeutic agents. In addition, another dual αvβ3 and αvβ5 integrin antagonist, SCH22153, has been shown to inhibit FGF2-induced angiogenesis and the tumor growth of melanoma cells injected into severe combined immunodeficient (SCID) mice (Kumar et al. 2001).

However, the first evidence of the potential of integrins as therapeutic agents in a 'human tumor situation' was provided by a SCID mouse-human model system with transplanted full-thickness human skin containing implanted human breast tumor cells (MCF cell line) (Brooks et al. 1995). The growing breast tumor cells induced a human angiogenic response as a consequence of the fact that they were in a human endothelial cell environment, confirmed by immunohistochemical (IHC) analysis using the LM609 MAb that targets human αvβ3 integrin. In turn, the intravenous administration of the LM609 antibody either prevented tumor growth or markedly reduced tumor cell proliferation. IHC analysis also showed that the LM609-treated tumors had a lower microvessel density and were less invasive than the tumors in the corresponding untreated control animals (Brooks et al. 1995). Using a panel of six human

Table 42.2. Summary of integrin antagonist activity in preclinical model systems

Angiogenesis/tumor model systems	Integrin antagonist	Integrin target	Reference(s)
Chick CAM	LM609 MAb	αvβ3	Brooks et al., 1994a, 1994b
Chick CAM	LM609 MAb Cyclic peptide RGDfV/EMD66203 P1F6	αvβ3 αvβ3 αvβ5	Friedlander et al. 1995
Chick CAM	Cyclic peptide RGDfV/EMD66203	αvβ3	Brooks et al. 1994b
Ocular angiogenesis	LM609 MAb	αvβ3	Friedlander et al. 1995
Mouse retina	Cyclic peptide RGDfV/EMD66203	αvβ3	Hammes et al. 1996
Rat aortic ring	CNTO 95	αv integrins	Trikha et al. 2004
SCID mouse human breast cancer Nude mouse	LM609 MAb	αvβ3	Brooks et al. 1995
Melanoma and solid tumor cell lines	17E6 MAb LM609 MAb EMD121974 (cilengitide)	αvβ3 αvβ3 αvβ3, αvβ5	Mitjans et al. 2000
Glioblastoma, medulloblastoma	EMD121974 (cilengitide)	αvβ3, αvβ5	MacDonald et al. 2001; Taga et al. 2002
Melanoma	CNTO 95	αv integrins	Trikha et al. 2004
Epidermoid, glioma, prostate	S247 plus RT	αvβ3	Abdollahi et al. 2005
Breast cancer	EMD121974 (cilengitide) plus RIT	αvβ3, αvβ5	Burke et al. 2002

melanoma cell lines (Table 42.3), the human pan-αv-blocking MAb 17E6, which recognizes only human αv integrins, blocked the in vivo growth of tumors derived from xenografts of human melanoma cells expressing the αvβ3 integrin in nude mice, but not that of αvβ3-negative tumor cells, in a dose-dependent manner (Mitjans et al. 2000). The cyclic RGD pentapeptide EMD 121974 (cilengitide) was also inhibitory for the most metastatic melanoma cell line, WM164 (Mitjans et al. 2000). The LM609 MAb, directed against both chick and human αvβ3 integrin, described in previous studies, was less effective than 17E6 in blocking tumor cell growth at identical doses. A control antibody 1D7 (anti-β3) was completely inactive, despite binding to the same receptor.

The pan-αv-blocking MAb 17E6 was also tested for its role in inhibiting the growth of a variety of αvβ3-negative human solid tumor cell lines with different patterns of αv integrin expression (Table 42.4). 17E6 failed to block the growth of tumors derived from the αvβ3-negative HT29 colon, NP18 pancreatic and MDA-MB-231, ZR75 and MCF-7 breast cancer cell lines in a mouse model system. These results clearly suggested that αvβ3 is the only αv integrin that is essential for the development of human malignant melanomas and that the other integrins on melanomas play only a secondary role, confirming the potential therapeutic value of inhibitors or antagonists of αvβ3 integrin in the treatment of malignant melanoma. Furthermore, since 17E6 does not recognize murine αvβ3

Table 42.3. Human melanoma cell lines used to assess the potential of anti-αv integrin therapy

Cell line	αv Integrin expression	Reference
WM164	αvβ3	Mitjans et al. 2000
M21	αvβ3	Cheresh and Klier 1986
M21-L	αvβ3 negative	Cheresh and Klier 1986
SKMel28	αvβ3	Mitjans et al. 2000
A375	αvβ3	Mitjans et al. 2000
KS-IMM	αvβ3	Albini et al. 1997

integrin the inhibitory effects on melanoma tumor growth were due to the direct anti-tumor activity of the integrin inhibitor and not the anti-angiogenic effects of inhibition of αvβ3 integrin. Thus, in the human clinical situation one might predict that the 17E6 and RGD peptide anti-tumor activities might be increased still further, with angiogenic human endothelial cells also a target (Mitjans et al. 2000).

In addition to the evidence of inhibition of melanoma cells described above, in a separate study cilengitide, which targets both human αvβ3 and αvβ5 integrin, was shown to induce apoptosis in human brain medulloblastoma and glioblastoma cell lines and suppress the growth of brain tumors following transplantation into nude mice (MacDonald et al. 2001; Taga et al. 2002). CNTO 95, the first fully human antibody to human αv integrins, has been shown to inhibit human melanoma cell adhesion, migration and invasion in vitro, to completely inhibit angiogenesis in a rat aortic ring angiogenesis assay, and to inhibit human melanoma xenograft tumors in nude mice (Trikha et al. 2004). The endogenous angiogenesis inhibitor tumstatin, the NC1 domain of the α3 chain of type IV collagen, has also been shown to mediate its anti-angiogenic and tumor-inhibitory properties via αvβ3 integrin in a variety of model systems (Hamano and Kalluri 2005; Sudhakar et al. 2003; Sund et al. 2005).

42.6.1.1 αv-Integrin-Targeting Agents in Combination with Other Treatment Modalities

Cilengitide has been shown to synergize with radioimmunotherapy (RIT) to increase the anti-tumor efficacy above that observed with either agent alone in a human breast cancer (HBT 3477) tumor model in nude mice (Burke et al. 2002). Interestingly the αvβ3 integrin antagonist alone did not alter tumor growth when compared with untreated mice, but when administered in combination with yttrium-90-labeled DOTA-peptide ChL6 MAb resulted in significantly more cures (44% vs 22%) than for RIT alone (p=0.02). Cyclic peptide in combination with RIT resulted not only in a significantly increased tumor uptake of the radiolabeled antibody but also in increased tumor and endothelial cell apoptosis concomitant with reduced tumor cell proliferation (Burke et al. 2002). Furthermore, this therapeutic advantage was achieved in the absence of an increase in RIT-induced toxicity, which indicates the true potential of such integrin-targeted therapy.

These observations were consistent with those reported for cilengitide in mouse tumor model systems, established by subcutaneous injection of colon cancer, melanoma and neuroblastoma cell lines, where regression of the implanted tumors was seen only when the integrin antagonist was combined with an antibody–cytokine fusion protein (ch14.18-IL2 or huKS1/4-IL-2) (Lode et al. 1999). Each therapeutic strategy when applied alone achieved at best a growth delay. Mice treated with the integrin antagonist cilengitide showed a 50% reduction in microvessel density in the tumor. Also, in addition to inhibition of the implanted tumor, sequential administration of the two agents induced a 1.5- to 2-log decrease in hepatic metastases compared to control mice in a murine neuroblastoma model system, again providing evidence of strong synergy between anti-angiogenic treatment strategies and immunotherapy (Lode et al. 1999). Thus, taken together, these preclinical data clearly suggest that αv integrin antagonists can be favorably combined with standard and other therapies for the treatment of localized tumors.

Table 42.4. Human tumor cell lines used to assess the potential of anti-αv integrin therapy

Cell line	Tumor origin	αv Integrin expression	Reference
HT29	Colon	αvβ3 negative αvβ5 positive αvβ6 positive	Kraft et al. 1999
NP18	Pancreas	αvβ3 negative αvβ5 positive αvβ6 negative	Villanueva et al. 1998
MDA-MB-231	Breast	αvβ3 low level expression αvβ5 positive	Meyer et al. 1998
ZR 75	Breast	αvβ3 negative αvβ5 positive	Meyer et al. 1998
MCF-7	Breast	αvβ3 negative αvβ5 positive	Meyer et al. 1998

The concurrent administration of the small-molecule, RGD-based, synthetic peptidomimetic αvβ3 integrin antagonist S247 (Harms et al. 2004; Shannon et al. 2004) and radiotherapy has been shown to enhance the anti-angiogenic and anti-tumor effects of radiotherapy in human prostate, glioma and epidermoid tumor xenografts in nude mice (Abdollahi et al. 2005). In vitro studies in human vascular endothelial cells (HUVECS) and human microvascular endothelial cells (HDMECs) showed that radiation increased αvβ3 integrin expression in a dose-dependent manner and S247 significantly inhibited HUVEC cell adhesion to vitronectin. In separate assays S247 was shown to inhibit HUVEC cell proliferation and migration and these anti-proliferative and anti-migratory effects were shown to be significantly enhanced by radiation. In vivo, in nude mice, S247 monotherapy and radiation monotherapy induced significant delays in tumor growth. However, the administration of radiation with concurrent S247 resulted in a significantly greater delay in tumor growth and was accompanied by a decrease in tumor vessel growth (Abdollahi et al. 2005).

One of the mechanisms proposed was that radiation induces an upregulation of αvβ3 integrin expression in endothelial cells, leading to the phosphorylation of Akt, which may provide the tumor with an escape mechanism from radiation-induced apoptosis. In the presence of the integrin antagonist S247, Akt phosphorylation is inhibited and apoptosis is no longer blocked (Fig. 42.1).

42.6.2 Importance of Integrin α5β1 in Angiogenesis: Preclinical Studies of Anti-Angiogenic Agents

Although αv integrins clearly have a critical role to play in angiogenesis, it seems that other integrin receptors and their ECM ligands may also regulate the angiogenic process. There is evidence that the integrin α5β1 and its ligand fibronectin are upregulated on blood vessels in human colon and breast tumor biopsies (Kim et al. 2000b). Angiogenesis induced by FGF2, TNF-α and IL-8, but not VEGF, was blocked by a variety of antibodies to the cellular binding domain of fibronectin (Kim et al. 2000b). Furthermore, addition of fibronectin or a proteolytic fragment of fibronectin to a chick CAM system enhanced angiogenesis in an α5β1-dependent fashion. Antibody, peptide and novel non-peptide antagonists of α5β1 blocked angiogenesis induced by FGF2, but not VEGF, in a human model of angiogenesis in SCID mice. Integrin α5β1 was also shown to be essential for tumor growth and tumor angiogenesis in the CAM assay. Taken together, these results

demonstrate a potential role for antagonists of α5β1 integrin in the inhibition of tumor growth and angiogenesis.

Collectively, the data presented in this review chapter support the potential of integrin antagonists as therapeutic agents (Table 42.5), and several are now in clinical development.

Integrin Antagonists in Clinical Development

The platelet glycoprotein GPIIb/IIIa (αIIbβ3) antagonist C7E3 (abciximab, Reo Pro) was the first integrin antagonist approved for use in the clinic and was designed to reduce the risk of ischemic complications following vascular surgery (Coller 1997). Significantly, it provides "proof of principle" for the development of other integrin antagonists for use in other clinical indications.

Among the specific mechanism-oriented targets proposed for anti-angiogenic therapy, the αvβ3 integrin and the VEGFs stand out. The VEGFs appear to be the main endothelial growth factors with high specificity for endothelial cells (Claffey and Robinson 1996), and αvβ3 integrin is the only integrin known to be expressed and upregulated with high selectivity on proliferating endothelial cells. Also, αvβ3 integrin is expressed on few other cell types except for tumor cells, smooth muscle cells and osteoclasts (Brooks et al. 1994a; Max et al. 1997). Considering the degree of redundancy of the endothelial growth factors postulated to participate in the initiation of tumor angiogenesis (Harris 1997), the αv integrins, particularly αvβ3 integrin, may represent even more attractive therapeutic targets than VEGFs, because endothelial cells critically depend on these integrins irrespective of the specific growth factors used to initiate the angiogenic process. Based on these findings, different integrin antagonists are in clinical development.

42.7.1 Cilengitide

Cilengitide, a salt of the homodetic, cyclic, RGD, low-molecular-mass pentapeptide cyclo-[Arg-Gly-Asp-DPhe-(NMeVal) (Merck KGaA, Darmstadt, Germany) is already being investigated in the clinic in several different oncology indications, e.g. recurrent glioma, pancreatic cancer, prostate cancer, malignant melanoma, and others. In clinical trials cilengitide is administered as a 1-h intravenous infusion. It has an IC_{50} for the inhibition of αvβ3 integrin receptor binding to vitronectin of 2 nM and an IC_{50} for αvβ5 integrin receptor binding of 120 nM.

In a phase I study in 37 patients with histologically or cytologically confirmed solid tumors refractory to standard treatment or for which no standard therapy was available, cilengitide was administered at a starting dose of 30 mg/m^2 (Eskens et al. 2003). Subsequent dose escalation was based on the pharmacokinetic data and the toxicities encountered. The dose levels studied were between 30 mg/m^2 and 1600 mg/m^2 in groups of three to six patients. Non-hematological toxicities were mild and never exceeded grade 2. Pharmacodynamic analysis showed there to be a tendency towards a correlation between levels of VEGF, sFLT-1, sTIE-2 and clinical outcome. Prolonged disease stabilization was seen in two patients with renal cell carcinoma (164 days for each patient) and one patient with colorectal carcinoma (168 days) (Eskens et al. 2003). These data demonstrated quite clearly that the αvβ3 and αvβ5 integrin antagonist cilengitide could be administered safely as palliative therapy to a range of cancer patients, with only mild non-hematological toxicity.

Subsequently, phase I trials and a phase II trial were conducted in patients with malignant gliomas and pancreatic cancer, respectively. A phase I trial in 52 assessable patients with recurrent gliomas at dose levels of between 120 mg/m^2 and 2400 mg/m^2, and six to seven patients per group, showed cilengitide to demonstrate minimal toxicity. Two patients demonstrated complete response (CR), three patients,

Table 42.5. Angiogenesis inhibitors that target integrins

Inhibitor	Class of agent	Integrin target	Reference
Arresten	Natural inhibitor of angiogenesis	α1β1, α2β1	Colorado et al. 2000
Canstatin	Natural matrix-derived inhibitor of angiogenesis	αvβ3, αvβ5	Kamphaus et al. 2000
RGDfV	Cyclic peptide	αvβ5	Friedlander et al. 1995
Cilengitide (EMD121974)	Cyclic RGD pentapeptide	αvβ3, αvβ5	Eskens et al. 2003; Raguse et al. 2004; Smith 2003[a]
Endostatin (Endostar [rh-endostatin, YH-16])	Natural inhibitor of angiogenesis (Recombinant endostatin)	α5β1	Sudhakar et al. 2003; Sun et al. 2005; Wickstrom et al. 2002[a]
Tumstatin	Natural inhibitor of angiogenesis fragment of type IV collagen	αvβ3	Maeshima et al. 2000, 2002
LM609	IgG1 MAb	Vitronectin/αvβ3 complex	Cheresh 1987
Vitaxin	Humanized LM609 monoclonal antibody	αvβ3	Gutheil et al. 2000a
Abciximab (ReoPro)	MAb	GP IIb/IIIa, αvβ3	EPILOG 1997
CNT095	Human pan-αv integrin MAb	αv integrins	Jayson et al. 2004[a]
SB-267268	non-peptidic antagonist	αvβ3, αvβ5	Wilkinson-Berka et al. 2006
SCH 221153	Peptidomimetic	αvβ3, αvβ5	Kumar et al. 2001
SU015	Tetraaza cyclic peptide	αvβ3, αvβ5	Mousa 2005
TA138/RP747		αvβ3	Mousa et al. 2005
S247	RGD-based peptidomimetic	αvβ3	Reinmuth et al. 2003
17E6	Human pan-αv integrin MAb	Pan-αv	Mitjans et al. 2000
Volciximab	Human α5β1 integrin MAb	α5β1	http://www.pdl.com/applications/pipeline.cfm?navId=54

[a]Clinical studies

partial response (PR) and four patients, stable disease (SD) (Nabors et al. 2004). Correlative biology analyses included perfusion MRI and plasma analysis for angiogenic factors. A statistically significant relationship was observed between tumor cerebral blood flow (CBF) and clinical response of any kind (Akella et al. 2004; Nabors et al. 2004). These data were considered to be encouraging in an aggressive tumor type for which there are few treatment options and led to a randomized phase II trial of cilengitide in patients with recurrent glioblastoma multiforme following failure of first-line therapy. In addition to this study the National Cancer Institute (NCI) in the US is sponsoring a number of trials, including a phase II trial of cilengitide in combination with rituximab and a phase I/II trial of cilengitide in combination with radiation therapy and temozolamide in patients with newly diagnosed glioblastoma multiforme. A phase II trial is also under way in patients with recurrent glioblastoma multiforme undergoing surgery for recurrent or progressive disease (www.clinicaltrials.gov/).

Meanwhile, a randomized phase II trial of cilengitide plus gemcitabine versus gemcitabine alone in 89 patients with advanced unresectable pancreatic cancer showed the combination of cilengitide and gemcitabine to be well tolerated with no adverse effects on the safety, tolerability and pharmacokinetics of either agent. Cilengitide was administered at 600 mg/m^2 twice weekly for 4 weeks and gemcitabine at 1000 mg/m^2 for 3 weeks followed by a week of rest per cycle. The planned treatment period was six 4-week cycles. The median overall survival was comparable between the treatment groups: 6.7 months for cilengitide and gemcitabine and 7.7 months for gemcitabine alone. The median progression-free survival times were 3.6 months and 3.8 months, respectively. The overall response rates were 17% and 14%, and the tumor growth control rates were 54% and 56%, respectively. There were therefore no clinically important differences regarding efficacy, safety and quality of life between patients receiving cilengitide and gemcitabine or gemcitabine alone.

However, the combination may have value for the treatment of other disease sites, and cilengitide (600 mg/m^2) in combination with gemcitabine administered on days 1 and 8 every 3 weeks for 5 months was shown to arrest the growth of a heavily pretreated squamous cell carcinoma of the head and neck, at fourth relapse, which had its origin in the upper left jaw (Raguse et al. 2004). The patient remained stable for 12 months on cilengitide maintenance therapy, demonstrating the clinical efficacy of cilengitide in a highly vascularized tumor. Other ongoing trials for cilengitide include two trials in patients with prostate cancer and one trial in patients with advanced solid tumors or lymphoma (www.clinicaltrials.gov/).

42.7.2 Vitaxin

A phase I study has evaluated the safety and pharmacokinetics of escalating doses (0.1–4.0 mg/kg/week) of 6-weekly infusions of vitaxin (Applied Molecular Evolution, San Diego, California), the humanized version of the LM609 anti-$\alpha v\beta 3$ antibody used in the early mechanistic studies, in cancer patients with stage IV solid tumors and an ECOG performance status 2 (Gutheil et al. 2000). Of 17 patients treated, 14 were evaluable for response. Doses of 1.0 mg/kg/week produced plasma concentrations which were sufficient to saturate the $\alpha v\beta 3$ receptor in preclinical studies. The half-life of vitaxin was in excess of 5 days with no accumulation over 6 weeks. One patient demonstrated a PR and seven patients achieved SD. One patient, who received vitaxin beyond the first cycle, achieved disease stabilization in excess of 22 months, demonstrating that $\alpha v\beta 3$ is a clinically relevant target. Vitaxin has also been studied in patients with refractory advanced solid tumors or lymphoma (www.clinicaltrials.gov/).

42.7.3 CNTO 95

An ongoing phase I trial is investigating the fully human pan-αv-integrin MAb CNTO 95 (Centocor Research and Development Inc) in 95 patients with solid tumors refractory to standard therapy (Jayson

et al. 2004). Patients receive CNTO 95 on days 0, 28, 35 and 42. An initial report on 13 patients, with a median number of two prior systemic treatment regimens, treated with 0.1 mg/kg (7 patients), 0.3 mg/kg (3 patients) and 1.0 mg/kg (3 patients) recorded no CNTO 95-related serious adverse events. Three ovarian cancer patients achieved stable disease (two patients at 0.3 mg/kg and one patient at 1.0 mg/kg) at day 42, and remain on extended CNTO 95 dosing. Dose escalation to a planned dose of 10.0 mg/kg continues. Again, these preliminary results are encouraging and provide further evidence of the relevance of αv integrins as therapeutic targets. CNTO 95 is also being investigated in patients with advanced melanoma in a phase I/II trial (www.clinicaltrials.gov/).

42.7.4 Endostatin

A phase I trial investigated the safety and pharmacokinetics of escalating doses (15, 30, 60, 120, 180, 300 and 600 mg/m^2/day) of recombinant endostatin (rh-endostatin or Endostar), which mediates its effects via α5β1 integrin (Herbst et al. 2002). Twenty-six patients with advanced solid tumors for which no standard treatment was available received treatment with rh-endostatin for a maximum of two 28-day cycles. A dose of 300 mg/m^2/day achieved an AUC associated with activity in preclinical models. In two patients evidence of anti-tumor activity was seen but no responses were observed. This study confirmed the safety of rh-endostatin in the clinic but clearly suggested that more trials were needed.

In a phase II trial (Kulke et al. 2003), 37 evaluable patients with advanced solid tumors were treated with a dose of 60 mg/m^2/day for 8 weeks. Twenty-one patients were escalated to a dose of 90 mg/m2/day. Overall, 41 patients received a median of 24 (range 1–52) weeks of therapy and 37 patients were evaluable for response. Two patients achieved minor radiological responses and 23 patients (62%) had SD as their best response. Again, this trial suggested that rh-endostatin may have activity.

More recently a placebo-controlled, phase III study in 486 assessable patients with advanced (stage IIIB and IV) non-small cell lung cancer showed the combination of rh-endostatin plus vinorelbine and cisplatin to achieve a response rate of 35.4%, compared with 19.5% for vinorelbine and cisplatin alone. The median times to progression were 6.3 months and 3.6 months, respectively. The clinical benefit rate was also greater for the rh-endostatin-containing treatment arm (73.3% versus 64.0%). In this study rh-endostatin conferred a significant and clinically meaningful improvement in response rate coupled with a favorable toxicity profile (Sun et al. 2005). A phase I study of an intratumoral injection of adenoviral vector-delivered human endostatin is under way (www.clinicaltrials.gov/).

Conclusion

Integrin-mediated cell adhesion is known to promote cell survival and integrin-dependent cell growth, while integrin antagonists induce apoptosis of adherent endothelial cells. For example, as described above de novo expression of αvβ3 integrin has been shown to occur in response to proangiogenic growth factors (Brooks et al. 1994b), facilitating increased endothelial cell interaction with the provisional ECM, preventing apoptosis and promoting cell growth. Integrin antagonists of αvβ3, on the other hand, initiate endothelial cell apoptosis, suppress angiogenesis and inhibit tumor growth. This and the evidence provided in this chapter in numerous model systems of the specific anti-angiogenic and anti-tumorigenic effects of a variety of integrin antagonists have led to their investigation in clinical trials as anti-angiogenic agents.

References

Abdollahi A, Griggs DW, Zieher H et al (2005) Inhibition of alpha(v)beta3 integrin survival signaling enhances antiangiogenic and antitumor effects of radiotherapy. Clin Cancer Res 11:6270–6279

Akella NS, Twieg DB, Mikkelsen T et al (2004) Assessment of brain tumor angiogenesis inhibitors using perfusion magnetic resonance imaging:quality analysis results of a phase I trial. J Magn Reson Imaging 20:913–22

Alavi A, Hood JD, Frausto R et al (2003) Role of Raf in vascular protection from distinct apoptotic stimuli. Science 301:94–96

Albini A, Paglieri I, Orengo G et al (1997) The beta-core fragment of human chorionic gonadotrophin inhibits growth of Kaposi's sarcoma-derived cells and a new immortalized Kaposi's sarcoma cell line. Aids 11:713–721

Boger DL, Goldberg J, Silletti S et al (2001) Identification of a novel class of small-molecule antiangiogenic agents through the screening of combinatorial libraries which function by inhibiting the binding and localization of proteinase MMP2 to integrin alpha(V)beta(3). J Am Chem Soc 123:1280–1288

Borges E, Jan Y, Ruoslahti E (2000) Platelet-derived growth factor receptor beta and vascular endothelial growth factor receptor 2 bind to the beta 3 integrin through its extracellular domain. J Biol Chem 275:39867–39873

Brakebusch C, Bouvard D, Stanchi F et al (2002) Integrins in invasive growth. J Clin Invest 109:999–1006

Brooks PC, Clark RA, Cheresh DA (1994a) Requirement of vascular integrin alpha v beta 3 for angiogenesis. Science 264:569–571

Brooks PC, Montgomery AM, Rosenfeld M et al (1994b) Integrin alpha v beta 3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. Cell 79:1157–1164

Brooks PC, Stromblad S, Klemke R et al (1995) Antiintegrin alpha v beta 3 blocks human breast cancer growth and angiogenesis in human skin. J Clin Invest 96:1815–1822

Buerkle MA, Pahernik SA, Sutter A et al (2002) Inhibition of the alpha-nu integrins with a cyclic RGD peptide impairs angiogenesis, growth and metastasis of solid tumours in vivo. Br J Cancer 86:788–795

Burke PA, DeNardo SJ, Miers LA et al (2002) Cilengitide targeting of alpha(v)beta(3) integrin receptor synergizes with radioimmunotherapy to increase efficacy and apoptosis in breast cancer xenografts. Cancer Res 62:4263–4272

Byzova TV, Goldman CK, Pampori N et al (2000a) A mechanism for modulation of cellular responses to VEGF: activation of the integrins. Mol Cell 6:851–860

Byzova TV, Kim W, Midura RJ et al (2000b) Activation of integrin alpha(V)beta(3) regulates cell adhesion and migration to bone sialoprotein. Exp Cell Res 254:299–308

Carmeliet P (2003) Angiogenesis in health and disease. Nat Med 9:653–660

Carron CP, Meyer DM, Pegg JA et al (1998) A peptidomimetic antagonist of the integrin alpha(v)beta3 inhibits Leydig cell tumor growth and the development of hypercalcemia of malignancy. Cancer Res 58:1930–1935

Cheresh DA (1987) Human endothelial cells synthesize and express an Arg-Gly-Asp-directed adhesion receptor involved in attachment to fibrinogen and von Willebrand factor. Proc Natl Acad Sci USA 84:6471–6475

Cheresh DA, Klier FG (1986) Disialoganglioside GD2 distributes preferentially into substrate-associated microprocesses on human melanoma cells during their attachment to fibronectin. J Cell Biol 102:1887–1897

Claffey KP, Robinson GS (1996) Regulation of VEGF/VPF expression in tumor cells: consequences for tumor growth and metastasis. Cancer Metastasis Rev 15:165–176

Coller BS (1997) Platelet GPIIb/IIIa antagonists: the first anti-integrin receptor therapeutics. J Clin Invest 100: S57–S60

Colorado PC, Torre A, Kamphaus G et al (2000) Anti-angiogenic cues from vascular basement membrane collagen. Cancer Res 60:2520–2526

Danen EH, van Rheenen J, Franken W et al (2005) Integrins control motile strategy through a Rho-cofilin pathway. J Cell Biol 169:515–526

De S, Razorenova O, McCabe NP et al (2005) VEGF-integrin interplay controls tumor growth and vascularization. Proc Natl Acad Sci USA 102:7589–7594

Eliceiri BP, Cheresh DA (1999) The role of alphav integrins during angiogenesis: insights into potential mechanisms of action and clinical development. J Clin Invest 103:1227–1230

Eliceiri BP, Cheresh DA (2000) Role of alpha v integrins during angiogenesis. Cancer J 6 [Suppl 3]:S245–S249

Eliceiri BP, Cheresh DA (2001) Adhesion events in angiogenesis. Curr Opin Cell Biol 13:563–568

Eliceiri BP, Paul R, Schwartzberg PL et al (1999) Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability. Mol Cell 4:915–924

EPILOG (1997) Platelet glycoprotein IIb/IIIa receptor blockade and low-dose heparin during percutaneous coronary revascularization. The EPILOG Investigators. N Engl J Med 336:1689–1696

Eskens FA, Dumez H, Hoekstra R et al (2003) Phase I and pharmacokinetic study of continuous twice weekly intravenous administration of Cilengitide (EMD 121974), a novel inhibitor of the integrins alphavbeta3 and alphavbeta5 in patients with advanced solid tumours. Eur J Cancer 39:917–926

Folkman J (1992) The role of angiogenesis in tumor growth. Semin Cancer Biol 3:65–71

Folkman J, Hanahan D (1991) Switch to the angiogenic phenotype during tumorigenesis. Princess Takamatsu Symp 22:339–347

Friedlander M, Brooks PC, Shaffer RW et al (1995) Definition of two angiogenic pathways by distinct alpha v integrins. Science 270:1500–1502

Friedlander M, Theesfeld CL, Sugita M et al (1996) Involvement of integrins alpha v beta 3 and alpha v beta 5 in ocular neovascular diseases. Proc Natl Acad Sci USA 93:9764–9769

Friedrich EB, Liu E, Sinha S et al (2004) Integrin-linked kinase regulates endothelial cell survival and vascular development. Mol Cell Biol 24:8134–8144

Garmy-Susini B, Jin H, Zhu Y et al (2005) Integrin alpha4beta1-VCAM-1-mediated adhesion between endothelial and mural cells is required for blood vessel maturation. J Clin Invest 115:1542–1551

Giancotti FG, Ruoslahti E (1999) Integrin signaling. Science 285:1028–1032

Good DJ, Polverini PJ, Rastinejad F et al (1990) A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. Proc Natl Acad Sci USA 87:6624–6628

Gutheil JC, Campbell TN, Pierce PR et al (2000) Targeted antiangiogenic therapy for cancer using Vitaxin: a humanized monoclonal antibody to the integrin alphavbeta3. Clin Cancer Res 6:3056–3061

Hamano Y, Kalluri R (2005) Tumstatin, the NC1 domain of alpha3 chain of type IV collagen, is an endogenous inhibitor of pathological angiogenesis and suppresses tumor growth. Biochem Biophys Res Commun 333:292–298

Hamano Y, Zeisberg M, Sugimoto H et al (2003) Physiological levels of tumstatin, a fragment of collagen IV alpha3 chain, are generated by MMP-9 proteolysis and suppress angiogenesis via alphaV beta3 integrin. Cancer Cell 3:589–601

Hammes HP, Brownlee M, Jonczyk A et al (1996) Subcutaneous injection of a cyclic peptide antagonist of vitronectin receptor-type integrins inhibits retinal neovascularization. Nat Med 2:529–533

Hanahan D, Folkman J (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 86:353–364

Harms JF, Welch DR, Samant RS et al (2004) A small molecule antagonist of the alpha(v)beta3 integrin suppresses MDA-MB-435 skeletal metastasis. Clin Exp Metastasis 21:119–128

Harris AL (1997) Antiangiogenesis for cancer therapy. Lancet 349 [Suppl 2]:SII13–SII15

Herbst RS, Hess KR, Tran HT et al (2002) Phase I study of recombinant human endostatin in patients with advanced solid tumors. J Clin Oncol 20:3792–3803

Hong YK, Lange-Asschenfeldt B, Velasco P et al (2004) VEGF-A promotes tissue repair-associated lymphatic vessel formation via VEGFR-2 and the alpha1beta1 and alpha2beta1 integrins. FASEB J 18:1111–1113

Hood JD, Cheresh DA (2002) Role of integrins in cell invasion and migration. Nat Rev Cancer 2:91–100

Hood JD, Frausto R, Kiosses WB et al (2003) Differential alphav integrin-mediated Ras-ERK signaling during two pathways of angiogenesis. J Cell Biol 162:933–943

Huang K, Andersson C, Roomans GM et al (2001) Signaling properties of VEGF receptor-1 and -2 homo- and heterodimers. Int J Biochem Cell Biol 33:315–324

Hynes RO (2002) Integrins: bidirectional, allosteric signaling machines. Cell 110:673–687

Jayson GC, Mullamitha S, Ton C et al (2004) Phase I study of CNTO 95, a full human monoclonal antibody to av integrins, in patients with solid tumors. J Clin Oncol 22:3119

Kamphaus GD, Colorado PC, Panka DJ et al (2000) Canstatin anovel matrix-derived inhibitor of angiogenesis and tumor growth. J Biol Chem 275:1209–1215

Kanno S, Oda N, Abe M et al (2000) Roles of two VEGF receptors, Flt-1 and KDR, in the signal transduction of VEGF effects in human vascular endothelial cells. Oncogene 19:2138–2146

Kerbel R, Folkman J (2002) Clinical translation of angiogenesis inhibitors. Nat Rev Cancer 2:727–739

Kerr JS, Slee AM, Mousa SA (2000) Small molecule alpha(v) integrin antagonists: novel anticancer agents. Exp Opin Invest Drugs 9:1271–1279

Kerr JS, Slee AM, Mousa SA (2002) The alpha v integrin antagonists as novel anticancer agents: an update. Exp Opin Invest Drugs 11:1765–1774

Kessler TA, Pfeifer A, Silletti S et al (2002) Matrix metalloproteinase/integrin interactions as target for anti-angiogenic treatment strategies. Ann Hematol 81 [Suppl 2]:S69–S70

Kim S, Bell K, Mousa SA et al (2000a) Regulation of angiogenesis in vivo by ligation of integrin alpha5beta1 with the central cell-binding domain of fibronectin. Am J Pathol 156:1345–1362

Kim S, Harris M, Varner JA (2000b) Regulation of integrin alpha vbeta 3-mediated endothelial cell migration and angiogenesis by integrin alpha5beta1 and protein kinase A. J Biol Chem 275:33920–33928

Klemke RL, Yebra M, Bayna EM et al (1994) Receptor tyrosine kinase signaling required for integrin alpha v beta 5-directed cell motility but not adhesion on vitronectin. J Cell Biol 127:859–866

Kraft A, Weindel K, Ochs A et al (1999) Vascular endothelial growth factor in the sera and effusions of patients with malignant and nonmalignant disease. Cancer 85:178–187

Kulke M, Bergsland E, Ryan DP et al (2003) A phase II, open-label, safety, pharmacokinetic, and efficacy study of recombinant human endostatin in patients with advanced neuroendocrine tumors. Proc Am Soc Clin Oncol 22 abstr 958

Kumar CC, Armstrong L, Yin Z et al (2000) Targeting integrins alpha v beta 3 and alpha v beta 5 for blocking tumor-induced angiogenesis. Adv Exp Med Biol 476:169–180

Kumar CC, Malkowski M, Yin Z et al (2001) Inhibition of angiogenesis and tumor growth by SCH221153, a dual alpha(v)beta3 and alpha(v)beta5 integrin receptor antagonist. Cancer Res 61:2232–2238

Lode HN, Moehler T, Xiang R, Jonczyk A, Gillies SD, Cheresh DA, Reisfeld RA (1999) Synergy between an antiangiogenic integrin alphav antagonist and an antibody-cytokine fusion protein eradicates spontaneous tumor metastases. Proc Natl Acad Sci U S A 96:1591–1596

MacDonald TJ, Taga T, Shimada H et al (2001) Preferential susceptibility of brain tumors to the antiangiogenic effects of an alpha(v) integrin antagonist. Neurosurgery 48:151–157

Maeshima Y, Colorado PC, Kalluri R (2000) Two RGD-independent alpha vbeta 3 integrin binding sites on tumstatin regulate distinct anti-tumor properties. J Biol Chem 275:23745–2350

Maeshima Y, Sudhakar A, Lively JC et al (2002) Tumstatin, an endothelial cell-specific inhibitor of protein synthesis. Science 295:140–143

Marmé D (2003) The impact of anti-angiogenic agents on cancer therapy. J Cancer Res Clin Oncol 129:607–620

Max R, Gerritsen RR, Nooijen PT et al (1997) Immunohistochemical analysis of integrin alpha vbeta3 expression on tumor-associated vessels of human carcinomas. Int J Cancer 71:320–324

Maxwell PH, Dachs GU, Gleadle JM et al (1997) Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. Proc Natl Acad Sci USA 94:8104–8109

Meyer T, Marshall JF, Hart IR (1998) Expression of alphav integrins and vitronectin receptor identity in breast cancer cells. Br J Cancer 77:530–536

Mitjans F, Meyer T, Fittschen C et al (2000) In vivo therapy of malignant melanoma by means of antagonists of alphav integrins. Int J Cancer 87:716–723

Mousa SA (2005) Alpha v integrin affinity/specificity and antiangiogenesis effect of a novel tetraaza cyclic peptide derivative, SU015, in various species. J Cardiovasc Pharmacol 45:462–467

Mousa SA, Mohamed S, Wexler EJ et al (2005) Antiangiogenesis and anticancer efficacy of TA138, a novel alphavbeta3 antagonist. Anticancer Res 25:197–206

Nabors LB, Rosenfeld SS, Mikkelsen T et al (2004) A phase I trial of EMD 121974 for treatment of patients with recurrent malignant gliomas. Neurooncology [Suppl] p 379

O'Reilly MS, Holmgren L, Shing Y et al (1994) Angiostatin: a circulating endothelial cell inhibitor that suppresses angiogenesis and tumor growth. Cold Spring Harb Symp Quant Biol 59:471–482

O'Reilly MS, Boehm T, Shing Y et al (1997) Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell 88:277–285

Petitclerc E, Boutaud A, Prestayko A et al (2000) New functions for non-collagenous domains of human collagen type IV. Novel integrin ligands inhibiting angiogenesis and tumor growth in vivo. J Biol Chem 275:8051–8061

Raguse JD, Gath HJ, Bier J et al (2004) Cilengitide (EMD 121974) arrests the growth of a heavily pretreated highly vascularised head and neck tumour. Oral Oncol 40:228–230

Rahimi N, Dayanir V, Lashkari K (2000) Receptor chimeras indicate that the vascular endothelial growth factor receptor-1 (VEGFR-1) modulates mitogenic activity of VEGFR-2 in endothelial cells. J Biol Chem 275:16986–16992

Rak J, Yu JL, Klement G et al (2000) Oncogenes and angiogenesis: signaling three-dimensional tumor growth. J Invest Dermatol Symp Proc 5:24–33

Reinmuth N, Liu W, Ahmad SA et al (2003) Alphavbeta3 integrin antagonist S247 decreases colon cancer metastasis and angiogenesis and improves survival in mice. Cancer Res 63:2079–2087

Reynolds AR, Reynolds LE, Nagel TE et al (2004) Elevated Flk1 (vascular endothelial growth factor receptor 2) signaling mediates enhanced angiogenesis in beta3-integrin-deficient mice. Cancer Res 64:8643–8650

Schneller M, Vuori K, Ruoslahti E (1997) Alphavbeta3 integrin associates with activated insulin and PDGFbeta receptors and potentiates the biological activity of PDGF. EMBO J 16:5600–5607

Seftor RE (1998) Role of the beta3 integrin subunit in human primary melanoma progression: multifunctional activities associated with alpha(v)beta3 integrin expression. Am J Pathol 153:1347–1351

Senger DR, Ledbetter SR, Claffey KP et al (1996) Stimulation of endothelial cell migration by vascular permeability factor/vascular endothelial growth factor through cooperative mechanisms involving the alphavbeta3 integrin, osteopontin, and thrombin. Am J Pathol 149:293–305

Senger DR, Claffey KP, Benes JE et al (1997) Angiogenesis promoted by vascular endothelial growth factor: regulation through alpha1beta1 and alpha2beta1 integrins. Proc Natl Acad Sci USA 94:13612–13617

Senger DR, Perruzzi CA, Streit M et al (2002) The alpha(1)beta(1) and alpha(2)beta(1) integrins provide critical support for vascular endothelial growth factor signaling, endothelial cell migration, and tumor angiogenesis. Am J Pathol 160:195–204

Serini G, Valdembri D, Bussolino F (2006) Integrins and angiogenesis: a sticky business. Exp Cell Res 312:651–658

Shannon KE, Keene JL, Settle SL et al (2004) Anti-metastatic properties of RGD-peptidomimetic agents S137 and S247. Clin Exp Metastasis 21:129–138

Silletti S, Kessler T, Goldberg J et al (2001) Disruption of matrix metalloproteinase 2 binding to integrin alpha vbeta 3 by an organic molecule inhibits angiogenesis and tumor growth in vivo. Proc Natl Acad Sci USA 98:119–124

Smith JW (2003) Cilengitide Merck. Curr Opin Invest Drugs 4:741–745

Stromblad S, Cheresh DA (1996) Integrins, angiogenesis and vascular cell survival. Chem Biol 3:881–885

Stupack DG, Cheresh DA (2002) Get a ligand, get a life: integrins, signaling and cell survival. J Cell Sci 115:3729–3738

Sudhakar A, Sugimoto H, Yang C et al (2003) Human tumstatin and human endostatin exhibit distinct antiangiogenic activities mediated by alpha v beta 3 and alpha 5 beta 1 integrins. Proc Natl Acad Sci USA 100:4766–4771

Sun Y, Wang J, Liu y et al (2005) Results of a phase III trial of endostar TM (Rh-endostatin, YH-16) in advanced non-small cell lung cancer (SCLC) patients. J Clin Oncol 23 abstr 7138

Sund M, Hamano Y, Sugimoto H et al (2005) Function of endogenous inhibitors of angiogenesis as endothelium-specific tumor suppressors. Proc Natl Acad Sci USA 102:2934–2939

Taga T, Suzuki A, Gonzalez-Gomez I et al (2002) alpha v-Integrin antagonist EMD 121974 induces apoptosis in brain tumor cells growing on vitronectin and tenascin. Int J Cancer 98:690–697

Trikha M, Zhou Z, Nemeth JA et al (2004) CNTO 95, a fully human monoclonal antibody that inhibits alphav integrins, has antitumor and antiangiogenic activity in vivo. Int J Cancer 110:326–335

Villanueva A, Garcia C, Paules AB et al (1998) Disruption of the antiproliferative TGF-beta signaling pathways in human pancreatic cancer cells. Oncogene 17:1969–1978

Wickstrom SA, Alitalo K, Keski-Oja J (2002) Endostatin associates with integrin alpha5beta1 and caveolin-1, and activates Src via a tyrosyl phosphatase-dependent pathway in human endothelial cells. Cancer Res 62:5580–5589

Wilkinson-Berka JL, Jones D, Taylor G et al (2006) SB-267268, a nonpeptidic antagonist of alpha(v)beta3 and alpha(v)beta5 integrins, reduces angiogenesis and VEGF expression in a mouse model of retinopathy of prematurity. Invest Ophthalmol Vis Sci 47:1600–1605

Thalidomide in Multiple Myeloma

43

Thomas M. Moehler, Jens Hillengass, Axel Glasmacher, Hartmut Goldschmidt

Abstract

Thalidomide (Thal) has antiangiogenic and immunomodulatory activity. Clinical research has provided clear evidence that Thal is one of the most active drugs for the treatment of multiple myeloma, leading to decrease of monoclonal protein of at least 50% in 30% of patients with relapsed or refractory multiple myeloma. Randomized trials based on a large body of evidence from phase II trials have determined that Thal significantly increases total response rate in combination regimens (dexamethasone and/or chemotherapy) for relapsed as well as newly diagnosed patients. Thal also increases time to response in combination therapy approaches. Thal has therefore been recognized by leading organizations as part of the treatment concept for patients with relapsed or refractory disease. Strict guidelines apply for the treatment and monitoring of Thal therapy to prevent its teratogenic effects and to monitor and prevent other potential adverse events as neuropathy and thrombosis. Additional randomized studies will now define the status of Thal for newly diagnosed patients and will form the basis for the approval of Thal in Europe and other countries worldwide.

CONTENTS

Introduction

Thalidomide [Thal; alpha-(N-phthalimido) glutarimide] was synthesized as a sedative drug in 1950 (Fig. 43.1). It also has anti-emetic activity and was marketed initially as the safest available sedative of its time due to lack of toxicity in human volunteers and lack of teratogenicity in murine animal models (Dredge et al. 2003). It became popular as a drug to counter the effects of morning sickness in Europe, Australia, Asia and South America, although it never received Food and Drug Administration (FDA) approval in the United States because of concerns about neuropathy (Dredge et al. 2003; Fullerton and Kremer 1961).

The teratogenic activity was first described independently by William McBride in Australia and Widukind Lenz in Germany, but the subsequent withdrawal of the drug was too late to prevent severe birth defects in 8,000–12,000 babies (Bartlett et al. 2004). This tragedy has overshadowed the use and development of Thal ever since.

Despite this severe complication, however, Thal never disappeared completely from the therapeutic market (Fig. 43.2).

In 1965 the Israeli dermatologist Jacob Sheskin reported the remarkable effectivity of Thal in patients with erythema nodosum leprosum (ENL) (Bartlett et al. 2004). This discovery led to the approval of Thal for the treatment of acute ENL by the FDA in 1998. The discovery of the immunomodulatory effects of Thal led to a number of clinical studies in the treatment of patients with autoimmune diseases such as rheumatoid arthritis, cutaneous lesions of systemic lupus erythematosus and Behcet's disease (Marriott et al. 1999; Hamza 1986; Atra and Sato 1993). Thal was also used to treat chronic graft-versus-host disease associated with allogeneic stem cell transplantation (Heney et al. 1991; Vogelsang et al. 1992).

The first reports on the investigation of Thal for cancer therapy date back to the 1960s (Olson et al. 1965), but this research was abandoned after reports on teratogenicity and first clinical trials with negative findings.

As described below in more detail. D'Amato/Folkman and coworkers discovered the antiangiogenic effect of Thal in 1994 and thereby provided a novel scientific basis for the investigation of Thal in cancer (D'Amato et al. 1994). There are several reasons why this description did not spark an immediate interest in this drug. The black history of Thal was certainly one aspect. In addition, Thal was not protected by patent rights and therefore pharmaceutical companies were concerned about the investment necessary to develop this drug. Another reason may be that the antiangiogenic effect as principal mechanism of cancer therapy was not unequivocally accepted at that time as it is nowadays (Folkman 1971, 2002). Finally, it was Bart Barlogie who discovered the antimyeloma effect of this drug by initially using it on

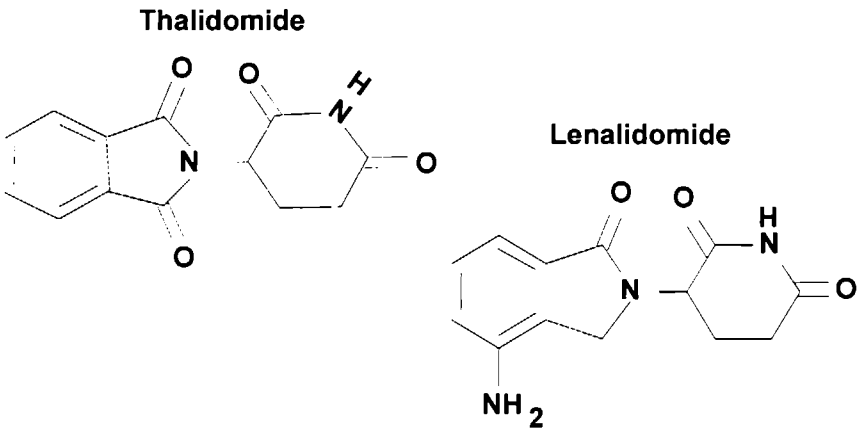


Fig. 43.1. Chemical structures of thalidomide and the thalidomide analog (immunomodulatory drug) lenalidomide (Revlimid)

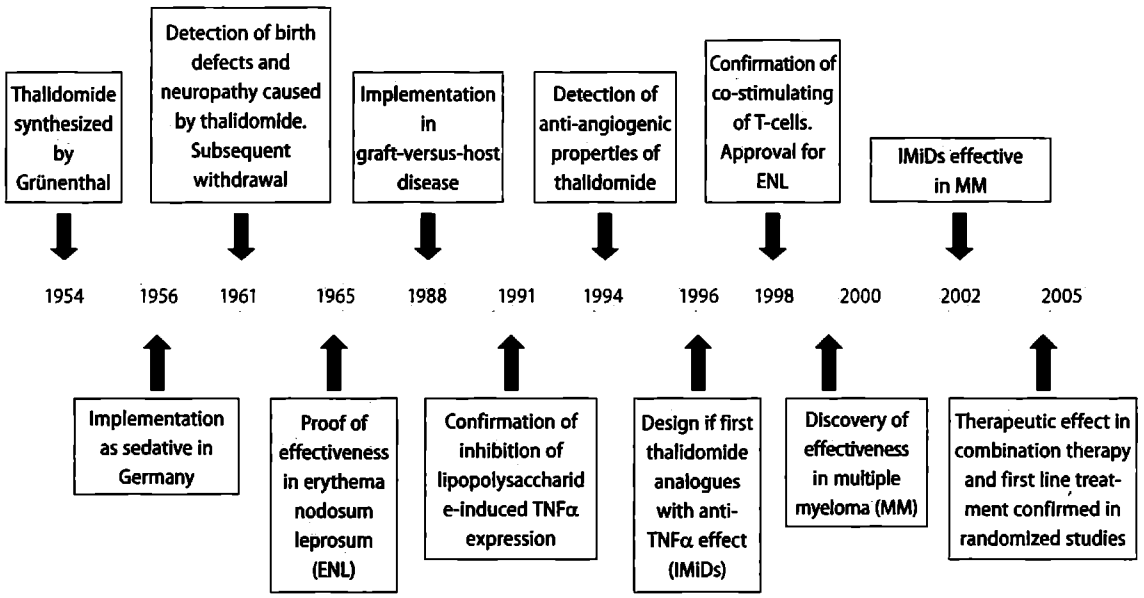


Fig. 43.2. Milestones in the clinical development of thalidomide (modified from Bartlett et al. 2004)

a compassionate use basis, supported by patients and their families, in 1998. The initial discovery of the therapeutic activity led to a clinical study in Little Rock, Arkansas which was published in 1999 (Singhal et al. 1999). This discovery initiated further clinical studies worldwide in multiple myeloma and other oncologic entities.

Chemistry and Pharmacokinetics

Thal [alpha-(N-phthalimido)glutarimide, $C_{13}H_{10}$, N_2O_4] is a glutamic acid derivative and contains a glutarimide moiety with a single chiral center. Thal consist of an racemic mixture of S(−) and R (+) enantiomers. The enantiomers can rapidly interconvert at physiologic pH and have different biologic effects, the S enantiomer being primarily responsible for the teratogenic effects and the R enantiomer for the sedative properties (Kumar et al. 2004; Teo et al. 2001; Eriksson et al. 2001). This finding indicated that purification of the R(+) isoform, although less effective in its ability to suppress TNF expression and antiangiogenic properties, would represent a safer way to use Thal. However, there is a rapid introversion of the two isomers under physiologic conditions, and the R(+) isoform was found to cause teratogenicity similar to the S(−) form in the New Zealand rabbit model (Teo 2005). Comparison of Thal pharmacokinetics and metabolite formation in mice, rabbits and multiple myeloma patients revealed widely different pharmacokinetic profiles. Elimination halflives were 0.5, 2.2 and 7.3 h for mice, rabbits and humans respectively (Chung et al. 2004). Interspecies comparison of high-performance liquid chromatography analysis of Thal metabolites revealed a higher proportion of hydroxylated metabolites in mice than in rabbits and undetectable levels in humans (Chung et al. 2004). Interspecies differences in pharmacokinetics and generation of metabolites are therefore a major reason for differences in biological effects.

At present, Thal is available only as an oral formulation. It is poorly soluble in water and nearly insoluble in ethanol. Studies in healthy volunteers and

HIV patients have indicated that the time to peak concentration after intake of 200 mg of Thal varies from 3 h to 6 h, indicating a slow absorption (Eriksson et al. 2001; Piscitelli et al. 1997). There is no indication that absorption is dependent on gender or age. There is some correlation between the dose and the peak concentration and the area under the curve but there is significant variability, probably reflecting the poor absorption (Teo et al. 2001). Thal does not bind significantly to plasma proteins and it has a large apparent volume of distribution. It was detected in semen after 4 weeks of therapy (Kumar et al. 2004). Thal is primarily metabolized by non-enzymatic hydrolysis in humans with an apparent mean clearance of 10 l/h for the (R) enantiomer and 21 l/h for the (S) enantiomer. This leads to a higher blood concentration of the (R) enantiomer than of the (S) enantiomer (Eriksson et al. 2001). The elimination half-life of both enantiomers is 4–7 h. The pharmacokinetics of patients with renal or hepatic dysfunction remains poorly understood. No induction of its own metabolism has been noted with prolonged use (Aweeka et al. 2003). Thal and its metabolites are eliminated in the urine.

Mechanism of Action

43.3.1 Thalidomide Inhibits Angiogenesis

Judah Folkman was one of the first researchers to associate tumor growth with angiogenesis (Folkman 1971). He and his coworkers had the hypothesis that the teratogenic effects of Thal were secondary to inhibition of blood vessel development in the developing fetal bud (d'Amato et al. 1994). Using a rabbit cornea micro-pocket assay, Folkman and his colleagues provided clear experimental evidence that Thal inhibits induction of angiogenesis by basic fibroblast growth factor (bFGF) (d'Amato et al. 1994). The molecular basis for the antiangiogenic effect has been under investigation for several years. Several mechanisms have been proposed, and future research has to determine which of them might be more basic and fundamental, possibly explaining secondary effects observed by others (Fig. 43.3).

Active bone marrow angiogenesis is a feature of progressive multiple myeloma (Moehler et al. 2003). Angiogenesis is an adverse prognostic factor for overall survival for myeloma patients (Moehler et al. 2003). Investigation of bone marrow microcirculation by dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) indicates that increased angiogenesis is also a feature of myeloma lesions with local destruction (Moehler et al. 2001a; Scherer et al. 2002).

Yabu et al. used a zebrafish model in which Thal induces vascular defects in major vessels during embryonic development (Yabu et al. 2005). According to their convincing experimental findings, which could be repeated in human umbilical vein endothelial cells (HUVECs), Thal induces an increase in neutral sphingomyelinase (nSMase) which leads to the generation of ceramide that is responsible for endothelial cell (EC) apoptosis directly or by down-regulation of vascular endothelial growth factor receptor (VEGF-R) and neuropilin, two critical receptors mediating angiogenesis. Blocking of nSMase completely inhibited the vascular defects induced by Thal in this model. nSMase-induced cellular apoptosis has been described in response to TNF-alpha and heat shock. The exact mechanism by which Thal activates nSMase was not described but is of great interest.

Another antiangiogenic mechanism is the down-modulation of angiogenic cytokines expressed by tumor cells or stroma cells in the tumor microenvironment.

The inhibition of proliferation of EC by Thal could be a secondary event subsequent to activation of EC apoptosis induced either directly or by down-regulation of VEGF-R and neuropilin (Yabu et al. 2005; Vacca et al. 2005; Ng et al. 2003). Further elucidation of the biochemical pathway of angiogenesis inhibition by Thal will provide important clues for the development of novel antiangiogenic drugs.

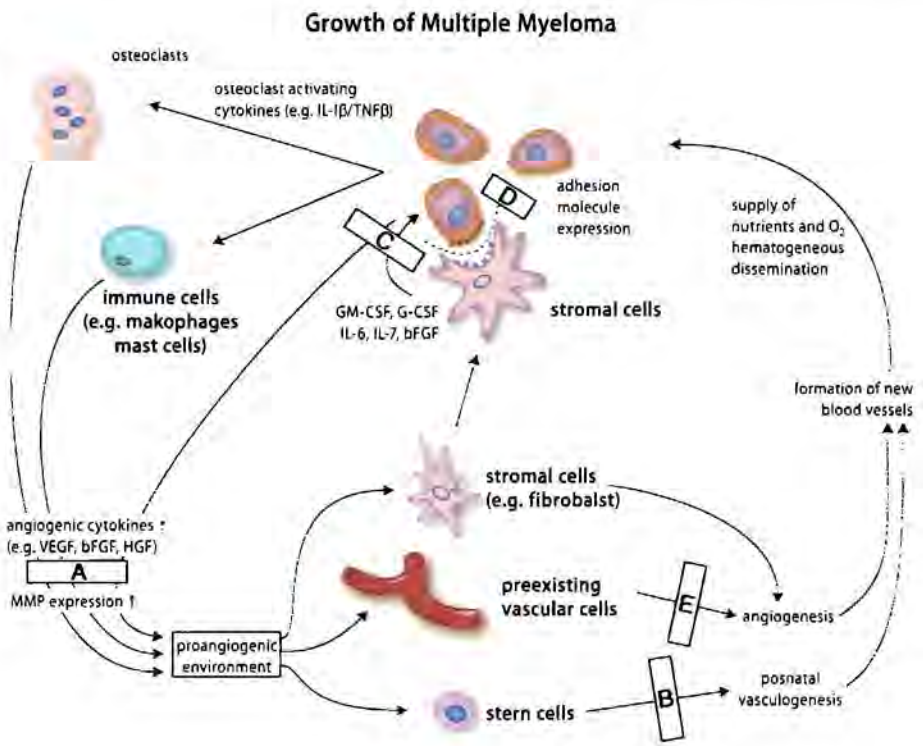


Fig. 43.3. Possible sites of interference of thalidomide with the angiogenic cascade in multiple myeloma (modified from Moehler et al. 2003)

The decrease of circulating endothelial cells induced by Thal could be a further independent antiangiogenic mechanism (Zhang et al. 2005). Zhang et al. found that circulating ECs (CECs), which were defined by the flow-cytometrically determined expression of CD34/CD146/CD105 and lack of CD11b expression, were significantly decreased after the administration of Thal (Zhang et al. 2005). This is an indication that Thal reduces the number of endothelial but possibly also of myelomonocytic cells in the peripheral blood that can support angiogenesis. The contribution of CECs or even circulating endothelial progenitor cells is a topic under intense debate in the scientific community (Urbich et al. 2003). Currently it is unknown to which extent bone marrow-derived stem cells actively differentiate into vascular endothelial cells at the peripheral sites of angiogenesis, as in tumor growth. In contrast, it is a widely accepted concept that myelomonocytic cells released from the bone marrow or the peripheral blood participate and promote angiogenesis in part by secreting angiogenic cytokines (Kaplan et al. 2005). Possible sites of interference of Thal with the angiogenesis cascade are summarized in Fig. 43.3.

Thal-induced remodeling of tumor vasculature could be demonstrated in murine tumor models and was found to result in radiosensitization (Ansiaux et al. 2005).

43.3.2 Immunomodulatory Activities

The ability of Thal to change and in particular decrease cytokine excretion has been reported for ECs but was also shown for monocytes (Fig. 43.4). Thal is a potent inhibitor of TNF-alpha production of lipopolysaccharide-stimulated human monocytes (Corral et al. 1996, 1999; Moreira et al. 1993). This inhibition is due to increased degradation of TNF-alpha mRNA by Thal (Moreira et al. 1993). Also other cytokines can be inhibited as interleukin-1 beta (IL-1-beta),

interleukin 6 (IL-6), granulocyte-macrophage colony stimulating factor (GM-CSF) (Corral et al. 1999). In contrast, interleukin 10 (IL-10) is stimulated (Corral et al. 1999). The effects of Thal on T-cells are opposite to the above-described effects on monocytes. Thal stimulates primary human T lymphocytes, inducing their proliferation, cytokine production and cytotoxic activity (Haslett et al. 1998). In peripheral mononuclear cells Thal induces a T-helper cell type 2 cytokine production, while concomitantly inhibiting T-helper cell type 1 cytokine production. Therefore Thal can no longer be referred to simply as a TNF-alpha inhibitor. The differential responses of Thal described above might explain the diverse beneficial and adverse clinical effects of Thal depending on the immunologic baseline parameters.

In addition to modulating the cytokine profile it was shown that Thal decreases the cell surface molecules ICAM-1, VCAM and E-selectin on HUVECs (Geitz et al. 1996). The decrease of these molecules involved in leukocyte recruitment is one explanation for the therapeutic effects in ENL. It has also been suggested that myeloma adhesion to ECs is diminished by this mechanism, leading to increased apoptosis of myeloma cells. Increasing activity of NK cells is a further mechanism of Thal's immunostimulatory effects (Davies et al. 2001).

43.3.3 Direct Effects of Thalidomide on Myeloma Cells

Thal has also a direct inhibitory effect on myeloma cells (Hideshima et al. 2000). Myeloma cells incubated in the presence of Thal undergo programmed cell death (Mitsiades et al. 2002; Hideshima and Anderson 2002) (Fig. 43.4). The molecular basis of this effect is not completely understood. A mechanism of apoptosis induction similar to that described for endothelial cells (see above) is possible but has to

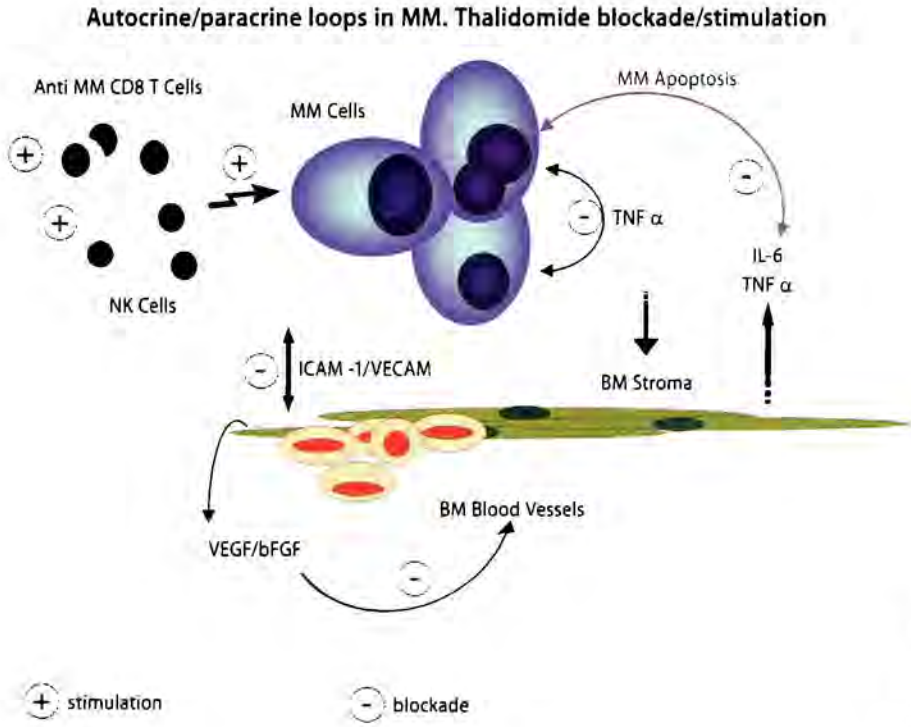


Fig. 43.4. Autocrine/paracrine loops in multiple myeloma: blockade or stimulation by Thal

be investigated in the future. Detection of apoptosis of myeloma cell cultures is considered as important experimental system to screen for anti-myeloma activity of Thal derivatives (Hideshima et al. 2000). Studies investigating the influence of Thal on myeloma cells also revealed that Thal is able to reverse the drug resistance of myeloma cells (Hideshima et al. 2000). In vivo the Thal-induced down-modulation of adhesion receptors on stroma cells is considered an additional factor that contributes to myeloma cell apoptosis (Geitz et al. 1996).

Table 43.1. Response rates (Glasmacher and von Lilienfeld-Toal 2005; Glasmacher et al. 2006)

Response	Reduction in monoclonal protein	No. of patients	% (95% CI)
Complete	>90/100%	18	1 (1–2)
Partial	>50%	370	29 (27–32)
Minor	26–50%	173	14 (12–16)
Stable disease	25→+25%	159	12 (11–14)
Progressive disease	>+25%	190	15 (13–17)
Not evaluable	Unknown	363	31 (28–33)

Thalidomide for Relapsed or Refractory Multiple Myeloma

43.4.1 Thalidomide As Single Agent for Relapsed or Refractory Myeloma

Initial reports on the activity of Thal in myeloma patients as described above initiated the discovery of Thal for cancer patients (Singhal et al. 1999). Thal is still mainly used for patients with multiple myeloma, but significant activity has been found in myelodysplastic syndrome and myeloid metaplasia as well as in selected solid tumors such as prostate cancer and Kaposi's sarcoma, as summarized below (Kumar et al. 2004).

The current status of phase II trials of Thal monotherapy was recently reviewed systematically. Table 43.1 summarizes the results of this meta-analysis with respect to response rates for Thal as single agent (Glasmacher and von Lilienfeld-Toal 2005; Kropff et al. 2003). The response rate for Thal as single agent was 29.4% for partial response (PR) and complete response (CR), and 43.2% when minor response (MR) was included. In this meta-analysis the median event-free survival (EFS; 12 trials including a total of 712 patients) was reported to be 12 months. The median overall survival (OAS) based on the data evaluation of 558 patients in 10 studies was 14 months (range 5–58 months). Graphical illustration of the results of PFS and OAS analysis is provided in Fig. 43.5.

With this activity profile Thal is one of the most active single agents in multiple myeloma. Thal's efficacy in relapsed and refractory patients is comparable with treatment regimens such as the combination of vincristine, doxorubicin and dexamethasone (Dex), which results in response rates of 39% (95% CI 32%–45%).

It has been reported that Thal at 50 mg is active. Doses exceeding 400 mg are associated with substantial increases in adverse effects (see below as well as summarized in Table 43.4 and Fig. 43.6) and cannot be recommended for that reason. Currently, therefore, the recommended dose range for the Thal treatment in myeloma patients is between 50 mg and 400 mg. Many individual clinicians have observed that if patients on low doses (e.g. 50 mg of Thal) develop relapse of multiple myeloma, disease progression can at least temporarily be controlled by increasing the dose of Thal. But still a clear dose–response relationship has not been established today.

In most clinical trials Thal was used in a dose of 50 mg or 100 mg at the beginning of treatment with stepwise increment to 400 mg. Barlogie et al. (2001) described a higher response rate and prolonged overall survival for patients receiving a cumulative dose of 42 g Thal over 3 months, equivalent to approximately 460 mg of Thal per day. Essentially the same

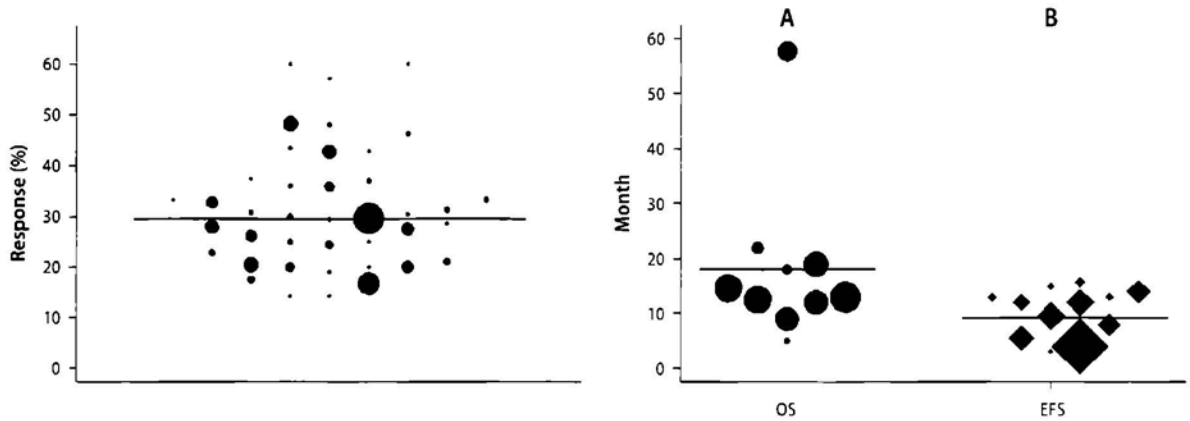


Fig. 43.5. Response rate (*left panel*) and overall survival (*OS*) and event-free survival (*EFS*) (*right panel*) in relapsed or refractory myeloma patients treated with Thal as single agent (Glasmacher and von Lilienfeld-Toal 2005; Glasmacher et al. 2006)

observation was made by other investigators, who described longer OAS or PFS for patients treated with a cumulative dose of 34.5 g over 3 months, equivalent to 400 mg per day, than for patients with lower doses (Moehler et al. 2001b; Neben et al. 2002).

The question of dosage was addressed by a randomized study comparing single-agent Thal at 100 mg and at 400 mg in relapsed or refractory myeloma patients (Yakoub-Agha 2006). Although there was a trend towards a small improvement in PFS in the 400 mg group, no significant difference between the two groups could be detected. Therefore 100 mg or 200 mg is the dosage currently considered optimal for patients with relapsed disease, whether as single-agent therapy or in a combination regimen. As described below, the 50 mg dose is recommended for most studies where prolonged treatment with Thal as maintenance therapy is used (Goldschmidt 2006).

43.4.2 Thalidomide in Combination with Corticosteroids and/or Chemotherapy for Relapsed or Refractory Myeloma: Clinical Phase II Data

The remarkable activity of Thal and a manageable risk profile (as described below) soon initiated clinical trials combining Thal with Dex or chemotherapy to investigate additive or synergistic effects. The initial trials had already provided evidence that Thal in addition with corticosteroids could achieve additional responses (Singhal et al. 1999; Glasmacher and von Lilienfeld-Toal 2005). One advantage of Thal in combination with cytotoxic drugs is that Thal has only a mild myelotoxic effect, if any at all. The choice of combination therapy with Thal has to be based at least in part on the potential additive adverse effects. In combination with anthracyclines, the sharp increase in thromboembolic complications necessitated the co-administration of low-molecular-weight heparins or other forms of anticoagulation (Barlogie et al. 2001; Minnema et al. 2004). The neuropathic effect of Thal excludes co-administration with vincristine.

Indeed, Thal in combination with Dex or chemotherapy achieved impressive response rates between 55% (CR/PR rate) and 79% (CR/PR rate) (Kropff et al. 2003; Moehler et al. 2001b; Dimopoulos and Anagnostopoulos 2003; Garcia-Sanz et al. 2004). Examples of phase II studies on the combination of Thal with chemotherapy in relapsed and refractory patients are summarized in Table 43.2. Comparison with historical data on chemotherapy combination indicated not only improved response rate for the Thal combination therapy but also improved PFS and OAS (Moehler et al. 2001b).

Based on the convincing data of Thal in relapsed and refractory multiple myeloma, regulatory authori-

ties in Australia, New Zealand, Turkey and Israel approved Thal Pharmion, which is associated with the PRMP (Pharmion Risk Minimization Program) security system similar to the STEPS program from Celgene established in the USA (see Sect. 43.9). Pharmion has now launched a large randomized, multinational, multicenter trial for single-agent Thal for patients with relapsed multiple myeloma comparing Thal (100, 200 or 400 mg) with single-agent Dex (OPTIMUM study) to generate data for approval of Thal.

Thalidomide for Treatment of Newly Diagnosed Patients

Thal's effectivity was confirmed in phase II and phase III trials in newly diagnosed patients, who showed more favorable responses than relapsed patients (Rajkumar et al. 2001, 2006). Trials for newly diagnosed patients concentrated on whether Thal improves the outcome based on the standard regimens for this patient group as MP, MD or VAD. These investigations revealed an increase of the response rate in comparison to the standard arm by approximately 20%. Examples of published trials are listed in Table 43.3. In addition, several researchers have reported that the response can be achieved significantly faster with the Thal combination (Goldschmidt 2006; Rajkumar et al. 2006; Ludwig 2006; Palumbo 2006; Cavo et al. 2005).

The remarkable improvement of effectivity of Thal in combination therapy has to be weighed against increased overall toxicity of these combination therapies and impact on EFS and OAS. Goldschmidt et al. showed that the improvement in response rate achieved by TAD (Thal/Adriamycin/Dex) induction therapy compared to VAD (vincristine/Adriamycin/Dex) was canceled out after the first high-dose melphalan (HD-Mel)treatment (Goldschmidt 2006). For that reason a number of investigators are currently reluctant to recommend Thal in newly diagnosed

Table 43.2. Combination of thalidomide with cyclophosphamide and dexamethasone for patients with relapsed or refractory disease (selection of phase II studies)

Study	Combination regimen	No. of patients	Response rate (CR+PR,%)
Moehler et al. (2001a, 2001b), including update 2003	Cy 1,600 mg/m^2 VP16 160 mg/m^2 T 400 mg/day Dex 160 mg	119	55 (46–65)
Kropff et al. (2003)	Cy 1,800 mg/m^2 T≤400 mg/day Dex 240 mg/m^2	60	70 (57–81)
Dimopoulos et al. (2003)	Cy 1,500 mg/m^2 T 400 mg/day Dex 160 mg/m^2	43	67 (51–81)
Glasmacher et al., data on file	Cy 800 mg/m^2 IDA 40 mg/m^2 T≥400 mg/day Dex 320 mg	39	59 (46–71)
Garcia-Sanz et al. (2004)	Cy 50 mg/day T≤800 mg/day Dex 160 mg	66	55 (42–67)

Cy, cyclophosphamide; Dex, dexamethasone; IDA, idarubicin; T, Thalidomide; VP16, etoposide

Table 43.3. Thalidomide combination therapy in newly diagnosed patients

Study	Regimen (R=randomized study)	No. of patients	Response rate standard (%)	Response rate thalidomide (%)
Elderly patients				
Palumbo (2006)	MP vs MPT (R)	102 in total	48	80
Ludwig (2006)	MP vs T-Dex (R)	64 vs 61	38	51
Before high-dose therapy				
Goldschmidt et al. (2006)	VAD vs TAD (R)	203 vs 203	63	80
Rajkumar et al. (2006)	Dex vs T-Dex (R)	104 vs 103	42	58
Cavo et al. (2005)	VAD vs T-Dex	100 vs 100	52	76
Barlogie et al. (2006)	TT2 ± Thal	345 vs 323	43%	62%

A, Adriamycin; D/Dex, dexamethasone; M, melphalan P, prednisone; T, thalidomide; TT2, total therapy 2; V, vincristine

patients that are eligible for HD-Mel and autologous blood stem cell transplantation (ABSCT) as a significant improvement for EFS and OAS in favor of the use of Thal during the induction therapy for newly diagnosed patients has not been confirmed in randomized studies (Goldschmidt 2006; Barlogie et al. 2006). Reasons to include Thal in the combination regimen for newly diagnosed patients eligible for HD-Mel/ABSCT outside of a clinical study could be VAD-refractory disease or necessity to induce a rapid response, e.g. in the case of impairment of renal function by cast nephropathy.

Thalidomide As Maintenance

Maintenance therapy in multiple myeloma is a very important issue, as most patients will suffer from relapse even after intensive therapy. So far only interferon alpha is approved for maintenance therapy (Myeloma Trialists' Collaborative Group 2001). A recent randomized trial has shown that alternate doses of prednisone 50 mg every other day can improve the PFS.

Many investigators have observed that days or weeks after withdrawal of a Thal therapy patients develop myeloma relapse. This observation, and the current practice of continuing a Thal maintenance treatment for patients responding to Thal or a Thal combination, make this a very important issue.

The IFM99-02 study investigated the role of Thal as maintenance therapy subsequent to HD-Mel/ABSCT with randomization of 580 patients into three arms: (A) no maintenance; (B) pamidronate; (C) Thal and pamidronate (Attal 2004). Data presented at the conference of the American Society of Hematology in December 2004 revealed significant improvements in EFS and PFS for arm C with the combination of Thal and pamidronate. The 40-months post-diagnosis probability of PFS was 70% (95% CI 42–80) for the Thal arm versus 53% (95% CI 37–65) in arm A and 52% (95% CI 36–68) in arm B.

Preliminary data from the GMMG (German speaking myeloma group)-HD3 trial investigating Thal (in a dose of 50 mg) versus interferon maintenance did not show a significant difference between the two arms, but adverse events were significantly less frequent in the Thal arm, with more patients on continuous maintenance after 1 year in the Thal arm (Goldschmidt et al., personal communication). Final data analyses from these and similarly designed studies are now awaited.

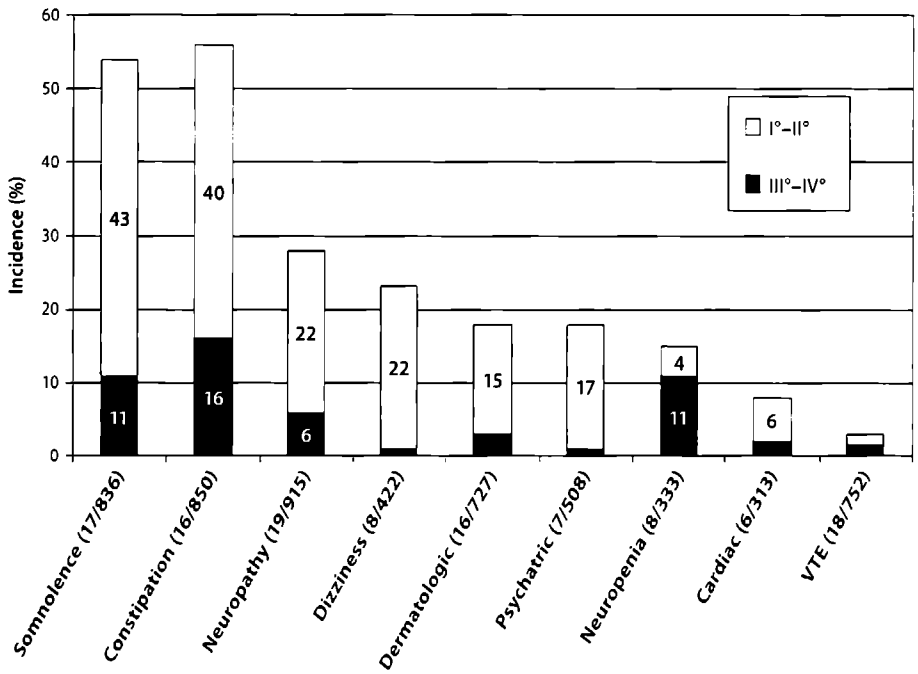


Fig. 43.6. Summary and frequency of grade I/II and grade III/IV adverse effects of thalidomide as single agent according to Glasmacher et al. (2006) and Glasmacher and von Lilienfeld-Toal (2005)

Development of Immunomodulatory Drugs and Thalidomide Analogs

Soon after the discovery of the beneficial clinical effects of Thal for multiple myeloma and other oncologic entities, a search for analogs with improved therapeutic efficacy and fewer adverse effects began. The drug discovery program for the first-generation Thal analogs used the Thal structural backbone as a template to synthesize compounds with increased immunological and anti-cancer properties. The second-generation analogs were focused on improved inhibition of TNF-alpha. It was found that analogs with an additional amino group added to the fourth carbon of the phthaloyl ring of Thal (4-amino analogs) displayed a 50,000 times greater ability to inhibit TNF-alpha in vitro than the parent compound (Fig. 43.1; example of lenalidomide/Revlimid/CC-5013) (Hideshima and Anderson 2002). Extensive preclinical testing finally led to the identification of CC-5013 and CC-4047 for further investigation in clinical trials (Teo 2005; Hideshima and Anderson 2002).

Third-generation Thal analogs (third-generation immunomodulatory drugs, IMiDs) are now in preclinical testing. The emphasis of work on these compounds has shifted from anti-TNF-alpha inhibition to their antiangiogenic and immunomodulatory effects.

The hope that non-teratogenic compounds will soon be in clinical use to substitute Thal has not been fulfilled. CC-5013 and CC 4047 are both teratogenic and have to be applied under strict guidelines.

CC-5013 (lenalidomide, Revlimid) is well advanced in clinical development. Several clinical studies have clearly shown that Revlimid is active as single agent for relapsed and refractory multiple myeloma (Richardson et al. 2002). In the first phase I study of Revlimid in relapsed MM (n=27), 42% of patients showed a greater than 25% reduction in M protein. In this study a maximum tolerated dose (MTD) of 25 mg/day was found due to grade 3 myelosuppression at 50 mg/day (Richardson et al. 2002).

In a phase I/II dose escalation trial of CC-4047 (Actimid) in patients with advanced multiple myeloma (n=18) in which 39% of the patients showed reduction in M protein greater then 25%, a MTD of

2 mg/day was defined due to neutropenia at higher dose (Schey et al. 2004).

Revlimid is far more advanced in clinical development than Actimid. In fact, Revlimid was approved by the FDA in 2006 for the treatment of refractory or relapsed multiple myeloma in combination with dexamethason. Recently, Revlimid received approval by the EMEA (European Medicines Agency) for the same indication (Bartlett et al. 2004). Further clinical studies provided evidence that Revlimid can also improve therapeutic efficacy in combination therapy settings for relapsed as well as newly diagnosed patients (Rajkumar 2006; Dimopoulos 2006). Dimopoulos et al. presented data from a randomized, placebo-controlled study comparing lenalidomide/Revlimid/CC-5013 in combination with Dex against Dex with placebo for relapsed/refractory multiple myeloma patients that included 351 patients. Revlimid led to a significant improvement of overall response rate (including CR and PR) from 24% with single-agent Dex to 59% in the combination arm. In addition, PFS was more than doubled from 4.7 months in the Dex group to 11.3 months in the combination group ($p<0.001$) (Dimopoulos 2006).

Common adverse effects of lenalidomide were hematotoxicity with grade III/IV neutropenia in 27% of patients and thrombocytopenia in 10% of patients. Furthermore, an increased incidence of thromboembolic events was noted (4% of patients) (Dimopoulos 2006). In contrast to Thal, peripheral neuropathy, constipation and somnolence were not associated with Revlimid.

Thalidomide in Non-plasma Cell Malignancies

The success of Thal in multiple myeloma triggered clinical investigation in other malignancies (Kumar et al. 2004).

Thal was found to induce objective responses in Kaposi sarcoma in 35–47% of patients in a dose range between 100 and 1000 mg per day (Little et al. 2000). Remarkable response rates between 27% and 53% were found for single-agent Thal in prostate cancer (Figg et al. 2001). Other phase II trials revealed responses in melanoma, brain tumors, melanoma and renal cancer, but response rates were under 10% in most trials. In breast cancer no objective responses were recorded (Baidas et al. 2000; Eisen et al. 2000).

Both Thal and Revlimid were recognized to have significant activity in a number of hematologic malignancies. One of the first discoveries was that Thal induced hematologic improvement in approximately 35% of patients with myelodysplastic syndrome. As a result of Thal treatment a significant number of patients achieved transfusion independence (Kumar et al. 2004). Strupp et al. showed in a limited number of patients that cytogenetic response can follow normalization of blood counts (Strupp et al. 2003). Recently Revlimid was approved for the treatment of myelodysplastic syndrome by the FDA.

Significant activity of Thal was also shown for myeloid fibrosis with myeloid metaplasia in several phase II trials (Kumar et al. 2004). A number of encouraging clinical effects were reported for T-cell lymphoma of the AILD type (angioimmunoblastic lymphoma with dysproteinemia), B-cell lymphomas and Hodgkin lymphomas, as well as acute myelogenous leukemia (Kumar et al. 2004).

Adverse Effects of Thalidomide

Thal has a number of adverse effects in addition to its well-known teratogenic potential. The frequency management and prevention of these adverse events has been recently reviewed (Ghobrial and Rajkumar 2003). In doses of 400 mg or less Thal's side effects are mild to moderate in severity and are generally well tolerated (Table 43.4). The most commonly encountered side effects are neurologic, with peripheral polyneuropathy mostly associated with prolonged use of Thal, sedation, fatigue, and tremor. Figure 43.6 summarizes the frequency and severity of adverse events as identified by a recent meta-

analysis of clinical trials with Thal as single agent (Glasmacher et al. 2006). Other common adverse effects are constipation and cutaneous changes, including dryness of skin, non-specific dermatitis, xerostomia and allergic skin reactions. In rare cases severe skin reactions as Stevens–Johnson syndrome, erythema multiforme or toxic epidermal necrolysis have been reported. Less common side effects include bradycardia, hearing disturbances and mild neutropenia.

Use of Thal in combination with drugs associated with thromboembolic disease results in a sharp increase in the risk of developing an thromboembolic event while on treatment. For that reason prophylactic anticoagulation has to be instituted in anthracycline combination therapy and has to be considered in other combinations, such as Thal/Dex (Barlogie et al. 2006).

If these adverse events are mild to moderate, i.e. WHO grade I or II toxicity, dosage of Thal is reduced by 50%. Further dose reduction to 50 mg is required if the adverse event is not resolved within 7 days. If there is still no resolution of the adverse event, Thal has to be discontinued. In the cases of WHO grade III and IV toxicity, discontinuation of Thal is mandatory. Re-administration of Thal following WHO grade III or IV toxicity in general is not recommended as the risk of induce life-threatening complications is high.

Regulatory Issues

Teratogenicity is the most feared adverse event and occurs when Thal is taken between days 27 and 40 of gestation (Lenz 1988). Therefore, national regulatory agencies in the USA, Europe, Australia and other countries have strict guidelines that are designed to control the use of Thal. For the USA Thal is marketed

Table 43.4. Adverse effects of thalidomide therapy

Frequent side effects	Less common side effects
Birth defects	Headache
Drowsiness and somnolence	Confusion
Skin rash	Malaise
Constipation	Asthenia
Peripheral neuropathy	Hyper- or hypoglycemia
Xerostomia	Tremor
Orthostatic hypotension and dizziness	Pruritus
Neutropenia<?11>	Peripheral edema Hepatitis (elevated serum transaminases) Stevens–Johnson syndrome Impotence Deep vein thrombosis Hearing disturbance Hair loss Loss of libido Nausea Fever Menstrual abnormalities Hypothyroidism

under the STEPS program by Celgene. A similar program is provided in Europe and other countries by Pharmion, namely the Pharmion Risk Minimization Program for Thal-Pharmion. Physicians, patients and pharmacies involved in the treatment of patients with multiple myeloma are registered within these programs and follow strict guidelines in the application and monitoring of Thal. Women in the child-bearing age group must undergo pregnancy testing before starting therapy and every 2-4 weeks during therapy. They must use two highly effective contraceptive methods. As Thal has been detected in the semen, male patients have to use a condom while under treatment even if they have had a successful vasectomy. All patients must continue these measures for at least 1 month after discontinuation of the drug. The security system is also instrumental in detect the other potential adverse effects.

References

Myeloma Trialists' Collaborative Group (2001) Interferon as therapy for multiple myeloma: an individual patient data overview of 24 randomized trials and 4012 patients. Br J Haematol 113:1020–1034

Ansiaux R, Baudelet C, Jordan BF, Beghein N, Sonveaux P, De WJ et al (2005) Thalidomide radiosensitizes tumors through early changes in the tumor microenvironment. Clin Cancer Res 11:743–750

Atra E, Sato EI (1993) Treatment of the cutaneous lesions of systemic lupus erythematosus with thalidomide. Clin Exp Rheumatol 11:487–493

Attal M (2004) Maintenance treatment with thalidomide after autologous transplantation for myeloma: first analysis of a prospective randomized study of the Intergroupe Francophone du Myelome (IFM 99 02). Session type: oral session. Harousseau J-L, Leyvraz S, Doyen C, Hulin C, Benboubker L, Facon T et al (eds). Blood 104, 155a. Ref type: generic

Aweeka F, Trapnell C, Chernoff M, Jayewardene A, Spritzler J, Bellibas SE et al (2001) Pharmacokinetics and pharmacodynamics of thalidomide in HIV patients treated for oral aphthous ulcers: ACTG protocol 251. AIDS Clinical Trials Group. J Clin Pharmacol 41:1091–1097

Baidas SM, Winer EP, Fleming GF, Harris L, Pluda JM, Crawford JG et al (2000) Phase II evaluation of thalidomide in patients with metastatic breast cancer. J Clin Oncol 18:2710–2717

Barlogie B, Desikan R, Eddlemon P, Spencer T, Zeldis J, Munshi N et al (2001) Extended survival in advanced and refractory multiple myeloma after single-agent thalidomide: identification of prognostic factors in a phase 2 study of 169 patients. Blood 98:492–494

Barlogie B, Tricot G, Anaissie E, Shaughnessy J, Rasmussen E, van RF et al (2006) Thalidomide and hematopoietic-cell transplantation for multiple myeloma. N Engl J Med 354:1021–1030

Bartlett JB, Dredge K, Dalgleish AG (2004) The evolution of thalidomide and its IMiD derivatives as anticancer agents. Nat Rev Cancer 4:314–322

Cavo M, Zamagni E, Tosi P, Tacchetti P, Cellini C, Cangini D et al (2005) Superiority of thalidomide and dexamethasone over vincristine-doxorubicin-dexamethasone (VAD) as primary therapy in preparation for autologous transplantation for multiple myeloma. Blood 106:35–39

Chung F, Lu J, Palmer BD, Kestell P, Browett P, Baguley BC et al (2004) Thalidomide pharmacokinetics and metabolite formation in mice, rabbits, and multiple myeloma patients. Clin Cancer Res 10:5949–5956

Corral LG, Haslett PA, Muller GW, Chen R, Wong LM, Ocampo CJ et al (1999) Differential cytokine modulation and T cell activation by two distinct classes of thalidomide analogues that are potent inhibitors of TNF-alpha. J Immunol 163:380–386

Corral LG, Muller GW, Moreira AL, Chen Y, Wu M, Stirling D et al (1996) Selection of novel analogs of thalidomide with enhanced tumor necrosis factor alpha inhibitory activity. Mol Med 2:506–515

D'Amato RJ, Loughnan MS, Flynn E, Folkman J (1994) Thalidomide is an inhibitor of angiogenesis. Proc Natl Acad Sci USA 91:4082–4085

Davies FE, Raje N, Hideshima T, Lentzsch S, Young G, Tai YT et al (2001) Thalidomide and immunomodulatory derivatives augment natural killer cell cytotoxicity in multiple myeloma. Blood 98:210–216

Dimopoulos M (2006) Study of lenalidomide plus dexamethasone versus dexamethasone alone in relapsed or refractory multiple myeloma (MM): Results of a Phase 3 Study (MM-010). Spencer A, Attal M, Prince M, Harousseau J-L, Dmoszynska A, Yu Z et al (eds). Blood 106, 6a. Ref type: generic

Dimopoulos MA, Anagnostopoulos A (2003) Thalidomide in relapsed/refractory multiple myeloma: pivotal trials conducted outside the United States. Semin Hematol 40:8–16

Dredge K, Dalgleish AG, Marriott JB (2003) Thalidomide analogs as emerging anti-cancer drugs. Anticancer Drugs 14:331–335

Eisen T, Boshoff C, Mak I, Sapunar F, Vaughan MM, Pyle L et al (2000) Continuous low dose thalidomide: a phase II study in advanced melanoma, renal cell, ovarian and breast cancer. Br J Cancer 82:812–817

Eriksson T, Bjorkman S, Hoglund P (2001) Clinical pharmacology of thalidomide. Eur J Clin Pharmacol 57:365–376

Figg WD, Dahut W, Duray P, Hamilton M, Tompkins A, Steinberg SM et al (2001) A randomized phase II trial of thalidomide, an angiogenesis inhibitor, in patients with androgen-independent prostate cancer. Clin Cancer Res 7:1888–1893

Folkman J (1971) Tumor angiogenesis: therapeutic implications. N Engl J Med 285:1182–1186

Folkman J (2002) Role of angiogenesis in tumor growth and metastasis. Semin Oncol 29:15–18

Fullerton PM, Kremer M (1961) Neuropathy after intake of thalidomide (distaval). Br Med J 5256:855–858

Garcia-Sanz R, Gonzalez-Porras JR, Hernandez JM, Polo-Zarzuela M, Sureda A, Barrenetxea C et al (2004) The oral combination of thalidomide, cyclophosphamide and dexamethasone (ThaCyDex) is effective in relapsed/refractory multiple myeloma. Leukemia 18:856–863

Geitz H, Handt S, Zwingenberger K (1996) Thalidomide selectively modulates the density of cell surface molecules involved in the adhesion cascade. Immunopharmacology 31:213–221

Ghobrial IM, Rajkumar SV (2003) Management of thalidomide toxicity. J Support Oncol 1:194–205

Glasmacher A, von Lilienfeld-Toal M (2005) The current status of thalidomide in the management of multiple myeloma. Acta Haematol 114 [Suppl 1]:3–7

Glasmacher A, Hahn C, Hoffmann F, Naumann R, Goldschmidt H, von Lilienfeld-Toal M et al (2006) A systematic review of phase-II trials of thalidomide monotherapy in patients with relapsed or refractory multiple myeloma. Br J Haematol 132:584–593

Goldschmidt H (2006) HOVON 50/GMMG-HD3-Trial: Phase III study on the effect of thalidomide combined with high dose melphalan in myeloma patients up to 65 years. Sonneveld P, Breitkreutz I, van der Holt B, Benner A, Barge RMY, Salwender H et al (eds). Blood 106, 128a. Ref type: generic

Hamza MH (1986) Treatment of Behcet's disease with thalidomide. Clin Rheumatol 5:365–371

Haslett PA, Corral LG, Albert M, Kaplan G (1998) Thalidomide costimulates primary human T lymphocytes, preferentially inducing proliferation, cytokine production, and cytotoxic responses in the CD8+ subset. J Exp Med 187:1885–1892

Heney D, Norfolk DR, Wheeldon J, Bailey CC, Lewis IJ, Barnard DL (1991) Thalidomide treatment for chronic graft-versus-host disease. Br J Haematol 78:23–27

Hideshima T, Anderson KC (2002) Molecular mechanisms of novel therapeutic approaches for multiple myeloma. Nat Rev Cancer 2:927–937

Hideshima T, Chauhan D, Shima Y, Raje N, Davies FE, Tai YT et al (2000) Thalidomide and its analogs overcome drug resistance of human multiple myeloma cells to conventional therapy. Blood 96:2943–2950

Kaplan RN, Riba RD, Zacharoulis S, Bramley AH, Vincent L, Costa C et al (2005) VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. Nature 438:820–827

Kropff MH, Lang N, Bisping G, Domine N, Innig G, Hentrich M et al (2003) Hyperfractionated cyclophosphamide in combination with pulsed dexamethasone and thalidomide (HyperCDT) in primary refractory or relapsed multiple myeloma. Br J Haematol 122:607–616

Kumar S, Witzig TE, Rajkumar SV (2004) Thalidomide: current role in the treatment of non-plasma cell malignancies. J Clin Oncol 22:2477–2488

Lenz W (1988) A short history of thalidomide embryopathy. Teratology 38:203–215

Little RF, Wyvill KM, Pluda JM, Welles L, Marshall V, Figg WD et al (2000) Activity of thalidomide in AIDS-related Kaposi's sarcoma. J Clin Oncol 18:2593–2602

Ludwig H (2006) Thalidomide-dexamethasone versus melphalan-prednisolone as first line treatment in elderly patients with multiple myeloma: an interim analysis. Drach J, Tóthová E, Gisslinger H, Linkesch W, Jaksic B, Fridik M et al (eds). Blood 106:231a. Ref type: generic

Marriott JB, Muller G, Dalgleish AG (1999) Thalidomide as an emerging immunotherapeutic agent. Immunol Today 20:538–540

Minnema MC, Breitkreutz I, Auwerda JJ, van der HB, Cremer FW, van Marion AM et al (2004) Prevention of venous thromboembolism with low molecular-weight heparin in patients with multiple myeloma treated with thalidomide and chemotherapy. Leukemia 18:2044–2046

Mitsiades N, Mitsiades CS, Poulaki V, Chauhan D, Richardson PG, Hideshima T et al (2002) Apoptotic signaling induced by immunomodulatory thalidomide analogs in human multiple myeloma cells: therapeutic implications. Blood 99:4525–4530

Moehler TM, Hawighorst H, Neben K, Egerer G, Hillengass J, Max R et al (2001a) Bone marrow microcirculation analysis in multiple myeloma by contrast-enhanced dynamic magnetic resonance imaging. Int J Cancer 93:862–868

Moehler TM, Neben K, Benner A, Egerer G, Krasniqi F, Ho AD et al (2001b) Salvage therapy for multiple myeloma with thalidomide and CED chemotherapy. Blood 98:3846–3848

Moehler TM, Ho AD, Goldschmidt H, Barlogie B (2003) Angiogenesis in hematologic malignancies. Crit Rev Oncol Hematol 45:227–244

Moreira AL, Sampaio EP, Zmuidzinas A, Frindt P, Smith KA, Kaplan G (1993) Thalidomide exerts its inhibitory action on tumor necrosis factor alpha by enhancing mRNA degradation. J Exp Med 177:1675–1680

Neben K, Moehler T, Benner A, Kraemer A, Egerer G, Ho AD et al (2002) Dose-dependent effect of thalidomide on overall survival in relapsed multiple myeloma. Clin Cancer Res 8:3377–3382

Ng SS, Gutschow M, Weiss M, Hauschildt S, Teubert U, Hecker TK et al (2003) Antiangiogenic activity of N-sub-

stituted and tetrafluorinated thalidomide analogues. Cancer Res 63:3189–3194

Olson KB, Hall TC, Horton J, Khung CL, Hosley HF (1965) Thalidomide (N-phthaloylglutamimide) in the treatment of advanced cancer. Clin Pharmacol Ther 40:292–297

Palumbo A (2006) Oral melphalan, prednisone and thalidomide for multiple myeloma. Bringhen S, Musto P, Caravita T, Capozzi R, Callea V, Cangialosi C et al (eds). Blood 106:230a. Ref type: generic

Piscitelli SC, Figg WD, Hahn B, Kelly G, Thomas S, Walker RE (1997) Single-dose pharmacokinetics of thalidomide in human immunodeficiency virus-infected patients. Antimicrob Agents Chemother 41:2797–2799

Rajkumar SV (2006) Combination therapy with lenalidomide plus dexamethasone (Rev/Dex) for newly diagnosed myeloma. Haymann S, Lacy MQ, Dispenzieri A, Geyer SM, Kabat B, Zeldenrust SR et al (eds). Blood 106:230a. Ref type: generic

Rajkumar SV, Dispenzieri A, Fonseca R, Lacy MQ, Geyer S, Lust JA et al (2001) Thalidomide for previously untreated indolent or smoldering multiple myeloma. Leukemia 15:1274–1276

Rajkumar SV, Blood E, Vesole D, Fonseca R, Greipp PR (2006) Phase III clinical trial of thalidomide plus dexamethasone compared with dexamethasone alone in newly diagnosed multiple myeloma: a clinical trial coordinated by the Eastern Cooperative Oncology Group. J Clin Oncol 24:431–436

Richardson PG, Schlossman RL, Weller E, Hideshima T, Mitsiades C, Davies F et al (2002) Immunomodulatory drug CC-5013 overcomes drug resistance and is well tolerated in patients with relapsed multiple myeloma. Blood 100:3063–3067

Scherer A, Wittsack HJ, Strupp C, Gattermann N, Haas R, Modder U (2002) Vertebral fractures in multiple myeloma: first results of assessment of fracture risk using dynamic contrast-enhanced magnetic resonance imaging. Ann Hematol 81:517–521

Schey SA, Fields P, Bartlett JB, Clarke IA, Ashan G, Knight RD et al (2004) Phase I study of an immunomodulatory thalidomide analog, CC-4047, in relapsed or refractory multiple myeloma. J Clin Oncol 22:3269–3276

Singhal S, Mehta J, Desikan R, Ayers D, Roberson P, Eddlemon P et al (1999) Antitumor activity of thalidomide in refractory multiple myeloma. N Engl J Med 341:1565–1571

Strupp C, Hildebrandt B, Germing U, Haas R, Gattermann N (2003) Cytogenetic response to thalidomide treatment in three patients with myelodysplastic syndrome. Leukemia 17:1200–1202

Teo SK (2005) Properties of thalidomide and its analogues: implications for anticancer therapy. AAPS J 7:E14–E19

Teo SK, Scheffler MR, Kook KA, Tracewell WG, Colburn WA, Stirling DI et al (2001) Thalidomide dose proportionality assessment following single doses to healthy subjects. J Clin Pharmacol 41:662–667

Urbich C, Heeschen C, Aicher A, Dernbach E, Zeiher AM, Dimmeler S (2003; Relevance of monocytic features for neovascularization capacity of circulating endothelial progenitor cells. Circulation 108:2511–2516

Vacca A, Scavelli C, Montefusco V, Di PG, Neri A, Mattioli M et al (2005) Thalidomide downregulates angiogenic genes in bone marrow endothelial cells of patients with active multiple myeloma. J Clin Oncol 23:5334–5346

Vogelsang GB, Farmer ER, Hess AD, Altamonte V, Beschorner WE, Jabs DA et al (1992) Thalidomide for the treatment of chronic graft-versus-host disease. N Engl J Med 326:1055–1058

Yabu T, Tomimoto H, Taguchi Y, Yamaoka S, Igarashi Y, Okazaki T (2005) Thalidomide-induced antiangiogenic action is mediated by ceramide through depletion of VEGF receptors, and is antagonized by sphingosine-1-phosphate. Blood 106:125–134

Yakoub-Agha I (2006) A multicenter prospective randomized study testing non-inferiority of thalidomide 100 mg/day as compared with 400 mg/day in patients with refractory/relapsed multiple myeloma: first results of the final analysis of the IFM 01–02 study. Hulin C, Doyen C, Benboubker L, Voillat L, Bouabdallah K, Guillerm G et al (eds). Blood 106:110a; Ref type: generic

Zhang H, Vakil V, Braunstein M, Smith EL, Maroney J, Chen L et al (2005) Circulating endothelial progenitor cells in multiple myeloma: implications and significance. Blood 105:3286–3294

Surrogate Markers of Angiogenesis

44

VALENTINA RAIA, PATRIZIA MANCUSO, FRANCESCO BERTOLINI

Abstract

Anti-angiogenic drugs have entered the anti-cancer drug arsenal, but there is a clear need to identify patients who are likely to benefit from this therapeutic strategy. Circulating endothelial cells (CECs) and progenitors (CEPs) have been reported to be modulated in cancer patients. At the preclinical level, CEC and CEP kinetics correlate well with several standard laboratory angiogenesis assays, which cannot be used in humans. At the clinical level, CEC kinetics and viability may correlate with clinical outcomes in cancer patients treated using anti-angiogenic therapeutic strategies. Thus, CEC and CEP measurement has potential as a surrogate marker for monitoring anti-angiogenic treatment/drug activity and helping to determine the optimal biological dosage of anti-angiogenic drugs.

CONTENTS

44.1 Introduction

"Molecularly targeted" anti-cancer drugs are perhaps the most significant recent advance in oncology therapeutics. However, despite the approval of some of these molecules for clinical use in the USA and Europe, the development of this class of drugs, including their anti-angiogenic agents, suffers from several handicaps. In fact, in many cases it is unclear whether the patient's cancer expresses the drug's target and whether the target is functionally contributing to cancer growth. Since in many cases these drugs are extremely expensive, and only some of the patients benefit from these novel therapeutics, there is a clear need to monitor the biological activity of these drugs, to select and stratify the patients who are likely to benefit from the treatment, and to determine each drug's optimal biological dose (OBD). This last point is related to the low frequency of ob-

jective tumor responses (tumor shrinkage) caused by such drugs, or the lack of dose-limiting toxicities (DLT) necessary to define a maximum tolerated dose (MTD), or expression of optimal therapeutic activity at doses below the MTD, when one of these parameters can be defined. These problems necessitate the development of alternative pharmacodynamic surrogate markers (Kerbel and Folkman 2002).

In this chapter we discuss several promising markers for use in monitoring targeted anti-angiogenic activity and debate their optimal therapeutic/biological dosing.

Circulating Endothelial Mature Cells and Progenitors

In the past, measurement of tumor angiogenesis to predict and/or assess the efficacy of anti-angiogenic therapies was mainly based on the evaluation of microvessel density (MVD). In this procedure, blood vessels of tumor samples are stained with antibodies and counted by light microscopy. However, this approach is invasive, it cannot be used to predict the efficacy of an anti-angiogenic drug (Hlatky et al. 2002), MVD of a tumor biopsy specimen might not equate with MVD of the whole tumor, and the correlation between MVD and clinical outcome is still uncertain in most tumor types (Weidner 2000).

It has been reported that circulating endothelial cells (CECs) are increased in the peripheral blood (PB) of patients affected by sickle cell anemia, cytomegalovirus or rickettsial infection, myocardial infarction, or endotoxinemia (Solovey et al. 1997; Grefte et al. 1993; Drancourt et al. 1992; Mutin et al. 1999; Lin et al. 2000). However, any attempt to quantify CECs has been hampered by two main factors: (1) CECs are rare events and their characterization requires a large amount of cells; (2) the lack of a specific antigen/monoclonal antibody that can unambiguously identify CECs.

In 2001, Mancuso et al. reported that CECs are increased in the peripheral blood of cancer patients, and first reported a flow cytometry technique to detect both CECs and the CEC subpopulation expressing markers associated with a progenitor cell activity, defined as circulating endothelial progenitors (CEPs). The details of the technique are summarized below.

44.2.1 Quantification of Human CECs by Flow Cytometry

In 2001, Mancuso et al. used a panel of human monoclonal antibodies including anti-CD45 to exclude hematopoietic cells, anti-CD31, -CD34, -CD36, -CD105, -CD106, -CD133, and -P1H12 (Solovey et al. 1997; Grefte et al. 1993; Drancourt et al. 1992; Mutin et al. 1999; Lin et al. 2000; George et al. 1991; St. Croix et al. 2000; Fornas et al. 2000; Rafii 2000; Khan et al. 2005; Li et al. 2000; Osborn et al. 1989; Elshal et al. 2005; Peichev et al. 2000; Salven et al. 2003), and appropriate analysis gates were used to enumerate resting and activated CECs and CEPs (Fig. 44.1). Monoclonal antibodies (Table 44.1) were conjugated with FITC, R-phycoerythrin (PE), PerCP or allophycocyanin (APC), and cell suspensions evaluated by a FACSCalibur equipped with a second red-diode laser (BD, San Jose, CA). Absolute cell numbers were calculated by reference fluorescent beads and „lyse-no-wash" procedures used to increase sensitivity and reproducibility (Fornas et al. 2000). After acquisition of at least 100,000 cells per PB sample, analyses were considered as informative when adequate numbers of events (i.e. >100, typically 300–400) were collected in the CEC enumeration gates. Resting CECs were defined as negative for hematopoietic marker CD45; positive for endothelial markers P1H12, CD31 and CD34; negative for activation markers CD105 and CD106; and negative for the progenitor marker CD133. Activated CECs were defined as $CD45^-$, $P1H12^+$, $CD31^+$, $CD34^+$, CD105 or $CD106^+$, $CD133^-$. According to Rafii (2000), CEPs were characterized by expression of CD133. Regarding activated CEC markers, high levels of CD105 (endoglin), a receptor for transforming growth factor beta (Li et al. 2000), and vascular cell adhesion

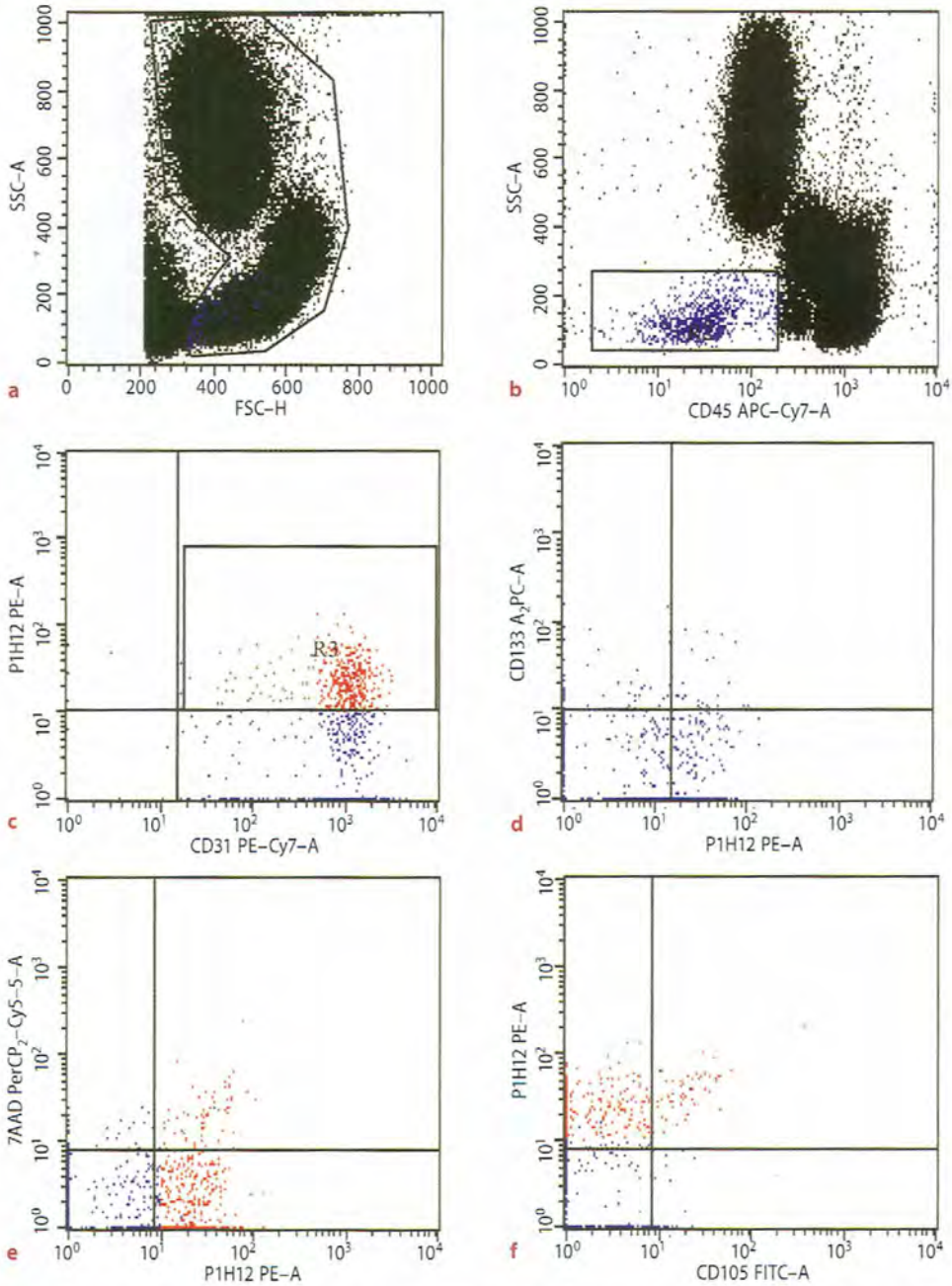


Fig. 44.1a–f. Six-color flow cytometry evaluation of circulating endothelial cells and endothelial progenitors. The use of monoclonal antibodies conjugated with six different fluorochromes allows the identification of mature, apoptotic, activated CECs and CEPs simultaneously. **a** An analysis gate (R1) restricted on lymphocytes and granulocytes is used to exclude platelets, dead cells, debris and microparticles. **b** A pan-leukocyte monoclonal antibody (anti-CD45) is used to exclude hematopoietic cells. R2 indicates negative CD45 and weakly positive CD45 cells and thereafter the expression of endothelial marker is performed on this population. **c** R3 indicates mature CECs evaluated by the co-expression of CD31 and P1H12. **d** CEPs are evaluated for their expression of CD133. **e** Apoptotic CECs are evaluated for their positivity for 7-AAD. **f** Activated CECs are evaluated for their positivity for CD105

Table 44.1. Target antigens, related cluster designations, antibody clones and conjugation of human monoclonal antibodies used for CEC and CEP enumeration

Antigen	Cluster designation	Antibody clone	Conjugation
PECAM-1	CD31	WM59 (Pharmingen BD)	FITC
gp 105–120	CD34	HPCA-2 (BD)	APC
LCA	CD45	2D1 (BD)	PerCP
Endoglin	CD105	8E11 (Euroclone)	FITC
VCAM-1	CD106	5110C9 (Pharmingen BD)	PE
AC133	CD133	AC133/1 (Miltenyi)	APC
P1H12	CD146	P1H12 (Chemicon)	PE

molecule-1 (CD106, VCAM-1; Osborn et al. 1989) are currently considered as hallmarks of activated endothelial cells either in vitro or in tissues undergoing angiogenesis. Activated endothelial cells also express increased adhesion molecules such as CD54 (intracellular adhesion molecule-1), CD62e (E-selectin), and markers of procoagulant activity such as CD142 (tissue factor; Khan et al. 2005).

The sensitivity and specificity of this procedure were evaluated by serial dilution of human umbilical cord endothelial cells (expressing an „activated CEC" phenotype) in the U-937 cell line. The detection limit of the procedure was 0.1 cell/μl, and specificity was >90%. Anti-CD34 antibodies conjugated with APC, when compared with PE, have decreased mean fluorescence intensity (Fornas et al. 2000). In any case, APC-conjugated anti-CD34 was useful to discriminate between the very tiny population of $CD45^-$ hematopoietic progenitors, which were $CD34^{+++}$(bright), and $CD34^+$ CECs.

Flow-cytometric enumeration of CECs is far from a standardized procedure, and several approaches have been published (George et al. 1991; Mancuso et al. 2001). Because of the lack of a marker that can unambiguously identify CECs and CEPs, multiparametric flow cytometry is the best technique to use to detect endothelial cells and discriminate them from cells with overlapping expression of antigens. First of all, the use of CD45 is necessary to exclude hematopoietic cells. For example, CD146 expression on activated T cells can be distinguished from CD146 on endothelial cells by co-staining with CD45 (Elshal et al. 2005). These antigens are present on T cells but not on CECs. CD34 expression on endothelial cells presents an interesting problem because this antigen is also found on hematopoietic stem cells. However, CD34 is brightly expressed on hematopoietic cells but has decreased mean fluorescence intensity on CECs, and flow cytometry readily detects this difference.

The discovery of CD133 (AC133), an antigen that identifies primitive stem cells, provided a means to dissect mature CECs from CEPs. CD34 could not be used for this purpose, because mature endothelial cells express this antigen (Rafii 2000). CD133, conversely, is not present on CECs or any mature endothelial cells.

Another very important issue regards the viability of CECs. Although there are various protocols for detection of CECs, consensus seems to be building towards the use of CD146 (P1H12, S-endo-1), CD45, and a viability stain such as propidium iodide or 7-AAD, perhaps in combination with additional endothelial markers such as CD31, as an appropriate means of identifying and enumerating CECs. Redundant endothelial markers increase the accuracy of endothelial cell detection, whereas exclusionary gating of $CD45^+$ cells and dead cells by the viability stain decreases the incidence of false-positive cells due to staining of lymphocytes (for CD146) or non-specific antibody binding by dead cells. Moreover, it is possible to discriminate between

viable and apoptotic CECs (Philpott et al. 1996) in order to evaluate the anti-angiogenic properties of different drugs.

44.2.2 Preclinical and Clinical Measurements of CECs and CEPs

In 2001, we measured CECs and CEPs in 76 newly diagnosed cancer patients (30 with lymphoma and 46 with breast cancer) and 20 controls (Mancuso et al. 2001). In these controls, mean values for resting and activated CECs/μl were 7.9 (95% confidence interval, CI, 4.7–11.1), and 1.2 (95% CI 0.1–2.3), respectively. In newly diagnosed patients, mean resting and activated CECs/μl were 39.1 (95% CI 16.8–61.4) and 6.8 (95% CI 5.0–8.6), i.e. increased fivefold ($P<0.0008$ vs control by ANOVA). CEC distribution was normal in controls and skewed in patients. Repeated CEC measurements in patients and controls indicated low longitudinal CEC variation. Evaluation of CD36 expression showed that in patients and controls at least half of the CECs were microvascular in origin. In patients and controls the count of resting and activated CECs did not correlate with the count of white cells, red cells or platelets.

VEGF is produced by the large majority of tumor cells and is involved in CEP mobilization. Correlation between MVD and VEGF and between MVD and CECs did not reach statistical significance. On the other hand, a positive correlation ($r=0.419$, $P=0.009$ by multiple regression) was found between CECs/μl and plasma VEGF. As shown in Fig. 44.2, a normal distribution of resting CECs, activated CECs and plasma VEGF was observed in controls, whereas a switch to increased VEGF, increased CECs and activated CEC phenotype was observed in cancer patients. The level of circulating VCAM-1, a glycoprotein produced by angiogenic endothelial cells, was significantly higher in patients than in controls. VCAM-1 strongly correlated with CECs/μl ($r=0.582$, $P<0.0001$) but not with MVD.

CEC numbers were found to be similar to control values in seven lymphoma patients who achieved

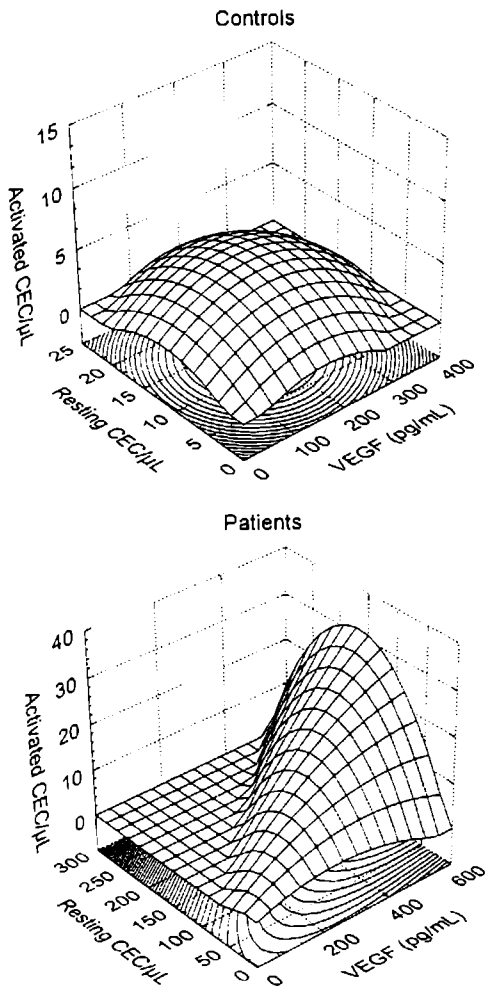


Fig. 44.2. Levels of VEGF, resting and activated CECs in healthy subjects and cancer patients. Normal distribution in controls (top); switch to increased VEGF, increased CECs, and activated CEC phenotype in cancer patients (bottom). (From Mancuso et al. 2001, modified)

complete remission after chemotherapy. In these patients, mean resting and activated CECs/μl were 12.8 (95% CI 4.0–21.6) and 0.8 (95% CI 0.4–1.2), respectively ($P=0.0001$ and 0.63 vs newly diagnosed patients and healthy controls, respectively). Furthermore, CECs were found to decrease in 13 breast cancer patients evaluated before and 24 h after quadrantectomy. Regarding CEPs, they were

<0.5/μl in all controls and newly diagnosed patients evaluated. Higher CEP counts were found in 4 out of 11 patients evaluated while recovering from high-dose-chemotherapy-induced aplasia and in 2 out of 7 healthy controls evaluated during the menstrual period. In these 6 cases, CEP values ranged from 1.1 to 9/μl.

This clinical observation prompted us to investigate whether CECs could be quantified in mice and used as surrogate markers of angiogenesis and anti-angiogenic activity of different drugs. CECs were enumerated using a panel of monoclonal antibodies reacting with murine CD45 (to exclude hematopoietic cells) and endothelial murine markers VEGF receptor 2, CD105, VE-cadherin, MECA-32, CD31 and CD34. Annexin V and 7AAD were used to depict apoptotic and dead cells (Fig. 44.3).

We used an animal model of human lymphoma (Monestiroli et al. 2001) to evaluate CEC kinetics and viability in cancer-bearing mice before and after drug therapy. We observed a stable CEC increase after lymphoma transplant, and the strong correlation observed between CECs and tumor volume indicates that CEC increase may parallel tumor progression. We found that in control animals most CECs seemed to have initiated an apoptotic program, whereas CEC viability was markedly improved in tumor-bearing mice (Monestiroli et al. 2001). Considering the anti-apoptotic properties of VEGF, known to be produced by cancer cells, this

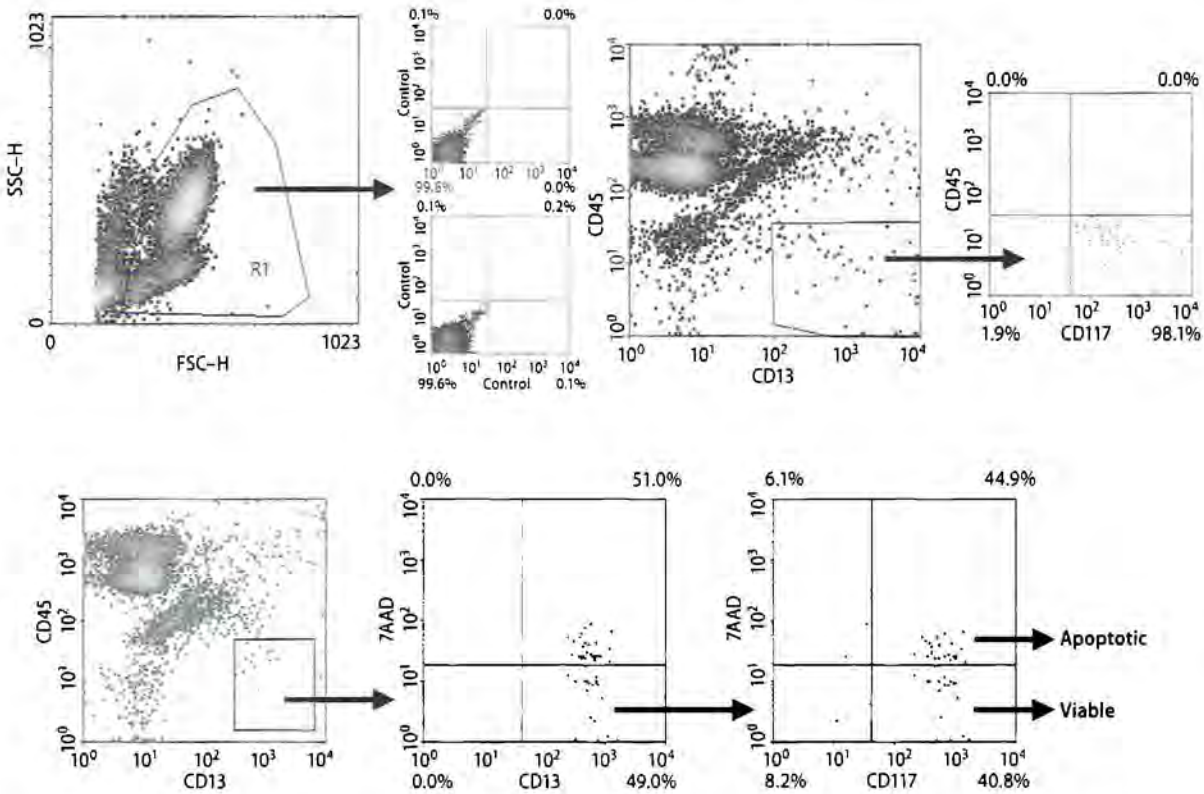


Fig. 44.3. Flow-cytometric evaluation of the number and viability of murine CECs and CEPs. (From Bertolini et al. 2003, modified). An analysis gate (R1) restricted on lymphocytes and granulocytes is used to exclude platelets, dead cells, debris and microparticles. Endothelial cells are defined in R2 as negative or weakly positive for CD45 (a pan-leukocyte marker) and positive for CD13

growth factor might play a relevant role in preserving CECs from apoptosis.

In a previous study (Bertolini et al. 2000), the frequency of endothelial apoptotic cells was found to be significantly increased in Namalwa lymphomas removed from NOD/SCID mice treated with the anti-angiogenic drug endostatin. We evaluated CEC viability before and after drug therapy, and found that the cytotoxic drug cyclophosphamide at MTD induced apoptosis of circulating hematopoietic and (to a lesser extent) endothelial cells (Monestiroli et al. 2001). Conversely, the anti-angiogenic drug endostatin specifically targeted endothelial and not hematopoietic cells. Thus, the measurement of CEC viability during clinical studies might help to define the balance between cytotoxic and anti-angiogenic activity of different drug schedules.

44.2.2.1 Genetic Heterogeneity in CEC and CEP Numbers: Implications for Their Validation as Cellular Surrogate Markers

To assess whether CEC and CEP measurement represents a valid surrogate approach for neovascularization, we collaborated with Robert Kerbel's laboratory in Toronto (Shaked et al. 2005). We examined eight inbred mouse strains and assessed sprouting angiogenesis using the classical corneal neovascular micropocket assay, the stimulus being 180 ng VEGF. Pellets showed a range of up to ~10-fold differences in the ability to cause angiogenesis, with strains such as 129/SvImJ found to have strong angiogenic ability and strains such as C57BL/6 J, C3H/HeJ and CD-1 showing a limited ability to induce angiogenesis. We next undertook an analysis of levels of CECs/CEPs [differentiated by means of the absence or the presence of CD177 (c-kit) expression, respectively] among the eight different inbred mouse strains representative of 'high', 'medium', and 'low' angiogenic responsiveness, as assessed by the corneal micropocket angiogenesis assay. A striking correlation was found between the angiogenic ability, assessed by the corneal neovascular micropocket assay, and the absolute number of CECs and CEPs (Fig. 44.4). Such a correlation suggests that both angiogenic and vasculogenic phenotypes are probably regulated by the same genetic heterogeneity among different mouse strains.

To show that the angiogenesis is not restricted to the assay and the ophthalmic environment, the Matrigel (subcutaneous) plug perfusion assay was used to measure the angiogenic ability in some of the mouse strains that were studied for levels of CECs and CEPs (Shaked et al. 2005). Again, the ratio between Matrigel plug fluorescence and plasma fluorescence was the highest (~0.0036) in the 129/SvImJ strain, which also showed the highest CEC and CEP levels. In contrast, the C57BL/6 J mouse strain, which demonstrated low angiogenesis/vasculogenic phenotype, was found to have a low ratio between Matrigel plug fluorescence and plasma fluorescence, and BALB/cJ mice demonstrated an intermediate range of angiogenic ability, with respect to intrinsic levels of CECs/CEPs and the Matrigel plug perfusion assay. These results confirmed that the correlation is not restricted to angiogenic responses induced in an ophthalmic microenvironment, or to the assay used.

To address the question of whether the basis of the genetic heterogeneity in levels of CECs and CEPs is, at least in part, regulated by known and validated molecular regulators of sprouting angiogenesis, we assessed levels of the cells in various mutant (knockout) or transgenic mouse models (Shaked et al. 2005). First, we evaluated a mutant of the C57BL/6 J mouse strain (that normally expresses low angiogenic ability relative to most other evaluated strains), which has a targeted disruption in the *tsp-1* gene. TSP-1 is a well-known endogenous inhibitor of angiogenesis (Kerbel and Folkman 2002). Both CECs and CEPs increased approximately fivefold in the TSP-1 null C57BL/6 J mice relative to the wild-type control mice. In addition, to confirm that TSP-1 has a direct effect on levels of CECs/CEPs, we undertook a pharmacologic 'knockout' approach in which TSP-1 null mice were treated on a daily basis with the anti-angiogenic drug, ABT-510, a peptide mimetic derived from the type I (properdin-like) repeats that mimics the interaction between TSP-1 and CD36 receptors. After 2 weeks of daily administration with

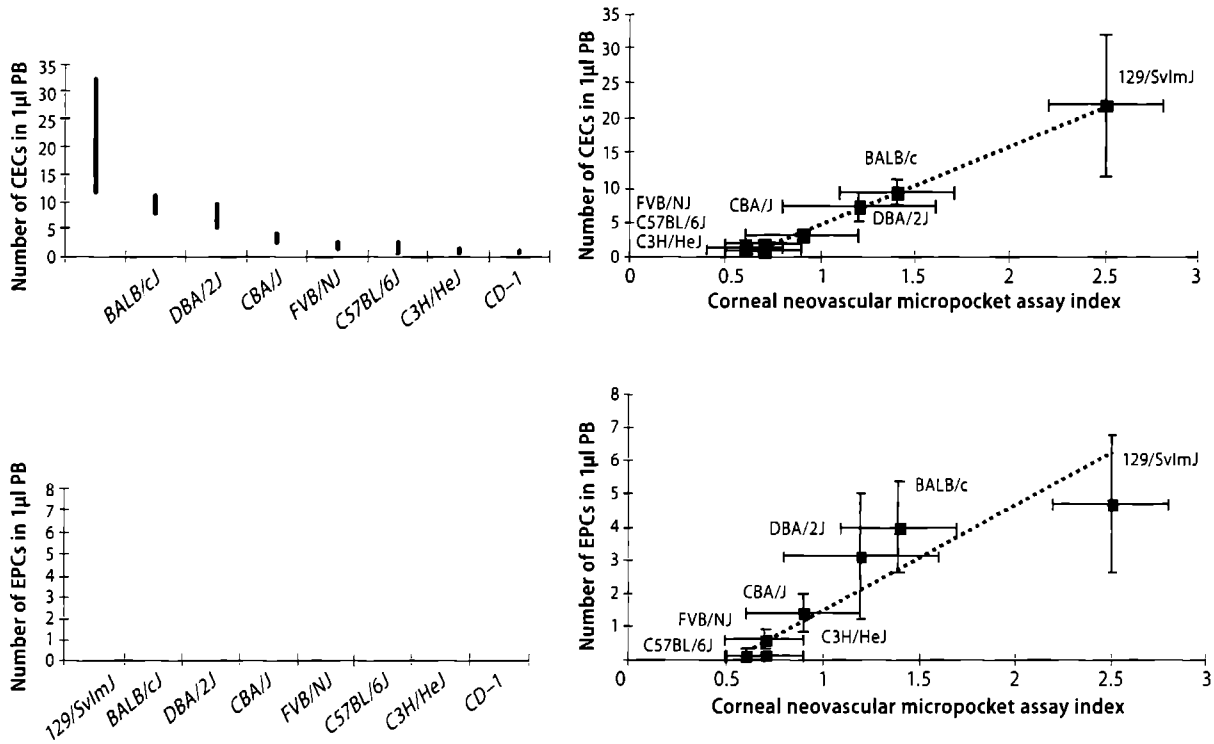


Fig. 44.4. The correlation between CECs and CEPs and the corneal neovascular micropocket assay index in different strains of mouse. (From Shaked et al. 2005, modified)

either ABT-510 peptide or the vehicle, mice were evaluated for CECs/CEPs. The treatment of TSP-1 null mice with ABT-510 results in a marked reduction of the intrinsically high CEC/CEP levels, which were reduced to basal values normally detected in wild-type control mice.

We next evaluated the effect of two different stimulatory regulators of angiogenesis, namely, VEGF and Tie-2, on intrinsic levels of CECs/CEPs (Shaked et al. 2005). To do so, we analyzed peripheral blood from either VEGF hypermorphic (VEGF-$A^{hi/+}$) or Tie-2 overexpressing mice. VEGF-$A^{hi/+}$ mice (received from Dr. Andras Nagy, University of Toronto, Canada) have a two- to threefold increase in circulating VEGF levels during embryonic development as well as in adulthood. The background of both of these mutant mouse strains is CD-1, which was previously shown to have low-grade angiogenic ability. Both VEGF-$A^{hi/+}$ mice and mice overexpressing Tie-2 receptor exhibit a seven- to eightfold increase in CEC levels in comparison to CD-1 wild-type control (p=7.8×10-5, p=0.001, respectively); similarly, there was a marked elevation in levels of CEPs (p=0.005, p=5.8×10-5, respectively). Interestingly, in the Tie-2-overexpressing mice, a distinct increase of approximately 60-fold in the levels of CEPs (from 0.1 cells/µl in the wild type to 6 cells/µl in mice overexpressing Tie-2) was observed.

In the light of several reports showing suppressive effects of various anti-angiogenic drugs on the CECs/CEPs, e.g. reduction in the viability and/or mobilization of CEPs, both preclinically (Capillo et al. 2003; Bertolini et al. 2003; Schuch et al. 2003) and clinically (Willett et al. 2004), we next asked whether CEC/CEP quantification might help to identify the OBD of anti-angiogenic drugs. Empir-

ical dose–response preclinical studies with DC101 [a rat monoclonal blocking antibody specific for the mouse VEGFR-2 (flk-1) receptor] (Kerbel and Folkman 2002) have shown that the optimal anti-tumor therapeutic dose of DC101 is in the range of 800 μg–1 mg/mouse, administered every 3 days. We investigated whether the evaluation of CECs/CEPs can, in principle, help establish an optimal dose of such an anti-angiogenic drug (Shaked et al. 2005). To do so, we first evaluated the viability of CEPs in two different tumor models. We tested the effect of DC101 in escalating doses (0–2000 μg/mouse/dose) on a syngeneic Lewis lung carcinoma in C57BL/6 J mice and on a human lymphoma (Namalwa) xenograft in NOD/SCID mice. We found that 800 μg/mouse of DC101 is the OBD in both the LL/2 C57BL/6 J model and the Namalwa lymphoma NOD/SCID model (Fig. 44.5), since it induced the lowest level of viable CEPs and the greatest decrease in tumor volume. These results were not altered by further increasing the DC101 dose to 2000 μg/mouse.

Furthermore, since the CEP levels are affected by the tumor growth, it is possible that the reduction in the CEP levels is due to the tumor volume rather than to the OBD of the drug. Therefore, we evaluated the viable CEPs in a correlation study in non-tumor-bearing mice (Shaked et al. 2005). First, we performed a correlation study with DC101 in both strains of tumor-free background mice we had al-

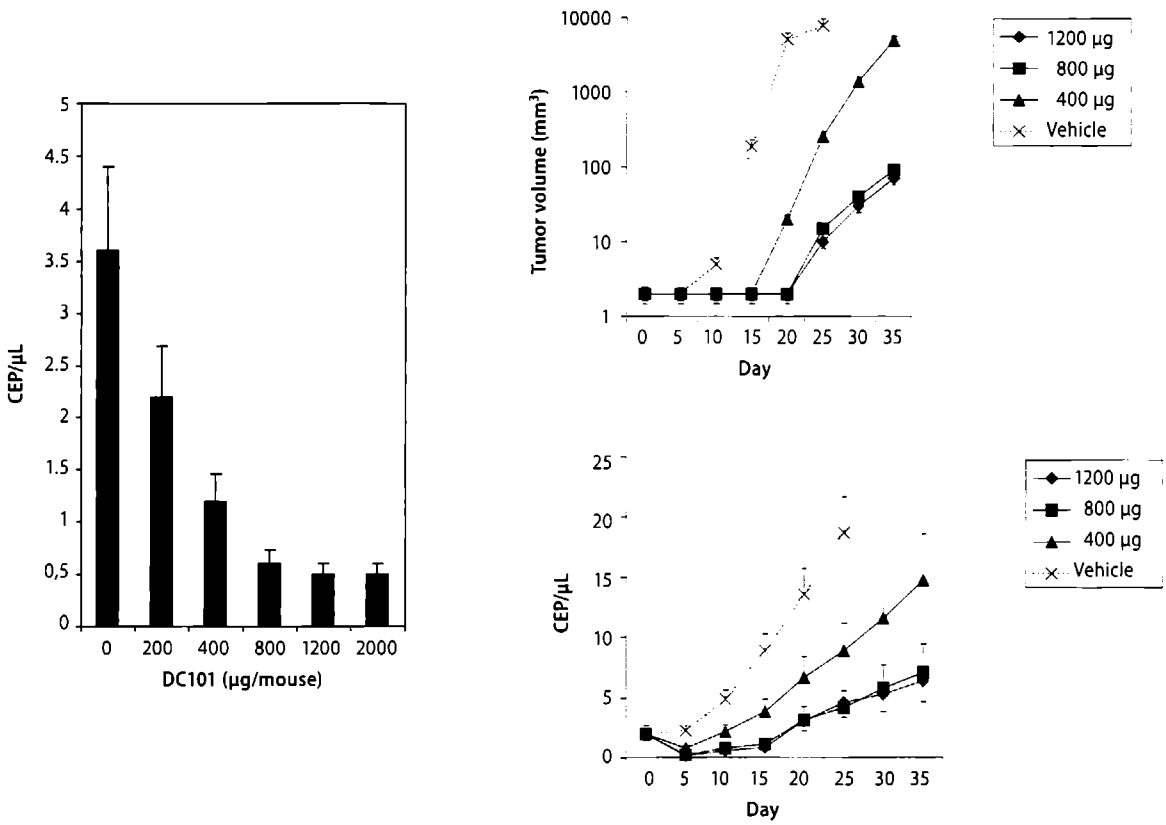


Fig. 44.5. Enumeration of CEPs as a surrogate biomarker to define the OBD of the anti-angiogenic drug DC101. In the Namalwa lymphoma NOD/SCID model (Bertolini et al. 2000), we found that a dose of 800 μg/mouse induced the lowest level of viable CEPs and greatest decrease in tumor volume. These results were not altered by further escalating the DC101 dose to 2000 μg/mouse. (From Shaked et al. 2005, modified)

ready tested (i.e. C57BL/6 J and NOD/SCID). In both cases, administration of DC101 in escalating doses showed that the optimal dose is 800 μg/mouse since it induced the greatest reduction in the viable CEPs that was not exceeded by higher doses. Second, we tested the viable CEPs in other strains and also with another drug – ABT-510, a TSP-1 mimetic peptide. Either 800 μg/mouse of DC101 or 60 mg/kg/day of ABT-510 are the optimal anti-angiogenic biological dose since they induced the lowest level of viable CEPs. This is similar to the reduction in the viable CEPs we obtained in both LL/2 C57BL/6 J and Namalwa NOD/SCID mice, and also consistent with the optimal dose of ABT-510 according to the manufacturer. In addition, we tested the OBD of either DC101 or ABT-510 in different strains to determine whether similar effects are detected in "high-angiogenic" strains such as 129/SvImJ or "low angiogenic" strains such as C57BL/6 J. Both DC101 and ABT-510 caused a marked reduction in the viable CEPs in all three strains, declining to basal levels of 0.1 viable CEPs/μl peripheral blood.

44.2.2.2 Dual Effects of Different Anti-Angiogenic Therapies on Circulating Endothelial Progenitors and Mature Cells

We investigated the correlation between CEC and CEP kinetics, their viability (assessed by 7AAD) and cancer growth in a number of preclinical models of cancer treated with different anti-angiogenic drugs and therapeutic strategies. In several murine models of lymphoma and solid cancer (melanoma, lung, prostate and breast cancer) we found that the administration of some drugs (endostatin, angiostatin and thalidomide) was associated with a reduction in the number of viable CECs and CEPs that paralleled the reduction in tumor growth compared to untreated controls. In the same murine models of cancer, the preclinical administration of some other drugs (such as molecules with anti-VEGFR2 activity) and some anti-angiogenic therapeutic strategies (metronomic chemotherapy using continuous, low doses of cyclophosphamide or vinblastine), were associated with an anti-cancer activity that paralleled a release of apoptotic CECs in circulation and an absence of CEP mobilization from the bone marrow. These preclinical models also indicated that the source of apoptotic CECs was most likely the tumor vasculature, because CECs were not increased in cancer-free mice treated with the same drugs (Mancuso et al. 2006).

The prognostic potential of CEC kinetic and viability was confirmed at the clinical level in 81 patients with advanced breast cancer receiving metronomic chemotherapy (cyclophosphamide plus methotrexate), where CEC levels were lower in patients for whom no overall clinical benefit (defined as a clinical response or a stable disease) was observed than in those who had a clinical benefit (p=0.015). This difference was due to an increased fraction of apoptotic CECs in patients with a clinical benefit. Univariate and multivariate analyses indicated that CEC values >11/μl were associated with longer progression-free survival (p=0.001) and improved overall survival (p=0.005, Mancuso et al. 2006).

The clinical value of CEC kinetics was also assessed in solid cancer patients (p=23) receiving different combinations of three drugs with anti-angiogenic activity (interferon-α, celecoxib and thalidomide). In this latter study, we observed, at variance with the former study on metronomic chemotherapy, a significant reduction of CECs in all patients. Patients with stable disease, however, still had a significantly different level of CECs (in particular of the apoptotic fraction), and the single patient who had a clinical response had a relevant increase in apoptotic CEC.

Folkman (2003) has indicated that in tumor angiogenic vessels the endothelial cell lining is continuously migrating, while in mature, quiescent vessels there is little or no endothelial cell turnover. This fits well with our preclinical and clinical findings on CEC kinetics. Our data, moreover, point to a novel dual effect of different classes of anti-angiogenic therapies on CECs and CEPs, and indicate that kinetics and viability of these surrogate biomarkers have potential to define OBDs of anti-angiogenic drugs and to serve as predictors of clinical response.

Real-Time Reverse-Transcription PCR as a New Tool to Study Angiogenesis

In addition to flow cytometry, our laboratory has studied how the quantitative investigation of gene expression might reflect the angiogenic status (Rabascio et al. 2004). We looked for genes selectively or particularly expressed by endothelial cells; in fact, the expression of these genes was expected to correlate to the amount of CECs in blood. Candidate genes studied wereVEGFR-2, VE-cadherin and Tie-2, and many are still under investigation. Together with genes supposed to mirror CEC values, the expression of the CD133 gene was studied to quantify CEPs.

VEGFR-2 is a receptor for VEGF; it is expressed on vascular endothelium, is involved in cell proliferation, migration and survival, and has a crucial role in the angiogenic process.

Tie-2, instead, is a receptor for angiopoietins; it is activated by Ang-1 and activated or inhibited by Ang-2, depending on tissues. Ang-1 is a strong modulator of vascular permeability, it stimulates vascular growth in many pathologic conditions, including some tumors, and it suppresses angiogenesis in other tumors and in the heart.

VEGFR-2 and Tie-2 mRNA expression in blood was not significantly increased in cancer patients compared to controls (Rabascio et al. 2004). Conversely, circulating VE-cadherin mRNA levels were increased in cancer patients compared to controls, and found to correlate with CEC viability. In this regard, Prandini et al. (2005) constructed transgenic mice carrying a gene (Lac-Z) under human VE-cadherin promoter control. They subcutaneously implanted lung carcinoma in these mice and investigated the presence of beta-galactosidase (in appropriate experimental conditions) and the binding of CD31 antibody in tumor sections. They thus demonstrated that VE-cadherin promoter is activated in tumor angiogenesis. These findings, together with those of Rabascio and colleagues (2004), support the hypothesis that VE-cadherin transcription is increased when a tumor angiogenic process occurs.

VE-cadherin is a molecule involved in the homotypic adhesion of endothelial cells and is expressed only on these cells. It is a trans-membrane protein interacting, by its intra-cytoplasmic region, with three proteins belonging to the *armadillo* family: beta-catenin, plakoglobin and p120. Beta-catenin and plakoglobin anchor VE-cadherin to cytoskeleton through a binding mediated by alpha-catenin and other molecules (Nieset et al. 1997). Interest in VE-cadherin is increasing because its role is not only mechanical. In fact, being susceptible to cell-to-cell contact, it seems to be involved in differentiation, cell growth and cell migration. Some researchers (Carmeliet et al. 1999) demonstrated VE-cadherin involvement in signal transduction pathway mediated by VEGF receptor 2; they proposed a model in which formation of VE-cadherin–beta-catenin–IP3 kinase–VEGFR-2 complex promotes cell survival through activation of Akt protein. As VE-cadherin has a role in CEC survival, it is reasonable that its circulating mRNA level correlates with CEC viability.

More recently, circulating mRNA levels of CD146, also known as P1H12, were found to be significantly higher in breast cancer patients than in controls, and these levels correlated with CEC numbers detected by flow cytometry (Fürstenberger et al. 2005).

By means of real-time reverse-transcription PCR (RT-PCR), many groups investigated gene expression in tissues instead of blood, looking for correlations with tumor angiogenesis. Kanda et al. (2005) evaluated the expression of 11 genes (total VEGF-A, VEGF121, VEGF165, VEGF189, VEGF-B, C and D, bFGF, dThdPase, MMP-2 and uPA) in 11 cervical carcinoma cell lines and 14 normal cervical tissues. Gene co-expression analysis revealed a significant correlation between angiogenic factors and proteinases in malignant but not in normal cervical samples. Gene expression levels of VEGF-C and MMP-2 correlated well with the number of tumor cells. VEGF-A splicing variants were increased in malignant compared to normal cervical samples but not associated with the invasive activity of the cells.

Using real-time quantitative PCR and in situ hybridization, Patel et al. (2005) showed that the expression of delta-like 4 ligand (DLL4) was up-regu-

lated almost ninefold within the vasculature of clear cell-renal cell carcinoma relative to normal kidney and was correlated with the expression of VEGF. DLL4 is a ligand that activates the Notch pathway, an intercellular signaling pathway involved in numerous biological processes including cell fate determination, cellular differentiation, proliferation, survival and apoptosis (Artavanis-Tsakonas et al. 1995).

Jiang's group examined the levels of expression (qualitatively and quantitatively) of tumor endothelial markers (TEMs) in human colorectal cancer (Rmali et al. 2005). TEMs are a newly discovered family of endothelial markers associated with tumor-specific angiogenesis. TEM-2 and -6 expressions were found to be similar in tumor and normal tissues. Conversely, TEM-1, -7, -7R and -8 were found to be overexpressed in colon cancer tissues compared to normal tissues.

44.3.1 A Real-Time RT-PCR Protocol for the Enumeration of Endothelial-Specific Genes

Blood samples are lysed with NH4Cl to remove red cells and stored with guanidine isothiocyanate at –80°C for RNA analysis. Total RNA is extracted with the QIAamp RNA blood extraction kit (Qiagen, Chatsworth, CA). To generate cDNA, total RNA is treated with RNase-Free Dnase set (Qiagen) to remove any contaminating genomic DNA. The Dnase-treated RNA (100 ng) is then converted into cDNA by murine leukemia virus reverse transcriptase (Life Technologies, Inc., Bethesda, MD).

To perform quantitative real-time RT-PCR, Rabascio et al. (2004) used the primers and probes listed in Table 44.2. VE-cadherin and beta-actin primers were originally designed using Primer Express software (Applied BioSystems, Foster City, CA). Primers for Tie-2, CD133, and VEGFR2 (SYBR green) were published elsewhere (Upalakalin et al. 2003). For each PCR evaluation, 9 µl of cDNA (diluted 1:3 in nuclease-free water) or plasmid product (serial dilutions), 10 µl of Universal PCR Master Mix (Applied BioSystems), 250 nM forward and reverse primer and 200 nM probe were added to a final volume of 20 µl. Amplification and detection were performed with the ABI Prism 7000 Sequence Detection Systems (Applied BioSystems). The thermal cycle used was 2 min at 50°C, 10 min at 95°C, and 50 cycles of 15 seconds denaturation at 95°C with 1 min annealing at 60°C. To assure the specificity of each primer set, amplicons generated from PCR reactions were evaluated for specific melting point temperatures using the first-derivative primer melting curve software supplied by Applied BioSystems.

After real-time PCR reaction, the appropriate threshold was fixed to determine the amplification cycle for each sample (Ct). Data were then analyzed

Table 44.2. Probes and primers used in Rabascio's study (Rabascio et al. 2004)

Beta-actin	Forward primer: 5'-TCACCCACACTGTGCCCATCT-3' Reverse primer: 5'-CAGCGGAACCGCTCATTGCCA-3' Probe: 5'-VIC-ATGCCCTCCCCCATGCCATCCTG-TAMRA-3'
VE-cadherin	Forward primer: 5'-AAGCCCTACCAGCCCAAAGT-3' Reverse primer: 5'-TTGCGGAGATCTGCAGGAC-3' Probe: 5'-FAM-TGTGAGAACGCTGTCCATGGCCAG-TAMRA-3'
Tie-2	Forward primer: 5'-GCTTGCTCCTTTCTGGAACTGT-3' Reverse primer: 5'-CGCCACCCAGAGGCAAT-3'
VEGFR-2	Forward primer: 5'-CACCACTCAAACGCTGACATGTA-3' Reverse primer: 5'-GCTCGTTGGCGCACTCTT-3'
CD133	Forward primer: 5'-TGGATGCAGAACTTGACAACGT-3' Reverse primer: 5'-ATACCTGCTACGACAGTCGTGGT-3'

as follows, comparing studied gene Ct and respective control gene Ct.

- $\Delta C^t = C^t$ of studied gene–C^t of control gene (beta-actin)
- $\Delta\Delta C^t = \Delta C^t$–number of total cycles (50)
- Final value = $2^{\wedge}$ $(-\Delta\Delta C^t)$

This protocol can be used to identify new surrogate markers for angiogenesis by real-time PCR.

Conclusions

Although increasing evidence correlates CECs/CEPs with other markers of angiogenesis and vascular disease (Schneider et al. 2005), questions remain regarding their precise definition, biology and origin. They may be damaged, necrotic–apoptotic, or viable, and could possess procoagulant and/or pro-inflammatory properties. Their phenotype (particularly in the coming era of six-color flow cytometry) may provide useful information, and there is growing evidence that CECs/CEPs may well be a novel biomarker, the measurement of which will have utility in various clinical settings related to cancer (Blann et al. 2005) and vascular diseases (Werner et al. 2005). At the present time, progress is hampered by the diversity of methodologies used to detect CEC/CEPs. Nevertheless, the number, viability and kinetics of CECs/CEPs predicts the clinical outcome in a number of diseases, suggesting their possible predictive/patient stratification role in the clinic (Rosenzweig 2005).

References

Artavanis-Tsakonas S, Matsuno K, Fortini ME (1995) Notch signaling. Science 268:225–232

Bertolini F, Fusetti L, Mancuso P, Gobbi A, Corsini C, Ferrucci PF, Martinelli G, Pruneri G (2000) Endostatin, an antiangiogenic drug, induces tumor stabilization after chemotherapy or anti-CD20 therapy in a NOD/SCID mouse model of human high-grade non-Hodgkin's lymphoma. Blood 96:282–287

Bertolini F, Paul S, Mancuso P et al (2003) Maximum tolerable dose and low-dose metronomic chemotherapy have opposite effects on the mobilization and viability of circulating endothelial progenitor cells. Cancer Res 63:4342–4346

Blann AD, Woywodt A, Bertolini F et al (2005) Circulating endothelial cells: biomarker of vascular disease. Thromb Haemost 93:228–s35

Capillo M, Mancuso P, Gobbi A et al (2003) Continuous infusion of endostatin inhibits differentiation, mobilization and clonogenic potential of endothelial cell progenitors. Clin Cancer Res 9:377–382

Carmeliet P, Lampugnani MG, Moons L, Breviario F, Compernolle V, Bono F, Balconi G, Spagnuolo R, Oosthuyse B, Dewerchin M, Zanetti A, Angellilo A, Mattot V, Nuyens D, Lutgens E, Clotman F, de Ruiter MC, Gittenberger-de Groot A, Poelmann R, Lupu F, Herbert J-M, Collen D, Dejana E (1999) Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. Cell 98:147–157

Drancourt M, George F, Brouqui P, Sampol J, Raoult D (1992) Diagnosis of Mediterranean spotted fever by indirect immunofluorescence of Rickettsia conorii in circulating endothelial cells isolated with monoclonal antibody-coated immunomagnetic beads. J Infect Dis 166:660–663

Elshal MF, Khan SS, Takahashi Y, Solomon MA, McCoy JP Jr (2005) CD146 (Mel-CAM), an adhesion marker of endothelial cells, is a novel marker of lymphocyte subset activation in normal peripheral blood. Blood. 106:2923–2924

Folkman J (2003) Fundamental concepts of the angiogenic process. Curr Mol Med 7:643–651

Fornas O, Garcia J, Petriz J (2000) Flow cytometry counting of CD34+ cells in whole blood. Nat Med 6:833–836

Fürstenberger G, von Moos R, Senn H-J, Boneberg E-M (2005) Real-time PCR of CD146 mRNA in peripheral blood enables the relative quantification of circulating endothelial cells and is an indicator of angiogenesis. Br J Cancer 93:793–798

George F et al (1991) Cytofluorometric detection of human endothelial cells in whole blood using S-Endo 1 monoclonal antibody. J Immunol Methods 139:65–75

Grefte A, van der Giessen M, van Son W, The TH (1993) Circulating cytomegalovirus-infected endothelial cells in patients with an active CMV infection. J Infect Dis 167:70–277

Hlatky L, Hahnfeldt P, Folkman J (2002) Clinical application of antiangiogenic therapy: microvessel density, what it does and doesn't tell us. J Natl Cancer Inst 94:883–893

Kanda K, Ueda M, Futakuchi H, Yamaguchi H, Mori K, Terai Y, Ueki M (2005) Transcriptional expression of the genes implicated in angiogenesis and tumor invasion in cervical carcinomas. Gynecol Oncol 98:453–461

Kerbel R, Folkman J (2002) Clinical translation of angiogenesis inhibitors. Nat Rev Cancer 2:727–739

Khan SS, Solomon MA, McCoy JP Jr (2005 Detection of circulating endothelial cells and endothelial progenitor cells by flow cytometry. Cytometry B Clin Cytom 64:1–8. Review

Li C, Hampson IN, Hampson L, Kumar P, Bernabeu C, Kumar S (2000) CD105 antagonizes the inhibitory signaling of transforming growth factor beta1 on human vascular endothelial cells. FASEB J 14:55–64

Lin Y, Weisdorf D.J, Solovey A, Hebbel RP (2000) Origins of circulating endothelial cells and endothelial outgrowth from blood. J Clin Invest 105:71–77

Mancuso P, Burlini A, Pruneri G, Goldhirsch A, Martinelli G, Bertolini F (2001) Resting and activated endothelial cells are increased in the peripheral blood of cancer patients. Blood 97:3658–3661

Mancuso P, Colleoni M, Calleri A, Orlando L, Maisonneuve P, Pruneri G, Agliano A, Goldhirsch A, Shaked Y, Kerbel RS, Bertolini F (2006) Circulating endothelial cell kinetics and viability predict survival in breast cancer patients receiving metronomic chemotherapy. Blood 108:452–9

Monestiroli S, Mancuso P, Burlini A, Pruneri G, Dell'Agnola C, Gobbi A, Martinelli G, Bertolini F (2001) Kinetics and viability of circulating endothelial cells as surrogate angiogenesis marker in an animal model of human lymphoma. Cancer Res 61:4341–4344

Mutin M, Canavy I, Blann A, Bory M, Sampol J, Dignat-George F (1999) Direct evidence of endothelial injury in acute myocardial infarction and unstable angina by demonstration of circulating endothelial cells. Blood 93:2951–2958

Nieset JE, Redfield AR, Jin F, Knudsen KA, Johnson KR, Wheelock MJ (1997) Characterization of the interactions of alpha-catenin with alpha-actinin and beta-catenin/plakoglobin. J Cell Sci 110:1013–1022

Osborn L, Hession C, Tizard R. R, Vassallo C, Luhowskyj S, Chi-Rosso G, Lobb R (1989) Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. Cell 59:1203–1211

Patel NS, Li J-L, Generali D, Poulsom R, Cranston DW, Harris AL (2005) Up-regulation of Delta-like 4 ligand in human tumor vasculature and the role of basal expression in endothelial cell function. Cancer Res 65:8690–8697

Peichev M et al (2000) Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. Blood 95:952–958

Philpott NJ, Turner AJC, Scopes J, Westby M, Marsch JCW, Gordon-Smith EC, Dalgleish AG, Gibson FM (1996) The use of 7-amino actinomycin D in identifying apoptosis: simplicity of use and broad spectrum of application compared with other techniques. Blood 87:2244–2251

Prandini MH, Dreher I, Bouillot S, Benkerri S, Moll T, Huber P (2005) The human VE-cadherin promoter is subjected to organ-specific regulation and is activated in tumour angiogenesis. Oncogene 24:2992–3001

Rabascio C, Muratori E, Mancuso P, Calleri A, Raia V, Foutz T, Cinieri S, Veronesi G, Pruneri G, Lampertico P, Iavarone M, Martinelli G, Goldhirsch A, Bertolini F (2004) Assessing tumor angiogenesis: increased circulating VE-cadherin RNA in patients with cancer indicates viability of circulating endothelial cells. Cancer Res 64:4373–4377

Rafii S (2000) Circulating endothelial precursors: mystery, reality, and promise. J Clin Invest 105:17–19

Rmali KA, Puntis MCA, Jiang WG (2005) Prognostic values of tumor endothelial markers in patients with colorectal cancer. World J Gastroenterol 11:1283–1286

Rosenzweig A (2005) Circulating endothelial progenitors cells as biomarkers. N Engl J Med 353:1055–1057

Salven P et al (2003) VEGFR-3 and CD133 identify a population of CD34+ lymphatic/vascular endothelial precursor cells. Blood 101:168–170

Schneider M, Tiwa M, Carmeliet P (2005) A surrogate marker to monitor angiogenesis at last. Cancer Cell 7:3–4

Schuch G, Heymach J, Nomi M et al (2003) Endostatin inhibits the vascular endothelial growth factor-induced mobilization of endothelial progenitor Cancer Res 63:8345–8350

Shaked Y, Bertolini F, Man S et al (2005) Genetic heterogeneity of the vasculogenic phenotype parallels angiogenesis: implications for cellular surrogate markers and analysis of antiangiogenesis. Cancer Cell 7:101–111

Solovey A, Lin Y, Browne P, Choong S, Wayner E, Hebbel RP (1997) Circulating endothelial cells in sickle cell anemia. N Engl J Med 337:1584–1590

St Croix B, Rago C, Velculescu V, Traverso G, Romans KE, Montgomery E, Lal A, Riggins G.J, Lengauer C, Vogelstein B, Kinzler KW (2000) Genes expressed in human tumor endothelium. Science 289:1197–1202

Upalakalin JN, Roberts MJ, Kocher O, Benjamin LE (2003) Blood markers for vasculogenesis increase with tumor progression in patients with breast carcinoma. Cancer Biol Ther 2:255–256

Weidner N (2000) Angiogenesis as a predictor of clinical outcome in cancer patients. Hum Pathol 31:403–405

Werner N, Kosiol S, Schiegl T et al (2005) Circulating endothelial progenitor cells and cardiovascular outcomes. N Engl J Med 353:999–1007

Willett CG, Boucher Y, di Tomaso E et al (2004) Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. Nat Med 10:145–147

Vascular Disrupting Agents in Cancer Therapy

45

GILLIAN M. TOZER, CHRYSO KANTHOU, DAVID J. CHAPLIN

Abstract

Vascular disrupting agents (VDAs) are distinguished from anti-angiogenic agents by their ability to cause a catastrophic vascular collapse in tumour tissue within minutes to hours of drug administration, leading to extensive tumour cell necrosis. Notwithstanding these effects, anti-angiogenic effects of VDAs can also be seen when they are administered in chronic dosing schedules. The largest group of VDAs is the tubulin-binding, microtubule-depolymerising combretastatins, with CA-4-P the lead compound. DMXAA is the other notable VDA currently in clinical trials. CA-4-P and DMXAA have different primary targets but are likely to have some mechanisms in common, notably involving the actin cytoskeleton. Extended vascular shut-down, which is necessary for tumour cell kill, requires a complex series of events which is only partially understood at present. Understanding and circumventing development of treatment resistance is also an important challenge. Nevertheless, phase I and II clinical trials have produced encouraging results for current VDAs and this approach holds the promise of providing a valuable new treatment choice to complement existing cancer therapy.

CONTENTS

Introduction

Therapeutic vascular targeting of tumours has concentrated on anti-angiogenic approaches, which aim to prevent the neovascularisation processes in tumours. A conceptually distinct approach is to target the established blood vessels in tumours, with the aim of causing a selective shut-down of tumour blood flow sufficient to induce extensive secondary tumour cell death. This anti-vascular approach has already achieved considerable success, with several low-molecular-weight drugs entering clinical trials in the past few years. The vascular shut-down that occurs following treatment with these drugs is extremely rapid, beginning within minutes of drug administration. Drugs that have these vascular disrupting properties are often known as vascular disrupting agents or VDAs. Relatively large intermittent doses of VDAs are usually required to produce their tumour vascular disrupting effects but anti-angiogenic effects may also be revealed if they are administered in a repeated dosing schedule.

The largest group of low-molecular-weight VDAs is the tubulin-binding combretastatins, which are structurally related to the classical tubulin-binding agent, colchicine. Several other classes of drugs have tumour vascular disrupting properties, including several compounds that were originally developed as anti-angiogenic agents. Development of novel targets for inducing tumour vascular disruption is also a thriving area of research. This chapter aims to provide an overview of the VDAs in development, a summary of the mechanisms of action of current VDAs, focusing on the combretastatins, the characteristics of the tumour vasculature that dictate susceptibility, and current clinical progress.

Current VDAs

45.2.1 Microtubule Depolymerising Agents

The classic tubulin-binding agent, colchicine (Fig. 45.1), is too toxic for clinical use but is long recognised as having tumour vascular damaging properties. Tubulin is also the target for a number of established anticancer drugs directed at tumour cells, including vincristine and vinblastine. These drugs bind to tubulin and prevent its polymerisation to form microtubules, thereby interfering with a range of important cellular processes, including mitosis and migration. In addition to cytotoxic effects against tumour cells, profound tumour vascular effects have been reported for vincristine and vinblastine but only at close to their maximum tolerated doses.

The Cape Bushwillow tree *Combretum caffrum* was the natural source of 17 combretastatins, some of which were moderately effective against tumour cells in vitro. Consequently, a soluble sodium phosphate salt of the most potent of them, combretastatin A-4 (CA-4), was developed (Pettit et al. 1995). The phosphated pro-drug, CA-4-P (disodium combretastatin A-4 3-0-phosphate; Fig. 45.1), is readily administered in vivo, where it is rapidly cleaved to CA-4 by the action of endogenous non-specific phosphatases. CA-4 binds to tubulin at or close to the colchicine binding site and has tumour vascular damaging effects at well-tolerated doses of CA-4-P in animal models. CA-4-P is now being developed as CA4 Prodrug by OXiGENE Inc. The relative lack of toxicity of CA-4-P most likely relates to a short plasma half-life for CA-4 and reversible binding kinetics, as opposed to the pseudo-irreversible binding of colchicine. This means that tissues are exposed to the drug for a relatively short time, which is nevertheless sufficient to cause vascular shut-down in susceptible tumour blood vessels. In animal models, this can be achieved within 20 min of drug exposure (Tozer et al. 2001).

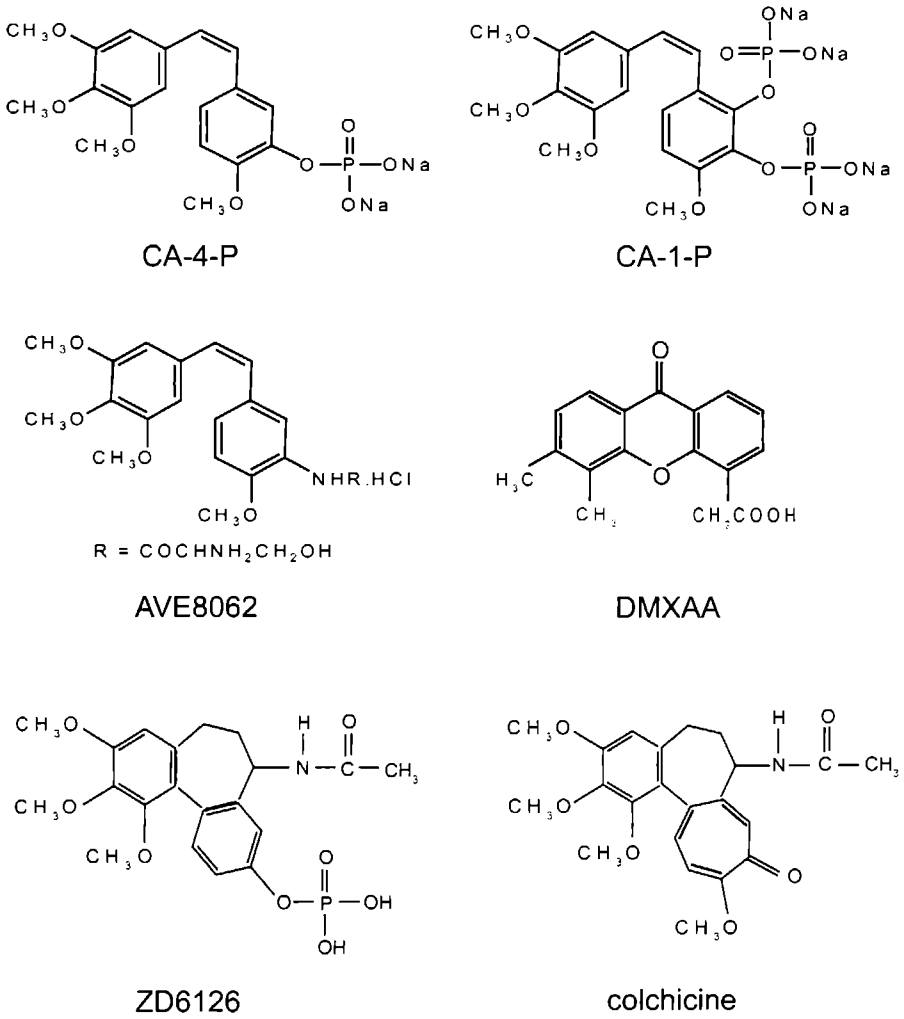


Fig. 45.1. Chemical structures of various low-molecular-weight vascular disrupting agents. CA-4-P and CA-1-P are the soluble phosphate prodrugs of the natural combretastatins, CA-4 and CA-1, respectively. AVE8062 is a synthetic combretastatin, which is also a prodrug. DMXAA is a derivative of flavone acetic acid. ZD6126 is a phosphate prodrug of N-acetyl colchinol, a derivative of colchicine. The structure of colchicine is shown for comparision

A second combretastatin, combretastatin A-1 (CA-1) (Pettit and Lippert 2000), is also being developed as the sodium phosphate salt, CA-1-P (Fig. 45.1), by OXiGENE Inc. (designated OXI4503). CA-1-P is more potent than CA-4-P in pre-clinical models and both compounds are currently being tested in clinical trials. A derivative of the combretastatins, the Aventis Pharma compound AVE8062 (Fig. 45.1), is also in clinical trials. This is also a pro-drug, the serine of which is cleaved by aminopeptidases to form the active component (Hori et al. 1999).

Other tubulin-binding agents with profound vascular effects in solid tumours include Auristatin PE (TZT1027), which is derived from dolastatin 10 (isolated from a marine mollusc) (Otani et al. 2000) and arsenic trioxide (Trisenox, Cell Therapeutics Inc.), which has many other effects as well as tubulin-binding properties and is most commonly used for the treatment of patients with acute promyelocytic leukaemia (APL) (Lew et al. 1999). An analogue of colchicine, N-acetylcolchinol-O-phosphate (ZD6126, Fig. 45.1), is undergoing pre-clinical and clinical testing by AstraZen-

eca (Davis et al. 2000), as is the compound ABT-751 at Abbott (Segreti et al. 2004). Newer microtubule disrupting agents undergoing pre-clinical testing that are active at or near the colchicine binding site include a series of chromenes developed by Maxim Pharmaceuticals Inc, (lead compound MX-116407) (Kasibhatla et al. 2004) and a diketopiperazine, NPI-2358, developed by Nereus Pharmaceuticals (http://www.nereuspharm.com). In addition to these agents, numerous synthetic analogues of the combretastatins are currently under development.

45.2.2 Flavonoids

DMXAA (5,6-dimethylxanthenone-4-acetic acid; Fig. 45.1) and FAA (flavone acetic acid) are members of a series of drugs, structurally distinct from the combretastatins, whose primary site of action is unknown but which have multiple antivascular actions including the induction of cytokines. Initial work found that FAA induced extensive haemorrhagic necrosis and reduced blood flow in animal tumour models, leading to the development of derivatives, one of which, DMXAA, is 16 times more potent than FAA and active in human tumours (Baguley 2003). DMXAA is being further developed as AS1404 by Antisoma plc (UK) and is currently in clinical trials.

45.2.3 Junctional Protein Disrupting Agents

Monoclonal antibodies targeted to the junctional protein VE-cadherin (vascular endothelial cadherin) are active against the tumour vasculature (Corada et al. 2002). N-cadherin (neural cadherin) is also involved with the structural integrity of blood vessels and its down-regulation prevents cell aggregation and induces apoptosis (Blaschuk and Rowlands 2000). In particular, N-cadherin is directly involved in regulating adhesion between the endothelial cells and pericytes of blood vessels. The novel cyclic pentapeptide N-cadherin inhibitor Exherin (ADH-1), developed by Adherex, is in phase I clinical trials and has been shown to cause haemorrhage in animal tumour models (http://www.adherex.com/). Exherin contains the cadherin cell adhesion recognition sequence His-Ala-Val, and low-molecular-weight antagonists of N-cadherin are currently under development.

45.2.4 Receptor Tyrosine Kinase Inhibitors

Vascular endothelial growth factor (VEGF) is a ubiquitous angiogenic growth factor in solid tumours and a major target for anti-angiogenic cancer therapy. A number of small-molecule VEGF receptor kinase inhibitors, with different inhibitory profiles, are now in clinical trials. VEGF acts as a survival factor for endothelial cells and its withdrawal can cause vascular damage. In addition, VEGF is a vasodilator, involving the synthesis/release of nitric oxide. As such, inhibitors of VEGF activity are likely to possess vascular disrupting, as well as anti-angiogenic, properties. Some vascular shut-down has been noted in spontaneous pancreatic islet tumours in transgenic (RIP-Tag2) mice, within 24 h of treatment with the Pfizer compound AG013736, a broad-spectrum receptor tyrosine kinase inhibitor (Inai et al. 2004). However, it is unlikely that inhibition of VEGF function has the very rapid tumour vascular disrupting properties of the other compounds discussed above. Vascular disruption may rely on the more protracted process of endothelial cell apoptosis, although this possibility remains to be investigated.

45.2.5 Vasoactive Agents

Tumours are exquisitely dependent on nitric oxide for maintenance of blood flow, and nitric oxide inhibitors can induce selective vascular shut-down in animal tumours. In certain dose schedules, this can also be translated into significant

tumour growth retardation, although this may be due to an anti-angiogenic rather than a vascular disrupting effect. CA-4-P and AVE8062 are clearly vasoconstrictive in tumour arterioles, and other vasoconstrictors acting at the same level of the microcirculation, such as epinephrine (adrenaline), can cause prolonged and selective tumour vascular shut-down. Other vasoactive agents such as the endothelin-1 agonist IRL1620 have potential for selective reduction of tumour blood flow but remain to be fully investigated.

45.2.6 Other Biological VDAs

Various peptides, antibodies, antibody fragments or growth factors are being designed to selectively bind to tumour blood vessels and induce coagulation and/or endothelial cell death (Thorpe 2004). As for the low-molecular-weight VDAs, these compounds are designed to induce rapid tumour vascular shut-down, leading to extensive tumour cell necrosis. Radio-isotopes, cytotoxic drugs and tissue factor for inducing coagulation have been conjugated to antibodies as vascular damaging strategies. Tumour endothelial cell targets that are currently being investigated include endoglin, integrins and VEGF receptors. In addition, a peptide fragment of fibrinogen, termed alphastatin, has been shown to possess some tumour vascular disrupting properties (Staton et al. 2004). Methods for identifying unique signatures on the tumour vasculature, which have the potential as targets for vascular disruption, include powerful bio-informatic analyses of published data and comparative analysis of gene expression on endothelial cells isolated from tumours and corresponding normal tissue (Neri and Bicknell 2005). Several putative targets have been identified, including the vascular developmental gene Robo4 and the tumour endothelial markers (TEMs). As well as developing armed peptides/antibodies to these targets, some of them may have a role in maintenance of tumour blood flow, with the potential for developing low-molecular-weight inhibitory VDAs.

Rapid Effects of VDAs

Hypotheses for the mechanism of action of VDAs have to account for their very rapid effects, initiated within minutes of drug administration (Fig. 45.2). Drug-induced vascular endothelial cell death is too slow a process to account for these rapid changes. Effective agents cause a prolonged period of vascular shut-down culminating in tumour cell death, manifested by extensive haemorrhagic tumour necrosis.

For CA-4-P, rapid vascular shut-down in vivo is likely to be associated with the morphological and functional changes in the endothelial cytoskeleton that occur in endothelial cells in vitro within minutes of exposure to this agent (Fig. 45.3; Kanthou and Tozer 2002). These include cell shape changes, assembly of actin stress fibres and actinomyosin contractility, formation of focal adhesions, disruption of cell–cell junctions, including those involving VE-cadherin, and an increase in monolayer permeability to macromolecules. In a subpopulation of cells, additional effects involve F-actin accumulation into surface blebs, with cells rounding up and stress fibres misassembling into a spherical band surrounding the cytoplasm, accompanied by malformed focal adhesions. The trigger for these changes is disruption of interphase microtubules following drug binding, which in turn leads to rapid cell signalling between the tubulin and actin cytoskeletons (Kanthou and Tozer 2002). Preliminary studies suggest that DMXAA also affects the endothelial cytoskeleton, resulting in a partial dissolution of actin filaments. However, unlike CA-4-P, the trigger does not appear to be disruption of interphase microtubules, which remain intact.

In vivo, an immediate effect of CA-4-P is to increase tumour vascular permeability to macromolecules, as calculated from the leakage kinetics of intravenously administered labeled dextrans (Fig. 45.4; see also Tozer et al. 2005). This is consistent with the in vitro data and may lead to a decrease in blood flow via a number of mechanisms. Firstly, protein leakage into the tumour interstitium disturbs the oncotic pressure differential between the

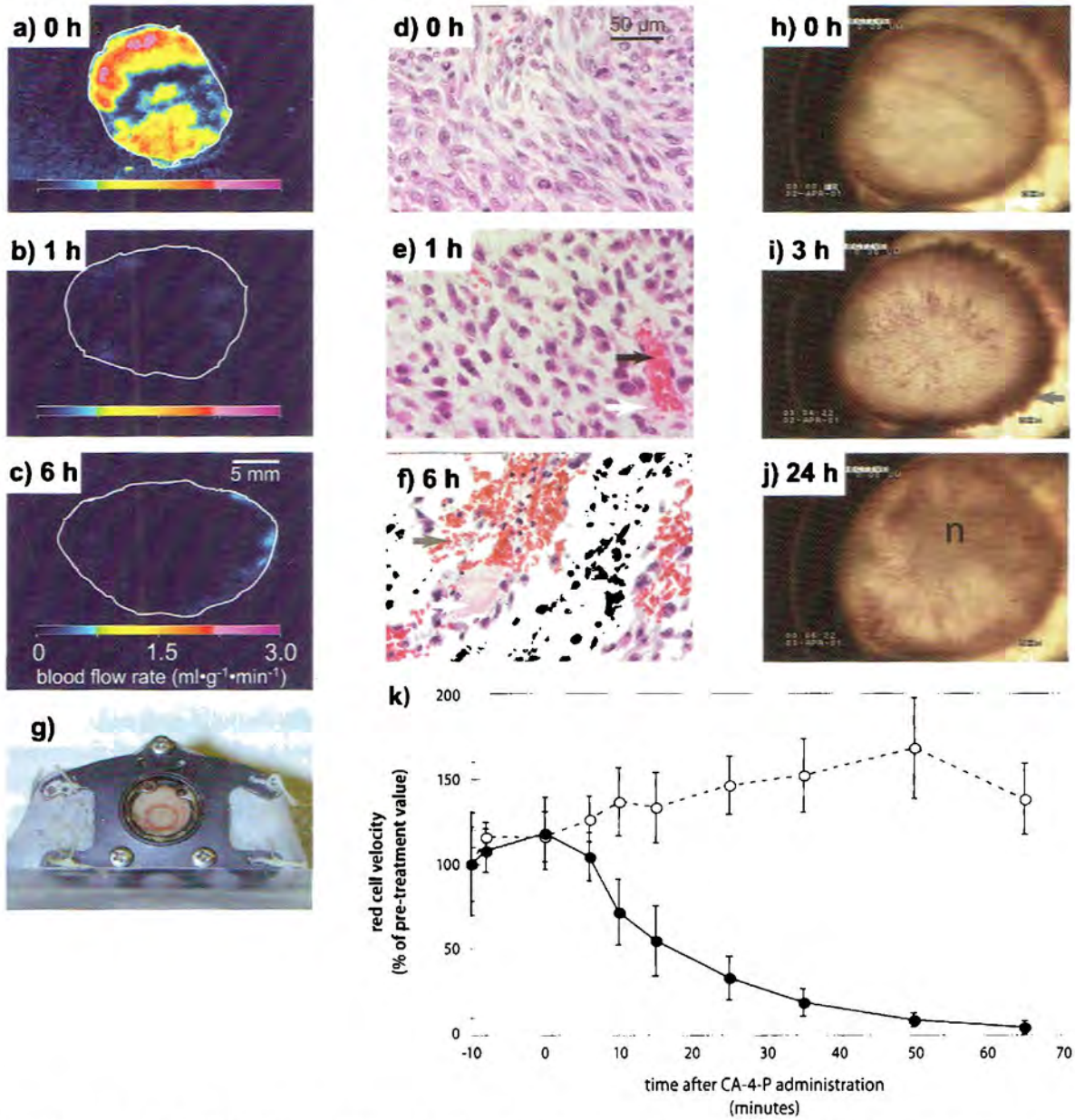


Fig. 45.2a–k. Rapid effect of a single moderate dose of CA-4-P on tumour blood flow rate in a rat sarcoma model. Times represent time after drug administration (10 mg/kg, approximately equivalent to the maximum tolerated dose in man). a–c Pseudo-colour images of blood flow rate computed from autoradiograms of 20-µm frozen sections from excised P22 tumours (implanted subcutaneously) following intravenous administration of the blood flow marker, 14C-labelled iodo-antipyrine (see Tozer et al. 1999). d–f Morphological images obtained from haematoxylin and eosin-stained 3-µm paraffin-embedded sections from a separate group of subcutaneous P22 tumours. *Black arrow*, congested vessel; *grey arrow*, haemorrhage; *white arrows*, coagulation. g Photograph of a tumour growing within a surgically implanted 'window' chamber for h–j intravital microscopy of tumour blood vessels. *Arrow*, haemorrhage; *n*, necrosis. Tumour is approximately 3 mm in diameter. k Red cell velocity measured in P22 tumours growing within window chambers

inside and outside of blood vessels, which may, in turn, disturb the hydrostatic pressure differential, leading to vessel narrowing. There is also some evidence that loss of fluid as blood passes through the tumour tissue bed leads to an increased haematocrit and consequently to increased blood viscosity and resistance to flow. If rounding up of tumour endothelial cells occurs in vivo, this could contribute to early vascular shut-down via a direct increase in geometric resistance to flow, as could the direct vasoconstrictive effect on tumour-supplying arterioles reported for CA-4-P and AVE8062 (Hori and Saito 2003; Tozer et al. 2001).

In addition to these primary effects, the organisation of tumour blood vessels, which generally contain numerous cross-links, leads to reduced efficiency and in many cases can result in catastrophic regional failure as a response to decreased flow rates. As blood flow falls below a critical level, red cells stack together to form rouleaux, increasing viscous resistance to flow and leading to further blood stagnation.

Signal Transduction Pathways

45.4.1 Vascular Permeability

Investigations of the signalling events that control vascular permeability are critical for understanding the initial rapid collapse of tumour blood flow in vivo. The signalling processes in endothelial cells that control vascular permeability are highly complex, endothelial paracellular permeability depending on both the integrity of intercellular junctions and actinomyosin contractility. Following exposure to CA-4-P, microtubule disruption triggers GTPase Rho- and Rho kinase-dependent signalling pathways that lead to the increased actin polymerisation, stress fibre formation, actinomyosin contractility, focal adhesion assembly and redistribution of junctional proteins described above. The importance of Rho and related proteins in the control of vascular permeability is well known, and the Rho inhibitor C3 exoenzyme or the Rho-kinase inhibitor Y-27632 attenuate both the development of these morphological changes in the actin cytoskeleton and CA-4-P-induced increase in endothelial monolayer permeability to macromolecules, thus providing a link between permeability and cytoskeletal remodelling (Kanthou and Tozer 2002). A further link is established from evidence that stabilisation of VE-cadherin / -catenin homophilic interactions in endothelial cells via gene transfer inhibits the CA-4-P-induced increase in monolayer permeability (Vincent et al. 2005). Exposure of NZM7 melanoma cells, cultured on Matrigel to induce expression of VE-cadherin, to DMXAA results in down-regulation of the VE-cadherin gene. Although this effect is not rapid enough to account for initial blood flow changes, it is further evidence for VE-cadherin being a target for VDA treatment. Phosphorylation of myosin light chain (MLC) in endothelial cells mediates the actinomyosin contractility and formation of focal adhesions and occurs within minutes of CA-4-P exposure, probably via Rho-kinase acting directly as a novel endothelial MLC kinase enzyme (Kanthou and Tozer 2002).

In addition to these pathways, CA-4-P rapidly activates stress-activated protein kinase-2/p38 (SAPK/p38), which is associated with the blebbing morphology of endothelial cells exposed to CA-4-P that can contribute to breakdown of endothelial cell integrity (Kanthou and Tozer 2002). Currently, little is known about these signal transduction pathways in vivo or the influence of tumour microenvironmental factors.

45.4.2 Endothelial Cell Death

Endothelial cell death is often erroneously considered to be a prerequisite for tumour vascular shut-down in vivo, even though it is too slow a process to account for the rapid vascular effects that characterise the lead VDA, CA-4-P. Nevertheless, extended exposure

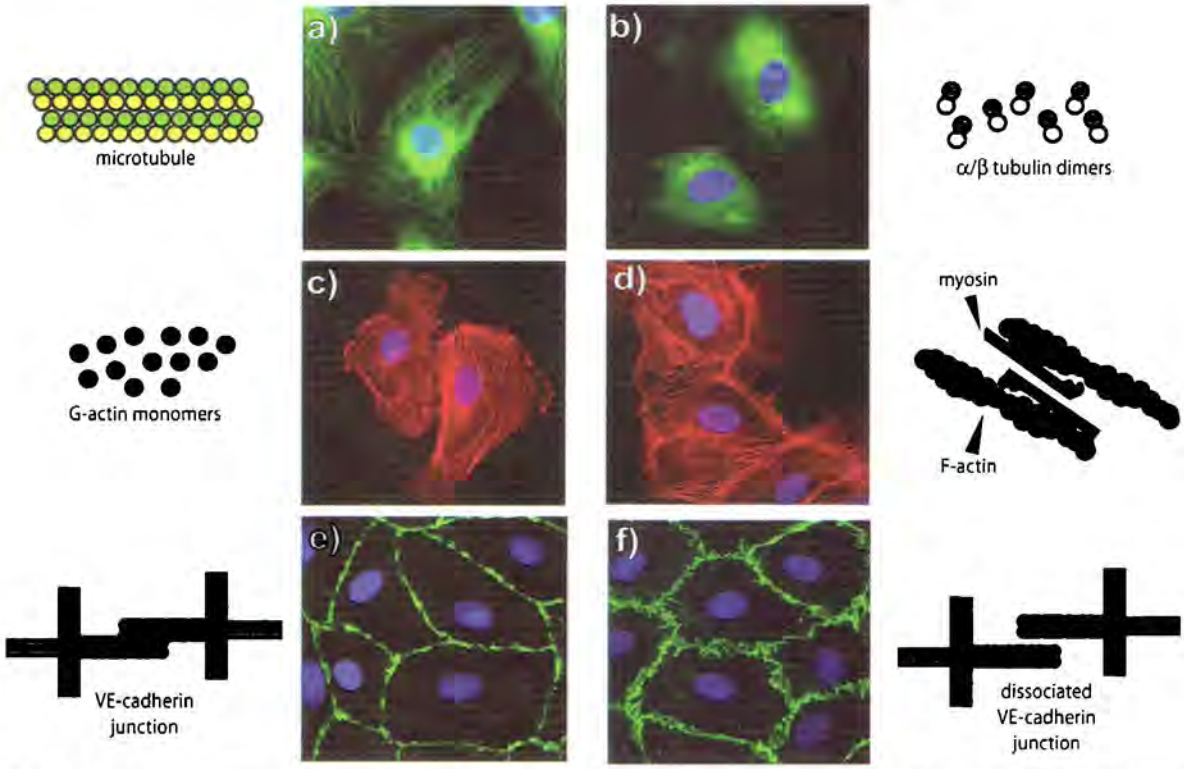


Fig. 45.3a–f. Photomicrographs of human umbilical vein endothelial cells (HUVECs) with or without CA-4-P treatment (100 nM, 30 min) and diagrams indicating associated cytoskeletal and junctional changes. Microtubules consist of alpha and beta tubulin heterodimers, which assemble together to form insoluble polymeric cylindrical structures. Disassembly of microtubules into soluble tubulin heterodimers occurs very rapidly in response to CA-4-P (a, b): immunofluorescence staining of β-tubulin. Actin is a globular protein (G-actin), which undergoes polymerisation to form filamentous actin (F-actin). Actin remodelling occurs in response to microtubule disruption by CA-4-P, resulting in the formation of contractile actin stress fibers, bundles of cross-linked F-actin interacting with phosphorylated myosin (c, d): staining of F-actin with fluorescent phalloidin. Endothelial adherens junctions are specialised cell–cell junctions, consisting of VE-cadherin protein, which control adhesion and permeability. Adherens junctions are disrupted in response to CA-4-P (e, f): immunofluorescence staining of VE-cadherin

to CA-4-P is clearly toxic to endothelial cells in vitro and therefore, if drug exposures are sufficient, endothelial cell death could contribute to the ultimate extent of vascular shut-down in vivo by preventing subsequent angiogenesis. For this reason, considerable effort has been made to elucidate the CA-4-P-induced signalling pathways that lead to endothelial cell death.

Extensive disruption of interphase microtubules in endothelial cells can lead to a rapid form of necrotic cell death, in which the blebbing morphology described above is an early manifestation (Kanthou and Tozer 2002). There is also evidence that disruption of VE-cadherin junctions by CA-4-P is downstream of a cell death pathway mediated by inhibition of PI3 K/Akt signalling (Vincent et al. 2005). However, mitotic spindles are at least as sensitive to CA-4-P as interphase microtubules, leading to anti-proliferative effects of the drug, which are linked to a cell death pathway (Kanthou et al. 2004). There is some confusion in the literature regarding the precise pathways involved here. However, most investigators agree that the primary cell death pathway in proliferating endothelial cells occurs via a caspase-independent mecha-

nism, which is nevertheless linked to apoptosis and dependent upon mitotic arrest. That is, death occurs as cells attempt to leave mitosis, such that relatively long drug exposures of many hours are required for significant cells to accumulate in mitosis and subsequently die.

45.5 Susceptibility of the Tumour Vasculature to VDAs

45.5.1 Comparison with Normal Tissues

Current VDAs produce a much greater blood flow reduction in tumours than in normal tissues (Tozer et al. 1999), and this forms the basis for their acceptance into clinical trials. Many tumours are characterised by regions of necrosis and hypoxia, which suggests that the blood supply is barely adequate to support tumour growth. Indeed tumour blood flow is characteristically highly heterogeneous, with blood in some vessels being practically stationary and/or periodically reversing in flow direction. Morphologically too, tumour blood vessels appear fragile and susceptible to disruption. Primarily, they are sinusoidal in appearance, with poor development of the vascular wall, often comprising of endothelial cells with poor cell–cell contacts and abnormal basement membrane. Mural support cells, in the form of pericytes, may be present but often make only poor contact with the vessel. Interestingly, in the rat, the normal spleen is relatively susceptible to blood flow reduction induced by CA-4-P, and this may be related to the sinusoidal nature of splenic blood vessels.

The precise reasons for tumour susceptibility to tubulin-binding agents such as CA-4-P remain

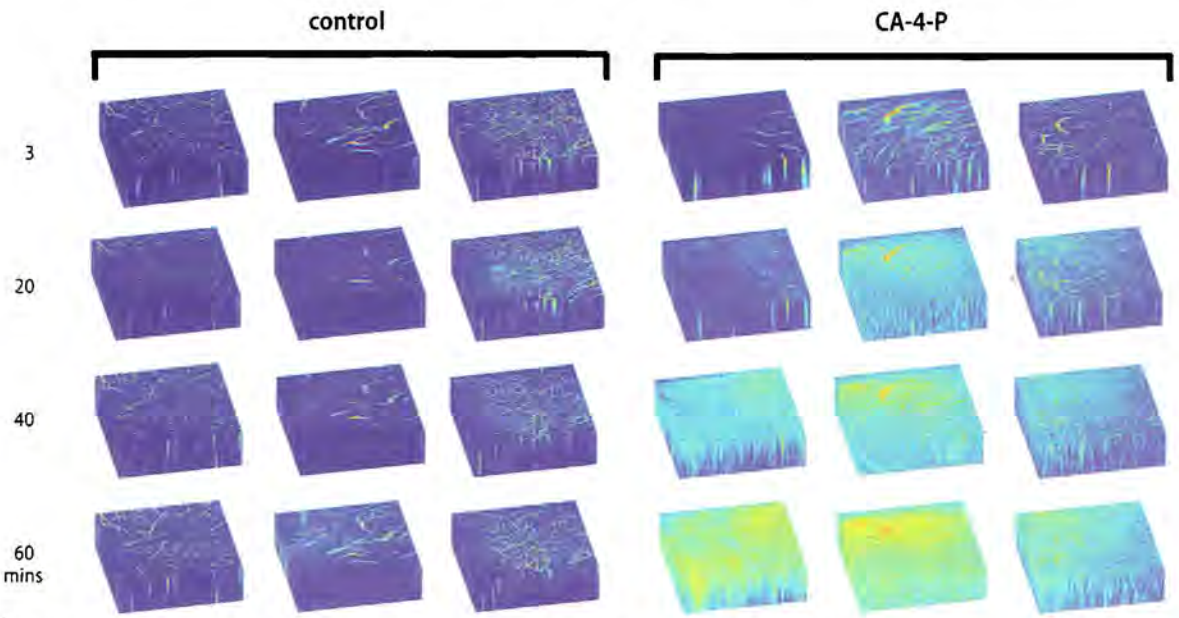


Fig. 45.4. CA-4-P (30 mg/kg) increases the leakage rate of 40-kDa dextran labelled with fluorescein isothiocyanate (FITC) from the bloodstream into tumour tissue. P22 rat sarcomas were grown in window chambers as shown in Fig. 45.2 and imaged at various times (as indicated) after intravenous administration of FITC-dextran with or without CA-4-P. 3D images, with no out-of-focus fluorescence, were obtained using multi-photon fluorescence microscopy (see Tozer et al. 2005). Each column represents images from a single tumour. Images courtesy of Dr Carlos Reyes-Aldosoro, University of Sheffield, UK

unclear. The relatively high endothelial proliferation rate in tumours is often cited as the crucial factor in tumour blood vessel sensitivity, but this is not as obvious as it might appear. Clearly, proliferating endothelial cells would be susceptible to cytotoxic effects of the drug, which we have seen are primarily dependent on cells entering mitosis. However, it is unclear how proliferative status would impact on the rapid blood flow shut-down that occurs in tumours but not in normal tissues and is the hallmark of vascular disrupting agents.

Other factors that may influence endothelial response to tubulin-binding agents, such as CA-4-P, include differential expression of tubulin isotypes, tubulin mutations, post-translational modifications of tubulin and types of microtubule-associated proteins (MAPs). These processes may well be influenced by the tumour microenvironment and contribute to tumour susceptibility to tubulin-binding agents. A recent study demonstrated differential expression of tubulin isotypes in several tumour cell lines cloned for their different degrees of resistance to CA-4-P (Wehbe et al. 2005). However, such variations within the tubulin cytoskeleton are unlikely to explain the susceptibility of the tumour vasculature to other VDAs such as DMXAA.

As we have seen, CA-4-P disrupts the integrity of endothelial cell junctions, especially those involving VE-cadherin. Furthermore, stabilisation of VE-cadherin interactions can prevent CA-4-P-induced increases in endothelial monolayer permeability in vitro. Endothelial cell–cell contacts are often poor in tumour blood vessels, suggesting the possibility that these may be further disrupted by VDAs more easily than the more organised junctions in normal tissues. A combination treatment of CA-4-P and a neutralising antibody to VE-cadherin in an animal tumour model produced better tumour growth retarding effects than either agent alone (Vincent et al. 2005), although it remains to be seen whether this is a synergistic effect or simply an additive one.

Other possible explanations for tumour blood vessel susceptibility to VDAs at the endothelial cell level focus on the tumour microenvironment and its influence on the cell signalling events described above. The most well known feature of the tumour microenvironment, severe hypoxia combined with hypoglycaemia, can induce endothelial monolayer permeability in vitro, linked to stimulation of various signalling pathways such as those involving protein kinase C (PKC), cGMP-dependent protein kinase (PKG) and p38. However, the interaction with VDAs is unknown.

The sensitivity of the tumour vasculature to VDAs may reside not at the level of the initial damaging insult but at the level of the response of the tissue to that insult (or a combination of the two). Factors such as heterogeneous blood flow rates, high vascular permeability and high interstitial fluid pressure are obvious candidates for exacerbating the initial vascular damaging effect. In tumour regions, where blood flow is already low, a further VDA-induced decrease in flow may have more dramatic effects than in normal tissues, partly because of the rapid rise in blood viscosity that occurs at very low flow rates. An increase in vascular permeability may also be catastrophic in a tumour, where the permeability and interstitial fluid pressure are already high. The maturity of the vascular wall, in terms of factors such as investment with pericytes, has an influence on vascular permeability. In addition, the presence of pericytes may confer stability in response to a damaging insult. There is some evidence that both pre-treatment vascular permeability and the maturity of blood vessel walls influence the tumour vascular response to CA-4-P in animal models.

Pharmacokinetics can also exacerbate the difference in the final damage inflicted on tumour versus normal tissue vasculature. Exposure to drug is undoubtedly higher in tumours than it is in normal tissues because of self-trapping as blood flow decreases and potential differences in phosphatase activity between tumour and normal tissue endothelial cells (Tozer et al. 1999).

Progress is now being made to elucidate the factors predicting blood vessel susceptibility to VDAs. This will enable selection of appropriate patients for current VDA treatment and open up new avenues for research into novel ways of targeting the established tumour vasculature.

45.5.2 Intratumoral Heterogeneity

A characteristic of all VDAs that have been tested in animal models is that they are more effective in the tumour centre than in the tumour periphery, initially in terms of the primary blood flow reduction achieved and consequently in terms of the extent of tumour necrosis (Fig. 45.5). This may also be the case in human tumours (Galbraith et al. 2003). The extent of VDA-induced tumour necrosis is dose dependent, and it has proved exceptionally difficult to eliminate tumour cells in the most peripheral tumour rim, such that at doses approaching the maximun tolerated, a viable tumour rim, a few cells wide, persists to re-populate the tumour after VDA treatment. This characteristic accounts for the fact that most VDAs given as single agents, produce only moderate effects on tumour growth; they need to be administered in combination with an appropriate second treatment to be effective.

The cause of the resistant tumour rim is intriguing. Although it is tempting to speculate that tumour cells residing in the tumour periphery acquire oxygen and nutrients from the surrounding undamaged normal tissue, thus surviving VDA treatment, this does not explain why blood flow is less compromised in these peripheral regions in the first place. Two important factors are likely to be interstitial fluid pressure and the vascular architecture in the two regions. Interstitial fluid pressure rises precipitously from the tumour periphery to the tumour centre, such that an increase in vascular permeability at the tumour centre may be catastrophic, whereas it is tolerated at the periphery. Small-calibre vessels are also more sensitive to shut-down than larger ones, and the proportion of these is often far higher at the centre than at the periphery. A complex vascular plexus often exists at the tumour periphery, compared with a rarefaction of the vascular bed at the tumour centre, so that in the event of extensive vascular damage, a residual flow is likely to persist at the periphery rather than at the centre. Indeed, this situation is often directly observed using microscopical techniques.

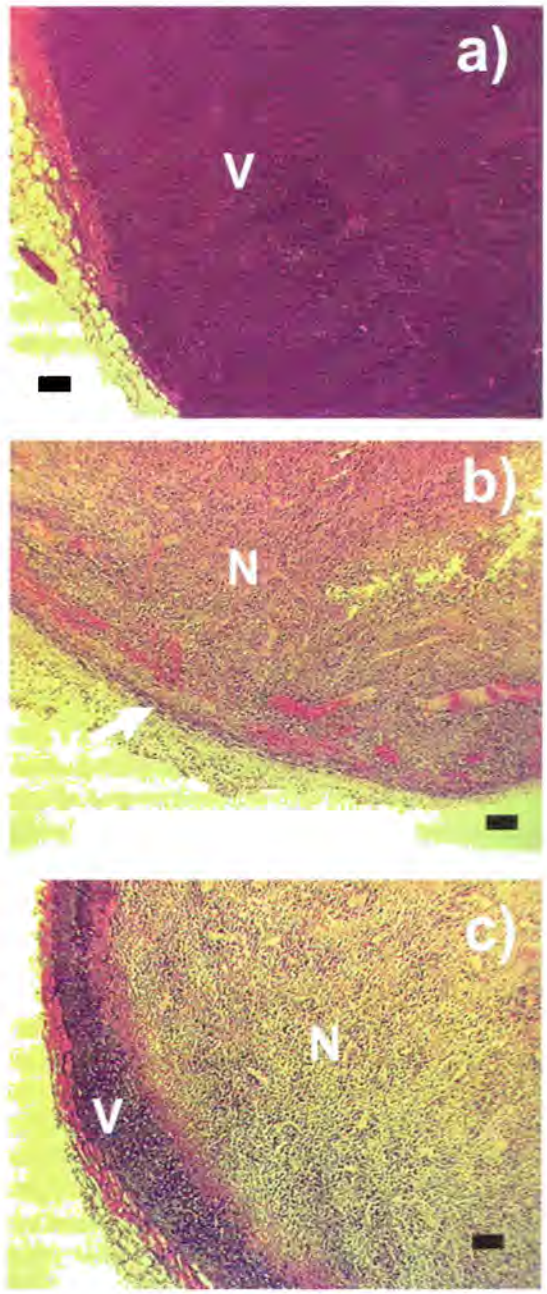


Fig. 45.5a–c. Tumour regrowth from the periphery, which is resistant to VDA treatment. Histological sections (stained with haematoxylin and eosin) from mouse mammary carcinoma (CaNT). a Control, untreated tumour; b, c 24 h and 72 h, respectively, after a single 100-mg/kg intraperitoneal dose of CA-4-P. V, viable tissue; N, necrotic tissue. *Scale bars* 200 μm. Images courtesy of Dr Sally Hill, Gray Cancer Institute

Prolonged Effects of VDAs

Following initial vascular shut-down, a whole series of events contribute to determining the duration of the shut-down and the amount of induced tumour cell necrosis. These include platelet activation, coagulation, haemorrhage, leukocyte–endothelial interactions, and rheological and anti-angiogenic effects. The induction of tumour haemorrhagic necrosis requires sustained inhibition of blood flow and this may be achieved in a number of ways.

45.6.1 Influence of Blood Cells

CA-4-P is active in tumours perfused ex vivo, with a saline-based perfusate, supporting the view that the primary event in vascular shut-down is a direct effect on the tumour vasculature (Tozer et al. 1999). However, a much larger vascular effect occurs in tumours under in vivo conditions, implicating an additional role for blood cells and/or systemic effects. In normal tissue inflammation, neutrophil adhesion to the endothelium, with subsequent generation of damaging oxidising species via the action of neutrophilic myeloperoxidase (MPO), causes vascular damage. Following treatment with CA-4-P, there is an up-regulation of adhesion molecules such as E-selectin on the surface of endothelial cells in vitro and an influx of neutrophils into tumours treated in vivo, which are likely to contribute to the vascular damaging and cytotoxic effects of the drug. Other immune effector cells such as macrophages are also found in tumours treated with VDAs but their influence on treatment outcome is unknown.

These considerations imply that there is not a simple relationship between extent and time course of blood flow reduction and tumour cell death, although several hours of tumour blood flow shut-down are clearly required to ensure significant tumour cell death. Tumour cell necrosis is not unequivocally demonstrable in histological sections until approximately 24 h after VDA treatment, so this time point is often chosen to determine efficacy of treatment.

Blood coagulation is normally evident in tumour blood vessels, within 1 h of VDA treatment, presumably consequent to endothelial cell damage. This clearly increases the viscous resistance to blood flow, completely blocking some vessels and extending the period of blood flow shut-down. However, anti-coagulants did not affect the initial vascular response of rodent tumours to AVE8062 (Nihei et al. 1999), consistent with the view that coagulation is not the initiating event in tumour vascular shut-down with combretastatins.

FAA, DMXAA, CA-4-P, colchicine and vinblastine all cause a rise in plasma levels of the principal hepatic metabolite of serotonin, 5-HIAA (5-hydroxyindole acetic acid), presumably as a result of platelet activation (Baguley et al. 1997 and personal communication). Serotonin is itself a vascular disrupting drug, acting through G-protein-coupled receptors to induce actin stress fibre formation, and it may therefore contribute to the net tumour vascular effects of all these agents.

45.6.2 Vascular Remodelling After VDA Treatment

Angiogenesis is dependent upon a functional cytoskeleton for both endothelial cell division and migration and is therefore susceptible to inhibition by tubulin-binding agents such as CA-4-P. Indeed, CA-4-P has been shown to inhibit migration and three-dimensional tube formation of endothelial cells in vitro (Ahmed et al. 2003), as well as endothelial cell proliferation. Since the primary mode of endothelial cell death following CA-4-P is linked to mitosis (see above), relatively long drug exposure times are required for cytotoxicity to be manifested, in order for a significant numbers of cells to enter mitosis and attempt to divide before dying. Clearance of the drug in vivo is rapid, making the importance of these effects in vivo unclear. The appearance of apoptotic and disintegrating tumour endothelial cells in solid tumours several hours after CA-4-P treatment may be a secondary effect of dying

tumour cells (from blood flow shut-down) rather than a primary effect of the drug in question. However, there is some evidence for an anti-angiogenic effect of multiple doses of CA-4-P in a rat rhabdomyosarcoma (Ahmed et al. 2003) and prevention of neovascularisation could also explain the increased efficacy of CA-4-P when given as a split-dose regime compared with the equivalent single dose (Hill et al. 2002). The influence of macrophage infiltration on these processes is unknown, but since these cells are known to be a primary source of angiogenic growth factors in tumours, their presence may well influence the kinetics of re-vascularisation following VDA treatment.

Unlike CA-4-P, the newer compound, CA-1-P (Oxi4503), was found to induce endothelial apoptosis by 1 h after treatment, in a hemangioendothelioma model (Sheng et al. 2004). This may relate to the formation of a particularly reactive metabolite following in vivo administration of CA-1-P but not CA-4-P (Kirwan et al. 2004). Similarly, DMXAA, in large single doses, can induce apoptosis in tumour endothelium within 30 min of treatment and inhibit angiogenesis induced by basic fibroblast growth factor (bFGF) in an in vivo model system. Despite this evidence that DMXAA can act as an anti-angiogenic agent, repeated dosing of lower drug concentrations or constant infusion over several days had no effect on tumour growth in an animal model (Zhao et al. 2003), suggesting that a high drug concentration is required to have any significant anti-angiogenic effect.

45.6.3 Cytokine Induction

DMXAA causes intratumoral increases in the activity of tumour necrosis factor alpha (TNF) (Ching et al. 1999). An initial hypothesis for the effect of FAA was that it was mediated by TNF induction, but data on the time course of tumour blood flow effects, as well as studies with knockout mice, were not consistent with this theory. TNF acts as a vascular disrupting agent in its own right (Kallinowski et al. 1989), and like many other vasoactive agents modifies the actin cytoskeleton and permeability through the Rho/Rac pathway. Cytokine induction is therefore likely to influence the extent of vascular shut-down but not act as the primary stimulus.

45.6.4 Direct Effects on Tumour Cells

In vitro studies have shown that, amongst normal cells, endothelial cells are particularly sensitive to CA-4-P. However, despite categorisation of CA-4-P and similar compounds as VDAs, the effects of these tubulin-binding drugs are not specific for endothelial cells. In particular, highly proliferative tumour cells are very susceptible to the anti-proliferative and cytotoxic actions of the drug, mediated by damage to the mitotic spindle. Indeed, in terms of the anti-proliferative action of CA-4-P, in vitro studies have shown that some tumour cell types are more sensitive to the drug than endothelial cells (Ahmed et al. 2003). Therefore, direct tumour cell toxicity may influence the final outcome of vascular targeted therapy. However, the extent to which direct tumour cell death contributes to the anti-tumour activity of CA-4-P and similar agents in vivo is likely to depend on tumour type, the specific agent used and dose/scheduling considerations. In particular, as for endothelial cells, long drug exposures are required to sustain mitotic arrest and induce cell death. For CA-4-P, which has a short plasma half-life, this is likely to be obtainable only with a repeated dosing schedule. The vascular versus direct effects of AVE8062 were investigated by comparing the growth response of tumours grown from wild-type c26 colon adenocarcinoma cells with that of tumours grown from counterpart c26/acr cells, which were cloned in vitro for their resistance to tubulin-binding agents (Nihei et al. 1999). Results showed similar growth responses to AVE8062 for the two cell types, suggesting that it was the vascular effects that dictated overall response. In summary, the combretastatins are clearly toxic to tumour cells if exposed for long enough, but this may not be achieved in clinically relevant dosing regimens.

Therapeutic Potential

Vascular disrupting agents administered as single agents produce only moderate growth retardation in animal tumours and human tumours xenografted into mice, due to rapid regrowth from the surviving tumour rim. However, well-tolerated doses of VDAs in mice can cause death of over 90% of tumour cells, encouraging the combination with a second treatment modality. Combined efficacy can be achieved if the two treatments provide spatial co-operation, independent toxicities or potentiation.

Spatial co-operation could be achieved by combining VDA treatment with a modality that targets the tumour periphery. Conventional chemotherapeutic agents and radiation are effective against highly proliferating and well-oxygenated cells, which are most evident in the periphery of tumours. In addition, blood flow tends to be more effective at the periphery, allowing ready access for blood-borne anti-cancer agents. The rationale for combining VDAs with conventional treatments is therefore apparent, and pre-clinical studies have indicated a benefit of combining CA-4-P, ZD6126 and DMXAA with radiotherapy and a range of chemotherapeutic agents, most notably the platinum drugs and taxanes (Fig. 45.6; see also Siemann and Horsman 2002; Siemann et al. 2002). There is some evidence that these improvements in tumour response can be achieved without any increased toxicity, therefore providing a true increase in therapeutic ratio. Novel biological anti-cancer agents, such as antibodies with potent binding characteristics, are often found to localise in the tumour periphery, with very little penetration into the tumour centre, resulting in poor efficacy. This is most likely a consequence of both poor delivery (low blood flow) and poor convective extravasation (high interstitial fluid pressure) at the centre of tumours. Spatial co-operation of high-molecular-weight biologicals with VDAs has been found for the combination of radio-immunotherapy and CA-4-P or DMXAA in pre-clinical studies (Pedley et al. 2002), and this is now being tested in clinical trials.

Scheduling is an important issue in combination treatments. In view of the tumour vascular shutdown following VDA treatment, it is advisable to avoid giving radiotherapy or chemotherapy shortly after VDA administration, when blood flow to tumour regions destined to survive the treatment is reduced. These areas will be hypoxic (and therefore radio-resistant) and will be poorly accessible for blood-borne chemotherapeutic agents. These considerations have been generally borne out by pre-clinical studies.

VDA-induced reduction in tumour blood flow can be exploited to 'trap' chemotherapeutic drugs in tumour tissue, thus providing potentiation of the drug effect. In most studies, it is difficult to separate this effect from any spatial co-operation or direct interaction of a VDA with a second agent. However, at least in one case (the combination of 5-fluorouracil with CA-4-P), effective tumour growth retardation has been achieved by a combined treatment, in the absence of any corresponding increase in tumour levels (trapping) of the drug (Grosios et al. 2000). In radiotherapy, damage to the tumour vasculature is increasingly recognised as being influential in determining tumour cell survival, but the interaction of radiation with current VDAs at the cellular level is unknown. There is some evidence that CA-4-P and ZD6126 have a major impact on the radiation-resistant hypoxic cell population, but the mechanism behind this effect requires further investigation.

The extensive ischaemic insult to tumours following VDA treatment results in extensive tumour cell hypoxia even in the surviving tumour rim (El-Emir et al. 2005). This raises the possibility of hypoxia-induced angiogenesis, with the concern that re-growing tumours will be particularly aggressive. An increase in expression of both VEGF and bFGF proteins in xenografted tumours following CA-4-P and CA-1-P treatment has been reported. These considerations led to investigations of the combination of VDAs with anti-angiogenic agents. In a pre-clinical study, the combination of ZD6126 with the VEGF receptor tyrosine kinase inhibitor, ZD6474, showed good enhancement in terms of tumour response (Siemann and Shi 2004). Similarly, administration of an anti-VEGF antibody 24 h prior

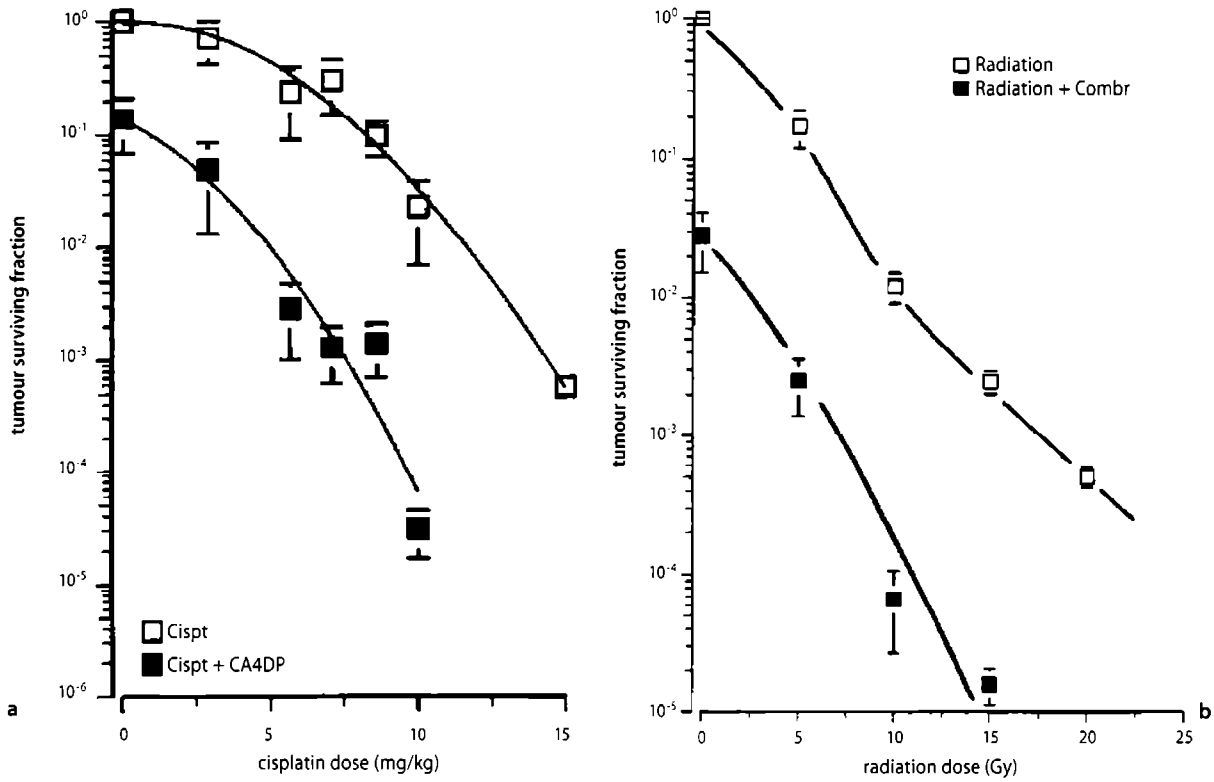


Fig. 45.6a,b. Effects of combining 100 mg/kg CA-4-P with a cisplatin and b radiation in the KHT sarcoma grown in C3H mice. Response was measured by clonogenic assay of tumour cells. CA-4-P was administered intraperitoneally, 1 h after drug or radiation treatment. Figures adapted, with permission, from Siemann et al. (2002) and Li et al. (1998)

to administration of either CA-4-P or CA-1-P markedly enhanced anti-tumour activity in an animal model (Shaked et al. 2006). CA-4-P also increased the surviving tumour cell expression of the glucose-regulated protein GRP78 (Dong et al. 2005). GRP78 is an endoplasmic reticulum-associated chaperone molecule which is inducible by severe glucose depletion, anoxia, and acidosis and is associated with drug resistance. This important finding illustrates the fact that the response of tumour cells to VDAs can have a major influence on treatment outcome and points to the potential impact of VDA treatment on development of drug resistance, which will require addressing in the future.

On the other hand, it may be possible to exploit the VDA-induced tumour ischaemia by combining VDA treatment with the so-called bioreductive drugs, which are activated under hypoxic conditions, or with other hypoxia-targeting strategies. In the case of bioreductive drugs, oxygen protects a pro-drug against reduction by reacting with an intermediate (usually a short-lived free radical) in its catabolism by cellular enzymes and thus restoring the drug to its original (pro-drug) form. In the absence of oxygen, the intermediate is reduced to an active form or the reduction process triggers release of an active effector molecule. An example is the combination of DMXAA with tirapazamine (Lash et al. 1998).

Recent innovations in drug delivery systems may impact on vascular targeting. Blood flow reductions in tumour regions that are destined to survive the treatment are a concern in terms of subsequent drug delivery, as well as acting as a stimulus for expression of growth-enhancing genes. The development

of nanoparticles that consist of a core and a pegylated-lipid envelope, for timed release of two different drugs, has been described as one approach to tackling this problem (Sengupta et al. 2005). In this case, vascular shut-down is instigated by release of CA-4 from the outer envelope and this is followed, once the nanoparticles are preferentially trapped in the tumour tissue, by release of a chemotherapeutic drug, doxorubicin, from the core. Another approach exploits CA-4 encapsulated in liposomes that incorporate specific peptide sequences on their surface for preferential targeting of irradiated tumour blood vessels via alphavbeta3 integrin (Pattillo et al. 2005). In this way, it is hoped to increase the selectivity of CA-4 to the tumour vasculature when used in combination with radiotherapy.

Clinical Trials

45.8.1 Microtubule Depolymerising Agents

Three phase I clinical trials with CA-4-P have been completed. These trials explored the safety and pharmacokinetics (PK) of three different intravenous (i.v.) administration schedules of CA-4-P as a single agent (i.e. single dose every 21 days, weekly for three consecutive weeks every 28 days and daily for five consecutive days every 21 days). The results from all the phase I trials have recently been reviewed (Young and Chaplin 2004). Doses as high as 114 mg/m^2 (weekly schedule) have been studied. Dose-limiting toxicities (DLTs) have been identified in all three trials, resulting in a maximum tolerated dose (MTD) in the range of 60–68 mg/m^2. DLTs have included dyspnoea, myocardial ischaemia, reversible neurological events and tumour pain. In general, across all three studies, the most frequently reported adverse events after a single intravenous injection of CA-4-P were mild (grade 1 or 2) nausea, vomiting, headache, fatigue and tumour pain. CA-4-P did not produce significant clinical chemistry or haematologic toxicity associated with other commonly used tubulin-binding agents such as the vinca alkaloids and taxanes, as well as colchicine. The other common toxicities associated with traditional cytotoxic drugs, such as stomatitis and alopecia, were not reported in these clinical trials.

In order to establish whether CA-4-P was reducing tumour blood flow, all three studies incorporated measurement of tissue uptake of i.v.-administered contrast agents, either using dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI; Fig. 45.7) or positron emission tomography (PET). Gadolinium diethylenetriaminepentaacetate (Gd-DTPA) was used as the contrast agent for DCE-MRI and ^{15}O-labelled water ($H_2{}^{15}O$) as the tracer for PET. Significant reductions in tumour uptake kinetics, consistent with tumour blood flow reduction, were observed in the majority of patients receiving doses at or above 52 mg/m^2 (Young and Chaplin 2004). Very few patients were evaluated for blood flow changes below this dose. Although patients receiving 10 mg/m^2 or less had no discernable blood flow changes detected using PET, low numbers of patients meant that it was not possible to establish a clear dose response between 10 and 52 mg/m^2. Recent PET studies have indicated significant tumour blood flow reductions at doses down to 30 mg/m^2. The blood flow changes seen in the tumour tissue appear to be selective, with no change in normal muscle perfusion being seen.

In terms of tumour response in the phase I studies, a complete response was seen in an anaplastic thyroid cancer and a partial response in a sarcoma. In addition, several minor responses in kidney and pancreas were observed as well as prolonged (>12 months) disease stabilisation in three patients, one with colon cancer and two with medullary thyroid cancer (Young and Chaplin 2004).

Based on the phase I data, several phase I/II clinical trials, in which CA-4-P is combined with chemotherapy, radiotherapy, anti-VEGF or antibody-based therapeutics, have been initiated. In these studies, dosing is expressed as the amount of free acid dosed rather than the sodium salt, as was used in the phase I single agent studies. These studies are currently us-

ing CA-4-P at doses of the free acid of 27–72 mg/m^2. These doses equate to 30–80 mg/m^2 of the sodium salt, which are known from the phase I studies to elicit vascular damaging activity.

Three phase I clinical trials with ZD6126, using similar dosing regimes to those used with CA-4-P, were recently completed. The results for weekly dosing have been published (Beerepoot et al. 2006). Dose-limiting toxicities at 28 mg/m^2 were hypoxia caused by pulmonary embolism and an asymptomatic decrease in left ventricular ejection fraction. No tumour responses were observed. Although the full results have not yet been published, some data have been reported as conference proceedings (Beerepoot et al. 2006). In the once every 21 days dosing regime, a maximum dose of 112 mg/m^2 was reached. The most common adverse events were pain, constipation, fatigue and dyspnoea. Dose escalation above 112 mg/m^2 was not pursued because of a variety of adverse events at this dose, including abdominal pain and hypertension. No tumour responses were reported. However, blood flow data, using DCE-MRI, established that at doses of 80 mg/m^2 and higher, ZD6126 treatment caused a 36–72% decrease

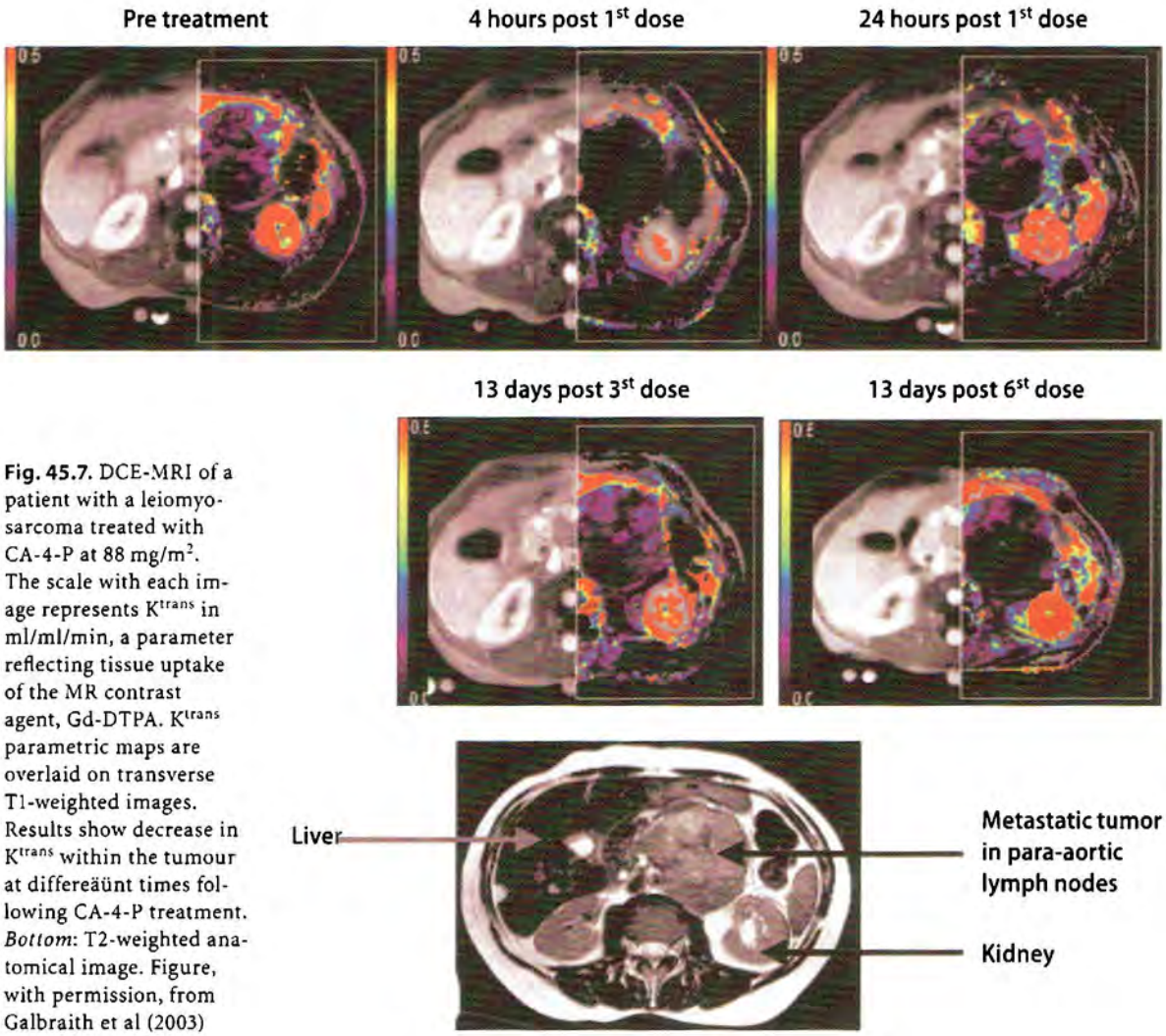


Fig. 45.7. DCE-MRI of a patient with a leiomyosarcoma treated with CA-4-P at 88 mg/m^2. The scale with each image represents K^{trans} in ml/ml/min, a parameter reflecting tissue uptake of the MR contrast agent, Gd-DTPA. K^{trans} parametric maps are overlaid on transverse T1-weighted images. Results show decrease in K^{trans} within the tumour at differeäünt times following CA-4-P treatment. *Bottom*: T2-weighted anatomical image. Figure, with permission, from Galbraith et al (2003)

in tumour blood flow in all patients studied, whilst no significant changes in muscle or spleen flow were observed (Evelhoch et al. 2004).

A summary of the ongoing clinical trials with AVE8062 has been reported as conference proceedings (Sessa et al. 2003). In the daily for 5 days and weekly dosing studies, the occurrence of four potentially drug-related vascular events (i.e. myocardial ischaemia, transient asymptomatic hypotension, transient cerebral ischaemia, and asymptomatic ventricular tachycardia, without residual clinical deficits) led to voluntary interruption of all trials. No vascular event was observed in the q21d schedule up to a 22 mg/m2 dose; thus, this trial was resumed after restricting eligibility criteria and increasing cardiovascular monitoring. No cardiac effects or tumour blood flow changes have been observed to date using this protocol.

There has been considerable focus on potential cardiovascular side effects of the tubulin-binding VDAs, based around their known vascular mechanism of action and the events seen in phase I, particularly with the recent data on AVE8062. Most work to elucidate the cardiovascular issues has been carried out with CA-4-P. In the reported phase I studies of CA-4-P, three cases of reversible myocardial effects were reported and these contributed to the establishment of the current MTD. In these patients, hypertension was evident prior to the myocardial effects occurring. As noted previously, microtubule depolymerising agents as a class can, in addition to their direct effects on endothelial cell morphology, induce arteriolar vasoconstriction. This presumably occurs via a direct effect on vascular smooth muscle cells, since it occurs even in endothelial cell denuded vessels (Platts et al. 1999). Also, it has been established that concomitant use of vasodilators with CA-4-P in rats can eliminate the hypertensive effect without altering the blood flow shut-down effects in the tumour (Honess et al. 2002), strongly suggesting that arteriolar vasoconstriction is not the primary mechanism of action for this agent. If, as is now believed, the vasoconstrictive actions of CA-4-P contribute to the DLTs, including cardiovascular ones, there is a possibility that the prophylactic use of vasodilators may not only alter its toxicity profile but also enable higher dose levels to be attained. This approach requires evaluation in a clinical setting. However, using the doses of CA-4-P chosen for phase I/II, cardiovascular effects, in terms of changes in blood pressure and heart rate, have been mild and reversible.

45.8.2 DMXAA

DMXAA entered clinical trials in 1996 and was administered as a 20-min intravenous infusion, in doses ranging from 6 to 4900 mg/m^2 (Jameson et al. 2003). Toxicities observed included tremor, confusion, slurred speech, anxiety, and urinary incontinence. Also, transient prolongation of cardiac QTc interval was seen in some of the patients who received doses at or above 2000 mg/m^2.

In terms of direct response, only two unconfirmed partial responses were observed, at doses of 1100 and 1300 mg/m^2. However, in terms of biological activity, over the dose range of 500–4900 mg/m^2, 9 of 16 patients had significant reductions in tumour blood flow, as measured by DCE-MRI, 24 h after the first dose of DMXAA (Galbraith et al. 2002). In addition, dose-dependent increases in the serotonin metabolite 5-hydroxyindoleacetic acid, a potential marker of vascular disruption with DMXAA in the mouse studies, were observed at doses exceeding 650 mg/m^2. These latter two findings indicate that DMXAA can act as a VDA in man at clinically achievable doses. Based on these findings, together with recent data showing no significant QTc changes at doses up to 1200 mg/m2, three phase II studies have been initiated in prostate, ovarian and non-small-cell lung cancer, where DMXAA is incorporated into taxane-based chemotherapy regimes.

45.8.3 N-Cadherin Antagonists

Data from the first phase I study of the N-cadherin antagonist ADH-1 (Exherin) have recently been reported (Jonker et al. 2005). Thirty-three patients

were treated. ADH-1 was administered as a bolus i.v. injection, doses from 50 to 840 mg/m^2 were evaluated and tumours characterised for their expression of N-cadherin. No responses or changes in blood flow were reported in patients with N-cadherin-negative tumours. Three patients with N-cadherin-positive tumours showed some evidence of antitumour activity, including a partial response in an oesophageal cancer. Although the MTD had not been reached, the most common adverse events at the doses used were nausea, fatigue and hot flushes. Some cardiovascular effects were seen but were thought to be related, at least in part, to prior cardiac history and injection rate. Based on the phase I data, a phase II trial has been initiated, focused on patients with N-cadherin positive tumours.

References

Ahmed B, van Eijk LI, Bouma-Ter Steege JC, van der Schaft DW, van Esch AM, Joosten-Achjanie SR, Lambin P, Landuyt W, Griffioen AW (2003) Vascular targeting effect of combretastatin A-4 phosphate dominates the inherent angiogenesis inhibitory activity. Int J Cancer 105:20–25

Baguley BC (2003) Antivascular therapy of cancer: DMXAA. Lancet Oncol 4:141–148

Baguley BC, Zhuang L, Kestell P (1997) Increased plasma serotonin following treatment with flavone-8-acetic acid, 5,6-dimethylxanthenone-4-acetic acid, vinblastine, and colchicine: relation to vascular effects. Oncol Res 9:55–60

Beerepoot LV, Radema SA, Witteveen EO et al (2006) Phase I clinical evaluation of weekly administration of the novel vascular-targeting agent, ZD6126, in patients with solid tumors. J Clin Oncol 24:1491–1498

Blaschuk OW, Rowlands TM (2000) Cadherins as modulators of angiogenesis and the structural integrity of blood vessels. Cancer Metastasis Rev 19:1–5

Ching LM, Goldsmith D, Joseph WR, Korner H, Sedgwick JD, Baguley BC (1999) Induction of intratumoral tumor necrosis factor (TNF) synthesis and hemorrhagic necrosis by 5,6-dimethylxanthenone-4-acetic acid (DMXAA) in TNF knockout mice. Cancer Res 59:3304–3307

Corada M, Zanetta L, Orsenigo F, Breviario F, Lampugnani MG, Bernasconi S, Liao F, Hicklin DJ, Bohlen P, Dejana E (2002) A monoclonal antibody to vascular endothelial-cadherin inhibits tumor angiogenesis without side effects on endothelial permeability. Blood 100:905–911

Davis PD, Hill SA, Galbraith SM, Chaplin DJ, Naylor MA, Nolan J, Dougherty GJ (2000) ZD6126: a new agent causing selective damage of tumor vasculature. Ninety-first Annual Meeting of the American Association for Cancer Research, vol 41, San Francisco

Dong D, Ko B, Baumeister P, Swenson S, Costa F, Markland F, Stiles C, Patterson JB, Bates SE, Lee AS (2005) Vascular targeting and antiangiogenesis agents induce drug resistance effector GRP78 within the tumor microenvironment. Cancer Res 65:5785–5791

El-Emir E, Boxer GM, Petrie IA, Boden RW, Dearling JL, Begent RH, Pedley RB (2005) Tumour parameters affected by combretastatin A-4 phosphate therapy in a human colorectal xenograft model in nude mice. Eur J Cancer 41:799–806

Evelhoch JL, LoRusso PM, He Z, DelProposto Z, Polin L, Corbett TH, Langmuir P, Wheeler C, Stone A, Leadbetter J, Ryan AJ, Blakey DC, Waterton JC (2004) Magnetic resonance imaging measurements of the response of murine and human tumors to the vascular-targeting agent ZD6126. Clin Cancer Res 10:3650–3657

Galbraith SM, Rustin GJ, Lodge MA, Taylor NJ, Stirling JJ, Jameson M, Thompson P, Hough D, Gumbrell L, Padhani AR (2002) Effects of 5,6-dimethylxanthenone-4-acetic acid on human tumor microcirculation assessed by dynamic contrast-enhanced magnetic resonance imaging. J Clin Oncol 20:3826–3840

Galbraith SM, Maxwell RJ, Lodge MA, Tozer GM, Wilson J, Taylor NJ, Stirling JJ, Sena L, Padhani AR, Rustin GJ (2003) Combretastatin A4 phosphate has tumor antivascular activity in rat and man as demonstrated by dynamic magnetic resonance imaging. J Clin Oncol 21:2831–2842

Grosios K, Loadman PM, Swaine DJ, Pettit GR, Bibby MC (2000) Combination chemotherapy with combretastatin A-4 phosphate and 5-fluorouracil in an experimental murine colon adenocarcinoma. Anticancer Res 20:229–234

Hill SA, Chaplin DJ, Lewis G, Tozer GM (2002) Schedule dependence of combretastatin A4 phosphate in transplanted and spontaneous tumour models. Int J Cancer 102:70–74

Honess DJ, Hylands F, Chaplin DJ, Tozer GM (2002) Comparison of strategies to overcome the hypertensive effect of combretastatin-A-4-phosphate in a rat model. Br J Cancer 86:S118

Hori K, Saito S (2003) Microvascular mechanisms by which the combretastatin A-4 derivative AC7700 (AVE8062) induces tumour blood flow stasis. Br J Cancer 89:1334–1344

Hori K, Saito S, Nihei Y, Suzuki M, Sato Y (1999) Antitumor effects due to irreversible stoppage of tumor tissue blood flow: evaluation of a novel combretastatin A-4 derivative, AC7700. Jpn J Cancer Res 90:1026–1038

Inai T, Mancuso M, Hashizume H, Baffert F, Haskell A, Baluk P, Hu-Lowe DD, Shalinsky DR, Thurston G, Yancopoulos GD, McDonald DM (2004) Inhibition of vascular endothelial growth factor (VEGF) signaling in cancer

causes loss of endothelial fenestrations, regression of tumor vessels, and appearance of basement membrane ghosts. Am J Pathol 165:35–52

Jameson MB, Thompson PI, Baguley BC, Evans BD, Harvey VJ, Porter DJ, McCrystal MR, Small M, Bellenger K, Gumbrell L, Halbert GW, Kestell P (2003) Clinical aspects of a phase I trial of 5,6-dimethylxanthenone-4-acetic acid (DMXAA), a novel antivascular agent. Br J Cancer 88:1844–1850

Jonker D et al (2005) Proceedings of the American Society of Clinical Oncology (ASCO), annual meeting, abstract 3038

Kallinowski F, Schaefer C, Tyler G, Vaupel P (1989) In vivo targets of recombinant human tumour necrosis factor-α: blood flow, oxygen consumption and growth of isotransplanted rat tumours. Br J Cancer 60:555–560

Kanthou C, Tozer GM (2002) The tumor vascular targeting agent combretastatin A-4-phosphate induces reorganization of the actin cytoskeleton and early membrane blebbing in human endothelial cells. Blood 99:2060–2069

Kanthou C, Greco O, Stratford A, Cook I, Knight R, Benzakour O, Tozer G (2004) The tubulin-binding agent combretastatin A-4-phosphate arrests endothelial cells in mitosis and induces mitotic cell death. Am J Pathol 165:1401–1411

Kasibhatla S, Gourdeau H, Meerovitch K, Drewe J, Reddy S, Qiu L, Zhang H, Bergeron F, Bouffard D, Yang Q, Herich J, Lamothe S, Cai SX, Tseng B (2004) Discovery and mechanism of action of a novel series of apoptosis inducers with potential vascular targeting activity. Mol Cancer Ther 3:1365–1374

Kirwan IG, Loadman PM, Swaine DJ, Anthoney DA, Pettit GR, Lippert JW 3rd, Shnyder SD, Cooper PA, Bibby MC (2004) Comparative preclinical pharmacokinetic and metabolic studies of the combretastatin prodrugs combretastatin A4 phosphate and A1 phosphate. Clin Cancer Res 10:1446–1453

Lash CJ, Li AE, Rutland M, Baguley BC, Zwi LJ, Wilson WR (1998) Enhancement of the anti-tumour effects of the antivascular agent 5,6-dimethylxanthenone-4-acetic acid (DMXAA) by combination with 5-hydroxytryptamine and bioreductive drugs. Br J Cancer 78:439–445

Lew YS, Brown SL, Griffin RJ, Song CW, Kim JH (1999) Arsenic trioxide causes selective necrosis in solid murine tumors by vascular shutdown. Cancer Res 59:6033–6037

Li L, Rojiani A, Siemann D (1998) Targeting the tumor vasculature with combretastatin A-4 disodium phosphate: effects on radiation therapy. Int J Radiat Oncol Biol Phys 42:899–903

Neri D, Bicknell R (2005) Tumour vascular targeting. Nat Rev Cancer 5:436–446

Nihei Y, Suzuki M, Okano A, Tsuji T, Akiyama Y, Tsuruo T, Saito S, Hori K, Sato Y (1999) Evaluation of antivascular and antimitotic effects of tubulin binding agents in solid tumor therapy. Jpn J Cancer Res 90:1387–1396

Otani M, Natsume T, Watanabe J, Kobayashi M, Murakoshi M, Mikami T, Nakayama T (2000) TZT-1027, an antimicrotubule agent attacks tumor vasculature and induces tumor cell death. Jpn J Cancer Res 91:837–844

Pattillo CB, Sari-Sarraf F, Nallamothu R, Moore BM, Wood GC, Kiani MF (2005) Targeting of the antivascular drug combretastatin to irradiated tumors results in tumor growth delay. Pharm Res 22:1117–1120

Pedley RB, El-Emir E, Flynn AA, Boxer GM, Dearling J, Raleigh JA, Hill SA, Stuart S, Motha R, Begent RH (2002) Synergy between vascular targeting agents and antibody-directed therapy. Int J Radiat Oncol Biol Phys 54:1524–1531

Pettit GR, Lippert JW III (2000) Antineoplastic agents 429. Syntheses of the combretastatin A-1 and combretastatin B-1 prodrugs. Anticancer Drug Des 15:203–216

Pettit GR, Temple C, Narayanan VL, Varma R, Simpson MJ, Boyd MR, Rener GA, Bansal N (1995) Antineoplastic agents 322. Synthesis of combretastatin A-4 prodrugs. Anticancer Drug Des 10:299–309

Platts SH, Falcone JC, Holton WT, Hill MA, Meininger GA (1999) Alteration of microtubule polymerization modulates arteriolar vasomotor tone. Am J Physiol 277:H100–H106

Segreti JA, Polakowski JS, Koch KA, Marsh KC, Bauch JL, Rosenberg SH, Sham HL, Cox BF, Reinhart GA (2004) Tumor selective antivascular effects of the novel antimitotic compound ABT-751: an in vivo rat regional hemodynamic study. Cancer Chemother Pharmacol 54:273–281

Sengupta S, Eavarone D, Capila I, Zhao G, Watson N, Kiziltepe T, Sasisekharan R (2005) Temporal targeting of tumour cells and neovasculature with a nanoscale delivery system. Nature 436:568–572

Sessa C et al (2003) Proceedings of the 96th annual meeting of the American Association for Cancer Research (AACR), abstract 5827

Shaked Y, Ciarrocchi A, Franco M et al (2006) Therapy-induced acute recruitment of circulating endothelial progenitor cells to tumors. Science 313 1785-1787

Sheng Y, Hua J, Pinney KG, Garner CM, Kane RR, Prezioso JA, Chaplin DJ, Edvardsen K (2004) Combretastatin family member OXI4503 induces tumor vascular collapse through the induction of endothelial apoptosis. Int J Cancer 111:604–610

Siemann DW, Horsman MR (2002) Enhancement of radiation therapy by vascular targeting agents. Curr Opin Invest Drugs 3:1660–1665

Siemann DW, Shi W (2004) Efficacy of combined antiangiogenic and vascular disrupting agents in treatment of solid tumors. Int J Radiat Oncol Biol Phys 60:1233–1240

Siemann DW, Mercer E, Lepler S, Rojiani AM (2002) Vascular targeting agents enhance chemotherapeutic agent activities in solid tumor therapy. Int J Cancer 99:1–6

Staton CA, Brown NJ, Rodgers GR, Corke KP, Tazzyman S, Underwood JC, Lewis CE (2004) Alphastatin, a 24-amino acid fragment of human fibrinogen, is a potent new inhibitor of activated endothelial cells in vitro and in vivo. Blood 103:601–606

Thorpe PE (2004) Vascular targeting agents as cancer therapeutics. Clin Cancer Res 10:415–427

Tozer GM, Prise VE, Wilson J, Locke RJ, Vojnovic B, Stratford MRL, Dennis MF, Chaplin DJ (1999) Combretastatin A-4 phosphate as a tumor vascular-targeting agent: early effects in tumors and normal tissues. Cancer Res 59:1626–1634

Tozer GM, Prise VE, Wilson J, Cemazar M, Shan S, Dewhirst MW, Barber PR, Vojnovic B, Chaplin DJ (2001) Mechanisms associated with tumor vascular shut-down induced by combretastatin A-4 phosphate: intravital microscopy and measurement of vascular permeability. Cancer Res 61:6413–6422

Tozer GM, Ameer-Beg SM, Baker J, Barber PR, Hill SA, Hodgkiss RJ, Locke R, Prise VE, Wilson I, Vojnovic B (2005) Intravital imaging of tumour vascular networks using multi-photon fluorescence microscopy. Adv Drug Deliv Rev 57:135–152

Vincent L, Kermani P, Young LM, Cheng J, Zhang F, Shido K, Lam G, Bompais-Vincent H, Zhu Z, Hicklin DJ, Bohlen P, Chaplin DJ, May C, Rafii S (2005) Combretastatin A4 phosphate induces rapid regression of tumor neovessels and growth through interference with vascular endothelial-cadherin signaling. J Clin Invest 115:2992–3006

Wehbe H, Kearney CM, Pinney KG (2005) Combretastatin A-4 resistance in H460 human lung carcinoma demonstrates distinctive alterations in beta-tubulin isotype expression. Anticancer Res 25:3865–3870

Young SL, Chaplin DJ (2004) Combretastatin A4 phosphate: background and current clinical status. Exp Opin Invest Drugs 13:1171–1182

Zhao L, Ching LM, Kestell P, Baguley BC (2003) Improvement of the antitumor activity of intraperitoneally and orally administered 5,6-dimethylxanthenone-4-acetic acid by optimal scheduling. Clin Cancer Res 9:6545–6550

Subject Index

B

C

D

E

F

G

H

M

N

O

P

R

S

T

U

V

W

Y

Z

Tumor angiogenesis is one of the most prominent mechanisms driving tumor development and progression. Since its discovery more than 30 years ago some of the most important signaling pathways linking specific angiogenic activities of the tumor cells to the fatal reactions of the patient's vascular system have been elucidated. As a consequence crucial targets for therapeutic intervention have been identified and validated.

Based on these efforts and achievements, targeted drug development programs have been implemented to interfere with tumor angiogenesis as an attractive strategy in cancer treatment. As a promising result the first targeted anti-angiogenic drugs have been approved for a variety of solid metastasizing cancers. The first generation of these molecules targets the two most prominent regulatory components of tumor angiogenesis: the vascular endothelial growth factor (VEGF), produced by tumor cells, and the VEGF receptor tyrosine kinase, which is expressed on vascular endothelial cells.

Part 1 of this volume describes the basic mechanisms of tumor angiogenesis. Beyond the VEGF/VEGF receptor system, additional tumor-angiogenic systems are presented as new potential targets for anti-angiogenic therapy. Part 2 reviews the efforts made in preclinical research to validate new targets and to show efficacy in animals. Part 3 is entirely devoted to the clinical development of the novel anti-angiogenic drugs and their current use in clinical practice.

Marmé
Fusenig
Editors
Tumor Angiogenesis
Basic Mechanisms and Cancer Therapy

ISBN 978-3-540-33176-6
9 783540 331766
springer.com