Angiogenesis Assays

A critical appraisal of current techniques

Carolyn A. Staton
Academic Unit of Surgical Oncology
University of Sheffield Medical School
Sheffield, UK

Claire Lewis
Academic Unit of Pathology
University of Sheffield Medical School
Sheffield, UK

Roy Bicknell
Division of Immunity and Infection
University of Birmingham
Birmingham, UK

John Wiley & Sons, Ltd

Angiogenesis Assays

Angiogenesis Assays

A critical appraisal of current techniques

Carolyn A. Staton
Academic Unit of Surgical Oncology
University of Sheffield Medical School
Sheffield, UK

Claire Lewis
Academic Unit of Pathology
University of Sheffield Medical School
Sheffield, UK

Roy Bicknell
Division of Immunity and Infection
University of Birmingham
Birmingham, UK

John Wiley & Sons, Ltd

Telephone (+44) 1243 779777

Email (for orders and customer service enquiries): cs-books@wiley.co.uk
Visit our Home Page on www.wileyeurope.com or www.wiley.com

Other Wiley Editorial Offices

John Wiley & Sons Inc., 111 River Street, Hoboken, NJ 07030, USA
Jossey-Bass, 989 Market Street, San Francisco, CA 94103-1741, USA
Wiley-VCH Verlag GmbH, Boschstr. 12, D-69469 Weinheim, Germany
John Wiley & Sons Australia Ltd, 42 McDougall Street, Milton, Queensland 4064, Australia
John Wiley & Sons (Asia) Pte Ltd, 2 Clementi Loop #02-01, Jin Xing Distripark, Singapore 129809
John Wiley & Sons Canada Ltd, 6045 Freemont Blvd, Mississauga, Ontario, L5R 4J3

Wiley also publishes its books in a variety of electronic formats. Some content that appears in print may not be available in electronic books.

Library of Congress Cataloging-in-Publication Data
Angiogenesis assays : a critical appraisal of current techniques /
[edited by] Carolyn Staton, Claire Lewis, Roy Bicknell.
p. ; cm.
Includes bibliographical references and index.

ISBN-13: 978-0-470-01600-8 (alk. paper)
ISBN-10: 0-470-01600-0 (alk. paper)

1. Blood-vessels–Growth. 2. Neovascularization. I. Staton, Carolyn.
II. Lewis, Claire E. III. Bicknell, R. J. (R. John)
[DNLM: 1. Neovascularization, Pathologic–physiopathology. 2.
Biological Assay–methods. 3. Models, Cardiovascular. 4.
Neovascularization, Physiologic–physiology. WG 500 A58425 2007]

QP102.A544 2007
612.1'3–dc22 2006031246

British Library Cataloguing in Publication Data

A catalogue record for this book is available from the British Library

ISBN-10 0 470 01600 0 ISBN-13 978-0-470-01600-8

Typeset in 10½/12½pt Times by Thomson Digital
Printed and bound in Great Britain by Antony Rowe Ltd., Chippenham, Wilts
This book is printed on acid-free paper responsibly manufactured from sustainable forestry in which at least two trees are planted for each one used for paper production.

Cover images provided by Carolyn A. Staton

Contents

Foreword

Heterogeneity of angiogenesis in disease: need for diverse approaches to study blood vessel growth and regression

The growth of new blood vessels from existing vessels, familiar to most as angiogenesis or neovascularization, has acquired an importance that would have been difficult to imagine a few years ago. The proven efficacy of recently approved drugs that block angiogenesis in tumours and age-related macular degeneration has heightened the visibility and relevance of research on blood vessel growth and regression. The promise of factors that stimulate functional revascularization of organs, starved of their blood supply by ischaemic vascular disease or other conditions, is also increasing. Success in the clinic has shifted into high gear the search for even more efficacious drugs. How can these agents be identified, screened and tested? How can their mechanism of action be determined? The seemingly ideal approach for evaluating agents would be through pre-clinical models of the targeted diseases. However, few pre-clinical models faithfully mimic human disease. Therefore, the search continues for faster, easier, more relevant ways of assessing agents that stimulate or inhibit angiogenesis.

The use of 'angiogenesis' as a generic term to describe vascular proliferation suggests a single process. Yet, angiogenesis occurs under many different conditions. Blood vessel growth is driven by multiple factors and occurs in varied settings. Are newly formed blood vessels the same regardless of the driving stimulus and environmental conditions in which they grow? Does each condition produce a homogeneous population of new blood vessels? Is angiogenesis in tumours the same as in eye disease, inflammation, or wound healing?

The answer to these questions is clearly, no. Examples of the heterogeneity of new blood vessels are accumulating at an increasing rate. The amount of angiogenesis driven by vascular endothelial growth factor (VEGF) is dose-dependent, and the phenotype of the new blood vessels is governed by local concentration. Greater concentrations of VEGF produce more abundant blood vessels and exaggerate vessel abnormalities. Lower concentrations of VEGF drive less angiogenesis and promote a more normal vessel phenotype. Most blood vessels in tumours have multiple, sometimes bizarre, abnormalities, and the types and severity of the abnormalities vary within each tumour and among tumours of different varieties. Blood vessels at sites of inflammation are leaky because tiny gaps form reversibly between endothelial cells, just as they do after a mosquito bite. In contrast, tumour vessels leak because of structural defects in the

endothelium, which may have multiple layers in some regions and an incomplete monolayer in others. Unlike new or remodelled blood vessels at sites of inflammation, which support high blood flow and robust influx of inflammatory cells, tumour vessel abnormalities may impair blood flow and repel entry of immune cells. When local blood flow in tumours is less than required for cell viability, necrosis results. Loss of blood flow to necrotic regions of tumour may redirect flow to adjacent viable regions that then grow even faster.

An important measure of blood vessel diversity stems from the chamaeleon-like characteristics of endothelial cells and mural cells (pericytes or smooth muscle cells), the two cell types that form the vasculature. In normal blood vessels, both types of cells adapt structurally and functionally to their environment to provide organ-specific features, such as impermeability of the blood–brain barrier, lymphocyte trafficking of high-endothelial venules, and efficient plasma filtration of renal glomeruli. The same chamaeleon-like, adaptive properties of endothelial cells and mural cells underlie the growth, remodelling, and heterogeneity of blood vessels at sites of disease.

Blood vessels that grow or undergo remodelling in disease reflect the integrated action of multiple angiogenic growth factors and inhibitors, substances that augment or limit blood flow, changing composition of the extracellular matrix, and other dynamic environmental conditions. Because of the diversity of conditions that influence angiogenesis in health and disease, newly formed blood vessels are heterogeneous. As a result, no single *in vitro* assay or *in vivo* model can simulate all forms of angiogenesis. Only a broad range of experimental models can mimic the spectrum of conditions that new blood vessels experience under different pathological circumstances.

The 19 chapters of this book review the attributes and limitations of *in vitro* assays, *in vivo* models and clinical settings for studying angiogenesis. Varied approaches are used to observe, characterize, compare, stimulate or block angiogenesis under different conditions. *In vitro* methods make it possible to examine endothelial cell proliferation, migration and tube formation, as well as to investigate effects of fluid shear stress and flow, membrane and intracellular signalling events, and – in co-culture experiments – interactions of mural cells with endothelial cells. Powerful *in vivo* models have been developed to study angiogenesis in normal and disease settings. Models range from the chick chorioallantoic membrane, mammalian cornea, implanted Matrigel plugs, subcutaneous air sacs and transgenic mouse models of cancer to real-time viewing of sprouting endothelial cells in transparent developing zebrafish or in tumours growing in subcutaneous windowed chambers. *In vivo* models also provide approaches for assessing the contribution of bone marrow-derived cells to growing blood vessels. In concert with clues from pre-clinical models, clinical research is searching for better ways to monitor the action of angiogenesis inhibitors or promoters in patients.

Given the momentum of research on angiogenesis and the broad-based development of agents to manipulate blood vessel growth, the future is exciting and

promising. But in moving forward, an ongoing challenge is to determine how to link properties of angiogenic blood vessels identified in pre-clinical assays to those in human disease. VEGF and multiple other factors clearly stimulate angiogenesis in the cornea and Matrigel plugs, but how do the new vessels compare with those at sites of angiogenesis in human cancer? Which assays provide the most meaningful information about angiogenesis in cancer, eye disease or inflammation? Which assays give a reliable fingerprint of blood vessel growth and remodelling driven *in vivo* by VEGF, PDGF, angiopoietins, ephrins, sphingolipids or chemokines, alone or in various combinations? Can data from *in vitro* or *in vivo* assays predict response to angiogenesis inhibitors in human disease? What biomarkers identified in pre-clinical assays can serve as meaningful readouts for actions of angiogenesis-related drugs in humans?

Better understanding of the process of angiogenesis and properties of newly formed blood vessels will lead to even more informative assays and biomarkers. These in turn will help in screening and evaluating of new, more efficacious drugs and other novel tools in vascular biology. Together, these advances will further the exploitation of vascular abnormalities as targets for drug delivery and the control of blood vessel growth and regression in health and disease.

Donald M. McDonald
San Francisco, California, USA
4 May 2006

Preface

Angiogenesis, the development of new blood vessels from the existing vasculature, is essential in normal developmental processes and in numerous pathological conditions such as diabetic retinopathy, rheumatoid arthritis, psoriasis and cancer. This process is a multi-factorial and highly structured sequence of cellular events comprising vascular initiation, formation, maturation, remodelling and regression which, under physiological conditions, are controlled and modulated to meet the tissue requirements. However, under pathological conditions this tight regulation is lost. As angiogenesis is a key player in over 70 different disease states there is a need to study this process in great detail for the development of future therapeutic strategies.

One of the most important technical challenges in studies of angiogenesis is selection of the appropriate assay. The ideal angiogenesis assay would be fast, easy, robust, with reliable readouts, automated computational analysis, multi-parameter assessment, including positive and negative controls and should relate directly to results seen in the clinic. Sadly, such a 'gold-standard' angiogenesis assay has yet to be developed. Endothelial cells whose migration, proliferation, differentiation and structural rearrangement is central to the angiogenesis process are commonly studied in *in vitro* assays, but they are not the only cell type involved in angiogenesis. Therefore the most translatable assays would include the supporting cells (e.g. pericytes, smooth muscle cells and fibroblasts), the extracellular matrix and/or basement membrane and the circulating blood. However, no *in vitro* assays exist which fully model all the components of this complex process. While *in vivo* assays have the components present, these are limited by species used, organ sites and lack of quantitative analysis.

Due to these technical challenges and the variety of assays being used between different laboratories, there is a need to highlight the details and limitations of each assay currently in use. In this book, therefore, we have invited experts in the use of a diverse range of assays to outline the key components and give a critical appraisal of the strengths and weaknesses of these assays. This book aims to provide the information to enable researchers in this field to make informed choices about the type of assays to use for their research and to recognise the limitations of these assays. As anti-angiogenic agents are now in clinical use a critical analysis of the biological end-points currently being used in clinical

trials to assess the efficacy of these drugs is included and the book finishes with a discussion of the direction future studies may take.

Carolyn A. Staton
Claire Lewis
Roy Bicknell

List of Contributors

Andrade, Silvia P.
Departments of Physiology and Biophysics
Institute of Biological Sciences
Federal University of Minas
Gerais-Av. Antonio Carlos 6627
Campus Pampulha
Cx Post 486 - CEP 31270-901
Belo Horizonte/MG, Brazil

Asai, Tomohiro
Department of Medical Biochemistry
School of Pharmaceutical Sciences
University of Shizuoka
Yada, Suruga-ku
Shizuoka 422-8526, Japan

Auerbach, Robert
Department of Zoology
University of Wisconsin
1117 West Johnson Street
Madison, WI 53706, USA

van Beijnum, Judy R.
Angiogenesis Laboratory
Research Institute for Growth and
Development (GROW)
Department of Pathology
Maastricht University and University
Hospital Maastricht
Maastricht, The Netherlands

Castoldi, Gianluigi
Haematology Section
Department of Biomedical Sciences
University of Ferrara
S. Anna Hospital
Corso Giovecca
203 44100 Ferrara, Italy

Chau, Cindy H.
Molecular Pharmacology Section
Medical Oncology Branch
Centre for Cancer Research
National Cancer Institute
National Institutes of Health
Bethesda, MD, 20892, USA

Cuneo, Antonio
Haematology Section
Department of Biomedical
Sciences
University of Ferrara
S. Anna Hospital
Corso Giovecca
203 44100 Ferrara, Italy

Dewhirst, Mark W.
Department of Radiation Oncology
Duke University School of Medicine
Durham, NC 27710, USA

Egginton, Stuart
Centre for Cardiovascular
Sciences and Department
of Physiology
The Medical School
The University of Birmingham
Birmingham B15 2TT, UK

Fan, Tai-Ping
Angiogenesis and TCM Laboratory
Department of Pharmacology
University of Cambridge
Tennis Court Road
Cambridge CB2 1PD, UK

Ferreira, Monica A. N. D.
General Pathology
Institute of Biological Sciences
Federal University of Minas
Gerais-Av. Antonio Carlos 6627
Campus Pampulha
Cx Post 486 - CEP 31270-901
Belo Horizonte/MG, Brazil

Figg, William D.
Molecular Pharmacology Section
Medical Oncology Branch
Centre for Cancer Research
National Cancer Institute
National Institutes of Health
Bethesda, MD 20892, USA

Griffioen, Arjan W.
Angiogenesis Laboratory
Research Institute for Growth and
Development (GROW)
Department of Pathology
Maastricht University and
University Hospital Maastricht
Maastricht, The Netherlands

Griffoni, Cristiana
Department of Experimental Biology
University of Bologna
Via Selmi 3
40126 Bologna, Italy

Hatziapostolou, Maria
Laboratory of Molecular Pharmacology
Department of Pharmacy
University of Patras
GR26504 Patras, Greece

Hillen, Femke
Angiogenesis Laboratory
Research Institute for Growth and
Development (GROW)
Department of Pathology
Maastricht University and University
Hospital Maastricht
Maastricht, The Netherlands

Hirschi, Karen K.
Baylor College of Medicine
One Baylor Plaza
Houston, TX 77030, USA

Hoffman, Robert M.
AntiCancer, Inc.
7917 Ostrow Street
San Diego, CA 92111, USA

Iorio, Rosa Anna
Department of Experimental Biology
University of Bologna
Via Selmi 3
40126 Bologna, Italy

Lang, Sven A.
Department of Surgery
University of Regensburg
93042 Regensburg, Germany

Laschke, Matthias W.
Institute for Clinical and Experimental
Surgery
University of Saarland
D-66421 Homburg/Saar, Germany

Lee, Wen-Sen
Department of Physiology
Taipei Medical University
Taipei 110, Taiwan

Lenaz, Patrizia
Department of Experimental Biology
University of Bologna
Via Selmi 3
40126 Bologna, Italy

Melotte, Veerle
Angiogenesis Laboratory
Research Institute for Growth and
Development (GROW)
Department of Pathology
Maastricht University and
University Hospital Maastricht
Maastricht, The Netherlands

Menger, Michael D.
Institute for Clinical and Experimental Surgery
University of Saarland
D-66421 Homburg/Saar, Germany

Mikolon, David
Moores UCSD Cancer Center
University of California
San Diego
3855 Health Science Drive
MC0803, USA

Mills Shaw, Kenna R.
National Institute of Child Health and Human Development
National Institutes of Health
9000 Rockville Pike, Bethesda
Maryland 20892, USA

Mousa, Shaker A.
Pharmaceutical Research Institute at Albany College of Pharmacy
106 New Scotland Avenue
Albany, NY 12208, USA

Nash, Gerard B.
Centre for Cardiovascular Sciences and Department of Physiology
The Medical School
The University of Birmingham
Birmingham B15 2TT, UK

Nix, Melissa K.
Baylor College of Medicine
One Baylor Plaza
Houston, TX 77030, USA

Oku, Naoto
Department of Medical Biochemistry
School of Pharmaceutical Sciences
University of Shizuoka
Yada, Suruga-ku
Shizuoka 422-8526, Japan

Papadimitriou, Evangelia
Laboratory of Molecular Pharmacology
Department of Pharmacy
University of Patras
GR26504 Patras, Greece

Polytarchou, Christos
Laboratory of Molecular Pharmacology
Department of Pharmacy
University of Patras
GR26504 Patras, Greece

Rigolin, Gian Matteo
Haematology Section
Department of Biomedical Sciences
University of Ferrara
S. Anna Hospital
Corso Giovecca
203 44100 Ferrara, Italy

Santi, Spartaco
Department of Experimental Biology
University of Bologna
Via Selmi 3
40126 Bologna, Italy

Shan, Siqing
Department of Radiation Oncology
Duke University School of Medicine
Durham, NC 27710, USA

Smiley, Shannon
Roswell Park Cancer Institute
Elm and Carlton Sreets
Buffalo
New York 14263, USA

Smith, Ewen J.
Tumour Targeting Group
Academic Unit of Pathology
University of Sheffield
Sheffield, S10 2RX, UK

Spisni, Enzo
Department of Experimental Biology
University of Bologna
Via Selmi 3
40126 Bologna, Italy

Staton, Carolyn A.
Academic Unit of Surgical Oncology
K-Floor, Royal Hallamshire Hospital
Glossop Road, Sheffield S10 2JF

Stoeltzing, Oliver
Department of Surgery
University of Regensburg
93042 Regensburg, Germany

Strillaci, Antonio.
Department of Experimental Biology
University of Bologna
Via Selmi 3
40126 Bologna, Italy

Stupack, Dwayne G.
Department of Pathology & Moores UCSD Cancer Center
University of California
San Diego
3855 Health Science Drive
MC0803, USA

Tomasi, Vittorio
Department of Experimental Biology
University of Bologna
Via Selmi 3
40126 Bologna, Italy

Vollmar, Brigitte
Department of Experimental Surgery
University of Rostock
Schillingallee 70
D-18055 Rostock, Germany

Weinstein, Brant, M.
National Institute of Child Health and Human Development
National Institutes of Health
9000 Rockville Pike
Bethesda
Maryland 20892, USA

Wong, Michael K. K.
Roswell Park Cancer Institute
Elm and Carlton Streets
Buffalo
New York 14263, USA

Yonezawa, Sei
Department of Medical Biochemistry
School of Pharmaceutical Sciences
University of Shizuoka
Yada, Suruga-ku
Shizuoka 422-8526, Japan

Zijlstra, Andries
Department of Cell Biology
The Scripps Research Institute
10550 North Torrey Pines Road
La Jolla, CA 92037, USA

1

Endothelial cell biology

Femke Hillen, Veerle Melotte, Judy R. van Beijnum and **Arjan W. Griffioen**

Abstract

Vascular endothelial cells are organized as a thin layer on the interior surface of all vessels and are known to function in a variety of important physiological processes. The interactions of endothelial cells with other cells and with the extracellular matrix are crucial in endothelial cell functions such as the initiation of coagulation, leukocyte adhesion and the selection of a leukocyte infiltrate, the angiogenesis cascade, and transport of molecules through the vessel wall by active or passive mechanisms. This chapter highlights these processes and describes endothelial cells, their heterogeneity, various isolation techniques and their use in *in vitro* models.

Keywords

endothelial cell morphology; endothelial cell functions; angiogenesis; isolation and culture; heterogeneity

1.1 Introduction

In 1661, Marcello Malpighi described for the first time the existence of capillaries in the mesenterium and the lung of a frog. The anatomical research of blood vessels was greatly advanced and stimulated by contributions of the pioneers in the development of microscopy, Antonie von Leeuwenhoek (1632–1723) and Jan Swammerdam (1637–1680), who developed with Friedrich Ruysch (1638–1731) the injection techniques for coloured solutions into the vessel lumen. Friedrich Gustav Jacob Henle introduced the expression 'epithelium' in 1837. Between 1841 and 1859, Henle, von Koelliker and Frey showed that the capillaries have their own wall, like a structureless skin with nuclei. A forceful discussion started about the origin, development and functions of endothelial cells, lasting until around

Angiogenesis Assays Edited by Carolyn A. Staton, Claire Lewis and Roy Bicknell

1930. For many years the endothelium was thought of as an inert single layer of cells that passively allowed the passage of water and small molecules across the vessel wall. In the 1920s and 1930s a new area began when Lewis and Shibuya published their first results on cultivation of endothelial cells. Between 1884 and 1950, 135 papers were published dealing with various cultivation techniques for endothelial cells (Thilo-Korner and Heinrich, 1983).

1.2 Morphology of the endothelium

As a monolayer lining the entire circulatory system, the endothelial cell surface consists of about 1 to 6×10^{13} cells and weighs approximately 1 kg (Cines *et al.*, 1998; Sumpio *et al.*, 2002). The whole circulatory system has a common basic structure and consists of three different layers: the *tunica intima* constitutes endothelium supported by a basement membrane and delicate collagenous tissue, an intermediate muscular layer which is named the *tunica media* and an outer supporting tissue layer called the *tunica adventitia* (Gallagher, 2005).

It is currently widely recognized that endothelial cells show a remarkable heterogeneity along the vascular tree, as a biological adaptation to local needs. This heterogeneity is most obvious at the morphological level. Based on the endothelium, vessel phenotype can be classified as continuous, fenestrated or discontinuous. These phenotypes relate to the differences in permeability displayed by various vascular beds. In *continuous capillaries* endothelial cells line the full surface of the vascular wall. This vessel type is found in most tissues. In *fenestrated capillaries* the endothelial cells have small openings, called fenestrae, about 80–100 nm in diameter. Their permeability is much greater than that of continuous endothelium type capillaries and they are found in the small intestine, endocrine glands and the kidney. Fenestrae are sheltered by a small, non-membranous, permeable diaphragm, and allow the rapid passage of macromolecules. The basement membrane of endothelial cells in fenestrated vessels is continuous over the fenestrae. *Discontinuous capillaries*, also called sinusoids, have a large lumen, many fenestrations with no diaphragm and a discontinuous or even absent basal lamina. Such vessels are found in the liver, spleen, lymph nodes, bone marrow and some endocrine glands (Cleaver and Melton, 2003; Ghitescu and Robert, 2002). Broad modulations even exist within each type of endothelium, for example, within the continuous endothelium, the extremes are the brain capillaries (with very few plasmalemmal vesicles) and the heart capillaries (rich in such vesicles) (Renkin, 1988). Beside this traditional classification, other distinguishing features are used, such as endothelial cell size or shape, orientation with respect to the direction of blood flow, complexity of inter-endothelial junctions, presence or absence of diaphragms on fenestrations and of plasmalemmal bodies, and composition of the vessel wall (Cleaver and Melton, 2003; Ghitescu and Robert, 2002).

In addition to morphological heterogeneity, there is also functional heterogeneity of endothelial cells, including roles in control of vasoconstriction and vasodilatation,

blood coagulation and anticoagulation, fibrinolysis, leukocyte homing, acute inflammation and wound healing, atherogenesis, antigen presentation and catabolism of lipoproteins.. Structural and functional diversity of endothelial cells is, as might be expected, the result of molecular differences between endothelial cell populations. These differences have been investigated between various populations of endothelial cells, such as those of arteries and veins (Lawson *et al.*, 2001; Wang *et al.*, 1998; Zhong *et al.*, 2000), large and small vessels (Muller *et al.*, 2002) and normal and tumour vessels (Carson-Walter *et al.*, 2001; St Croix *et al.*, 2000).

In the mature vascular system, the endothelium is supported by mural cells that express characteristics specific to their localization. The arteries and veins are surrounded by single or multiple layers of vascular smooth muscle cells, whereas the smallest capillaries are partially covered by solitary cells referred to as pericytes (Gerhardt and Betsholtz, 2003). Smooth muscle cells maintain the integrity of the vessel and provide support for the endothelium. They control blood flow by contracting or dilating in response to specific stimuli.

Smooth muscle cells synthesize the connective tissue matrix of the vessel wall, which is composed of elastin, collagen and proteoglycans. Like endothelial cells, smooth muscle cells show a very low level of proliferation in the normal artery but proliferate in response to vessel injury.

Pericytes are associated with capillaries and post-capillary venules. They provide structural support to the endothelial cells and mediate endothelial cell function. Pericytes constitute a heterogeneous population of cells and their ontogeny is not well understood. Differences in pericyte morphology and distribution among vascular beds suggest tissue-specific functions. The number of pericytes also varies among different tissues and among vessels at different sites. Pericytes are plastic and have the capacity to differentiate into other mesenchymal cell types, such as smooth muscle cells, fibroblasts and osteoblasts (Jung *et al.*, 2002).

Arteries and veins

A well-known anatomical and physiological distinction between vessels is that of arteries and veins (Carmeliet, 2003). Arterial vessels carry afferent circulation and are exposed to the highest pressure and flow and are characteristically surrounded by a thick medial layer consisting mostly of vascular smooth muscle cells. In contrast, venous vessels carry efferent circulation with low pressure, have less surrounding smooth muscle, and possess specialized structures, such as valves, to ensure blood flow in a single direction. Although differences in fluid dynamics within the circulation play an important role in determining the characteristic structure of an artery or vein, recent evidence suggests that the identity of endothelial cells lining these vessels is established before the onset of circulation by genetic mechanisms during embryonic development (Lawson *et al.*, 2002; Wang *et al.*, 1998). Several breakthrough discoveries have led to our current understanding of the molecular difference between arterial and venous endothelial

cells. In 1998, the group of Anderson showed that EphrinB2 and EphB4 were specific markers for arterial and venous endothelial cells, respectively (Sato, 2003), which showed for the first time that the arterial–venous distinction had a genetic basis. Consequently, in the cardiovascular system, EphrinB2 expression is restricted to the arteries, smooth muscle cells, pericytes and mesenchyme that surround sites of vascular remodelling. EphB4 is expressed predominantly on venous and lymphatic endothelial cells (Harvey and Oliver, 2004). The difference between arteries and veins is also guided by *gridlock* (grl), an artery-restricted gene that is expressed in the lateral posterior mesoderm and acts downstream of the notch signalling pathway (see below). The gridlock gene was first described by Weinstein and Fishman in 1995 (Weinstein *et al.*, 1995; Zhong *et al.*, 2000). In 2001, the same researchers observed that the Notch signalling pathway is regulated by the earlier described gridlock gene. In mammals, four different Notch receptors (Notch 1–4) have been cloned and characterized and these receptors bind to five ligands (Jagged 1 and 2 and Delta-like 1, 3 and 4). The Notch pathway is activated when endothelial cells adopt a venous phenotype but when this pathway is inhibited by the gridlock gene, endothelial cells assume the arterial fate. Among the potential molecules that may act upstream of the Notch pathway to induce arterial differentiation is vascular endothelial growth factor (VEGF). Most recently, three independent groups discovered that VEGF act as an inducer of the arterial fate of endothelial cells (Harvey and Oliver, 2004). In zebrafish it was discovered that the sonic Hedgehog pathway, which lies upstream of VEGF, also functions in regulating the arterial fate of endothelial cells (Sato, 2003). Since their isolation in the early 1990s, members of the Hedgehog family of intercellular signalling proteins have been recognized as key mediators of many fundamental processes of embryonic development. Several studies suggest an important role for sonic Hedgehog, in particular, during blood vessel development. Recent work has shown that sonic Hedgehog can promote angiogenic blood vessel growth in part by inducing the expression of vascular endothelium growth factor, and as well as angiopoietin-1 and -2. These observations suggest that sonic Hedgehog may cooperate with vascular-specific growth factors during the development of the embryonic vasculature.

1.3 Endothelial cell adhesion and interactions

Endothelial cells have an important function in the interaction with each other and with a large variety of other cells, among which are pericytes, smooth muscle cells and leukocytes, as well as with the extracellular matrix. To accomplish these functions endothelial cells are equipped with a variety of different adhesion molecules.

Endothelial cell–cell interactions

Cell–cell-interactions are important for the regulation of tissue integrity, and the generation of barriers between different tissues and body compartments. Individual

cells are anchored together through adhesion junctions, organized in three categories: tight junctions, adherens junctions and gap junctions (Bazzoni and Dejana, 2004; Dejana, 2004). The adhesion molecules that function in these structures as well as several other molecules important in cell–cell adhesion will be discussed. The intercellular interactions, mediated by these adhesion receptors, are important in the regulation of intracellular signalling.

Adherens and tight junctions both share the same binding feature. In both types of junctions, adhesion is mediated by transmembrane proteins that promote homophilic interactions and form a zipper-like structure along the cell border (Figure 1.1).

Tight junctions are responsible for regulating paracellular permeability and play a role in maintaining cell polarity by subdividing the plasma membrane into an

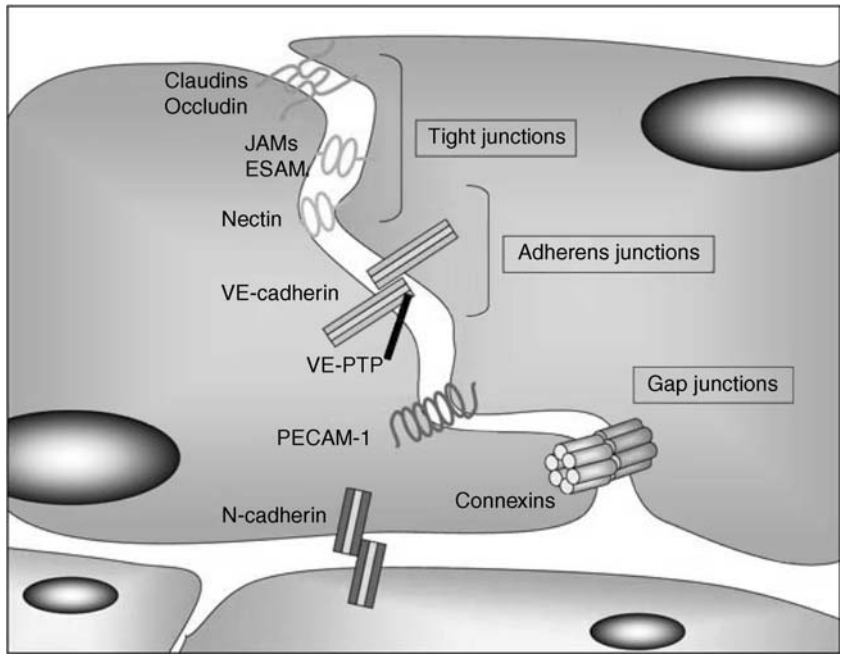

Figure 1.1 Endothelial cell–cell junctions transmembrane adhesive proteins between endothelial cells are organized in three classes. Members of the tight junctions are claudins, occludin, junctional adhesion molecules (JAMs) and endothelial cell selective adhesion molecule (ESAM). The adherens junctions are represented with adhesion molecules like vascular endothelial cadherin (VE-cadherin), which, through its extracellular domain, is associated with vascular endothelial protein tyrosine phosphatase (VE-PTP). Nectin has a role in the organization of both tight junctions and adherens junctions. Outside these junctional zipper-like molecules, platelet endothelial cell adhesion molecule (PECAM) participates to endothelial cell–cell adhesion. In endothelial cells, neuronal cadherin (N-cadherin) is connecting endothelial cells to pericytes and smooth muscle cells. Gap junctions are composed of arrays of small channels that permit small molecules to shuttle from one cell to another and thus directly link the interior of adjacent cells. Adapted from a figure by Dejana (2004). (A colour reproduction of this figure can be viewed in the colour section towards the centre of the book).

apical and a basolateral side. These structures are located at the boundary between apical and basolateral domains. The main function of tight junctions is their barrier function. The adhesion molecules that form these stuctures have a molecular architecture that is highly complex. Zonula-occludens-1 (ZO-1) was first discovered in 1986 and is perhaps the most extensively studied tight junction molecule (Dejana, 2004). Other important tight junction proteins are occludins, claudins (Schneeberger and Lynch, 2004), junctional adhesion molecules (JAMs; Keiper *et al.*, 2005) and endothelial cell selective adhesion molecule (ESAM; Hirata *et al.*, 2001).

Adherens junctions are important in the regulation of contact inhibition of cell growth, transendothelial migration of leukocytes and solutes, and in the organization of new vessels during angiogenesis (Bazzoni and Dejana, 2004). They are distributed in all blood and lymphatic vessels. Endothelial cells express an important key player in these structures, a member of the cadherin family, called vascular endothelial cadherin (VE-cadherin; Vincent *et al.*, 2004). VE-cadherin forms dimers that then undertake a second head-to-head dimerization with another VE-cadherin dimer on an adjacent cell. Through its extracellular domain, VE-cadherin is associated with a vascular endothelial protein tyrosine phosphatase (VE-PTP). The latter molecule binds through its cytoplasmatic tail to components like β-catenin, plakoglobulin and P120, that through signalling mediate cell shape and polarity. Nectin and its cytoplasmatic binding partner afadin are also present on endothelium, but little is known about their specific function. They carry out a role in both adherence and tight junctions (Takai and Nakanishi, 2003).

Gap junctions allow the passage of small molecular weight solutes and ions from cell to cell. These intercellular junctions allow direct electrical and metabolic communication between endothelial cells, between endothelial cells and smooth muscle cells and between endothelial cells and lymphocytes or monocytes (Nilius and Droogmans, 2001). Because ions can flow through them, gap junctions permit changes in membrane potential to pass from cell to cell which are constructed as a hexamer of transmembrane proteins called connexins. Through the variable use of several isoforms of connexins, there is variability in functional cell–cell interactions.

Endothelial cells have also other cell-specific homophilic adhesion proteins at the intercellular contacts. Two of the most studied are platelet endothelial cell adhesion molecule-1 (PECAM-1) and S-endo-1, both members of the immunoglobulin superfamily. The amino-terminal immunoglobulin-like domain of PECAM-1 is involved in homophilic binding on adjacent cells. Other domains of this molecule are involved in heterophilic adhesive interactions with several ligands such as $\alpha_v\beta_3$, CD38 and several proteoglycans (Jackson, 2003). S-endo-1 (also termed CD146, Mel-CAM, MCAM, MUC18 or A32 antigen) is a membrane glycoprotein involved in homophilic cell–cell interactions, but its binding partner is still unknown (Bardin *et al.*, 2001).

Another member of the cadherin family, N-cadherin, with the same type of dimerization, can be found at comparable levels to VE-cadherin in most endothelial cells. In contrast to VE-cadherin, N-cadherin is localized at the basal side of endothelial cells and is in contact with pericytes or smooth muscle cells.

Endothelial cell–matrix interactions

Maintenance of the integrity of the vessel wall is one of the most important functions accomplished through interactions between the vascular endothelium and the surrounding matrix. The sub-endothelium, a protein rich matrix underneath the endothelial cells, is crucial in the preservation of optimal endothelial cell functioning. Specific matrix ligands and receptors on the membrane contribute to the maintenance of an intact endothelial cell layer. The extracellular matrix (ECM) is organized in two layers, one of which is composed of a vascular basement membrane or basal lamina and smooth muscle cells, and the other is composed of interstitial matrix. The basement membrane consists of a network of molecules such as collagen IV, laminin, heparin sulphate proteoglycans and nidogen/entactin (Kalluri, 2003), whereas typical components of the interstitial matrix are fibrillar collagens and glycoproteins such as fibronectin (Iivanainen *et al.*, 2003). The extracellular matrix not only has a mechanical role in supporting and maintaining tissue architecture but can also be described as a dynamic structure that regulates migration, proliferation and differentiation of endothelial cells. Under normal physiological conditions in resting tissues, endothelial cells have a slow turnover and adhesive interactions with the extracellular matrix are stable. When angiogenic stimuli are present, one of the first events to occur is the production of specific proteases (matrix metalloproteinases) by endothelial cells that are capable of degrading matrix components. This causes specific molecular interactions between vascular endothelial cells and the surrounding microenvironment to change, paving the way for the formation of new blood vessels.

These interactions with the extracellular matrix occur mainly through integrins and heparan sulphate proteoglycans. Integrins are heterodimeric transmembrane proteins that consist of an α and a β subunit. There are 18 known α and eight known β subunits which form at least 24 different heterodimers in mammals. These molecules recognize ECM components and are expressed by all adhesive cells (Iivanainen *et al.*, 2003). Integrin-mediated cellular adhesion to ECM leads to intracellular signalling and modulates endothelial cell adhesion by targeting matrix degrading enzymes to the site of sprouting. For example, integrin $\alpha_v\beta_3$, the integrin that is the best characterized for its role in angiogenesis, interacts directly with MMP-2 (Brooks *et al.*, 1996). Another function of integrins is the regulation of the activity of a number of angiogenic and antiangiogenic factors, for example, $\alpha_v\beta_3$ directly associates with, and regulates the signalling of, vascular endothelial growth factor (VEGF) receptor 2. In addition, $\alpha_v\beta_3$ is induced in endothelial cells by angiogenic growth factors such as VEGF and bFGF. Other antiangiogenic molecules, such as endostatin, angiostatin and thrombospondin, that are natural components of the ECM, can also bind to $\alpha_v\beta_3$ and disrupt the endothelial cell–extracellular matrix interactions. Finally, it is known that many signalling pathways activated by integrins are also directly or indirectly activated by growth factors (Li *et al.*, 2003; Stupack and Cheresh, 2004).

A second group of endothelial receptors are the cell surface heparan sulphate proteoglycans (HSPGs) (Iivanainen *et al.*, 2003). Many matrix components have

heparin binding motifs that mediate the interaction with cell surface HSPGs. This group of cell surface adhesion molecules consist of a core protein that is covalently linked to heparin sulphate-type glycosaminoglycan side-chains. There are two main HSPG gene families that are present in the membrane of cells: the syndecans and glypicans. Syndecans are transmembrane molecules that signal through various pathways by their cytoplasmic tail. Glypicans do not have a hydrophobic transmembrane or cytoplasmic domain and are anchored to the cell surface at the extracellular site by a glycosyl-phosphatidyl-inositol (GPI) anchor. This anchor gives glypicans the potential to participate in intracellular signalling. HSPGs can also contribute to signalling by interaction with other matrix receptors that anchor directly to the cytoskeleton and serve as an integrin co-receptor. Endothelial cells express syndecan-1, syndecan-4, glypican-1 and glypican-4. Other membrane glycoproteins, which carry heparan sulphate side-chains and are present on endothelial cells, are betaglycan and CD44. Syndecan-1 and 4 are known to be induced during neovascularization during wound repair (Gallo *et al.*, 1996).

In normal physiological conditions endothelial cells are quiescent and bound to the ECM. The structure of the ECM is complex and highly cross-linked, and only certain domains of the matrix components can bind to endothelial cells. Due to an angiogenic response, induced by VEGF, bFGF, PDGF and several chemokines, pericytes are detached, endothelial cells are dislodged from the blood vessels by degrading and invading through the ECM and detach from the adhesive components. The proteolytic degradation of the ECM is mediated by matrix proteinases. Their role in physiological and tumour-associated angiogenesis has been widely investigated. The best characterized enzymes, important in the degradation of both the vascular basement membrane and the underlying ECM, are the matrix metalloproteinases (MMPs) (Iivanainen *et al.*, 2003). MMPs are a family consisting of 22 members of zinc-dependent endopeptidases that can degrade ECM, cytokines, chemokines and their receptors. Based on their structure and substrate specificity, they are classified into several subgroups: collagenases, stromelysins, matrilysins, gelatinases and membrane type MMPs. Most of them are secreted as zymogens that will be activated by other MMPs or serine proteinases. After detachment of endothelial cells, MMPs can promote migration and proliferation of endothelial cells. In the initial step of angiogenesis a fibrin gel, a provisional matrix generated from fibrinogen leakage, is polymerized and endothelial cells attach to these provisional matrix components including fibrin, vitronectin, fibronectin, collagen I and thrombin.

Pro-angiogenic factors like VEGF and bFGF, produced by macrophages and tumour cells, are captured in the ECM and require matrix metalloproteinases such as MMP2 and MMP9 for mobilization of the growth factors and the initiation of angiogenesis. MMPs are predominantly secreted by stromal and immune cells. MMP-mediated degradation can be a positive and negative regulator of tumour angiogenensis (Sottile, 2004). At early degradation specific domains of matrix components like collagen, laminin and fibronectin provide pro-angiogenic signals. When degradation reaches completion, fragments like endostatin, arrestin, canstatin, tumstatin and other collagen fragments exert anti-angiogenic properties.