The Vascular Endothelium II

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Preface

It was with great pleasure that I accepted the invitation of Springer to edit this book. My association with the vascular endothelium covers a large part of my scientific career and, as with any good long-standing relationship, it has had moments of great excitement and periods of laborious construction. It has sometimes been difficult but has never given me cause for despondency. Indeed, in the last quarter of a century, research on the vascular endothelium has been very productive and its results have contributed, arguably more than any others, to unravelling the mystery of cardiovascular disease, its origin, its development, its complications and its prevention or treatment once it has developed.

I am very happy that Annie Higgs agreed to join me in this task. Over the years we have collaborated closely and, as always, she has shouldered the brunt of the work and has made sure that things get done to everybody's satisfaction. We have also been fortunate in that the scientists who have made some of the most significant contributions in the field agreed to write chapters; as a result, we have produced two volumes which is a good representation of our knowledge in early 2006. We are, however, aware that the field has expanded beyond all expectation and that there may have been some oversight in the covering of a specific area or some aspect of it. This is compounded by the speed at which knowledge is being generated, with more than 4,100 papers concerning the endothelium published in 2005.

These volumes are organised in such a way that the early chapters discuss the structure, development and function of the normal vascular endothelium. The subsequent chapters consider conditions that lead to disruption of vascular physiology, while the later chapters deal with specific pathologies and their treatment. The final chapter describes various gene-therapy strategies for the treatment of vascular pathologies. Interestingly, although this field of research can now be considered mature, it continues to generate a great deal of new information at a time when some of its fruits are having a direct impact on clinical medicine. This is clearly exemplified in the contents of most of the chapters.

The concept of endothelial dysfunction, although mooted many years ago, has come to the fore and has been very useful in defining a situation which may exist long before the overt signs of vascular diseases can be identified. Although

endothelial dysfunction is likely to comprise a variety of disturbances, it is interesting that these days it is almost exclusively measured as a decrease in nitric oxide (NO)-dependent vascular dilatation, either induced by suitable pharmacological agonists or by increases in blood flow. Oxidative stress, which is associated with the genesis of endothelial dysfunction, is a loose term used to define an imbalance between the release of oxygen-derived free radicals and the anti-oxidant systems of the body. Many years ago our work established that reactive oxygen species are important in reducing the local concentrations of both prostacyclin and NO. It is now clear that free radicals also affect other homeostatic systems in the vasculature. However, many things remain to be clarified, especially the origin of oxidative stress in early disease.

The absence of one of these mediators, in this case not NO, but prostacyclin, has been discussed in the scientific and popular press for the past couple of years. The reason is that it is very likely that the cardiovascular side effects which have led to the withdrawal from the market of the anti-inflammatory class of drugs known as COX II inhibitors are due to their inhibitory action on the generation of prostacyclin by the vasculature, leading to a pro-thrombotic situation. The fact that reducing prostacyclin formation in the vasculature leads eventually to cardiovascular events validates the concept we proposed in 1976 that a balance between the generation of thromboxane A_2 by the platelets and prostacyclin by the vessel wall is significant in defining the pro- or anti-thrombotic status of the cardiovascular system. Previously, the only evidence available came from the action of low-dose aspirin which, by inhibiting platelet thromboxane A_2 without affecting prostacyclin, leads to an anti-thrombotic situation. This raises the issue about the status of a cardiovascular system in which both prostacyclin and thromboxane A_2 are inhibited following long-term administration of the classical COX I inhibitors, something which we are only now beginning to address.

The above are just a few considerations that exemplify the problems and challenges that occupy a great deal of our attention today. They show that the vascular endothelium has moved a long way from the "cellophane wrapper" described by early vascular biologists to being recognised as an organ with a variety of functions, some of which, I am sure, remain to be defined. What has yet to be discovered promises to be as exciting and rewarding as that which we already know.

London, S. Moncada
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Haemostasis

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Abstract When the continuity of the vascular endothelium is disrupted, platelets and fibrin seal off the defect. Haemostatic processes are classified as primary (mainly involving platelets) and secondary (mainly related to fibrin formation or blood coagulation). When the blood clot is no longer required for haemostasis, the fibrinolytic system will dissolve it. The pivotal ligand for initial platelet recruitment to injured vessel wall components is von Willebrand factor (vWF), a multimeric protein present in the subendothelium and in plasma, where it is conformationally activated by shear forces. Adhering activated platelets recruit additional platelets, which are in turn activated and form a platelet aggregate. Coagulation is initiated by a reaction, activating factors IX and X. Once critical amounts of factor Xa are generated, thrombin generation is initiated and soluble fibrinogen is converted into insoluble fibrin. Excessive thrombin generation is prevented via inhibition by antithrombin and also via downregulation of its further generation by activation of the protein C pathway. Activation of the fibrinolytic system results from conversion of the proenzyme plasminogen into the active serine proteinase plasmin by tissue-type or urokinase-type plasminogen activators. Plasmin digests the fibrin component of a blood clot. Inhibition of the fibrinolytic system occurs at the level of the plasminogen activator (by plasminogen activator inhibitors) or at the level of plasmin (by α_2-antiplasmin). Together, these physiological processes act to maintain normal functioning blood vessels and a non-thrombotic state.

Keywords Haemostasis · Thrombosis · Bleeding · Platelets · Coagulation · Fibrinolysis

1 Introduction

Integrity of the vascular wall is a prerequisite for normal functioning blood vessels and for maintenance of a non-thrombotic state. When the continuity of the vascular endothelium is disrupted, platelets and fibrin seal off the defect, and the fibrinolytic system dissolves the blood clot. The endothelial cells, which form a monolayer lining the inner surface of blood vessels, synthesise and release activators and inhibitors of platelet aggregation, blood coagulation and fibrinolysis and thus play an active role in the regulation of these systems by providing both procoagulant and anticoagulant substances.

Vessel wall injury exposes subendothelial matrix and collagen fibres to flowing blood; circulating platelets adhere to these structures and initiate arrest of blood flow. Both subendothelial and circulating vWF play an important role in platelet adhesion to sites of injury, in particular in the arterial circulation, where shear forces conformationally activate vWF. Adhering activated platelets recruit additional platelets from the flowing blood, which are in turn activated

via secondary amplification loops resulting in the formation of a platelet aggregate. Activation of the coagulation cascade on the platelet surface results in the formation of a fibrin network that provides a matrix for cell migration, thus supporting wound healing.

In the current model of blood coagulation, the extrinsic PTase reaction initiates coagulation (Broze 1995a, b). Once critical amounts of factor Xa (required for the initiation of thrombin generation) are formed, the extrinsic PTase reaction is efficiently turned off by the tissue factor pathway inhibitor (TFPI), and further formation of thrombin is maintained via positive feedback mechanisms involving thrombin-induced activation of factors V, VIII and XI. Thrombin converts fibrinogen to fibrin. Excess thrombin is efficiently inhibited by its physiological inhibitor antithrombin and downregulates its own generation via stimulation of the protein C pathway.

The fibrinolytic system generates a serine proteinase, plasmin, that degrades fibrin into soluble fibrin degradation products, and thus plays an important role in the dissolution of blood clots and in the maintenance of a patent vascular system.

2
Platelets in Haemostasis

Platelet recruitment to injured vessel wall components depends on several platelet receptors. Strong adhesion to fibrillar collagens I and III, localised in the deeper layers of the vasculature, is determined by specific collagen receptors such as glycoprotein (GP)VI and GP $\alpha_2\beta_1$ integrin (see Sect. 2.3). Platelets adhere to subendothelial vWF. This is a multimeric protein, synthesised by endothelial cells (Jaffe et al. 1974) and stored in specialised inclusion particles, the Weibel–Palade bodies. vWF is released in the circulation and deposited in the subendothelium. Although platelets have several integrin receptors that mediate adhesion to extracellular matrix-associated fibronectin and laminin (Bastida et al. 1987; Hindriks et al. 1992), vascular wall-associated vWF appears to be the pivotal ligand for initial platelet recruitment.

2.1
Von Willebrand Factor

The central role of vWF in haemostasis is supported by several observations. First, vWF is associated with collagen VI in the subendothelium (Rand et al. 1993). Upon de-endothelialisation, subendothelial vWF becomes a potent vascular ligand triggering platelet rolling and tethering. Second, circulating vWF contributes to haemostasis in a dual manner: It carries factor VIII and binds to vascular collagens exposed to the blood stream (Bolhuis et al. 1981). Third, it

participates in platelet–endothelial cell interactions and is thus at the interface between haemostasis and inflammation (Theilmeier et al. 2002).

2.1.1 Structure of vWF

The mature vWF subunit (~250 kDa) consists of four types of repeating functional domains arranged in the following sequence: D′-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK (Shelton-Inloes et al. 1986). The binding site for factor VIII is located within the D′ domain, and that for platelet GPIbα within the A1 domain. A platelet integrin $\alpha_{IIb}\beta_3$ binding sequence Arg-Gly-Asp (RGD) is located in the C1 domain, and the main collagen binding site is located in the A3 domain (Romijn et al. 2003).

The A1 domain is structurally shaped by a disulphide bridge between Cys^{509} and Cys^{695} (Sugimoto et al. 1991). X-ray diffraction studies of the A1 domain revealed a globular shape, comprising a central core constituted of six hydrophobic β-strands, surrounded by six amphipathic α-helices (Celikel et al. 1998; Jenkins et al. 1998). Analysis of naturally occurring loss-of-function mutations, together with mutagenesis and GPIbα peptide docking studies (Cruz et al. 2000; Matsushita et al. 2000; Bonnefoy et al. 2003), has identified a central front groove on the A1 domain next to strand β3, as part of the binding site for GPIbα. Recently, the crystal structure of a gain-of-function A1 domain mutant in complex with the amino-terminal domain of GPIbα (also containing a gain-of-function mutation) confirmed that the frontal part of A1 constitutes the contact area for GPIbα. Furthermore, two distinct areas of tight interaction were revealed, the first and most extensive contact site located near the top of A1, the second involving residues near the bottom face of A1 (Huizinga et al. 2002). In a shear stress field, vWF A1 domains undergo a conformational change, triggering binding to GPIb (Ruggeri 1993; Siedlecki et al. 1996).

The A3 domain (aa 920–1,111) contains the major binding site for fibrillar collagens I and III. Unlike the I domain of integrin chains α_1, α_2, α_{10} and α_{11}, the A3 domain lacks a functional metal ion dependent adhesion site (MIDAS) motif (Pietu et al. 1987). Binding to collagen occurs via residues located in the strand β_3 and the loop $\alpha_3\beta_4$ in the lower half of the front face of A3 (Romijn et al. 2003).

2.1.2 Function of vWF

In small arterioles, in stenosed arteries and at atherosclerotic plaques in partially occluded arteries, platelet adhesion occurs, controlled by elevated fluid shear stress. GPIb/IX/V on flowing platelets interacts with immobilised vWF, initiating platelet tethering to the damaged area (Savage et al. 1992). During translocation, the platelet is progressively activated and adheres by forming

tight bonds between platelet–membrane-activated integrins and vessel wall components, such as collagen and vWF. Subsequently, circulating platelets recognise adhesive molecules (mainly vWF and fibrinogen) on already adhered platelets and initiate platelet aggregation. At elevated shear forces, platelet recruitment and thrombus growth are mainly dependent on platelet binding to vWF, although fibrinogen binding to platelet integrin $\alpha_{IIb}\beta_3$ is also required for thrombus consolidation (Savage et al. 1992; Ni et al. 2000). In the absence of flow, soluble vWF A1 domain sequences are not available for interaction with GPIb, but when exposed to wall shear rates exceeding 600 s^{-1} (Wu et al. 2000), soluble vWF acquires affinity for platelet GPIb and subendothelial vWF is activated. Thus, both subendothelial and collagen-bound vWF participate in platelet recruitment (Sixma et al. 1991).

2.1.3 vWF-GPIb/IX/V Interactions in Arterial Thrombogenesis

Epidemiological studies uncovered a link between elevated plasma vWF levels and the incidence of heart disease caused by arterial thrombosis (Folsom et al. 1997). The plasma of patients with acute myocardial infarction exhibit elevated plasma vWF concentrations and support enhanced shear-induced platelet activation (SIPA), suggestive of a causative role for vWF in acute coronary thrombosis (Goto et al. 1999). Moreover, upregulated vWF antigen contributing to platelet recruitment has been found in atherosclerotic plaques, after balloon angioplasty (Bosmans et al. 1997) or collar placement (De Meyer et al. 1999) and in hyperplastic intima of autogenous arterial grafts (Qin et al. 2001).

2.2 The GPIb Complex as a Platelet Receptor for vWF

The GPIb/IX/V receptor is assembled from four gene products in a hetero-oligomeric complex in the platelet membrane.

2.2.1 GPIb/IX/V Organisation

The GPIb unit is composed of covalently linked GPIbα (~145 kDa) and GPIbβ (~22 kDa) subunits. GPIb is non-covalently associated to GPIX (~17 kDa) and GPV (~82 kDa). GPIb/IX/V seems to be specifically expressed by megakaryocytes and platelets, although treatment of cultured endothelial cells with cytokines has been reported to induce GPIbα messenger RNA (mRNA) expression (Rajagopalan et al. 1992). GPIb/IX/V is expressed on the platelet surface at about 25,000 copies per platelet, each complex assembling with an apparent molecular ratio of 2:2:2:1 (GPIbα:GPIbβ:GPIX:GPV). GPV occupies

the central position, although it does not seem to be needed for GPIb membrane stabilisation and function. GPIbβ and GPIX are required for the correct processing and membrane surface expression of intact GPIb/IX/V. GPIbα is composed of 610 residues, oriented in the platelet membrane with its amino terminus in the extracellular space. It has a large extracellular domain, a single transmembrane domain and a short cytoplasmic tail. The GPIbα subunit contains the binding site for vWF located within a globular amino-terminal domain (~residues 1–290), characterised by leucine-rich-repeats (Shen et al. 2000; Huizinga et al. 2002).

2.2.2 GPIb Mediated Platelet Activation

The cytoplasmic domain of GPIbα (96 residues) binds to filamin-1 and to the adaptor protein 14.3.3ζ (Williamson et al. 2002). These interactions anchor the membrane complex to the cytoskeleton, contributing to the control of dynamic interactions between sheared platelets and vWF. This domain transduces signals, resulting in activation of the integrin $\alpha_{IIb}\beta_3$ (GPIIbIIIa) (Yap et al. 2000). A binding site for 14.3.3ζ also exists on the GPIbβ chain, and binding is controlled via phosphorylation of Ser^{166} by a protein kinase A. Finally, binding sites for calmodulin have been described both on GPIbβ and GPV (Andrews et al. 2001). The mechanism by which vWF binding to GPIb/IX/V mediates $\alpha_{IIb}\beta_3$ activation is poorly understood. It involves protein tyrosine phosphorylation (Syk and Src), activation of protein kinase C and phosphoinositol 3 (PI3) kinase, elevation of the intracellular calcium concentration and synthesis of thromboxane A_2 (Wu et al. 2003). Activation is dependent on co-associated transmembrane proteins, such as the FcR γ-chain and FcγRIIA, containing an immunoreceptor tyrosine-based activation motif (ITAM). Recent studies suggest that the p85 subunit of PI3 kinase mediates GPIb-related activation signals and activates Src independently of the enzymatic activity of the PI 3-kinase (Wu et al. 2003).

2.3 Platelet Collagen Receptors

Collagens are structural proteins found in many tissues including the vascular wall. Containing collagen-like domains, the collagen superfamily consists of some 20 members and some 10 additional proteins. The most abundant collagens in the vascular extracellular matrix are type I and III. These are organised in fibrils, providing extracellular strength to the vascular system. Other collagens, present in smaller amounts in the vessel wall, are fibrillar collagen V, the network-forming collagens type IV and VIII, the beaded filament-forming microfibrillar collagen VI, and fibril-associated collagens, with interrupted triple helices, type XII and VIV. Collagen type IV is a major component of the base-

ment membrane underlying the endothelium, and together with types I and III, it represents the most reactive collagen with regard to platelet activation (Madri et al. 1980; Palotie et al. 1983).

2.3.1 Integrin $\alpha_2\beta_1$ Structure

Integrin $\alpha_2\beta_1$ (GPIaIIa, VLA-2 or CD49b/CD29) is expressed on endothelial cells, fibroblasts, lymphocytes and platelets. On platelets, $\alpha_2\beta_1$ mainly serves as a collagen receptor, whereas on endothelial and epithelial cells it reacts both with collagen and laminin (Elices and Hemler 1989). On platelets, $\alpha_2\beta_1$ has a low density, approximately 1,000–2,900 copies per platelet. It is an integrin, composed of two non-covalently linked transmembrane polypeptides α and β, with the overall shape of a globular head standing on two long legs, ending in a pair of single-pass transmembrane helices and short cytoplasmic tails (Humphries 2000). The α_2-subunit is 1,181 amino acids long (~165 kDa). The short C-terminal α_2 cytoplasmic tail contains a highly conserved GFFKR motif, important for integrin activation (Wang et al. 2003) and binding of several intracellular proteins such as F-actin and calreticulin (Rojiani et al. 1991).

Common to several integrin α-chains, the α_2-subunit contains a 200-amino-acid inserted domain (I-domain) between the second and the third repeat, probably presented on the upper surface of the β-propeller. This I-domain is homologous to the vWF A-domains and recapitulates many of the ligand binding properties of the parent integrin. Unique to the α_2 I-domain is an additional short α-helix, called a C-helix, at the top of the domain in close proximity to the MIDAS. Both mutagenesis and crystallography studies showed that the MIDAS-motif is required for ligand binding (Emsley et al. 2000). The β_1-subunit has a molecular weight of 130 or 110 kDa under reducing or non-reducing conditions, respectively. Like the α_2-subunit, the β-chain is a type I transmembrane protein with a large extracellular domain, a single passing transmembrane region and a short cytoplasmic tail. The extracellular part further contains four cysteine-rich epidermal growth factor (EGF)-like repeated segments that all have a high number of internal disulphide bridges. This domain has endogenous disulphide isomerase activity that might be responsible for regulating conformational changes in the integrin (Lahav et al. 2003).

2.3.2 Recognition Site in Collagen for Integrin $\alpha_2\beta_1$

The sequence GFOGER was identified as the ligand for $\alpha_2\beta_1$ in the CB3 peptide of collagen type I (Knight et al. 1998), whereas two weaker recognition sites, GLOGER and GASGER, were found (Xu et al. 2000). Sequence alignment of the α-chains of collagen type I and III showed that GAOGER and GLSGER occupy the same position. Another GAOGER motif was found in collagen type III

at the same position as the GFOGER in collagen type I. An additional $\alpha_2\beta_1$-binding sequence, GMOGER, was identified in the same position relative to GFOGER and GAOGER in collagen type I and type III respectively (Morton et al. 1989; Knight et al. 2000). The spatial distribution of these $\alpha_2\beta_1$-recognition sites is strongly preserved between the fibrillar collagens, suggesting that their organisation might have a role in platelet binding and signalling. The GFOGER peptide induced spreading of platelets through activation of Src and Syk family kinases, leading to tyrosine phosphorylation of PLCγ2 (Inoue et al. 2003). This pathway is very similar to the one utilised by GPVI (see below).

2.3.3 GPVI on Platelets

GPVI is a 63-kDa type I transmembrane glycoprotein belonging to the immunoglobulin (Ig) superfamily. In contrast to integrin $\alpha_2\beta_1$, GPVI is restricted to platelets and megakaryocytes (Jandrot-Perrus et al. 2000). It consists of 319 amino acids with two extracellular Ig-like domains formed by disulphide bonds, followed by a highly glycosylated stem of approximately 60 amino acids. The transmembrane region contains an arginine residue, critical for interaction with the FcR γ-chain (Zheng et al. 2001). The 51-amino-acid cytoplasmic domain contains binding sites for calmodulin (Andrews et al. 2002) and Src homology SH3 binding proteins (Suzuki-Inoue et al. 2002). The GPVI-Fc complex probably operates as dimers on the platelet surface, since pairs of GPO motifs separated by three or four intervening triplets interact best with the receptor.

Recently, the putative primary collagen binding site was localised to the apical area of GPVI, where the first Ig-like domain meets the interdomain linker, with lysine 59 as a crucial residue (Smethurst et al. 2004). GPVI does not require a specific recognition site, as it strongly binds to the collagen-related-peptide (CRP), which consists of a triple helical polymer of ten GPO triplets. In contrast, triple helical GPP polymers are very poor ligands. Platelet adhesion starts in the presence of one GPO triplet and gradually increases up to four GPO triplets. Only a slight additional increase is observed with CRP (ten GPO triplets). In the three-dimensional structure of collagen fibres, single GPO triplets of one strand may neighbour those of adjacent strands and thus constitute the required GPVI recognition motif (Farndale et al. 2003).

GPVI is non-covalently associated with the signal-transducing FcR γ-chain, also belonging to the Ig superfamily (Tsuji et al. 1997).This association is required both for surface expression and for the functional activity of GPVI. The cytoplasmic tail of GPVI contains a proline-rich domain that binds to the SH3 domains of the tyrosine kinases Fyn and Lyn (Quek et al. 2000). Cross-linking of GPVI by ligand binding may bring the SH3-associated kinases to the FcR γ-subunit, enabling phosphorylation of the ITAM of the FcR γ-subunit. This leads to binding and activation of tyrosine kinase, Syk, which further signals through a cascade of tyrosine phosphorylations in which the adaptor

molecules LAT (linker for activation of T cells) and SLP-76 play an important role. The result is an activation of several effector molecules such as PLCγ2 and PI3 kinase, finally leading to activation of protein kinase C and Ca^{2+} mobilisation from internal stores (Nieswandt and Watson 2003).

2.4 Secondary Platelet Recruitment and Aggregation

Bound adhering platelets are activated via transducing signals delivered to collagen and GPIb receptors by bound collagens and vWF, respectively. The vWF-mediated platelet activation is a consequence of shear stress-induced Ca^{2+} influx and is aspirin-insensitive (Kroll et al. 1996). Activated adhering platelets undergo morphological modifications associated with platelet spreading and the secretion of their granular contents. Thus, the released thromboxane A_2, serotonin, vWF and fibrinogen will further activate neighbouring platelets, finally resulting in glycoprotein $\alpha_{IIb}\beta_3$ receptor inside-out activation. This membrane receptor thus acquires the capacity to react with fibrinogen and to support platelet aggregation. Fibrinogen binding to $\alpha_{IIb}\beta_3$ is mediated primarily via the fibrinogen γ-carboxyterminal dodecapeptide as well as by its Arg-Gly-Asp (RGD) sequences (Steiner et al. 1989). Whereas initial platelet–vWF–collagen interactions are co-ordinated primarily via $\alpha_2\beta_1$ and GPIb, these interactions are consolidated via $\alpha_{IIb}\beta_3$, creating stable bonds between platelets (Savage et al. 1996).

Platelet degranulation releases nucleotides, such as adenosine diphosphate (ADP) and ATP, strongly amplifying platelet activation during the secondary recruitment phase of flowing platelets. The rapid secretion of nucleotides is capable of triggering even Ca^{2+}-dependent platelet activation steps involved in the permanent activation of the first layer of adhering platelets. Purines and pyrimidines act by interacting with distinct cell-surface receptors. Purinergic receptors were first recognised by Burnstock et al. (1978). They were divided into two classes: At P1 purinoceptors, adenosine is the principal natural ligand, while P2 purinoceptors recognise both purine and pyrimidine nucleotides, namely ATP, ADP, uridine triphosphate (UTP) and UDP (Abbracchio and Burnstock 1994).

The extensive and heterogeneous group of P2 receptors is subdivided into P2X ligand-gated cation channels and G protein-coupled P2Y receptors (Fredholm et al. 1997). The two main types of purinoceptors for extracellular nucleotides operate on different scales of time and distance. P2X receptors act within milliseconds whereas P2Y receptors trigger second-messenger cascades (Communi et al. 2000) that amplify and prolong the duration of the signal over hundreds of milliseconds or even seconds. Platelets have two P2Y receptors whose combined action is required for full activation and aggregation in response to ADP (Gachet 2001). One of these, $P2Y_1$, is coupled to the heterotrimeric guanosine triphosphate (GTP)-binding protein G_q and to

phospholipase C-β activation; it induces mobilisation of cytoplasmic Ca^{2+} and mediates shape change followed by an initial wave of rapidly reversible aggregation. The other receptor, $P2Y_{12}$ (Cattaneo et al. 1997), is negatively coupled to adenylyl cyclase through G_i; it mediates progressive and sustained platelet aggregation in the absence of shape change and plays an important role in the potentiation of secretion induced by several agonists via its interaction with released ADP. This process is independent of the formation of large aggregates and of thromboxane A_2 synthesis. $P2Y_{12}$-mediated activation of the PI3 kinase pathway contributes to stabilise thrombin-induced platelet aggregates (Trumel et al. 1999), although thrombin and thrombin-related peptides can cause platelet aggregation independently of G_i signalling (Kim et al. 2002). Comparison of the relative potency of $P2Y_1$ and $P2Y_{12}$ during experimental thrombosis in gene-deficient mouse models has demonstrated the central role of $P2Y_{12}$, the receptor inactivated by thienopyridines (see Sects. 2.6 and 2.5.2). Thrombosis studies in gene-deficient mice and in a mouse model overexpressing the ion channel $P2X_1$ have demonstrated that platelet activation also depends on contributions by degranulated ATP during shear stress-controlled events and during collagen-induced platelet aggregation (Hechler et al. 2003; Oury et al. 2003).

2.5 Lessons from Disease: Loss and Gain of Function

2.5.1 Bernard–Soulier Syndrome and Platelet-Type Von Willebrand Disease

Mutations affecting GPIb/IX/V integrity are associated with a prolonged bleeding time. In Bernard–Soulier syndrome, mutations in the GPIbα, GPIbβ or GPIX gene may affect the transport of the protein chains to the cellular membrane, leading to deficient vWF binding. Alternatively, non-functional mutations in *GPIb*α may allow normal transport, while resulting in defective platelet aggregation and reduced platelet adherence to subendothelium, especially at high shear stress (Hayashi and Suzuki 2000). In platelet-type von Willebrand disease (VWD), the bleeding tendency is due to gain-of-function mutations Gly233Val or Met239Val, in the GPIbα subunit. The effects of these mutations resemble type 2B VWD (see below). vWF spontaneously binds to the platelets, leading to depletion of large vWF multimers from the circulation and to moderate thrombocytopaenia and bleeding (Tait et al. 2001).

2.5.2 Von Willebrand Disease

VWD is an inherited bleeding disorder classified in three main groups according to biosynthesis defects and protein dysfunction. In type 1 VWD, vWF displays a partial quantitative deficiency with normal multimerisation. Patients with type 2 VWD manifest qualitative deficiencies categorised into four

variants: 2A, 2B, 2M and 2N. Type 2A VWD shows an absence of large vWF multimers due to defective vWF multimerisation in the Golgi or to increased proteolytic degradation in the plasma (Lyons et al. 1992). vWF from type 2B patients has an increased affinity for platelet GPIbα and shows spontaneous binding of multimers to platelets in vivo. This paradoxically results in a haemostatic defect due to large vWF multimer clearance from the circulation and intermittent thrombocytopaenia. vWF type 2M mutations cause defective binding to platelets, without dysfunctional multimerisation. Most 2M mutations are located in the A1 domain of vWF, compatible with defective binding to GPIbα. vWF type 2N mutations affect factor VIII binding, resulting in a reduced factor VIII stabilisation. This bleeding tendency resembles mild haemophilia A. Finally, type 3 VWD, the most severe subtype, is characterised by the absence of plasma, tissue or cellular vWF. Type 3 VWD is caused by frameshift, deletion and nonsense mutations (Sadler 1998).

2.5.3
Collagen Receptor Deficiency and Bleeding

Evidence for the importance of integrin $\alpha_2\beta_1$ in platelet function was obtained in a patient with mild bleeding problems related to strongly reduced expression of integrin $\alpha_2\beta_1$ (Nieuwenhuis et al. 1985). Platelet aggregation in response to various types of collagen and adhesion to collagen under static and flow conditions was markedly reduced, and the few platelets that adhered failed to spread. In this and in a second female patient, symptoms disappeared after menopause, accompanied by normalisation of the $\alpha_2\beta_1$ expression, suggesting that the gene defect was located in the promoter region of $\alpha_2\beta_1$ chains, the bleeding defect thus rather reflecting defective hormone regulation of gene expression. In a 66-year-old man with a myeloproliferative disorder and prolonged bleeding time but no bleeding history, a deficient collagen-induced aggregation and aberrant adhesion to collagen were found, due to an acquired deficiency in integrin $\alpha_2\beta_1$ (Handa et al. 1995). The first patient with a GPVI deficiency (Sugiyama et al. 1987) suffered from autoimmune thrombocytopaenia caused by antibodies against a 65-kDa protein (i.e. GPVI) that was present in healthy individuals but absent in the patient. His platelets failed to respond to collagen. A few additional patients were described with low GPVI expression levels, suffering from mild bleeding problems and with platelets responding poorly to collagen (Moroi et al. 1989) or to CRP (Kehrel et al. 1998). The molecular basis for these GPVI deficiencies is, however, poorly defined.

2.6
Inhibition of Platelet Deposition on the Vessel Wall

Pharmacological inhibition of platelet deposition onto damaged vessel wall structures is potentially antithrombotic. However, adhering platelets also re-

lease vasoactive substances and growth factors, predominantly the platelet-derived growth factor (PDGF), promoting smooth muscle cell activation and migration (Ferns et al. 1991). Vessel wall injury thus not only predisposes to thrombosis but also initiates neointima formation, resulting in vessel wall thickening and eventually in stenosis, a problem encountered in about one-third of patients undergoing a percutaneous transluminal coronary angioplasty (PTCA) (Glazier et al. 1989). Neointima formation is impaired in thrombocytopaenic animals, in agreement with the progression of restenosis, under control by platelet-derived vasoactive substances (Friedman et al. 1977). Therefore, such receptor–ligand interactions involved in platelet adhesion to the vessel wall may represent interesting targets. These include collagen, vWF and fibronectin (Melis et al. 2004).

At present, during acute coronary interventions, $\alpha_{IIb}\beta_3$ antagonists have become the standard treatment to block platelet aggregation. Yet $\alpha_{IIb}\beta_3$ antagonists have a poor effect on the deposition of (single) platelets and therefore have a poor outcome in the prevention of restenosis (Nguyen and Harrington 2003). In contrast, the potent inhibition by $\alpha_{IIb}\beta_3$ antagonists causes a bleeding risk, which narrows their therapeutic window and requires careful patient monitoring. Furthermore, poor bioavailability and immune-mediated thrombocytopaenia, in about 1% of patients treated, precludes the chronic use of these antagonists.

Anti-adhesive anti-platelet drugs with antithrombotic potential, reducing neointima formation, have been studied in animal models. The murine anti-human GPIbα monoclonal antibody 6B4 (Cauwenberghs et al. 2001) prevented arterial thrombosis in a baboon model of femoral artery stenosis, without prolonging the bleeding time (Wu et al. 2002). Even in combination with a neutralising anti-human $\alpha_{IIb}\beta_3$ antibody, a strong antithrombotic effect was achieved without bleeding time prolongation. Likewise, the mouse anti-vWF monoclonal antibody AJvW-2 is a potent inhibitor of GPIbα–vWF interactions. In vitro and ex vivo, AJvW-2 inhibits SIPA, as well as high shear stress-induced platelet adhesion and aggregation onto surface coated collagen (Kageyama et al. 1997). It also inhibits the enhanced SIPA in platelet-rich plasma of patients suffering from acute coronary syndromes (Eto et al. 1999). In several animal models, AJvW-2 prevents both arterial and venous thrombosis; it exerts a protective effect during neointima formation after balloon injury in the guinea-pig (Kageyama et al. 2000) due to inhibition of platelet deposition on the vessel wall. Its antithrombotic effect is not accompanied by a bleeding time prolongation, in contrast to that of the $\alpha_{IIb}\beta_3$ antagonist lamifiban, studied in parallel, or the widely used anti-$\alpha_{IIb}\beta_3$ antibody abciximab.

Drugs such as aspirin (inactivating cyclooxygenase, thus eliminating thromboxane A_2 production by thromboxane A_2 synthase in platelets) and thienopyridines (inactivating $P2Y_{12}$ via reactive metabolites that couple to a critical thiol of the receptor) inhibit specific amplification pathways of platelet activation and are efficient in the primary and secondary prevention of thrombosis, at

the expense, however, of a well-defined bleeding risk. Blood platelet activation relies on the synergistic interplay of several activation pathways, and it is clear that selected combinations of inhibitors of separate pathways offer the potential of inhibiting thrombosis to a variable degree, with variable effects on the haemostatic balance. Anti-adhesive inhibition of GPIbα–vWF interactions and of collagen receptor–collagen interactions may have the potential to control thrombosis by inhibiting primarily arterial thrombosis, while maintaining an acceptable bleeding risk.

3
Coagulation System

Haemostatic processes are traditionally divided in two parts: Primary haemostasis mainly involves platelets (see Sect. 2), and secondary haemostasis mainly relates to fibrin formation or blood coagulation following an extrinsic or intrinsic pathway (MacFarlane 1964). This model, although still valuable for laboratory diagnosis of haemostatic abnormalities, has recently been revised based on (1) the discovery of TFPI (Rapaport 1989; Broze et al. 1990), (2) the activation of factor XI by thrombin (Gailani and Broze 1991; Naito and Fujikawa 1991), (3) the finding that primary and secondary haemostatic processes strongly interact and (4) the notion that tissue factor may be blood-borne (Giesen et al. 1999).

In the current model of coagulation, the extrinsic tenase reaction initiates coagulation by activating factors IX and X. Platelets play a crucial role in the exposure of tissue factor and deliver the first trace amounts of activated factor V. Once critical amounts of factor Xa, required for the initiation of thrombin generation, are formed, the extrinsic tenase reaction is efficiently turned off by TFPI, and further formation of thrombin is maintained via positive feedback mechanisms involving thrombin-induced activation of the plasma factors V, VIII and XI. Excess thrombin is efficiently inhibited by its physiological inhibitor antithrombin and downregulates its own further generation via stimulation of the protein C pathway.

3.1
Structure of the Main Procoagulant and Anticoagulant Proteins

Procoagulant and anticoagulant proteins are composed of multiple domains, which have a high degree of structural and functional homology (Table 1; Colman et al. 1994; Bloom et al. 1994).

3.1.1
Signal Peptide

Both procoagulant and anticoagulant proteins in plasma are initially synthesised with a signal peptide. This short (usually very hydrophobic) peptide,

Table 1 Overview of the main procoagulant and anticoagulant proteins with some of their properties

	Function or main substrate of the active form	M_r (kDa)	Chain composition	Plasma conc. (µg/ml)	$t_{1/2}$ (h)	Domains				
						Gla	EGF	Kringle	Catalytic	Other
Zymogens										
Prothrombin	Fibrinogen, factor XIII	72	Sc, 581 AA	100	72	10 AA	None	2	Ser protease	
Factor VII	Factor X, factor IX	50	Sc, 406 AA	0.5	5	10 AA	2	None	Ser protease	
Factor X	Prothrombin	59	Tc, 254 AA, 139 AA	8	32	11 AA	2	None	Ser protease	
Factor IX	Factor X	56	Sc, 415 AA	5	24	12 AA	2	None	Ser protease	
Factor XI	Factor IX	160	Tc, 607 AA each	5	72	None	None	None	Ser protease	
Factor XII	Factor XI	80	Sc, 596 AA	30	60	None	2	1	Ser protease	
Protein C	Factor Va, factor VIIIa	62	Tc, 262 AA, 155 AA	3–5	7	9 AA	2	None	Ser protease	
Cofactors										
Tissue factor	Extrinsic tenase cofactor	45	Sc, 263 AA	Cell-bound	–	None	None	None	–	2 Barrel-like structures Transmembrane module Cytoplasmic tail
Factor V	Prothrombinase cofactor	330	Sc, 2196 AA	7–10	12	None	None	None	–	A1, A2, B, A3, C1, C2

Table 1 (continued)

	Function or main substrate of the active form	M_r (kDa)	Chain composition	Plasma conc. (μg/ml)	$t_{1/2}$ (h)	Domains				
						Gla	EGF	Kringle	Catalytic	Other
Factor VIII	Intrinsic tenase cofactor	280	Tc, 1313 AA, 684 AA	0.2	12	None	None	None	–	A1, A2, B, A3, C1, C2
Protein S	Cofactor for activated protein C	75	Sc, 635 AA	20	42	11 AA	4	None	–	Sex hormone-binding globulin-like module
Thrombo-modulin	Cofactor for protein C activation	60	Sc, 557 AA	Cell-bound	–	None	6	None	–	Lectin-like module Hydrophobic region Transmembrane module Cytoplasmic tail
Inhibitors										
Antithrombin	Inhibitor of thrombin and factor Xa	58	Sc, 432 AA	125	48	None	None	None	–	
Tissue factor pathway inhibitor	Inhibitor of extrinsic tenase and factor Xa	42	Sc, 276 AA	0.1	–	–	–	–	–	Kunitz domains 1, 2 and 3

Sc, single-chain; Tc, two-chain; AA, number of amino acids

required for translocation of the growing polypeptide chain into the endoplasmatic reticulum, is cleaved off prior to secretion.

3.1.2 Propeptide/γ-Carboxyglutamic Acid-Rich Domain

All vitamin K-dependent proteins (prothrombin, factors VII, IX and X, protein C and protein S), contain a γ-carboxylation recognition site located on the propeptide domain between the signal peptide and the γ-carboxyglutamic acid-rich domain (Gla domain). This site directs γ-carboxylation of the γ-carboxyglutamic acid residues located in the adjacent, approximately 40-residue-long Gla domain. After carboxylation of the Gla domain, which is crucial for the Ca^{2+}-mediated binding of vitamin K-dependent proteins to negatively charged membranes, the propeptide is cleaved off.

3.1.3 Epidermal Growth Factor Domain

Several procoagulant and anticoagulant proteins contain two or more EGF-like domains. These domains consist of about 43 to 50 amino acid residues, and their structure is determined by three characteristic disulphide bonds. The function of EGF-like domains in many coagulation proteins, although not fully understood, appears to be in the formation of protein complexes. The EGF-like domains in factor VII are important for the binding to tissue factor. The second EGF-like domain of factor IX contains a binding site for activated factor VIII. The second EGF-like domain of protein C is involved in the binding of protein S. The binding sites on thrombomodulin for protein C and thrombin are located on the fourth and fifth EGF-like domains respectively.

3.1.4 Kringle Domain

Kringle domains consist of about 100 amino acids, and their structure is determined by three disulphide bonds. These domains are involved in interactions with other proteins. Only two procoagulant proteins, prothrombin and factor XII, contain kringle domains. The second kringle of prothrombin probably contains the main binding site for activated factor V.

3.1.5 Catalytic Domain

The catalytic domain of all procoagulant enzymes contains an active site and an internal core that is similar to that of trypsin. Conversion of an inactive proenzyme to an active enzyme depends on limited proteolysis and, for some

proteins, on cleavage of so-called activation peptides. The active site of all clotting enzymes (as with all serine proteases) contains a catalytic triad consisting of serine, aspartic acid and histidine.

3.1.6 Pseudosubstrates

The natural inhibitors of coagulation, antithrombin and TFPI, are pseudosubstrates with high affinity for their specific target enzymes. Antithrombin is a single-chain globular molecule which depends on heparin to obtain its optimal inhibitory conformation required for docking and locking the catalytic centre of its target enzymes, thrombin and factor Xa. It forms 1:1 stoichiometric complexes which are rapidly cleared from the circulation. TFPI is a single-chain molecule with three Kunitz domains, which contain about 58 residues and three characteristic disulphide bonds. They act as pseudosubstrates for their target serine proteases. The first Kunitz domain of TFPI inhibits the factor VIIa/tissue factor complex, whereas the second inhibits factor Xa; the function of the third Kunitz domain is unknown.

3.2 Procoagulant Mechanisms

3.2.1 Initiation of Coagulation

Tissue factor is the vascular trigger required to initiate coagulation (Rapaport and Rao 1995). In healthy blood vessels, tissue factor is mainly located in the extracellular matrix beneath and between endothelial cells and therefore appears to form a protective lining around blood vessels, capable of activating blood coagulation after vascular injury (Drake et al. 1989).

Tissue factor binds to factor VIIa and accelerates the activation of factor IX and factor X by factor VIIa (Rapaport and Rao 1995). The physiological importance of tissue factor has been confirmed by the finding that disruption of the tissue factor gene in mice is associated with impaired vascular development and lethal embryonic bleeding (Carmeliet et al. 1996; Bugge et al. 1996). Association of tissue factor with phospholipids is required for significant procoagulant activity (Nemerson 1995). Relipidation experiments with recombinant tissue factor have shown that both phosphatidylcholine and phosphatidylethanolamine support the procoagulant properties of tissue factor, whereas phosphatidylserine is inactive.

At high tissue factor concentrations, factor X is mainly activated by the factor VIIa–tissue factor complex, whereas at low concentrations, factor IXa-/factor VIIIa-dependent activation becomes more pronounced (Osterud and Rapaport 1977; Marlar et al. 1982). Factor X activation by the extrinsic tenase reaction is responsible for the initiation phase. Activated factor X activates

prothrombin on a phospholipid surface upon association with its cofactor, activated factor V, secreted from the α-granules of activated platelets (Gould et al. 2004). The cleavage of prothrombin is sequential. In a first stage, meizothrombin is generated (Krishnaswamy et al. 1986); this active enzyme remains attached to the phospholipid surface. Subsequent removal of fragments including the Gla domain of prothrombin results in soluble thrombin that diffuses away from the catalytic surface.

3.2.2 Blood-Borne Tissue Factor

Endothelial cells themselves have little or no tissue factor activity, but it can be strongly induced in vitro by endotoxin, thrombin, fibrin and several cytokines, as well as by shear stress and hypoxia (Contrino et al. 1997; Lin et al. 1997; Rapaport 1989; Nemerson 1995). Both monocytes and natural killer cells have also been found to upregulate tissue factor expression in endothelial cells (Napoleone et al. 1997). However, it is doubtful whether this phenomenon occurs in pathological conditions in vivo. Tissue factor is highly concentrated in the areas surrounding the cholesterol clefts of diseased coronary vessels (Fuster et al. 1997; Nemerson 1995), but whether it initiates thrombus formation after plaque rupture is doubtful. Recent work indicates that platelets adhering to a ruptured plaque effectively prevent contact between the plaque tissue factor and the blood (Hathcock and Nemerson 2004). In addition, it was shown that when native human blood is allowed to flow over a glass coverslip at high shear, platelets adhere to the coverslip and biologically active tissue factor containing microparticles adheres to the platelet layer (Giesen et al. 1999). Until recently, tissue factor was believed to be located essentially extravascularly; now, however, the surprising concept of blood-borne tissue factor has emerged. Monocytes and possibly polymorphonuclear leucocytes are the source of these tissue factor-positive microparticles, which are transferred to the adhering platelets (Rauch et al. 2000; see Fig. 1).

During platelet activation the α-granule membranes, containing P-selectin (CD62P) (Johnston et al. 1989), fuse with the plasma membrane which becomes decorated with P-selectin. Surface P-selectin then interacts with CD15 (a leucocyte membrane-bound carbohydrate known as sialyl Lewis X) or with P-selectin glycoprotein ligand 1 (Sako et al. 1993), also on leucocytes.This interaction results in the formation of conjugates between activated platelets and leucocytes or leucocyte microparticles. Under normal conditions, most cell surface tissue factor is encrypted, which means that it binds factor VIIa but is not capable of initiating coagulation. Encrypted tissue factor allows circulating tissue factor-positive monocytes to be present in the circulation in the absence of generalised coagulation (Maynard et al. 1975). However, when the phospholipids in the monocyte plasma membrane are scrambled by cal-

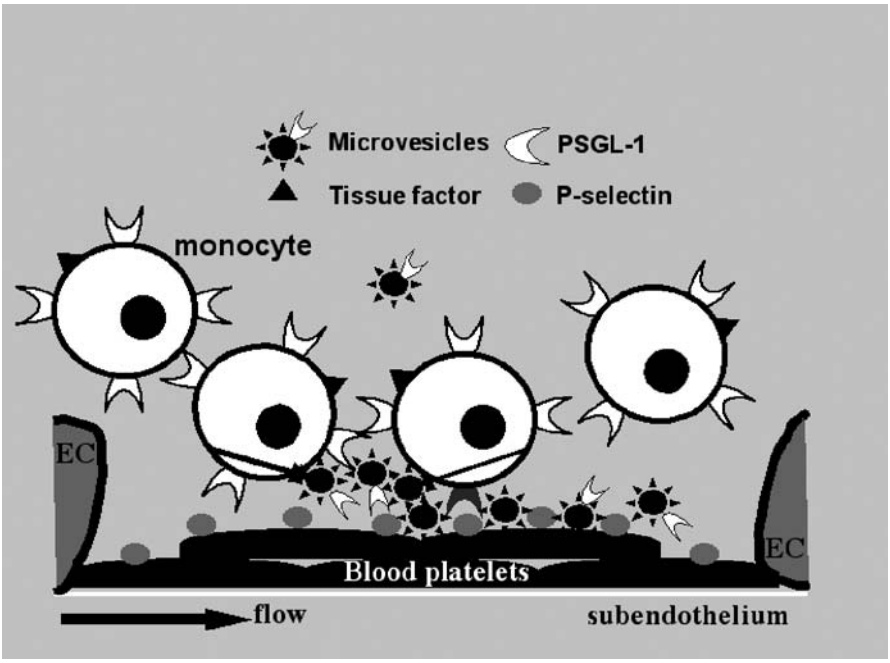

Fig. 1 Blood-borne tissue factor. Platelets rapidly adhere to injured vessels and expose P-selectin. Surface P-selectin then interacts with PSGL-1 (P-selectin glycoprotein ligand-1) on monocytes and monocyte-derived microvesicles, delivering blood-borne tissue factor

cium ionophore, allowing binding of clotting factors as described above, tissue factor becomes de-encrypted and coagulation ensues (Bach and Rifkin 1990). The transfer of tissue factor-positive microparticles to the surface of a spread platelet that has bound clotting factors therefore allows thrombin generation.

The co-localisation of platelets, blood-borne tissue factor and fibrin in blood flowing over an ex vivo surface has recently been visualised in real time (Balasubramanian et al. 2002). Furie et al. (2001) used intravital confocal microscopy of the microcirculation of living mice to study thrombosis induced by laser injury. Co-localisation of platelets, leucocytes and fibrin was observed. Their preliminary experiments have shown that thrombus formation is significantly reduced in mice either deficient in P-selectin or in P-selectin glycoprotein ligand 1.

If platelet–leucocyte interaction via P-selectin is the cellular basis for intravascular thrombus formation, then inhibition of P-selectin function seems an attractive therapeutic strategy that is currently being actively pursued. Both anti-P-selectin antibodies (Palabrica et al. 1992; Downing et al. 1997) and recombinant soluble P-selectin glycoprotein ligand 1 (Khor et al. 2000) are being evaluated. In primate models, pretreatment with a blocking monoclonal antibody to P-selectin accelerated pharmacological thrombolysis of

arterial thrombosis (Toombs et al. 1995) and reduced stasis-induced venous thrombosis (Downing et al. 1997).

3.2.3 Propagation of Coagulation

Both meizothrombin and thrombin are responsible for the propagation phase of coagulation. Meizothrombin, by lateral diffusion on the phospholipid surface, effectively activates factor V and factor XI (Tans et al. 1994; von dem Borne et al. 1997). Thrombin causes further platelet activation and factor XI activation on the platelet surface (Walsh 2001), and dissociates factor VIII from von Willebrand factor and activates it (Vlot 1998). Activated factor VIII binds to the phospholipid surface through its C_2 domain. Factor VIIIa is the cofactor for factor IXa and is required for the propagation phase induced by the intrinsic tenase reaction. Recent studies show that factor Xa generation via the intrinsic tenase reaction occurs after that of the extrinsic tenase reaction, as it requires thrombin-dependent activation of factor VIII (Butenas et al. 1997).

The propagation phase, involving the intrinsic tenase reaction, consists of a new burst of factor X activation which leads, on the one hand, to the formation of a factor Xa-TFPI complex shutting down the extrinsic tenase reaction (Broze et al. 1990, Broze 1995a, b) and, on the other hand, to an explosive generation of thrombin (see Fig. 2). The propagation phase of coagulation results in a high local concentration of thrombin that converts fibrinogen into a fibrin network that is stabilised through covalent cross-linking by thrombin-activated factor XIII.

Endothelial cells may promote the propagation phase of coagulation in several ways. They synthesise and bind factor V, and its expression on the endothelial cell surface is enhanced by mechanical injury (Annamalai et al. 1986). Endothelial cells also contain factor VIII (Kadhom et al. 1988); although its cellular localisation is not clearly identified, it is conceivable that factor VIII is stored in the Weibel–Palade bodies associated with vWF, since both are concomitantly released upon infusion of DDAVP (1-deamino-8-D-arginine vasopressin). Thrombin-activated endothelial cells release vWF that plays a role in platelet adhesion, whereas concomitant release of factor VIII may cause an increased concentration at the site of thrombus formation.

3.2.4 Blood Coagulation as a Surface-Catalysed Process

With the exception of fibrinogen and prothrombin, the coagulation factors are trace proteins (see also Table 1). For efficient interactions they need to be concentrated on a cell surface. The main physiological catalytic surface is a layer of phospholipid containing negatively charged phospholipids such as phosphatidylserine. Phosphatidylserine normally is sequestered in the inner

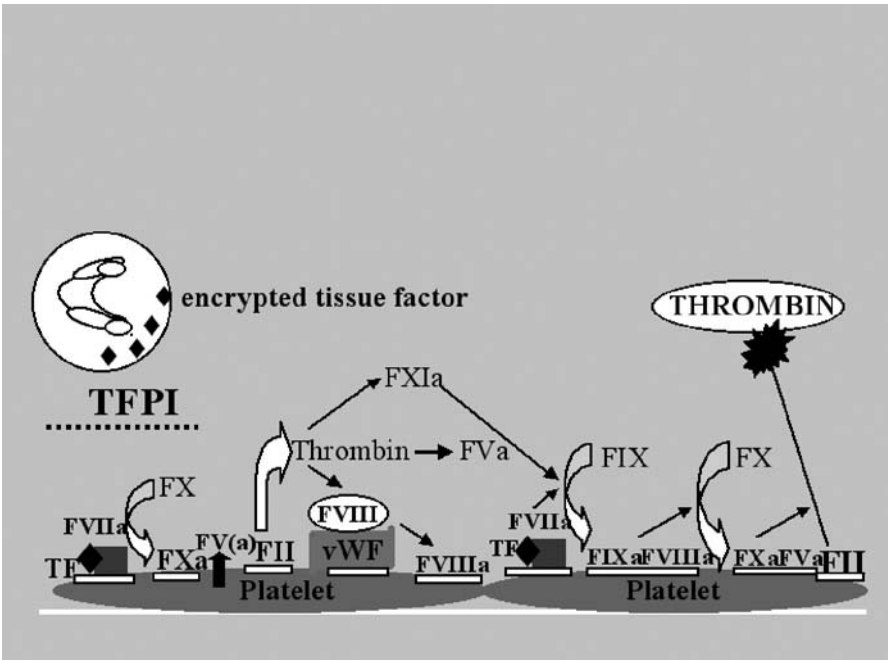

Fig. 2 Blood coagulation as a surface-catalysed process. For efficient interactions, procoagulant factors need to be concentrated on a cell surface enriched in phosphatidyl serine. FVIIa binds to blood-borne tissue factor on activated platelets and initiates coagulation by generating limited amounts of FXa. This enzyme together with FVa, released from platelets, generates the first traces of thrombin, which amplifies its own generation by activating FV, FVIII and FIX. As soon as a critical concentration of FXa is formed, TFPI (tissue factor pathway inhibitor) inhibits the extrinsic tenase reaction. The end result of the process is an explosive generation of thrombin

leaflet of a cellular phospholipid bilayer. Upon activation of cells, in particular of platelets, phospholipid scrambling occurs (Sims and Wiedmer 2001). Cell surface-exposed phosphatidylserine following scrambling serves as a receptor for the vitamin K-dependent coagulation factors (prothrombin, factors VII, IX and X), for factor V and for factor VIII (Heemskerk et al. 2002). Glutamic acid (glu) residues at the NH_2-terminal ends of vitamin K-dependent coagulation factors are carboxylated to ɣ-carboxyglutamic acid (gla) residues (Stenflo et al. 1974). This "gla-domain" anchors these proenzymes to the negatively charged phospholipid membrane in a Ca^{2+}-dependent manner. Factors V and VIII are protein cofactors that facilitate the interaction of the vitamin K-dependent (pro)enzymes. Their sequence contains six sequential domains arranged in the order A_1-A_2-B-A_3-C_1-C_2. They bind to phospholipid through the C_2 domain by the burial of hydrophobic residues within the phospholipid bilayer; these hydrophobic residues are surrounded by positively charged residues that

interact with the negatively charged phospholipid head groups (Pratt et al. 1999). Activated factor V is secreted from the α-granules of activated platelets and binds with high affinity to the phospholipid surface (Gould et al. 2004). Factor VIII is concentrated on activated platelets via its carrier protein, vWF (Kawasaki et al. 1999). The latter, subjected to shear stress, binds to activated platelets through their membrane GPIb/IX/V and GPIIb/IIIa complexes (Ruggeri 1997). Finally, the proenzyme factor XI also binds to the platelet GPIb/IX/V complex, where it is activated by meizothrombin or thrombin (Tans et al. 1994; von dem Borne 1997; Walsh 2001).

Thus, primary and secondary haemostasis interact strongly. Upon adhesion to and spreading on collagen, activated platelets assemble on their surface a number of proenzymes and protein cofactors that interact efficiently through lateral diffusion on the phospholipid surface, resulting in thrombin generation and further platelet activation.

3.3 Anticoagulant Mechanisms

The formation of thrombin and the deposition of fibrin on the surface of quiescent endothelial cells is impaired by several pathways.

The extrinsic tenase activity is inhibited by the Kunitz-type inhibitor, TFPI (Broze et al. 1990; Broze 1995a, b; Rapaport 1989) and by the serine protease inhibitor, antithrombin (Rapaport and Rao 1995; van 't Veer and Mann 1997). Both inhibitors neutralise factor VIIa only when it is bound to tissue factor. The mature full-length TFPI is a 43-kDa protein with an acidic NH_2-terminal region followed by three tandem Kunitz-type protease inhibitory domains and a basic COOH-terminal region. TFPI inhibits the extrinsic tenase reaction via a two-step mechanism. In the first step, factor Xa is inhibited by binding to an arginine residue in the reactive centre of the second Kunitz domain. In the second step, the TFPI/factor Xa complex forms a quaternary complex with factor VIIa/tissue factor in which factor VIIa binds to a lysine residue in the reactive centre of the first Kunitz domain. TFPI is predominantly located in the endothelial cell extracellular matrix, where it is bound to heparan sulphate or other glycosaminoglycans. The plasma concentration of TFPI (2 nM) is increased several-fold after intravenous injection of heparin. Plasma TFPI has a lower molecular weight (34–41 kDa) than its endothelium-bound form and appears to be truncated at the COOH-terminal end. It circulates bound to lipoproteins and has substantially lower factor Xa inhibitory activity than the full-length form. The physiological importance of TFPI has been extensively studied in animal models. Infusion of high concentrations of TFPI prevents thrombosis and intravascular coagulation following tissue factor or endotoxin infusion in rabbits (Broze 1995). Neutralisation of TFPI by polyclonal antibodies promotes tissue factor-induced intravascular coagulation (Broze 1995a; Rapaport and Rao 1995). Targeted TFPI gene disruption has recently been

shown to cause intrauterine lethality in mice due to yolk sac haemorrhages or fatal bleeding, compatible with a consumptive coagulopathy (Huang et al. 1997).

Antithrombin, in the presence of heparin, rapidly inhibits the extrinsic tenase reaction (Broze 1995a; Huang et al. 1997; van 't Veer and Mann 1997). In solution, this inhibitory pathway is probably equivalent to the TFPI-dependent inhibition of factor VIIa/tissue factor, whereas on cell surfaces TFPI-dependent inhibition is much faster (Broze 1995a; van 't Veer and Mann 1997). Antithrombin is the major thrombin-inactivating protein (Beresford and Owen 1990). This serpin also inactivates factors Xa, IXa, XIa and kallikrein. Antithrombin only displays its full inhibitory activity in the presence of heparin or other sulphated glycosaminoglycans which are synthesised and expressed by endothelial cells. Some cell surface heparan sulphate proteoglycans may be involved in thrombin–antithrombin interactions (Mertens et al. 1992). Heparan sulphate proteoglycans are also a major constituent of the extracellular matrix, which explains why the thrombogenicity of balloon-injured vessels can be abolished by treatment with antithrombin, whereas heparin is ineffective (Frebelius et al. 1994).

Another endothelial cell-dependent anticoagulant pathway involves the integral membrane glycoprotein thrombomodulin (Esmon 1995). Its physiological importance is well established and supported by gene disruption studies (Rosenberg 1997). Thrombomodulin consists of a lectin-like NH_2-terminal domain, followed by six EGF-like domains, a serine–threonine-rich domain, a transmembrane domain and a short cytoplasmic tail. The fifth and sixth EGF-like domains are essential for thrombin binding, while the calcium-dependent binding of protein C requires the linker region between the third and fourth EGF-like domain. Thrombomodulin has both direct and indirect anticoagulant properties. The direct anticoagulant action of thrombomodulin involves binding, neutralisation and degradation of thrombin (Esmon 1995). Thrombomodulin accounts for about half of the thrombin-binding sites on endothelial cells. Agents such as endotoxin, interleukin-1 and tumour necrosis factor, which stimulate tissue factor activity, downregulate thrombomodulin activity by suppressing its transcription. Thrombomodulin-bound thrombin cannot cleave fibrinogen and cannot activate factor V, factor XIII or platelets and is rapidly endocytosed and degraded (Esmon 1993, 1995).

The indirect anticoagulant action of thrombomodulin involves the generation of activated protein C (Esmon 1989). The zymogen protein C, a vitamin K-dependent protein, is activated by thrombin, and this activation is accelerated up to 20,000-fold by thrombomodulin. Activated protein C has anticoagulant properties by inhibiting factors Va and VIIIa. This reaction is moderately catalysed at the endothelial cell surface by protein S, another vitamin K-dependent cofactor that is synthesised and expressed by endothelial cells in the liver (Dahlback 1991). Protein S binds to the endothelial cell membrane and to protein C, forming a cell surface-bound complex. Due to the

exposure of negatively charged phospholipids, activated platelets may provide the appropriate surface for the inactivation of factors Va and VIIIa. However, activated protein C is also active on endothelial cells, probably involving the recently described endothelial cell protein C receptor (Fukudome and Esmon 1994). Protein S not only functions as a cofactor in the protein C pathway, but also directly inhibits the prothrombinase and tenase reactions on phospholipid vesicles, platelets and human endothelial cells or matrices (Heeb et al. 1993, 1994; Koppelman et al. 1995; van Wijnen et al. 1996).

Other potential anticoagulant proteins include annexin V, protease nexin 1 (PN-1) and protease nexin 2 (PN-2), their roles as endothelial anticoagulants are, however, not firmly established. Annexins are a family of non-glycosylated proteins that bind calcium and phospholipids. Annexin V is localised in the endothelium of venous and arterial blood vessels (van Heerde et al. 1995); it preferentially binds to phosphatidylserine, thereby preventing the assembly of activated coagulation factors on phospholipid surfaces. Anticoagulant properties of annexin V have been reported on phospholipid vesicles, platelets and endothelial cells. PN-1 is a serpin that inhibits thrombin, plasmin, urokinase, activated protein C, kallikrein, factor Xa and trypsin (Bombeli et al. 1997). It is localised on the surface of vascular endothelial cells, fibroblasts and platelets. PN-1 bound to cell surfaces or endothelial cell matrix retains its inhibitory properties for thrombin but not for urokinase or plasmin. Inhibition of thrombin and factor Xa by PN-1 is accelerated by heparin, whereas that of plasmin is not. PN-2 is the secreted form of the transmembrane amyloid β-protein precursor. It is abundantly present in the α-granules of platelets but also in monocytes and endothelial cells (van Nostrand et al. 1992). PN-2 is a much more potent inhibitor of factors IXa and XIa than of thrombin and may be involved in the regulation of the intrinsic tenase reaction on endothelium (Schmaier et al. 1993).

4
Fibrinolysis

4.1
Regulation of Physiological Fibrinolysis

The fibrinolytic system (Fig. 3) comprises an inactive proenzyme, plasminogen, that can be converted to the active enzyme plasmin that degrades fibrin by two immunologically distinct physiological plasminogen activators: tissue-type (t-PA) and urokinase-type (u-PA) plasminogen activator. Inhibition of the fibrinolytic system may occur either at the level of the plasminogen activators, by specific plasminogen activator inhibitors (PAI-1 and PAI-2), or at the level of plasmin, mainly by α_2-antiplasmin (Collen and Lijnen 1991). The main biochemical properties of these components are summarised in Table 2. The

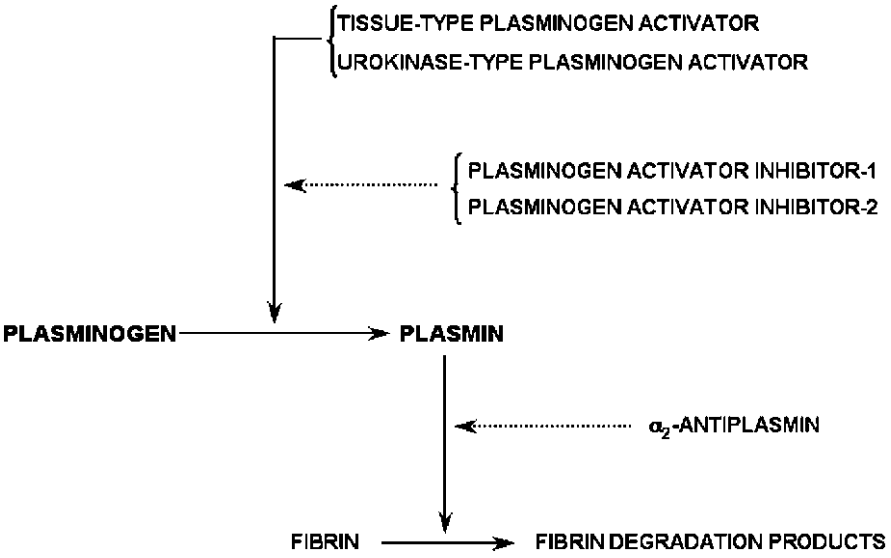

Fig. 3 Schematic representation of the fibrinolytic system

Table 2 Biochemical properties of the main components of the fibrinolytic system

	M_r (kDa)	Chain composition	Carbohydrate content (%)	Amino acids	Catalytic triad or reactive site	Plasma concentration (mg/l)
Plasminogen	92	1	2	791	His603, Asp646, Ser741	200
Plasmin	85	2	2	±715	His603, Asp646, Ser741	–
t-PA	68	1/2	7	527	His322, Asp371, Ser478	0.005
u-PA	54	1/2	7	411	His204, Asp255, Ser356	0.008
α_2-Antiplasmin	67	1	13	464	Arg364-Met365	70
PAI-1	52	1	ND	379	Arg346-Met347	0.05
PAI-2	47	1	ND	393	Arg358-Thr359	<0.005

ND, not determined

fibrinolytic system thus is regulated by controlled activation and inhibition, but also by increased or decreased synthesis and/or secretion of t-PA and of PAI-1, primarily from the vessel wall (Lijnen et al. 2000a).

Impaired fibrinolysis—due to a defective synthesis and/or release of plasminogen activators, a deficiency or functional defect in plasminogen, or in-

creased levels of inhibitors of plasminogen activators or plasmin—is associated with thrombosis. In turn, excessive fibrinolysis resulting from increased levels of t-PA or from α_2-antiplasmin or PAI-1 deficiency may result in bleeding complications.

4.1.1 Plasminogen Activation by t-PA

In the absence of fibrin, t-PA is a poor enzyme, but the presence of fibrin strikingly enhances the activation rate of plasminogen. Fibrin indeed provides a surface to which t-PA and plasminogen adsorb in a sequential and ordered way, yielding a cyclic ternary complex (Hoylaerts et al. 1982). Plasminogen binding to fibrin involves the lysine-binding sites in its kringle structures, whereas binding of t-PA to fibrin is mediated via its finger and kringle 2 domains. Formation of this complex results in an enhanced affinity of t-PA for plasminogen, yielding up to three orders of magnitude higher efficiencies for plasminogen activation. Increased binding of both enzyme and substrate to degrading fibrin is mediated in part by COOH-terminal lysine residues generated by plasmin cleavage. Their interaction with lysine-binding sites on t-PA and plasminogen may allow an improved alignment and allosteric changes enhancing the rate of plasminogen activation (Thorsen 1992).

Consequently, proteins that remove COOH-terminal lysine residues from the fibrin surface, such as the thrombin activatable fibrinolysis inhibitor (TAFI), may have an antifibrinolytic action (Nesheim et al. 1997). TAFI is a 60-kDa single-chain protein, identical to plasma procarboxypeptidase B, that occurs at a concentration of 75 nM and is activated by thrombin, trypsin or plasmin.

4.1.2 Plasminogen Activation by u-PA

u-PA is secreted as a single-chain molecule (scu-PA) that is converted to a two-chain moiety (tcu-PA) by plasmin. In contrast to tcu-PA, scu-PA displays very low activity towards low molecular weight chromogenic substrates, but it appears to have some intrinsic plasminogen-activating potential, which represents 0.5%, or less of the catalytic efficiency of tcu-PA (Lijnen et al. 1990). Other investigators, however, have claimed that scu-PA has no measurable intrinsic amidolytic or plasminogen activator activities. In plasma, in the absence of fibrin, scu-PA is stable and does not activate plasminogen; in the presence of a fibrin clot, scu-PA, but not tcu-PA, induces fibrin-specific clot lysis (Gurewich et al. 1984). The fibrin specificity of scu-PA does not require its conversion to tcu-PA, nor its binding to fibrin, but is mediated by enhanced binding of plasminogen to partially digested fibrin (Fleury et al. 1993).

4.1.3
Inhibition of Plasmin by α_2-Antiplasmin

α_2-Antiplasmin forms an inactive 1:1 stoichiometric complex with plasmin. The inhibition involves two consecutive reactions: a fast, second-order reaction producing a reversible inactive complex, followed by a slower first-order transition resulting in an irreversible complex. The second-order rate constant of the inhibition is very high ($2–4 \times 10^7$ $M^{-1}s^{-1}$), but this high inhibition rate is dependent on the presence of a free lysine-binding site and active site in the plasmin molecule and on the availability of a plasminogen-binding site and reactive site peptide bond in the inhibitor. The half-life of plasmin molecules on the fibrin surface, which have both their lysine-binding sites and active site occupied, is estimated to be two to three orders of magnitude longer than that of free plasmin (Wiman and Collen 1978).

4.1.4
Inhibition of Plasminogen Activators by PAI-1

PAI-1 reacts very rapidly with t-PA and with tcu-PA, with second-order inhibition rate constants of the order of 10^7 $M^{-1}s^{-1}$, and it does not react with scu-PA (Kruithof 1988). Like other serpins, PAI-1 inhibits its target proteinases by formation of a 1:1 stoichiometric reversible complex, followed by covalent binding between the hydroxyl group of the active site serine residue of the proteinase and the carboxyl group of the P1 residue at the reactive centre ("bait region") of the serpin.

PAI-1 occurs as an active inhibitory form that spontaneously converts to a latent form, due to insertion of part of the reactive centre loop in the major β-sheet of PAI-1, which is thereby not accessible to the target enzyme (Mottonen et al. 1992). Another molecular form of intact PAI-1 has been isolated that does not form stable complexes with t-PA but is cleaved at the P1-P′1 peptide bond ("substrate PAI-1") (Declerck et al. 1992).

4.2
Pharmacology of Plasminogen Activators

Following intravenous administration of wild-type recombinant t-PA (rt-PA) to man, it is cleared from the circulation with an initial half-life of 4–8 min. Clearance is the result of interaction with several receptor systems. Liver endothelial cells have a mannose receptor which recognises the high mannose-type carbohydrate side-chain at Asn^{117} in the kringle 1 domain, whereas liver parenchymal cells contain a calcium-dependent receptor which interacts mainly with the growth factor domain of t-PA (Otter et al. 1992; Kuiper et al. 1996). In addition, the low-density lipoprotein receptor-related protein (LRP), expressed in

high copy number on hepatocytes, binds free t-PA and complexes with PAI-1 (Orth et al. 1992; Bu et al. 1992).

The recommended dose of rt-PA [alteplase, Activase (Genentech, South San Francisco), Actilyse (Boehringer Ingelheim, Ingelheim)] for the treatment of acute myocardial infarction was 100 mg administered as 60 mg in the first hour (of which 6–10 mg was given as a bolus over the first 1–2 min), 20 mg over the second hour and 20 mg over the third hour. Later it was proposed to give the same total dose of 100 mg but "front loaded", starting with a bolus of 15 mg followed by 50 mg in the next 30 min and the remaining 35 mg in the following hour (Neuhaus et al. 1989). In the GUSTO trial, a dose of 15 mg intravenous bolus of alteplase followed by 0.75 mg/kg over 30 min (not to exceed 50 mg) and then 0.50 mg/kg over 60 min (not to exceed 35 mg) was utilised (GUSTO Investigators 1993). In the COBALT (1997) trial, double bolus administration of rt-PA (50 mg given 30 min apart) was evaluated in patients with myocardial infarction. Whichever regimen is used, it is important to co-administer intravenous heparin during and after alteplase treatment. For catheter-directed local thrombolysis with alteplase in patients with recent peripheral arterial occlusion, a dose of 0.05–0.10 mg/kg per hour over an 8-h period is used.

During thrombolytic therapy, there is a vast excess of t-PA over PAI-1 in the circulation, but critical lysis occurs at the surface of an arterial thrombus, where the local PAI-1 concentration can be very high. Therefore, mutants with resistance to PAI-1 may be useful to reduce re-occlusion. In addition, mutants with a prolonged half-life allow efficient thrombolysis by bolus administration at a reduced dose (Collen and Lijnen 2003).

The main mechanism of removal of u-PA from the blood is by hepatic clearance. Scu-PA is taken up in the liver via a recognition site on parenchymal cells and is subsequently degraded in the lysosomes (Kuiper et al. 1992). Following intravenous infusion of recombinant scu-PA (saruplase) in patients with acute myocardial infarction, a biphasic disappearance was observed with an initial half-life in plasma of 8 min (Van de Werf et al. 1986).

With a preparation containing 160,000 IU/mg of saruplase, the dose used successfully in patients with acute myocardial infarction was 20 mg given as a bolus and 60 mg over the next 60 min, immediately followed by an intravenous heparin infusion (20 IU/kg per hour) for 72 h (PRIMI Study 1989). In the LIMITS Study in patients with acute myocardial infarction, the same dose regimen of saruplase was used, but with a prethrombolytic heparin bolus of 5,000 IU and an i.v. heparin infusion for 5 days starting 30 min after completion of thrombolysis (Tebbe et al. 1995). A recombinant glycosylated form of scu-PA (A-74187) has been evaluated in patients with acute myocardial infarction, using 60 or 80 mg monotherapy or 60 mg primed with a preceding bolus of 250,000 IU of recombinant tcu-PA, always combined with aspirin and i.v. heparin (Weaver et al. 1994).

4.3 Role of Fibrinolysis in Arterial Restenosis

Vascular interventions for the treatment of atherothrombosis induce restenosis of the vessel within 3–6 months in 30%–50% of treated patients. Arterial stenosis may result from remodelling of the vessel wall (such as occurs predominantly after balloon angioplasty) or from accumulation of cells and extracellular matrix in the intimal layer (such as occurs predominantly after intraluminal stent application).

Proteinases from the plasminogen/plasmin system participate in the proliferation and migration of smooth muscle cells (SMC), and in matrix remodelling during arterial wound healing. To assess their role in SMC migration and neointima formation, a perivascular electric injury model in the mouse has been extensively used (Carmeliet et al. 1997a). In this model, surgically exposed femoral arteries are injured perivascularly via delivery of an electric current, which destroys all medial SMC, denudes the injured segment of intact endothelium and transiently induces platelet-rich mural thrombosis. A vascular wound-healing response results that is characterised by degradation of the mural thrombus, transient infiltration of the vessel wall by inflammatory cells and progressive removal of the necrotic debris. Topographic analysis reveals repopulation of the media and accumulation in the neointima of SMC, originating from the uninjured borders and progressing into the necrotic centre. Within 3 weeks after injury, a neointima is formed that contains up to 12 layers of smooth muscle α-actin-immunoreactive cells. Evans blue staining in injured arteries reveals progressive re-endothelialisation from the uninjured borders.

This electric injury model has been applied to wild-type mice and to mice with deficiency of the main components of the plasminogen/plasmin system. At 1 week after vascular injury in wild-type mice, t-PA activity in arterial sections or extracts was not significantly altered, whereas u-PA activity levels were two- to threefold higher than control at 2 days after injury of the femoral artery. Prolonged fibrin overlay with femoral or carotid artery sections from t-PA-deficient mice revealed that the fibrinolytic activity in injured versus control segments was markedly enhanced. This activity was reduced by approximately 50% upon inhibition of u-PA (Lijnen et al. 1998).

In plasminogen-deficient mice, wound healing was significantly impaired with delayed removal of necrotic debris, reduced leucocyte infiltration and SMC accumulation, and decreased neointima formation. SMC accumulated at the uninjured borders but failed to migrate into the necrotic centre (Carmeliet et al. 1997b). Neointima formation and neointimal cell accumulation were also reduced in u-PA-deficient and in combined t-PA-and u-PA-deficient arteries but not in t-PA-deficient arteries. Similar to the plasminogen-deficient arteries, SMC accumulated at the uninjured borders but failed to migrate into the necrotic centre in u-PA-deficient and in the double-deficient arter-

ies (Carmeliet et al. 1997c). Proliferation of SMC and re-endothelialisation were not affected by a deficiency in plasminogen, u-PA or t-PA. Thus, u-PA and plasminogen play a significant role in vascular wound healing and arterial neointima formation after injury, most likely by promoting cellular migration. In this model, binding of u-PA to its cellular receptor u-PAR is not required to provide sufficient pericellular u-PA-mediated plasmin proteolysis to allow cellular migration into a vascular wound (Carmeliet et al. 1998).

Deficiency of PAI-1, the main inhibitor of both u-PA and t-PA, in contrast, improved vascular wound healing in this model. SMC migrated more rapidly from the uninjured borders into the necrotic centre of the arterial wound than in wild-type SMC. When PAI-1-deficient mice were intravenously injected with replication-defective adenovirus expressing human PAI-1, plasma PAI-1 antigen levels increased in a dose-dependent fashion and luminal stenosis was significantly suppressed. By impairing cellular migration, PAI-1 thus plays an inhibitory role in vascular wound healing and arterial neointima formation after electric injury (Carmeliet et al. 1997d). In contrast, in murine models of vascular injury induced by ferric chloride, rose bengal or copper, a positive overall correlation was observed between PAI-1 levels and neointima formation (Konstantinides et al. 2001; Eitzman et al. 2001; Ploplis et al. 2001). These discrepancies may be explained to some extent by subtle differences in the genetic background of the mice strains or by differences in the experimental models. PAI-1 binds with high affinity to its cofactor vitronectin (VN), which stabilises its activity and mediates binding to fibrin clots. PAI-1 and VN play a role in the thrombotic response to ferric chloride-induced carotid artery injury in mice by preventing premature thrombus dissolution and embolisation. The effect of PAI-1 and VN on restenosis after vascular injury may depend on which phase of the wound healing response and what part of the vasculature are analysed; a critical feature may be the presence or absence of thrombus/fibrin (Konstantinides et al. 2002). Thus, PAI-1 may inhibit neointima formation in the absence of fibrin, but enhance it in the presence of fibrin. Indeed, the mechanical and electric injury models are usually associated with only transient thrombosis, in contrast to the prominent thrombotic reaction in the injury models induced with ferric chloride, rose bengal or copper.

In wild-type mice and in mice deficient in α_2-antiplasmin—the main physiological plasmin inhibitor—the neointimal and medial areas at 1–3 weeks after electric injury of the femoral artery were similar, resulting in comparable intima/media ratios. Nuclear cell counts in cross-sectional areas of the intima of the injured region were also comparable. Fibrin deposition was not significantly different in arteries of both genotypes at 1 day after injury, and no mural thrombosis was detected at 1 week after injury. Thus, α_2-antiplasmin does not seem to play a major role in SMC migration and neointima formation after vascular injury in mice (Lijnen et al. 2000b).

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Vascular Endothelium and Blood Flow

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Abstract Major advances have been made over the last decade towards the elucidation of the molecular mechanisms involved in the endothelium-dependent regulation of vascular tone and blood flow. While the primary endothelium-derived vasodilator autacoid is nitric oxide, it is clear that epoxyeicosatrienoic acids and other endothelium-derived hyperpolarising factors, as well as endothelin-1 and reactive oxygen species, play a significant role in the regulation of vascular tone and gene expression. This review is intended as an overview of the signalling mechanisms that link haemodynamic stimuli (such as shear stress and cyclic stretch) and endothelial cell perturbation to the activation of enzymes generating vasoactive autacoids.

Keywords Fluid shear stress · Mechanotransduction · Nitric oxide synthase · PECAM-1 · Tyrosine phosphatase

1 Introduction

In order to adapt to the varying metabolic demands of the tissues, the circulatory system has evolved a variety of control mechanisms that act in concert to maintain an adequate blood flow. At a given blood pressure, the blood flow to each organ is determined by its vascular resistance, which is adjusted by central and local mechanisms affecting the tone of the smooth muscle cells in small terminal arteries and large and small arterioles. Endothelial cells situated at the interface between blood and the vessel wall play a crucial role in controlling vascular tone and homeostasis, particularly in determining the expression of pro- and anti-atherosclerotic genes. Many of these effects are mediated by changes in the generation and release of the vasodilator nitric oxide (NO) in response to haemodynamic stimuli exerted on the luminal surface of endothelial cells by the streaming blood (wall shear stress) and the cyclic strain of the vascular wall, which results from the pulsatile changes in blood pressure (for review see Busse and Fleming 2003). NO is, however, not the only endothelium-derived vasodilator autacoid, and shear stress and cyclic stretch are also known to stimulate the formation of prostacyclin (PGI_2) and the endothelium-derived hyperpolarising factor (EDHF).

2 Endothelial Cells and the Modulation of Vascular Tone by Haemodynamic Stimuli

2.1 Nitric Oxide

NO is generated by endothelial NO synthase (eNOS), the activity of which can be regulated by Ca^{2+}-dependent and Ca^{2+}-independent processes. Indeed, although eNOS was originally classified as a Ca^{2+}/calmodulin-dependent enzyme with a low but measurable activity at resting levels of intracellular Ca^{2+} ($[Ca^{2+}]_i$) (Busse and Mülsch 1990), it is now evident that eNOS can be activated by certain stimuli without a sustained increase in $[Ca^{2+}]_i$ being necessary. The most important of these stimuli is the fluid shear stress generated by the viscous drag of blood flowing over the endothelial cell surface and in response to the application of shear stress to endothelial cells, NO production is enhanced two- to fourfold over basal values and is maintained as long as the stimulus is applied. The magnitude and the kinetics of the shear stress-induced activation of eNOS therefore differ markedly from those observed in response to a Ca^{2+}-elevating agonist, which can elicit a transient but 10- to 20-fold increase in NO output. This does not imply that shear stress is without effect on $[Ca^{2+}]_i$ in endothelial cells, as Ca^{2+} transients have been detected repeatedly

(Kuchan and Frangos 1994; Kanai et al. 1995; Ayajiki et al. 1996; Hoyer et al. 1998). However, in both cultured endothelial cells (Kuchan and Frangos 1994; Fleming et al. 1994; Corson et al. 1996; Fleming et al. 1998) and in isolated arteries (Ayajiki et al. 1996), there is a discrepancy in the time course of the Ca^{2+} response and the time course of the shear stress-induced production of NO so that the former is transient (in the order of seconds to minutes) and the latter maintained, indicating that a sustained increase in $[Ca^{2+}]_i$ is not essential for the shear stress-induced activation of eNOS. To highlight this difference in the dependency on Ca^{2+}, the shear stress-induced activation of eNOS was referred to as "Ca^{2+}-independent". However, this is—strictly speaking—not the case, since the chelation of $[Ca^{2+}]_i$ also abolishes the shear stress-induced increase in eNOS activity. Rather, the shear stress-induced increase in NO production is associated with eNOS phosphorylation and an increase in the sensitivity of the enzyme to Ca^{2+}, so that the enzyme can be activated at resting Ca^{2+} levels (Dimmeler et al. 1999; Fulton et al. 1999).

It is now accepted that the changes in the phosphorylation of several serine and threonine (and possibly also tyrosine) residues regulate NO production in response to Ca^{2+}-elevating agonists as well as to haemodynamic stimuli such as cyclic stretch and fluid shear stress (Fleming and Busse 2003; Boo and Jo 2003). Most is known about the role played by Ser^{1177}, which is situated in the reductase domain of the enzyme, and Thr^{495}, which is situated in the calmodulin (CaM)-binding domain, in the regulation of NO production. The phosphorylation of these sites appears to play a reciprocal role in the regulation of eNOS activity, as Ser^{1177} becomes phosphorylated in response to endothelial cell activation while Thr^{495} is constitutively phosphorylated but dephosphorylated upon stimulation as a consequence of the activation of phosphatases (Harris et al. 2001; Michell et al. 2001; Fleming et al. 2001a). The dephosphorylation of Thr^{495} facilitates the Ca^{2+}-dependent association of CaM with eNOS (Fleming et al. 2001a) while the phosphorylation of eNOS on Ser^{1177} increases NO output in an apparently Ca^{2+}-independent manner (Dimmeler et al. 1999; Fulton et al. 1999). The kinases responsible for the phosphorylation of eNOS on Ser^{1177} vary with the stimuli applied. Phosphorylation has been attributed to the activation of the Ca^{2+}/CaM-dependent kinase II in bradykinin-stimulated endothelial cells (Fleming et al. 2001a) but to Akt in cells stimulated with vascular endothelial growth factor (VEGF) (Fulton et al. 1999) or hepatocyte growth factor (HGF) (Makondo et al. 2003), and to Akt in cells exposed to fluid shear stress (Dimmeler et al. 1999; Fisslthaler et al. 2000). As the maintained production of endothelium-derived NO in response to fluid shear stress is a Ca^{2+}-independent process (Kuchan and Frangos 1994; Ayajiki et al. 1996), it is generally assumed that the phosphorylation of eNOS on Ser^{1177} plays the predominant role in regulating eNOS activity in response to haemodynamic stimuli.

Relatively little is known about the initial steps in the signalling cascade that determine eNOS activity in response to fluid shear stress other than that

a tyrosine phosphorylation-dependent step (and thus at least one tyrosine kinase) is involved (Ayajiki et al. 1996; Berk et al. 1995). Approximately 6 years ago it was reported that the phosphatidylinositol 3-kinase (PI 3-K) and its downstream effector, the kinase Akt, are activated in endothelial cells exposed to shear stress and that Akt is able to phosphorylate eNOS, thus enhancing its activity (Go et al. 1998; Dimmeler et al. 1999; Fulton et al. 1999; Gallis et al. 1999). While there is no doubt of the importance of Akt in regulating NO production in response to growth factors, a detailed study of the role of the adaptor protein Gab1 (Grb2-associated binder 1) has revealed that the original concept that Akt is responsible for the shear stress-induced activation of eNOS needs to be revised.

2.1.1 Gab1 and SHP2

Gab1 is an adapter protein that belongs to the insulin receptor substrate-1 family and is a mammalian homologue of the *Drosophila* protein Daughter of Sevenless, which is involved in multiple signalling events mediated by cytokine and tyrosine kinase receptors, including that of the HGF receptor c-Met (Weidner et al. 1996). Gab1 is known to translocate to the plasma membrane in response to fluid shear stress (Osawa et al. 2002) and can bind to platelet endothelial cell adhesion molecule-1 (PECAM-1) which has also been implicated in mechanotransduction (see Sect. 4.3). PECAM-1 also associates with other proteins such as the tyrosine phosphatase SHP2 (Cunnick et al. 2001; Gu and Neel 2003) and the p85 subunit of PI 3-K (Nishida and Hirano 2003; Gu and Neel 2003). Specific mutation of the pleckstrin homology domain of Gab1 revealed that it was possible to abrogate the shear stress-induced phosphorylation of Akt without affecting the phosphorylation and activation of eNOS. The shear stress-induced phosphorylation and activation of eNOS was, however, causally linked with the Gab1-dependent activation of the tyrosine phosphatase SHP2 inasmuch as a Gab1 mutant to which SHP2 cannot bind, as well as a dominant-negative SHP2 mutant, abrogated the shear stress-induced activation of eNOS in cultured as well as in native endothelial cells (Fig. 1). Which kinase is then responsible for the shear stress-induced phosphorylation of eNOS? The most likely candidate is protein kinase (PK)A, as a specific PKA inhibitor abrogated the shear stress-induced activation of eNOS in cultured and native endothelial cells (Dixit et al. 2005). At first sight, the latter observations appeared to contradict a report that Gab1 regulates the phosphorylation and activation of eNOS via Akt, as the down-regulation of Gab1 (using a siRNA approach) and a Gab1 mutant that was unable to bind PI 3-K attenuated both processes (Jin et al. 2005). However, preventing the shear stress-induced activation of PI 3-K would be expected to affect the activation of both Akt and PKA (Boo and Jo 2003). Additionally, the global down-regulation of Gab1 would be expected to affect both the activation of

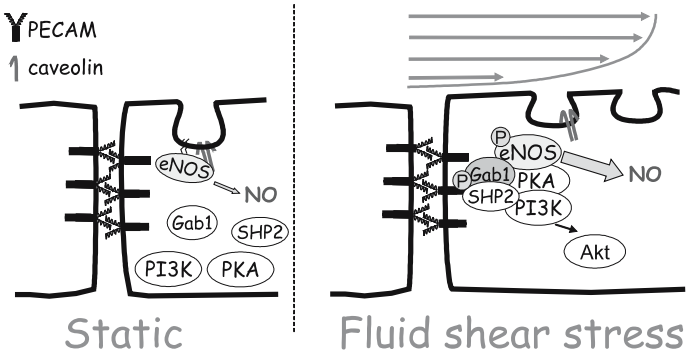

Fig. 1 Shear stress-induced assembly of the eNOS signalosome. In cells not exposed to haemodynamic stimuli, eNOS is located in caveolae bound to caveolin and generates only small amounts of NO. Upon cell stimulation with fluid shear stress, one of the early events is the rapid tyrosine phosphorylation of PECAM-1 and its association with the scaffolding protein Gab1 and the tyrosine phosphatase SHP2. The latter proteins then form part of the eNOS signalosome together with protein kinase A (*PKA*), and the p85 subunit of the PI 3-K and Akt. However, it is PKA rather than Akt that is responsible for the phosphorylation of eNOS on Ser^{1177} and its activation

Akt, which requires the translocation of Gab1 to the plasma membrane, and the association of SHP2 with Gab1, which is dependent upon the phosphorylation of Tyr^{627} and which does affect the shear stress-induced phosphorylation of eNOS.

A role for PKA rather than Akt in the shear stress-induced phosphorylation of eNOS Ser^{1177} has been suggested previously on the basis of experiments using specific inhibitors and dominant-negative mutants of Akt (Boo et al. 2002). Although it is unclear how the activity of PKA is stimulated, SHP2 seems to be involved in this process (Dixit et al. 2005). It is not known whether SHP2 acts at the level of the adenylyl cyclase or at that of PKA in shear stress-stimulated endothelial cells, as there is no information available regarding a role for SHP2 in the regulation of adenosine 3′5′ cyclic monophosphate (cyclic AMP) production or kinase activity. However, it is tempting to suggest that SHP2 can directly regulate PKA and thus eNOS activity, since shear stress does increase PKA activity but is reported not to increase endothelial cyclic AMP levels (Malek et al. 1993) and PKA, and the A-kinase anchor protein 121 and SHP2 are reported to coexist as a signalosome complex (Cardone et al. 2004).

2.1.2 Caveolin and Hsp90

When considering the regulation of eNOS activity by adaptor proteins or molecular scaffolds (or both), it is impossible to ignore the roles played by caveolin-1 and heat shock protein 90 (Hsp90).

In endothelial cells, eNOS has been localised to caveolae, small invaginations of the plasma membrane rich in cholesterol. Membrane cholesterol depletion impairs agonist-induced relaxation due to alteration in caveolar structure and disruption of the eNOS signalling complex (Everson and Smart 2001), while hypercholesterolaemia is reported to attenuate NO output by enhancing the interaction of caveolin-1 and eNOS (Feron et al. 1999). The association of these two proteins is generally reported to attenuate enzymatic activity, and an increase in NO production is linked to their dissociation (García-Cardena et al. 1996; Michel et al. 1997). Indeed, the application of fluid shear stress is reported to elicit the rapid dissociation of eNOS and caveolin, thus permitting the binding of calmodulin (Rizzo et al. 1999). As mentioned already, the response to shear stress/increased blood flow is biphasic, consisting of an initial Ca^{2+}-dependent peak followed by a maintained Ca^{2+}-independent plateau phase. In cultured endothelial cells, the latter phase of NO production is reported to be linked to changes in the expression and distribution of caveolin in endothelial cells as well as enhanced caveolae density, the end result being enhanced sensitivity to mechanical stimuli (Rizzo et al. 2003).

Hsp90, on the other hand, acts as a scaffold for the binding of Akt and possibly other components of the eNOS signalosome, and the association of eNOS and Hsp90 has been demonstrated in response to fluid shear stress (García-Cardena et al. 1998).

2.1.3 eNOS Expression

Elucidation of the mechanisms and factors determining transcription of the eNOS gene under different physiological/pathophysiological conditions has long been considered central for a thorough understanding of alterations in vascular NO production. Although numerous studies have concentrated on the activity of the eNOS promoter and changes in eNOS messenger RNA (mRNA) expression, the stimuli associated with the most pronounced effects on eNOS protein levels [i.e. oestrogen, tumour necrosis factor-α (TNF-α) and shear stress] are now appreciated to regulate post-transcriptional processes which mainly determine eNOS mRNA stability.

Given the list of transcription factors that bind to the eNOS promoter, it is hardly surprising that eNOS mRNA levels in cultured and native endothelial cells can be modulated by numerous stimuli. Oestrogen is a good example of a hormone whose beneficial cardiovascular effects have been linked to an improvement of vascular function, mainly as a consequence of enhanced NO production. Numerous investigations have assessed the effects of oestrogen on eNOS expression, and although 17β-oestradiol has been reported by numerous investigators to up-regulate eNOS mRNA and protein levels in cultured endothelial cells (Kleinert et al. 1998; Yang et al. 2000), an equal number of investigators have found no convincing effect or even a decrease in eNOS ex-

pression (Macritchie et al. 1997). The best demonstrations of a link between oestrogen and eNOS expression have been made using animal models, but even in those, chronic changes in oestrogen levels have been reported to increase as well as decrease eNOS levels (Wang et al. 1997; Pelligrino et al. 2000; Jayachandran et al. 2001; McNeill et al. 1999; Teichert et al. 2000; Rupnow et al. 2001).

Shear stress is also known to increase eNOS expression, and arterial levels of fluid shear stress tend to increase eNOS mRNA and protein levels between two- and sixfold (Uematsu et al. 1995; Harrison et al. 1996; Ziegler et al. 1998a) while, in contrast to initial reports (Awolesi et al. 1995), cyclic stretch does not affect eNOS expression. The response to shear stress is thought to underlie the increase in enzyme expression detected in exercising animals (Laughlin et al. 2001; Johnson et al. 2001; Kojda et al. 2001) as well as in humans (Testa et al. 2000; Taddei et al. 2000). Interestingly, although the increase in eNOS mRNA and protein has been repeatedly shown, it generally fails to turn up as a positive hit in microarray studies looking at the effects of fluid shear stress on protein expression in endothelial cells (García-Cardena et al. 2001; McCormick et al. 2001; Dai et al. 2004).

The sequence in some important regions of the human and murine eNOS promoters is highly conserved (Teichert et al. 1998), and using promoter-reporter insertional transgenic murine lines containing 5,200 bp of the native murine eNOS promoter directing transcription of a nuclear-localised β-galactosidase, it has been possible to map eNOS promoter activity in adult animals (Teichert et al. 2000). Examination of β-galactosidase expression in the heart, lung, kidney, liver, spleen and brain of such mice revealed a robust signal in large and medium-sized blood vessels. However, the eNOS promoter was apparently silent in arterioles, capillaries and venules (Teichert et al. 2000), a finding which may reflect the fact that smaller blood vessels regulate tone via NO-independent mechanisms.

2.2 Prostacyclin

A key enzyme in the synthesis of prostanoids is prostaglandin H synthase or cyclooxygenase (COX). Two isoforms of COX have been identified, COX-1 and COX-2, and although they catalyse the same reaction, they have distinct pharmacological profiles and biological roles (Loll and Garavito 1994). COX-1 is constitutively expressed, while COX-2 is expressed in response to pro-inflammatory stimuli (for review see Gimbrone et al. 2000; Davidge 2001). The expression of both enzymes in endothelial cells is modulated by shear stress, with the regulation of COX-2 depending on the magnitude of the shear stress applied, whereas the regulation of COX-1 protein levels seems to be independent of the shear stress amplitude (Topper et al. 1996; McCormick et al. 2000).

The availability of the COX substrate, arachidonic acid, which is released from membrane phospholipids by phospholipase A_2 (PLA_2), is the rate-limiting step in prostaglandin synthesis by endothelial cells and other cell types. PGI_2 is the major product of the subsequent COX-1 pathway in all arterial and venous endothelial cells studied so far (Schrör 1985), although other prostaglandins (PGE_2, $PGF_{2\alpha}$ and PGD_2) are also synthesised by some cultured microvascular endothelial cells (Alhenc-Gelas et al. 1982). As a consequence of the Ca^{2+}-sensitivity of PLA_2, the synthesis of PGI_2 by endothelial cells, like that of NO, is a Ca^{2+}-dependent process (Chang et al. 1987). This co-dependency on Ca^{2+} means that in most circumstances both NO and PGI_2 are released; however, the activation of PLA_2 appears to require higher Ca^{2+} levels than are necessary to increase NO synthesis (Lückhoff et al. 1988). The differential sensitivity of the autacoid-generating pathways to Ca^{2+} may also explain why cyclic stretch is a much better stimulus for PGI_2 production than shear stress, as cyclic stretch does induce a marked increase in endothelial $[Ca^{2+}]_i$ (Rosales et al. 1997). Although PGI_2 is generated by endothelial cells exposed to fluid shear stress, and its generation is negatively regulated by NO (Osanai et al. 2000), it is not clear how the activity of PLA_2 and/or COX-1 can be modulated in the absence of a maintained increase in $[Ca^{2+}]_i$. Although COX-1 is not phosphorylated, its activity is reportedly regulated by several potentially phosphorylatable associated proteins (Marcelin-Jimenez and Escalante 2001). It is therefore feasible that the production of PGI_2 in shear stress-stimulated endothelial cells is regulated by dynamic changes in the COX-1 protein complex.

2.3 The Endothelium-Derived Hyperpolarising Factor

The term EDHF does not describe a single factor but rather a variety of mechanisms that regulate endothelial cell membrane potential. Initially, an EDHF was supposed to be a factor generated in the endothelium that diffuses out of the cells to stimulate Ca^{2+}-dependent K^+ channels (K^+_{Ca}) on the underlying smooth muscle cells, thus eliciting hyperpolarisation and inducing relaxation in an NO- and PGI_2-independent manner. It is now generally accepted that the initial event in all of the EDHF-mediated responses characterised to date is not the hyperpolarisation of smooth muscle cells but the hyperpolarisation of endothelial cells mediated by the opening of small and intermediate conductance K^+_{Ca} channels (for review see Busse et al. 2002). Four main mechanisms have been proposed to account for EDHF-mediated responses:

1. The efflux of K^+ from endothelial cells to activate either inwardly rectifying K^+ channels or the Na^+K^+ATPase on smooth muscle cells (Edwards et al. 1998).

2. The generation of a vasodilator epoxyeicosatrienoic acid (EET) by a cytochrome P450 (CYP) epoxygenase (Campbell et al. 1996; Fisslthaler et al. 1999).

3. The generation of hydrogen peroxide (H_2O_2) (Matoba et al. 2000, 2002).
4. The transmission of an electrical signal involving gap junctional communication and cyclic AMP (Chaytor et al. 1997; Griffith and Taylor 1999). At least in the microcirculation, endothelium-dependent hyperpolarisation may be mediated by low electrical resistance coupling via myo-endothelial gap junctions (Coleman et al. 2001).

Most of the potential EDHFs—or rather the mechanisms referred to above—are difficult to study directly, as responses are generally best observed when NOS and COX enzymes are inhibited. An additional complication is that in many cases conclusions about EDHF are made based on the sensitivity of NO- and PGI_2-independent responses to K^+ channel blockers. One exception is the CYP-dependent EDHF, as more or less specific CYP inhibitors are now available and molecular techniques make it possible to up- or down-regulate CYP expression in vivo and in isolated arteries. In fact, although native endothelial cells express CYP epoxygenases of the 2C family (which generate vasodilator EETs), protein and mRNA expression decreases dramatically in culture so that within 24 h of cell isolation CYP activity is barely detectable. Interestingly, the most effective way to re-induce the expression of the protein and to enhance CYP activity is to expose cultured endothelial cells to either cyclic stretch or fluid shear stress (Fisslthaler et al. 2001; Fisslthaler et al. 2003). Moreover, given that pulsatile stretch can elicit the generation of a CYP-dependent EDHF in native endothelial cells (Popp et al. 1998; Fleming et al. 2001b), and increase the production of EETs by cultured cells (Fisslthaler et al. 2001), this stimulus may be responsible for the maintenance of CYP expression in vivo. Data regarding the role of EDHF in responses to shear stress are rare, but it has recently been possible to detect EETs released from arterioles exposed to fluid shear stress, but surprisingly only in female $eNOS^{-/-}$ mice (Huang et al. 2005).

There is, however, a dark side to the CYP epoxygenase-mediated regulation of vascular tone, as several of these enzymes are able to generate superoxide anions (O_2^-) which react with NO and therefore decrease the concentration of the radical that is able to activate the soluble guanylyl cyclase (Fleming et al. 2001c). Not only does the increase in O_2^- production affect vascular tone, but it also alters the consequences of CYP activation on the activity of the transcription factor, nuclear factor κB (NF-κB) and the expression of adhesion molecules. Moreover, in humans with manifest coronary artery disease, a CYP epoxygenase inhibitor was able to restore acetylcholine-induced, NO-mediated vasodilatation (Fichtlscherer et al. 2004). Ischaemia–reperfusion injury in a rat model has also been linked to an increase in the generation of O_2^- by a CYP epoxygenase (Granville et al. 2004). However, it is unclear what determines the switch between a CYP-derived vasodilator such as an EET and the CYP-derived generation of O_2^-. It has been suggested that the activity of a CYP epoxygenase

may also account for the H_2O_2-like EDHF reported to mediate the flow-induced vasodilatation of human coronary arterioles (Miura et al. 2003), but this point remains to be addressed specifically.

2.4 Endothelin-1

Endothelin-1 (ET-1) is one of a family of 21-amino-acid peptides that are synthesised in several cell types, including the vascular endothelium. The production and release of ET-1 is stimulated by many hormonal and metabolic factors, as well as by hypoxia, and most importantly by shear stress and cyclic stretch. Once released, ET-1 binds to the endothelin receptors (ET_A and ET_B), the ET_A receptors on vascular smooth muscle cells mediating vasoconstriction, and the ET_B receptors on the endothelium linked to NO and PGI_2 formation. Several lines of evidence indicate that NO impairs ET production/action. For example, blockade of NO formation magnifies the ET-1-induced constriction of various vascular beds, and acute pressor responses caused by NOS inhibitors in vivo are blunted by selective ET_A or dual ET_A/ET_B receptor blockade (for review, see Lavallee et al. 2001). On the other hand, the shear stress-induced decrease in ET-1 expression is mediated by the activation of eNOS (Kuchan and Frangos 1993; Morawietz et al. 2000), and inhaled NO can decrease plasma levels of ET-1 in humans (Wagner et al. 2004).

The release of ET-1 from human endothelial cells exposed to laminar shear stress varies with time in a biphasic manner. Physiological levels of shear stress modulate ET-1 gene expression in endothelial cells, inducing an early transient up-regulation followed by a sustained suppression (Malek and Izumo 1992; Kuchan and Frangos 1993). The picture is rather different in cells exposed to pulsatile pressure changes in combination with pulsatile shear stresses typical of those present in regions prone to the development of atherosclerotic plaques. In the latter situation, eNOS levels fail to increase to the extent observed in cells exposed to laminar flow only, and ET-1 mRNA levels increase rather than decrease with time (Ziegler et al. 1998b). The generation of NO and ET-1 is not only determined by changes in shear stress but can also be modulated by increased pressure/cyclic strain (Hishikawa et al. 1995; Gan et al. 2000; Qiu and Tarbell 2000). For example, while increased strain increases ET-1 production, this stimulus has little or no effect on the expression of eNOS, leading to the hypothesis that elevated intravascular pressure (such as in hypertension) would selectively enhance the generation of the potent vasoconstrictor. Some evidence has been obtained using isolated perfused arteries and veins to suggest that this may be the case, and both preproendothelin-1 mRNA abundance and intravascular ET-1 peptide content were reported to be strongly up-regulated by raising the intraluminal pressure for up to 12 h (Lauth et al. 2000). While it is clear that eNOS inhibition prevents the down-regulation of preproendothelin-1 mRNA by shear stress, it has been reported that chronic

exposure to fluid shear stress up-regulates the expression of the ET_B receptor, also via an NO-dependent pathway (Morawietz et al. 2000).

2.5 Oxygen-Derived Free Radicals

Over the last 10 years, we have come to recognise just how important the regulation of free radical production within the vascular wall really is for the regulation of vascular tone. Superoxide anions not only determine the bioavailability of NO but also largely regulate the formation of H_2O_2, as well as that of the potent oxidant peroxynitrite ($ONOO^-$). It is beyond the scope of this review to detail potential radical sources within the vascular wall or the chemistry of the interactions that occur; these have all been carefully detailed in a series of excellent recent reviews (Wolin et al. 2002; Thomas et al. 2003; Touyz 2004; Brandes and Kreuzer 2005).

The study of radicals in the vascular wall is complicated by the fact that several different enzymes with specific sub-cellular localisations and mechanisms of activation can all generate oxygen-derived free radicals and contribute to the oxidative burden. These include eNOS, enzymes of the respiratory chain, some cytochrome P450 monooxygenases, xanthine oxidase and NADPH (nicotinamide adenine dinucleotide phosphate, reduced) oxidases. The latter enzymes have been of particular interest given the availability of NADPH oxidase subunit knockout mice and the accumulating evidence that the generation of reactive oxygen species by NADPH oxidases triggers their release by the other enzymes, particularly from eNOS (Landmesser et al. 2003).

2.5.1 eNOS

All of the NOS isoforms generate O_2^- and H_2O_2 under specific conditions, i.e. lower than optimal concentrations of the essential co-factor tetrahydrobiopterin (BH_4) or the substrate L-arginine. The uncoupling of NOS basically means that the transport of electrons to the ferrous-haem-O_2 species generated during the stepwise activation of O_2 by NOS does not occur fast enough to prevent their oxidative decay; this results in the generation of reactive oxygen species (Stuehr et al. 2001). Indeed, in the absence of substrate and pterin, the oxyferrous complex decays to the ferric state and generates O_2^- (Bec et al. 1998). The loss of an electron results in the oxidation of BH_4 to the trihydrobiopterin (BH_3) radical, a species that has can be detected by electron paramagnetic resonance (EPR) spectroscopy (Hurshman et al. 1999; Bec et al. 2000; Kuzkaya et al. 2003). Initial experiments concentrated on inducible NO synthase (iNOS), but BH_4 has since been shown to exert the same function in all of the NOS isoforms, although the rates of BH_4 radical formation differ with the isoform studied so that neuronal (n)NOS > iNOS > eNOS (Wei et al. 2005).

Although all of the NOS isoforms can generate O_2^-, most of the information relating to the consequences of NOS uncoupling is related to eNOS. Levels of BH_4 decrease rapidly in cell culture, and cultured endothelial cells generally have such low BH_4 levels that eNOS is largely uncoupled. The latter phenomenon may be related to the slow formation of NO by eNOS and poor stability of its BH_4 radical (Wei et al. 2005). Indeed, it has been well appreciated for several years that it is difficult to measure NO by EPR spectroscopy without pretreating the cells with vitamin C to increase BH_4 levels, a process which markedly decreases oxidative stress in endothelial cell cultures at the same time as increasing NO production (Huang et al. 2000; Smith et al. 2002).

The in vivo evidence for a role of eNOS uncoupling in the phenomenon of endothelial dysfunction (a term that actually describes endothelial cell activation and a decrease in bioactive levels of NO) is convincing and has been linked to a lack (or rather to the oxidation) of BH_4. Supplementation with BH_4 restores endothelial function in isolated canine coronary arteries (Cosentino and Katusic 1995) and in rats with diabetes (Shinozaki et al. 1999; Shinozaki et al. 2000; Bagi and Koller 2003), as well as in healthy subjects following oral glucose challenge (Ihlemann et al. 2003) and in patients with hypercholesterolaemia (Stroes et al. 1998). High concentrations of vitamin C can also restore NO-dependent responses in ApoE-deficient mice (d'Uscio et al. 2003) and in humans with coronary artery disease (Gokce et al. 1999). This property of vitamin C can be attributed to its ability to reduce the oxidation of BH_4.

2.5.2 NADPH Oxidase

What causes the oxidation of BH_4 that ultimately leads to eNOS uncoupling? O_2^- generated by the NADPH oxidase are candidate oxidants, as NADPH oxidase activity is known to be increased in situations associated with a manifest endothelial dysfunction (Lassegue and Griendling 2004). Moreover, eNOS uncoupling can be induced in wild-type mice treated with deoxycorticosterone acetate (DOCA)-salt to induce hypertension, but the same phenomenon cannot be demonstrated in mice lacking the NADPH oxidase subunit p47phox (Landmesser et al. 2003). The second BH_4 oxidant that needs to be considered is the product of the reaction of NO with O_2^-, i.e. $ONOO^-$. Peroxynitrite is thought to be a more potent BH_4 oxidant than O_2^- (Laursen et al. 2001) and oxidises BH_4 to BH_3, which can be reduced back to BH_4 by ascorbate (Kuzkaya et al. 2003). In cultured bovine aortic endothelial cells in which eNOS was uncoupled by treating cells with a $ONOO^-$ donor and an inhibitor of BH_4 synthesis, O_2^- production was decreased and NO production increased by ascorbate and BH_4 supplementation (Kuzkaya et al. 2003).

Fluid shear stress and cyclic stretch are reported to affect NADPH oxidase subunit expression as well as enzyme activity and radical production (Brandes and Kreuzer 2005), but given the overall benefits of this stimulus on endothelial

cell biology, it remains to be determined whether or not the activation of the NADPH oxidase under physiological conditions can contribute to oxidative stress in vivo. One of the most important issues determining the response of endothelial cells to haemodynamic stimuli is the range of forces to which they are exposed. Indeed so-called "arterial" levels of shear stress are thought to be vasculo-protective and to enhance NO production and maintain the anti-atherogenic phenotype of the endothelium. Deviations from this optimal level of stimulation, such as at bifurcation points where shear stress can oscillate markedly or even drop to zero, generally result in endothelial cell activation and the induction of an inflammatory response that eventually promotes atherogenesis. The NADPH oxidase is thought to make a major contribution to the latter processes, and oscillatory flow leads to a sustained increase in radical production of endothelial cells (De Keulenaer et al. 1998), which is not observed in cells obtained from p47phox-deficient mice (Hwang et al. 2003). Elevated blood pressure or cyclic strain (or the two together), on the other hand, is an excellent stimulus for the activation of the NADPH oxidase. High pressure increases translocation and phosphorylation of p47phox in isolated arteries (Oeckler et al. 2003; Ungvari et al. 2003). Moreover, vessels removed from animals subjected to prolonged hypertension exhibit increased NADPH oxidase-dependent radical formation and endothelial dysfunction, which is sensitive to PKC inhibitors, as well as inhibitors of the epidermal growth factor (EGF) receptor tyrosine kinase, and Rac (Jung et al. 2004).

3
Endothelial and Vascular Smooth Muscle Cells

Classically, NO formed by eNOS freely diffuses from its generation site to smooth muscle cells, where it activates soluble guanylyl cyclase producing guanosine 3′5′ cyclic monophosphate (cyclic GMP). Subsequently, cyclic GMP activates both PKG and PKA leading to smooth muscle relaxation. However, NO may influence vascular tone by additional mechanisms.

3.1
NO and 20-HETE

One of the more interesting recent suggestions is that NO can modulate the production of a vasoconstrictor eicosanoid independently of cyclic GMP. The eicosanoid in question is 20-hydroxyeicosatetraenoic acid (20-HETE), which is an ω-hydroxylation product of arachidonic acid generated by CYP 4A enzymes in vascular smooth muscle cells, where it is thought to mediate the development of myogenic tone (Harder et al. 1997). Indeed, CYP 4A mRNA and protein are expressed in vascular smooth muscle cells (Harder et al. 1994), and 20-HETE is endogenously produced by smooth muscle cells from small

renal and cerebral vessels in response to stretch or increases in transmural pressure (Ma et al. 1993; Harder et al. 1997; Carroll et al. 1996) and is not affected by COX inhibition and endoperoxide/thromboxane receptor antagonism. Once formed, 20-HETE acts as an intracellular second messenger and increases smooth muscle tone by inhibiting large conductance K^+_{Ca} channels, inducing depolarisation and increasing $[Ca^{2+}]_i$, probably by activating L-type Ca^{2+} channels (Ma et al. 1993; Zou et al. 1996; Imig et al. 1996). NO can modulate the formation of 20-HETE (Alonso-Galicia et al. 1997) by binding and inactivating the P450 haem moiety in much the same way that it inhibits the EET-generating epoxygenase enzymes (Bauersachs et al. 1996). This effect may account for the cyclic GMP-independent vasodilator effects of NO in certain arterial preparations (Takeuchi et al. 1996) and may also explain the observation that NO donors inhibit L-type channel activity (Schobersberger et al. 1997). There are, however, differences in the sensitivity of 20-HETE-generating enzymes to NO, and although CYP 4A2 contributes greatest to haemodynamic responses in the kidney, CYP 4A3 contributes greatest to tubular responses following NO inhibition (Hercule et al. 2003).

3.2 EDHF-Dependent Dilatation

Several mechanisms can account for the NO- and PGI_2-independent dilatation of different vascular beds. The CYP-derived arachidonic acid metabolite(s) implicated in the EDHF response are the EETs, in particular 5,6-, 11,12- and 14,15-EET. When applied exogenously, 11,12-EET and 5,6-EET induce K^+_{Ca} channel inhibitor-sensitive relaxations of endothelium-denuded arteries and activate iberiotoxin-sensitive, large conductance K^+_{Ca} (BK_{Ca}) channels in native and cultured smooth muscle cells.

The fact that EETs activate iberiotoxin-sensitive BK_{Ca} channels has frequently been used as an argument against the involvement of an EET in the EDHF-mediated response, since EDHF-dependent relaxation is more universally sensitive to the combination of charybdotoxin and apamin than to iberiotoxin (Zygmunt and Högestätt 1996) and the smooth muscle cells from many freshly isolated arteries do not express the BK_{Ca} channel (Gauthier et al. 2002). However, the EDHF-mediated relaxation of porcine coronary arteries that is sensitive to the CYP 2C9 inhibitor sulphaphenazole and the CYP 2C antisense approach is insensitive to iberiotoxin and sensitive to charybdotoxin and apamin, suggesting that the role of EETs in the EDHF phenomenon may not simply be related to the activation of BK_{Ca} channels. For example, patch clamp experiments performed in the cell-attached mode i.e. when the channel under investigation is shielded from the extracellular medium by the pipette, have shown that EDHF is still able to activate K^+_{Ca} channels. Thus, it would appear that this factor activates K^+ channels in an indirect manner, possibly involving membrane-associated second messengers or that binding to the in-

tracellular rather than the extracellular portion of the channel is necessary for activation (Popp et al. 1996). In favour of the second-messenger hypothesis is the observation that EETs activate K^+_{Ca} channels in coronary smooth muscle cells via a $G_s\alpha$-mediated mechanism (Li and Campbell 1997); this phenomenon is observed only in the presence of added GTP or GTP and ATP, and may alter the sensitivity of these channels to Ca^{2+} (Baron et al. 1997). The activation of PKA by 11,12-EET is reported to be an important mechanism responsible for afferent arteriole vasodilatation (Imig et al. 1999), and there is evidence suggesting that K^+_{Ca} channels are modulated by cyclic AMP and PKA (Esguerra et al. 1994; Desaphy and Joffre 1998). EETs also play a key role in modulating intracellular Ca^{2+} levels in endothelial cells as well as the endothelial cell hyperpolarisation that occurs because of the subsequent activation of low and intermediate conductance K^+_{Ca} channels. This implies that CYP-derived EETs are basically intracellular amplifiers of the endothelial hyperpolarisation response, and it goes a long way towards explaining why EDHF appears to have different properties in different vessels. Indeed, an agonist-induced Ca^{2+} transient as well as the activation of small and intermediate conductance K^+_{Ca} channels can be demonstrated in endothelial cells from a wide range of arteries, a phenomenon that results in an EDHF-mediated relaxation. EETs simply potentiate all of these responses, most probably by directly affecting the open probability of non-selective cation channels of the transient receptor potential (Trp) family. Some of these channels possess an arachidonic acid binding site and can be activated by EETs (Watanabe et al. 2003).

In addition to activation of the different K^+_{Ca} channels, an additional problem with the "EET is the EDHF" hypothesis was that EETs are lipophilic. Thus, simple diffusion from the endothelium to the media down a concentration gradient would be too slow to account for the rapidly initiated, EDHF-mediated hyperpolarisation and relaxation. With the model outlined here, however, which redefines EETs as intracellular messengers modulating endothelial cell hyperpolarisation, there is no need for EETs to diffuse to vascular smooth muscle cells to activate BK_{Ca} channels in order to be involved in EDHF-dependent vasodilatation. Indeed, although CYP metabolites can be released from the cells in which they are generated, the vast majority are not usually present in a free form, and more than 90% of the EETs detected in plasma are esterified to the phospholipids of circulating lipoproteins, in particular to low-density lipoprotein, high-density lipoprotein and very low-density lipoprotein (Karara et al. 1992). An alternative is that, in addition to amplifying the endothelial cell hyperpolarisation, EETs facilitate the transfer of this hyperpolarisation from one cell type to another. In endothelial cells in culture (Popp et al. 2002) and in the isolated hamster gracilis muscle (Hoepfl et al. 2002), EETs are able to do just that and facilitate gap junctional communication via a PKA-dependent process (Fig. 2).

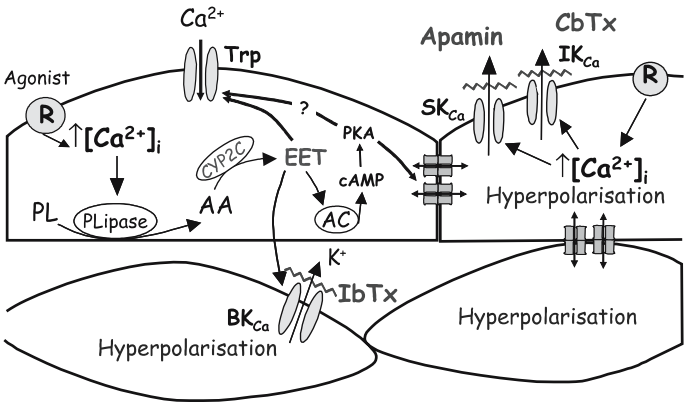

Fig. 2 EETs and their role in modulating EDHF responses. In CYP-expressing endothelial cells, cell stimulation (for example via the binding of an agonist to its receptor, *R*) induces a discrete increase in $[Ca^{2+}]_i$ which activates phospholipases (*PLipase*) to release arachidonic acid (*AA*) from membrane-bound phospholipid (*PL*). The AA is metabolised by CYP 2C epoxygenases to EETs which can directly affect the open probability of Trp channels to further increase $[Ca^{2+}]_i$ and thus amplify the effects of cell stimulation on the opening of Ca^{2+}-dependent K^+ (K_{Ca}) channels. Such a mechanism accounts for the EET-dependent activation of small and intermediate K_{Ca} which are sensitive to charybdotoxin (*CbTx*) and apamin. EETs also facilitate the transfer of hyperpolarisation between adjacent endothelial cells and between the endothelium and the underlying vascular smooth muscle by a mechanism involving the activation of the adenylyl cyclase (*AC*), the production of cyclic AMP (*cAMP*) and activation of PKA. EETs can also directly activate iberiotoxin (*IbTx*)-sensitive large conductance K_{Ca} (*BK*$_{Ca}$) channels on vascular smooth muscle cells to induce hyperpolarisation and relaxation

3.3 Ascending Dilatation

The resistance network that controls blood flow to skeletal muscle comprises terminal arterioles, as well as larger arterioles and the small (feeder) arteries from which they derive. These arteries and arterioles are functionally connected and, in a contracting skeletal muscle, low PO_2 and vasoactive metabolites elicit a local response and initiate a conducted vasodilatation that "ascends" the vascular tree to induce the simultaneous vasodilatation of the feed arteries as well as branch arteries and thus increase blood flow. This co-ordinated longitudinal transmission of vasomotor responses is essential to achieve optimal organ perfusion (Pohl and de Wit 1999). This conducted or ascending vasodilatation can travel bi-directionally, but upstream sites are generally studied since the vasoactive compounds applied in superfused systems are unable to directly affect upstream sites. The amplitude of the conducted vasodilatation is generally smaller than that of the local response, and although there is a gradual decline in the conducted vasodilatation along some arterioles, there is no obvious decay of the conducted response along feeder arteries.

Conducted responses have been intensively investigated and, although the exact mechanism remains to be clarified, the ascending dilatation elicited in hamster cheek pouch arterioles by bradykinin and acetylcholine has been attributed to a wave of NO release (Doyle and Duling 1997; Budel et al. 2003). These reports are, however, not so easy to reconcile with the fact that both the NO wave and the EDHF response are apparently dependent on endothelial cell hyperpolarisation (Budel et al. 2003). Moreover, most researchers have been unable to detect a major role for NO in conducted vasodilatation, which is generally not affected by NOS inhibitors (Hungerford et al. 2000; Welsh and Segal 2000; Hoepfl et al. 2002) and the response is apparently intact in eNOS-deficient mice (de Wit et al. 2000). Weighing up the available data, it appears that changes in membrane potential are central to the phenomenon of ascending dilatation. Responses are generally attributed to the propagation of a hyperpolarisation along the vascular wall (Welsh and Segal 1998) that is either linked to the actions of an EDHF (Welsh and Segal 2000; Hoepfl et al. 2002) or to the direct transmission of an electrical signal between vascular cells (Xia and Duling 1995, 1998; Yamamoto et al. 1999; Coleman et al. 2001). Over the past few years, evidence has accumulated to suggest that homocellular (Emerson and Segal 2000a; Looft-Wilson et al. 2004) as well as heterocellular (Emerson and Segal 2000b; Yashiro and Duling 2000; Emerson and Segal 2001) gap junctional communication is involved in the phenomenon of conducted dilatation. (For recent reviews on this topic, see Figueroa et al. 2004; Thomas and Segal 2004.)

4
Mechanotransduction

The first step in the process that translates a physical stimulus into an increase in endothelium-derived autacoid production was initially thought to be the activation of a specific mechanoreceptor (such as a mechano-sensitive ion channel or a component of the glycocalyx), but current thinking tends towards classifying the entire endothelial cell cytoskeleton as a mechanoreceptor (for recent reviews, see Helmke et al. 2003; Davies et al. 2003; Ingber 2003). Thus, the application of a mechanical stimulus can be transmitted through the entire cell by the cytoskeleton to activate signal transduction cascades in "signalling hot spots".

4.1
Caveolae and Caveolin

Cell membrane invaginations, the so-called caveolae, or "little caves", are approximately 50–100 nm in diameter and are found in many of the cells within the cardiovascular system (endothelial cells, smooth muscle cells, macrophages, cardiac myocytes and fibroblasts). Over the last few years, it has become clear that caveolae are involved in the trafficking of proteins and

contain a large variety of signal transduction molecules, a phenomenon which means that, following cell activation, these cellular microdomains contain a large number of active signalling molecules. Moreover, chronic exposure to shear stress is reported to increase caveolae formation and thus amplify cellular responses to changes in physical stimulation (Park et al. 1998; Rizzo et al. 2003; Boyd et al. 2003).

Given that eNOS can associate with caveolin-1, it follows that much more is known about the role of caveolae in the regulation of NO production than that of PGI_2 or an EDHF. However, caveolae are relatively difficult to study, and initial reports were limited to showing that the application of fluid shear stress to either cultured endothelial cells (Park et al. 1998) or to an in situ lung preparation (Rizzo et al. 1999) elicited the activation of signalling cascades within caveolin-rich membrane domains. More recently, cholesterol-depletion (Park et al. 1998; Lungu et al. 2004) or cholesterol-loading experiments (Feron et al. 1999) have been used to demonstrate the effects of caveolae disturbance on shear stress-induced signalling and NO production.

Caveolin-1-deficient (Cav-$1^{-/-}$) mice are viable and lack caveolin-1 protein expression and plasmalemmal caveolae. In addition, the lung parenchyma (an endothelial cell-rich tissue) shows hypercellularity with thickened alveolar septa and an increase in the number of VEGF receptor (VEGF R2; Flk-1)-positive endothelial cells (Razani et al. 2001) as well as enhanced microvascular permeability (Schubert et al. 2002). eNOS activity is up-regulated in Cav-$1^{-/-}$ mice, and isolated arterial rings both fail to establish a steady contractile tone and demonstrate markedly enhanced relaxant responses to acetylcholine. Closer evaluation revealed that the basal release of NO in endothelial cells from Cav-$1^{-/-}$ endothelial cells was approximately 30% higher than from wild-type cells and was accompanied by a much higher intracellular cyclic GMP content (Drab et al. 2001). Thus, eNOS becomes hyperactive in the absence of caveolin-1 (Drab et al. 2001; Razani et al. 2001). There are, unfortunately, no data available regarding the flow-induced activation of eNOS in Cav-$1^{-/-}$ mice.

4.2 Focal Adhesion Contacts

Exposure of cultured endothelial cells to shear stress and cyclic stretch results in cytoskeletal rearrangements and redistribution of focal adhesion proteins, site-specific focal adhesion kinase (FAK) phosphorylation, small GTPase activation and barrier regulation in human pulmonary endothelial cells (Shikata et al. 2005). The phosphorylation and activation of FAK has been implicated in responses to shear stress and in endothelial cell migration (Li et al. 1997). Shear stress increases the tyrosine phosphorylation and the kinase activity of FAK as well as its association with growth factor receptor binding protein 2 (Grb2) in a rapid and transient manner, and has been linked to the mitogen-activated protein kinase signalling pathway. Confocal microscopic observations further

demonstrated that FAK tends to cluster with the vitronectin receptor near the abluminal side of endothelial cells (Li et al. 1997). This association is of potential functional importance, as stimulation of the vitronectin receptor initiates a flow-mediated dilatation (Fox and Frame 2002).

Fluid shear stress enhances endothelial cell migration in the direction of flow, and has been correlated with the rapid recruitment of FAK to focal adhesions. Under flow conditions, endothelial cells migrate with new focal adhesions being concentrated at the leading edge in the direction of flow, these are then subsequently disassembled after the rear of the cell moves over them (Li et al. 2002). The activation of FAK may be differentially regulated by steady versus pulsatile stretch, and in isolated rabbit aortae constant stretch was reported to activate FAK, whereas pulsatile stretch did not (Lehoux et al. 2005). For more detailed information on the role of focal adhesions, the reader is advised to see two excellent reviews on this topic (Davies 1995; Chen et al. 2004).

Although shear stress and cyclic stretch can activate signalling cascades within caveolae (Rizzo et al. 1999) and focal adhesion contacts (Vuori 1998; Tamada et al. 2004), the lateral zone of cell–cell adhesion is thought to be the major signal transduction site for fluid shear stress (Noria et al. 1999; Kano et al. 2000; Fujiwara et al. 2001; Davies et al. 2003).

4.3 Cell–Cell Contacts and PECAM-1

PECAM-1 (CD31) is concentrated at cell–cell contacts and undergoes homophilic binding between adjacent endothelial cells. It was initially attributed to a function in the regulation of leucocyte transmigration, cell migration, cell adhesion and angiogenesis (DeLisser et al. 1997), but more recently PECAM-1 was found to contain two intracytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs), centred around Tyr^{663} and Tyr^{686}, which has led to a reconsideration of its role in cell signalling (Newman 1999). Although the fact that PECAM-1 possesses ITIMs would tend to suggest that any potential role in signalling would be modulatory rather than activatory, PECAM-1 is known to become rapidly tyrosine-phosphorylated following the application of fluid shear stress to endothelial cells grown under static conditions (Harada et al. 1995; Osawa et al. 1997). As this response cannot be mimicked by Ca^{2+}-elevating agonists or growth factors, it was suggested that PECAM-1 may represent a mechanoreceptor, or part of such a complex, on the endothelial cell surface (Osawa et al. 1997; Osawa et al. 2002). Thus, PECAM-1 appears to be exquisitely sensitive to fluid shear stress and is situated in a subcellular compartment that is rich in many of the signalling molecules known to be activated following the application of fluid shear stress to endothelial cells, including eNOS (Govers et al. 2002).

In confluent cultures of human endothelial cells, the application of fluid shear stress (12 dynes cm^{-2}) elicits the rapid and maintained tyrosine phosphorylation of PECAM-1. The kinase responsible for this effect most probably

belongs to the Src-family of non-receptor tyrosine kinases but is unlikely to be Src itself (Arai et al. 1997). Indeed, Src- and Csk-related protein tyrosine kinases can phosphorylate PECAM-1 (Cao et al. 1998), and c-Src can be co-precipitated with PECAM-1 from shear stress-stimulated endothelial cells (Osawa et al. 1997).

In addition to their co-localisation at the endothelial cell plasma membrane (Govers et al. 2002), PECAM-1 and eNOS have also been reported to physically associate in endothelial cells. However, the reports regarding the shear stress-induced alterations in this liaison are contradictory. For example, one study reports that eNOS and PECAM-1 interact in cultured endothelial cells maintained under static conditions and that the application of fluid shear stress elicits the rapid disassociation of the complex (Dusserre et al. 2004). Other reports describe the time-dependent association of eNOS with PECAM-1 (Fleming et al. 2005). The reasons for these discrepant findings are currently unclear but may be related to the time frame in which the experiments were performed. Indeed, the dissociation was reported to occur between 15 and 60 s after the application of shear stress, i.e. during the initial Ca^{2+}-dependent phase of the response, while the enhanced association of PECAM-1 and eNOS occurred from 5–60 min after the application of shear stress, i.e. within the secondary Ca^{2+}-independent phase of the shear stress response. Indeed, the shear stress-induced tyrosine phosphorylation of PECAM-1 is a delayed phenomenon relative to the activation of Ras (Gudi et al. 2003) or of K^+ channels (Olesen et al. 1988).

The role of PECAM-1 in mechanotransduction has recently been addressed by down-regulating PECAM-1 in human endothelial cells using a small interfering RNA (siRNA) approach, but again the results of this intervention are contradictory. One group reported that the loss of PECAM-1 is without consequence on the shear stress-induced phosphorylation of Akt (Sumpio et al. 2005), while another reports a significant attenuation of the phosphorylation of Akt and eNOS as well as a decrease in eNOS activity (Fleming et al. 2005). In addition, both groups assessed the shear stress-induced phosphorylation of Akt in endothelial cells from PECAM-$1^{-/-}$ mice, again obtaining discrepant results. Indeed, the phosphorylation of Akt was similar in PECAM-$1^{-/-}$ endothelial cells and in cells into which the enzyme was re-introduced. But while this fact provides a strong argument against a significant role for PECAM-1 in transducing the shear stress (Sumpio et al. 2005), a direct comparison of the shear stress-induced phosphorylation of Akt in endothelial cells from wild-type versus PECAM-$1^{-/-}$ mice revealed that the loss of the adhesion molecule correlates with an attenuated response (Fleming et al. 2005). More recently, flow-dependent vasodilatation was reported to be attenuated in isolated skeletal muscle arterioles from PECAM-$1^{-/-}$ compared to the same preparation from wild-type mice, although agonist-induced NO production was similar in vessels from both strains (Bagi et al. 2005).

Interestingly, while a decrease in PECAM-1 expression has been linked to an attenuated activation of Akt and eNOS, other pathways such as the shear

stress-induced activation of the AMP-activated protein kinase (AMPK) are apparently unaffected (Fleming et al. 2005). Links have previously been made between PECAM-1 and the PI 3-K/Akt pathway, as PI 3-K can associate with PECAM-1 in neutrophils (Pellegatta et al. 1998), and in a subset of CD14-positive circulating leucocytes the ligation of PECAM-1 leads to the activation of Akt and to the induction of Bcl-2 and Bcl-X (Ferrero et al. 2003).

The fact that a tyrosine kinase must be activated in order for PECAM-1 to be phosphorylated means it is unlikely that PECAM-1 acts as a mechanoreceptor per se. Indeed, fluid shear stress elicits the tyrosine phosphorylation of PECAM-1 mutants that lack the transmembrane domain of the adhesion molecule and which do not localise to the lateral membranes or participate in cell–cell homophilic PECAM-1 binding (Kaufman et al. 2004). Rather, it is more likely that PECAM-1 modulates endothelial cell activation in response to shear stress by virtue of its function as a scaffold for the binding of signalling molecules such as the tyrosine phosphatase SHP2 and the scaffolding protein Gab1 (for recent reviews, see Newman and Newman 2003; Ilan and Madri 2003). Interestingly, Gab-1 translocates from the cytoplasm to endothelial cell junctions in response to flow (Osawa et al. 2002), and a dominant-negative form of the non-receptor protein-tyrosine kinase Fer, which can phosphorylate PECAM-1, SHP2 and Gab1 in vitro, is reported to prevent the engagement-dependent phosphorylation of PECAM-1 (Kogata et al. 2003). A role for Gab-1 in the shear stress-induced phosphorylation and activation of Akt and activation of eNOS has also been recently reported (Jin et al. 2005), although in the latter study the Src-dependent phosphorylation of Gab-1 was linked to that of the VEGF R2 rather than to the tyrosine phosphorylation of PECAM-1.

The conclusions of both studies indicating that a physical association exists between eNOS and PECAM-1 (Dusserre et al. 2004; Fleming et al. 2005) are based on co-immunoprecipitation experiments, but currently no information is available to indicate whether this interaction is direct or mediated by other adaptor or scaffolding proteins. Moreover, although alterations in the association of the two proteins have been temporally correlated to changes in cellular NO output, there is currently no direct evidence demonstrating that the activity of the PECAM-1-associated eNOS is any different from that of the dissociated enzyme. Summarising the available data, it seems that PECAM-1 is implicated in the shear stress-induced activation of Akt and eNOS but that this is not the only mechanotransduction pathway activated by shear stress in endothelial cells.

4.4
Haemodynamic Stimuli and Crosstalk with Growth Factor Receptors

A number of growth factor receptors, as well as some G protein-coupled receptors, have been implicated in responses to haemodynamic stimuli. Initially

it was suggested that fluid shear stress and stretch stimulate the release of agonists for these receptors from endothelial cells; however, this concept went out of fashion as researchers began to appreciate that crosstalk can take place between signalling pathways at an intracellular level. Shear stress in particular has been linked with the enhanced tyrosine phosphorylation of the VEGF (Chen et al. 1999; Lee and Koh 2003) and insulin receptors (Kim et al. 2001) as well as of Tie2 (Lee and Koh 2003).

VEGF R2 is rapidly tyrosine-phosphorylated in response to endothelial cell stimulation by shear stress and this is paralleled by its association with the adaptor protein Shc, the clustering of the receptor, activation of extracellular signal-regulated kinases and c-Jun N-terminal kinases, as well as the recruitment of PI 3-K (Chen et al. 1999; Wang et al. 2002; Jin et al. 2003). The latter response has been linked to the shear stress-induced activation of eNOS (Jin et al. 2003). VEGF R2 has also been reported to translocate to the nucleus of stimulated cells and to bind the adherens junction molecules vascular endothelial (VE)-cadherin and β-catenin to the endothelial cytoskeleton (Shay-Salit et al. 2002). The shear stress-induced activation of VEGF R2 has also been implicated in the differentiation of VEGF R2-expressing embryonic stem cells, inasmuch as cell density increased more markedly than in cells maintained under static conditions. Furthermore, the treated cells expressed the endothelial cell markers VE-cadherin and PECAM-1, formed tube-like structures in collagen gels and developed an extensive tubular network significantly faster than the static controls (Yamamoto et al. 2005).

Both fluid shear stress and insulin stimulate tyrosine and serine phosphorylation of the insulin receptor substrate-1 (IRS-1) and increase IRS-1-associated PI 3-K activity and eNOS phosphorylation. Somewhat unexpectedly, TNF-α inhibited the shear stress-induced activation of IRS-1 and almost totally inhibited NO production (Kim et al. 2001), a phenomenon that was also observed in endothelial cells treated with free fatty acids composed of palmitic, oleic or linoleic acids. The effects of the free fatty acid preparations were linked to an increase in the activity of the IκB kinase β, which regulates the activation of the transcription factor NF-κB (Kim et al. 2005).

Differences have been reported in the kinetics of growth factor receptor activation by shear stress, and while cell stimulation produced a rapid, marked and sustained Tie2 phosphorylation (Lee and Koh 2003), it elicits a rapid but only slight and transient phosphorylation of the insulin receptor and VEGF R2 (Chen et al. 1999; Lee and Koh 2003).

The idea that mechanical stimuli activate growth factor receptors has recently resurged, and compressive stress, which shrinks the lateral intercellular space surrounding epithelial cells, is reported to trigger cellular signalling via autocrine binding of EGF family ligands to the EGF receptor (Tschumperlin et al. 2004). In the latter study, a mathematical analysis was used to calculate that the concentration of EGF receptor ligands shed from the cell surface would be sufficient to initiate receptor signalling (Tschumperlin et al. 2004).

However, although the activation of the EGF receptor has been reported in epithelial (Correa-Meyer et al. 2002) and endothelial cells exposed to cyclic stretch (Oeckler et al. 2003), no information is available on its role in the shear stress-induced activation of endothelial cells.

4.5 The Kallikrein–Kinin System

Endogenously formed kinins have also been proposed to participate in the regulation of vascular tone. As a B_2 kinin receptor antagonist decreases the vasodilator response to increased blood flow in human arteries, it has been suggested that activation of vascular B_2 receptors by either circulating or locally formed kinins is involved in flow-induced vasodilatation (Groves et al. 1995). Further support for a role of endogenously generated kinins in mediating the response to haemodynamic stimuli has been obtained using tissue kallikrein-deficient mice in which flow-mediated vasodilatation was attenuated (by about a third) relative to wild-type animals (Bergaya et al. 2001; Meneton et al. 2001). In an intriguing twist to this story, however, the presence of functional angiotensin II AT_2 receptors is necessary to observe the contribution of the vascular kallikrein–kinin system to flow-dependent dilatation (Bergaya et al. 2004).

In this chapter, we have described a number of pathways implicated in co-ordinating vascular responses to haemodynamic stimuli. From the variety of mechanisms listed, it is evident that responses to haemodynamic stimuli are not sensed by a single specific mechanoreceptor which activates a defined mechano-chemical signalling pathway. Rather, there is a high degree of redundancy in the system with several membrane-bound events (e.g. the tyrosine phosphorylation of PECAM-1, EGFR or VEGF R2) all of which lead to alterations in autacoid production. Thus, the endothelial response to haemodynamic stimuli most likely reflects an integrated response of multiple signalling networks at different subcellular locations.

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Biomechanical Modulation of Endothelial Phenotype: Implications for Health and Disease

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Abstract The functional phenotypic plasticity of the vascular endothelium relies on the ability of individual endothelial cells to integrate and transduce both humoral and biomechanical stimuli from their surrounding environments. Increasing evidence strongly suggests that biomechanical stimulation is a critical determinant of endothelial gene expression and the functional phenotypes displayed by these cells in several pathophysiological conditions. Herein we discuss the types of biomechanical forces that endothelial cells are constantly exposed to within the vasculature, explain how these biomechanical stimuli serve as regulators of endothelial function and discuss the increasing evidence that "atherosclerosis-protective" or "atherosclerosis-prone" haemodynamic environments can be important causative factors for atherogenesis via the differential regulation of endothelial transcriptional programmes.

Keywords Endothelium · Atherosclerosis · Haemodynamic forces · Transcriptional programmes

1 Introduction

The flow of blood throughout the branched tubular array that constitutes the vertebrate cardiovascular system generates distinct haemodynamic forces.

These biomechanical stimuli influence vascular morphogenesis during vertebrate development and also regulate vascular function and remodelling in the adult organism. The vascular endothelium—the single-cell-thick lining of blood vessels—is exquisitely sensitive to these biomechanical stimuli and, as part of its normal functioning, continuously senses and transduces distinct haemodynamic environments into rapid cellular responses, as well as long-lasting changes in gene expression, leading to acquisition and maintenance of distinct endothelial phenotypes (Gimbrone et al. 2000; Traub and Berk 1998). Haemodynamic forces are therefore increasingly being recognised as important modulators of endothelial function in health, and as pathophysiological stimuli in various vascular disease states.

The three major haemodynamic forces experienced by the vascular endothelium are the frictional *shear stresses* imparted to its surface by flowing blood; the pulsatile *cyclic stretching* of the vessel wall; and the compressive *hydrostatic pressures* generated with every cardiac cycle (Fig. 1; Davies 1995). Some haemodynamic environments—characterised by laminar blood flow, moderate pulsatile stretching and moderate amounts of hydrostatic pressure—generate physiological patterns of biomechanical stimulation that serve as important influences in the maintenance of normal endothelial function. In

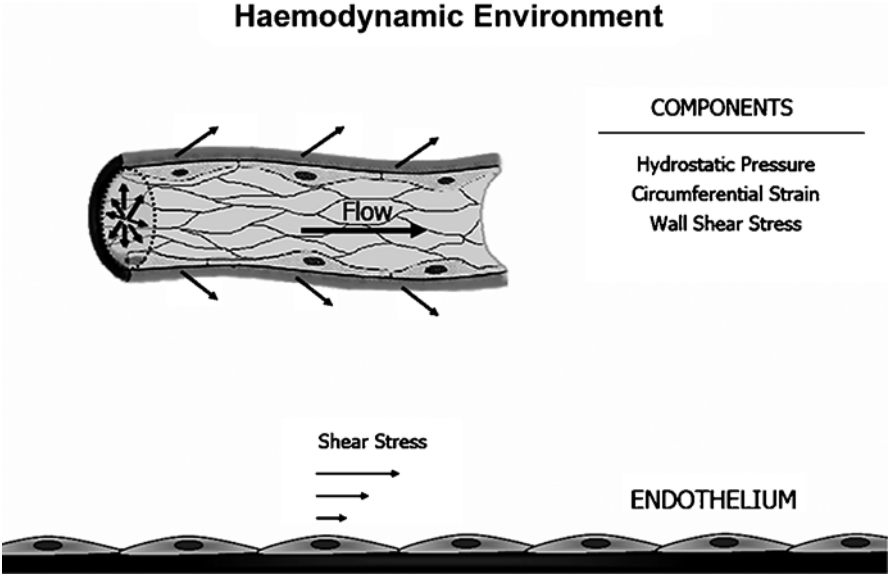

Fig. 1 Haemodynamic environment of blood vessels. The vascular endothelial cells that comprise the lining of all blood vessels are subjected to three types of biomechanical forces generated by the flow of blood. Hydrostatic pressures, acting perpendicular to the vessel wall; circumferential strains, generated within the vessel wall due to cyclic deformation; and shear stresses, frictional forces imparted directly to the surface of endothelial cells, due to the bulk flow of blood, a viscous fluid

other haemodynamic environments, however, such as those seen in hypertensive vessels or those present in regions where atherosclerotic plaques initially form, local patterns of biomechanical stimulation can trigger the genesis of disease by altering the functional phenotype of vascular endothelium.

This chapter will focus on certain aspects of the distinct haemodynamic environments present in different regions of the human vasculature, explain how these biomechanical stimuli serve as key regulators of endothelial function and discuss the increasing evidence that "atherosclerosis-protective" or "atherosclerosis-prone" haemodynamic environments can be important causative factors in the aetiology of this disease.

2
Haemodynamics at the Interface Between Blood and the Vessel Wall

The single-cell-thick layer of endothelial cells which lines the inner surface of blood vessels constitutes an important interface between blood and all of the tissues of the body. These cells provide an antithrombotic container for blood, and also participate in the regulation of vascular tone, permeability, angiogenesis, inflammation and coagulation. Like all living tissues, endothelial cells are exposed to external physical forces, including gravity, osmotic pressure, fluid and air pressure and tension within and between cells, but what makes endothelial cells unique is their exposure to haemodynamic forces—those complex fluid mechanical forces generated specifically by the action of the heart and the flow of blood (Davies 1995). How endothelial cells actually sense and transduce these haemodynamic forces into mechanical stimuli remains a source of intensive research and debate (reviewed in Davies 1995). Various studies have provided evidence for stretch-activated (Lansman et al. 1987) and shear-sensitive cell membrane ion channels (Olesen et al. 1988), mechanically sensitive proteins like integrins (Shyy and Chien 2002), cytoskeletal (Ingber 1993; Wang et al. 1993), nuclear (Ou et al. 2002) and possibly glycocalyx mechanosensing and mechanotransducing events (Weinbaum et al. 2003), mechanosignalling via G proteins (Clark et al. 2002; Gudi et al. 1996, 1998) and mitogen-activated protein kinase (MAPK) cascades (Berk et al. 1995). It is now clear that, as a consequence of their ability to sense and transduce mechanical forces, endothelial cells can respond to patterns of biomechanical stimulation derived from these three fundamental haemodynamic forces: shear stress, pulsatile strain and hydrostatic pressure.

2.1
Fluid Shear Stresses Modulate Endothelial Structure and Function

Whereas all layers of the vascular wall experience hydrostatic pressure and axial stretching, only endothelial cells come into contact with significant levels

of shear stress, the frictional drag of blood flow acting parallel to the vascular lining (Davies 1995). While the fluid mechanics associated with the flow of blood (a viscous fluid) are complex, in general, vascular shear stress increases with elevated rates of flow, greater blood viscosity and smaller vessel diameter. Accordingly, shear stress varies temporally, based on cardiac output and the cardiac cycle, and spatially, based on regional differences in vessel geometry. Large arteries, for instance, are defined by a high-pressure, pulsatile flow with shear stresses that cycle with systole and diastole, peaking at relatively high levels (10–70 dynes/cm^2). Veins, on the other hand, experience much lower shear stresses (on the order of 1–2 dynes/cm^2) and a lesser degree of force variation with each pulsation. Furthermore, neighbouring cells within the same vessel sometimes experience remarkably different patterns of these frictional forces, the best example being certain geometries within arteries (e.g. branch points, curvatures) prone to the development of atherosclerosis. These localised regions are characterised by unusually low time-averaged shear stresses that result from disturbed laminar flows (Asakura and Karino 1990; Caro et al. 1969, 1971; Dai et al. 2004; Gimbrone et al. 2000; Zarins et al. 1983).

The first experimental evidence of the importance of shear stress as a biologically active force came from the observation that endothelial cells change shape structurally in response to in vitro models of flow (Dewey et al. 1981). Subsequent reports, in the early 1980s, established that shear stress also modulates endothelial fluid phase endocytosis (Davies et al. 1984) and the regulation of prostacyclin (PGI_2, Frangos et al. 1985) and tissue plasminogen activator expression (Diamond et al. 1989), thus providing critical evidence for the idea that mechanical forces could influence pathophysiologically relevant aspects of endothelial function.

There is now an extensive literature on fluid shear stresses and their influence on endothelial function. For technical reasons, almost all of the studies begin with the artificial condition of culturing endothelial cells in a static fluid milieu; then after a period of equilibration, the cells are exposed to an applied shear stress by way of any number of experimental apparatuses designed to control fluid motion. Endothelial responses to shear stress in these experiments can be grouped according to time, with some of the first changes occurring within seconds to minutes, and others—in particular gene expression and protein synthesis—occurring over longer periods of time (hours to days). As reviewed by Davies (1995) and confirmed by a number of recent studies (Chen et al. 2001; Chiu et al. 2005; Dekker et al. 2002; Garcia-Cardena et al. 2001; Grabowski et al. 2001; McCormick et al. 2001; Peters et al. 2002; Yamawaki et al. 2003), within the first minute of the application of unidirectional flow, endothelial cells activate flow-sensitive potassium and calcium channels as well as signalling cascades involving 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) generation, leading to release of immediate-acting molecules like nitric oxide (NO) and prostacyclin (PGI_2). Within the first hour, additional signalling cascades are activated, including those involving G proteins, MAP kinases and

transcription factors like nuclear factor of κ light polypeptide gene enhancer in B cells (NF-κB) and c-*jun*. Functionally, basic fibroblast growth factor (bFGF) and platelet-derived growth factor B (PDGF-B) are also upregulated, and fluid-phase endocytosis increases. Over the course of 6 h, mechanosensitive gene transcription begins to ramp up, with increased expression of genes important in both inflammation and anti-inflammation, anti-coagulation and the regulation of vascular tone. After about 6 h, endothelial cells then start adapting to the chronic presence of flow, with the downregulation of genes associated with acute changes, and steady expression of what are now recognised as "shear-responsive" genes. Given that endothelial cells in vivo are constantly exposed to some level of shear stress, the relative importance of these early versus late effects observed in vitro remains a fundamental and unresolved question. Conceivably, the acute changes are likely to reflect basic mechanosensing mechanisms at the cellular level, while the subsequent adaptations are likely to reflect more long-term, flow-sensitive and adaptive phenotypes that may have important pathophysiological significance.

The presence of distinct types of shear stresses within the human vascular tree has inspired investigators to design devices to mimic these localised haemodynamic environments in vitro. Thus, early experiments demonstrated that endothelial cells are differentially responsive to laminar versus turbulent shear stress (Davies et al. 1986). While turbulent flow is rarely encountered in the normal cardiovascular system in vivo, this flow pattern provides a biomechanically well-defined comparative stimulus for steady laminar flow in in vitro studies. Several studies have examined low levels of shear as the variable of interest (Gerszten et al. 1996; Mohan et al. 1999); other studies have examined pulsatile unidirectional flow (Helmlinger et al. 1995), oscillatory flow (Chappell et al. 1998; De Keulenaer et al. 1998; Ku et al. 1985; Mazzolai et al. 2002; Ziegler et al. 1998), disturbed flow (Brooks et al. 2002) and turbulent flow (Garcia-Cardena et al. 2001). More recently, "atheroprone" and "atheroprotective" waveforms derived from atherosclerosis-susceptible and atherosclerosis-resistant regions of the human carotid artery have been programmed into mechanical devices (Fig. 2) to reproduce more faithfully the local haemodynamic milieu of the endothelial lining in the different regions of this arterial system in vivo (Dai et al. 2004). Taken together, data derived from these experiments have demonstrated that endothelial cells respond differentially to distinct patterns of shear stress at the level of morphological changes, activation of signal transduction cascades, and differential regulation of gene expression programmes, strongly suggesting that specific types of shear stress may have important pathophysiological implications.

Using high-throughput, microarray-based experimental approaches, a number of endothelial-expressed genes have been found to be "shear-responsive" (Chen et al. 2001; Dai et al. 2004; Dekker et al. 2002; Garcia-Cardena et al. 2001; McCormick et al. 2001; Ohura et al. 2003; Peters et al. 2002; Warabi et al. 2004; Wasserman et al. 2002). The challenge in interpreting all of these data,

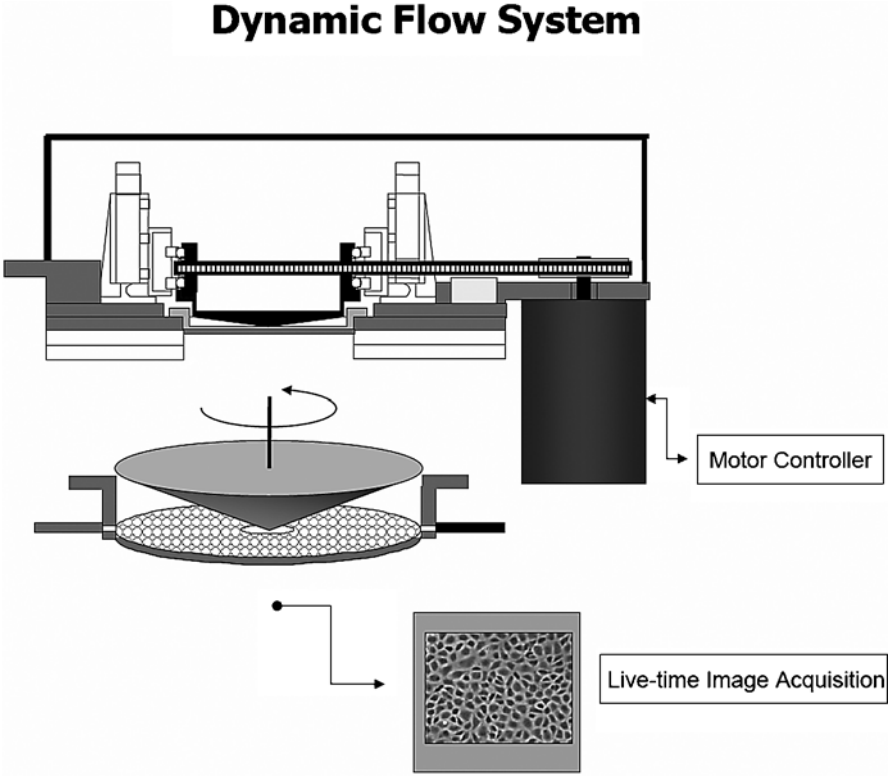

Fig. 2 Dynamic flow system (DFS). This fluid mechanical system is a cone-and-plate device that mounts onto a microscope stage, allowing direct visualisation of the endothelial cells, cultured on a transparent surface, during the application of defined fluid shear stresses. The rotation of the cone (and the resultant shear stress profile) is precisely controlled via a timing belt connection to a computerised programmable stepper motor (see Blackman et al. 2002)

however, has been the lack of standardisation between studies, wherein cell types, protocols, flow apparatuses, shear stress intensities, microarray platforms and statistical techniques vary greatly. Nevertheless, these experiments have clearly demonstrated that endothelial cells are not only able to sense and transduce shear stress but, perhaps more importantly, they are able to respond differentially to distinct types of haemodynamic environments via discrete changes in gene expression.

3
Haemodynamics as a Local Risk Factor for Atherogenesis

Atherosclerosis is a potentially life-threatening disease of large arteries that is strongly associated with systemic risk factors such as hypercholesterolaemia,

hypertension, diabetes and hyperhomocysteinaemia. In the face of these systemic risk factors, certain regions of the arterial vasculature nonetheless remain relatively resistant to the development of atherosclerotic lesions (Gimbrone et al. 2000). There is increasing evidence that the haemodynamically distinct environments in these arterial geometries appear to exert a protective influence on the endothelial lining; however, the mechanisms of this "atheroprotection" remained poorly characterised.

Physical and computational models have identified these vascular regions as exposed to uniform laminar flows; in contrast, regions prone to the development of atherosclerosis lesions are exposed to low time-average shear stress, a high oscillatory shear index, and steep temporal and spatial gradients of shear stress. In an effort to unveil mechanistic links between haemodynamics and atherogenesis, research over the past decade has focussed on emulating these flow characteristics in vitro and then delineating the unique signalling pathways and downstream changes at the level of endothelial cell structure and function that are induced. The initial observations of the differential and sustained upregulation of certain "atheroprotective" genes (e.g. endothelial NO synthase, cyclooxygenase-2 and manganese superoxide dismutase) in endothelial cells exposed to laminar shear stress, but *not* turbulent shear stress (Topper et al. 1996), led to a guiding hypothesis for the field. It posits that the steady laminar shear stresses characteristic of atherosclerosis-resistant regions elicit the expression of "atheroprotective genes", whereas the altered shear stresses generated by disturbed flow present in atherosclerosis-prone regions elicit the expression of "atheropathogenic genes", suppression of "atheroprotective genes" or both at once (Topper et al. 1996). To begin to test this hypothesis, several laboratories have used transcriptional profiling to capture the gene expression signatures of endothelial cells exposed to distinct types of biomechanical stimuli (Brooks et al. 2002; Dai et al. 2004; Garcia-Cardena et al. 2001) in order to identify potential atheroprotective genes.

Dai and co-workers recently developed an experimental in vitro model system in which cultured human endothelial cells can be exposed to well-defined shear stress waveforms that accurately simulate those present in vivo in atherosclerosis-susceptible and atherosclerosis-resistant regions of the human carotid artery (Fig. 3; Dai et al. 2004). This study demonstrated that endothelial cells exposed to wall shear stresses present in atherosclerosis-susceptible arterial geometries acquire a pro-inflammatory phenotype, expressing several pathophysiologically important chemokines and chemokine receptors. This atheroprone waveform also elucidated a dysregulation of the expression and organisation of cytoskeletal and junctional proteins. In addition, a differential activation of the NF-κB pathway and the enhanced cytokine-inducible cell surface expression of vascular cell adhesion molecule (VCAM)-1 in endothelial cells preconditioned by exposure to the atheroprone waveform compared with the atheroprotective waveform was observed in this study (Dai et al. 2004).

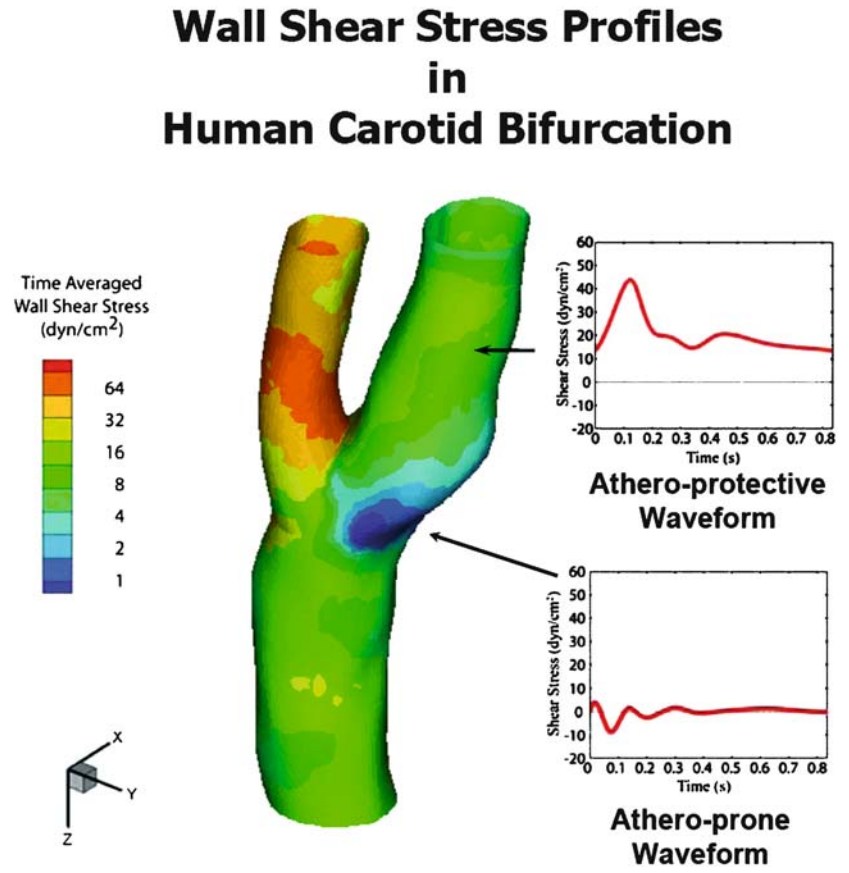

Fig. 3 Prototypic "atheroprotective" and "atheroprone" waveforms representative of those present in atherosclerosis-resistant and atherosclerosis-prone regions of the human carotid artery bifurcation. Representing distinct pathophysiological biomechanical stimuli, these two waveforms were applied to cultured human endothelial monolayers using the dynamic flow system

Taken together, the distinct transcriptional programmes and the suppressed anti-inflammatory response evoked in endothelial cells exposed to atheroprotective flow motivated us to search for key regulators of this flow-dependent endothelial atheroprotection. Thus, our group first sought to identify all the transcription factors differentially upregulated by atheroprotective flow, and validate the differential expression of such genes by real-time Taqman polymerase chain reaction (PCR). One of the most robustly upregulated transcription factors arising from this analysis was the Kruppel-like factor 2 (KLF2). The biology of this gene, its flow-dependent expression in vascular endothelium, and the possibility that KLF2 may constitute a "master regulator" of the atheroprotective endothelial phenotype will be discussed in the next section.

4
The Transcription Factor KLF2 and Atherogenesis

4.1
KLF2

KLF2 is a zinc-finger-containing transcription factor implicated in T cell quiescence (Kuo et al. 1997a), adipogenesis (Banerjee et al. 2003; Wu et al. 2005) and lung development (Wani et al. 1999). In the vasculature, KLF2 is endothelial-specific, and generation of KLF2-null mouse embryos resulted in embryonic lethality at approximately embryonic day (E)12.5–E14.5, the most pronounced gross phenotype being intra-embryonic haemorrhaging near the aortic outflow tract and into the abdomen at E12.5, and profuse bleeding into the amniotic cavity in embryos surviving to E13.5 (Kuo et al. 1997b). Using the endothelial marker platelet endothelial cell adhesion molecule (PECAM)-1, the authors found no evidence of abnormal vasculogenesis (capillary plexus formation) or angiogenesis (capillary sprouting) at E12.5, arguing against a role for these early vascular processes in mediating the KLF2-null phenotype. In contrast, later stages of vascular development, including vessel wall maturation of the umbilical vessels and dorsal aortae, were found to be severely defective before signs of haemorrhaging, and were characterised histologically by impaired smooth muscle recruitment to the endothelium. The resulting impairment in the tunica media was associated with aneurismal dilatation of these larger vessels. Because KLF2 was found to be restricted to the endothelium, this phenotype implies a non-autonomous role of KLF2 for signalling between the endothelium and surrounding pericytes which is yet to be defined. KLF2 thus appears to function as an endothelium-restricted critical regulator of vessel wall maturation and stability during development.

4.2
Regulation of KLF2 by Haemodynamic Forces

KLF2 was first shown to be one of a small number of genes upregulated in cultured human endothelial cells exposed to laminar shear stress for 7 days when compared to static (no flow) culture conditions (Dekker et al. 2002). Using in situ hybridisation of adult human arteries, these authors demonstrated that KLF2 expression was restricted to the endothelium within the vessel wall, and that the pattern of expression correlated with predicted types of shear stress. Specifically, endothelial expression of KLF2 was highest in regions that are predicted to be exposed to laminar shear stress, and thus resistant to atherosclerosis, and comparatively absent in atheroprone regions exposed to non-laminar shear stress (e.g. bifurcations). Based on these observations, Dekker et al. hypothesised a potential role for KLF2 in atherogenesis (Dekker et al. 2002). The response of KLF2 expression to laminar shear stress in vitro

has since been confirmed by other groups including our own (Huddleson et al. 2004; SenBanerjee et al. 2004).

Furthermore, complex modelling of the arterial waveforms characteristic of the in vivo atherosclerosis-resistant (atheroprotective flow) and atherosclerosis-prone (atheroprone flow) regions of the human carotid artery also demonstrates a selective upregulation of KLF2 by atheroprotective flow as determined by transcriptional profiling (Fig. 4; Dai et al. 2004). Dissection of the basic biomechanical parameters relevant to KLF2 induction revealed an increased effect of pulsatile flow compared to non-pulsatile laminar flow, and no KLF2 response to cyclic stretch (Dekker et al. 2005). In vivo studies in *silent heart* mutant zebrafish, which lack blood flow yet remain viable and functional for several days, demonstrate the absence of vascular endothelial KLF2a (the

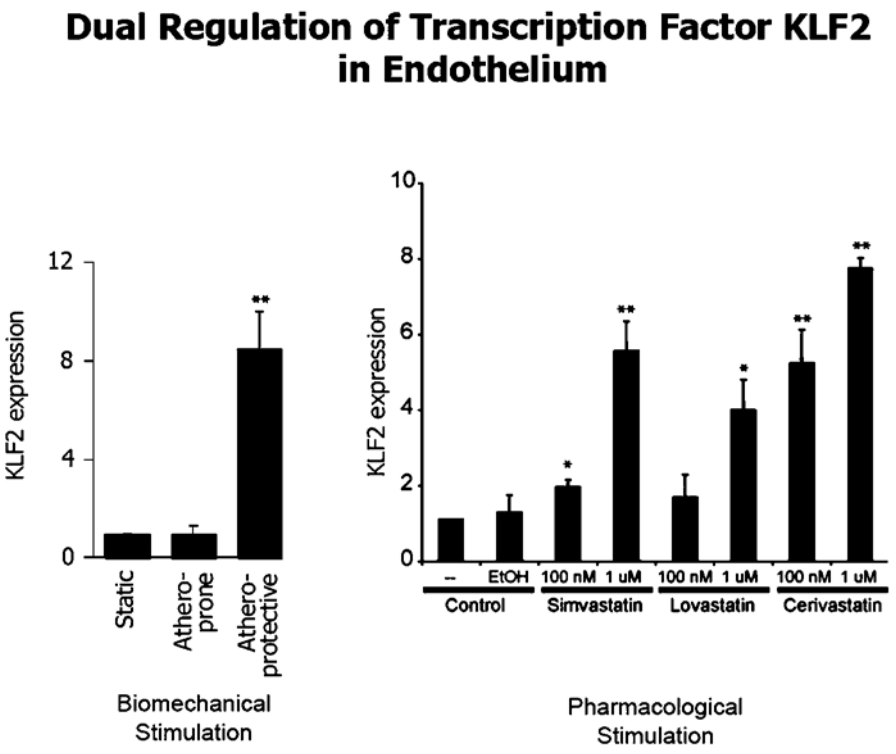

Fig. 4 Expression of the transcription factor KLF2 is differentially responsive to distinct haemodynamic environments and is regulated by statins. Human umbilical vein endothelial cells (HUVECs) were cultured under static (no flow), "atheroprone" or "atheroprotective" flow conditions for 24 h (*left panel*). HUVECs were also cultured for 24 h in normal medium, medium with ethanol vehicle, or 100-nM and 1-μM concentrations of simvastatin, lovastatin and cerivastatin (*right panel*). Kruppel-like factor-2 (*KLF2*) mRNA expression was measured by real-time Taqman PCR. All data are expressed as mean+/−SE

homologue of the mammalian KLF2) expression in the aorta and cardinal vein (Parmar et al. 2005a). These observations demonstrate that endothelial KLF2 expression is dependent on blood flow in vivo and that this mode of regulation may be evolutionarily conserved.

Further in vitro analyses performed in our laboratories have revealed that the mechanisms linking haemodynamic forces and KLF2 expression involve activation of a MEK5/ERK5/MEF pathway and that MEK5 activation is indeed necessary and sufficient for the flow-mediated upregulation of KLF2 in vascular endothelium. These results are consistent with a model in which flow activates MEK5, which in turn phosphorylates ERK5, resulting in the activation of the MEF2 family of transcription factors at the KLF2 promoter. Interestingly, we demonstrated that binding of the MEF2 members to the KLF2 promoter is found under static conditions and is not substantially altered upon the exposure of endothelial cells to flow, indicating that this transcription factor appears to bind tonically to the KLF2 promoter and act as a "switch" by receiving upstream signals (Parmar et al. 2005a).

4.3
Regulation of KLF2 Expression by Statins

Statins, which are the most commonly prescribed class of lipid-lowering agents and were originally designed as inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A, have recently been discovered also to upregulate KLF2 expression in cultured human endothelial cells at pharmacologically relevant doses in vitro (Fig. 4; Parmar et al. 2005b; Sen-Banerjee et al. 2005). Statins block the production of mevalonate, which forms two major downstream products known as isoprenoids: farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). Both of these isoprenoids can prenylate distinct sets of proteins in the cell to enable their proper localisation and signalling. The statin-mediated upregulation of KLF2 in human umbilical vein endothelial cells (HUVECs) is dependent on the depletion of GGPP (Parmar et al. 2005b; Sen-Banerjee et al. 2005), which is well known to prenylate several members of the Rho superfamily. Importantly, upregulation of KLF2 is critical for many statin-dependent transcriptional changes in endothelial cells, thus implicating KLF2 in the so call "pleiotropic" beneficial cardiovascular effects of this class of cardiovascular drugs (Liao and Laufs 2005).

4.4
Downstream Transcriptional Targets and Functions of KLF2 in Endothelium

Recent work from our laboratory utilising a "systems biology approach" involving the systematic analyses of large datasets has unveiled KLF2 as a critical integrator of the global transcriptional responses of human endothelial cells to atheroprotective shear stress stimulation. KLF2 overexpression experiments

revealed the global programmes downstream of this transcription factor, including blood vessel development, inflammation, thrombosis and vascular tone (Parmar et al. 2005a). Thus, for example, KLF2 was able to repress the induction of 32 cytokines/chemokines in response to pro-inflammatory stimuli, in addition to promoting a robust anti-inflammatory programme involving the production of 15-deoxy-δ (12, 14) prostaglandin J_2, as well as the protective cytokines elafin and interleukin (IL)-11. KLF2 also regulates a co-ordinated anti-thrombotic phenotype by upregulating thrombomodulin and downregulating tissue factor. Importantly, aside from endothelial NO synthase and thrombomodulin, none of the direct transcriptional targets of KLF2 in endothelium is known. We have also demonstrated that KLF2 is responsible for the flow-mediated anti-adhesive endothelial phenotype in context of the pro-inflammatory stimulus IL-1β. Endothelial-leucocyte adhesion is thought to be a critical step at the earliest stages of inflammation, and inhibition of this process by KLF2 may be an important mechanism underlying atheroprotection at sites exposed to atheroprotective flow. Another important cellular protective mechanism of flow on endothelial cells is a conferred resistance to oxidative stress. KLF2 upregulation under flow also appears to be critical for this protective phenotype in vitro (Parmar et al. 2005a).

5 Conclusions

Endothelial cells are uniquely responsive to particular patterns of haemodynamic forces, especially shear stress. Under normal, physiological conditions, these forces serve as important signals to preserve the antithrombotic, anti-inflammatory, anti-oxidative stress and anti-apoptotic flow-responsive phenotype of the vascular endothelium (Fig. 5). Critical testing of the "atheroprotective gene hypothesis" will depend upon refinement of in vitro fluid mechanical models, genetic loss and gain-of-function experimental strategies in animal models, and the validation of expression patterns of candidate atheroprotective genes in the setting of human vascular pathobiology. The recent development of reliable methods for endothelial cell isolation from different regions of the pig aorta and the subsequent amplification of transcripts from a small number of cells and their analysis by complementary DNA (cDNA) microarrays hold much promise for the identification of atheroprotective genes from non-cultured endothelium (Passerini et al. 2004). Moreover, as we define in greater detail the components and mechanisms of mechano-homeostasis in vascular endothelium, targeting therapies to these mechanisms may prove to be an exciting and novel strategy for the treatment of diseases in which endothelial dysfunction plays a pathogenic role.

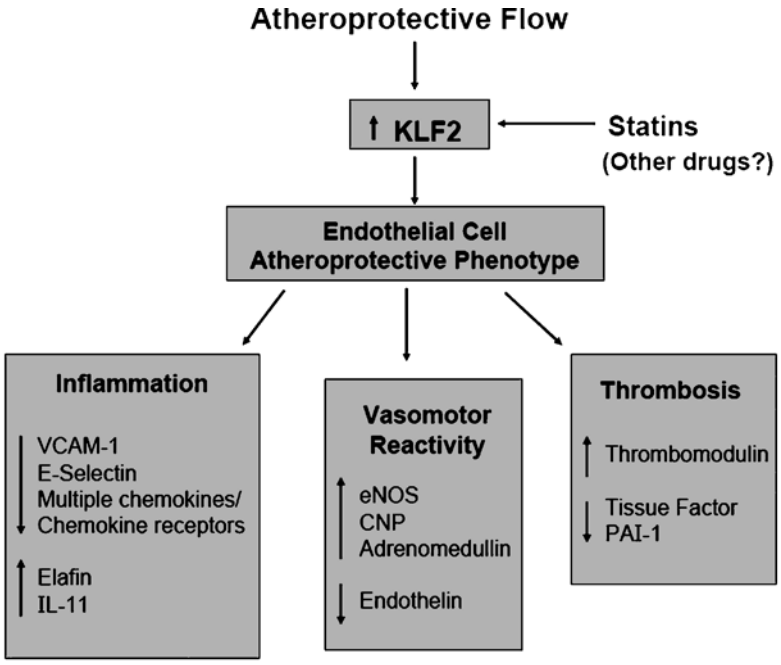

Fig. 5 KLF2 is a key transcriptional integrator of the endothelial atheroprotective phenotype. The exposure of endothelial cells to atheroprotective flow leads to the upregulation of KLF2 expression. A similar upregulation of KLF2 is observed when endothelial cells are cultured in the presence of statins (see Fig. 4). The activation of this transcription factor (and others yet to be defined) evokes an "atheroprotective" endothelial cell phenotype. The identification of KLF2-dependent transcriptional programmes involved in the regulation of multiple endothelial functions (including inflammation, vascular tone and thrombosis) strongly suggests a critical role for this biomechanically regulated transcription factor in endothelial atheroprotection. *CNP*, C-type natriuretic peptide

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Leucocyte-Endothelial Interactions in Health and Disease

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Abstract The emigration of leucocytes into the tissue as a crucial step in the response to inflammatory signals has been acknowledged for more than 100 years. The endothelium does not only represent a mechanical barrier between blood and tissue, the circulatory system also connects different organ systems with each other, thus allowing the communication between remote systems. Leucocytes can function as messengers and messages at the same time. Failure or dysregulation of leucocyte-endothelial communication can severely affect the integrity of the organism. The interaction between leucocytes and the vascular endothelium has been recognised as an attractive target for the therapy of numerous disorders and diseases, including excessive inflammatory responses and autoimmune diseases, both associated with enormous consequences for patients and the health care system. There is promising evidence that the success rate of such treatments will increase as the understanding of the molecular mechanisms keeps improving. This chapter reviews the current knowledge about leucocyte-endothelial interaction. It will also display examples of both physiological and dysregulated leucocyte-endothelial interactions and identify potential therapeutical approaches.

Keywords Adhesion molecules · Transmigration · Leucocyte trafficking · Inflammation

1 Introduction

The microcirculatory system is essential for the maintenance of homoeostasis in a healthy organism, with its persistent exposition to both exogenous and endogenous threats. However, it is also a major player in innumerable diseases with severe consequences for affected patients. Recently it has become evident that, under certain circumstances, the microcirculation itself must be considered a target organ and may require both monitoring and therapeutical intervention (Ince 2004).

For many years the interaction between circulating leucocytes and the vascular endothelium has been acknowledged as one of the key events within the microvascular compartment involved in both protecting and damaging tissues. Leucocyte-endothelial interactions mediate immune surveillance, acute and chronic inflammation and wound repair, as well as thrombosis and its resolution. An impaired communication between leucocytes and endothelium can cause a severe immunodeficiency, as displayed in patients with leucocyte adhesion deficiency (LAD) syndromes (Bunting et al. 2002). However, excessive adhesive interactions lead to excessive accumulation of leucocytes in the tissue. Atherosclerosis, reperfusion injury, inflammatory bowel disease and acute lung injury are only a few characteristic examples.

It is not surprising that various challenges at different sites of the organism elicit distinct mechanisms for the communication between blood leucocytes and endothelial cells (Andonegui et al. 2002; Bowden et al. 2002; Olson et al. 2002). However, there are general sequence-dependent mechanisms which are shared by most of them. As early as in the nineteenth century, a detailed description of the leucocyte adhesion cascade was provided (Cohnheim 1889). Molecular changes and structural rearrangements in blood leucocytes (DiVietro et al. 2001; Simon et al. 2000b) and endothelial cells (Burns et al. 2000; Shaw et al. 2001) are necessary to initiate adhesive contact. A variety of participating molecules such as adhesion molecules, chemoattractants, and their ligands and receptors are employed to orchestrate the multistep cascade of cell capture, rolling, firm adhesion and finally, transmigration into the tissue (Butcher 1991; Ley 1989).

2 Structural Requirements for the Leucocyte-Endothelium Interaction

2.1 Multiple Steps: The Adhesion Cascade

Under resting conditions, blood leucocytes and vascular endothelial cells are not adhesive. Leucocyte adhesion requires an initial binding event which is mostly dependent on the expression of the selectin family of adhesion molecules.

This first step of the adhesion cascade is termed capture or tethering (Ley et al. 1995). In this phase, the rapid formation of bonds results in leucocyte rolling if new bonds are formed before the initially formed bonds disrupt. Rolling is mostly observed in postcapillary venules, although it also occurs in capillaries of certain areas such as in the lung (Downey et al. 1993; Walker et al. 1995), arterioles (Kunkel et al. 1997) and arteries (Eriksson et al. 2001). Rolling leads to a close contact between leucocytes and endothelial cells. It is a prerequisite for the following steps of firm adhesion and arrest which are mainly dependent on members of the integrin superfamily of adhesion receptors on leucocytes. Blocking rolling by antibodies or by gene deletion attenuates leucocyte adhesion and transmigration significantly (Forlow et al. 2000). Slowly rolling leucocytes become activated by mediators of the inflamed endothelium, particularly by chemokines presented on the endothelium, launching a complex machinery of intracellular signalling pathways.

Leucocytes undergo structural rearrangements, allowing for a directed migration to the site of inflammation. The process of transmigration is also under the control of adhesion molecules, although this step is far from being well understood. However, there is good evidence that there are specialised adhesion molecules "guiding" the leucocytes through their transendothelial path. Platelet-endothelial cell adhesion molecule (PECAM)-1, vascular cell adhesion molecule (VCAM)-1, vascular endothelial (VE)-cadherin, CD99 and junctional adhesion molecules (JAM) are highly expressed in areas of interendothelial junctions, the preferred site of leucocyte emigration (Burns et al. 1997) and may assist the migration process (Martin-Padura et al. 1998; Su et al. 2002). β_1 integrins may play a role not only for the migration through the endothelium but also for the penetration through the subendothelial basal membrane and for the subsequent transit of leucocytes through the extravascular environment (Shang et al. 1999; Shang and Issekutz 1997; Werr et al. 1998).

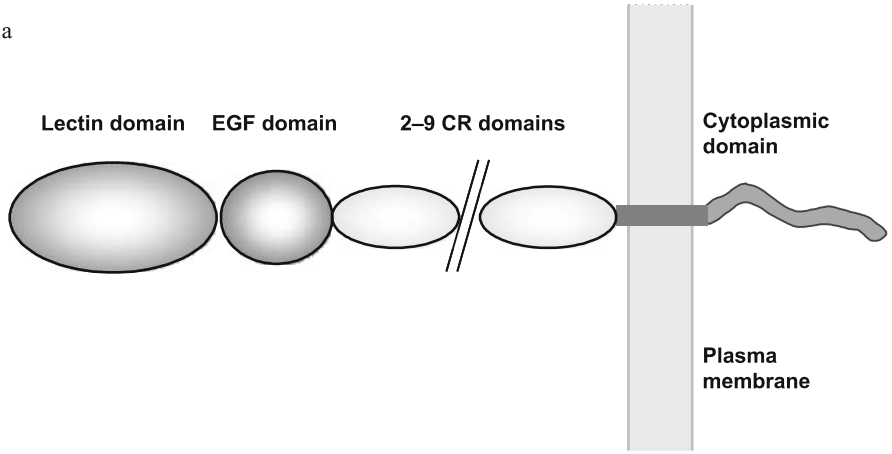
The complexity of the adhesion cascade reflects the need to exactly control leucocyte-endothelial interactions. The system is robust but the disruption of two or more participants can cause severe consequences for vital functions of the organism (Bullard et al. 1996; Forlow et al. 2000). Selectins and integrins are the most studied adhesion molecules. Their unique structure and their abundant expression at most sites of the microcirculation make them ideal candidates to regulate the communication between blood cells and endothelium.

2.1.1 Selectins and Their Ligands

Selectins are transmembrane type I glycoproteins. Three selectins have been described: E-, L- and P-selectin, originally termed after the cell where they were detected first (endothelium-leucocytes-platelets) (Kansas 1996). Selectins function as rolling receptors for leucocytes ("brakes") but they also have sig-

nalling functions. Selectins share a significant (>50%) structural homology (Fig. 1). The extracellular part of selectins consists of a sugar-binding lectin domain on the N-terminus, followed by a domain with homologies to epidermal growth factor (EGF) and a sequence of so-called short consensus repeats (SCR) which share homology with complement-binding receptors. In contrast to the extracellular domain, the short cytoplasmic and the transmembrane domains do not share any homology. However, they are highly conserved in the same selectin of different species, reflecting a characteristic function of each single selectin. In fact, while the lectin domain is indispensable for the binding to glycan ligands, the cytoplasmic region is essential to target different intracellular structures and the plasma membrane. The cytoplasmic tail of L-selectin is connected to cytoskeletal structures, such as α-actinin, stabilising cell adhesion under shear stress and enhancing adhesive properties (Dwir et al. 2001). P-selectin requires the cytoplasmic tail to be directed to the intracellular

a



b **Approximate amino acid identity**

Among same selectin between species	**72%**	**60% (P > 90%)**	**40%**	**80% (L > 95%)**	**>75%**
Among different selectins in same species	**52%**	**47%**	**35%**	**None**	**None**

Fig. 1 a,b Structure of selectins. **a** Selectins are composed of an N-terminal lectin domain, an epidermal growth factor (*EGF*) domain, two (L-selectin), six (E-selectin) or nine (P-selectin) consensus repeats with homology to complement regulatory (*CR*) proteins, a transmembrane domain, and a cytoplasmic domain. **b** This panel shows amino acid sequence identity within each domain, among different species (human, mouse and cow) (*top row*) and among different selectins in the same species (*bottom row*). (Modified from Kansas 1996)

granules (Modderman et al. 1998). In contrast, the SCR region seems to play only a minor role in cell adhesion (Mehta et al. 1997).

2.1.1.1 L-Selectin

L-selectin is constitutively expressed on most blood leucocytes, including neutrophils, most monocytes and lymphocytes as well as on haematopoietic cells in the bone marrow. Upon stimulation, L-selectin is shed from the cell surface and appears in a soluble form in the blood. The physiological role of L-selectin shedding is unclear, but preventing excessive adhesion and cell migration might be the most likely function. L-selectin shedding leads to increased neutrophil but not lymphocyte rolling velocity (Galkina et al. 2003; Walcheck et al. 1996). In neutrophils, L-selectin shedding reduces the activation of lymphocyte function-associated antigen (LFA-1) and Mac-1 and their binding to intercellular adhesion molecule (ICAM)-1 (Hafezi-Moghadam et al. 2001). It has been suggested that soluble L-selectin might serve as an early marker of various inflammatory conditions, such as bacterial infection or diabetes (Kretowski et al. 2000; Wilson et al. 2001). However, its clinical relevance is unclear.

L-selectin is crucial for leucocyte capturing and rolling, particularly, but not exclusively, on high endothelial venules (HEVs) of lymph nodes and Peyer's patches. Mice deficient in L-selectin or one of its ligands exhibit severe defects in lymphocyte rolling in lymphoid venules (van Zante et al. 2003) and Peyer's patches (Arbones et al. 1994; Kunkel et al. 1998). L-selectin may also be involved in the "homing" of bone marrow-derived progenitor endothelial cells to the site of tissue damage, and might therefore be important for the neovascularisation of ischaemic or otherwise injured areas (Biancone et al. 2004). L-selectin is an important mediator of leucocyte migration during inflammation. In different animal models, mice lacking L-selectin show impaired neutrophil recruitment to sites of inflammation (Lewinsohn et al. 1987; Olson et al. 2002; Tedder et al. 1995).

2.1.1.2 E-Selectin

The constitutive expression of E-selectin on vascular endothelium is negligible except in the skin. However, expression is upregulated upon stimulation with cytokines such as tumour necrosis factor (TNF)-α or interleukin (IL)-1 as well as in patients with chronic inflammatory diseases such as chronic skin infections or rheumatoid arthritis. E-selectin mediates leucocyte adhesion and rolling to sites of inflammation. E-selectin is re-internalised, but a low concentration of the soluble form can be found in the plasma of patients after undergoing cardiopulmonary bypass surgery (Eikemo et al. 2004); its clinical relevance is unknown. Mice lacking E-selectin show only a moderate

phenotype. Thus, it has been suggested that E-selectin functions overlap with other adhesion molecules and indeed, mice lacking both E- and P-selectin exhibit severly reduced leucocyte rolling (Bullard et al. 1996). In addition, E-selectin-deficient mice reconstituted with CD18-deficient bone marrow show a severe defect in leucocyte adhesion (Forlow et al. 2000). In TNF-α-treated venules, neutrophil arrest is mediated by both E-selectin and CXC chemokines which have overlapping functions (Smith et al. 2004).

2.1.1.3 P-Selectin

In contrast to L- and E-selectin, P-selectin is stored in secretory α-granules in platelets and in Weibel-Palade bodies in endothelial cells (McEver et al. 1989), allowing P-selectin to be rapidly expressed on the cell surface. Endothelial secretagogues like histamine, thrombin and complement C5a induce rapid expression of P-selectin, which then leads to leucocyte rolling and migration to inflammatory sites (Mayadas et al. 1993). Trauma-induced leucocyte rolling, as seen after surgical preparation of the mouse cremaster muscle for intravital microscopy, is almost exclusively dependent on P-selectin (Ley et al. 1995). In addition, both leucocyte rolling and adhesion is largely P-selectin dependent in a model of autoimmune encephalomyelitis (Kerfoot and Kubes 2002). Elevated concentrations of soluble P-selectin can be found in the plasma of patients with unstable angina (Parker et al. 2001) and many other cardiovascular diseases. Soluble P-selectin levels predict heart attacks and other adverse cardiovascular events, similar to C-reactive protein.

2.1.1.4 Selectin Ligands

Different selectin ligands have been described, the most important being P-selectin glycoprotein ligand 1 (PSGL-1). During inflammation, PSGL-1 mediates adhesion of leucocytes to already adherent cells (secondary capturing) (Sperandio et al. 2003). Recombinant PSGL-1 is able to block leucocyte rolling on all three selectins (Hicks et al. 2003). Interestingly, in this study selectin-induced inflammation could be reduced by recombinant (r)PSGL-1 at a much lower dose than that was needed to block rolling, suggesting alternative selectin functions. Consistent with these findings, adhesion to P-selectin can induce activation of β_2 integrins in neutrophils (Ma et al. 2004) and leads to activation of both neutrophils and monocytes. In addition, PSGL-1 engagement by P-selectin is able to induce activation of the mitogen-activated protein (MAP)-kinases extracellular signal-regulated kinase (ERK)-1 and ERK-2, small GTPases, and secretion of IL-8 (Hidari et al. 1997).

Ligands for L-selectin consist of a group of *O*-glycosylated glycoproteins,

which include GlyCAM-1 and CD34 in the mouse and podocalyxin and CD34 in the human (Rosen 2004). Binding of these ligands by L-selectin requires sialylation, fucosylation and sulphation of their mucin-like domains. The monoclonal antibody MECA-79 recognises this set of L-selectin ligands on HEVs which are referred to as peripheral lymph node addressin (PNAds). MECA-79 is also able to block lymphocyte binding to peripheral lymph node HEVs in vitro and inhibits lymphocyte homing to lymph nodes in vivo (Streeter et al. 1988).

Patients suffering from leucocyte adhesion deficiency type II (LAD II) fail to synthesise effective selectin ligands due to defective cellular fucose transport (Luhn et al. 2001a). These patients have recurring bacterial infections of the mucosal membranes and the skin. Oral substitution of fucose has been effective in some, but not all, patients (Wild et al. 2002).

2.1.2 Integrins and Their Ligands

Integrins are transmembrane molecules containing two non-covalently associated subunits, α and β. Integrins were originally described as pure adhesion receptors mediating firm leucocyte adhesion. However, recent work has revealed many functions beyond adhesion, including linkage between the extracellular matrix and the cytoskeleton and activation of many intracellular signalling pathways (Takagi and Springer 2002). All known integrins share a common heterodimeric structure, containing an α- and a β-subunit. There have been 19 different integrin α-subunits and 8 different β-subunits described so far, forming at least 25 αβ heterodimers, each of them with distinct functions depending on the cell type they are expressed on, the ligand they bind to, and the signalling pathway they activate (Hynes 1992). The regulatory mechanisms dictating the expression of specific integrin patterns on different leucocytes are not well understood.

2.1.2.1 β_2 Integrins

β_2 integrin expression is restricted to leucocytes, and each subtype of leucocyte expresses one or more members of the β_2 integrin family. Four different heterodimers are formed: LFA-1 ($\alpha_L\beta_2$; CD11aCD18), Mac-1 ($\alpha_M\beta_2$; CD11bCD18), p150.95 ($\alpha_X\beta_2$; CD11cCD18) and $\alpha_D\beta_2$ (CD11dCD18). The extracellular portion of the α-chain includes seven N-terminal homologous repeats organised into a β-propeller structure. The I domain, essential for ligand binding, resides within the third repeat. The membrane-proximal repeats contain a calcium-binding site which might be important for the orientation of the β-propeller as well as for the association with the β-chain (Springer 1997). The cytoplasmic tail of the α-chain contains a GFFKR motif which is involved in the α/β association and in ligand recognition as shown by GFFKR deletion experiments

(Lu and Springer 1997). The extracellular part of the β-chain contains an I-like domain, which corresponds with the I domain of the α-chain, both together representing the integrins' binding site for their ligands. Like the α-chain, the cytoplasmatic tail of the β-subunit is highly conserved and crucial for the linkage with the cytoskeleton.

β_2 integrins are involved in leucocyte adhesion and transmigration, as shown in various experimental models using blocking strategies. LFA-1 was originally described as a major factor in lymphocyte trafficking ("lymphocyte function-associated antigen"). In fact, LFA-1 is predominantly used for lymphocyte emigration (Li et al. 1996). However, in some but not all inflammatory models, neutrophil migration is also LFA-1-dependent (Ding et al. 1999). LFA-1 can also mediate leucocyte rolling as shown in LFA-1 knockout mice (Dunne et al. 2001; Dunne et al. 2002; Henderson et al. 2001). In addition, LFA-1 has recently been shown to actively participate in neutrophil migration through the endothelium by forming ring-like clusters at the neutrophil-endothelial junction (Shaw et al. 2004). As neutrophils migrate through the endothelial layer, these clusters crawl from the leading edge to the neutrophil uropod, always maintaining contact with their major ligand ICAM-1.

Mac-1 is expressed on neutrophils and monocytes. It is stored in secretory, secondary and tertiary granules, ready to be immediately translocated to the plasma membrane upon cell activation (Borregaard et al. 1994). In addition to ICAM-1, Mac-1 has several other ligands, including complement factor C3bi, coagulation factor Xa, fibrinogen, denatured protein and different bacterial proteins. In various models, Mac-1 has been shown to participate in the migration of leucocytes to sites of inflammation. Neutrophil migration across the intestinal epithelium is Mac-1-dependent (Zen et al. 2002). In contrast, Mac-1-deficient mice did not show a defect in neutrophil migration into the intraperitoneal cavity in a thioglycollate-induced peritonitis model (Lu et al. 1997). Besides leucocyte-endothelial interaction, Mac-1 also mediates leucocyte migration through platelet monolayers on a vascular thrombus (Diacovo et al. 1996) by binding to the platelet counter receptor glycoprotein Ibα. Thus, Mac-1 might be an important molecule in perpetuating (and complicating) atherosclerosis (see Sect. 5.1).

The role of the other two β_2 integrins, p150.95 and $\alpha_D\beta_2$, is not completely defined. p150.95 is expressed on the surface of natural killer (NK) and dendritic cells as well as on lymphocyte subsets. $\alpha_D\beta_2$ is expressed on myeloid cells, macrophages and eosinophils. It binds to ICAM-3 and VCAM-1. Recent work suggested that $\alpha_D\beta_2$ is involved in the recruitment of monocytes and neutrophils into lesions of spinal cord injury (Saville et al. 2004), but this remains to be confirmed.

Humans with either absence or functional defects in β_2 integrins (leucocyte adhesion deficiency type I; LAD I) exhibit recurrent bacterial infections that are often life-threatening. These patients are unable to recruit neutrophils or monocytes to sites of inflammation (Anderson and Springer 1987). Since its

original description, several variants of LAD I have been reported. In addition to the absence, they include functional mutations of the β_2 chain.

2.1.2.2 α_4 Integrins

The α_4 chain is a type I transmembrane glycoprotein. It has a cytoplasmic GFFKR motif but no extracellular I domain; α_4 associates with β_1 or β_7 subunits to form $\alpha_4\beta_1$ (CD49d/CD29; VLA-4) or $\alpha_4\beta_7$ integrin. VLA-4 is expressed on haematopoietic stem cells, monocytes, eosinophils, NK cells, lymphocytes, and, at low levels, on neutrophils. It binds to VCAM-1 and fibronectin. VLA-4 is associated with haematopoietic tissues such as bone marrow and embryonic liver (Jaspers et al. 1995). VLA-4 is also crucial for the embryogenic development of the vasculature and the heart, as shown in VLA-4-deficient mice (Sengbusch et al. 2002). Further, VLA-4 mediates leucocyte recruitment to atherosclerotic sites and neointimal growth (Barringhaus et al. 2004). Hence, it might be an attractive target to avoid both early and late complications of atherosclerosis. VLA-4 has also been demonstrated to mediate neutrophil rolling in the absence of LFA-1, indicating overlapping functions of both (Henderson et al. 2001). In a model of chronic vasculitis, adhesion of neutrophils to the inflamed endothelium has been shown to be VLA-4-dependent. Interestingly, the adhesion was not blocked by antibodies against VCAM-1, suggesting evidence for an alternative endothelial ligand (Johnston et al. 2000).

$\alpha_4\beta_7$ is expressed on haematopoietic progenitor cells and lymphocytes. It binds to MAdCAM-1 (mucosal addressin-cell adhesion molecule 1), which is found on high endothelial venules of Peyer's patches, mesenteric lymph nodes and endothelial cells of (inflamed) lamina propria venules, suggesting a critical role in mucosal immunity. In fact, $\alpha_4\beta_7$ has been shown to participate in T cell homing into gut-associated lymphoid tissues (Petrovic et al. 2004; Wagner et al. 1996). Concordantly, acute graft-versus-host disease after bone marrow transplantation, characterised by the infiltration of T cells into the gut, can be effectively attenuated by blocking $\alpha_4\beta_7$ (Snider and Liang 2001).

2.1.2.3 Integrin Ligands

ICAM-1 (CD54) is anchored to the endothelium by a transmembrane domain and a short cytoplasmic tail, and it contains five extracellular immunoglobulin domains. ICAM-1 is constitutively expressed on resting endothelial cells. Upon stimulation, e.g. by cytokines or chemokines, ICAM-1 is highly upregulated (Dustin et al. 1986) and mediates both rolling and firm adhesion of leucocytes by binding to its major ligands Mac-1 and LFA-1. ICAM-1 can be shed from the surface and found soluble in the plasma where it may have a prognostic value for cardiovascular diseases (see Sect. 5.1). Elevated levels of ICAM-1 have

been found in children with sepsis (Whalen et al. 2000). However, anti-ICAM-1 strategies have yielded mixed results (Welty-Wolf et al. 2001; Xu et al. 1994).

VCAM-1 (CD106) is an endothelial ligand for α_4 integrins. Human VCAM-1 contains six or seven immunoglobulin domains. Under resting conditions, VCAM-1 expression is very low or absent. IL-1, TNF or bacterial lipopolysaccharide (LPS) rapidly induces endothelial expression in a NF-κB-dependent way (Carlos et al. 1990). VCAM-1 expression can be found in various inflammatory disorders, including atherosclerosis (Nakashima et al. 1998) or acute lung injury (Muller et al. 2002). In addition, VCAM-1 is involved in the homing of haematopoietic progenitor cells to the bone marrow (Mazo et al. 1998; Papayannopoulou and Craddock 1997). In mice, VCAM-1 deficiency is lethal, most likely because of a defective placentation and an impaired fusion of the allantois to the chorion, resulting in abnormal umbilical cords, coronary vessels and epicardium (Gurtner et al. 1995; Kwee et al. 1995).

Like ICAM-1 and VCAM-1, PECAM-1 belongs to the family of immunoglobulins. In addition to its expression on endothelial cells, it can be found on platelets and on most leucocytes. Endothelial PECAM-1 is preferentially located within the interendothelial junctions between endothelial cells. In some models, PECAM-1 is involved in transendothelial migration of leucocytes. Antibody blocking strategies revealed migration defects in various inflammatory models in vitro and in vivo. PECAM-1-deficient mice show migration defects when backcrossed into FVB/n, SJL and the outbred strain Swiss Webster (Schenkel et al. 2004), but not C57BL/6 mice (Duncan et al. 1999). Interestingly, leucocytes in mice deficient for PECAM-1 accumulate between the endothelial layer and the basal membrane, indicating that PECAM-1 interaction capacitates leucocytes for migration through the subendothelial matrix (Duncan et al. 1999; Wakelin et al. 1996).

2.1.2.4 Inside-Out Integrin Signalling

Integrins need activation in order to bind to their ligands. This feature appears to assure specificity of leucocyte binding at sites of inflammation. Different intracellular signals can lead to a conformational change in the integrin structure (inside-out signalling). Subsequently, integrins open their binding sites, and ligands are able to bind. This conformational shape change was originally described for $\alpha_V\beta_3$ but might apply for other integrins too (Fig. 2). The intracellular signals leading to activation of integrins vary among the different integrins and cell types. They include lipid, cytokine and chemokine signalling molecules (Bouaouina et al. 2004; Feldhaus et al. 2002; Grabovsky et al. 2000). Lack of these activation mechanisms can cause impaired leucocyte adhesion and transmigration, even in the presence of integrin expression (Morgan et al. 1997). Further, signalling from other adhesion molecules such as selectins, induced by either ligand binding or cross-linking, are involved in integrin

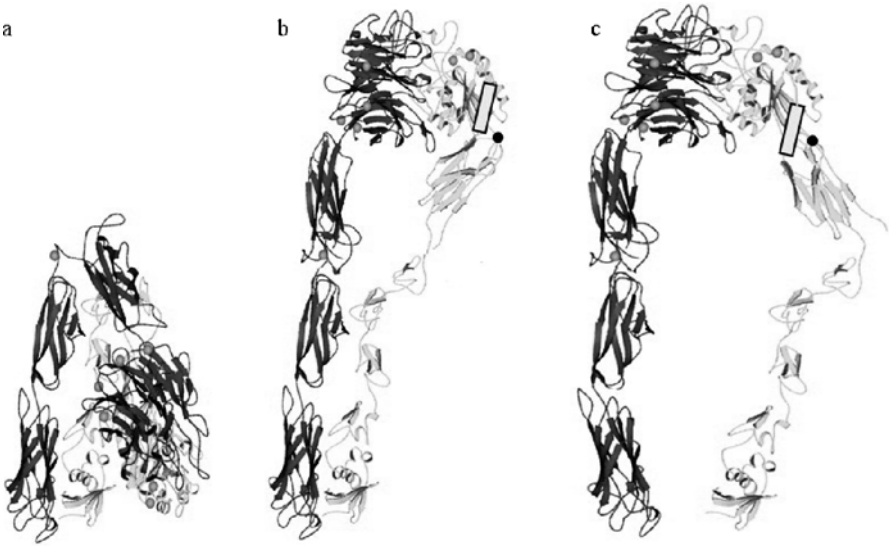


Fig. 2 a–c Model of integrin activation with three conformations of the β_3 extracellular domain: **a** bent conformation with low affinity; **b** extended conformation with closed headpiece; **c** extended conformation with open headpiece. The *yellow cylinder* indicates the I-like domain α_7-helix which is moved downward during conformational change. (Modified from Luo et al. 2004)

activation (Simon et al. 1999). The mechanisms by which integrin inside-out activation is regulated are not completely understood. Small GTPases such as Rho might be involved in chemokine-induced β_2 signalling (Giagulli et al. 2004) but additional cell- and integrin-specific pathways might be employed.

2.1.2.5 Outside-In Integrin Signalling

Engagement of integrins with specific ligands or cross-linking of integrins can cause activation of intracellular signalling pathways, leading to different cellular responses (outside-in signalling). For instance, ligation of CD11b or CD11c, but not CD11a, with antibodies has been shown to induce transcriptional upregulation of chemotactic chemokines IL-8, macrophage inflammatory protein (MIP)-1α and MIP-1β (Rezzonico et al. 2001). Thus, outside-in signalling seems to be important for amplifying leucocyte activation upon adhesion. Integrin outside-in signalling can promote leucocyte cell differentiation (Shi et al. 2004), actin polymerisation (Feldhaus et al. 1998), release of superoxide (Walzog et al. 1994), eosinophil degranulation (Kato et al. 1998) and T cell activation (Doucey et al. 2003).

3
Mechanisms of Transendothelial Migration

Migration of leucocytes through the endothelial layer requires their detachment from the apical endothelial surface. Thus, modulation of selectin and integrin bonds is necessary. Leucocytes must break bonds with the apical surface of the endothelium and form new bonds within the interendothelial junctions, the preferential site of emigration. In fact, the activation of neutrophils has been shown to attenuate their binding to P-selectin (Lorant et al. 1995). Upon activation, PSGL-1 on leucocytes moves to the uropod, facilitating migration by disruption of selectin bonds at the leading edge (Bruehl et al. 1997). Accordingly, integrin-mediated leucocyte binding can be modulated by a mechanism that involves RhoA kinase-dependent alterations of the cytoskeleton, as shown for neutrophils and T lymphoblasts (Liu et al. 2002). At the same time, membrane protrusions and new adhesive contacts are formed at the leading edge of a migrating cell. β_1 integrin engagement activates GTPases Cdc42 and Rac1, thus promoting directional movement (Price et al. 1998).

Once the emigration of leucocytes at the apical surface of interendothelial junctions has been initiated, substantial rearrangements in the endothelial cells are necessary to allow leucocytes to traverse. As mentioned, PECAM-1 is located at endothelial junctions and is involved in the transmigration of neutrophils, monocytes and NK cells. CD99 is an *O*-glycosylated transmembrane protein expressed on both endothelial cells and haematopoietic cells. Like PECAM-1, it is concentrated at endothelial junctions. It mediates monocyte migration by homophilic interaction between CD99 on monocytes and on endothelial cells (Schenkel et al. 2002). Interestingly, CD99 was found to function distal of PECAM-1, suggesting a sequential role in migration.

Other adhesion molecules are found at the borders between confluent endothelial cells. Junctional adhesion molecules (JAM) belong to the immunoglobulins, and they are co-expressed with tight junctions, suggesting a role as a "gate keeper". In fact, JAM-3 (or JAM-C) has recently been shown to mediate neutrophil migration in a model of thioglycollate-induced peritonitis (Chavakis et al. 2004). In addition, JAM-3 was found at epithelial intercellular junctions of the intestine where it mediated Mac-1-dependent neutrophil migration (Zen et al. 2004). Distinct functions in leucocyte migration have also been reported for JAM-1 (T cells and neutrophils) and JAM-2 (lymphocytes) (Johnson-Leger et al. 2002; Ostermann et al. 2002).

In addition to their route between endothelial cells (paracellular migration), leucocytes can migrate through individual endothelial cells (transcellular migration). ICAM-1 and VCAM-1 are highly expressed at these sites of extravasation, surrounding transmigrating leucocytes and guiding them through the endothelial layer ("transmigratory cup") (Carman and Springer 2004).

4
Leucocyte-Endothelial Interaction in Health

The process of cell adhesion and migration is essential in response to inflammatory challenges. Nonetheless, leucocyte-endothelial interactions occur without inflammation. The development of the immune system and the maintenance of a balanced haematopoietic system are pivotal functions of a healthy organism. Regulation of both would not be possible without cell adhesion and migration. Failure of leucocytes to appropriately adhere and migrate can lead to severe consequences, as impressively displayed in both humans and mice with structural or functional deficiencies in the adhesion process.

4.1
Constitutive T Cell Trafficking

In order to develop immune responses, naïve T cells must have direct contact with pathogen-derived antigens. T cells migrate to secondary lymphoid organs such as lymph nodes, spleen, or mucosa-associated lymphoid tissue where antigen-presenting cells such as dendritic cells concentrate to present these antigens. T cell migration to lymphoid organs ("homing") occurs continuously, even under resting conditions, and therefore requires constitutive expression of adhesion molecules on the vascular endothelium of secondary lymphoid tissue. In contrast, lymphocyte migration into peripheral tissue is only observed in response to an inflammatory stimulus.

Circulating T cells enter lymph nodes and Peyer's patches through specialised HEVs (Mebius et al. 1996). HEVs in lymph nodes express peripheral-node addressin (PNAd), while HEVs in Peyer's patches express MAdCAM-1. Lymphocytes bind to PNAd through L-selectin, which mediates rolling. Accordingly, the absence of L-selectin in mice is associated with a severe defect in homing to peripheral lymph nodes, where L-selectin is the major adhesion molecule (Arbones et al. 1994). Further adhesion molecules are involved in lymphocyte homing. LFA-1 on lymphocytes binds to ICAM-1 and ICAM-2 on peripheral and mesenteric lymph nodes, both of which have shown overlapping functions (Lehmann et al. 2003).

In Peyer's patches, in mesenteric lymph nodes and on endothelial cells of lamina propria venules, engagement of $\alpha_4\beta_7$ integrin is required to achieve firm adhesion of lymphocytes. $\alpha_4\beta_7$ binds to MadCAM-1 and hence, this bond is critical for the immune response against pathogens entering the mucosal membranes of the gut. Mice lacking the β_7 chain exhibit severe impairment in the formation of the gut-associated lymphoid tissue, most likely due to a decreased lymphocyte migration into the tissue (Wagner et al. 1996).

After encountering the lymphoid tissue, T cells get activated and subsequently antigen differentiate into antigen-specific effector cell clones, which are then released into the circulation. In order to reach the pathogenic or-

ganism, effector cells must leave the circulation at the site of the antigenic or microbial threat (Butcher and Picker 1996). In addition, these cells must be prevented from recirculation and homing back to the peripheral lymph nodes. T cells downregulate homing receptors such as L-selectin and upregulate ligands for endothelial adhesion molecules which are expressed at the site of inflammation. For instance, functional PSGL-1, a major ligand for endothelial P- and E-selectin, is highly expressed on T helper-1 (Th1) cells and crucial for the migration into skin (Austrup et al. 1997) and peritoneum (Xie et al. 1999). α_E integrin has recently been shown to be correlated with the emigration of lymphocytes to sites of inflammation (Huehn et al. 2004). α_E-positive cells exhibited a high expression of E/P-selectin-binding ligands, as well as LFA-1, a major ligand of ICAM-1 and important for lymphocyte trapping in the lung (Lehmann et al. 2003).

4.2 Haematopoietic Homeostasis

The number of circulating leucocytes is tightly regulated, and a balance between leucocyte production, distribution and elimination is achieved. At least four different compartments are involved in this system: (1) the bone marrow as the site of leucocyte production and storage, (2) the circulating pool, (3) the marginated pool and (4) the tissue pool. Evidence that cell adhesion plays a major role in maintaining the steady state in leucocyte count originated from the observation of transgenic mice with deficiencies in one or more adhesion molecules. Mice lacking ICAM-1, CD18 integrins, selectins or combinations of them show mild to severe neutrophilia (Forlow et al. 2001). Similar to LAD-I, patients suffering from LAD II exhibit persistent leukocytosis which can be reduced to normal levels by oral administration of fucose in some cases (Luhn et al. 2001b).

The mechanisms accounting for elevated leucocyte counts in subjects deficient in certain adhesive functions are not completely defined. However, neutrophils have a short life-time in the vascular compartment (~7 h) and migrate constitutively into peripheral tissues, including the skin, gut and mucous membranes (Bicknell et al. 1994). This migration appears in a random pattern and is mainly mediated by β_2 integrins and their endothelial ligands. Increased concentrations of granulopoiesis-mediating factors such as granulocyte colony-stimulating factor (G-CSF) and IL-17 have been demonstrated in adhesion molecule-deficient mice. Interestingly, reconstitution of CD18-deficient mice ($CD18^{-/-}$) with only 10% of $CD18^{+/+}$ bone marrow was able to maintain neutrophil homeostasis, suggesting that transmigrated neutrophils might directly influence (i.e. inhibit) granulopoiesis via an IL-17/G-CSF-dependent pathway (Forlow et al. 2001). IL-17 is produced by neutrophil-regulatory T cells (Tn), and its release is under the control of IL-23, which is secreted by dendritic cells and macrophages. Phagocytosis of apoptotic neutrophils attenuates IL-23 se-

cretion. Deficient neutrophil emigration into the tissue disrupts this (negative) feedback circuit and results in elevated neutrophil counts (Stark et al. 2005).

Haematopoietic progenitor cells (HPC) continuously home to the bone marrow. Both E- and P-selectin have been shown to mediate rolling of HPC in the bone marrow microvasculature. In addition, VCAM-1 is involved in this process, and blocking all three molecules abolished rolling almost completely (Mazo et al. 1998). Interestingly, both endothelial selectins and VCAM-1 are expressed constitutively on bone marrow endothelium, while in other organs they only appear upon stimulation. This highlights the important role of these molecules in homing to the bone marrow. Homing was also reduced when ligands of the endothelial adhesion molecules, i.e. PSGL-1 and α_4 integrins, were blocked (Katayama et al. 2003). However, blocking α_4 integrins had more effect than blocking VCAM-1, suggesting other ligands of α_4 to be involved in this process. In addition to the $\alpha_4\beta_1$/VCAM-1 pathway, the interaction between $\alpha_4\beta_7$ integrin and MAdCAM-1 has recently been demonstrated to contribute to HPC homing (Katayama et al. 2004).

5
Leucocyte-Endothelial Interactions in Diseases

While physiological leucocyte trafficking is a prerequisite for the organism to maintain vital functions and protect the body from the ongoing threat of infectious agents, it leads to damage when dysregulated and uncontrolled. Excessive leucocyte recruitment contributes to the development and perpetuation of both acute and chronic inflammatory diseases, including atherosclerosis, diabetes mellitus, rheumatoid arthritis and many others with tremendous personal and economic consequences. Drugs targeting leucocyte adhesion have recently been approved by the FDA for the therapy of psoriasis (efalizumab, which inhibits the binding of CD11a to ICAM-1) and relapsing multiple sclerosis (natalizumab, a monoclonal antibody to α_4 integrins). After confirmation of one fatal case and one additional case of severly disabling progressive multifocal leukoencephalopathy (PML) in patients receiving natalizumab for multiple sclerosis, the clinical trial was halted. As of February 2006, the FDA has lifted the clinical hold on trials of natalizumab for patients with multiple sclerosis. Biogen-IDEC can now resume administration of natalizumab to patients with relapsing-remitting multiple sclerosis who had previously been treated with the drug in clinical trials. Other applications might follow as further research in this area is conducted (Table 1). This chapter cannot provide a comprehensive description of all leucocyte-endothelial-related disorders. Instead, it will focus on the significance of leucocyte-endothelial interactions in some exemplary diseases, with particularly emphasis on potentials for therapeutic approaches.

Table 1 Clinical trials targeting leucocyte–endothelial interaction

Target	Drug	Indication	Effects
Selectin antagonists			
E-, P- and L-selectin antagonists	TBC-1269 (bimosiamose)	Asthma	Attenuates late asthmatic reaction
		Psoriasis	Reduces severe psoriasis lesions
	CY-1503	Ischaemia-reperfusion injury in infant heart surgery	no effect
Anti-E-selectin antibody	CDP-850	Psoriasis	no effect
P- and L-selectin antagonists	rPSGL-1-Ig	MI	no effect
Integrin antagonists			
Anti-β_2 integrin antibody	Rovelizumab	MI	no effect
		Stroke	no effect
		MS	no effect
	Erlizumab	MI	no effect
Anti-α_L (LFA-1) antibody	Odulimomab	GVH	Attenuation of GVH
		Renal transplantation	Prevention of graft rejection
	Efazulimab (FDA-approved)	Psoriasis	Decrease in psoriasis area
		Asthma	no effect
		RA	no effect
Anti-α4 (CD49d) antibody	Natalizumab	MS	Positive effect in relapsing MS
		IBD	Positive
Anti-$\alpha_4\beta_7$ antibody	MLN02	IBD	no effect
ICAM-1 (CD54)			
Antisense nucleotide to ICAM-1	ISIS2302	IBD, RA	no effect
Anti-ICAM-1 antibody	Enlimomab	Stroke	Negative (worsened outcome)

IBD, inflammatory bowel disease; ICAM-1, intercellular adhesion molecule 1; MI, myocardial infarction; MS, multiple sclerosis; GVH, graft-versus-host disease; RA, rheumatoid arthritis

5.1 Atherosclerosis

Atherosclerosis is an inflammatory disease of the wall of large arteries. Interactions between blood leucocytes and the endothelium have been shown to be a crucial step in the development of atherosclerotic lesions. Monocytes and T cells seem to be central in this process. Attracted by local secretion of chemoattractants such as monocyte chemoattractant protein (MCP)-1 (CCL2), oxidised LDL (oxLDL), or TNF-α, cell adhesion and migration is initiated. Once migrated into the arterial wall, monocytes are able to ingest lipids, particularly oxLDL, differentiate into foam cells and stimulate proliferation of smooth muscle cells, ultimately leading to the typical structural alterations seen in atherosclerotic plaques. T cells in the vessel wall stimulate this process by production of various cytokines such as interferon (IFN)-γ. Reduced blood flow, i.e. decreased shear stress, appears at specific arterial sites such as branches or bifurcations and causes upregulation of endothelial adhesion molecules such as ICAM-1 and VCAM-1 (Nagel et al. 1994). Disturbance of the physiological blood flow might reduce the production of local nitric oxide (NO), the major endogenous vasodilator, and lead to an increased expression of E-selectin, ICAM-1 and VCAM-1 (Zampolli et al. 2000).

ICAM-1 has been studied extensively, and earlier observations have suggested that ICAM-1 might play a major role in atherosclerosis. In mice gene-deficient for apolipoprotein E (apo E), ICAM-1 is constitutively expressed at sites of increased susceptibility to atherosclerotic lesions (Nakashima et al. 1998). In addition, the authors of this study demonstrated an increased expression of VCAM-1 at these sites, suggesting that those molecules might mediate the recruitment of monocytes and T cells. Consistently, mice lacking ICAM-1, P-selectin, CD18 or a combination of these show reduced atherosclerotic lesions induced by a pro-atherogenic diet and apo E-deficiency (Collins et al. 2000; Nageh et al. 1997). Another study, using mice deficient for the LDL-receptor and a mutant VCAM-1 confirmed the essential role of VCAM-1 in atherogenesis (Cybulsky et al. 2001). However, ICAM-1 was found to be less important in this model. A study in an isolated mouse carotid artery preparation revealed that rolling of mononuclear cells occurred on areas of atherosclerotic lesions from apo E-deficient mice, whereas no rolling was observed in wild-type mice (Ramos et al. 1999). Rolling in this model was dependent on P-selectin and VCAM-1 as well as on their ligands, PSGL-1 and α_4 integrin. In vivo observations using intravital microscopy confirmed the essential role of endothelial selectins in leucocyte rolling and adhesion to atherosclerotic lesions (Eriksson et al. 2001).

Activated platelets are found in atherosclerotic plaques. They secrete P-selectin, which mediates adhesion between platelets and monocytes leading to the release of pro-inflammatory chemokines, and which promotes VCAM-1-dependent monocyte adhesion to the endothelium and thus accelerates

atherosclerosis (Huo et al. 2003). In addition, there is evidence that secondary accumulation of leucocytes in a vascular thrombus might further perpetuate the inflammatory response. The initial leucocyte rolling on platelets is P-selectin-dependent. It is followed by firm adhesion and migration mediated by binding of leucocyte Mac-1 to platelet glycoprotein Ibα (Ehlers et al. 2003). The fact that Mac-1-deficient mice exhibit reduced neointimal thickening after vascular injury demonstrates the significance of Mac-1 in vascular injury (Simon et al. 2000a).

There is evidence that in humans similar mechanisms underlie the development of atherosclerosis. Focal expression of ICAM-1, VCAM-1, PECAM-1, E-selectin and P-selectin has been demonstrated in atherosclerotic plaques (Davies et al. 1993; Johnson-Tidey et al. 1994). Levels of soluble adhesion molecules, most likely derived from proteolytic cleavage from the cell surface, have been suggested to serve as independent predictive markers for cardiovascular events (Mulvihill et al. 2002). In particular, soluble ICAM-1 has been attributed a specific role as a prognostic factor in apparently healthy subjects. However, their clinical value, i.e. their sensitivity and-even more important-their specificity, appears at least questionable. P-selectin is implicated in atherothrombosis by mediating leucocyte-endothelium, leucocyte-platelet and platelet-platelet interactions (Merten et al. 2000; Vestweber and Blanks 1999). Interestingly, gallic acid has recently been shown to inhibit P-selectin-dependent platelet-leucocyte interactions, providing one potential explanation for the cardioprotective effects of red wine (Appeldoorn et al. 2005).

In addition to already established therapeutic concepts such as seeking to lower risk factors, targeting cell adhesion seems to be an attractive approach. Antibody strategies aiming at the blockade of VCAM-1 have been successfully used to reduce neointimal formation after carotid injury in mice (Oguchi et al. 2000). In addition, blockade of $\alpha_4\beta_1$, the ligand of VCAM-1, was able to reduce the intimal hyperplasia in endarterectomised carotid arteries (Lumsden et al. 1997). The migration of monocytes to atherogenic sites of the arterial wall is crucial in the initiation of the inflammatory process. Apo E knockout mice lacking MCP-1 or its receptor CCR-2 exhibit significantly reduced atherosclerotic lesions (Boring et al. 1998; Gosling et al. 1999). Moreover, lesions can be reduced by administration of a specific CCR-2 receptor inhibitor in apo E-deficient mice (Yamashita et al. 2002). Given the central role of macrophages and foam cells in the initiation and perpetuation of atherosclerosis, they might be potential targets for therapeutic approaches in the future (Li and Glass 2002).

5.2 Ischaemia-Reperfusion Injury

Disorders associated with either local or systemic ischaemia, such as myocardial infarction, stroke or sepsis, have a significant impact on mortality and morbidity of affected patients. Substantial efforts have been made to develop

therapeutic strategies aiming at the early reperfusion and thus re-oxygenation of the ischaemic area. Unfortunately, even after the successful reconstitution of blood flow, the ischaemic tissue faces a new challenge: reperfusion injury (RI). RI is characterised by an excessive inflammatory response, and it has been reported for almost every organ system, with severe consequences for the functional and structural integrity of the tissue (Lehr et al. 1993). Despite effective reperfusion, some capillaries might remain ischaemic ("no-reflow"). Depending on the duration and the extent of the ischaemic event, this might lead to a definitive occlusion of capillaries (Manciet et al. 1994). A high rate of left heart failure is associated with the occurrence of RI and no-reflow after acute myocardial infarction (Ito et al. 1996). RI is responsible for the majority of tissue injury in lung transplantation (Ross et al. 2000). In addition, organ failure such as acute renal failure is frequently observed upon successful cardiovascular resuscitation and represents another clinical complication of RI.

Several mechanisms may contribute to RI and no-reflow, including rheological factors (haemoconcentration and thrombosis), vasomotor dysregulation (vasoconstriction) and oedematous swelling of capillaries (sodium- and calcium-influx due to a disturbed endothelial integrity). In addition, adhesion and migration of activated leucocytes into the vessel wall have been recognised as a major mediator of RI. Early studies suggested neutrophils to be crucial, since depletion of neutrophils was able to attenuate myocardial (Romson et al. 1983), intestinal (Grisham et al. 1986), renal (Singbartl and Ley 2000) and skeletal (Korthuis et al. 1988) RI. Indirect effects rather than mechanical occlusion seem to be largely responsible for the disruption of the leucocyte-mediated capillary integrity, and cell adhesion has been studied extensively in various models of RI.

In various organs, ischaemia-reperfusion rapidly upregulates P-selectin in postcapillary venules, indicating a major role in mediating RI (Basile et al. 2000; Eppihimer et al. 1997; Naka et al. 1997). Blocking strategies have confirmed an important role for P-selectin (Kanwar et al. 1998; Singbartl et al. 2000). NO is the most important vasodilator, and the concentration of NO in ischaemic capillaries is reduced significantly (Webb et al. 2004). The lack of NO, therefore, does not only attenuate the capillary's ability to vasodilate but also induces the adhesion of leucocytes to the capillary wall. Activation of the endothelial NO synthase (eNOS), e.g. by administration of glucocorticoids, is able to reduce both myocardial infarct size and the number of adherent cells after local ischaemia (Hafezi-Moghadam et al. 2002). The mechanism underlying the protective effect of NO is not well understood.

L-selectin has been suggested to mediate reperfusion-induced leucocyte adhesion in heart (Ma et al. 1993), liver (Martinez-Mier et al. 2000), intestine (Andrews et al. 1997), muscle (Yan et al. 2000) and skin (Mihelcic et al. 1994). However, this effect might be organ-specific and less important than P-selectin. E-selectin is upregulated after RI. This upregulation is delayed for several hours, suggesting that E-selectin might be involved in maintaining leucocyte

recruitment at sites of RI, a fact that makes E-selectin particularly attractive for therapeutic use in already established RI (Singbartl and Ley 2000). In humans, the role of E-selectin has been confirmed in liver transplantation, where the expression of E-selectin correlates with the extent of RI (Mueller et al. 1996).

The role of β_2 integrins in RI has been studied extensively. Early work reported a significant contribution of CD18 to RI in various models (Hernandez et al. 1987; Vedder et al. 1988). In further studies, Mac-1 has been shown to be the predominant β_2 integrin in RI. Expression of Mac-1 is upregulated after ischaemia-reperfusion and its functional relevance has been confirmed in both antibody and gene blocking strategies (Jaeschke et al. 1993; Soriano et al. 1999). In contrast, LFA-1 seems to have only a minor role.

ICAM-1, the major endothelial ligand for Mac-1 and LFA-1, has been reported to protect animals from RI in various organs, including heart (Palazzo et al. 1998), lung and liver (Colletti et al. 1998), kidney (Dragun et al. 1998), intestine (Horie et al. 1996), striated muscle (Nolte et al. 1994) and skin (Tosa et al. 1998).

The promising preclinical data led to several phase II and III clinical trials in various RI-dependent disorders, including shock, myocardial infarction, ischaemic stroke, pulmonary thrombectomy and neonatal cardiopulmonary bypass. However, despite beneficial trends in some trials, overall results were rather disappointing (Harlan and Winn 2002). Both anti-ICAM-1 and anti-CD18 strategies failed to improve organ function and outcome after myocardial infarction and after renal transplantation (Rusnak et al. 2001; Salmela et al. 1999). In fact, the administration of an anti-ICAM-1 antibody in ischaemic stroke was not only associated with more adverse events but also with an increased mortality (Sherman et al. 2001). Major differences between the experimental set-up and the human disease, particularly a more prolonged ischaemic period in patients, might be the most likely explanation for the apparent failure of anti-adhesive strategies. This emphasises the role of cell adhesion in a very early phase of RI and therapeutic approaches might therefore be likely to succeed in acute events only.

5.3 Inflammatory Bowel Disease

Inflammatory bowel diseases (IBD), i.e. Crohn's disease and ulcerative colitis, are chronic inflammatory diseases of the gut associated with a dysregulated mucosal activation in the presence of various antigens in the luminal flora, ultimately leading to the destruction of the intestinal integrity. Several, mostly immunosuppressive therapeutic approaches have been clinically tested. However, serious side-effects limit their application, and surgical intervention is still necessary in many cases.

A key event in the initiation and perpetuation of IBD is the infiltration of type 1 helper-T-cells (Th1; Crohn's disease) or type 2 helper-T-cells (Th2; ulcerative colitis) into the mucosa, both resulting in the release of certain cy-

tokines and chemokines such as interferon-γ and IL-2 (Th1) or transforming growth factor β and IL-5 (Th2) (Fuss et al. 1996). Subsequent activation of macrophages leads to the release of further cytokines (IL-12, IL-18) and thus to a self-sustaining cycle. In addition, the secretion of macrophage-derived cytokines, e.g. TNF, IL-1 or IL-6, results in recruitment of leucocytes from the vascular space, which is essential for maintaining the inflammatory process. Selectins have been shown to be upregulated in the setting of experimental IBD, and blocking of P-selectin attenuated leucocyte adhesion but had no effect on tissue damage in ulcerative colitis (Sans et al. 2001). Cyclosporin A, given in patients with severe ulcerative colitis, is known to block lymphocyte activation. Recent work, however, has shown that in an experimental model of colitis, it also reduces the expression of ICAM-1 and VCAM-1 in colonic venules (Soriano-Izquierdo et al. 2004). Thus, attenuated leucocyte infiltration might be another beneficial effect of cyclosporin A. Specific research interest has arisen in MAdCAM-1, a member of the immunoglobulin family, because of its expression on Peyer's patch HEVs, mesenteric lymph nodes and endothelial cells of lamina propria venules. It is upregulated in the setting of IBD and selectively binds to $\alpha_4\beta_7$ integrins on lymphocytes (Berlin et al. 1993); this engagement is typical for lymphocyte homing in the gut. Antibodies to block the $\alpha_4\beta_7$-MadCAM-1 interaction were successfully tested in cotton-top tamarins, a South American primate which spontaneously develops chronic colitis (Hesterberg et al. 1996).

Natalizumab is a compound which is able to block both $\alpha_4\beta_7$-MadCAM-1 and $\alpha_4\beta_7$-VCAM-1 interactions in humans (the latter one is a crucial step for lymphocyte migration in multiple sclerosis; see Sect. 2.1.2.3). It has shown promise in phase II trials of patients with Crohn's disease (Sandborn and Yednock 2003).

Besides their function in leucocyte migration, adhesion molecules also contribute to lymphocyte activation. ICAM-1 is upregulated in IBD and induces a co-stimulatory signal in antigen-presenting T cells (Vanseventer et al. 1990). However, ICAM-1 blocking strategies have failed to prove a beneficial effect in two clinical trials (Sandborn and Yednock 2003).

There is a great body of evidence to suggest that neutrophils, in addition to lymphocytes, play a role in IBD. CXCR2 and its ligands, essential for neutrophil recruitment into the lung (see the following section), are upregulated in rectal biopsies of patients with active IBD and appear to be involved in the initial recruitment of neutrophils to the inflamed gut. Blocking strategies have been successfully tested in experimental IBD (Ajuebor et al. 2004). Their impact in humans is currently unknown.

5.4 Acute Lung Injury: A Different Story?

Leucocyte adhesion and transmigration in the systemic microcirculation follow common mechanisms in most tissues. However, in the lung these rules

seem to be different. Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), ALI's more severe form, are some of the major challenges in intensive care medicine, with a high mortality and no specific therapy. ALI/ARDS is characterised by an excessive inflammatory reaction in response to various direct and indirect stimuli, such as pneumonia, acid aspiration, sepsis and many others. An excessive neutrophil influx into all lung compartments is characteristic in the early phase of the disease (Abraham 2003), resulting in a destruction of the alveolar-capillary membrane with severe consequences for the gas exchange in both animals and humans. In several studies, neutropaenia has been shown to attenuate the extent of lung damage significantly (Abraham et al. 2000; Azoulay et al. 2002).

Various animal models have been employed to improve our understanding of the molecular mechanisms of leucocyte trafficking in the lung (Reutershan and Ley 2004). The unique structure of the pulmonary microcirculation influences leucocyte-endothelial interaction fundamentally. Due to the small diameter of lung capillaries, leucocytes are in close contact to the endothelium, even under resting conditions, resulting in a "marginated pool" of leucocytes ready to migrate immediately in response to an inflammatory stimulus (Doerschuk et al. 1987). In contrast to the systemic circulation, the pulmonary capillaries constitute the major site of leucocyte recruitment (Gebb et al. 1995). Consequently, selectins, essential to slow down leucocytes and to initiate leucocyte rolling in the systemic circulation, seem to be dispensable in the lung. In fact, even the absence or blockade of all three selectins was not able to inhibit neutrophil migration in response to LPS or *Streptococcus pneumoniae* (Burns et al. 2001; Mizgerd et al. 1996). Nevertheless, selectins might have a function in the lung, one distinct from capturing and rolling (Hayashi et al. 1999; Kubo et al. 1999; Olson et al. 2002).

Integrins, however, are involved in pulmonary leucocyte trafficking. β_2 integrins (CD18) are the most studied integrins and have been shown to mediate lung injury when induced by *Escherichia coli*, *Pseudomonas aeruginosa*, cobra venom factor, IgG immune complex, IL-1 or intratracheally administered lipopolysaccharide (LPS) (Doerschuk et al. 2000). However, CD18-independent migration has been described. Leucocytes in patients with complete deficiency of CD11/CD18 (leucocyte adhesion deficiency type I) fail to migrate into the gut and lymphatic organs but show a near-normal migration into the lung (Hawkins et al. 1992). In addition, various animal models of lung injury revealed CD18-independent mechanisms using blocking antibodies and gene-targeted mice. In chimeric mice reconstituted with neutrophils from wild-type ($CD18^+$) and CD18-deficient ($CD18^-$) mice, significantly more $CD18^+$ neutrophils migrated in response to LPS or *Pseudomonas Aeruginosa* (CD18-dependent), while there was no difference between $CD18^+$ and $CD18^-$ neutrophils in response to *S. pneumoniae* (CD18-

independent) (Mizgerd et al. 1999). The regulation of CD18-independent migration is poorly understood, but other members of the integrin family, such as the β_1 integrins very late antigen-4 and -5, might be involved (Burns et al. 2001).

ICAM-1 is the major ligand of CD18 in the lung, and it has been suggested that CD18-dependent migration might in fact be an ICAM-1-dependent mechanism, and most likely mediated by an endothelial upregulation of ICAM-1 (Burns et al. 1994). In addition, expression of ICAM-1 is regulated by NF-κB-dependent IL-1 and TNF-α, both of which are required in CD18-dependent pathways. PECAM-1 has been suggested to be required for neutrophil recruitment into the lung (Vaporciyan et al. 1993), but this has not been confirmed (Tasaka et al. 2003).

In human lung injury, ICAM-1, VCAM-1 and E-selectin are strongly upregulated in patients who have died from ARDS (Muller et al. 2002). In addition, CD18 expression on neutrophils is higher after incubation with bronchoalveolar lavage fluid (BAL) from ARDS patients who have received a conventional compared to a lung-protective ventilation (Zhang et al. 2002). The authors concluded that reduced neutrophil activation might be one of the beneficial effects of ventilation with low tidal volumes.

Besides adhesion molecules, chemokines have a significant impact for the leucocyte-endothelial interaction in acute lung injury. IL-8 has been extensively studied and is known to mediate neutrophil influx in ARDS patients (Miller et al. 1992) as well as in many animal models. In ARDS, IL-8-anti-IL-8 complexes are found in the BAL, which largely account for the chemotactic activity and might also be useful as a prognostic marker (Kurdowska et al. 2002). CXCR2, one of two IL-8 receptors in humans, is essential in various animal models of acute lung injury. Mice lacking CXCR2 are protected from neutrophil influx in hyperoxia-, ventilator- and LPS-induced lung injury (Belperio et al. 2002; Sue et al. 2004; Reutershan et al. 2006). The murine CXCR2 ligands KC (CXCL1) and MIP-2 (CXCL2/3) are strongly upregulated in LPS-induced lung inflammation (Jeyaseelan et al. 2004) and contribute to the generation of a chemotactic gradient. KC, produced by alveolar macrophages and type II pneumocytes, is transported selectively from the airspace into the blood and might function as a leucocyte primer in the circulation (Quinton et al. 2004). KC might also mediate the release of new neutrophils from the bone marrow, most likely by desensitising the bone marrow neutrophil response to stromal cell-derived factor (SDF)-1, a CXCR4-ligand which is thought to be responsible for the retention of neutrophils in the bone marrow (Martin et al. 2003; Suratt et al. 2004). Other chemokines exist and might play a role in pulmonary leucocyte trafficking. ENA-78 is chemotactic for neutrophils and can be found in the BAL of ARDS patients (Goodman et al. 1996). However, the role of this and other chemokines has yet to be elucidated.

6 Conclusion and Future Directions

The interaction between blood leucocytes and the vascular endothelium is essential to ensure an intact innate and adaptive immune system and maintenance of a haematopoietic homeostasis. Its failure can result in severe immunodeficiency, as impressively displayed in patients with LAD. On the other hand, uncontrolled or dysregulated leucocyte adhesion and migration can cause numerous disorders in any organ system, with enormous consequences for the integrity of crucial body functions. The latter set of inflammatory disorders is much more relevant for medical practice and public health. Modulating leucocyte adhesion seems to be an attractive approach to combat a variety of diseases. However, specificity is a major concern when interfering with a system involved in countless regulatory mechanisms and pathways. The first drugs targeting the leucocyte-endothelial interaction have recently been approved. New compounds will be developed as our understanding of the underlying molecular mechanisms continues to grow.

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Endothelial Cell Dysfunction, Injury and Death

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Abstract Vascular endothelial cells (ECs) perform a number of functions required to maintain homeostasis. Inflammation can cause EC injury and death which disrupt these processes and result in endothelial dysfunction. Three common mediators of EC injury in inflammation are macrophage-derived cytokines, such as tumour necrosis factor (TNF); neutrophil-generated reactive oxygen species (ROS) and cytolytic T lymphocytes (CTL). Here we describe the distinct but overlapping biochemical pathways of injury elicited by these different agents.

Keywords Apoptosis · Necrosis · Cytolytic T lymphocytes · Tumour necrosis factor · Reactive oxygen species

1 Introduction

The endothelial cell (EC) lining of the vascular system—while displaying variations in the properties of ECs among different tissues and among different types of vascular segments (e.g. arterial, microvascular, venous) within particular tissues—performs a number of important, common functions that warrant consideration of the EC as a specific cell type. These common functions include: regulation of underlying smooth muscle cell (SMC) tone; control of transendothelial cell movement of fluid, solutes and macromolecules; maintenance of blood fluidity; presentation of a non-interactive surface to the formed

elements (e.g. erythrocytes, leucocytes and platelets) of the blood; and display of tissue antigens to circulating memory T cells as part of immune surveillance (Choi et al. 2004). The constitutive functions of ECs are dependent upon active metabolic processes, and all may be impaired by EC injury such as that which occurs in inflammation. For example, ECs normally produce nitric oxide (NO), a mediator that, among other effects, reduces basal SMC tone (Stamler et al. 1994). Injured ECs may produce inadequate quantities of NO which increases SMC tone and causes pathological elevation of blood pressure (Panza et al. 1990). This particular perturbation is commonly referred to as "endothelial dysfunction". However, other behaviours of the EC may also exhibit dysfunction. For example, endothelial injury may lead to loss of the EC barrier that retains plasma proteins within the intravascular space, leading to vascular leakiness (Joris et al. 1990). Failure to block intravascular coagulation, leading to thrombosis, is yet another example of endothelial dysfunction (Aird 2003). The ultimate form of injury is one which causes cell death, and dead ECs are globally dysfunctional, failing in performance of all normal functions. However, not all injury is lethal and there is a spectrum of EC injury and concomitant endothelial dysfunction. Importantly, endothelial dysfunction is a common feature of many disease states and is a harbinger of adverse cardiovascular outcomes (Endemann and Schiffrin 2004).

The idea that injury can cause endothelial dysfunction is clear, but confusion may arise because of phenotypic overlap with another type of EC response to stimuli known as "endothelial activation" (Pober 1988). As originally defined, endothelial activation referred to a state in which ECs, responding to various mediators, acquire the capacity to perform new functions. Stimuli that produce activation include autacoids and cytokines. Tumour necrosis factor (TNF) is a prototypical inflammatory cytokine, and TNF-activated ECs display new surface adhesion molecules and chemokines that lead to recruitment and activation of circulating leucocytes. Thus, while a normal EC may produce signals that minimise leucocyte activation, such as NO release (Kubes et al. 1991), and a dysfunctional EC that produces too little NO may fail to adequately inhibit leucocyte binding to the EC surface, an activated EC has been modified to actively promote leucocyte binding and transendothelial migration. This kind of activation is a desirable response at the site of local infection where leucocytes mediate host defence, but can become pathological in an inflammatory disease such a rheumatoid arthritis. The key point is that activated and dysfunctional ECs may share a common feature, namely enhanced recruitment of leucocytes. Nevertheless, activated ECs are not necessarily injured and the outcome, as in the case of leucocyte recruitment at a site of infection, may actually be beneficial. Similarly, a healthy EC will display on its luminal surface anti-coagulant heparan sulphate moieties that bind anti-thrombin III and actively inhibit coagulation, whereas antibody-mediated injury may produce endothelial dysfunction by triggering the shedding of heparan sulphate and promoting thrombosis (Platt et al. 1991). A TNF-activated EC may synthesise

and display tissue factor, an initiating catalyst for thrombosis (Nawroth and Stern 1986). In the case of TNF, this activation of coagulation can be an important part of the mechanism by which infectious organisms may be locally contained. The point is again that an activated EC and a dysfunctional EC may contribute to a common result, namely thrombosis.

While dysfunction is generally a result of injury and activation is normally associated with host defence, this distinction can become blurred. For example, host defence mechanisms may cause EC injury, and both processes are often present in the same vessel segment or even the same EC. For example, the same EC that sheds heparan sulphate may express tissue factor. Even more confusing is that some agents generally associated with activation, such as TNF, may in certain contexts cause EC injury and dysfunction. For example, TNF not only induces tissue factor, leucocyte adhesion molecules, and chemokines (Pober 1988), but it also destabilises the messenger RNA (mRNA) molecules encoding endothelial nitric oxide synthase (eNOS), the enzyme responsible for NO synthesis in the EC (Yoshizumi et al. 1993). Since TNF can act in an endocrine manner (i.e. at a distance), it may promote host defence at a local site and simultaneously cause endothelial dysfunction elsewhere in the vasculature.

In this chapter, we will focus on the biochemistry of EC injury and death, i.e. the processes which underlie dysfunction. We will begin the discussion with a consideration of apoptotic vs other forms of cell death in the EC. We will specifically review common major pathways of EC injury that occur in various forms of inflammation. We will first describe the response of ECs to TNF, a key macrophage-derived effector molecule, which as we have noted can be both an EC activator and a mediator of EC dysfunction. We will then consider injury by reactive oxygen species (ROS), a major effector pathway of phagocytic leucocytes, especially neutrophils. Finally, we will review injury mediated by immune effector T cells, a major component of the adaptive immune response. Since many responses to injury are cell type-specific, we will emphasise those features that seem to distinguish ECs from other cell types.

2
Apoptotic and Non-apoptotic Death in Endothelial Cells

About 30 years ago, developmental biologists observed that certain cell populations underwent programmed cell death as an embryo matured (Kerr et al. 1972). The pattern of death, designated as apoptosis, included membrane vesiculation as well as nuclear condensation and fragmentation. Apoptosis differed from the patterns of cell death that had been described as occurring in damaged tissues, which were more typically associated with cellular and nuclear swelling and often accompanied by inflammation. Oncosis is a more precise but not widely used term for death by swelling (Majno and Joris 1995). Such non-apoptotic cell death is now commonly called necrotic cell death, although

it should be appreciated that necrosis originally was used to describe injury to tissues rather than to cells, and necrotic tissues may contain both necrotic (i.e. oncotic) cells and apoptotic cells. Biochemically, apoptosis involves early loss of polarity of the plasma membrane lipid phosphatidylserine, normally confined to the inner leaflet of the bilayer; this change is detectable by binding of certain proteins, such as annexin V. Apoptotic cells also undergo cleavage of nuclear DNA into histone-covered segments of approximately 200 bp known as nucleosomes. These may be detected by gel electrophoresis of DNA or by enzyme-linked immunosorbent assay (ELISA) for released nucleosomes. Apoptotic cells are frequently phagocytosed by neighbouring cells and often disappear without eliciting an inflammatory response (Saraste and Pulkki 2000).

In the 1990s the biochemical processes underlying apoptosis were elucidated (Blagosklonny 2000). The key feature is that the morphological changes which constitute apoptosis depend upon cleavage of particular cellular proteins by a family of structurally related intracellular proteases. These enzymes have an activated cysteine in their catalytic site and cleave protein substrates at aspartyl residues; they are collectively known as active *c*ysteinyl *asp*artyl-directed prote*ases* or caspases. Nucleosome formation is caused by proteolytic attack on the protein inhibitor of a specific DNAse, thereby releasing the active enzyme from an inactive complex. The caspase substrate is called inhibitor of caspase-activated DNAse or iCAD and the nuclease is known as CAD. Apoptotic death has become equivalent to caspase activation, and other forms of cell death may thus be classified as caspase-independent.

ECs may undergo apoptosis or necrosis (oncosis), depending on the stimulus. EC apoptosis has a special relationship to coagulation and thrombosis. When ECs apoptose, the process of plasma membrane vesiculation releases lipid-rich microparticles (vesicles) into the circulation that can serve as a platform for assembly of coagulation factors (Bombeli et al. 1997; Mallat et al. 2000). Newly synthesised tissue factor is often sequestered intracellularly, where it cannot interact with factor VIIa—a phenomenon called "encryption". Tissue factor within ECs may be "de-encrypted" by apoptosis, further enhancing coagulation (Greeno et al. 1996). EC death may activate coagulation by other means as well. While healthy ECs are normally unreactive with platelets, they elaborate a basement membrane that can bind and activate platelets. Under normal circumstances, ECs completely cover this basement membrane and thus prevent platelets from adhering. However, injured ECs may detach from the vessel wall. When individual ECs detach, their previously occupied space is quickly (or even concomitantly) covered by spreading of viable neighbouring cells (Reidy and Schwartz 1981). This process can attenuate the pro-coagulant effects of the subendothelial basement membrane so long as it is limited to a small fraction of the EC lining. More severe damage is likely to lead to extensive loss of ECs; under these circumstances, platelets are exposed to the basement membrane and thrombosis will ensue (Walker et al. 1983).

3 TNF-Mediated Injury

TNF was originally discovered as a factor produced by macrophages in endotoxin-stimulated rabbits that could cause haemorrhagic necrosis of experimental tumours (Carswell et al. 1975). TNF is now recognised as a major effector of macrophage-mediated host defence and tissue injury. TNF is also made by cells of the adaptive immune system, including T cells, natural killer (NK) cells and mast cells. Vascularised tumours are particularly susceptible to destruction by TNF, and the mechanism of action involves thrombosis and vascular EC injury (Palladino et al. 1987). Since thrombosis preferentially occurs in tumour vessels rather than in normal tissues, it seems likely that ECs which line tumour vasculature are more responsive to TNF actions than other ECs. The best explanation is that TNF-mediated activation of ECs, measured as elaboration of tissue factor, is enhanced by interactions with tumour-derived mediators such as vascular endothelial growth factor (VEGF) (Clauss et al. 1990). The effect of TNF on tumour vasculature resembles that caused by TNF to a normal EC during profound systemic disturbances such as sepsis. In the case of acute sepsis, interferon-ɣ (IFN-ɣ) has been identified as a key co-factor which potentiates TNF actions (Heinzel 1990). IFN-ɣ not only enhances certain TNF activation responses, such as display of adhesion molecules that bind leucocytes (Doukas and Pober 1990; Lechleitner et al. 1998), but it also enhances TNF-mediated EC death (Wallach et al. 1999; Wang et al. 1999). In contrast to IFN-ɣ, VEGF may actually reduce TNF-mediated injury (Alavi et al. 2003).

Over the last decade, much attention has been paid to elucidating the biochemical signalling pathways that trigger cell death in response to TNF (Wallach et al. 1999; Fig. 1).

The death response is typically initiated by TNF binding to its type I receptor (TNF-R1, also known as CD120a). TNF-R1 is sometimes called death receptor (DR)-1 and shares with a few other members of the TNF receptor superfamily an intracellular domain referred to as a death domain (DD). DDs mediate protein–protein interactions with other DD-containing proteins. For example, Fas (CD95, also known as DR-2) is a DD-containing transmembrane receptor that, upon binding of its trimeric ligand to its extacellular regions, recruits a cytosolic adaptor called Fas-associated death domain protein (FADD) through DD interactions to its intracellular domain. It is not clear if the ligand initiates this response by oligomerising Fas receptors or if instead it induces a conformational change in pre-associated receptor trimers. In addition to its DD, FADD also contains a so-called death effector domain (DED) that binds to the pro-domain of pro-caspase 8. FADD-bound pro-caspase 8 undergoes auto-catalytic activation, a process involving proteolysis, and active caspase 8 is released to the cytosol where it can proteolytically activate effector caspases, such as caspase 3, leading to apoptosis. The complex of FADD with (pro)caspase 8 is known as death-inducing signalling complex (DISC). In hu-

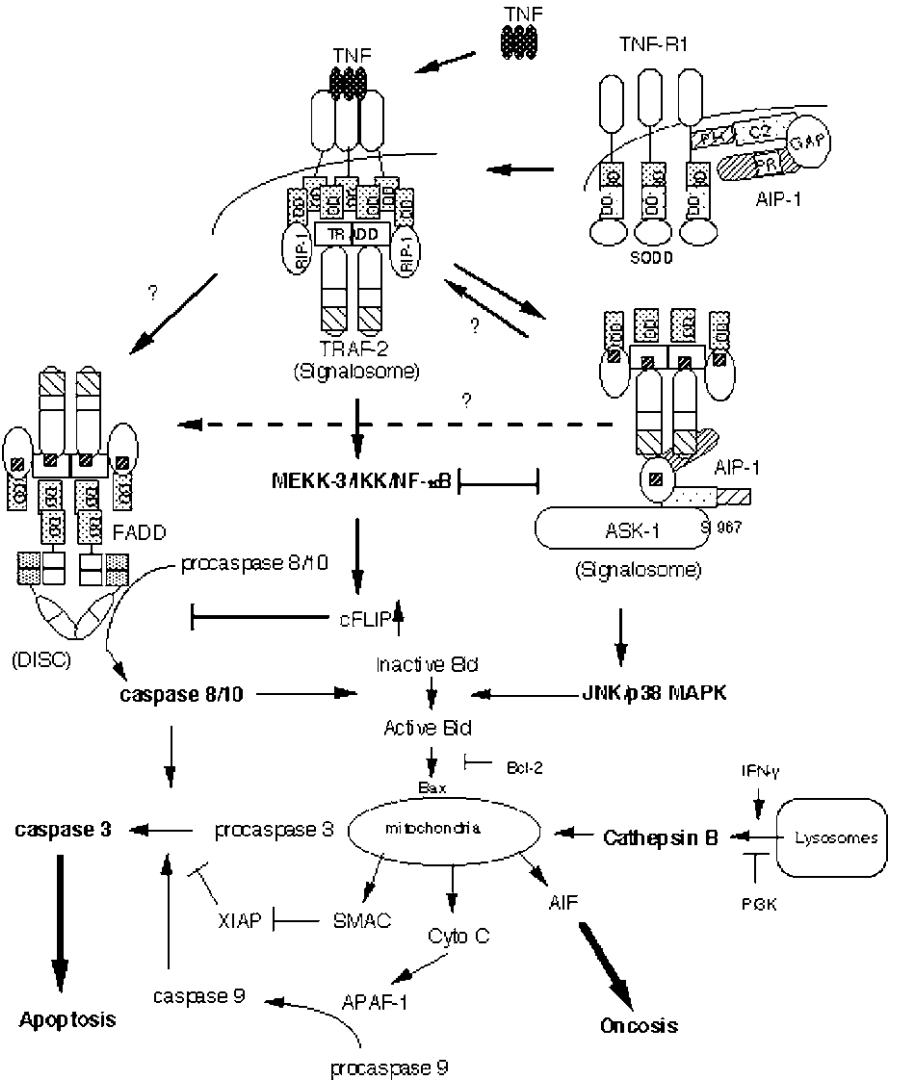
mans, the DISC may activate caspase 10 as well as 8; the role of this seeming redundancy is unknown.

While formation of a DISC is sufficient to initiate apoptosis in many cells, it is insufficient to do so in others. In some cell types there exists an amplification pathway involving various member of the Bcl-2 gene family (Yin 2000). In general, pro-apoptotic members of this family, such as Bax or Bak, may homodimerise in the mitochondrial membrane, leading to release of mitochondrial proteins into the cytosol that can initiate cell death. Anti-apoptotic members of the Bcl-2 family, such as Bcl-2, Bcl-X_L or A1, antagonise this effect by forming heterodimers with Bax or Bak. In general, anti-apoptotic members of the family, such as Bcl-2, contain characteristic "BH" domains numbered 1–4, whereas pro-apoptotic members contain only BH domains 1–3. Other members of the family can serve as initiators of Bax or Bak dimerisation. Of relevance to DISC signalling, caspase 8 can proteolytically activate a cytosolic member of the Bcl-2 family, known as Bid, and activated Bid interacts with Bax, causing mitochondria to release death-inducing molecules. Bid contains only one of the four structural domains found in Bcl-2 and is known as a "BH-3 only" member of the family.

The best-characterised molecules released from mitochondria by Bax dimerisation are a protein called apoptosis-inducing factor (AIF), a protein called either second mitochondrial-derived activator of caspases or direct inhibitor of apoptosis (IAP)-binding protein with low pI (SMAC/Diablo), and cytochrome *c*

Fig. 1 Overview of TNF signalling in the EC. TNF binding to TNF-R1 leads to the formation of three distinct signal-inducing complexes that activate different responses. The initial recruitment of TRADD, RIP-1 and TRAF-2 to TNF-R1 forms a signalosome that activates the MEKK-3/IKK/NF-κB pathway. The initial signalosome complex rapidly dissociates from TNF-R1. The recruitment of ASK-1 and AIP-1 to the signalosome blocks the NF-κB pathway and instead activates MEKKs which lead to activation of JNK and p38 MAPK. At later times, one or both of these signalosomes recruit FADD, forming a DISC that converts pro-caspases 8 and 10 to active caspases 8 and 10, respectively. Active caspases 8 and 10 convert pro-caspase 3 to active caspase 3, leading to apoptosis. Both the NF-κB and the JNK/p38 MAPK pathways activate new gene transcription. Genes activated by the NF-κB pathway are pro-inflammatory and/or anti-apoptotic, inducing proteins that inhibit caspase activation (such as cFLIP, an inhibitor of DISC function). Sustained activation of JNK and p38 MAPK is generally pro-apoptotic. Both caspase 8 and JNK can convert inactive Bid to active Bid, which in turn interacts with Bax to release death-inducing proteins, such as cytochrome *c*, SMAC and AIF, from the mitochondria. This response is antagonised by Bcl-2. Released cytochrome *c* forms a complex with APAF-1 in the cytosol, known as an apoptosome that converts pro-caspase 9 to active caspase 9. Caspase 9, like caspases 8 and 10, initiates apoptosis by activating caspase 3. SMAC, by sequestering XIAP (an inhibitor of caspase 3), enhances this process. AIF initiates non-apoptotic (oncotic) death. The release of cathepsin B from lysosomes to the cytosol is promoted by IFN-γ or by PI3-K inhibitors. As-yet-uncharacterised TNF-R1 signals activate cytosolic cathepsin B to release death-inducing proteins from mitochondria. (For abbreviations see text)

(Saelens et al. 2004). AIF, contrary to the name, initiates cell death but does not activate caspases, so death is non-apoptotic. SMAC/Diablo works by binding to and sequestering X chromosome-linked inhibitor of apoptosis protein (XIAP), a natural inhibitor of caspase 3. Thus, release of SMAC/Diablo into the cytosol potentiates activated caspase 3. Cytochrome *c* initiates apoptosis by binding to a cytosolic protein called apoptosis activating factor (APAF)-1, and the cytochrome *c*–APAF-1 complex binds pro-caspase 9, promoting its auto-catalytic activation in an ATP-dependent manner. This complex is sometimes called an apoptosome and essentially functions like a DISC. Active caspase 9 is released



from the apoptosome and, like active caspase 8, will proteolytically activate effector caspases such as caspase 3. Cell types, like hepatocytes, that require mitochondrial amplification to respond to Fas ligand are protected from apoptosis by expression of Bcl-2, whereas cell types in which DISC activation is sufficient to cause cell death are not.

The FADD-mediated death pathway can also be triggered by binding of TNF-related apoptosis-inducing ligand (TRAIL) to TRAIL-R1 or TRAIL-R2, sometimes called DR4 and DR5, respectively. However, the injury and death responses initiated by TNF appear to be more complex than these activated by Fas ligand or TRAIL. Genetic experiments have linked TNF-R1-initiated cell death to FADD, but TNF-R1, despite having a DD, does not bind FADD and there are no biochemical data showing the presence of TNF-R1 in a FADD-containing DISC. Instead, TNF-R1 associates, in the absence of ligand, with a DD-containing protein called silencer of death domains (SODD) and, upon TNF binding, releases SODD and binds an adaptor protein called TNF receptor-associated death domain protein (TRADD) (Wallach et al. 1999). TRADD is a key mediator of the TNF-activation response in the EC and other cell types (Siwkowski et al. 2004). In ECs TRADD remains associated with TNF-R1 only for a brief period (minutes), after which the receptor-TRADD complex is internalised and dissociates (Jones et al. 1999). During this process, TRADD is in some fashion "altered" (perhaps by clustering on the ligand-bound receptor). Due to this alteration, TRADD is able to recruit two other adaptor proteins, known as receptor-interacting protein-1 (RIP-1), a serine-threonine kinase that binds to TRADD via its own DD, and TNF receptor associated factor-2 (TRAF-2), an adaptor protein that is an E3 ubiquitin-ligase which does not contain a DD.

The TRADD/RIP-1/TRAF-2 complex, known as a "signalosome", is able to activate a number of signalling cytosolic kinases belonging to the mitogen-activated protein kinase kinase kinase (MAP3K) family. MAP3K activation does not require RIP kinase activity but in some cases involves the E3 ligase activity of TRAF-2, which attaches ubiquitin to protein substrates via a linkage that favours signalling rather than targeting proteins for degradation. One of the targets of the signalosome, namely MEKK-3 (a member of MAPK kinase kinase), phosphorylates and activates the inhibitor of κB kinase (IKK) complex (Yang et al 2001). Alternatively, TRAF-2/RIP-1 may directly recruit IKK to TNF-R1 and in this way induce IKK activation (Devin et al. 2001).

Once activated, IKK phosphorylates inhibitor of κB (IκB)-α, -β, -ε proteins. The various IκB molecules are typically cytosolic proteins that bind to various nuclear factor κB (NF-κB) family members, retaining them in the cytosol. IκB phosphorylation initiates IκB ubiquitination and proteolytic degradation, releasing the sequestered forms of NF-κB to enter the nucleus and activate transcription of genes associated with inflammation and cell survival (Kucharczak et al. 2003). A key cell survival gene induced in ECs by NF-κBr is c-FLICE inhibitory protein (c-FLIP, FLICE being an older name for caspase 8). c-FLIP

prevents FADD-mediated activation of caspase 8 within the DISC (Bannerman et al. 2004). Some other NF-κB-responsive anti-apoptotic proteins in the EC include A1, a molecule related to Bcl-2 that inhibits Bax actions on mitochondria, members of the IAP family that act as caspase inhibitors, and A20, an enzyme that reverses TRAF-mediated ubiquitination and stops TNF signalling.

The TRADD/RIP-1/TRAF-2 complex can also bind and activate a MAP3K enzyme known as apoptosis signalling kinase-1 (ASK-1) (Matsukawa et al. 2004). The recruitment and activation of ASK-1 and the associated ASK-interacting protein (AIP)-1 to the signalling complex prevents activation of IKK and the NF-κB pathway and instead favours activation of several MAP2Ks that lead to phosphorylation and activation of c-Jun N-terminal kinases (JNKs) and p38 MAP kinases (Zhang et al. 2004a; Fig. 1). In other words, ASK-1 and AIP-1 binding converts the signalosome from an activator of NF-κB to an activator of JNK/p38 MAPK. The pathway leading to JNK but not p38 MAPK activation appears to require the E3 ligase activity of TRAF-2 (Habelhah et al. 2004). When transiently activated, JNKs contribute to the transcription of pro-inflammatory genes by phosphorylating activating protein (AP)-1, a transcription factor typically formed from a heterodimer of c-Jun and c-Fos. However, when JNK activation is sustained much beyond 30 min, it promotes an alternative proteolytic activation of Bid that, like caspase 8 cleavage, can lead to apoptosis (Deng et al. 2003). p38 MAPK can also contribute to apoptosis, but the mechanism is less clear than for JNK. One recently described effect observed in UV-irradiated keratinocytes involves stimulating translocation of Bax to the mitochondria (Van Laethem et al. 2004). Both JNK and p38 may also contribute to transcriptional activation of death-inducing molecules such as Fas and Fas ligand in hepatocytes (Wang et al. 2004) or of p53 in prostatic carcinoma cells (Shimada et al. 2003). p53, a sensor of DNA damage, is an alternative activator of mitochondrial release of death-inducing molecules like cytochrome *c*. Thus, the general effect of JNK or of p38 MAPK activation is pro-apoptotic. In contrast, the MEKK-3/IKK/NF-κB pathway is generally anti-apoptotic. We have already noted that recruitment of ASK-1 and AIP-1 to the signalosome blocks NF-κB activation. The IKK/NF-κB pathway induces proteins that negatively modulate TNF-mediated JNK activation (Tang et al. 2001). The balance between the two forms of the signalosome may determine whether TNF kills or activates ECs.

Following a delay of several hours, activated TRADD, now clearly dissociated from TNF-R1, may recruit FADD and form a DISC similar to that activated by Fas (Michaeu and Tschopp 2003; Fig. 1). Because the TNF-induced DISC is not formed until several hours after the activation of NF-κB, levels of c-FLIP are usually too high to permit effective activation of caspase 8 in the EC and many other cell types (Madge et al. 2003). c-FLIP normally has a short half-life, and cells, including ECs, may be killed by TNF if c-FLIP levels are reduced by blocking its ongoing synthesis, for example with an inhibitor of new

RNA synthesis such as actinomycin D, an inhibitor of new protein synthesis such as cycloheximide, selective inhibition of NF-κB activation (e.g. by over-expression of a non-phosphorylatable form of IκB-α), or a specific antisense oligonucleotide. In normal human EC, Bcl-2 does not protect ECs treated with TNF plus cycloheximide from apoptosis, suggesting that when c-FLIP levels are reduced caspase 8 activation by the DISC is sufficient to cause apoptosis without involvement of Bid cleavage or mitochondrial release of death-inducing proteins.

There are alternative agents which may sensitise ECs to killing by TNF, including low levels of ceramide (Slowik et al. 1997) or the phosphatidylinositol 3-kinase (PI3-K) inhibitor Ly294002 (Madge et al. 2003). In these instances, over-expression of Bcl-2 is protective, implying a need for amplification of the death signal by mitochondria. In this case, caspase 8 may activate Bid. However, two recent observations have suggested that these agents actually enable TNF to initiate a caspase-independent and Bid-independent death pathway. First, killing of human ECs by TNF plus ceramide or Ly294002 cannot be prevented using caspase inhibitory peptides such as zVAD.fmk, an agent that readily blocks killing by TNF plus cycloheximide (Slowik et al. 1997; Madge et al. 2003). Second, the TNF plus Ly294002 response involves lysosomal release (by Ly294002) and subsequent activation (by TNF) of cathepsin B, a protease that activates release of death factors from mitochondria independently of caspase 8 (Madge et al. 2003). Caspases are activated in this process, but only after mitochondria release cytochrome *c*, and caspase inhibition does not prevent cell death, which is possibly mediated by AIF.

The relevance of ceramide or LY294002 to TNF killing in vivo is unclear. Of obvious pathophysiological significance, we have recently observed that IFN-γ can potentiate both apoptotic and non-apoptotic TNF-initiated death pathways, requiring simultaneous blockade of caspases and cathepsin B to avoid EC death (Li and Pober 2005). The effect of IFN-γ on the caspase pathway appears to depend on an increase in pro-caspase 8 rather than on reduced expression of c-FLIP (Li et al. 2002); this may explain why Bcl-2 expression can prevent caspase-mediated cell death in TNF plus IFN-γ-treated human ECs but not in TNF plus cycloheximide-treated ECs. The effect of IFN-γ on the cathepsin B pathway appears to depend on IFN-γ-initiated release of cathepsin B from lysosomes (Li and Pober 2005). Cathepsin B normally operates at acidic pH, the environment within the lysosome. The mechanism by which TNF allows cytosolic cathepsin B to operate at neutral pH is unknown.

There are a number of additional pathways of TNF killing whose relevance to the EC is unclear at present. First, RIP-1, initially identified as a signalling molecule which participates in the signalosome complex that leads to activation of NF-κB (Kelliher et al. 1998), can also trigger non-apoptotic T cell death (Holler et al. 2000). In contrast to NF-κB activation, the death-inducing

activity of RIP-1 does appear to require its kinase activity. Second, TNF-R1 may also interact with an adapter protein called factor associated with neutral sphingomyelinase (FAN); binding of FAN to TNF-R1 is independent of TRADD (Adam-Klages et al. 1996). As the name implies, FAN can activate neutral sphingomyelinase activity, releasing ceramide from membrane sphingomyelin (Adam-Klages et al. 1996), and ceramide by itself can kill ECs via a mitochondrial-dependent death pathway (Slowik et al. 1996). At lower doses, ceramide potentiates killing by TNF as described above. However, FAN appears to be expressed at very low levels in unactivated human umbilical vein (HUV) ECs, and treatment with TNF does not release ceramide. It remains possible that FAN can be induced to higher levels by some as-yet-uncharacterised signal or that it is operative in other human EC types. It has also been observed that in some cell systems TNF can induce ROS in a pathway involving either the mitochondrial electron transport system (Goossens et al. 1999) or arachidonic acid metabolism (O'Donnell et al. 1995; Woo et al. 2000). We will discuss how ROS may kill cells in the next section of the chapter.

It is important to remember that TNF may also induce survival pathways that prevent killing. We have already discussed how TNF activates NF-κB to induce several anti-apoptotic proteins. Another anti-apoptotic effect of TNF involves the activation of PI3-K, an enzyme that converts phosphatidylinositol 4,5 bisphosphate to phosphatidylinositol 3,4,5 trisphosphate. The latter form of this plasma membrane lipid serves as a docking site for cytosolic enzymes containing so-called pleckstrin homology domains. These include phosphatidylinositol-dependent kinases (PDK)-1 and -2 and Akt (also known as protein kinase B). PDKs (or perhaps other lipid-binding protein kinases) contribute to the phosphorylation and activation of Akt on the inner leaflet of the plasma membrane. Thus, by activating PI3-K, TNF activates Akt (Madge and Pober 2000). Akt is a serine-threonine kinase that has been shown to phosphorylate and inactivate a number of pro-apoptotic factors such as glycogen synthase kinase 3, BAD, caspase 9 and Forkhead transcription factors in ECs. Akt together with heat shock protein (Hsp)90 also forms a complex with ASK-1 that inhibits ASK-1 activity (Zhang et al. 2005a). In other words, Akt is an anti-apoptotic enzyme. In some ECs, the activation of PI3-K and Akt may be mediated by signalling through TNF-R2 (CD120b) rather than TNF-R1. In bovine aortic ECs, TNF-R2 is associated with a cytosolic tyrosine kinase called endothelial/epithelial tyrosine kinase (Etk); TNF binding activates to Etk to phosphorylate vascular endothelial growth factor receptor 2 (VEGF-R2), leading to PI3-K activation (Zhang et al. 2003). In HUVECs (and the HUVEC-derived cell line, EA.hy926) signalling through TNF-R1 will activate PI3-K. TNF-R1 does not interact with Etk, and the mechanism for activating PI3-K is unknown; TNFR1-mediated PI3K activation appears to require lipid rafts or caveolae, suggesting that it may similarly involve crosstalk with VEGF-R2 or other receptors found in caveolae (D'Alessio et al. 2005).

4
Injury by Reactive Oxygen Species

The term ROS includes a number of highly reactive moieties that are generated from the partial reduction of molecular oxygen. We have already noted that such species can be generated as by-products by mitochondria or by arachidonate metabolism (O'Donnell et al. 1995; Goossens et al. 1999; Woo et al. 2000). The most potent pathway for generating ROS is catalysed by the enzyme phagocyte oxidase (Phox) complex, which is expressed by neutrophils and macrophages or the homologous non-phagocyte oxidase (Nox) complex, which is expressed in vascular cells. Much of the damage produced by neutrophils is mediated by ROS. Phox or Nox complexes reduce molecular oxygen to superoxide (O_2^-). Superoxide is often converted to hydrogen peroxide (H_2O_2), a reaction catalysed by superoxide dismutase (Cai et al. 2003). A variant low output Pbox is the major pathway for generating H_2O_2 in human ECs (Li and Shah 2003); eNOS may also generate H_2O_2 instead of NO, especially when tetrahydrobiopterin is limiting (Vasquez-Vivar et al. 2003). Human ECs do not express xanthine oxidase, the major generating system for H_2O_2 in rodents. The amount of ROS made by these endogenous systems is much smaller than that generated by neutrophils (via Phox), and ROS injury in humans often requires neutrophil recruitment and activation (i.e. acute inflammation).

ROS may react directly with cellular macromolecules or combine with NO to form even more reactive peroxynitrite. Reaction of cellular macromolecules with peroxide or peroxynitrite radicals results in cell injury; these changes are collectively described as "oxidative stress". Oxidative stress activates biochemical pathways that result in specific cellular responses. A convenient model for studying those responses involves adding an oxidant, such as H_2O_2 or oxidised low-density lipoprotein, to cultured human ECs. When ECs are treated with H_2O_2 in vitro, several changes may result, depending on the concentration. At low levels, cells retract and lose membrane organisation so that luminal, abluminal and junctional proteins may diffuse into other regions of the plasma membranes (Bradley et al. 1995). These changes are reversible. Addition of H_2O_2 also appears to antagonise TNF responses (Bradley et al. 1993). This is caused by activation of TNF-α converting enzyme (TACE), a metalloproteinase that causes shedding of TNF-R1, TNF-R2 and certain other cell surface macromolecules (Madge et al. 1999). [The term TNF-α is sometimes used to distinguish TNF from lymphotoxin (LT, sometimes called LT-α), a structurally related cytokine once designated as TNF-β. LT, which is made by activated T cells, binds to the same receptors and activates the same responses in ECs as TNF (Pober et al. 1987).] At somewhat higher levels of H_2O_2, ECs will undergo apoptosis. If the H_2O_2 levels are increased further, then cellular necrosis (oncosis) may result instead of the orderly death that normally accompanies apoptosis. For example, at high doses of H_2O_2, EC nuclei may swell and the plasma membrane may burst instead of displaying typical apoptotic features

such as nuclear condensation, nuclear fragmentation and plasma membrane vesiculation (J.S. Pober, unpublished observations). ECs that are sub-lethally injured by H_2O_2 are generally dysfunctional. However, chemical destruction of NO by H_2O_2 reduces the effectiveness of NO as a signalling molecule, resulting in endothelial dysfunction independent of actual cell injury.

The biochemical responses that couple oxidative stress to apoptosis are increasingly well understood (Fig. 2). Many of the responses are mediated by p38 MAPK and JNK, and ASK-1 appears to be an important effector molecule in this response (Matsukawa et al. 2004). Oxidants may activate ASK-1 in at least two different ways that are distinct from the AIP-1 pathway utilised by TNF (Fig. 2). First, oxidants may reduce the quantity of reduced glutathione or of reduced thioredoxin in the cell; these proteins normally bind to ASK-1

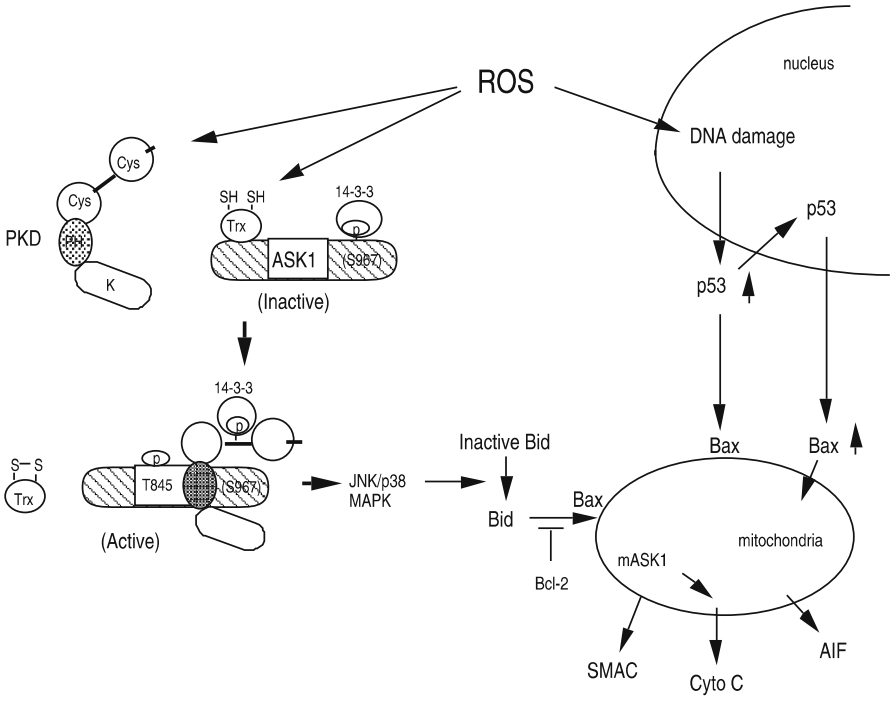


Fig. 2 Overview of ROS signalling in ECs. ROS can cause EC death by at least three different pathways. First, ROS may activate PKD which, in turn, activates ASK-1, leading to JNK-and p38 MAPK-initiated death responses. Second, ROS may directly activate ASK-1 by oxidising natural inhibitors of the enzyme, such as thioredoxin. This pathway may be particularly relevant for activating a sub-population of ASK-1 molecules which are localised within mitochondria. Third, ROS can produce DNA damage. Cycling cells sense DNA damage and express p53. p53 may directly interact with Bax to initiate release of death-inducing proteins (cytochrome *c*, SMAC and AIF) from mitochondria and also acts as a transcription factor to increase the level of proteins (e.g. Bax) that trigger cell death

and keep it in an inactive state (Liu and Min 2002; Zhang et al. 2004b). Second, oxidants may activate protein kinase D (PKD), which in turn can interact with and activate ASK-1 (Zhang et al., 2005b). Oxidative activation of ASK-1 in the cytosol leads to sustained activation of p38 MAPK and JNK and results in mitochondrial release of death-inducing proteins in a process dependent on Bax. A subset of ASK-1 molecules are actually localised within mitochondria in ECs, and mitochondrial ASK-1 may be activated by ROS to directly cause mitochondrial release of death-inducing proteins independent of JNK (Zhang et al. 2004b). In some instances, ECs may show an alternative oxidative stress response triggered by oxidative damage of DNA (Norbury and Zhivotovsky 2004). This depends on induction of the protein p53 (Aoki et al. 2001). Once induced, p53 responds to DNA damage by causing mitochondrial release of death factors, probably through Bax, and can also induce (by acting as a transcription factor) other proteins that augment mitochondrial responses, including Bax itself.

As we have noted for TNF-induced injury, ECs have mechanisms to resist ROS-induced injury. We have already mentioned that reduced glutathione or thioreductase must be depleted before injury ensues. These molecules are abundant in vivo and are unlikely to be exhausted unless large amounts of ROS are generated, e.g. by activated leucocytes. ECs also make enzymes that contribute to oxidative resistance such as haemoxygenase-1 and mitochondrial manganese superoxide dismutase. Interestingly, many of these protective proteins are controlled by NF-κB and are induced by TNF. Other pathways, such as Janus kinase/signal transducer and activator of transcription (JAK-STAT) or extracellular signal regulated kinase (ERK) signalling, activated by interleukin (IL)-6 or IL-11, may also contribute to resistance of ECs to oxidative injury (Waxman et al. 2003). The protective proteins induced or activated by these pathways have not been identified.

5 T Cell-Mediated Injury

When pathogens are not contained by the defence mechanisms of innate immunity, host defence may be strengthened by recruitment of the adaptive immune system. Adaptive immunity both potentiates the effector mechanisms of innate immunity, e.g. by secretion of activating cytokines that enhance neutrophil and macrophage function, and recruits additional types of effector cells, notably effector T lymphocytes. T effector cells may be divided into cytokine-producing T helper (T_H) cells and cytolytic T lymphocytes (CTL). In general, T_H are $CD4^+$-expressing T cells that are activated by the recognition of cognate antigenic peptide displayed in the surface of a specialised antigen-presenting cell (APC) in association with class II major histocompatibility complex (MHC) molecules. T_H cells may be subdivided into those that

specialise in activating mononuclear phagocytes and secrete IFN-ɣ and LT (known as T_H1) and those that specialise in activating eosinophils and secrete IL-4, IL-5 and IL-13 (known as T_H2). CTL, which typically express CD8 instead of CD4, are activated by recognition of cognate peptide antigen in association with class I MHC molecules. CTL secrete cytokines (usually IFN-ɣ and LT but sometimes T_H2-type cytokines) but also express death-inducing signals. For example, CTL contain secretory lysosome-derived granules that contain proteins which, upon release, can deliver lethal signals to target cells (Lieberman 2003). Important effector molecules within their cytolytic granules are perforin, granzyme A and granzyme B (Fig. 3). Perforin acts primarily to facilitate uptake of granzymes into the cytosol or nucleus of the target cells. Granzymes are active serine proteases. Killing by granzyme A is incompletely understood but involves DNA degradation. Granzyme B, like caspases, cleaves aspartyl residues in target proteins and, although the proteins cleaved by granzyme B extensively overlap with those cleaved by caspases, the granzyme B and caspase cleavage sites within these proteins are generally different. Specifically, granzyme B can proteolytically activate certain caspases such as caspase 8 and caspase 3. The activation of caspase 3 by granzyme B is often incomplete and full activation is blocked by the presence of XIAP. Caspase 8 or granzyme B (or both caspase 8 and granzyme B) cleavage of Bid—effecting the release of SMAC/Diablo from the mitochondria—sequesters XIAP and promotes full activation of caspase 3 (Roberts et al. 2003; Fig. 3). Overall, granzyme B-initiated death is typically apoptotic, depending on the activated caspases. Both T_H and CTL may, upon antigen recognition, display surface ligands for death receptors such as Fas ligand or TRAIL, providing an alternative pathway that can also lead to apoptotic death of target cells (Trapani 1998).

ECs are prime targets for injury and death by effector T cells (Choi et al. 2004). This is because ECs display high levels of both class I and class II MHC molecules (which may depend upon tonic exposure to low levels of IFN-ɣ) and also provide additional features, including expression of co-stimulators and adhesion molecules. These characteristics allow for effective T cell activation and stable binding of T cells to ECs, necessary for exocytosis and delivery of granzymes. The most important co-stimulator expressed by human ECs is LFA-3 (CD58), the principal ligand for T cell CD2. Human ECs generally lack CD80 and CD86, the ligands for T cell CD28, but do express a number of additional co-stimulators that are especially relevant for interactions with effector T cells (and effector memory T cells), namely ICOS ligand, 4-1BB ligand and Ox40 ligand. They also express adhesion molecules that preferentially engage counter-receptors on effector and effector-memory T cells, namely intercellular adhesion molecule (ICAM)-1 (CD54, which interacts with T cells LFA-1, also known as CD11a/CD18) and vascular cell adhesion molecule (VCAM)-1 (CD106, which interacts with T cell VLA-4, also known as CD49d/CD29). A central point is that many of these molecules define the characteristics of

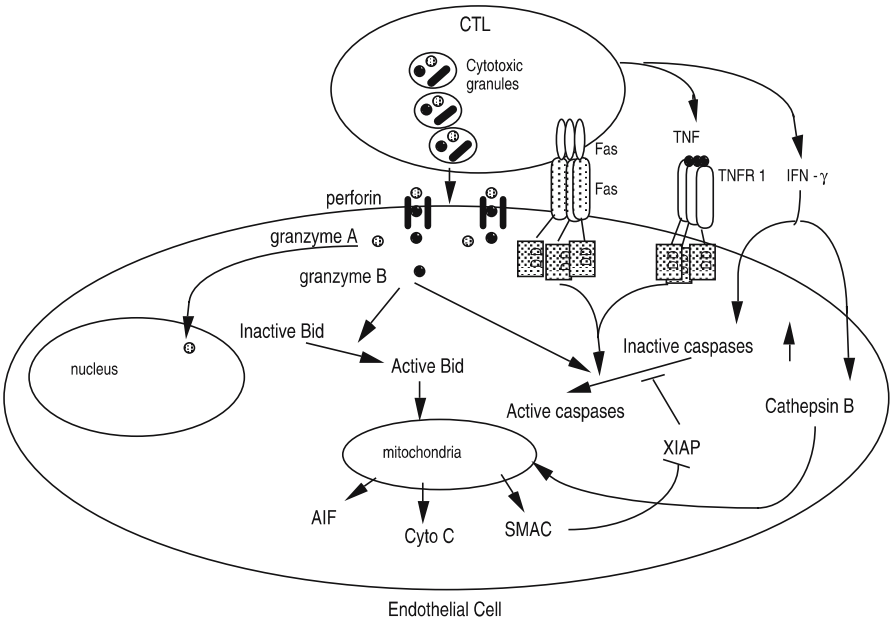


Fig. 3 Overview of CTL signalling in ECs. CTL may kill ECs through three distinct mechanisms. First, CTL granules contain death-inducing protein complexes. Perforin in these complexes promotes uptake of other granule proteins, notably granzymes A and B, into target cells. Granzyme A triggers DNA degradation through an incompletely understood pathway. Granzyme B proteolytically activates caspases 3 and 8 to initiate apoptosis. Granzyme B (or activated caspase 8) can also activate Bid, triggering mitochondrial release of death-inducing proteins. SMAC binding to XIAP may be particularly important for complete activation of caspase 3 by granzyme B. Second, CTL express death receptor ligands such as Fas ligand, with trigger formation of a FADD-containing DISC, initiating caspase 8 activation independently of granzyme B. Third, CTL release cytokines, such as TNF and IFN-ɣ, which produce cell death by pathways described in Fig. 1

effector (and effector memory) T cells, and these are precisely the T cells that bind to and injure ECs.

T cell-derived cytokines may produce EC dysfunction independent of overt injury. For example, we have already noted that TNF can diminish expression of eNOS mRNA and protein. The response of ECs to activated effector T cells, which is in part dependent on TNF, is also dependent on IFN-ɣ. These same two cytokines can act in concert to reorganise the EC cytoskeleton and cell junctions, resulting in loss of permselectivity (also known as vascular leak) (Stolpen et al. 1986). Both responses could be viewed as part of the pro-inflammatory activation response, since NO inhibits leucocyte recruitment and vascular leak permits plasma proteins, such as fibrinogen and fibronectin, to enter the tissue and form a provisional scaffolding on which leucocytes can attach and

crawl. However, even though these responses can be part of host defence when localised, when systemic they result in EC dysfunction. Furthermore, as we noted earlier, the combination of TNF and IFN-ɣ may trigger EC death via caspase- and cathepsin B-initiated pathways (Li and Pober 2005).

ECs are susceptible to death by CTL through granule exocytosis (Zheng et al. 2000; Zheng et al. 2002). This may be a major mechanism of allograft injury in which graft ECs are recognised as if they were infected by virus by a significant fraction of host CTL. Direct recognition of foreign MHC molecules is a molecular cross-reaction in which foreign peptide-specific T cells are triggered by complexes of self or foreign peptides bound to non-self MHC molecules. In vitro, ECs may be protected by peptide inhibition of granzyme B or by drugs that block secretory lysosomal exocytosis (e.g. concanamycin). Human ECs are also protected by over-expression of Bcl-2, suggesting that mitochondrial release of SMAC/Diablo and efficient activation of caspase 3 is critical for cell death. Surprisingly, porcine ECs are not protected from granzyme B killing by Bcl-2 protein, implying that granzyme B acts differently on substrates in pig ECs than in human ECs. IL-11, acting through a JAK-STAT3 pathway, also appears to protect human ECs from CTL in vitro (Mahboubi et al. 2000) and in vivo (Kirkiles-Smith et al. 2004). In the latter system, at least some of the cytoprotective effect derives from induction of survivin, an IAP family member.

6 Summary and Conclusions

In this chapter, we have summarised pathways of injury in ECs mediated by TNF (a major pathway of macrophage-mediated injury), by ROS (a major effector of neutrophil-mediated injury) and by effector T cells; collectively, these mechanisms represent major pathways of inflammation-induced injury. We have also emphasised the connections between EC injury and dysfunction, additionally pointing out the connections between EC activation and dysfunction. Our underlying assumption is that the prevention of EC injury and death can be a strategy to prevent endothelial dysfunction, a major contributor to cardiovascular disease. A key conclusion of this review is that several independent pathways are involved in these responses, and there is unlikely to be a "magic bullet" that will protect ECs from all types of injury. It also must be considered that, while it may appear beneficial to prevent EC death in settings of acute injury, a global approach that blocks cell death pathways could promote survival of mutated and potentially cancerous cells. Nevertheless, the recent and ongoing elucidation of specific death pathways opens new opportunities to intervene in a selective manner to reduce cardiovascular dysfunction and disease.

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Principles and Therapeutic Implications of Angiogenesis, Vasculogenesis and Arteriogenesis

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Abstract The vasculature is the first organ to arise during development. Blood vessels run through virtually every organ in the body (except the avascular cornea and the cartilage), assuring metabolic homeostasis by supplying oxygen and nutrients and removing waste products. Not surprisingly therefore, vessels are critical for organ growth in the embryo and for repair of wounded tissue in the adult. Notably, however, an imbalance in angiogenesis (the growth of blood vessels) contributes to the pathogenesis of numerous malignant, inflammatory, ischaemic, infectious and immune disorders. During the last two decades, an explosive interest in angiogenesis research has generated the necessary insights to develop

the first clinically approved anti-angiogenic agents for cancer and blindness. This novel treatment is likely to change the face of medicine in the next decade, as over 500 million people worldwide are estimated to benefit from pro- or anti-angiogenesis treatment. In this following chapter, we discuss general key angiogenic mechanisms in health and disease, and highlight recent developments and perspectives of anti-angiogenic therapeutic strategies.

Keywords Angiogenesis · Vasculogenesis · Arteriogenesis · Angiogenic disorders · Vessel growth · Endothelial progenitors · Haematopoietic progenitors · Guided navigation · Anti-angiogenic therapy

1 Angiogenic Disorders

After birth, angiogenesis still contributes to organ growth, but during adulthood most blood vessels remain quiescent; angiogenesis only occurs in the cycling ovary and placenta during pregnancy. However, endothelial cells (ECs) retain the remarkable ability of dividing rapidly in response to a physiological stimulus, such as hypoxia and inflammation. Angiogenesis is also reactivated during wound healing and repair. In many disorders, however, this stimulus becomes excessive, and the balance between stimulators and inhibitors is disturbed, resulting in an angiogenic switch. The best-known conditions in which angiogenesis is switched on are malignant, ocular and inflammatory disorders, but many additional processes are affected-such as atherosclerosis, asthma, diabetes, cirrhosis, multiple sclerosis, endometriosis, acquired immunodeficiency syndrome (AIDS), bacterial infections and autoimmune diseases (Table 1). In obesity, adipose tissue may also show excessive growth. A high-fat diet induces an angiogenic gene programme in fat (Li et al. 2002) and angiogenic factors stimulate adipogenesis, while treatment of obese mice with anti-angiogenic agents results in weight reduction and adipose tissue loss (Rupnick et al. 2002). Viral and bacterial pathogens carry angiogenic genes of their own (Meyer et al. 1999), or induce the expression of angiogenic genes in the host (Harada et al. 2000). The human herpesvirus-8 transforms ECs and causes Kaposi's sarcoma in human immunodeficiency virus (HIV)-1-infected AIDS patients (Barillari and Ensoli 2002).

In other diseases, such as ischaemic heart disease or pre-eclampsia, the angiogenic switch is insufficient, thereby causing EC dysfunction, vessel malformation and regression, or preventing revascularisation, healing and regeneration (Table 2). In the skin, age-dependent reductions in vessel density and maturation cause vessel fragility, leading to hair loss and the development of purpura, telangiectasia, angioma and venous lake formation (Chang et al. 2002). A progressive loss of the microvasculature in elderly people has been implicated in nephropathy (Kang et al. 2001), bone loss (Martinez et al. 2002) and impaired re-endothelialisation after arterial injury (Gennaro et al. 2003). Diabetes, atherosclerosis and hyperlipidaemia also impair vessel growth

Table 1 Diseases characterised or caused by abnormal or excessive angiogenesis

Organ	Disease in mice or humans
Numerous organs	Cancer (activation of oncogenes; loss of tumour suppressors) and metastasis; infectious diseases (pathogens that express angiogenic genes (Meyer et al. 1999), induce angiogenic programmes (Harada et al. 2000) or transform ECs (Barillari and Enzoli 2002; Wang et al. 2004)); vasculitis and angiogenesis in auto-immune diseases such as systemic sclerosis, multiple sclerosis and Sjögren's syndrome (Kirk and Karlik 2003; Ohno et al. 2004; Storkebaum et al. 2004)
Vasculature	Vascular malformations (Tie-2 mutation (Vikkula et al. 1996)); DiGeorge syndrome (low VEGF/Nrp-1 expression, (Stalmans et al. 2003)); hereditary haemorrhagic telangiectasia (mutation of endoglin or ALK (van den Driesche et al. 2003; Lebrin et al. 2005)); cavernous haemangioma (loss of Cx37/40 (Simon and McWhorter 2002)); cutaneous haemangioma (VG5Q mutation (Lambrechts and Carmeliet 2004; Tian et al. 2004)); transplant arteriopathy and atherosclerosis (Kahlon et al. 1992; Khurano et al. 2005; Nakano et al. 2005)
Skin	Psoriasis (high VEGF and Tie2 (Xia et al. 2003; Leong et al. 2005; Voskas et al. 2005)); warts (Harada et al. 2000); allergic dermatitis (high VEGF and PlGF (Oura et al. 2003; Agha-Majzoub et al. 2005)); scar keloids (Yang et al. 2003; Gira et al. 2004); pyogenic granulomas; blistering disease (Brown et al. 1995); Kaposi's sarcoma in AIDS patients (Barillari and Enzoli 2002); systemic sclerosis (Distler et al. 2004)
Adipose tissue	Obesity (angiogenesis induced by fat diet); weight loss by angiogenesis inhibitors; anti-VEGFR2 inhibits preadipocyte differentiation via effects on ECs (Fukumura et al. 2003); adipocytokines stimulate angiogenesis (Shibata et al. 2004))
Eye	Persistent hyperplastic vitreous syndrome (loss of Ang-2 (Hackett et al. 2000; Gale et al. 2003) or $VEGF^{164}$ (Stalmans et al. 2002)); diabetic retinopathy; retinopathy of prematurity (Campochiaro 2004); choroidal neovascularisation (Campochiaro 2004) (TIMP-3 mutation (Qi et al. 2003))
Bone, joints	Arthritis and synovitis (Arima et al. 2005; Lainer and Brahn 2005; Szekanecz et al. 2005; Taylor and Sivakumar 2005); osteomyelitis (Hausman and Rinker 2004); osteophyte formation (Luttun et al. 2002); HIV-induced bone marrow angiogenesis (Patsouris et al. 2004)

Table 1 (continued)

Organ	Disease in mice or humans
Lung	Primary pulmonary hypertension (BMPR-2 mutation; somatic EC mutations (Yeager et al. 2001; Humbert and Trembath 2002; Voelkel et al. 2002)); asthma (Bai and Knight 2005), nasal polyps (Gosepath et al. 2005); rhinitis (Kirmaz et al. 2004); chronic airway inflammation (Baluk et al. 2005), cystic fibrosis (Shute et al. 2003)
Gastro-intestinal tract	Inflammatory bowel disease (ulcerative colitis (Konno et al. 2004)); liver cirrhosis (Ward et al. 2004; Fernandez et al. 2005; Medina et al. 2005)
Reproductive system	Endometriosis (Hull et al. 2003; Groothuis et al. 2005); uterine bleeding, ovarian cysts (Abd el Aal et al. 2005); ovarian hyperstimulation (LeCouter et al. 2001)
Kidney	Diabetic nephropathy (Yamamoto et al. 2004; Schrijvers et al. 2005)

BMPR-2, bone morphogenic protein-2

(Van Belle et al. 1997; Waltenberger 2001; Tepper et al. 2002), whereas hypertension causes microvascular rarefaction (Boudier 1999). Reduced angiogenic signalling causes pulmonary fibrosis (Koyama et al. 2002) and emphysema (Kasahara et al. 2000). The delayed healing of gastric or aphthous oral ulcerations has been attributed to the ability of invading pathogens to produce angiogenesis inhibitors-in particular after *Helicobacter pylori* infections (Jenkinson et al. 2002). Besides its vascular activity, vascular endothelial growth factor (VEGF) is also trophic for nerve cells, cardiac muscle fibres and lung epithelial cells, further explaining why insufficient VEGF levels contribute to cardiac failure, respiratory distress and motor neuron degeneration, reminiscent of amyotrophic lateral sclerosis (Table 2). At present, angiogenesis has been implicated in more than 70 disorders, and the list is ever-growing.

2 Modes of Vessel Growth

In the developing embryo as well as in adult tissues, key events and distinct mechanisms exist to establish and maintain a functional vascular network (Fig. 1). Endothelial progenitor cells (EPCs) arising from various embryonic regions or from adult bone marrow can form vessels in a process referred to as vasculogenesis. Angiogenesis denotes the process in which budding from preexisting vessels gives rise to sprouts of new blood vessels, while arteriogenesis refers to the stabilisation of these new sprouts by mural cells such as pericytes and smooth muscle cells (SMCs)-arteriogenesis is critical for the new vasculature to become stable, mature and functional. Collateral vessel growth repre-

Table 2 Diseases characterised or caused by insufficient angiogenesis or vessel regression

Organ	Disease in mice or humans	Angiogenic mechanism
Nervous system	Alzheimer's disease	Vasoconstriction, microvascular degeneration and cerebral angiopathy due to EC toxicity by amyloid-β (de la Torre 2004; Zlokovic 2005)
	Amyotrophic lateral sclerosis; diabetic neuropathy	Impaired perfusion and neuroprotection, causing motoneurone or axon degeneration due to insufficient VEGF production (Oosthuyse et al. 2001; Lambrechts et al. 2003; Azzouz et al. 2004; Storkebaum and Carmeliet 2004; Storkebaum et al. 2005)
	Stroke	Correlation of survival with angiogenesis in brain (Krupinski et al. 1994); stroke due to arteriopathy (Notch-3 mutations (Kalimo et al. 2002))
Vasculature	Diabetes	Characterised by impaired collateral growth (Waltenberger 2001) and angiogenesis in ischaemic limbs (Rivard et al. 1999), but enhanced retinal neovascularisation secondary to pericyte drop-out (Caldwell et al. 2005)
	Hypertension	Microvessel rarefaction due to impaired vasodilatation or angiogenesis (Boudier 1999; Kubis et al. 2002; Sane et al. 2004)
	Atherosclerosis	Characterised by impaired collateral vessel development (Van Belle et al. 1997)
	Restenosis	Impaired re-endothelialisation after arterial injury (Gennaro et al. 2003)
Heart	Ischaemic heart disease, cardiac failure	Imbalance in capillary-to-cardiomyocyte fibre ratio due to reduced VEGF levels (Jesmin et al. 2005; Shiojima et al. 2005)
Gastro-intestinal tract	Gastric or oral ulcerations	Delayed healing due to production of angiogenesis inhibitors by pathogens (*H. pylori*) (Jenkinson et al. 2002; Kim et al. 2004)
	Crohn's disease	Characterised by mucosal ischaemia (Konno et al. 2004; Hatoum et al. 2005)

Table 2 (continued)

Organ	Disease in mice or humans	Angiogenic mechanism
Bone	Osteoporosis, impaired bone fracture healing	Impaired bone formation due to age-dependent decline of VEGF-driven angiogenesis (Martinez et al. 2002); angiogenesis inhibitors prevent fracture healing (Yin et al. 2002); osteoporosis due to low VEGF (Pufe et al. 2003); healing of fracture non-union is impaired by insufficient angiogenesis (Hausman and Rinker 2004)
Skin	Hair loss	Retarded hair growth by angiogenesis inhibitors (Yano et al. 2001)
	Skin purpura, telangiectasia, and venous lake formation	Age-dependent reduction of vessel number and maturation (SMC drop-out) due to EC telomere shortening (Chang et al. 2002)
	Systemic sclerosis, lupus	Insufficient compensatory angiogenic response (Mackiewicz et al. 2002)
Reproductive system	Pre-eclampsia	EC dysfunction, resulting in organ failure, thrombosis and hypertension due to deprivation of VEGF by soluble Flt1 (Maynard et al. 2003; Levine et al. 2004)
	Menorrhagia (uterine bleeding)	Fragility of SMC-poor vessels due to low Ang-1 production (Hewett et al. 2002)
Lung	Neonatal respiratory distress syndrome (RDS)	Insufficient lung maturation and surfactant production in premature mice with low HIF-2/VEGF (Compernolle et al. 2002); low VEGF levels in human neonates also correlate with RDS (Tsao et al. 2005)
	Pulmonary fibrosis, emphysema	Alveolar EC apoptosis upon VEGF inhibition (Kasahara et al. 2000; Tang et al. 2004b; McGrath-Morrow et al. 2005)
Kidney	Nephropathy (ageing; metabolic syndrome); glomerulosclerosis; tubulointerstitial fibrosis	Characterised by vessel dropout, microvasculopathy and EC dysfunction (low VEGF; high TSP1) (Kang et al. 2001; Gealekman et al. 2004; Long et al. 2005); recovery of glomerular/peritubular ECs in glomerulonephritis, thrombotic microangiopathy and nephrotoxicity is VEGF-dependent (Schrijvers et al. 2004)

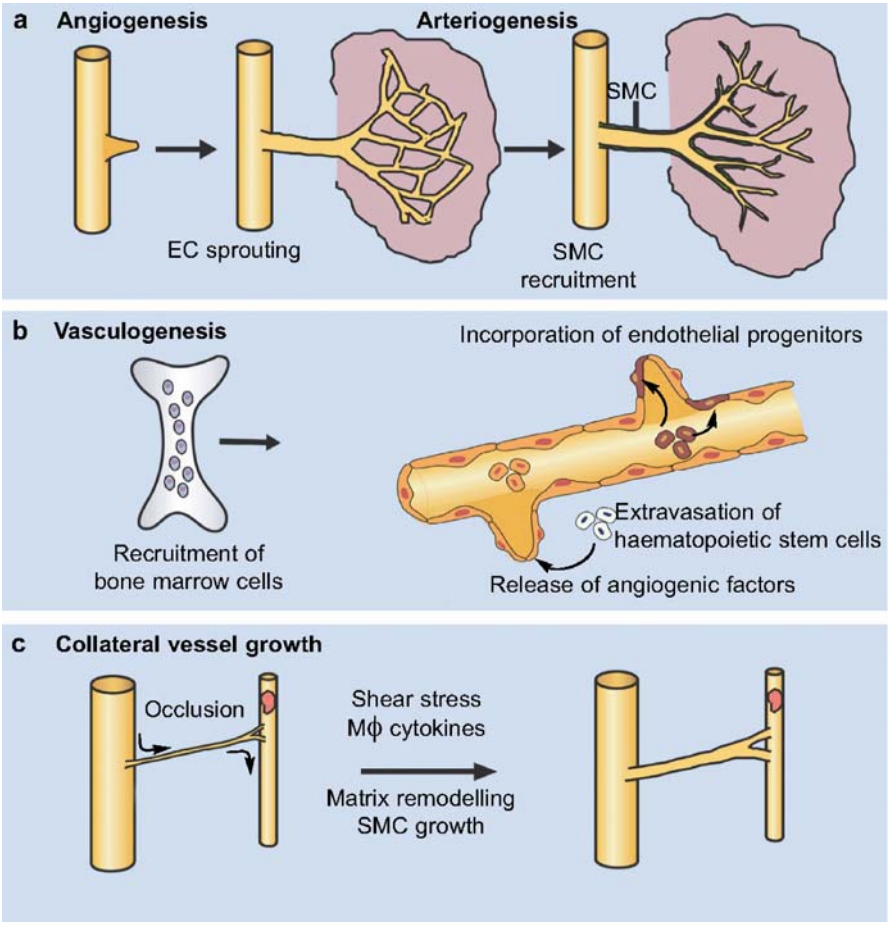

Fig. 1 a–c Mechanism of vessel growth. **a** Angiogenesis denotes the sprouting of new endothelial cell-lined vessels from pre-existing vessels; arteriogenesis refers to the subsequent stabilisation of nascent vessels via recruitment of smooth muscle cells. **b** Vasculogenesis refers to the recruitment of bone marrow-derived endothelial progenitors, which are incorporated into nascent vessels or stimulate new vessel growth by releasing pro-angiogenic factors. **c** Collateral vessel growth denotes the expansive growth of pre-existing collateral vessels upon occlusion of a supply vessel, for instance by a thrombus. Recruitment of macrophages and monocytes to the shear stress-activated endothelium plays a critical role in this process

sents the formation of collateral bridges between arterial networks and remodelling of pre-existing vessels after occlusion of a main artery-this type of vessel growth is of major therapeutic importance. A fine-tuned interplay between molecular signals in a spatial and temporal manner is necessary for these essential events to occur. We will now discuss these individual steps in more detail.

3 Vasculogenesis

Vasculogenesis has now been documented in the embryo and adult. In the embryo, mesoderm-derived EPCs give rise to the first embryonic blood vessels. In the adult, EPCs originating from the bone marrow enter the circulation and are recruited to sites of neovascularisation. These two different events will be discussed separately in this chapter. In addition, we will discuss the exciting recent insights that haematopoietic progenitors also contribute to the formation of new vessels in the embryo and adult.

3.1 Endothelial Progenitors in the Embryo

In the yet-avascular embryo, blood vessels emerge through recruitment of separate mesodermal precursors at distinct locations in the mesoderm (Fig. 2). In amniotes in particular, the first blood vessels arise in the extra-embryonic

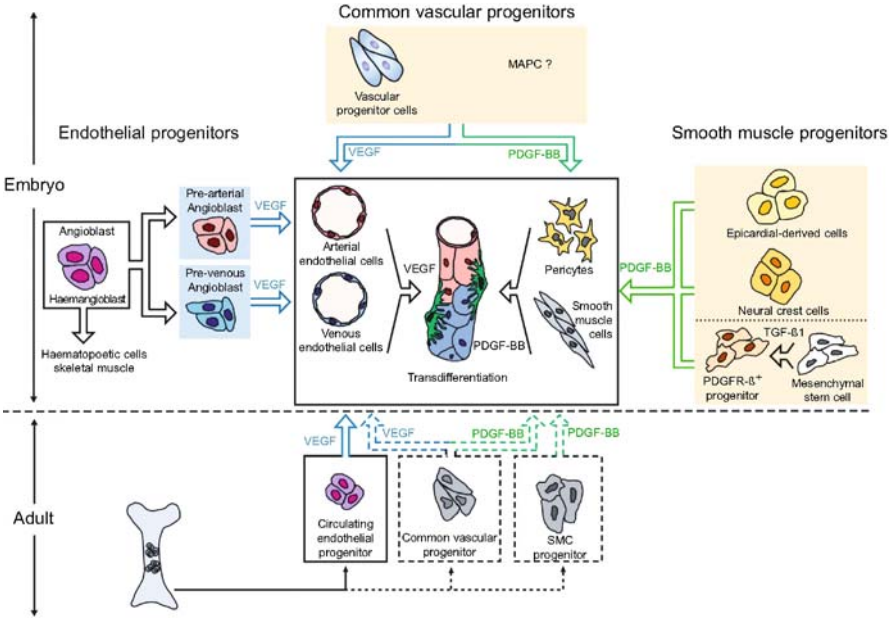

Fig. 2 Vascular progenitors in the embryo and adult. Endothelial, smooth muscle and common vascular progenitors contribute to vascular development in the embryo. Smooth muscle cells have different origins, as indicated. Recently, endothelial and smooth muscle progenitors, derived from the bone marrow, have also been demonstrated in the adult. The effect of VEGF, platelet-derived growth factor (PDGF)-BB and other growth factors on these progenitors is indicated

mesoderm of the yolk sac, when mesenchymal cells aggregate to form blood islands. The inner cells form primitive blood cells, while the outer boundaries give rise to precursors for ECs. Vascular progenitors that contribute to the formation of the major embryonic vascular system also derive from the intra-embryonic mesoderm, and differentiate to form the dorsal aorta, cardinal veins and vitelline plexus. Within the embryo, the different mesodermal compartments vary in their vasculogenic capacity; the splanchnopleural and the paraxial mesoderm are the richest in endothelial precursor cells. Grafting experiments in quail and chick embryos suggest the existence of two distinct lineages of EPCs (Pardanaud et al. 1996). A first lineage, derived from the paraxial mesoderm, is known to have solely angioblastic capacity. A second bipotential haemangioblastic precursor cell line is derived from the splanchnopleural mesoderm, and differentiates to both endothelial and haematopoietic cells. Although no ultimate proof has been provided, the close proximity of differentiating haematopoietic and ECs at sites of both extra- and intra-embryonic vasculogenesis (de Bruijn et al. 2000) suggests the existence of a bipotential mesodermal precursor cell for both systems, the haemangioblast (Choi 2002; Ribatti et al. 2002; Rafii and Lyden 2003).

A common origin for blood and ECs is further suggested by molecular links between the embryonic precursors of the angiogenic and haematopoietic lineages, which share expression of a number of different genes, such as *CD34*, *CD133*, *PECAM-1* (encoding platelet EC adhesion molecule-1), c-*Kit* and *Sca-1*. In addition, in vitro experiments have shown that a transient population of so-called blast colony-forming cells (BL-CFC) can be derived from embryonic stem cell cultures (Kennedy et al. 1997; Nishikawa et al. 1998). BL-CFCs are responsive to VEGF and contain both endothelial and haematopoietic precursors. Strikingly, expression of mesodermal genes precedes the expression of genes marking early stage endothelial and haematopoietic development in these embryonic stem cell lines (Kennedy et al. 1997; Fehling et al. 2003), thus recapitulating the gene expression sequence observed in the yolk sac in vivo. Moreover, isolation of single cells expressing VEGF-receptor 2 (VEGFR-2; also foetal liver kinase 1, Flk-1) that can give rise to both endothelial and haematopoietic cells in vitro strongly suggests the existence of a common progenitor for the two lineages, even though it does not rule out the possibility that this progenitor is actually a more primitive multipotent precursor.

Although the molecular players determining the critical early steps of haemangioblast differentiation in the blood islands are not yet fully elucidated, several genes have so far been implicated in this process, including *Ets-1*, *Fli-1*, *Vezf-1* and *VEGFR-2*, along with the transcription factor Tal-1 (T cell acute leukaemia, also *Scl*) and members of the GATA, Hox and inhibitor of differentiation (Id) protein families (Rafii and Lyden 2003).

VEGFR-2 is expressed on both mature ECs and endothelial precursors, and embryos deficient in VEGFR-2 display an early arrest in haemangioblast

differentiation (Shalaby et al. 1995). In contrast, loss of VEGF induces severe vascular defects, but ECs still differentiate in the absence of VEGF (Carmeliet et al. 1996). Whether this implies that VEGF-C, which also binds to VEGFR-2, might be involved in haemangioblast differentiation remains to be determined. Tal-1 is involved in early cell fate determination of the haemangioblast, most likely by exerting combinatorial effects with VEGFR-2 (Visvader et al. 1998; Ema et al. 2003). In contrast, targeted inactivation of VEGFR-1 (also fms-like tyrosine kinase 1, Flt-1) does not prevent haemangioblast differentiation, but leads to vascular disorganisation, most likely due to an excess in EPCs (Fong et al. 1995; Kearney et al. 2002).

Endodermal signals may also regulate vasculogenesis in the adjacent mesoderm. Recent studies in *Xenopus* and in avian embryos suggest that the endoderm regulates the assembly of angioblasts to vascular tubes-rather than the specification of the haemangioblast lineage-and that sonic hedgehog (Shh) signalling is the key mediator involved in this interaction (Vokes and Krieg 2002; Vokes et al. 2004). By contrast, genetic studies in zebrafish show that the endoderm regulates the directional migration of the angioblasts to the midline (Jin et al. 2005).

3.2 Endothelial Progenitors in the Adult

Until recently, neovascularisation in the adult has been primarily attributed to sprouting angiogenesis. However, the isolation of putative EPCs from circulating mononuclear cells in the peripheral blood of adult humans and the demonstration that such EPCs home to sites of neovascularisation and contribute to this process introduced the concept of "post-natal vasculogenesis" (Figs. 1 and 2). EPCs, isolated from human peripheral blood mononuclear cells (PB-MNCs), express VEGFR-2 and CD34 (Asahara et al. 1997). Various other cell surface markers [c-kit, CXCR4, von Willebrand's factor (vWF), CD31, CD146] have been identified on EPCs (Rafii and Lyden 2003). Expression of AC133, an orphan receptor that is specifically expressed on immature EPCs, is lost upon differentiation into more mature ECs (Iwami et al. 2004).

EPCs should be distinguished from circulating ECs (CECs) in the peripheral blood, which are sloughed off due to shedding from the existing vasculature and enter the circulation as a result of traumatic or infectious vascular injury, or tumour growth. Unlike EPCs, mature CECs do not express the stem cell marker AC133 (Rafii and Lyden 2003). Not all circulating CECs are viable, and the fraction of apoptotic CECs in the peripheral blood increases upon treatment of tumour-bearing mice with anti-angiogenic agents. Besides bone marrow-derived EPCs, multipotent adult progenitor cells (MAPCs) with angioblastic potency have been identified in the bone marrow (Reyes et al. 2002), while tissue-specific stem cells might also exist. In skeletal muscle, myo-endothelial progenitors might differentiate locally into muscle or ECs (Tamaki et al. 2002).

In the bone marrow niche, EPCs are likely to reside in close association with haematopoietic stem cells and stromal cells. Though not yet fully elucidated, these cells are possibly involved in promoting local EPC proliferation and transmigration across the bone marrow/blood barrier via secretion of VEGF, placental growth factor (PlGF) and other angiogenic factors (Tordjman et al. 2001). Mobilisation of EPCs from the bone marrow, as well as their recruitment to sites of adult vasculogenesis, involves a number of similar cues that also regulate EC sprouting (angiogenesis), such as VEGF (Asahara et al. 1999), fibroblast growth factor-2 (FGF-2), PlGF (Carmeliet et al. 2001; Rafii and Lyden 2003) platelet-derived growth factor-CC (PDGF-CC; Li et al. 2005), and angiopoietin-1 (Ang-1; Hattori et al. 2001), in addition to other factors such as metalloproteinases and adhesion molecules (Rafii and Lyden 2003). A chemoattractant for haematopoietic progenitor cells (HPCs), the chemokine stromal cell-derived factor-1α (SDF-1α), also induces migration of EPCs (some of which express the SDF-1α receptor CXCR-4; Yamaguchi et al. 2003) and enhances VEGF-mediated proliferation of ECs (Peichev et al. 2000; Neuhaus et al. 2003), while inhibition of SDF-1α blocks EPC recruitment to tumours (Guleng et al. 2005).

To what extent EPCs contribute to vascular growth remains an outstanding question. Apart from differentiating to mature ECs, which are incorporated as building blocks in the endothelial layer (Peichev et al. 2000) in nascent vessels, mononuclear cells might also create, together with accessory cells derived from the bone marrow, a pro-angiogenic microenvironment to facilitate neovascularisation. For instance, CD34-expressing precursor cells, mobilised from the bone marrow, stimulate vascularisation in myocardial infarcts both via vasculogenic in situ vessel formation and via stimulation of angiogenic sprouting by local induction of angiogenic growth factor secretion (Kocher et al. 2001).

The relative numeric contribution of bone marrow-derived EPCs to adult organ and tumour neovascularisation is highly variable. In different experimental settings of pathological angiogenesis, incorporation of EPCs into the growing vasculature has been reported to be remarkably high (Garcia-Barros et al. 2003) or negligibly low (Rajantie et al. 2004; Ziegelhoeffer et al 2004). Apart from differences in the genetic background of mouse strains used for those studies, the variability might also reflect differences of spontaneous and xenografted tumours in their dependence on bone marrow-derived endothelial precursors (Ruzinova et al. 2003). Mathematical models have been suggested to calculate-and possibly predict-the contribution of EPCs to tumour neovascularisation (Stoll et al. 2003).

Despite these unresolved questions, the concept of post-natal vasculogenesis offers challenging clinical perspectives for the treatment of cardiovascular disorders and cancer. Mobilisation of EPCs from the bone marrow is enhanced in patients with ischaemic conditions (Shintani et al. 2001), and levels of circulating EPCs have been introduced as a valuable clinical parameter for cardiovascular risk assessment (Hill et al. 2003). In tumour-bearing mice, EPC

(and CEC) levels in peripheral blood correlate with the anti-angiogenic effect of angiogenesis inhibitors on tumour angiogenesis and growth (Shaked et al. 2005), suggesting EPCs (and CECs) as a useful biomarker for dose finding and monitoring the effect of anti-angiogenic treatment in cancer (see Sect. 6.1).

3.3 The Endothelial/Haematopoietic Connection: An Emerging Theme

In the embryo, haematopoietic stem cells (HSCs) migrate into avascular areas and attract sprouting ECs by releasing angiogenic factors such as Ang-1 (Takakura et al. 2000). In the adult, bone marrow-derived haematopoietic cells-expressing markers such as Sca-1, c-Kit, CXCR4 and VEGFR1-become recruited, often together with EPCs, to tumours or ischaemic tissues in response to VEGF and PlGF (Hattori et al. 2002; Rafii and Lyden 2003; Grunewald et al. 2005; Orimo et al. 2005). These angio-competent cells extravasate around nascent vessels, where they are retained by SDF-1α, and stimulate growth of resident vessels by releasing angiogenic factors such as VEGF, PlGF and angiopoietin-2 (Ang-2; Ceradini et al. 2004; Butler et al. 2005; Okamoto et al. 2005). In other cases, these cells function as haemangioblasts, producing both haematopoietic and endothelial progenitors, which give rise to new blood vessels (Rafii and Lyden 2003). Furthermore, in response to PlGF released by tumour cells, VEGFR-1-expressing haematopoietic bone marrow progenitors home to tumour-specific pre-metastatic sites, where they recruit tumour cells and EPCs; anti-VEGFR1 antibodies prevent the formation of such pre-metastatic niches (Riba et al. 2005).

3.4 Arterial and Venous Cell Fate Specification

Arteries and veins have evolved as anatomically distinct but closely interconnected blood vessel types. The structural differences between arteries and veins were attributed to different flow dynamics and distinct physiological requirements. But, evidence has recently emerged that molecular differences between arterial and venous ECs already exist even before blood vessels are formed, and that complex genetic pathways are responsible for this arterial versus venous specification. The expression of the ligand ephrinB2 in arteries and of the Eph receptor tyrosine kinase EphB4 in veins occurs before the onset of circulation (Gerety et al. 1999; Gerety and Anderson 2002). This indicates that while ephrins are essential for proper distinction between arterial and venous cells, they are not required for the initial fate decision that distinguishes arterial and venous endothelial progenitors.

Lineage tracking in zebrafish embryos indicates that angioblasts in the lateral posterior mesoderm receive signals from the notochord and the ventral

endoderm, and become restricted to the aorta or trunk vein (Zhong et al. 2001). Studies in zebrafish and *Xenopus* indicate that Shh, produced by the notochord, specifies arterial EC fate (Lawson and Weinstein 2002: Lawson et al. 2002). Indeed, formation of the aorta is impaired in zebrafish embryos mutant for *sonic you* (*syu*), the zebrafish homologue of Shh (Chen et al. 1996; Brown et al. 2000) or after morpholino knock-down of Shh (Lawson et al. 2002). Shh induces the expression in the adjacent somites of VEGF which, in turn, drives arterial differentiation of angioblasts. In the chick, the early extra-embryonic blood islands contain a mixture of subpopulations of cells expressing Nrp-1 and Nrp-2, which subsequently become lineage markers of arteries and veins, respectively (Herzog et al. 2005). This suggests that even early angioblasts may already be committed to either the arterial or venous lineage. Further evidence that VEGF has a role stems from findings that, when released from Schwann cells, it induces arterial specification of vessels, tracking alongside these nerves (Mukouyama et al. 2002) and that Nrp-1, a receptor selective for the $VEGF^{165}$ isoform, is expressed in arterial beds (Moyon et al. 2001; Stalmans et al. 2002). VEGF also determines arterial EC specification after birth in the heart and retina, where the matrix-binding $VEGF^{188}$ isoform is critical for arterial development (Stalmans et al. 2002; Visconti et al. 2002).

The Notch pathway acts downstream of VEGF in arterial EC specification (Lawson et al. 2002). Notch signalling is initiated when the Notch receptors (Notch 1-4) are activated by their ligands Jagged-1, Jagged-2 and Delta-like-1, -3 and -4 (Alva and Iruela-Arispe 2004). During vascular development, defects in Notch signalling disrupt normal arterial-venous differentiation, resulting in loss of artery-specific markers (e.g. ephrinB2) and ectopic expression of venous markers (e.g. Flt-4) in the aorta (Lawson et al. 2001). Conversely, over-activation of Notch suppresses differentiation of vessels to veins. Furthermore, Hey2, a transcription factor that is induced by Notch signalling, confers features of arterial EC gene expression on vein-derived ECs, up-regulating arterial-specific genes, including *ADHA1* (aldehyde dehydrogenase 1 family, member A1), *EVA1* (epithelial V-like antigen 1) and *keratin 7*, while suppressing vein-specific genes, such as *GDF* (growth and differentiation factor), *lefty-1* and *lefty-2* (Chi et al. 2003). The hairy-related transcription factor gridlock is required for the early assignment of arterial endothelial identity (Zhong et al. 2000). Zebrafish lacking this protein show a disrupted assembly of the aorta in the posterior part of the body (Zhong et al. 2000, 2001), while gridlock over-expression caused a similar disruption of the vein without affecting the artery (Zhong et al. 2001). All these genetic findings appear to refute the hypothesis that physiological cues are responsible for arterio-venous differentiation. However, even after ECs attain a specific arterial or venous phenotype late in embryonic development, this genetic programme still remains remarkably plastic (Moyon et al. 2001).

3.5
Tissue-Specific Endothelial Cell Differentiation

ECs in different organs acquire highly specialised properties which permit these cells to optimally perform specific functions within each tissue and organ (Ruoslahti and Rajotte 2000). For instance, ECs in the brain are tightly linked to each other and are surrounded by numerous peri-endothelial cells which constitute a barrier that protects brain cells from potentially toxic blood-derived molecules. The development of the blood-brain barrier requires the interactions between astroglial cells that express glial fibrillary acidic protein, pericytes and adequate angiotensinogen levels (Lindahl et al. 1998). The tight junctional complex between ECs consists of numerous integral membrane and cytosolic proteins, belonging to the families of cadherins, occludins, claudins and membrane-associated guanylate kinase homologous proteins (Rubin and Staddon 1999; Tsukita and Furuse 1999). In contrast, vessels in endocrine glands lack these tight junctions. Their endothelium is rather discontinuous and fenestrated, allowing high volume molecular and ion transport as well as hormone trafficking. Overall, the factors that regulate acquisition of specific endothelial properties are largely unknown. However, it appears that the interaction with the host environmental extracellular matrix, in concert with VEGF, plays a major role (Risau 1995; Andries et al. 1998). Besides vascular cell heterogeneity in distinct organs, ECs within the same organ can even be heterogeneous. In the heart, for instance, ECs in distinct locations of the coronary vascular tree differ in their expression of the endothelial constitutive nitric oxide (NO) synthase isoform (Andries et al. 1998), brain-derived neurotrophic factor (Donovan et al. 2000) or adhesion molecules (Derhaag et al. 1996, 1997). Even within a single vessel, ECs may have distinct cell fates. For example, three types of ECs, each with a distinct cell fate, build the inter-segmental vessels in the zebrafish embryo (Childs et al. 2002).

Recently, genetic studies in mice, zebrafish and *Xenopus* have started to unravel the transcriptional code that determines EC fate (Brown et al. 2000; Liao et al. 2000). This code involves basic helix-loop-helix (bHLH) transcriptional activators [hypoxia-inducible factor (HIF)-2α, stem cell leukaemia factor, Tfeb; Carmeliet 1999] as well as Id repressors as demonstrated by the perturbation of developmental and tumour-associated angiogenesis in mice lacking Id-1/3 (Neufeld et al. 2002). However, there are other mechanisms determining endothelial heterogeneity and organ-specific angiogenesis. For instance, the activity of VEGF and Ang-1 varies in different tissues. Low permeability tumours over-express Ang-1 and/or under-express VEGF or PlGF, whereas tumours with high permeability lack Ang-1 but over-express Ang-2 (Jain and Munn 2000). Another example is the effect of Ang-1, which stimulates angiogenesis in the skin but suppresses vascular growth in the heart (Suri et al. 1998; Visconti et al. 2002).

Moreover, tissue-specific angiogenic factors determine the angiogenic switch restrictedly in particular organs. A striking example of this novel class of cues comprises the endocrine gland-derived VEGF (EG-VEGF) and Bv8, which selectively affect EC growth and differentiation (fenestration) in endocrine glands (LeCouter et al. 2001, 2002, 2004; Ferrara et al. 2004b). Other organ-specific angiogenic factors include Bves and fibulin-2 in the heart (Wada et al. 2001), and glial-derived neurotrophic factor in the brain. That ECs in different tissues are distinct is further suggested by their considerably different response to anti-angiogenic factors. Indeed, ECs in endocrine glands rapidly loose their fenestrations and even become apoptotic in response to VEGF inhibitors, resulting in a 70% loss of the microvasculature in these organs. In contrast, the microvasculature in other organs is much more resistant to such pruning in response to anti-VEGF therapy (Tang et al. 2004a; Kamba et al. 2005). Malignant cells also induce ECs in tumour vessels to acquire a distinct fate and express unique markers ("vascular zip codes") that are absent or barely detectable in quiescent blood vessels of normal tissue (Ruoslahti 2002, 2004). Tumour cells also change the responsiveness of ECs to cues-for instance, epidermal growth factor (EGF) up-regulates its receptors in tumour-associated vessels, thereby making these ECs responsive to the mitogenic activity of EGF-a finding of significant therapeutic relevance.

4
Angiogenesis

Once a nascent primitive vascular labyrinth has been formed through vasculogenesis, it successively expands and remodels into a more complex, hierarchically and stereotypically organised network of larger vessels, ramifying into smaller vessels. This process, referred to as angiogenesis, involves sprouting, bridging and intussusceptive growth from pre-existing vessels, navigation and guidance, as well as remodelling and pruning. We will now discuss this process by viewing it as a step-wise progression of the following orderly series of events (Fig. 1). In response to angiogenic factors released in nearby hypoxic regions, activated ECs induce extracellular matrix (ECM) degradation, proliferate and navigate towards these angiogenic cues, sprouting into new vessels. Supported by surrounding SMCs and pericytes, nascent vessels consequently stabilise, mature and acquire specified functional properties to accommodate local requirements-namely arteriogenesis (Fig. 3).

4.1
Vascular Permeability and Extracellular Matrix Degradation

Water and nutrients move from blood to tissues across the walls of capillaries and venules. The wall of blood vessels is composed of ECs and mural cells,

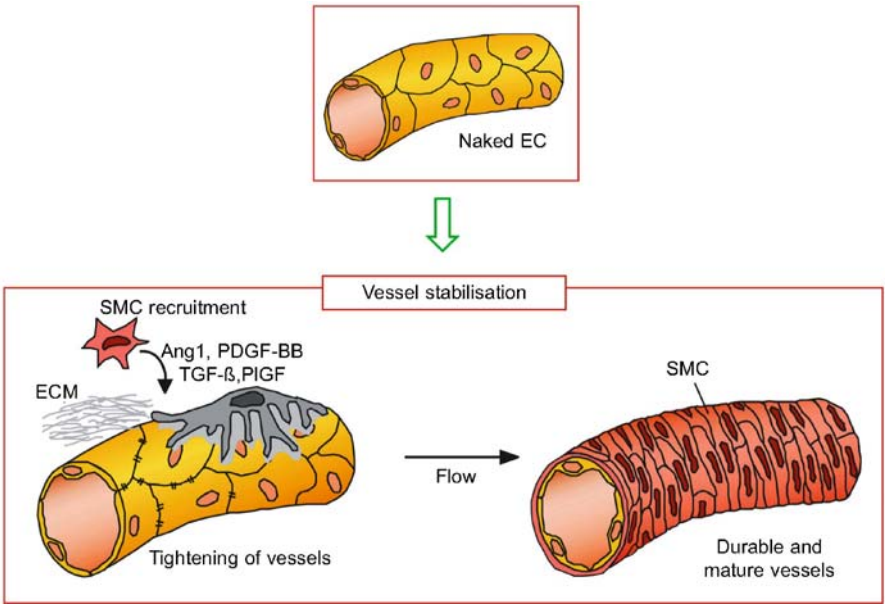

Fig. 3 Vessel maturation and stabilisation. When vessels sprout, they initially consist of naked endothelial cell channels. These nascent vessels become stabilised by recruitment of smooth muscle cells and pericytes (a process called arteriogenesis), deposition of extracellular matrix and tightening of cell junctions. Blood flow plays a critical role in making these vessels durable

namely pericytes and SMCs, which are embedded in an ECM. The expression of cell adhesion molecules in quiescent vessels-such as vascular endothelial cadherin (VE-cadherin) in adherent junctions and claudins, occludins and JAM-1 in tight junctions-provides mechanical strength and tightness to the vessel wall and establishes a permeability barrier. Between vascular cells, an interstitial matrix of collagen-I and elastin provides visco-elasticity and strength to the vessel wall. The ECM is responsible for the contacts between ECs and the surrounding tissue, and thus prevents vessels from collapsing. This stable vessel structure must be first destabilised before new vessels can sprout-a process which we will now describe.

The key angiogenic factor VEGF was originally discovered by H. Dvorak as a potent vascular permeability factor. When vascular permeability increases, plasma proteins (such as fibronectin, fibrinogen, etc.) extravasate and provide a provisional matrix for migrating ECs. Vascular leakage results from changes in EC fenestration, a redistribution of intercellular adhesion molecules, such as PECAM-1 and VE-cadherin, as well as from alterations in the cell membrane, a process involving Src kinase (Eliceiri et al. 1999; Gale and Yancopoulos 1999). However, excessive vascular leakage can result in pathological outcomes, such as circulatory collapse, intracranial hypertension, formation of peritoneal ad-

hesions, metastasis or blindness. Moreover, it contributes to the pathogenesis of chronic inflammatory disorders such as psoriasis and myocardial or brain infarction, and leads to increased interstitial fluid pressure in tumours (Jain 2005). Therefore, changes in vascular permeability need to be tightly regulated. Ang-1, a ligand of the endothelial Tie-2 receptor, is a natural inhibitor of vascular permeability. It tightens vessels by affecting the endothelial junctional molecules PECAM, VE-cadherin and occludins (Gamble et al. 2000; Thurston et al. 2000), thus counteracting the VEGF effect and protecting against abnormal plasma leakage.

To emigrate from their resident site, ECs need to loosen their inter-endothelial contacts, relieve peri-endothelial cell support and break down the surrounding ECM. Ang-2, an inhibitor of Tie-2 signalling and antagonist of Ang-1, is involved in the destabilisation of mature vessels by detaching SMCs and loosening the underlying matrix (Maisonpierre et al. 1997; Gale and Yancopoulos 1999). Proteolytic degradation of the ECM is mediated by several proteinase families, i.e. plasminogen activators [such as urokinase plasminogen activator (uPA) and its inhibitor, PAI-1], matrix metalloproteinases [MMPs and tissue inhibitors of metalloproteinases (TIMPs)], chymases, heparanases, tryptases, cathepsins and kallikreins (and their inhibitor kallistatin; Kostoulas et al. 1999; Luttun et al. 2000; Berchem et al. 2002; Jackson 2002; Miao et al. 2002; Yousef and Diamandis 2002). Proteinases also liberate matrix-bound angiogenic growth factors, such as FGF-2, VEGF, insulin-like growth factor-1 (IGF-1), transforming growth factor-β (TGF-β) and tumour necrosis factor-α (TNF-α), and proteolytically activate angiogenic chemokines such as interleukin-1β (IL-1β; Coussens et al. 1999; Bergers et al. 2000). In addition, proteinases cleave VEGF into shorter isoforms which differ in their solubility, receptor binding and biological activities (Park et al. 1993). In contrast, proteinases involved in the breakdown of ECM also play a role in the resolution of angiogenesis, as they liberate matrix-bound inhibitors [thrombospondin-1 (TSP-1), canstatin, arrestin, tumstatin, angostatin, endostatin, cleaved anti-thrombin III, platelet factor 4, arresten, endorepellin; Nyberg et al. 2005] and inactivate angiogenic cytokines such as SDF-1α (Orimo et al. 2005).

When considering the critical role of ECM in vessel growth and maintenance, it is conceivable that proteolytic remodelling of the ECM must occur in a balanced manner. Indeed, excessive breakdown removes critical support and guidance cues for migrating ECs and, consequently, impairs angiogenesis; on the other hand, insufficient degradation prevents vascular cells from leaving their original site. This concept was illustrated by genetic studies of the plasminogen and MMP systems. Indeed, loss of PAI-1 suppressed pathological angiogenesis in tumours, ocular and other disorders, whereas adenoviral PAI-1 gene transfer reverted this phenotype (Bajou et al. 1998; Lambert et al. 2001; Devy et al. 2002). Conversely, plasmin proteolysis is required for angiogenesis, as vascularisation of ischaemic hearts was reduced in uPA-deficient mice (Heymans et al. 1999), while tumour vascularisation was impaired in

plasminogen-deficient mice (Bajou et al. 2001). Similarly, pathological angiogenesis was decreased in mice lacking components of the MMP system, such as MMP-2 and MMP-9, whereas over-expression of membrane-type (MT)-MMP-1 results in highly vascularised tumours (Sounni et al. 2002). A fine-tuned balance between proteinases and their inhibitors is therefore crucial and might explain why the uPA inhibitors PAI-1 and MT-MMP-1 are risk factors for a poor prognosis in several cancers (Bajou et al. 1998; Luttun et al. 2000).

4.2 Endothelial Budding and Sprouting

Once the physical barriers are dissolved, proliferating ECs migrate to distant sites. This is a complex, tightly regulated process, requiring the involvement of numerous stimulators and inhibitors. For reasons of brevity, we will only review some key signals. The most important signalling of all involves VEGF, which via binding its receptor VEGFR-2, regulates embryonic, neonatal and pathological angiogenesis in a strict dose-dependent manner. The latter phenomenon is exemplified by genetic studies. Indeed, loss of a single VEGF allele results in lethality due to early embryonic vascular defects (Carmeliet et al. 1996; Ferrara et al. 1996), while reduction of VEGF levels by only 25% impairs spinal cord perfusion and causes motor neuron degeneration, reminiscent of amyotrophic lateral sclerosis (Oosthuyse et al. 2001). Several additional gene-manipulating studies in mice, zebrafish and *Xenopus* have documented the principal role of VEGF in vascular development and illustrated its potential to stimulate new vessel growth (Cleaver and Krieg 1998; Nasevicius et al. 2000; Liang et al. 2001; Stalmans et al. 2003). Conditional inactivation of VEGF after birth or expression of particular VEGF isoforms in knock-in mice revealed that VEGF is requisite for vascular expansion during post-natal growth in various organs (e.g. kidney, bone, heart and retina) and, when insufficiently available, causes tissue ischaemia, impaired growth and organ failure (Carmeliet et al. 1999b; Haigh et al. 2000; Maes et al. 2002; Mattot et al. 2002; Stalmans et al. 2002; Eremina et al. 2003). On the other hand, over-expression of VEGF, for instance in the skin of transgenic mice, stimulates abundant cutaneous capillary growth and an inflammatory skin condition resembling psoriasis (Xia et al. 2003). Numerous studies also established VEGF as a key angiogenic player in cancer (Ferrara 2002). Because of its predominant role, VEGF is currently being evaluated for both pro- and anti-angiogenic therapy (Ferrara 2000a, b).

Gene targeting studies in mice have elucidated the functional role of PlGF, a homologue of VEGF, which binds to VEGFR-1. Loss of PlGF-while not causing any vascular defects during embryonic development, reproduction or normal adult life-impaired angiogenesis and plasma extravasation in pathological conditions, including ischaemia, inflammation and cancer (Carmeliet et al. 2001). The important role of PlGF in pathological angiogenesis is further evidenced by findings that PlGF stimulates angiogenesis and collateral growth (see below)

in the ischaemic heart and limb of wild-type mice (Luttun et al. 2002) and, in combination with VEGF, in the ischaemic heart of a mouse model resistant to the VEGF treatment alone (Autiero et al. 2003). PlGF contributes to the angiogenic switch in pathological conditions by affecting, directly and indirectly, multiple cell types (Fig. 4).

First, PlGF has direct effects on ECs by inducing its own signalling via VEGFR-1 and by amplifying VEGF-driven angiogenesis (Carmeliet et al. 2001; Autiero et al. 2003). Second, PlGF-by directly stimulating SMCs and fibroblasts, which express VEGFR-1-recruits SMC around nascent vessels and thus promotes vessel maturation and stabilisation (Green et al. 2001; Ishida et al. 2001; Luttun et al. 2002; see Sect. 5). Third, PlGF stimulates the mobilisation of VEGFR-1-positive HSC/HPCs from the bone marrow (Carmeliet et al. 2001; Lyden et al. 2001) and, indirectly via up-regulation of VEGF expression, recruitment of VEGFR-2-positive EPCs to the site of neovascularisation (Hattori et al. 2001, Luttun et al. 2002). At such sites they promote new vessel growth by directly incorporating into the vessel wall and/or by creating a pro-angiogenic microenvironment through the release of angiogenic molecules (Rehman et al. 2003). Furthermore, PlGF can also recruit HPCs to distant sites to form pre-metastatic niches. Fourth, PlGF is chemo-attractive for monocytes and macrophages, which express VEGFR-1 (Sawano et al. 2001; Luttun

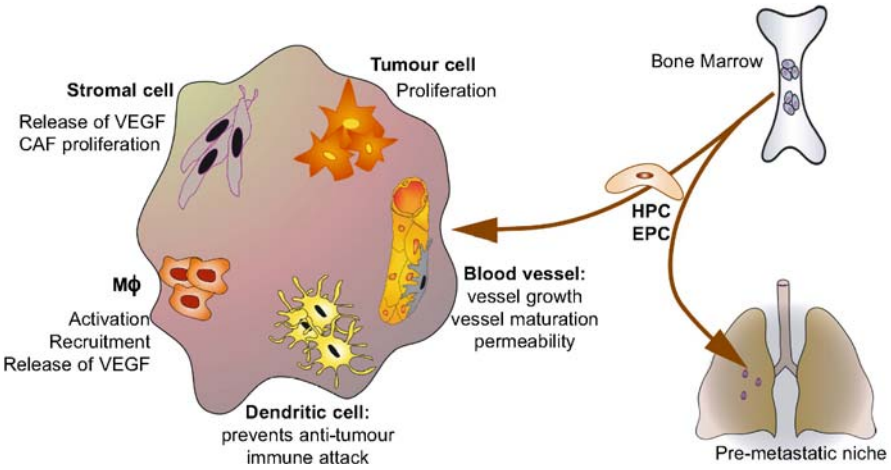

Fig. 4 PlGF is a master switch of pathological angiogenesis and stimulates tumour vascularisation and growth by affecting multiple cell types. Within the tumour environment, PlGF stimulates either vascular cells (endothelial and smooth muscle cells) or non-vascular cells (monocytes/macrophages, stromal cells and dendritic cells). PlGF may also affect VEGFR-1-expressing tumour cells directly. In addition, PlGF stimulates the mobilisation and recruitment of VEGFR-1-positive HSC/HPCs from the bone marrow to the primary tumour and pre-metastatic niches. It remains to be determined whether PlGF also directly affects EPC recruitment by interacting with its receptor VEGFR-1 on EPCs

et al. 2002)-activated macrophages are a rich source of a variety of angiogenic molecules (Autiero et al. 2003) and also produce PlGF, thereby providing a positive feedback.

The role of PlGF and VEGFR-1 in both endothelial and haematopoietic lineages explains why blocking VEGFR-1 more efficiently suppresses inflammatory angiogenic disorders (atherosclerosis, arthritis) than blocking VEGFR-2 (Luttun et al. 2002). Similar effects would thus be expected when VEGFR-1 activation is prevented by PlGF inhibitors or antibodies. VEGF-B is another homologue of VEGF, but its angiogenic activities remain to be determined.

Another angiogenic signalling system involved in vessel growth and stabilisation comprises the Tie-2 receptor, which binds the angiopoietins (Ang-1 and Ang-2). Ang-1, via phosphorylation of Tie-2, is chemotactic for ECs, induces vascular sprouting, stimulates EC survival, mobilises EPCs and HSC/HPCs, and stabilises networks initiated by VEGF, presumably by stimulating the interaction between endothelial and peri-endothelial cells (Suri et al. 1996, 1998; Gale and Yancopoulos 1999; Hattori et al. 2001). All these activities may explain why Ang-1 stimulates vessel growth in skin, ischaemic limbs, gastric ulcers and in some tumours (Suri et al. 1998; Shim et al. 2002; Plank et al. 2004). However, Ang-1 also suppresses angiogenesis in other tumours and the heart (Ahmad et al. 2001; Visconti et al. 2002). In fact, Ang-1 may restrain vessel sprouting by tightening vessels via effects on junctional molecules (Thurston et al. 2000), by recruiting pericytes and by promoting endothelial-mural cell interactions as an adhesive protein (Carlson et al. 2001). Ang-2 in concert with VEGF is also angiogenic and has been proposed to stimulate the growth of immature (SMC-poor) tumour vessels by loosening the endothelial-peri-endothelial cell interactions and degrading the ECM via up-regulation of proteinases, thereby counteracting the activity of Ang-1 (Ahmad et al. 2001; Etoh et al. 2001; Gale et al. 2002). However, the angiogenic activity of Ang-2 seems to be contextual as well since, in the absence of VEGF, Ang-2 causes EC death and induces vessel regression (Maisonpierre et al. 1997).

Several additional factors regulate the proliferation of ECs. Integrins are heterodimeric cell surface receptors of specific ECM molecules which, by bidirectionally transmitting signals between the outside and inside of vascular cells, assist vascular cells to build new vessels in co-ordination with their surroundings (Hood and Cheresh 2002; Hynes 2002). The $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins have long been considered to regulate the angiogenic switch positively (Lee and Juliano 2004), because their pharmacological antagonists which are currently being evaluated in clinical trials suppress pathological (i.e. tumour) angiogenesis (McNeel et al. 2005). Furthermore, a combination of antibodies against $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins reduces tumour vascularisation (Senger et al. 2002). However, genetic deletion studies suggest that vascular integrins inhibit, rather than stimulate, tumour angiogenesis (Reynolds et al. 2002). This inhibitory activity may be attributable to the ability of these integrins to suppress VEGFR-2-mediated EC survival, trans-dominantly block other inte-

grins, or mediate the anti-angiogenic activity of angiogenesis inhibitors such as tumstatin, endostatin, and canstatin (Carmeliet 2002; Reynolds et al. 2002; Hamano et al. 2003; Sudhakar et al. 2003, 2005; Lee and Juliano 2004, Magnon et al. 2005). Thus, while these genetic insights do not invalidate the promising (pre)clinical results obtained with integrin antagonists for cancer treatment, a better understanding of whether and in which conditions integrins play positive or negative roles in tumour angiogenesis is desirable.

FGFs stimulate EC growth directly and, by recruiting pro-angiogenic mesenchymal and inflammatory cells, also indirectly (Carmeliet 2000a). Though PDGF-BB has been documented to stimulate microvascular sprouting of ECs, its main activity is to recruit pericytes and SMCs around nascent vessel sprouts, thereby stimulating vessel maturation and stabilisation, and increasing vessel perfusion (Lindahl et al 1998, 1999; see Sect. 5). Molecules such as TGF-β1, activin-A and TNF-α stimulate or inhibit EC growth, depending on the context (Pepper 1997; Gohongi et al 1999; Guo et al. 2000).

Chemokines are another interesting class of molecules, capable of stimulating or inhibiting EC growth, depending on the type of receptor they activate. Chemokines binding CXCR2 and CXCR4 are angiogenic (e.g. GRO-α, GRO-ɣ, ENA-78, GCP-2, IL-8, SDF-1α, 9E3, eotaxin, I-309, MCP-1, fractalkine), while chemokines binding CXCR3 (e.g. PF-4, MIG, IP-10, ITAC, BCA-1, SLC/6Ckine) have angiostatic activity (Bernardini et al. 2003). At least two of those have received increasing recognition. IL-8 is expressed in several tumours and inflammatory conditions, and is even up-regulated in tumours after anti-VEGF therapy, while anti-IL-8 antibodies block tumour growth (Mizukami et al. 2005). Furthermore, emerging evidence indicates that SDF-1α stimulates angiogenesis via direct effects on ECs, as well as via recruitment of bone marrow-derived EPCs and HPCs both in ischaemic and malignant tissues (Ceradini et al. 2004; Butler et al. 2005); antagonists of SDF-1α block tumour growth (Guleng et al. 2005; see above).

EGF is a mitogen for epithelial cells and is over-expressed in various tumours. While it does not regulate vascular development, it has been implicated in tumour angiogenesis. Indeed, EGF induces the expression of its own receptors in ECs and is mitogenic for EGFR-positive ECs. In addition, EGF indirectly stimulates tumour angiogenesis by inducing the release of VEGF and the expression of VEGF receptors in tumour vessels (van Cruijsen et al. 2005). Another growth factor, hepatocyte growth factor (HGF), stimulates angiogenesis when exogenously administered (Jiang et al. 2005). Other molecules are capable of stimulating EC growth in vitro or angiogenesis in experimental models, but their endogenous role in angiogenesis during development or disease often remains incompletely determined. Some examples include erythropoietin, IGF-1, neuropeptide-Y, leptin, Thy-1, tissue factor, interleukins and others (Carmeliet 2003a).

Angiogenic sprouting is a complex process, requiring a finely tuned balance between activators and inhibitors. Some of the endogenous angiogenesis

inhibitors that are currently being evaluated for clinical use include angiostatin, endostatin, anti-thrombin III, interferon-β, leukaemia inhibitory factor and platelet factor 4, tumstatin, C-terminal hemopexin-like domain of MMP-2 (PEX) and vasostatin (O'Reilly et al. 1994, 1997; Carmeliet 2000b, 2003a; Nyberg et al. 2005).

4.3 Vascular Lumen Formation

Sprouting ECs assemble into solid cords which then undergo tubulogenesis to form vessels with a central lumen. Little is known about how lumen formation is regulated in vivo. Gene targeting studies revealed that VEGF co-ordinatedly regulates vessel size and guidance. VEGF exists in different isoforms with distinct affinities for the ECM. Thus, $VEGF^{121}$ is diffusible, $VEGF^{189}$ binds to the matrix, whereas $VEGF^{165}$ has an intermediate profile (in mice, all VEGF isoforms are shorter by one residue). By virtue of their distinct affinities, the isoforms produce a gradient with $VEGF^{120}$ acting over a long-range and $VEGF^{188}$ over a short range (Ruhrberg et al. 2002; Gerhardt et al. 2003). In the mouse retina, a gradient of matrix-bound VEGF produced by astrocytes guides endothelial tip cells and regulates lumen formation of nascent vessels. Indeed, an alteration of the gradient by loss-of-function manipulation led to a reduction in vessel branching and a concomitant increase in vessel lumen size, while a gain-of-function induced the opposite vascular changes (Gerhardt et al. 2003). Further evidence for a role of VEGF gradients in tip cell guidance and vessel lumen regulation was deduced from analysis of three mouse lines (the $VEGF^{120}$, $VEGF^{164}$ and $VEGF^{188}$ lines), each engineered to express a single VEGF isoform. $VEGF^{164}$ mice are normal, but $VEGF^{120}$ or $VEGF^{188}$ mice exhibit serious vascular remodelling defects (Carmeliet et al. 1999b; Stalmans et al. 2002). Vessels in $VEGF^{120}$ mutants are enlarged, stunted and exhibit reduced branching. Their tip cell filopodia extend chaotically in all directions, which is thought to cause lumen enlargement at the expense of directed branch formation and elongation. These defects presumably result from replacement of the normal VEGF gradient by a non-directional deposition of the highly diffusible $VEGF^{120}$. In $VEGF^{188}$ mice, a shortage of diffusible VEGF causes the opposite phenotype, i.e. supernumerary branches at the expense of luminal enlargement.

In combination with VEGF, Ang-1 also augments lumen diameter (Suri et al. 1998). Egfl7, a recently identified endothelial-derived secreted factor, is expressed at high levels in the developing embryo. Knock-down of Egfl7 in zebrafish embryos revealed that Egfl7 specifically blocks vascular tubulogenesis (Parker et al. 2004). Other molecules involved in the control of lumen formation are the integrins α5, β1 and αvβ3 (Bayless et al. 2000), probably because of their interaction with the surrounding ECM. Finally, thrombospondin-1 (TSP-1) and tubedown-1 (tbdn-1) suppress vascular lumen formation (Gendron et al. 2000).

4.4 Guided Navigation of Vessels

During evolution, organisms have come to perform more specialised tasks, requiring an increased supply of nutrients by blood vessels. Wiring of blood vessels into functional circuits is therefore of utmost importance. The complexity of this task is underscored by the high degree of orderly patterning of the vascular networks. Five centuries ago, Andreas Vesalius illustrated the parallels in the stereotyped branching patterns of vessels and nerves. Today, evidence is emerging that vessels, which arose later in evolution than nerves, co-opted several of the organisational principles and molecular mechanisms that evolved to wire up the nervous system. The choreographed morphogenesis of both networks suggests that they are directed by genetically programmed mechanisms. Specialised endothelial "tip" cells are present at the forefront of navigating vessels and share many similarities with axonal growth cones (Gerhardt et al. 2003). They extend and retract numerous filopodia in saltatory fashion to explore their environment, suggesting that they direct the extension of vessel sprouts. The key function of the tip cells appears to be to "pave the path" for the subjacent "stalk" ECs. Tip cells proliferate minimally, whereas stalk cells proliferate extensively while migrating in the wake of the tip cell, thus permitting extension of the nascent vessel.

Guidance of embryonic vessels requires local guidance cues that instruct them to navigate along specific paths to reach their correct targets. Nerves and vessels face similar challenges in finding their trajectories, which are staked out with multiple checkpoints that divide navigation over a long trajectory into a series of shorter decision-making events (Autiero et al. 2005). Axons and vessels often take advantage of one another to follow the same path. In some cases, vessels produce signals (such as artemin and neurotrophin 3) that attract axons to track alongside the pioneer vessel (Honma et al. 2002; Kuruvilla et al. 2004). Conversely, nerves may also produce signals such as VEGF to guide blood vessels (Mukouyama et al. 2002). Very recent evidence reveals that the same cues that control axon guidance also function to pattern blood vessels. Four families of axon guidance cues, acting over a short-range (cell- or matrix-associated signals) or long-range (secreted diffusible signals), have been identified, namely netrins and their deleted in colorectal cancer (DCC) and Unc5 receptors, semaphorins and their neuropilin and plexin receptors, Slits and their Robos receptors and ephrins and their Eph receptors (reviewed in Dickson 2002; Carmeliet 2003b; Huber et al 2003). A role for Unc5b and netrin1a in vessel guidance was identified by analysis of the developing intersegmental vessels (ISV) in zebrafish embryos. Pathfinding of these vessels is stereotyped and believed to be genetically programmed by an interaction of attractive and repulsive cues. In control embryos, ISVs sprout from the dorsal aorta to the dorsolateral roof of the neural tube. After knock-down of Unc5b or netrin1a, ISVs exhibit supernumerary, often ectopically located filopodial

extensions (Lu et al. 2004), and the dorsal trajectory of most ISVs is irregular, with numerous extra branches deviating from the normal stereotyped path (Fig. 5). These findings suggest a tight control of ISV navigation by netrin family members.

Cross-talk between semaphorins and their receptors (e.g. neuropilins and plexins) is also necessary for ISVs to select the appropriate branching site along the dorsal aorta and to follow the pathway along the somite boundaries (Miao et al. 1999; Serini et al. 2003; Shoji et al. 2003; Torres-Vazquez et al. 2004; Gu

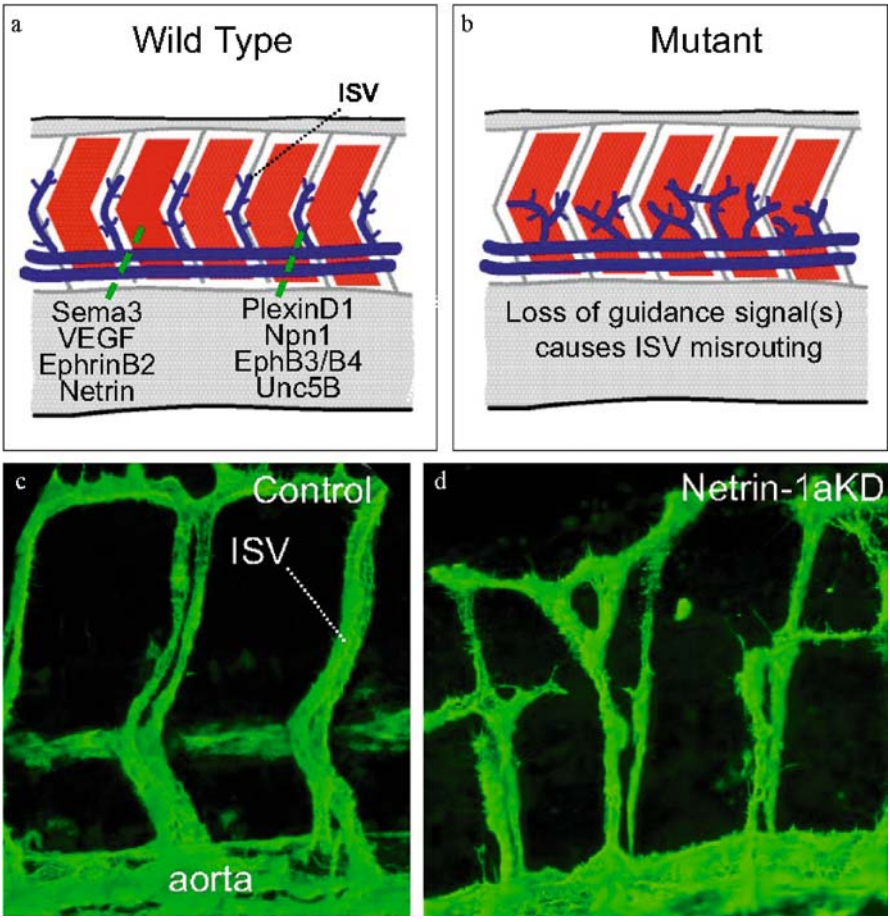

Fig. 5a–d Role of guidance signals in intersomitic vessel guidance. **a** Schematic representation of the zebrafish embryo trunk, showing the somites (*red*) producing the indicated guidance cues and intersomitic vessels (ISV) (*blue*). **b** In the absence of these guidance cues, the ISVs are misrouted. **c** This diagram shows the stereotyped pattern of ISVs in zebrafish embryos [the endothelial cells express an enhanced green fluorescent protein (eGFP) transgene]. **d** After knock-down (*KD*) of *netrin1a*, the ISVs are misguided

et al. 2005). A vascular-specific Robo homologue, Robo4, has been identified (Park et al. 2003). In vitro, Slit2 is able to repel ECs and Robo4 may mediate this effect (Park et al. 2003). A Robo4 knock-down study in zebrafish showed that some Robo4-expressing ISVs failed to sprout from the dorsal aorta or arrested midway through their dorsal migration path, whereas others deviated from their normal dorsal trajectory (Bedell et al. 2005). Repulsive ephrin-Eph signals provide short-range guidance cues for vessels to navigate through tissue boundaries. For instance, ephrinB2 repels EphB3/EphB4-expressing ISVs from entering somites (Wang et al. 1998; Adams et al. 1999; Oike et al. 2002). Understanding this process in the primitive zebrafish embryo may have therapeutic implications, as many of these guidance signals are excessively expressed in tumours, which characteristically develop a chaotic, misguided vasculature (Autiero et al. 2005; Carmeliet and Tessier-Lavigne 2005).

4.5 Vessel Maintenance

When vessels sprout, they initially consist of naked EC channels. Once assembled in new vessels, these ECs become quiescent and survive for years. The importance of endothelial survival is demonstrated by the finding that diminished endothelial survival causes vascular dysfunction and regression in the embryo as well as in the adult (Carmeliet et al. 1999a; Gerber et al. 1999; Baffert et al. 2005). The molecular mechanisms enabling a confluent endothelium to maintain its physiological function in various vascular beds for long periods of time is still unclear. However, some insights have been obtained from in vivo and in vitro studies. For instance, Ang-1 promotes endothelial survival, while in the absence of angiogenic stimuli Ang-2 suppresses endothelial survival, thus contributing to the regression of tumour vessels (Suri et al. 1998; Gale and Yancopoulos 1999; Holash et al. 1999). Haemodynamic forces are also essential for the maintenance of the vascular integrity in different vascular beds, as physiological shear stress reduces endothelial turnover and abrogates TNF-α-mediated endothelial apoptosis (Dimmeler et al. 1996). Flow is critical too for maintaining vessel branches, as hypoperfused sprouts often regress.

But perhaps the most critical survival factor for quiescent ECs in the adult is VEGF. Thus, when VEGF levels are reduced, for instance by exposure of premature babies to hyperoxia, retinal vessels regress (Alon et al. 1995; Meeson et al. 1999). The recent clinical experience with VEGF inhibitors has revealed that these anti-angiogenic agents may cause rare but important adverse effects such as thrombosis, hypertension, bleeding and renal dysfunction (Hurwitz et al. 2004). Some of these adverse effects of anti-VEGF therapy can be explained by the requirement of threshold levels of VEGF for the survival and maintenance of quiescent vessels in healthy organs. For instance, the thrombotic risk may be related to the reduced release of fibrinolytic components (Pepper et al. 2001), the increased release of fibrinolytic inhibitors and pro-coagulants (Ma

et al. 2005), the reduced release of NO (an inhibitor of platelet aggregation and vasospasms, Yang et al. 1996), and EC dysfunction resulting from deprivation of VEGF vessel maintenance signals (Gerber et al. 1999; Kamba et al. 2005). The hypertension is probably attributable to reduced vasodilatation by NO, and possibly to pruning of normal vessels (Sane et al. 2004), while proteinuria and glomerulonephritis may be related to the maintenance role of VEGF in podocyte functioning (Eremina et al. 2003). Bleeding in centrally located cavitary necrotic lung tumours is likely to be due to vessel disintegration. As mentioned above, VEGF inhibitors cause the microvasculature to regress by 70% in endocrine organs, further highlighting the importance of VEGF as a maintenance cue for quiescent vessels in healthy organs (Tang et al. 2004a; Kamba et al. 2005).

5
Arteriogenesis

Establishment of a functional vascular network requires that nascent vessels-formed by vasculogenesis and angiogenesis-mature into durable, stable, non-leaky and functional vessels (Fig. 3). This stabilisation requires recruitment of SMCs, generation of an ECM and specialisation of the vessel wall for structural support and regulation of vessel function-the process of arteriogenesis (Jain 2003). Endothelial channels are covered by multiple layers of SMCs in the proximal part of larger vessels, and by single pericytes in smaller distal vessels. The coverage of vessels by mural cells (pericytes and SMCs) not only regulates EC proliferation, survival, differentiation and haemostatic control, but also assists ECs in acquiring specialised functions to accommodate various needs in different vascular beds. Moreover, interstitial matrix components, generated by mural cells, interconnect ECs and provide blood vessels with visco-elastic properties. SMCs provide structural integrity of the vessel wall. These multifunctional cells contract spontaneously or in response to agonists, maintaining intravascular pressure and tissue perfusion.

5.1
Smooth Muscle Progenitor Cells

A striking feature of SMC biology is the considerable heterogeneity in SMC origin, both during embryonic development and in the adult vasculature (Fig. 2). They can differentiate from ECs, from mesenchymal cells or from bone marrow progenitors and macrophages. For instance, the first SMCs in the dorsal aorta and the SMC-like myofibroblasts in the prospective cardiac valves transdifferentiate from the endothelium (Nakajima et al. 1997; Gittenberger-de Groot et al. 1999). Cardiac neural crest cells are the source of SMC of the large thoracic blood vessels and the proximal coronary arteries (Creazzo et al. 1998;

Gittenberger-de Groot et al. 1999). Mural cells from the distal coronary arteries are recruited from the epicardial layer (Gittenberger-de Groot et al. 1999), whereas coronary vein SMCs are derived from the atrial myocardium (Dettman et al. 1998).

In the adult, the recruitment of SMCs is accomplished by both the division of pre-existing SMCs and the differentiation of bone marrow-derived SMC progenitors, as exemplified in heterotypic cardiac and aortic transplantation in mice (Hillebrands et al. 2001; Saiura et al. 2001). In addition, fibroblasts can differentiate into myofibroblasts, which in turn differentiate into vascular SMCs in response to biochemical or mechanical cues. In humans, SMC progenitors could be identified in the mononuclear fraction of the peripheral blood (Simper et al. 2002). However, the numeric contribution of bone marrow-derived SMCs to vessel growth or maturation of vessels is still controversial. In some animal studies, bone marrow-derived SMCs contributed substantially (10%–50%) to neointima formation and re-endothelialisation in the context of transplant atherosclerosis, balloon injury and primary atherosclerosis (Religa et al. 2002; Sata et al. 2002; Caplice et al. 2003). Other reports, however, demonstrated a more modest (1%–10%) contribution of bone marrow-derived SMCs during these events (Li et al. 2001; Hillebrands et al. 2002). Moreover, SMCs were shown to transdifferentiate from circulating EPCs and mature ECs (Frid et al. 2002; Simper et al. 2002).

5.2 Smooth Muscle Cell Recruitment, Growth and Differentiation

Recruitment of mural cells to nascent vessels is achieved by the involvement of several regulatory pathways (Fig. 3). The PDGF family comprises four family members (i.e. PDGF-A to D) which bind, with distinct selectivity, the receptor tyrosine kinases PDGFR-α and -β, expressed on ECs and SMCs. PDGF-BB and its receptor PDGFR-β play essential roles in the stabilisation of nascent blood vessels. By releasing PDGF-BB, ECs stimulate growth and differentiation of PDGFR-β-positive mesenchymal progenitors and recruit them around nascent vessels (Betsholtz 2004). Absence or insufficient recruitment of periendothelial cells in mouse embryos lacking PDGF-B or PDGFR-β increases endothelial growth and permeability, enlarges vessel size and enhances fragility, resulting in bleeding, impaired perfusion and hypoxia (Lindahl et al. 1998; Hellstrom et al. 1999). The subsequent increase in VEGF levels further aggravates vascular permeability and oedema, and promotes haemangioma formation. Similar neovascularisation occurs in the retina of diabetic subjects, when their pericytes are killed by toxic metabolites.

Vessels in tumours are covered by fewer pericytes than in normal tissues (Eberhard et al. 2000; Morikawa et al. 2002; Ostman 2004). These mural cells differentiate from pools of c-Kit$^+$Sca-1$^+$VEGFR1$^+$ perivascular progenitor cells, which are mobilised from the bone marrow in response to PDGF-B (Song

et al. 2005). When PDGFs are over-expressed, tumour vessels are covered by more mural cells and tumour growth is accelerated (Ostman 2004). Conversely, when PDGFR-β signalling is inhibited, fewer pericytes are recruited, tumour vessels are dilated and EC apoptosis is increased. Combined administration of receptor tyrosine kinase inhibitors (RTKIs), targeting VEGFRs and PDGFR-β, increases the anti-angiogenic effect, even in the often-intractable late stage of solid tumours (Bergers et al. 2003). PDGR-β inhibitors also destabilise the larger SMC-covered vessels, which supply bulk flow to tumours, and thereby render them more susceptible to EC-specific inhibitors.

The Tie-2/Ang and PlGF/VEGFR-1 signalling systems (see Sect. 4.2) are also involved in vessel stabilisation and maintenance. Ang-1, by binding the Tie-2 receptor and counteracting Ang-2 activity, recruits pericytes and promotes the interaction between nascent endothelial channels and mural cells by serving as an adhesive ECM-associated and α5-integrin binding protein (Carlson et al. 2001; Xu and Yu 2001). A precise balance of Tie-2 signals thus seems critical, as a hereditary dysfunction of Tie-2 in humans induces vascular malformations, characterised by enlarged vessels with reduced SMC coverage (Vikkula et al. 1996). PlGF, via binding to VEGFR-1, directly affects SMCs and fibroblasts which express VEGFR-1, but may also indirectly influence SMC proliferation and migration via cytokine release from activated ECs (Luttun et al. 2002). Through these effects, PlGF recruits SMCs around nascent vessels, thereby stabilising them into mature, durable and non-leaky vessels (Vikkula et al. 1996; Green et al. 2001; Ishida et al. 2001; Luttun et al. 2002).

The multifunctional cytokine TGF-β1 promotes vessel maturation by stimulating ECM production and by inducing differentiation of mesenchymal cells to mural cells (Pepper 1997; Chambers et al. 2003). It is expressed in a number of different cell types, including endothelial and peri-endothelial cells and, depending on the context and concentration, both pro- and anti-angiogenic properties have been ascribed to TGF-β1 (Gohongi et al. 1999). Gene targeting studies in mice underscore the importance of TGF-β1, its receptors (RI, RII and endoglin) and the downstream signalling molecules activin receptor-like kinase (ALK)-1 and ALK-5 in the initial phases of resolution and maturation of angiogenesis (Pepper 1997; Weinstein et al. 2000). Hereditary haemorrhagic telangiectasia (HHT), which is characterised by telangiectasia and arteriovenous malformations, has been associated with loss-of-function mutations of endoglin (HHT-1) and ALK-1 (HHT-2, Begbie et al. 2003). However, the precise underlying mechanisms of vascular defects in HHT lesions remain unresolved, as the respective roles of ALK-1 (with Smad1 and Smad5) and ALK-5 (with Smad2 and Smad3) in vessel maturation are debated (Goumans et al. 2002; Lamouille et al. 2002; van den Driesche et al. 2003).

A special type of arteriogenesis represents the enlargement of pre-existing collateral arterioles upon occlusion of a supply artery in the myocardium and peripheral limbs (Fig. 6). This process has been termed “adaptive arteriogenesis” or “collateral vessel growth” and differs significantly from the mecha-

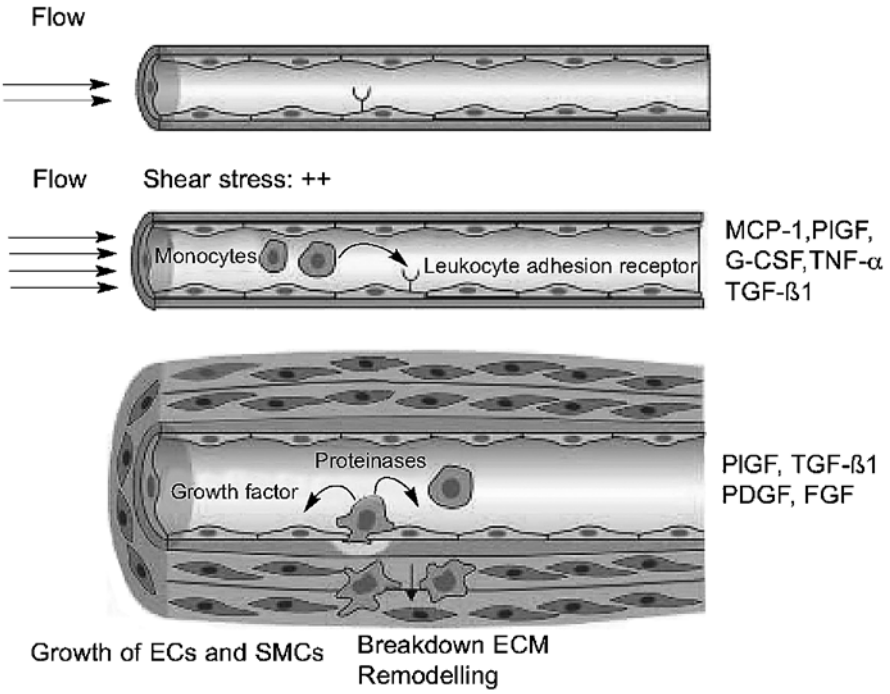

Fig. 6 Mechanism of collateral vessel growth. Upon occlusion of a supply feeder, the flow through of pre-existing collateral vessel increases, which activates the endothelium. As a result, endothelial cells secrete chemoattractants for monocytes and express leucocyte adhesion receptors, onto which leucocytes bind. After extravasation, monocytes produce endothelial and smooth muscle cell mitogens and proteinases to remodel the vessel wall, resulting in enlargement of the collateral vessel. A number of molecules known to stimulate this process are indicated

nisms of angiogenesis (Helisch and Schaper 2003). As a result of increased collateral blood flow and shear stress, activated ECs express monokines, including monocyte chemotactic protein 1 (MCP-1), and monocyte adhesion molecules, such as intracellular adhesion molecule 1 (ICAM-1). By producing growth factors and proteinases (uPA and MMPs), the recruited monocytes infiltrate and proteolytically remodel the vessel wall, enabling SMCs to migrate and divide. The importance of inflammatory cells in collateral vessel growth is underscored by studies in which depletion of monocytes impairs, whereas delivery of monocytes enhances, vessel enlargement (Heil et al. 2002; Kamihata et al. 2002). Cytokines attracting monocytes or prolonging their life span (such as MCP-1, granulocyte-macrophage colony-stimulating factor, TGF-β1 and TNF-α) enhance collateral growth, whereas anti-inflammatory cytokines (such as IL-10) act as inhibitory molecules (Buschmann et al. 2001; Hoefer et al. 2002; van Royen et al. 2002; Voskuil et al. 2003). PlGF also enhances

collateral growth, not only by recruiting monocytes but also by stimulating EC and SMC growth (Luttun et al. 2002; Pipp et al. 2003; see Sect. 4.2). Delivery of FGF, FGF-4 or basic FGF stimulates collateral growth, in part via up-regulating PDGFR-expression (Cao et al. 2003; Pipp et al. 2003). However, VEGF alone appears to affect capillary angiogenesis more efficiently than collateral growth, explaining, at least in part, why results of clinical trials have not been more positive (Isner 2002; Helisch and Schaper 2003). Co-administration of VEGF with additional molecules such as PDGF, PlGF or Ang-1 may therefore enhance its therapeutic potential for the treatment of ischaemic heart and limb disease (Chae et al. 2000).

6 Therapeutic Implications

Over the last decade, intensive efforts have been undertaken to develop therapeutic strategies to promote revascularisation of ischaemic tissues or to inhibit angiogenesis in cancer, ocular, joint or skin disorders. Over 500 million people worldwide have been estimated to benefit from either pro- or anti-angiogenic therapy. Unfortunately, clinical trials testing the pro-angiogenic potential of VEGF or FGF have not met the expected results (Isner 2002; Simons 2005). While some of this failure is attributable to suboptimal delivery strategies, stimulation of durable and functional vessel growth is a more formidable challenge than previously anticipated. Novel strategies involving transplantation of bone marrow-derived cells or the delivery of molecules capable of stimulating the growth of not only distal capillaries but also of proximal collateral conduit vessels will be required in the future (Chae et al. 2000; Luttun et al. 2002; Cao et al. 2003; Pipp et al. 2003; Ferrara and Kerbel 2005; Simons 2005).

Most efforts to date have been focussed on developing anti-angiogenic agents, blocking the activity of VEGF (Table 3). The first two VEGF antagonists-the anti-VEGF antibody bevacizumab (Avastin, Genentech; Ferrara et al. 2004a, 2005) and a $VEGF^{165}$ aptamer (Macugen, Eyetech; Cunningham et al. 2005)-have recently been FDA-approved for the treatment of malignant and ocular disease, respectively. Bevacizumab provides an overall survival benefit in colorectal, breast and lung cancer patients when combined with conventional chemotherapy. Meanwhile, monotherapy with the multi-targeted receptor tyrosine kinase inhibitors (RTKIs) sorafenib (Bayer and Onyx) or sunitinib (Pfizer), which target ECs as well as cancer, mural, stromal and haematopoietic cells, demonstrates clinical benefit in certain cancers (Branca 2005; Marx 2005; Rini et al. 2005). However, as angiogenesis is a tightly regulated process dependent on the complex interplay of numerous molecules, identifying the key targets for drug development remains challenging, and thus a number of outstanding questions still remain to be addressed. How do we clinically assess the efficacy of anti-angiogenic therapies? Is administration of a single angiogenic

Table 3 Anti-angiogenic agents

Drug	Target	Class	Development
Bevacizumab	VEGF	MAb	FDA-approved
Anti-PlGF	PlGF	MAb	Preclinical
Anti-VEGFR-1	VEGFR-1	MAb	development
IMC-C1121b	VEGFR-2	MAb	Phase I
VEGF-trap	VEGF, PlGF, VEGF-B, -C (?), -D (?)	Protein	Phase I–II
AEE788	VEGFR-2, EGFR	RTKI	
AG-013736	VEGFR, PDGFR, c-kit	RTKI	
AMG 706	VEGFR-1,-2, PDGFR, c-kit	RTKI	
AZD2171	VEGFR-1,-2,-3	RTKI	
CEP-7055	VEGFR-1,-2	RTKI	
CHIR258	VEGFR-1,-2, FGFR-1	RTKI	
CP-547632	VEGFR-2	RTKI	
GW786034	VEGFR-2	RTKI	
KRN-951	VEGFR-1,-2,-3, PDGFR, c-kit	RTKI	
OSI-930	VEGFR-1,-2,-3, c-kit	RTKI	
XL999	FGFR, VEGFR-1,-2,-3, PDGFR	RTKI	
ZK-CDK	VEGFR-1,-2,-3; CDKs	RTKI	
ZD6474	VEGFR-2, EGFR	RTKI	
PTK787/ZK 2258	VEGFR-1,-2,-3, PDGFR-β, c-kit	RTKI	Phase III
SU11248	VEGFR-2,-3, PDGFR- β, c-kit	RTKI	
BAY (43-9006)	VEGFR-1,-2,-3, PDGFR, c-kit, Raf	RTKI	

MAb, monoclonal antibody; RTKI, receptor tyrosine kinase inhibitor

molecule sufficient, or do tumours easily find escape routes to switch on alternative angiogenic programmes? Will long-term anti-angiogenic treatment, and in particular combinatorial anti-angiogenic treatment, cause toxicity?

6.1 Potential Biomarkers for Anti-angiogenic Therapy

Reliable biomarkers need to be established to validate the efficacy of anti-angiogenic therapy, to identify responsive patients and optimal doses, to predict efficacy of regimens that include anti-angiogenic agents, and to detect and prevent tumour escape. Traditional chemotherapy drugs are used at the maximum tolerated dose, which is determined by toxicity, and typically results in a reduction of the cross-sectional diameter of a tumour when measured on serial computed tomography scans (Simon et al. 1997). It is tempting to consider

tumour size as a marker of response to anti-angiogenic treatment. However, major functional, structural, cellular and molecular changes can occur in tumours in response to VEGF blockade without a significant reduction of tumour volume (Tong et al. 2004; Willet et al. 2004, 2005; Winkler et al. 2004). Thus, in the absence of an overt cytotoxic effect of anti-angiogenic treatment, other surrogate markers that do not rely on tumour regression must be identified. Some of the candidate markers include classic diagnostic or prognostic biomarkers, as well as newly developed, target- and mechanism-based biomarkers (Park et al. 2004).

Biopsy of tumour tissue is routinely carried out, but is invasive and not readily feasible for some tumours. It represents a prognostic method offering great potential to identify valuable markers for therapeutic efficacy, including the evaluation of protein expression, microvascular density, perivascular cell coverage of tumour vessels, cell proliferation and apoptosis, as well as genomic analysis during anti-angiogenic treatment (Willet et al. 2004, 2005; Ince et al. 2005). Measurements of interstitial fluid pressure (IFP) and tissue oxygenation are parameters that reflect vascular function and delivery of therapeutics. Changes in IFP and tissue oxygen levels might represent valuable surrogate markers of efficacy and vessel normalisation during anti-angiogenic treatment (Willet et al. 2004, 2005).

Less invasive methods include determining changes in growth factor protein concentrations in bodily fluids. For instance, plasma levels of VEGF have been found at higher levels in many tumours and in metastatic disease, and have long been regarded as a potential surrogate marker of cancer growth and anti-angiogenic drug efficacy (Bocci et al. 2004). However, many studies of its use as a surrogate marker have not proved its reliability (Brower 2003). Meantime, new research on tumour growth markers is shifting away from measuring levels of VEGF to measuring its effects, such as the recruitment of progenitor cells from the bone marrow to the tumour where they contribute to neovascularisation. Indeed, recent studies indicate that circulating progenitor cells might represent a reliable surrogate marker of anti-angiogenic therapy (Rafii et al. 2002; Willet et al. 2004, 2005; Beaudry et al. 2005; Blann et al. 2005; Shaked et al. 2005). However, their population is heterogeneous and their concentration in whole blood in humans is very low (Shaked et al. 2005). Measuring, therefore, the number of viable and apoptotic CECs might be more feasible (see Sect. 3.2).

Non-invasive techniques including dynamic contrast-enhanced magnetic resonance imaging, spectral imaging and positron emission tomography have the potential to measure functional parameters and offer surrogate markers regardless of tumour type and localisation (Jennens et al. 2004; Collins 2005; Liu et al. 2005; Miller et al. 2005a). The improvement of these techniques for monitoring the spatial and temporal changes in tumour blood flow, vascular structure, permeability and tumour metabolism (Cohen et al. 1994; Wu et al. 1994; Pham et al. 1998; Herbst et al. 2002; Pahernik et al. 2002; Morgan et al.

2003; Willet et al. 2004; Xie et al. 2004) might allow us to assess more precisely the efficacy of anti-angiogenic treatment.

6.2 Escape from Anti-angiogenic Therapy

Despite promising successes, cancer patients receiving a single class of angiogenesis inhibitors still die. Does that suggest the strategy is insufficient, or does it evoke resistance; if so, how can we avoid resistance? Emerging evidence indicates that treatment with a single anti-angiogenic molecule is more challenging than anticipated, and may not suffice to combat the wide array of angiogenic factors produced by cancer cells and the growing tumour. Redundancy of alternative angiogenic factors in anti-angiogenic monotherapies, up-regulation of angiogenic or anti-apoptotic factors, and heterogeneity of vascular dependency among tumour subpopulations are possible mechanisms that may contribute to the development of acquired drug resistance during anti-angiogenic treatment (Kerbel et al. 2001, Broxterman et al. 2003; Miller et al. 2005b). Indeed, inhibition of a single target leads to up-regulation of additional angiogenic factors. For instance, PlGF is up-regulated after anti-VEGF therapy (Willett et al. 2005), VEGF after anti-VEGFR-2 or anti-EGFR administration (Bocci et al. 2004; Viloria-Petit and Kerbel 2004; Bianco et al. 2005), and IL-8 after HIF-1 deletion (Mizukami et al. 2005). Mutant tumour-cell clones, such as those lacking p53, are able to survive in hypoxic conditions, and their reduced vascular dependence impairs the anti-angiogenic response (Yu et al. 2002). Tumour stromal cells, upon prolonged anti-VEGF treatment, switch to other pro-angiogenic factors (PDGF-B, ephrinB), allowing regrowth of the tumour vasculature (Huang et al. 2004). Furthermore, the PDGFR-α signalling pathway has recently been identified as an alternative mechanism for stromal fibroblast recruitment when tumour cells are deficient in VEGF production (Dong et al. 2004). End-stage tumours might contain more pericyte-coated vessels, explaining why EC-targeted therapies fail to induce regression of established tumours, while targeting pericytes in addition to ECs was more effective (Bergers and Hanahan 2002; Bergers et al. 2003).

Thus, combined treatment of anti-angiogenic agents with distinct complementary mechanisms of action, targeting other angiogenic molecules and targeting not only ECs but also other pro-angiogenic cell types may offer advantages of increased efficacy-at least if toxicity is not a concern (see the following section). Another advantage is that such combinations may give the tumour less chance to escape from anti-angiogenic treatment. Exploring strategies to delay, minimise or even avoid resistance to anti-angiogenic agents might further increase the benefit of anti-angiogenic treatments.

6.3 Adverse Effects of Anti-angiogenic Therapy

As anti-angiogenic agents will hopefully be delivered earlier and earlier to more and more patients for less advanced and life-threatening disease, probably in combination with additional medications, the safety of anti-angiogenic treatment is a topic of emerging importance. Apart from side-effects experienced in clinical trials (Hurwitz et al. 2004), pharmacological and genetic studies in mice revealed that inhibition of VEGF-driven angiogenesis might be expected to cause many more adverse effects (Marx et al. 2002). Fortunately, such toxicity has not yet been observed in humans, but it may emerge in conditions where genetic or iatrogenic predisposition increase the risk. Some of the unwanted toxicity of VEGF/VEGFR-2 targeting agents can be explained by the requirement of threshold levels of VEGF for the survival and maintenance of quiescent vessels in healthy organs (see Sect. 4.5; Gerber et al. 1999; Eremina et al. 2003; Tang et al. 2004a; Baffert et al. 2005; Kamba et al. 2005). A better understanding of why the PlGF/VEGFR-1 pathway is effective only in pathological but not in physiological angiogenesis (Carmeliet et al. 2001; Luttun et al. 2002; Autiero et al. 2003) should therefore be relevant for the design of safe therapeutic strategies. The challenge now involves developing these novel anti-angiogenic strategies and optimising combinatorial treatment regimens to fully exploit the therapeutic potential of angiogenesis inhibition.

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Endothelial Cell Senescence

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Abstract The wear and tear processes that are thought to contribute to human ageing may play an important role in the development of vascular diseases. One such process is cellular senescence. In endothelial cells the senescent phenotype can be induced by a number of factors, including telomere damage, oxidative stress and sustained mitogenic stimulation. Several lines of evidence indicate that endothelial cell senescence may be relevant to vascular disease. In this chapter we examine the causes, mechanisms and regulation of endothelial cell senescence as they emerge from studies in cell culture. We also describe the senescent phenotype and discuss its pathophysiological implications. We review the evidence for the occurrence of endothelial cell senescence in vivo and examine findings in animal models of ageing and human genetic disorders that argue for and against a role of endothelial cell senescence in age-related vascular pathology. Finally, we address the particular case of endothelial progenitor cell senescence and discuss the relevance of this phenomenon for angiogenesis and vascular repair.

Keywords Ageing · Atherosclerosis · β-Galactosidase · Stress · Telomere

1 Introduction

Most normal somatic cells have a limited potential for proliferation, a property that is classically manifested upon serial passage in culture. When this potential is exhausted, cells enter into a permanent and phenotypically distinctive non-dividing state referred to as "replicative senescence" (reviewed by Campisi 2001). Replicative senescence is now recognised as a specific case of a more general phenomenon termed "cellular senescence", which can also be induced by a number of external cellular insults, most strikingly by those causing intracellular oxidative stress (reviewed by Ben Porath and Weinberg 2004; Serrano and Blasco 2001; Toussaint et al. 2000).

Senescence has been implicated in organismal ageing and in age-related pathologies (Hayflick 2003). While definitive proof that this process is an underlying cause of ageing is still lacking, several studies have now provided conclusive evidence that senescence is not just a cell culture phenomenon, but that it also occurs in vivo (Dimri et al. 1995; Satyanarayana et al. 2003; Schmitt et al. 2002). Data from various groups, including our own, indicate that this is also true for endothelial cells (Fenton et al. 2001; Minamino et al. 2002; Vasile et al. 2001). Furthermore, there is now evidence to suggest that endothelial cell senescence contributes to the dysfunction of the endothelium and to the progression of atherosclerosis and its clinical sequelae.

2
Mechanisms of Senescence

Senescence can be triggered by the loss of telomere integrity or by telomere-independent events. However, it should be emphasised that this division is not absolute, since there is significant overlap between the downstream signalling events activated by different senescence-triggering factors.

2.1
Telomere-Dependent Senescence

2.1.1
Telomere Shortening and Replicative Senescence

When endothelial cells from different vascular beds are grown in culture they show a limited capacity to divide, becoming senescent after 40–60 population doublings (Maciag et al. 1981; Rosen et al. 1981; Thornton et al. 1983; van der Loo et al. 1998; Wagner et al. 2001). Senescence due to extended propagation in cell culture was discovered in fibroblasts more than 40 years ago and has since been associated with progressive shortening of telomeres. For a historic account of the developments in this field see the reviews by Greider (1998) and Hayflick (2003). Telomeres are high-order structures formed by DNA and specialised proteins that cap the chromosome ends. Telomeres protect the chromosome from nuclease attack and end-to-end fusions. In addition, they are involved in chromosomal positioning and segregation during mitosis (Blackburn 2001). In human cells, telomeric DNA consists of a 5- to 15-kb double-stranded stretch of the repeated motif TTAGGG ending in a G-rich single-stranded 3′ overhang of 100–250 nucleotides. This 3′ overhang is thought to fold back and invade the adjacent double-stranded region, forming a structure called the "T-loop", which may provide functional stability to the telomere (Griffith et al. 1999).

Synthesis of telomeres is a complex process requiring a specialised reverse transcriptase called telomerase (see Sect. 2.1.2 below) in addition to the conventional DNA replication machinery. According to the "telomere hypothesis of senescence", in cells that lack telomerase (or when telomerase levels are very low), telomeres shorten with each round of cell division as a consequence of the so-called "end-replication problem", i.e. the inability of conventional DNA polymerases to complete the synthesis of a linear DNA molecule (Olovnikov 1996). This model proposes that telomere shortening constitutes a mechanism that counts cell divisions and that will trigger senescence when one or more telomeres are reduced to a critical length that will be incompatible with a stable telomeric structure. Senescence resulting from telomere shortening has been also called "intrinsic senescence" in order to distinguish it from senescence that has its origins in external stimuli and that occurs when telomeres are still

functional (Itahana et al. 2004). Telomere shortening was first demonstrated experimentally in fibroblasts grown in culture (Harley et al. 1990) and has since been documented in a variety of cultured cell types, including endothelial cells (Chang and Harley 1995; Kurz et al. 2004). In replicating fibroblasts and endothelial cells, telomere shortening occurs at a rate of about 50–100 bp per round of cell division. Telomere shortening has also been described in vivo in association with ageing and pathological conditions (Aikata et al. 2000; Hastie et al. 1990; Rufer et al. 1999; Vaziri et al. 1993; see also Sect. 4.2.3 below). Substantial support for the telomere hypothesis of senescence has been provided by experiments in which reconstitution of telomerase by gene transfer stabilised telomere length and extended the replicative capacity of fibroblasts and retinal pigment epithelial cells (Bodnar et al. 1998; Vaziri and Benchimol 1998).

2.1.2 Telomerase

Telomerase is a ribonucleoprotein complex that catalyses the addition of TTAGGG repeats to the 3′ end of telomeric DNA (reviewed by Collins and Mitchell 2002; Greider 1996). Human telomerase is composed of various subunits, two of which are essential for its activity, namely the catalytic reverse transcriptase protein subunit or hTERT (for human telomerase reverse transcriptase) and the RNA template subunit or hTR (for human telomerase RNA). In most cell types hTR is constitutively expressed, whereas hTERT is tightly regulated in its expression. In human post-natal life, substantial levels of telomerase activity can be detected in the germline (Wright et al. 1996) and in some somatic tissues that undergo proliferative renewal such as haematopoietic lineages, the epidermis and the intestine (Forsyth et al. 2002). In addition, telomerase is vastly up-regulated in the majority of tumour cells (Kim et al. 1994).

Telomerase is also expressed by normal endothelial cells (Hsiao et al. 1997; Kurz et al. 2003; Vasa et al. 2000), although levels of activity in proliferating cells are about 40 times lower than those measured in a typical cancer cell line (Kurz et al. 2003). In agreement with its behaviour in immortal cell lines and lymphocytes (Forsyth et al. 2002), telomerase activity in endothelial cells is growth-regulated. Thus, hTERT messenger RNA (mRNA) levels and activity are elevated in actively cycling cells but are down-regulated in endothelial cells growth-arrested in culture or in the quiescent endothelium in vivo (Kurz et al. 2003). Endothelial cell mitogens (Kurz et al. 2003), nitric oxide (NO) (Vasa et al. 2000) and oxidative stress (Haendeler et al. 2003; Haendeler et al. 2004; Kurz et al. 2004) have been implicated in the modulation of telomerase in these cells. Regulation appears to occur at multiple levels, including transcription (Kurz et al. 2003), post-translational Akt-mediated phosphorylation (Breitschopf et al. 2001) and intracellular localisation (Haendeler et al. 2003).

In our own studies we have found that among a series of individual endothelial cell mitogens, fibroblast growth factor-2 (FGF-2) was the only one with the capacity to induce telomerase activity on its own. This effect correlated with the up-regulation of the mRNA levels for the transcription factor Sp1 and for hTERT itself, suggesting that FGF-2 signals the trans-activation of *hTERT* via an Sp1-mediated mechanism (Kurz et al. 2003). Indeed, Sp1 is known to activate *hTERT* in co-operation with the transcription factor c-Myc, both of which have multiple binding sites in the core promoter region of *hTERT* (Kyo et al. 2000).

In endothelial cell cultures, telomerase activity decreases as a function of replicative age (Kurz et al. 2004a; Vasa et al. 2000). In one study, NO donors were shown to counteract this phenomenon and also to reduce senescence, apparently by stimulation of telomerase activity (Vasa et al. 2000). In this study, addition of the NO synthase inhibitor N^G-monomethyl-L-arginine reduced telomerase activity, but the same group could not confirm this finding in another study (Breitschopf et al. 2001). Thus, the role of NO on the regulation of telomerase is still open to investigation.

2.1.3 The Loss of Telomere Integrity

Many studies suggest that it is not the length of the telomeric DNA duplex per se, but rather the loss of the structural integrity of the telomere that determines the onset of senescence. As will be discussed below (see Sect. 2.2.2), dysfunctional telomeres trigger senescence after they are registered by the cell cycle checkpoint machinery as a permanent form of DNA damage (von Zglinicki et al. 2005).

To maintain telomere integrity, apart from a minimum length of TTAGGG repeats, at least two other elements are required, namely the 3′ overhang and functional telomere-binding proteins (Blackburn 2001; de Lange 2002). In human cells, two proteins—telomere repeat binding factor (TRF)-1 and TRF-2—bind to duplex telomeric DNA repeats where they form multi-protein complexes by recruiting additional regulatory elements. The TRF-1 complex is a telomere length regulator (Smogorzewska and de Lange 2004), whereas the primary function of TRF-2 is to protect chromosome ends (van Steensel et al. 1998). Both inhibition of TRF-2 expression (Smogorzewska and de Lange 2002) and erosion of the 3′ overhang (Stewart et al. 2003) have been associated with the induction of senescence in fibroblasts. However, the view that 3′ overhang loss is a signal for senescence has recently been challenged by another laboratory (Chai et al. 2005).

The role of TRF-2 has also been investigated in endothelial cells. Minamino et al. (2002) have shown that the introduction of a dominant-negative version of this protein into human aortic endothelial cells resulted in growth arrest and a senescent phenotype. Similarly, Spyridopoulos et al. (2004) have shown

that endothelial progenitor cell premature senescence in culture is associated with a loss of TRF-2 rather than with telomere shortening, and that both TRF-2 loss and senescence could be prevented by statins.

Increasing evidence indicates that telomerase plays a role in the control of telomere integrity beyond its involvement in telomere-length maintenance. In our own work we found that, while endothelial cells stimulated to grow with FGF-2 restored telomerase activity and attained a normal replicative life-span, cells grown with vascular endothelial growth factor-A (VEGF-A) did not re-activate telomerase and underwent senescence prematurely (Kurz et al. 2003; Trivier et al. 2004). Intriguingly, we noted that the effect of FGF-2 in extending replicative life-span could not be attributed to a decrease in the rate of telomere shortening. To explain this phenomenon, we proposed that the low levels of telomerase found in human endothelial cells play an active role in delaying senescence by a mechanism that preserves telomere function independently of overall telomere length maintenance (Kurz et al. 2003; Kurz and Erusalimsky 2003). A recent study investigating the relationship between telomerase and telomere structure and function in normal human fibroblasts lends further support to this model of telomerase function in somatic human cells (Masutomi et al. 2003). This study demonstrated the presence of telomerase in fibroblasts. It further showed that although levels of expression were extremely low, they prevented premature senescence. In line with our observations in endothelial cells, this work also showed that inhibition of telomerase expression had no effect on the rate of telomeric DNA erosion in the double-stranded region. In contrast, these low levels of telomerase were required to maintain the 3′ overhang. In agreement with this alternative mode of action of telomerase, ectopic expression of *hTERT* has been shown to extend the life-span of human umbilical vein endothelial cells (HUVEC) and bovine corneal endothelial cells, even though telomeres in these cells continued to shorten beyond the length of their senescent counterparts (Veitonmaki et al. 2003; Yang et al. 1999). In addition, it is important to point out that further studies in exponentially growing fibroblasts suggest that, beyond its effects on telomeres, telomerase participates in the regulation of the DNA damage response, probably through effects on the chromatin state (Masutomi et al. 2005). Whether or not telomerase is required in endothelial cells to maintain the telomeric 3′ overhang or some other aspect of telomere structure (or another function) is unknown at present. A diagram summarising the different factors known to affect telomere integrity in endothelial cells is shown in Fig. 1.

2.1.4
The Role of Oxidative Stress in Telomere Dynamics

Work with cultured fibroblasts has shown that oxidative stimuli accelerate telomere erosion, probably due to the generation of single-strand breaks in the telomeric DNA (reviewed in von Zglinicki 2002).

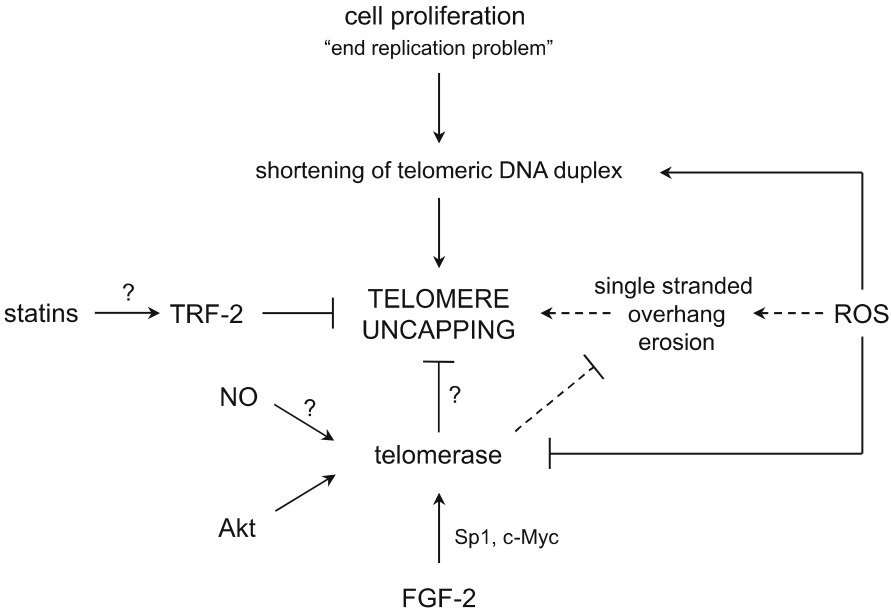

Fig. 1 Factors affecting telomere integrity in endothelial cells. *Broken arrows* indicate presumed connections. *Question marks* denote unknown mechanism of action. For a detailed description see text

More recently, evidence has been accumulating that oxidative stress can also accelerate the loss of telomere integrity and the onset of senescence in endothelial cells. This effect could be demonstrated by manipulating the intracellular redox environment through different mechanisms, including adding a stable vitamin C analogue to the culture medium (Furumoto et al. 1998), raising intracellular reactive oxygen species (ROS) levels with homocysteine (Xu et al. 2000) and interfering with the glutathione redox-cycle (Kurz et al. 2004). An additional effect observed in endothelial cells during even mild oxidative stress was a rapid down-regulation of telomerase activity (Kurz et al. 2004). This is consistent with other work showing that substances which both induce oxidative stress and have pro-atherogenic properties, such as oxidised low-density lipoprotein (LDL) or tumour necrosis factor (TNF)-α, also reduced telomerase activity (Breitschopf et al. 2001).

2.2 Telomere-Independent Senescence

A number of cellular insults can induce senescence in a relatively rapid fashion. This acute form of senescence, sometimes termed "stress-induced premature senescence" (Toussaint et al. 2000), is normally considered unrelated to telomere damage (Chen et al. 2001). Within the factors that induce telomere-

independent senescence, those that may be most relevant to the endothelium in vivo include oxidative stress, radiation-induced genomic DNA damage and bioenergetic stress. Oncogenic stress, on the other hand, has been shown to cause senescence of endothelial cells in culture, and while as such it may not be pertinent to the in vivo situation, it can provide important clues as to the pathways involved in the orchestration of the senescence programme in these cells.

2.2.1 Oxidative Stress

A number of atherogenic and inflammatory stimuli as well as mitochondrial dysfunction may lead to an increase in the levels of ROS, thus causing intracellular oxidative stress. As shown in Fig. 2, ROS can induce senescence by acting at multiple levels. Besides affecting telomeres (see Sect. 2.1.4 above) they can attack genomic DNA. ROS can also damage mitochondrial DNA and other components of this organelle. Mitochondria by themselves generate ROS during normal aerobic metabolism, and some studies suggest that when their normal function is impaired they may augment ROS output, thus increasing

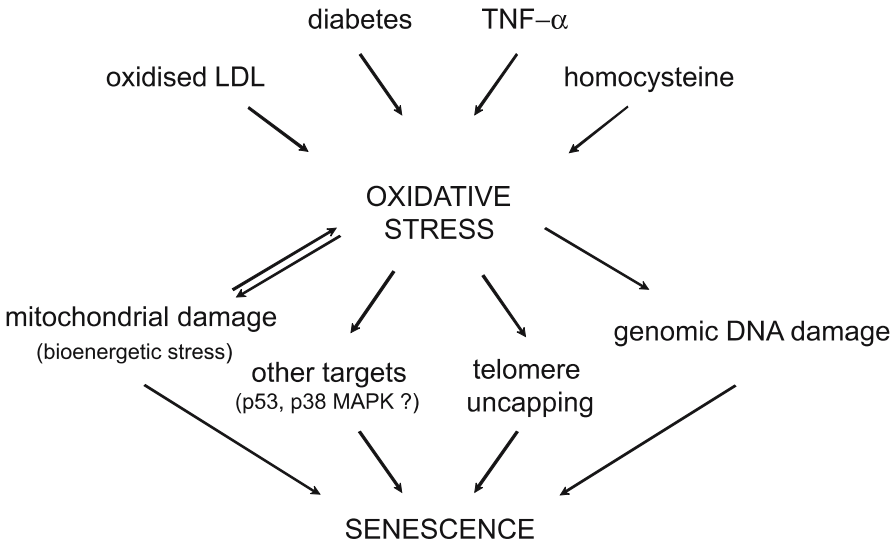

Fig. 2 Putative targets of oxidative stress that may induce senescence in endothelial cells. Pro-inflammatory and pro-atherogenic stimuli may increase ROS formation and cause intracellular oxidative stress. ROS can damage genomic and telomeric DNA. ROS can also damage mitochondria, which may form more ROS to generate more damage. Dysfunctional mitochondria in turn may lead to bioenergetic stress. p53 and stress-activated protein kinases such as p38 are also potential targets of additional oxidative stress-activated pathways leading to senescence

the oxidative burden of the cell (reviewed in Balaban et al. 2005). In addition, ROS can be mediators of oncogenic stress and in turn activate cytosolic stress response kinases and other signalling molecules which have been implicated in senescence responses, as detailed in the following sections.

2.2.2 DNA Damage

In addition to disruption of telomeres, several other forms of chromosomal damage can activate the senescence programme. Thus, chromatin decondensation caused by inhibition of histone deacetylases and DNA damage induced by radiation, oxidising compounds, alkylating agents and drugs that generate double strand breaks have all been shown to cause senescence in various cellular contexts (reviewed in von Zglinicki et al. 2005). In the case of endothelial cells, morphological, functional and molecular changes consistent with the induction of a senescent phenotype have been observed in cultured cells that survived ionising radiation-induced DNA damage (Gajdusek et al. 2001). This observation raises the possibility that radiation-induced endothelial cell senescence may be one of the pathogenic mechanisms underlying the adverse effects that radiotherapy has on the vasculature.

In fibroblasts the senescence response triggered by DNA damage or by telomere uncapping appears to be mediated by various signalling events which are also known to be involved in the classical DNA damage response pathway, including the formation of DNA damage foci, activation of ATM/ATR (ataxia-telangiectasia-mutated/ataxia-telangiectasia-mutated and Rad3-related) and Chk1/Chk2 protein kinase family members, and p53 up-regulation (d'Adda di Fagagna et al. 2003; Herbig et al. 2004). The relevance of this pathway to endothelial cells has been highlighted recently by the work of Spyridopoulos et al. (2004) who showed that the DNA damage checkpoint kinase-2 (Chk2), a downstream kinase which is activated by the formation of DNA damage foci, was up-regulated concomitantly with the down-regulation of TRF-2 during the onset of endothelial progenitor cell senescence.

2.2.3 Bioenergetic Stress

Insufficient energy output results in bioenergetic stress. This phenomenon may stem from a variety of conditions, including increased energy demand, restriction of nutrient or oxygen supply (ischaemia and hypoxia) and mitochondrial dysfunction. Bioenergetic stress causes an increase in intracellular AMP levels, and this in turn leads to the activation of the AMP-activated protein kinase (AMPK), an enzyme which plays a central role in the control of intracellular energy metabolism (Carling 2004). AMP binding to the enzyme promotes its phosphorylation by the tumour suppressor LKB1, resulting in full activation

(Hardie 2004). Once activated, the enzyme turns off biosynthetic pathways and at the same time turns on catabolic pathways, thus restoring ATP levels. Bioenergetic stress and persistent activation of AMPK have recently also been implicated in the induction of senescence in cultured fibroblasts (Wang et al. 2003; Zwerschke et al. 2003). Conversely, the loss of LKB1 has been previously shown to prevent stress-induced senescence of mouse embryonic fibroblasts (Bardeesy et al. 2002). While these studies underscore the significance of AMPK in the induction of senescence, the precise mechanism underlying this effect is currently unclear. In this respect, the finding that AMPK can phosphorylate p53 suggests a possible link (Imamura et al. 2001).

At present there is no direct evidence that bioenergetic stress induces senescence in endothelial cells. Nevertheless, the notion that these cells may be exposed in vivo to insults that are likely to decrease their energetic output suggests that this may be an important pathological mechanism. In this regard, the complex interplay between levels of NO, oxygen availability and the redox environment may have an important role (Moncada and Erusalimsky 2002; see also Sect. 3.4; S. Moncada and E.A. Higgs, volume I). More recently it has been claimed that AMPK can be activated by mitochondria-derived ROS (Zou et al. 2004), raising the possibility that oxidative stress-induced senescence also may act in part through this pathway.

2.2.4 Oncogenic Stress

Forced expression of certain activated oncogenes has been shown to induce senescence in various primary cell types (reviewed in Ben Porath and Weinberg 2005; Serrano and Blasco 2001). In endothelial cells this phenomenon has been observed when active forms of Akt (Miyauchi et al. 2004), Ras (Spyridopoulos et al. 2002) or Rac1 (Deshpande et al. 2003) were over-expressed. Promotion of senescence by Akt was postulated to be mediated by the phosphorylation and consequent inactivation of the transcription factor Foxo3a, followed by the down-regulation of one of its target genes, *Mn-superoxide dismutase*. This was shown to affect the redox balance of the cell, leading to an increase in ROS which in turn stimulated p53 activity and p21 expression (Miyauchi et al. 2004). Over-expression of an oncogenic form of Ras has also been shown to increase the expression of p21 and to cause senescence in bovine aortic endothelial cells (Spyridopoulos et al. 2002). Although not demonstrated in endothelial cells, this effect of Ras could also have been mediated by an increase in ROS; indeed, this appears to be the case in fibroblasts (Lee et al. 1999). Similarly, in an independent study, introduction of an activated form of the small GTPase family member Rac1 into HUVEC was shown to induce oxidative stress and premature senescence, apparently by a mechanism involving the production of ceramide and mitochondrial ROS (Deshpande et al. 2003). However, at present other mechanisms for the generation of ROS, including Rac1-induced activa-

tion of a NADPH oxidase (Li and Shah 2004) cannot be excluded. In addition, work in fibroblasts showing that Rac1 lies downstream of Ras in stimulating the production of ROS (Irani et al. 1997) suggests that the Rac1 pathway may be also relevant to Ras-induced senescence in endothelial cells. Taken together these findings suggest that persistent activation of growth signalling pathways in endothelial cells may result in dysregulation of the cellular redox balance and culminate in oxidative stress-induced premature senescence. The pathophysiological significance of these findings lies in the fact that pro-atherogenic conditions, such as hyperinsulinaemia, chronic inflammation and hypercholesterolaemia, are known to activate Akt- and Ras-mediated signalling in endothelial cells.

2.3 Integration of Senescence Signals

Extensive work with mammalian fibroblasts has implicated the tumour suppressors p53, p16 and the retinoblastoma protein (RB) in the induction of senescence (Campisi 2001). p16 induces senescence by inhibiting the activity of the cyclin-dependent kinases CDK4 and CDK6, which would otherwise phosphorylate and inactivate RB (Fig. 3). Hypophosphorylated RB binds to E2F family members and shuts down the transcription of its targets; these targets are normally required for cell cycle progression. p53 induces senescence through up-regulation of its downstream transcriptional target p21. p21 in turn inhibits cyclin E/CDK2, another CDK complex which phosphorylates RB (Fig. 3). Thus, RB is activated either by p53/p21 or by p16. In human cells both the p53/p21 and p16 pathways can induce senescence, acting independently or in parallel. While both arms seem to be implicated in most forms of senescence, fulfilling partially redundant roles, the relative contribution of each depends on the cellular context and the initial triggers (Ben Porath and Weinberg 2004; Herbig and Sedivy 2006). In fibroblasts, activation of the p53/p21 pathway is dominant in the response to telomere and DNA damage, whereas activation of the p16 pathway occurs primarily during stress-induced premature senescence. Nevertheless, it should be emphasised that p16 is also involved in the response to telomere damage that occurs during replicative senescence (Jacobs and de Lange 2004). Some studies have shown that up-regulation of p16 occurs shortly after the up-regulation of p21, suggesting that this is a means to ensure the maintenance of the senescence phenotype (Stein et al. 1999). However, exceptions to this model of temporally ordered p21 and p16 accumulation have been reported (Brookes et al. 2004). In addition, it should be noted that in some contexts p53 could induce senescence by an unknown mechanism that does not require p21 or RB.

The senescence response mediated by p53 is controlled by two upstream pathways (Fig. 3). One is the classical DNA damage response pathway. In this pathway, damage to the telomere or other parts of the chromosomal DNA

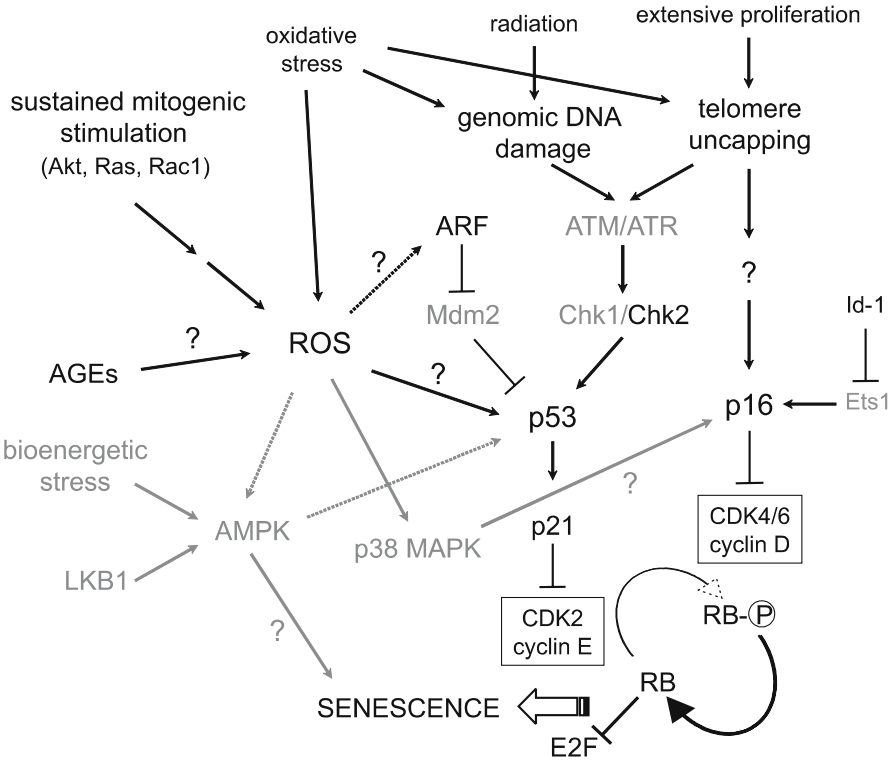

Fig. 3 Integration of senescence signals in endothelial cells. Signalling intermediates demonstrated to be involved in endothelial cell senescence are noted in *black*. Pathways and proteins implicated in the senescence response of other cell types but not confirmed in the response of endothelial cells are represented in *grey*. *Broken arrows* indicate presumed connections. *Question marks* denote unknown mechanism of induction. For a detailed description see text

activates the ATM/ATR and ChK1/ChK2 kinases which stabilise p53 through phosphorylation. The other pathway involves the alternative reading frame (ARF) product of the *INK4a* locus (the same locus that encodes p16). ARF acts by sequestering the E3 ubiquitin ligase Mdm2 to the nucleolus, thus preventing the proteolytic degradation of p53. The ARF pathway does not appear to be implicated in replicative senescence; instead, ARF is induced by cellular stresses.

A limited number of studies have examined the relationship of the p53/p21 and p16 pathways with the induction of replicative (telomere-dependent) senescence in endothelial cells. In bovine corneal endothelial cells this phenomenon was associated with the up-regulation of p53, occurring concomitantly with a reduction in telomere length (Whikehart et al. 2000). Furthermore, in bovine capillary endothelial cells (Veitonmaki et al. 2003) and HUVEC

(Wagner et al. 2001), both p21 and p16 were shown to be over-expressed and RB was found mostly in its hypophosphorylated form. Conversely, in one of these studies it was shown that ectopic expression of *hTERT* led to repression of both p21 and p16 and maintenance of RB in the hyperphosphorylated state, thus allowing the cells to bypass senescence during increasing population doublings, even though telomeres continued to shorten (Veitonmaki et al. 2003). In line with these findings, Tang et al. (2002) showed that the helix-loop-helix protein Id-1 delays the onset of replicative senescence in human endothelial cells by repressing p16 expression; this effect is probably mediated by the Id-1-mediated downregulation of the p16 transactivator Ets1 as shown in fibroblasts (Ohtani et al. 2001; Fig. 3). Finally, both p21 and p16 were also up-regulated in human aortic endothelial cells when senescence was induced by expressing a TRF-2-dominant negative mutant (Minamino et al. 2002). Taken together these findings suggest that in endothelial cells both the p53/p21 and p16 pathways participate in the senescence response induced by the loss of telomere integrity (Fig. 3). In contrast to this tenet, however, work by Freedman and Folkman (2005) in HUVEC has questioned the importance of p16 for the induction of replicative senescence, postulating instead that the critical factor is loss of CDK2 activity.

Other studies have also attempted to clarify the involvement of these pathways in the induction of stress-induced premature senescence in endothelial cells. In one study with human cells, activation of the p53/p21 pathway was shown to be required for Akt-induced senescence (Miyauchi et al. 2004). In this case, while the level of phosphorylated RB was decreased, p16 was not elevated. Similarly, in another study p21 up-regulation was shown to be involved in Ras-induced senescence of bovine aortic endothelial cells (Spyridopoulos et al. 2002). However, while this study showed that p21 is instrumental for the induction of growth arrest, it also suggested that additional signalling events may be required for the implementation of the full senescent phenotype (Spyridopoulos et al. 2002). In agreement with this possibility, studies in HUVEC using the advanced glycation end-product (AGE) glycated collagen to induce premature senescence have implicated both the ARF arm of the p53/p21 pathway and the p16 pathway in the process (Chen et al. 2002, 2006; see also Fig. 3). Furthermore, while this work confirmed that in endothelial cells both pathways act through RB, it suggested that p53, besides its role in the induction of p21, also fulfils a permissive function in the initiation of senescence by p16 (Chen et al. 2006). Finally, while work in fibroblasts has suggested that the up-regulation of p16 by oncogenic and oxidative stress is implemented via activation of the mitogen-activated protein kinase (MAPK) p38 (Iwasa et al. 2003; Wang et al. 2002), the role of this stress-activated protein kinase in endothelial cell senescence has not been investigated. The fact that ROS are known to activate p38 MAPK in this cell type (Griendling et al. 2000) warrants further exploration of this possibility.

3
The Senescent Phenotype

Apart from the alterations related to the block in cell replication described in Sect. 2.3, senescent cells show other characteristic changes in gene expression, morphology and function (Campisi 2001). Some of these changes may also be important in affecting the progression of atherosclerosis and its clinical sequelae.

3.1
Morphological Changes

Endothelial cells that have undergone replicative senescence in culture show distinctive changes in morphology such as an increase in size, polymorphic nuclei, flattening and vacuolisation (Rosen et al. 1981; van der Loo et al. 1998). Consistent with these striking morphological differences, comparison of gene expression patterns between young and senescent cells reveals changes in the expression levels of proteins associated with cytoskeletal function and cellular architecture (Chang et al. 2005; Kamino et al. 2003; Shelton et al. 1999; Vasile et al. 2001).

3.2
Senescence-Associated β-Galactosidase

A common feature of senescent cells is the presence of senescence-associated β-galactosidase (SA-β-gal) (Dimri et al. 1995). We have previously demonstrated that this activity is a manifestation of an increase in lysosomal mass (Kurz et al. 2000). This probably reflects the accumulation of autophagic vacuoles in the senescent cell containing non-degradable intracellular macromolecules and organelles. The detection of SA-β-gal is associated not only with replicative senescence and with a reduction in telomeric structural integrity but also with senescence induced by other mechanisms. The increase in lysosomal mass has an uncertain underlying cause and its pathophysiological significance in the arterial wall has not been investigated to date.

3.3
Pro-atherogenic and Pro-thrombotic Changes

Upon replicative senescence in culture, endothelial cells over-express interleukin-1α (Maier et al. 1990) and the intercellular adhesion molecule ICAM-1 (Maier et al. 1993; Shelton et al. 1999). The latter is a surface molecule that promotes monocyte adhesion. In vitro senesced endothelial cells also produce plasminogen activator inhibitor-1 (PAI-1) (Comi et al. 1995; Grillari et al. 2000; Shelton et al. 1999), a protein which decreases fibrinolytic activity. These features are

also characteristic of the pro-inflammatory/pro-thrombotic phenotype of endothelial cells found in atherosclerotic human arteries (Davies et al. 1993; Lupu et al. 1993; Moyer et al. 1991; Schneiderman et al. 1992; van der Wal et al. 1992).

Further studies have shown that the levels of ICAM-1 and PAI-1 expression also increase when telomere shortening (and hence senescence) is accelerated in culture by homocysteine treatment (Xu et al. 2000). Similarly, ICAM-1 expression increases when telomere function is inhibited by over-expressing a dominant-negative form of TRF-2, and conversely it decreases when telomerase is over-expressed (Minamino et al. 2002). Other studies revealed that senescent cells undergo changes in the expression of proteins associated with the remodelling of the extracellular matrix (Chang et al. 2005; Grillari et al. 2000; Kamino et al. 2003; Shelton et al. 1999). Finally, Vasile et al. (2001) have shown that early passage human dermal microvascular endothelial cells degrade more atherogenic lipoproteins than their senescent counterparts, suggesting that the latter have a reduced capacity to metabolise atherogenic lipids.

All together these studies indicate that the accumulation of senescent cells in the arterial wall may contribute to both the initiation and progression of atherosclerosis.

3.4 Changes Affecting Endothelium-Dependent Vasodilatation

Nitric oxide bioavailability is critical to normal endothelial function (see S. Moncada and E.A. Higgs, volume I). Advanced age leads to impairment of endothelial NO production (Tschudi et al. 1996), which contributes to age-related endothelial dysfunction. A number of studies have investigated whether endothelial senescence may be involved in this phenomenon. These studies have established that senescent endothelial cells have lower levels of endothelial NO synthase (eNOS) activity and produce decreased levels of NO (Hoffmann et al. 2001; Matsushita et al. 2001; Sato et al. 1993). This phenotypic change was also brought about after senescence was induced in aortic endothelial cells by introducing the dominant-negative form of TRF-2 (Minamino et al. 2002). In contrast, endothelial cells bypassing senescence by ectopically expressing hTERT displayed no decline in eNOS expression or NO production (Matsushita et al. 2001; Minamino et al. 2002). One of the most potent inducers of eNOS expression in endothelial cells is laminar shear stress. The effect of shear stress on eNOS expression was also markedly blunted in senescent endothelial cells, but again could be rescued by hTERT over-expression. The same was true of the NO-mediated inhibition of monocyte adhesion to the endothelial monolayer (Matsushita et al. 2001). Levels of another important vasodilator, prostacyclin, were also found to be reduced in endothelial cultures undergoing senescence in vitro (Nakajima et al. 1997).

In contrast to endothelial cells that became senescent after successive rounds of replication, endothelial cells undergoing premature senescence due to oxidative stress actually increased eNOS expression, despite the fact that their ability to produce NO was reduced (Chen et al. 2002). Similarly, in the aortic wall of ageing rats in vivo, while NO production was impaired, eNOS was upregulated about sevenfold (van der Loo et al. 2000). This reduction in NO bioavailability was attributed to an increase in mitochondrial-derived ROS and concomitant production of peroxynitrite (van der Loo et al. 2000). While on the one hand these findings would be consistent with the occurrence of stress-induced senescence in the vasculature, it is important to point out that senescent endothelial cells produce higher levels of ROS than their younger counterparts (Unterluggauer et al. 2003) Hence, oxidative stress is not only a stimulus for senescence but also an outcome of this process. This concept is further substantiated by evidence showing that senescent HUVEC underexpress the so-called senescence evasion factor (SNEV) (Grillari et al. 2000), a protein associated with cellular life-span extension as well as with resistance to oxidative stress and DNA damage (Voglauer et al. 2006).

3.5 Senescence and Apoptosis

The activation of the p53 pathway has been implicated not only in senescence but also in apoptosis (Wahl and Carr 2001), raising the possibility that the two processes may be interrelated. This relationship between senescence and apoptosis has been investigated in various cell types, leading in some cases to conflicting observations. Thus, cultured fibroblasts and keratinocytes were shown to become resistant to apoptosis upon senescence (Chaturvedi et al. 1999; Wang 1995) while the opposite effect has been reported for endothelial cells (Wagner et al. 2001). Other studies suggest that endothelial cell senescence by itself does not result in apoptosis, but rather it increases the sensitivity of these cells to apoptotic stimuli such as TNF-α and oxidised LDL (Hoffmann et al. 2001; Spyridopoulos et al. 2002). In one case this effect was attributed to the reduced levels of NO present in senescent cells (Hoffmann et al. 2001; see Sect. 3.4 above), and it is consistent with the notion that physiological concentrations of NO promote cell survival (Moncada and Erusalimsky 2002).

Studies in HUVEC examining the effects of p53, p21 and p16 expression on the induction of apoptosis or senescence indicate that the p53/p21 pathway is involved in the induction of both phenomena, whereas the p16 pathway is only involved in the induction of senescence (Chen et al. 2006). In addition, these studies showed that the primary role of the p53/p21 pathway is in the control of apoptosis. Thus, these findings suggest that in endothelial cells, whether or not apoptosis occurs in association with senescence may also depend in part on the relative levels of expression of each of these mediators.

4
Senescence of Endothelial Cells In Vivo

In tissues such as skin, for which the rates of cell turnover are relatively high, the presence of senescent fibroblasts and keratinocytes, once identified (Dimri et al. 1995), has not been called into question. In contrast, in the case of the endothelium, for which cellular turnover is generally considered to be extremely low (Hobson and Denekamp 1984; Schwartz et al. 1980), the suggestion that senescent cells might accumulate in vivo has been a matter of controversy.

4.1
Early Studies of Endothelial Regeneration in Animal Models

Early studies using animal models of aortic and carotid artery endothelial regeneration showed that, while small denuded areas could be rapidly and totally repaired with new endothelial cells (Reidy and Schwartz 1981), large denuded areas were not completely repopulated (Reidy et al. 1982, 1983), even after a long period of time (Clowes et al. 1986). Initially it was suggested that the cessation of growth was the result of endothelial cell senescence occurring following a period of sustained regeneration (Reidy et al. 1982). However, this interpretation was later abandoned when it was shown that incompletely regenerated endothelium, although unable to continue to re-populate the remaining area of denudation, was still capable of replication after it was wounded a second time (Reidy et al. 1983). Further studies also indicated that complete regeneration of large areas of endothelium was possible, provided that denudation of the vessel wall was performed without trauma to the media (Lindner et al. 1989). Thus, these early experiments indicated that injury-induced endothelial cell proliferation did not deplete the replicative capacity of the endothelium around the denuded area.

4.2
Endothelial Cell Senescence in Ageing and Vascular Pathology

Ageing is associated with endothelial dysfunction (Celermajer et al. 1994; Tschudi et al. 1996; Zeiher et al. 1993), impaired angiogenesis (Rivard et al. 1999), defective endothelial repair capacity (Gennaro et al. 2003) and an increasing prevalence of atherosclerosis (Eggen and Solberg 1968; Strong and McGill 1962; Weingand et al. 1986). The reasons for these associations are still unclear, but endothelial cell senescence has been increasingly implicated as a possible cause.

Animal studies have revealed that in areas of vascular transitions, such as bifurcations and branching points, the rate of endothelial cell replication is increased (Caplan and Schwartz 1973; Wright 1968). These sites, which in humans correspond to the “atherosclerosis-prone areas” (Stary et al. 1992), are

subjected to changes in haemodynamic forces of shear and stretch that may act as a source of chronic injury to the endothelium (Glagov et al. 1988). Hence, in these locations the endothelium may respond with an increase in turnover, in an attempt to maintain its integrity (Langille et al. 1986). This suggests that, in vivo, as chronological age advances, areas with high endothelial cell turnover could be increasingly covered by clusters of senescent cells. Furthermore, the vasculature is chronically exposed to a variety of oxidative burdens, including oxidative metabolites released from activated phagocytes, modified lipoproteins, and various types ROS generated by vascular cells themselves (Harrison et al. 2003; Sorescu et al. 2001; Witztum and Steinberg 2001), all of which may accelerate the process of senescence in vivo. Consistent with this possibility, as summarised in the remainder of this section, several lines of evidence point to an age-associated emergence of senescent endothelial cells in vivo, and to a prevalence of this phenomenon in areas of the vasculature which are more susceptible to the development of atherosclerosis.

4.2.1 Morphological and Cytogenetic Evidence

Ultrastructural studies demonstrate that endothelial cells covering the surface of aortic, carotid and coronary atheromatous lesions at various stages of disease progression display morphological features reminiscent of senescent cells grown in culture (see Sect. 3.1), the most conspicuous of which is the presence of giant cells (Burrig 1991; Repin et al. 1984). An increase in the incidence of large cells, often showing multiple nuclei, has also been described in primary cultures derived from aortae affected by atherosclerosis (Antonov et al. 1986; Tokunaga et al. 1989). Cell size and the frequency of aneuploidy have been reported to increase also as a function of donor age in endothelial cells of the human abdominal aorta (Aviv et al. 2001). Furthermore, Minamino et al. (2002) reported that SA-β-gal-positive human coronary endothelial cells present on the surface of atherosclerotic plaques appear flattened and enlarged, in contrast to the round shape of endothelial cells in non-diseased regions.

4.2.2 Identification of In Vivo Senescence by SA-β-gal Staining

In our laboratory we have used a balloon endothelial denudation model as a mechanism for promoting vascular cell replication, in order to look for vascular cell senescence in non-atheromatous rabbit carotid arteries. Six weeks after a single denudation we found SA-β-gal-positive cells in both the neointima and the media of the injured vessel, and immunohistochemical analysis identified these as endothelial and vascular smooth muscle cells (Fenton et al. 2001). We also found that a second denudation resulted in

a marked acceleration in the accumulation of senescent cells. Around the same time, Vasile et al. (2001) reported the occurrence of endothelial cells overlying human aortic atherosclerotic plaques, which expressed SA-β-gal activity and which showed differential gene expression consistent with a senescent phenotype. Minamino et al. (2002) extended these findings, confirming the presence of SA-β-gal-positive endothelial cells in atherosclerotic plaques of coronary arteries. SA-β-gal staining was also used to reveal the presence of senescent endothelial cells in the aorta of diabetic rats (Chen et al. 2002).

4.2.3 Telomere Shortening In Vivo

Telomere length has been measured in specimens of intimal tissue and endothelial cells obtained from different regions of the vasculature and from donors of different ages (Aviv et al. 2001; Chang and Harley 1995; Okuda et al. 2000). Collectively, these studies indicate that telomeres in the endothelium shorten with age and that this erosion is more pronounced in the atherosclerosis-prone areas. However, it should be noted that discrepancy exists with regard to the rates of attrition. In one study the rate of shortening was calculated to be 87 bp per year for the intima of the internal mammary artery compared to 147 bp per year for the intima of the iliac artery (Chang and Harley 1995). In contrast, another study reported rates of 15 and 28 bp year for the intima of the proximal and distal abdominal aorta, respectively (Okuda et al. 2000). Despite these quantitative differences, these findings support the tenet that endothelial cells in atherosclerosis-prone vessels manifest increased rates of turnover.

A recent paper by Ogami et al. (2004) examined the relationship between telomere length and coronary artery disease. The authors demonstrated that telomeres in diseased coronary endothelium were shorter than in non-diseased age-matched specimens.

5 Telomere Length as a Hereditable Determinant of Cardiovascular Disease

Telomere length varies considerably between individuals of the same age, both in neonates and in adults. Genetic studies performed in twins have demonstrated that these inter-individual differences are to a large extent genetically determined (Slagboom et al. 1994), and the hereditability of telomere length has since been confirmed in three other cohorts (Jeanclos et al. 2000; Nawrot et al. 2004; Vasa-Nicotera et al. 2005). The replicative capacity (ex vivo) and the telomere length of human cells decline with increasing age of the donor (Allsopp et al. 1992). Does this mean that individuals with shorter telomeres might

be at increased risk of developing age-related degenerative diseases as a result of having a reduced capacity to regenerate their tissues in response to life-long wear and tear? Research investigating the association between telomere length in peripheral white blood cells (WBC) and a number of cardiovascular disease states or their risk factors seem to support this concept (Table 1). These studies have relied on the use of WBC as a surrogate for endothelium because vascular tissue specimens from large numbers of patients are not readily available. Two reports demonstrating a strong intra-individual correlation between telomere lengths of WBC and fibroblasts or synovial cells from the same donor provide evidence to support this strategy (Friedrich et al. 2000; von Zglinicki et al. 2000). Nonetheless, a caveat remains, since to date no reports comparing WBC and endothelial cell telomere length from the same donor have been published.

Table 1 shows that, apart from age, risk factors which have been associated with shorter WBC telomere length include male sex, smoking, high pulse pressure, insulin resistance and body mass index (Gardner et al. 2005; Jeanclos et al. 2000; Nawrot et al. 2004). In one small study, WBC telomere length was shown to be shorter in patients with severe coronary disease than age-matched controls (Samani et al. 2001). In a larger case-control study, the same researchers also found a significant association between shorter WBC telomere length and the risk of premature (<50 years of age) myocardial infarction (Brouilette et al. 2003). Similarly, carotid atherosclerosis has been associated with shorter telomeres, when hypertensive patients with and without carotid artery plaques were compared (Benetos et al. 2004). Looking beyond atherosclerosis, it was recently demonstrated that patients with degenerative calcific aortic valve stenosis, an archetypal age-related disorder, have shorter WBC telomeres than controls matched for age, sex and the presence or absence of coronary disease (Kurz et al. 2006). Shorter telomeres have also been associated with vascular dementia (von Zglinicki et al. 2000). To date only one study has examined the prognostic relevance of telomere length in a longitudinal fashion. In this study of 143 healthy individuals older than 60 years of age, shorter telomeres in WBC were associated with a 3.2-fold higher mortality rate from cardiovascular disease (Cawthon et al. 2003).

Although the studies summarised in Table 1 appear to delineate a consistent line of evidence, it needs to be emphasised that these studies show only an association, without actually demonstrating that telomere shortening is a primary abnormality rendering organisms more susceptible to atherosclerotic risk factors. One could argue that the shorter WBC telomere length found in atherosclerotic vascular disease might instead reflect the increased turnover of WBCs associated with the chronic systemic inflammatory state in this disease. According to this concept, shorter WBC telomeres in age-related vascular disease would be a secondary epiphenomenon without any causal involvement.

Table 1 Conditions or diseases associated with short telomere length in peripheral white blood cells

Condition/disease	No. of patients	Age (mean or range)	Control population	*p*-Value	Reference
Cardiovascular mortality	143	>60	N/A (supra- vs sub-median groups)	0.008	Cawthon et al. (2003)
Coronary atherosclerosis	10	42–72	Normal coronary arteries (*n*=20)	0.002	Samani et al. (2001)
Premature myocardial infarction (<50 years)	203	47 vs 47	Healthy controls (*n*=180)	<0.0001	Brouilette et al. (2003)
Vascular dementia	41	18–98	Healthy controls (*n*=73)	<0.001	von Zglinicki et al. (2000)
Hypertensives with carotid atherosclerosis	73	60 vs 64	Hypertensives with no carotid atherosclerosis (*n*=90)	0.03	Benetos et al. (2004)
Age-related calcific aortic stenosis	64	77 vs 76	No aortic stenosis (*n*=129)	0.0003	Kurz et al. (2006)
Increased pulse pressure	98	18–44	N/A (correlation)	0.0032	Jeanclos et al. (2000)
Smokers	82	15–80	Non-smokers (*n*=189)	0.014	Nawrot et al. (2004)
Male sex	119	15–80	Females (*n*=152)	0.028	Nawrot et al. (2004)
Insulin resistance	49	21–44	N/A (correlation)	<0.001	Gardner et al. (2005)
Body mass index	70	21–44	N/A (correlation)	<0.001	Gardner et al. (2005)

N/A, not applicable

6
Models of Ageing to Examine the Role of Senescence in Vascular Pathology

6.1
Animal Models

During the past few years a number of genetically manipulated mice models displaying a premature ageing phenotype have been generated. Among these, mice with deletions of genes involved in genome maintenance represent the largest group (Hasty et al. 2003). In most of these models, an increase in cellular senescence has been observed, but anomalies of the vascular system have not been reported. In contrast, vascular phenotypes have been identified in some mouse models of ageing associated with alterations in redox homeostasis. For instance, the $p66^{shc}$ knock-out mouse shows an increased life-span (Migliaccio et al. 1999), a reduced development of early atherogenesis in response to a high-fat diet (Napoli et al. 2003) and a reduction in the age-related loss of endothelial function (Francia et al. 2004). However, reports on cellular senescence in the $p66^{shc}$ knock-out mice are lacking. Conversely, premature senescence, accelerating ageing and early atherogenesis in response to a Western-type diet have been found in the so-called senescence-accelerated mouse (Fenton et al. 2004). Finally, a mutant mouse defective in the *klotho* gene, which encodes a single-pass transmembrane protein, has been developed (Kuro-o et al. 1997). The phenotype of this mouse includes a reduced life-span and a wide range of typical age-related disorders, including atherosclerosis. A secreted form of Klotho has been detected in mouse and human blood (Imura et al. 2004) that might function as a circulating anti-ageing hormone (Kurosu et al. 2005). Indeed, recent evidence suggests that Klotho protein binds to a cell-surface receptor and counteracts insulin/insulin-like growth factor-1-stimulated intracellular signalling (Kurosu et al. 2005), resulting in an increased resistance to oxidative stress (Yamamoto et al. 2005). Furthermore, Klotho protein has now been shown to reduce oxidative stress-induced senescence in HUVEC (Ikushima et al. 2006). However, the occurrence of endothelial cell senescence in Klotho-mutant mice has yet to be reported.

Two different telomerase knock-out mice, $mTR^{-/-}$ and $mTERT^{-/-}$ (targeting the RNA component or the reverse transcriptase component, respectively) have been generated (Blasco et al. 1997; Yuan et al. 1999). Detailed studies in $mTR^{-/-}$ mice have shown the development of senescence-related organ failure in a number of systems after several inbred generations (Rudolph et al. 1999). However, a spontaneous vascular phenotype has not been detected in these mice thus far (reviewed in Serrano and Andres 2004).

The lack of a relationship between senescence and vascular disease in most murine models of accelerated ageing should be interpreted with caution in view of the fact that (1) mice do not naturally develop atherosclerosis and (2) telomere biology differs significantly between mice and humans, with

mice having much longer telomeres and constitutive telomerase activity. In order to overcome these limitations, Poch et al. (2004) investigated the phenotype of late generation telomerase-deficient mice which had been crossbred with apolipoprotein E-null mice. The latter, when fed a high-cholesterol diet, develop extensive arterial atheromatosis, and are considered an established model of human atherosclerosis. These double-knock-out mice with short telomeres proved to be resistant to the development of atheromatosis, which was thought to be a result of replicative immuno-senescence in these animals. Indeed, proliferation of macrophages and lymphocytes, two cellular elements which are instrumental in atherogenesis, was markedly impaired in these animals.

Neovascularisation and vascular repair has also been investigated in mouse mutants displaying premature ageing. Franco et al. (2002) demonstrated that angiogenesis in Matrigel implants as well as tumour angiogenesis was reduced in late generation telomerase-deficient mice, resulting in decreased tumour growth. Similarly, in the Klotho mutant mouse, tissue angiogenesis was impaired in response to hind limb ischaemia (Shimada et al. 2004).

6.2 Human Genetic Disorders

A number of human genetic disorders are associated with abnormal telomere homeostasis and premature ageing. One of the most conspicuous pathological features in two of these syndromes, Werner syndrome and Hutchinson–Gilford progeria, is the occurrence of severe generalised atherosclerosis at a young age. Patients with Werner syndrome have an average life expectancy of 40–50 years and also suffer from ageing of the skin, cataracts and diabetes in young adulthood. Werner syndrome is caused by mutations in the *wrn* gene, which encodes a RecQ DNA helicase (Yu et al. 1996). Cells from Werner patients are characterised by slow proliferation, premature senescence due to accelerated telomere erosion, and genomic instability, features which result from incomplete lagging strand replication of sister chromatids (Crabbe et al. 2004). Patients with Hutchinson–Gilford progeria develop a wizened old appearance when very young and usually die of coronary disease by age 10. The defect involves the gene *lmna*, which encodes the nuclear envelope proteins lamins A and C, although it remains unclear how this results in the phenotype. These children are born with telomeres which are only about half the length of those in age-matched controls (Allsopp et al. 1992).

In contrast to the above two syndromes, two other well-characterised inherited disorders associated with short telomeres and features of premature ageing, dyskeratosis congenita and ataxia telangiectasia, do not feature accelerated development of atherosclerosis. Two different genetic variants of dyskeratosis congenita exist. The X-linked form has a mutation in the dyskerin gene, which is involved in ribosomal DNA processing and telomerase function,

while the autosomal dominant form has a defect in the gene for the telomerase RNA component. Dyskeratosis congenita patients have reduced telomerase activity and short telomeres, and usually die in young adulthood of bone marrow failure, cancer or pulmonary complications (Marciniak et al. 2000). Patients with the autosomal recessive disorder ataxia telangiectasia have mutations in *atm* (*ataxia telangiectasia mutated*), which encodes a protein involved in DNA damage repair. They suffer from neurological degeneration, premature ageing and increased neoplasia. Cells from these patients display accelerated telomere shortening in vitro, probably due to an impairment in the repair of oxidative damage to telomeric DNA (Tchirkov and Lansdorp 2003). The absence of atherosclerosis in patients with either of these two disorders would appear to argue against a role for telomere-based cell senescence in atherogenesis.

7 Endothelial Progenitor Cell Senescence

Mathematical models integrating endothelial cell turnover, response to oxidative stress, and the sequelae of telomere shortening and senescence predict that by the age of approximately 65 years critical defects in the endothelial monolayer of the vasculature would become a relevant problem (Op den Buijs et al. 2004). These models also predict that such a problem would be significantly alleviated if endothelial progenitor cells (EPCs) could home to the vasculature at a rate of about 5%/year, a rate that was inferred from data obtained from experimental systems of vascular remodelling (Crosby et al. 2000). Nonetheless, while bone-marrow-derived, circulating EPCs are thought to make an important contribution to post-natal tissue vascularisation (Rafii and Lyden 2003), their role in the maintenance of endothelial integrity in the vasculature is less well established.

EPCs appear to be sensitive to atherosclerotic risk factors which reduce their number in the circulation (Vasa et al. 2001) and increase their propensity to undergo premature senescence when placed in culture (Hill et al. 2003; Imanishi et al. 2005a). The ex vivo life-span of these cells can be extended by transient transfection of hTERT (Murasawa et al. 2002) and by adding statins (Assmus et al. 2003) or oestrogen (Imanishi et al. 2005b) to the culture medium. The effects of statins in prolonging life-span appears to be mediated by inhibiting isoprenylation which, by some as-yet-undefined mechanism, prevents the loss of TRF-2 (Spyridopoulos et al. 2004; See also Sect. 2.1.3). The effect of oestrogen, on the other hand, was associated with an increase in telomerase activity (Imanishi et al. 2005b), although a causal relationship in this case was not established.

The increased susceptibility of EPCs to undergo premature senescence when placed in culture suggests that these cells have a diminished capacity to withstand oxidative stress. In line with this possibility, it has been shown recently

that oxidized LDL accelerated EPC senescence in vitro and that this effect was accompanied by a reduction in telomerase activity; both these effects were prevented by atorvastatin (Imanishi et al. 2004). As statins have also been reported to reduce oxidative stress, it is possible that this property may be in part responsible for its effect on the life-span of EPCs. The importance of oxidative stress in EPC senescence has been further highlighted by studies showing that angiotensin II accelerates the onset of EPC senescence and reduces telomerase activity; both these effects are inhibited by addition of superoxide dismutase (Imanishi et al. 2005c).

8 Concluding Remarks

Research carried out over the last 5 years has shown that endothelial cell senescence occurs in vivo. In cell culture, initiation of the senescence programme depends on the integrity of telomeres but can also be induced by other mechanisms, including the up-regulation of certain oncogenes and oxidative stress. A stress-based mechanism and a telomere-based mechanism often seem to act in synergy, dictating the timing of the onset of senescence. Ultimately, senescence is implemented via activation of the p53/p21, p16, or both pathways, and possibly via other cellular stress response pathways. The question of whether these mechanisms are responsible for the emergence of senescent cells in the endothelium in vivo has not been clearly answered.

Both genetic and environmental factors influence telomere length. Although shorter telomeres seem to be associated with a number of age-related cardiovascular disorders, their causal involvement remains unproven. Levels of telomerase in endothelial cells are very low, but this activity appears to be essential to maintain cellular replicative capacity in vitro. Whether telomerase activity prevents senescence in endothelial cells by maintaining the 3′ overhang (or by another mechanism) and whether this is relevant for the maintenance of a fully functional endothelium in vivo are unknown at present.

Despite these uncertainties, increasing evidence implicates cellular senescence in atherosclerosis initiation and progression, and supports the concept that the number of vascular cell replications that take place in the vessel wall increases not only as a function of chronological age, but also as a function of the haemodynamic or biochemical stress to the vascular bed. This would link vascular cell senescence to the "response to injury" hypothesis of atherosclerosis (Ross 1993).

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The Vascular Endothelium in Hypertension

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Abstract The vascular endothelium plays a fundamental role in the basal and dynamic regulation of the circulation. Thus, it has a crucial role in the pathogenesis of hypertension. A spectrum of vasoactive substances is synthesised in the endothelium; of these, nitric oxide (NO), prostacyclin (PGI_2) and endothelin (ET)-1 are the most important. There is a continuous basal release of NO determining the tone of peripheral blood vessels. Systemic inhibition of NO synthesis or scavenging of NO through oxidative stress causes an increase in arterial blood pressure. Also, the renin–angiotensin–aldosterone system has a major role in hypertension as it has a direct vasoconstrictor effect and important interactions with oxygen free radicals and NO. Prostacyclin, in contrast to NO, does not contribute to the maintenance of basal vascular tone of conduit arteries, but its effect on platelets is most important. ET acts as the natural counterpart to endothelium-derived NO and

has an arterial blood pressure-raising effect in man. Anti-hypertensive therapy lowers blood pressure and may influence these different mediators, thus influencing endothelial function. In summary, due to its position between the blood pressure and smooth muscle cells responsible for peripheral resistance, the endothelium is thought to be both victim and offender in arterial hypertension. The delicate balance of endothelium-derived factors is disturbed in hypertension. Specific anti-hypertensive and anti-oxidant treatment is able to restore this balance.

Keywords Endothelium · Hypertension · Nitric oxide · Endothelin · Oxidative stress

The vascular endothelium synthesises and releases a spectrum of vasoactive substances and therefore plays a fundamental role in the basal and dynamic regulation of the circulation (Lüscher and Vanhoutte 1990). Due to its strategic anatomical position, the endothelium is constantly exposed to the different risk factors for atherosclerosis.

1 Endothelial Vasoactive Substances

The endothelium—probably the largest and most extensive tissue in the body—forms a highly selective permeability barrier and is a continuous, uninterrupted, smooth, and non-thrombogenic surface. The endothelium synthesises and releases a broad spectrum of vasoactive substances (Fig. 1), including nitric oxide (NO), prostacyclin (PGI_2) and endothelin (ET)-1.

NO prevents leucocyte adhesion and migration into the arterial wall, smooth muscle cell proliferation, and platelet adhesion and aggregation, i.e. key events in the development of atherosclerosis (Bhagat et al. 1996; Bhagat and Vallance 1997; Boulanger and Lüscher 1990; Fichtlscherer et al. 2000; Hingorani et al. 2000; Ross 1999). NO, synthesised by NO synthase (NOS), is released from endothelial cells mainly in response to shear stress produced by blood flow (Anderson and Mark 1989; Furchgott and Zawadzki 1980; Joannides et al. 1995a, b; Palmer et al. 1988a, b; Rubanyi et al. 1986; Stamler et al. 1994; Vallance et al. 1989), leading to relaxation of vascular smooth muscle cells (Fig. 1; Palmer et al. 1988a). ET-1 acts as the natural counterpart to endothelium-derived NO (Lüscher et al. 1990). In addition to its arterial blood pressure-raising effect in man (Kiely et al. 1997; Vierhapper et al. 1990), ET-1 induces vascular and myocardial hypertrophy (Barton et al. 1998; Ito et al. 1991; Yang et al. 1999), which are independent risk factors for cardiovascular morbidity and mortality (Bots et al. 1997; Kannel et al. 1969; O'Leary et al. 1999). ET-1 stimulates the release of inflammatory mediators such as interleukin (IL)-1, IL-6 and IL-8, thereby antagonising the anti-inflammatory effects of NO. NO itself plays an important role in clinical systemic inflammatory syndromes when the inducible isoform of the NO-generating enzyme, iNOS, is activated.

2
Nitric Oxide in Hypertension

2.1
Biological Actions

NO, originally described as endothelium-derived relaxing factor (EDRF), is released from endothelial cells in response to shear stress produced by blood flow, and in response to activation of a variety of receptors (Fig. 1; Anderson and Mark 1989; Furchgott and Zawadzki 1980; Rubanyi et al. 1986; S. Moncada and E.A. Higgs, volume I). NO is a free radical gas—with a half-life in vivo of a few seconds—that is readily able to cross biological membranes (Furchgott and Zawadzki 1980; Palmer et al. 1987; Stamler et al. 1992). After diffusion from endothelial to vascular smooth muscle cells, NO increases intracellular cyclic guanosine monophosphate (cGMP) concentrations by activation of the enzyme guanylate cyclase, leading to relaxation of the smooth muscle cells (Palmer et al. 1988a).

NO is synthesised by NOS from L-arginine (Palmer et al. 1988a). The conversion from L-arginine to NO can be inhibited by false substrates for the NOS, e.g. by N^G-monomethyl-L-arginine (L-NMMA) (Palmer et al. 1988b). Since there is a continuous basal release of NO determining the tone of peripheral

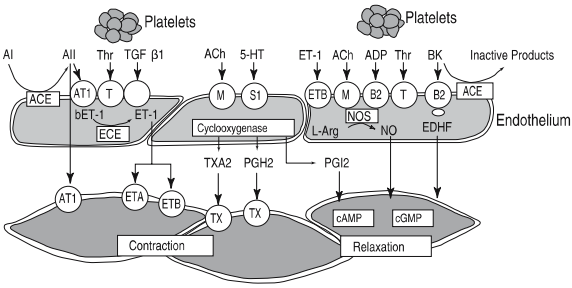

Fig. 1 Endothelium-derived vasoactive substances. Nitric oxide (NO) is released from endothelial cells in response to shear stress and to activation of a variety of receptors. NO exerts vasodilating and anti-proliferative effects on smooth muscle cells and inhibits thrombocyte aggregation and leucocyte adhesion. Endothelin-1 (ET-1) exerts its major vascular effects—vasoconstriction and cell proliferation—through activation of specific ET_A receptors on vascular smooth muscle cells. In contrast, endothelial ET_B receptors mediate vasodilatation via release of NO and prostacyclin. Additionally, ET_B receptors in the lung were shown to be a major pathway for the clearance of ET-1 from plasma. *ACE*, angiotensin-converting enzyme; *ACh*, acetylcholine; *AII*, angiotensin II; AT_1, angiotensin 1 receptor; *BK*, bradykinin; *COX*, cyclooxygenase; *ECE*, endothelin converting enzyme; *EDHF*, endothelium-derived hyperpolarising factor; ET_A and ET_B, endothelin A and B receptor; *ET-1*, endothelin-1; *L-Arg*, L-arginine; PGH_2, prostaglandin H_2; PGI_2, prostacyclin; *S*, serotoninergic receptor; *Thr*, thrombin; *T*, thromboxane receptor; TXA_2, thromboxane; *5-HT*, 5-hydroxytryptamine (serotonin). Modified from Lüscher and Noll (1997)

Table 1 Haemodynamic effects of NO synthase inhibition in healthy volunteers (modified after Spieker et al. 2000a)

	Baseline	L-NMMA	(mg/kg/min)
		0.3	1.0
SBP	134±7	152±5	150±3*
DBP	73±4	87±5	85±5 †
SVR	1114±124	1413±145*	1973±203‡
HR	67±4	70±6	63±6
CI	3.5±0.3	3.1±0.2*	2.3±0.2§
SVI	53±6	48±6	38±5†
CVP	4±0.7	3.6±0.4	4.3±0.05
B/min	23.1±3.5	14±4.5	18.6±5.5

*$p<0.05$, †$p<0.01$, ‡$p<0.001$, §$p<0.0001$, for each data point compared with baseline values
Abbreviations: B/min, sympathetic bursts per minute; CI, cardiac index ($l \cdot min^{-1} \cdot m^2$); CVP, central venous pressure (mmHg); DBP, diastolic blood pressure (mmHg); HR, heart rate (beats/min); L-NMMA, N^G-monomethyl-L-arginine; SBP, systolic blood pressure (mmHg); SVI, stroke volume index ($ml \cdot min^{-1} \cdot m^2$); SVR, systemic vascular resistance ($dyn \cdot s^{-1} cm^{-5}$)

blood vessels, systemic inhibition of NO synthesis causes an increase in arterial blood pressure (Anderson and Mark 1989; Palmer et al. 1988a, b; Rubanyi et al. 1986; Vallance et al. 1989). There are three types of NOS: two constitutive and one inducible isoform. The former, which are present in endothelial cells and neurons, are therefore called endothelial NOS (eNOS) and neuronal NOS (nNOS), respectively. The inducible form (iNOS) is an important inflammatory mediator expressed in macrophages, vascular smooth muscle and other cells in response to immunological stimuli (Palmer et al. 1992). NO has also anti-thrombogenic, anti-proliferative and leucocyte adhesion-inhibiting effects, and influences myocardial contractility (Anderson and Mark 1989; Joannides et al. 1995a, b; Vallance et al. 1989). The haemodynamic effects of pharmacological NO inhibition include an increase in blood pressure and a decrease in cardiac output (Table 1).

2.2 NO in Experimental Models of Hypertension

Endothelium-derived NO-mediated vascular relaxation is impaired in spontaneously hypertensive animals (Table 2; Diederich et al. 1990; Dohi et al. 1990; Lüscher and Vanhoutte 1986; Lüscher et al. 1986). Thus, the bioavailability of NO is reduced. Surprisingly, the NO pathway is paradoxically up-regulated in the resistance circulation and the heart of spontaneously hypertensive rats (SHR) (Kelm et al. 1992; Nava et al. 1998). Adult SHR possess a higher eNOS

Table 2 The nitric oxide (NO) pathway in selected experimental models of arterial hypertension

Animal model	Alteration in NO pathway
Spontaneously hypertensive rats (SHR)	Up-regulation
Stroke-prone SHR (SHRSP)	Up-regulation, but reduced bioavailability
Dahl salt-sensitive rats	Down-regulation
Two-kidney, one clip experimental hypertension (Goldblatt hypertension)	Impaired stimulated NO release, intact basal NO release
DOCA salt hypertensive rats	Impaired basal NO release

activity than their normotensive counterparts (Nava et al. 1995). Very young pre-hypertensive SHR have, in contrast, similar eNOS activity to young normotensive rats without a genetic background for hypertension, indicating that the increased activity of eNOS in adult SHR is indeed related to hypertension (Fig. 2). Moreover, the plasma concentrations of the oxidative product of NO metabolism, nitrate, are higher in hypertensive rats than in normotensive controls (Nava et al. 1998). These results indicate that the basal release of NO is increased in hypertensive rats.

Thus, it appears that in SHR there must be a factor blunting the haemodynamic effect of NO (Grunfeld et al. 1995). Indeed, NO production is increased in stroke-prone SHR (SHRSP), but bioavailability is reduced (McIntyre et al. 1997). Direct in situ measurement of NO release by a porphyrinic microsensor in SHRSP confirmed that hypertension is associated with increased NO

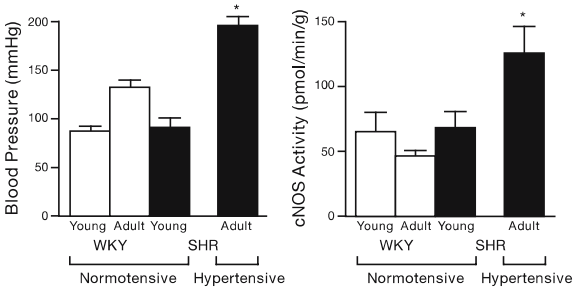

Fig. 2 Increased activity of constitutive nitric oxide synthase in cardiac endothelium of spontaneously hypertensive rats (SHR, *black bars*). Adult SHR possess a higher activity of constitutive nitric oxide synthase (NOS) than their normotensive counterparts (Wistar Kyoto rats, WKY; *open bars*). Very young pre-hypertensive SHR have, in contrast, lower constitutive NOS activity than the normotensive, indicating that the increased activity of NOS in adult SHR is indeed related to hypertension. Modified from Nava et al. (1995)

decomposition by superoxide anions, i.e. free oxygen radicals (Fig. 3; Tschudi et al. 1996). Nevertheless, a further increase of NO by inhibition of arginase, an enzyme which degrades L-arginine, the substrate of NO production by eNOS, has been shown to improve endothelial function and prevent the development of arterial hypertension in SHR (Demougeot et al. 2005).

In other models of hypertension—i.e. in Dahl salt-sensitive rats, in two-kidney, one clip experimental hypertension, and in desoxycorticosterone acetate (DOCA)-salt hypertensive rats—endothelium-dependent relaxation is also impaired (Table 2; Dohi et al. 1991; Hayakawa et al. 1993; Hirata et al. 1995; Lee et al. 1995; Lüscher et al. 1987a). In high-renin arterial hypertension such as the two-kidney, one-clip model there is impaired stimulated NO release but intact basal NO release (Artigues-Varin et al. 2002). Augmented NO production may serve as a counteracting system against the activation of the angiotensin receptor (AT_1) in this high-renin model of hypertension (Cervenka et al. 2002).

NO production by eNOS is reduced rather than up-regulated in Dahl salt-sensitive rats (Fig. 3; Hayakawa et al. 1993; Kakoki et al. 1999; Ni et al. 1999). L-Arginine, the substrate of NO production by eNOS, normalises blood pressure and simultaneously increases urinary excretion of nitrate, the degradation product of NO, in Dahl salt-sensitive rats (Chen and Sanders 1991, 1993; Chen et al. 1993; Hu and Manning 1995). Further mechanisms contribute to the pathogenesis of salt-sensitive hypertension. These include:

– Decreased expression of endothelial ET_B receptors, which mediate NO release (Hirata et al. 1995; Kakoki et al. 1999; Matsuoka et al. 1997)

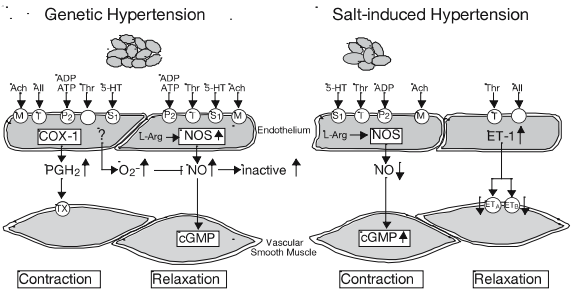

Fig. 3 Heterogeneity of endothelial dysfunction in experimental hypertension. In spontaneous hypertension (*left panel*) nitric oxide synthase (NOS) is upregulated and nitric oxide (NO) is inactivated by superoxide anions. In addition, the production of thromboxane (TXA_2) and prostaglandin H_2 (PGH_2) is increased. In salt-related hypertension (*right panel*), NO production is reduced and the endothelin (ET) system is upregulated. *ACE*, angiotensin-converting enzyme; *ACh*, acetylcholine; *AII*, angiotensin II; AT_1, angiotensin 1 receptor; *cGMP*, cyclic guanosine monophosphate; *COX*, cyclooxygenase; ET_A and ET_B, endothelin A and B receptor; *ET-1*, endothelin-1; *L-Arg*, L-arginine; *M*, muscarinergic receptor; O_2^-, superoxide anion; PGI_2, prostacyclin; *S*, serotoninergic receptor; *T*, thrombin receptor; *Thr*, thrombin; *TX*, thromboxane receptor; *5-HT*, 5-hydroxytryptamine (serotonin). Modified from Spieker et al. (2000b)

– Altered expression of the constitutive brain NOS (nNOS) as well as the iNOS isoform, possibly leading to alterations in renal sympathetic nervous activity and sodium handling (Deng and Rapp 1995; Ikeda et al. 1995; Rudd et al. 1999; Simchon et al. 1996)

Low functional levels of nNOS in the Dahl salt-sensitive rat may indeed contribute to its salt-sensitivity (Tan et al. 1999). In other low-renin models of hypertension, such as the DOCA salt-sensitive rat, there is augmented vascular superoxide production mediated via an ET_A/NADPH oxidase pathway (Li et al. 2003).

2.3 Nitric Oxide in Human Hypertension

There are several techniques for the assessment of NO bioavailability in man. Most often, flow-mediated vasodilatation (FMD) of the brachial artery—a marker of endothelial function—is assessed by high-resolution ultrasonography (Fig. 4). Alternatively, endothelium-dependent or -independent vasomotion in response to intra-arterially infused vasoactive substances is assessed using venous occlusion plethysmography. Among the most often used endothelium-dependent vasodilators are acetylcholine and serotonin (5-hydroxytryptamine). Sodium nitroprusside or nitroglycerin serves as an endothelium-independent vasodilator. Recently, new guidelines for assessment of endothelial function and dysfunction have been published, underlining the importance of standardised methods (Deanfield et al. 2005).

Endothelial dysfunction plays a crucial role in arterial hypertension (Brunner et al. 2005). Endothelium-dependent vasodilatation in response to acetylcholine is impaired in patients with arterial hypertension, both in the forearm circulation (Fig. 5; Creager and Roddy 1994; Hirooka et al. 1992; Linder et al. 1990; Panza et al. 1990, 1993a, b, c 1994; Taddei et al. 1994, 1995, 1997a) and in the coronary vascular bed (Egashira et al. 1995; Treasure et al. 1993). Especially in populations at low risk, endothelial function measured by FMD is related to the principal cardiovascular risk factors (Witte et al. 2005). There is a strong correlation between endothelium-dependent vasodilatation in the human forearm and coronary vascular beds (Anderson et al. 1995; Takase et al. 1998).

Basal NO activity is decreased in hypertensive patients (Calver et al. 1992). Furthermore, urinary excretion of the metabolic oxidation product of NO, ^{15}N nitrate, after administration of ^{15}N-labelled arginine (i.e. the substrate for the generation of NO) is reduced in hypertensive patients compared to normotensive controls (Fig. 5; Forte et al. 1997). Thus, whole-body NO production in patients with essential hypertension is diminished under basal conditions. In line with these findings, the vasoconstrictor response to L-NMMA, an inhibitor of NO synthesis, is significantly less in hypertensive patients compared

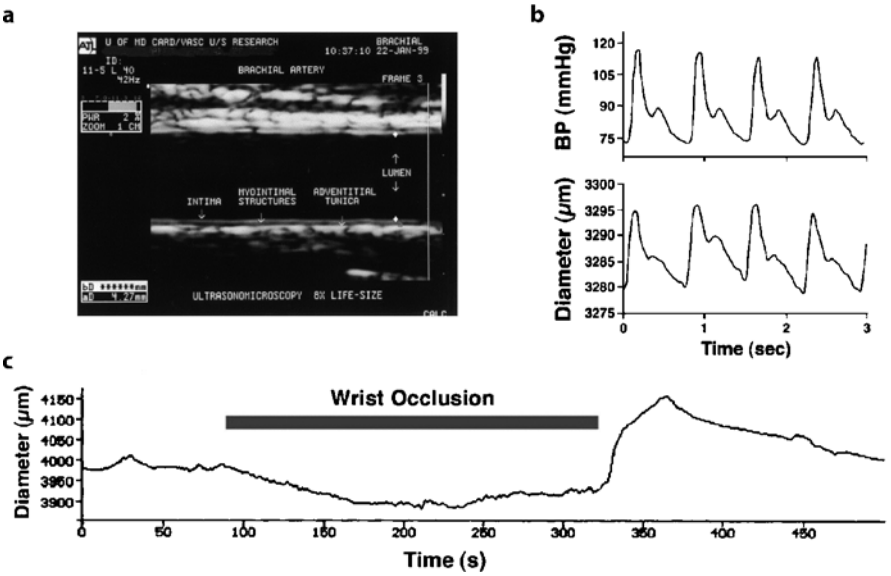

Fig. 4 Flow-mediated vasodilatation of the brachial artery is measured by high-resolution ultrasonography (**a**). With the use of echo-tracking, arterial diameter can be measured on a beat-to-beat basis (**b**). After establishing stable baseline conditions, flow-mediated vasodilatation is measured after release of a blood pressure cuff placed around the wrist and inflated to suprasystolic pressure for 5 min (**c**). The resulting hyperaemic blood flow to the hand after release of the wrist cuff leads to a more or less pronounced vasodilatation of the brachial artery, which is mediated by endothelium-derived nitric oxide (NO)

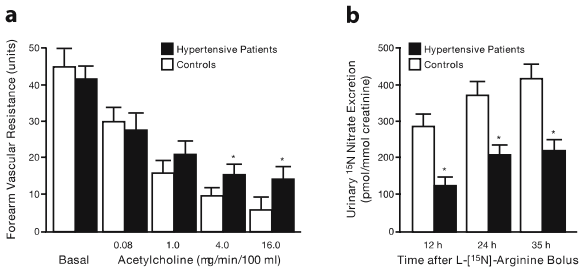

Fig. 5 a,b Endothelial dysfunction in arterial hypertension. **a** Patients with hypertension exhibit decreased endothelium-dependent vasodilatation in response to acetylcholine compared to normotensive controls. Modified from Linder et al. (1990). **b** Cumulative urinary excretion of ^{15}N nitrate after administration of ^{15}N-labelled arginine, i.e. the substrate for enzymatic production of NO. Urinary excretion of the metabolic oxidation product of NO, nitrate, is reduced in hypertensive patients compared to normotensive controls. These data show that whole-body NO production in patients with essential hypertension is diminished under basal conditions. Modified from Forte et al. (1997)

with normotensives, whereas there is no difference between hypertensives and normotensives in the response to noradrenaline, an endothelium-independent vasoconstrictor (Calver et al. 1992; Taddei et al. 1999a).

Normotensive offspring of hypertensive parents exhibit impaired endothelium-dependent vasodilatation to acetylcholine (Taddei et al. 1992). Vasoconstriction in response to an inhibitor of NO synthesis is also decreased in such subjects, indicating impaired basal synthesis of NO (McAllister et al. 1999). Thus, derangement of endothelial function in hypertension is likely to be caused in part by genetic factors, and is not just a consequence of elevated blood pressure (although the haemodynamic factor makes an important contribution) (Millgard and Lind 1998).

NO has a direct effect on vascular tone but, in addition, there is growing evidence that NO influences vascular tone by interaction with the central autonomic nervous system, resulting in sympatho-inhibitory effects in animals (Lewis et al. 1991) and in humans (Lepori et al. 1998). This indirect effect may also play an important role in the pathogenesis of arterial hypertension (Sartori et al. 2005).

3
Oxidative Stress in Hypertension

Oxidative stress plays an important role in the pathogenesis of hypertension (Fig. 6). Superoxide anion (O_2^-), an oxygen radical, can scavenge NO to form peroxynitrite ($ONOO^-$), effectively reducing the bioavailability of endothelium-derived NO (Fig. 7; Rubanyi and Vanhoutte 1986; Tschudi et al. 1996). In addition, O_2^- can act as a vasoconstrictor (Auch-Schwelk et al. 1989; Cosentino et al. 1994; Katusic et al. 1993; Katusic and Vanhoutte 1989). Nicotinamide adenine dinucleotide (NADH) dehydrogenase, a mitochondrial enzyme of the respiratory chain, seems to be a major source of O_2^- (Turrens and Boveris 1980). Expression of NAD(P)H oxidase in human coronary artery smooth muscle cells is up-regulated by pulsatile stretch, generating increased oxidative stress (Hishikawa et al. 1997). Another source of O_2^- is cyclooxygenase (COX) (Kontos et al. 1985). In contrast, xanthine oxidase, another generator of superoxide anions, does not appear to play a significant role in essential hypertension (Cardillo et al. 1997; Hishikawa et al. 1997).

Paradoxically, NOS (i.e. the NO generating enzyme) can also produce O_2^- (Cosentino et al. 1998; Kerr et al. 1999; Stroes et al. 1998). Production of O_2^- in SHRSP, an experimental model of genetic hypertension, can be prevented by NOS inhibition (Kerr et al. 1999). Administration of exogenous tetrahydrobiopterin (BH_4), an essential cofactor for NOS, can reduce excess O_2^- in the aorta of SHRSP (Kerr et al. 1999). In pre-hypertensive SHR, the calcium ionophore A23187 (a receptor-independent activator of NOS)-stimulated pro-

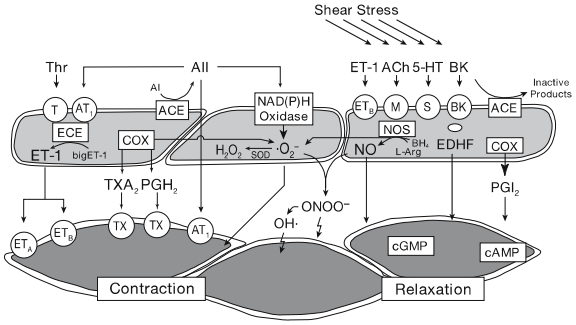

Fig. 6 Role of oxidative stress in the pathogenesis of endothelial dysfunction in hypertension. Superoxide anion, generated by angiotensin II-activated NAD(P)H oxidase, by dysfunctional NO synthase, and by cyclooxygenase, can scavenge the vasodilator NO to form the highly reactive peroxynitrite. Peroxynitrite can damage cell membranes and oxidise lipids. In addition, superoxide anion can act as a vasoconstrictor. *ACE*, angiotensin-converting enzyme; *ACh*, acetylcholine; *AII*, angiotensin II; AT_1, angiotensin 1 receptor; BH_4, tetrahydrobiopterin; *BK*, bradykinin; *COX*, cyclooxygenase; *ECE*, endothelin-converting enzyme; *EDHF*, endothelium-derived hyperpolarising factor; ET_A and ET_B, endothelin A and B receptor; *ET-1*, endothelin-1; H_2O_2, hydrogen peroxide; *L-Arg*, L-arginine; *NAD(P)H oxidase*, nicotinamide adenine dinucleotide oxidase; O_2^-, superoxide anion; *OH*, hydroxyl radical; $ONOO^-$, peroxynitrite; PGH_2, prostaglandin H_2; PGI_2, prostacyclin; *S*, serotoninergic receptor; *SOD*, superoxide dismutase; *Thr*, thrombin; *TXA*, thromboxane receptor; TXA_2, thromboxane; *5-HT*, 5-hydroxytryptamine (serotonin). Modified from Spieker et al. (2000b)

duction of O_2^- was significantly higher than in control rats. NO release was reduced in SHR aortas, with opposite results in the presence of exogenous BH_4. Thus, dysfunctional endothelial NOS may be a source of O_2^- in pre-

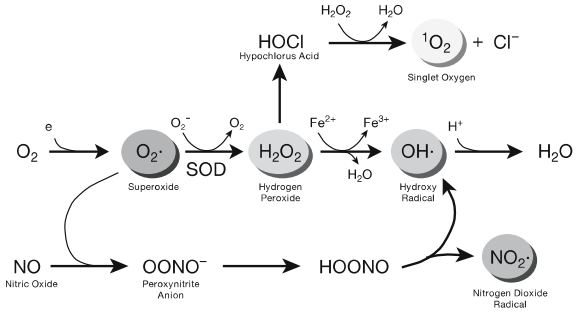

Fig. 7 Superoxide anion (O_2^-), an oxygen radical, is detoxified by superoxide dismutase (SOD), forming H_2O_2 which is further metabolised by catalase. However, the reaction between the two radicals O_2^- and NO is three times faster than the detoxification of O_2^- by SOD. Depending on the relative concentrations of NO and SOD, there may be a propensity for O_2^- to preferentially react with NO. O_2^- can scavenge NO to form peroxynitrite ($ONOO^-$), effectively reducing the bioavailability of endothelium-derived NO

hypertensive SHR and may contribute to the development of hypertension and its vascular complications (Cosentino et al. 1998; Jameson et al. 1993).

O_2^- is finally detoxified by superoxide dismutase (SOD), forming H_2O_2 which is further metabolised by catalase (Fridovich and Freeman 1986). However, the reaction between the two radicals O_2^- and NO is three times faster than the detoxification of O_2^- by SOD (Thomson et al. 1995). Depending on the relative concentrations of NO and SOD, there may be a propensity for O_2^- to react preferentially with NO, resulting in decreased bioavailability of NO. In SHR aortas, SOD (Sekiguchi et al. 2004) or the oral administration of potent anti-oxidants such as flavonoids (Machha and Mustafa 2005) is able to improve endothelium-dependent relaxation. This underlines the importance of scavenging free oxygen radicals, as the imbalance between oxidative stress and the anti-oxidant defence mechanism is considered a major factor in the development of hypertension.

The gene for cytosolic SOD (i.e. SOD1) is located on the 21q22.1 region of chromosome 21 (Levanon et al. 1985). Patients with Down's syndrome (trisomy 21) have an extra copy of the SOD gene. Because of gene dosage excess, their SOD activity is 50% greater than in the diploid population, leading to reduced O_2^- levels (De La Torre et al. 1996). Patients with Down's syndrome have lower blood pressure levels, indicating a major role for O_2^- in the regulation of arterial blood pressure. Furthermore, the normal age-associated increase of blood pressure is absent in patients with Down's syndrome (Morrison et al. 1996).

3.1 The Renin–Angiotensin–Aldosterone System

The renin–angiotensin system plays a major role in hypertension (Fig. 1; Goldblatt et al. 1934; C. Dimitropoulou et al., volume I). Apart from the direct vasoconstrictor effects of angiotensin II (ANG II), there are important interactions between ANG II, oxygen radicals, and NO. Indeed, ANG II stimulates the generation of O_2^- by increasing the expression of the NAD(P)H oxidase gene (*p22phox* and others) and increasing the activity of NAD(P)H oxidase (Fukui et al. 1997; Laursen et al. 1997; Rajagopalan et al. 1996; Zafari et al. 1998). The vasoconstrictor effect of ANG II is enhanced in the absence of NO, and diminished during co-infusion of anti-oxidant vitamin C (Dijkhorst-Oei et al. 1999). Thus, the vasoconstrictor effect of ANG II is modulated by reactive oxygen species, mainly O_2^-, and their interaction with endothelium-derived NO (Fig. 6). In addition, ANG II-induced oxidative stress results in the activation of several pro-inflammatory transcription factors (Cheng et al. 2005). Statins, hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, ameliorate ANG II-induced hypertension and vascular inflammatory response independently of cholesterol reduction (Dechend et al. 2001). Inhibition of NO synthesis by oral L-NAME increases the activity of the renin–angiotensin

system and ANG II concentration (Vandermeersch et al. 2003). Studies suggest that the protective effects of angiotensin-converting enzyme inhibitors on the ANG II-induced inflammatory response are linked to the improvement of NO bioavailability (Chen et al. 2003). Furthermore, ANG II increases the production of ET in the blood vessel wall, which exerts vasoconstriction and induces proliferation of the vascular smooth muscle cells (Moreau et al. 1997).

4 Prostaglandins in Hypertension

PGI_2 is another endothelium-derived relaxing factor that is released in response to shear stress (Fig. 1; Koller and Kaley 1990; Okahara et al. 1998; Pohl et al. 1986; Rubanyi et al. 1986). PGI_2 is synthesised by COX from arachidonic acid (Moncada et al. 1976). PGI_2 increases intracellular cyclic adenosine monophosphate (cAMP) in smooth muscle cells and platelets. In contrast to NO, PGI_2 does not contribute to the maintenance of basal vascular tone of large conduit arteries (Joannides et al. 1995a). Instead, its platelet inhibitory effects are most important. The synergistic effect of PGI_2 and NO enhances the anti-platelet activity (Radomski et al. 1987).

Depending on the animal model of hypertension and the vascular bed, endothelium-dependent contractions to acetylcholine, a muscarinic receptor-dependent stimulator of NO synthesis, have been documented (Fig. 3). Since this response is inhibited by COX inhibitors and thromboxane receptor antagonists, the most likely contractile factors are thromboxane A_2 and prostaglandin H_2 (Küng and Lüscher 1995; Noll et al. 1997).

Interactions between COX products and NO have been demonstrated (Yang et al. 1991). Celecoxib, a selective COX-2 inhibitor, was able to improve endothelial function and reduce oxidative stress (Hermann et al. 2003) as well to reduce cellular inflammation in a model of salt-sensitive hypertensive rats (Hermann et al. 2005). In humans, selective inhibition of COX-2 by celecoxib lowers C-reactive protein levels and improves endothelial function in patients with coronary artery disease (Chenevard et al. 2003). Short- (3 h) and long-term (1 week) inhibition of COX-2 by celecoxib restores endothelial function in hypertensive patients (Widlansky et al. 2003), whereas rofecoxib has no effect (Title et al. 2003; Verma et al. 2001). In hypertensive patients, indomethacin, a COX inhibitor, significantly increased the response to acetylcholine, an effect that could be blocked by co-infusion of L-NMMA, an inhibitor of NO synthesis (Taddei et al. 1997b). Therefore, COX inhibition restores NO-mediated vasodilatation in essential hypertension, suggesting that COX-dependent substances can impair NO bioavailability. COX is indeed a source of the NO scavenger O_2^- (Kontos et al. 1985).

5
Endothelium-Derived Hyperpolarising Factor

Inhibitors of the L-arginine pathway do not prevent all endothelium-dependent relaxations (Richard et al. 1990). Since under these conditions vascular smooth muscle cells become hyperpolarised, an endothelium-dependent hyperpolarising factor (EDHF) of unknown chemical structure has been proposed (Fig. 1; Taylor and Weston 1988; Vanhoutte 1987). There is evidence that a calcium-dependent potassium channel on endothelial or smooth muscle cells is important in mediating endothelium-dependent hyperpolarisation, a mechanism that is impaired in arterial hypertension (Edwards et al. 1998; Fujii et al. 1992; Van de Voorde et al. 1992). Endothelium-dependent hyperpolarisation may also be involved in the compensation for the impaired NO system in patients with essential hypertension (Taddei et al. 1999b; Takase et al. 1996).

As EDHF remains unidentified, its involvement in regulating vascular reactivity is defined as the response that persists in the presence of combined inhibition of NO and PGI_2 synthesis. The relative contribution of the mediators to endothelium-dependent dilatation (NO, prostacyclin and EDHF) is inversely related to vessel calibre. NO- and PGI_2-mediated responses are more important in conduit vessels, whereas EDHF is more prominent in resistance arteries (Shimokawa et al. 1996).

A recent study in $eNOS^{-/-}$ and $COX^{-/-}$ mice shows that EDHF is the predominant endothelium-derived relaxing factor in female mice, whereas NO and PGI_2 are predominant mediators in male mice (Scotland et al. 2005). The disruption of both eNOS and COX genes resulted in elevated blood pressure in male mice, whereas the female mice were protected against hypertension, indicating that EDHF may contribute to the lower incidence of cardiovascular disease in pre-menopausal women (Scotland et al. 2005).

6
The Endothelin System

Over a decade ago, a novel vasoconstrictor peptide synthesised by vascular endothelial cells was identified (Hickey et al. 1985; Yanagisawa et al. 1988; see A.P.Davenport and J.J. Maguire, volume I). The ET family consists of three closely related peptides—ET-1, ET-2, and ET-3—which are converted by ET-converting enzymes (ECE) from "big endothelins" originating from large pre-proendothelin peptides cleaved by endopeptidases (Ikegawa et al. 1990; Ohnaka et al. 1993; Rossi et al. 1995; Shimada et al. 1994; Takahashi et al. 1993). The ET peptides are not only synthesised in vascular endothelial and smooth muscle cells, but also in neural, renal, pulmonal and some circulatory cells holding the genes for ETs (Inoue et al. 1989a, b). The chemical structure of the ETs is closely related to neurotoxins (sarafotoxins) produced by scorpions and snakes (Fleminger et al. 1989; Kloog et al. 1988). Factors modulating the

expression of ET-1 are shear stress, adrenaline, ANG II, thrombin, inflammatory cytokines (tumour necrosis factor α, interleukin-1 and -2), transforming growth factor β and hypoxia (Barton et al. 1997; Boulanger and Lüscher 1990; Boulanger et al. 1992; Dohi et al. 1992; Hieda and Gomez-Sanchez 1990; Kanse et al. 1991; Kohno et al. 1989; Kourembanas et al. 1991; Miyamori et al. 1991; Ohta et al. 1990; Shirakami et al. 1991; Woods et al. 1998; Yoshizumi et al. 1989). ET-1 is metabolised by a neutral endopeptidase that also cleaves natriuretic peptides (Abassi et al. 1992, 1993).

Imbalance of endothelium-derived relaxing and contracting substances disturbs the normal function of the vascular endothelium (Lüscher 1990; Lüscher and Vanhoutte 1990). ET acts as the natural counterpart to endothelium-derived NO (Fig. 1), which exerts vasodilating, anti-thrombotic, and anti-proliferative effects, and inhibits leucocyte adhesion to the vascular wall (Boulanger and Lüscher 1990). In addition to its arterial blood pressure-raising effect in man (Kiely et al. 1997; Vierhapper et al. 1990), ET-1 induces both vascular and myocardial hypertrophy (Barton et al. 1998; Ito et al. 1991; Yang et al. 1999), which are independent risk factors for cardiovascular morbidity and mortality (Bots et al. 1997; Kannel et al. 1969; O'Leary et al. 1999). Indeed, in patients with essential hypertension, carotid wall thickening and left ventricular mass correlate with reduced endothelium-dependent vasodilatation (Ghiadoni et al. 1998; Perticone et al. 1999a).

ET-1 has a paracrine rather than an endocrine mode of action, which is reflected by plasma levels of ET-1 in the picomolar range (Sorensen 1991; Wagner et al. 1992). Infusion of an ET receptor antagonist into the brachial artery or systemically in healthy humans leads to vasodilatation, indicating a role of ET-1 in the maintenance of basal vascular tone (Haynes and Webb 1994; Haynes et al. 1996). When ET-1 itself is infused, vasoconstriction follows a brief phase of vasodilatation, which may be explained by relaxation of smooth muscle cells caused by ET_B receptor-mediated release of the vasodilators NO and PGI_2 (Fig. 1). In addition, ET-1 may exert effects on the central and autonomic nervous systems and alter baroreflex function (Chapleau et al. 1992; Donckier et al. 1991; Gardiner et al. 1990; Kannan et al. 1994; Knuepfer et al. 1989; Lysko et al. 1991; Mosqueda-Garcia et al. 1998; Nakamoto et al. 1991; Nambi et al. 1990; van den Buuse and Itoh 1993; Yang et al. 1990a, b). In the kidney, sodium re-absorption is modulated (Sorensen et al. 1994) and aldosterone secretion is regulated by ET-1 (Fig. 8; Rossi et al. 2003).

6.1 The Endothelin System in Hypertension

The ET system is activated in several but not all animal models of arterial hypertension (Barton et al. 1998; Doucet et al. 1996; Hocher et al. 1995, 1996, 1999; Lariviere et al. 1993a, b, 1995; Li et al. 1994; Miyauchi et al. 1989; Schiffrin et al. 1995a). Correspondingly, ET plasma levels have been reported to be elevated

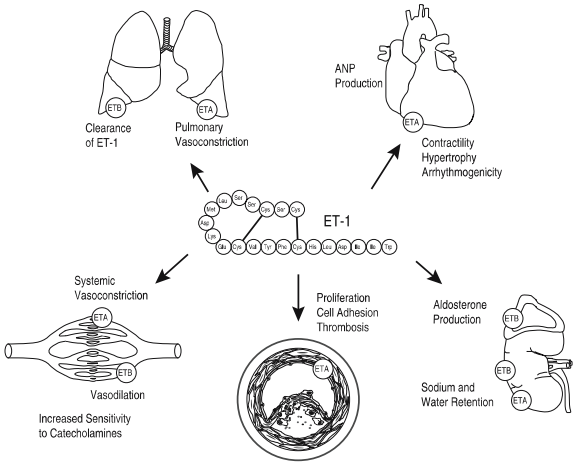

Fig. 8 Pathophysiological role of endothelin (ET)-1. In the heart, ET-1 contributes to contractility. In addition to its vasoconstrictor effects in the systemic and pulmonary circulation, ET-1 leads to hypertrophy of myocardial and smooth muscle cells. The pulmonary circulation is an important source of ET-1, but is also involved in the clearance of ET-1. In the kidney, ET-1 regulates sodium and water excretion. Modified from Spieker et al. (2001)

in certain patients with essential hypertension (Saito et al. 1990), but this observation is controversial (Miyauchi et al. 1992; Taddei et al. 1999a). The causal role of ET-1 in the pathogenesis of hypertension thus remains unclear (Haynes et al. 1998). As ET has pro-inflammatory, hypertrophic and pro-fibrotic properties in the heart, kidney and blood vessels, it seems to play a predominant role in mediating complications of hypertension (Schiffrin 2005).

Because most ET-1 synthesised in endothelial cells is secreted abluminally, it might attain a higher concentration in the vessel wall than in the plasma. Indeed, significant correlations have been found between the amount of immunoreactive ET-1 in the tunica media and (1) blood pressure, (2) total serum cholesterol and (3) the number of atherosclerotic sites (Rossi et al. 1999). In blood vessels of healthy controls, ET-1 was detectable almost exclusively in endothelial cells, whereas in patients with coronary artery disease, arterial hypertension or both, sizeable amounts of ET-1 were detectable in the tunica media of different types of arteries (Rossi et al. 1999). Furthermore, there is evidence that certain gene polymorphisms of ET-1 and ET receptors could be associated with blood pressure levels (Nicaud et al. 1999; Sharma et al. 1999; Stevens and Brown 1995). Even at very low concentrations of ET-1, interactions between ET-1 and adrenergic mediators lead to enhanced vasoconstriction (Fig. 9; Yang et al. 1990b).

Moreover, in hypertensive patients, intra-arterial infusion of various $ET_{A/B}$ receptor antagonists caused significantly greater vasodilatation than in normotensive subjects (Fig. 10; Cardillo et al. 1999, 2004; Taddei et al. 1999a). However, these findings remain controversial (Ferro et al. 2002; Nohria et al.

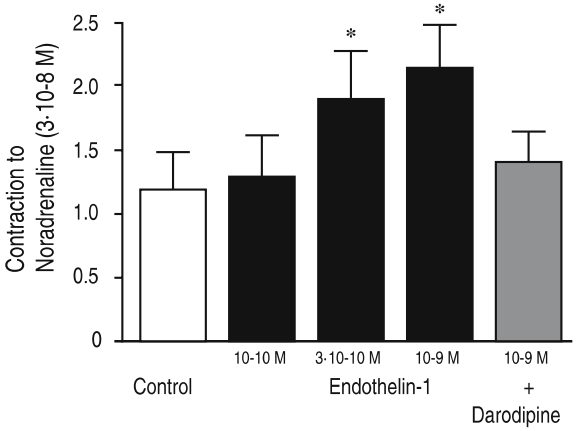

Fig. 9 Threshold concentrations of endothelin-1 potentiate contractions to noradrenaline in human arteries. In mammary artery rings, the contractions to noradrenaline were potentiated by threshold and low concentrations of endothelin-1. The calcium antagonist darodipine prevented the potentiation of the response to noradrenaline evoked by endothelin-1. Modified from Yang et al. (1990b)

2003). If plasma levels of ET-1 are similar in normotensive and hypertensive patients, then increased sensitivity to endogenous ET-1 must be postulated. Indeed, sensitivity to endogenous and exogenous ET-1 is increased in hypertensive patients (Nohria et al. 2003; Taddei et al. 1999a). One of the major functional consequences is impaired exercise-induced vasodilatation in hypertensive subjects, both in the coronary and the peripheral circulation (Fig. 11; Frielingsdorf et al. 1996; Linder et al. 1990; Nohria et al. 2003; Panza et al. 1990). Decreased bioavailability of NO may be involved in this phenomenon, since NO antagonises some of the effects of ET-1.

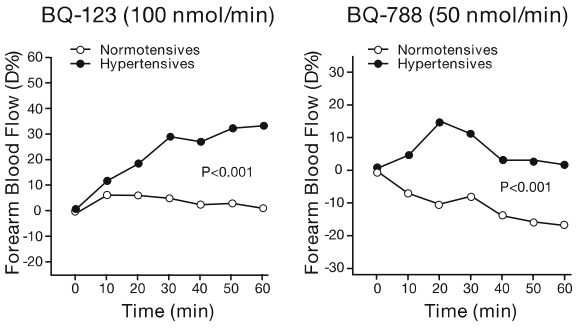

Fig. 10 Forearm blood flow responses to intra-arterial infusion of the selective ET_A receptor antagonist BQ-123 (100 nmol/min), and the ET_B receptor antagonist BQ-788 (50 nmol/min) in hypertensive patients and normotensive controls. The vasodilator response to endothelin antagonism is significantly enhanced in hypertensives. Modified from Cardillo et al. (1999)

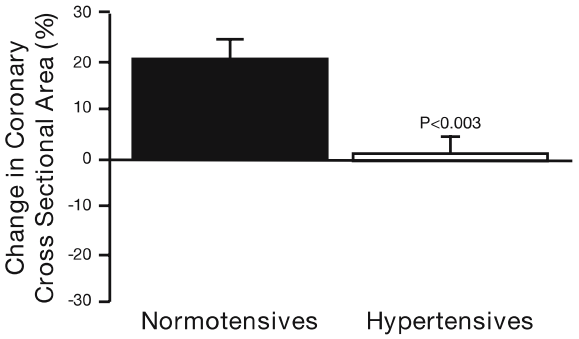

Fig. 11 Coronary luminal area change during exercise in hypertensive patients and normotensive control subjects. Exercise-induced coronary vasodilatation is impaired in hypertensives. Modified from Frielingsdorf et al. (1996)

7
Effects of Anti-hypertensive Therapy on the Vascular Endothelium in Hypertensive Patients

In hypertensive animals, most classes of anti-hypertensive drugs (e.g. calcium-channel blockers, ACE-inhibitors, AT_1 receptor antagonists) improve endothelium-dependent vasodilatation (Boulanger et al. 1994; d'Uscio et al. 1998; Dohi et al. 1994; Lüscher et al. 1987b; Maeso et al. 1998; Rodrigo et al. 1997; Takase et al. 1996; Tschudi et al. 1994). Surprisingly and in contrast to animal experiments, anti-hypertensive therapy cannot consistently restore impaired endothelium-dependent vasodilatation in patients with arterial hypertension (Creager and Roddy 1994; Hirooka et al. 1992; Linder et al. 1990; Panza et al. 1990, 1993a, b, c, 1994, 1995; Taddei et al. 1994, 1995, 1997a). However, depending on the anti-hypertensive drug and its pharmacological profile, improvements in endothelium-dependent vasodilatation can be achieved (Table 3; Creager et al. 1992; Dawes et al. 1999; Ghiadoni et al. 2000, 2003; Hirooka et al. 1992; Lyons et al. 1994; Millgard et al. 1998; Millgard and Lind 1998; Panza et al. 1993c; Perticone et al. 1999b; Schiffrin and Deng 1996; Schiffrin et al. 1995a; Schiffrin et al. 1995b; Sudano et al. 1998; Taddei et al. 1994, 1997c, 1998a; Yavuz et al. 2003). The multifactorial aetiology of essential hypertension as well as the duration of blood pressure elevation may explain certain inconsistent results of different investigators (Cockcroft et al. 1994; Perticone et al. 1998).

Several calcium channel blocking agents have been successful in improving endothelial function in human hypertension (Table 3). The anti-oxidative properties of an anti-hypertensive drug are important, since oxidative stress plays a central role in the pathophysiology of human hypertension. The endothelial function of patients with hypertension is improved by acute administration of ascorbic acid, an anti-oxidant vitamin, which protects against the decomposition of NO by O_2^- (Taddei et al. 1998b). Scavenging of reactive oxygen species by

Table 3 Effect of antihypertensive therapy on endothelial function in patients with arterial hypertension

Reference	Antihypertensive therapy	Duration of treatment	NO-release agonist/ antagonist	Improvement in endothelium-dependent vasomotion
	ACE inhibitors			
Hirooka et al. 1992	Captopril	Acute	ACh	Yes
Creager et al. 1992	Captopril	7–8 weeks	MCh	No
	Enalapril	7–8 weeks	MCh	No
Taddei et al. 1998a	Lisinopril	Acute	ACh	No
			Bk	Yes
		1 and	ACh	No
		12 month	Bk	Yes
Lyons et al. 1994	Enalapril	6 weeks	L-NMMA	Yes
Millgard et al. 1998	Captopril	Acute	MCh	Yes
		3 months	MCh	Yes
Schiffrin et al. 1995b	Cilazapril	1 and 2 years	ACh	Yes
Yavuz et al. 2003	Enalapril	6 months	FMD	Yes
Ghiadoni et al. 2003	Perindopril	6 months	FMD	Yes
	ANG II antagonist			
Ghiadoni et al. 2000	Candesartan	2 months	ACh	No
		12 months	ACh	Yes*
Bragulat et al. 2003	Irbesartan	6 months	ACh	Yes*
Yavuz et al. 2003	Losartan	6 months	FMD	No
Ghiadoni et al. 2003	Telmisartan	6 months	FMD	No
	β-Blocker			
Schiffrin and Deng 1996	Atenolol	2 years	ACh	No
Dawes et al. 1999	Nebivolol	Acute	L-NMMA	Yes
Ghiadoni et al. 2003	Nebivolol	6 months	FMD	No
Ghiadoni et al. 2003	Atenolol	6 months	FMD	No

Table 3 (continued)

Reference	Antihypertensive therapy	Duration of treatment	NO-release agonist/ antagonist	Improvement in endothelium-dependent vasomotion
	Ca antagonists			
Hirooka et al. 1992	Nifedipine	Acute	ACh	No
Millgard et al. 1998	Nifedipine	Acute	MCh	No
Sudano et al. 1998	Nifedipine	6 months	ACh	Yes
Schiffrin and Deng 1996	Nifedipine	Chronic	ACh	Yes
Ghiadoni et al. 2003	Nifedipine	6 months	FMD	No
Taddei et al. 1997c	Lacidipine	2 and 8 month	ACh and Bk	Yes
Lyons et al. 1994	Amlodipine	6 weeks	L-NMMA	Yes
Perticone et al. 1999b	Isradipine	2 and 6 month	ACh	Yes
Ghiadoni et al. 2003	Amlodipine	6 months	FMD	No
	Other			
Panza et al. 1993c	Various (diuretics, verapamil, β-blockers, clonidine, α-methyldopa)	Chronic vs 2 weeks withdrawal	ACh ACh	No No
Taddei et al. 1994	Potassium	Acute	ACh	Yes

Abbreviations: ANG II, angiotensin II; ACE, angiotensin converting enzyme; ACh, acetylcholine; Bk, bradykinin; Ca, calcium; FMD, flow-mediated vasodilatation; L-NMMA, N^G-monomethyl-L-arginine; MCh, methacholine; NO, nitric oxide *This effect was paralleled by an enhanced endothelium-independent vasodilatation to sodium nitroprusside

anti-oxidants may become an important therapeutic strategy (Nakazono et al. 1991; Tschudi et al. 1996), since chronic treatment with vitamin C is in fact able to lower blood pressure in patients with hypertension (Duffy et al. 1999).

Treatment with candesartan, an AT_1 receptor antagonist, reduced the vasodilator response to the mixed $ET_{A/B}$ receptor antagonist TAK-044 that was initially more pronounced in hypertensive patients than in normotensive con-

trols (Ghiadoni et al. 2000). This was paralleled by a reduction in circulating plasma ET-1 levels. Furthermore, the impaired vasoconstrictor response to L-NMMA in hypertensives was augmented by anti-hypertensive treatment. Thus, the ANG II receptor blocker candesartan improves the basal release of NO and reduces vasoconstriction to endogenous ET-1 in the forearm of hypertensive patients. Irbesartan, another AT_1 receptor antagonist, has also been investigated in hypertensive patients. Long-term irbesartan treatment enhanced both endothelium-dependent and -independent vascular vasodilatation responses. In addition, irbesartan restored the vasoconstrictor capacity of L-NMMA, suggesting a direct effect on tonic NO release, and decreased ET-1 production (Bragulat et al. 2003). However, other AT_1 receptor antagonists such as telmisartan and losartan did not improve endothelium-dependent vasodilatation in hypertensive patients (Ghiadoni et al. 2003; Yavuz et al. 2003).

Interestingly, infusion of nebivolol, but not other β-blockers, intra-arterially in the forearm of healthy subjects is associated with an increase in forearm blood flow (Cockcroft et al. 1995). The increase in forearm blood flow achieved by nebivolol can be prevented by co-infusion of L-NMMA. Similar results have been obtained in the human venous circulation (Bowman et al. 1994). This strongly suggests that nebivolol stimulates the formation of NO in the vasculature and may therefore have an interesting haemodynamic profile which—unlike other β-blockers—leads to peripheral vasodilatation in addition to the classical β-blocking effects on the sympathetic nervous system, heart rate and cardiac contractility (Van Nueten and De Cree 1998; Wallin et al. 1984). Indeed, nebivolol also causes NO-dependent vasodilatation in hypertensive patients (Dawes et al. 1999). However, this favourable effect did not last during chronic treatment (6 months) with this new type of β_1-blocker (Ghiadoni et al. 2003).

The effects of newer anti-hypertensive agents—e.g. ET receptor antagonists, ECE inhibitors, and inhibitors of neutral endopeptidases cleaving natriuretic peptides—on endothelial function in hypertension are awaited.

8 Conclusions

The vascular endothelium, synthesising and releasing vasoactive substances, plays a crucial role in the pathogenesis of hypertension. Due to its position between the blood pressure and smooth muscle cells responsible for peripheral resistance, the endothelium is thought to be both victim and offender in arterial hypertension. The delicate balance of endothelium-derived factors, which is disturbed in hypertension, can be restored by specific anti-hypertensive and anti-oxidant treatment.

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Vascular Endothelium and Atherosclerosis

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Abstract Atherosclerosis depends critically on altered behavior of the intrinsic cells of the artery wall, the endothelial cells and smooth muscle cells, and inflammatory leukocytes that join them in the arterial intima during the atherogenic process. The homeostatic properties of the normal endothelium contribute importantly to maintenance of aspects of arterial health including the appropriate regulation of blood flow, a basal anti-inflammatory state, promotion of fibrinolysis while opposing blood coagulation, and control of the balance of cellular proliferation and death. Alterations in these endothelial homeostatic mechanisms contribute critically to atherogenesis, the progression of this disease, and ist complications. Recent advances have highlighted novel molecular mechanisms that regulate the atheroprotective functions of normal endothelial cells that go awry during atherogenesis. Therapeutic strategies that alter the course of atherosclerosis may act by combating endothelial dysfunction.

Keywords Immunity · Inflammation · Leukocytes · Thrombosis

1 Introduction

Not so long ago, pathologists viewed atherosclerosis as a degenerative process characterised by accumulation of lipid in the artery wall, akin to a cholesterol storage disease. We now recognise that atherosclerosis depends critically on altered behaviour of the intrinsic cells of the artery wall, the endothelial cells and smooth muscle cells, and inflammatory leucocytes that join them in the arterial intima during the atherogenic process. Many early workers considered the endothelial cell as a passive lining cell involved primarily in regulating the transport of solutes across the blood-tissue interface. Altschul famously focussed on the endothelial cell as more than a passive barrier in the context of atherosclerosis (Altschul 1954). The dawn of the era of cell biology of atherosclerosis, heralded by the ability to grow homogeneous populations of vascular cells in vitro, hastened appreciation of the manifold regulatory roles of the endothelial cell in vascular diseases including atherosclerosis (Jaffe et al. 1973; Gimbrone et al. 1974).

The late Russell Ross championed the cell biological concept of atherogenesis. In the mid-1970s, he postulated a key role of endothelial cells in the cascade of alterations that leads to the formation of atheromatous plaques. In the original formulation of the "response to injury" hypothesis, Ross and colleagues theorised that a denuding injury to the endothelial cell in response to risk factors such as hyperhomocysteinaemia might represent a key initial step in atherogenesis. According to this scheme, areas of endothelial denudation would provoke platelet adherence and degranulation (Ross and Glomset 1976). The release of mitogenic materials from the platelets, including platelet-derived growth factor, would beckon smooth muscle cells to enter the intima, promote their proliferation, and lead to atherosclerotic plaque progression.

Subsequent morphological examinations of experimental atherosclerosis in various species revealed the essential integrity of the luminal endothelial lining while lesions formed (Joris et al. 1983; Faggiotto et al. 1984). Such observations, when performed on carefully perfusion-fixed tissues, challenged the concept that a frank denuding injury to the endothelium incited atherosclerosis. In the 1980s, the concept of qualitative alterations in the properties of the endothelium collectively denoted "endothelial dysfunction" flourished, supplanting the prior notion of desquamative endothelial injury as the first step in atherogenesis (Gimbrone 1981). Altered endothelial homeostasis rather than denudation emerged as an early aspect of atherogenesis. The pivotal property of the endothelial cells to show two faces, Janus-like, accounts for its essential and salubrious role in homeostasis and in host defences, but also its ability to promote pathological states such as atherosclerosis (Fig. 1). The concept of endothelial dysfunction initially embraced inappropriate interaction with blood leucocytes and disequilibrated

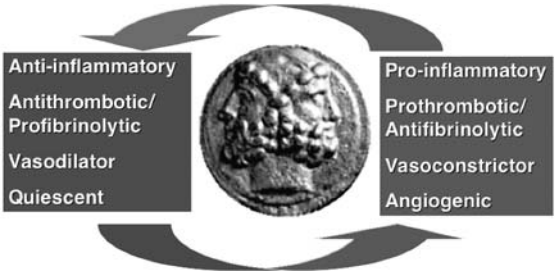

Fig. 1 The two faces of the endothelial cell. Like Janus, the Roman god of thresholds or portals, the endothelial cell has two faces. Strategically positioned at the interface of the blood and the tissues, the endothelial cell likewise must cast a gaze forward and backwards. The duality between the homeostatic properties (*left*) and the functions involved in host defences, tissue injury and many diseases (*right*) also evokes the two faces of Janus. See text for fuller explanation. After Libby (1987)

haemostatic and fibrinolytic properties. With the discovery of endothelial-dependent vasodilatation, the term endothelial dysfunction evolved to encompass derangements in vasodilator capacity by the arterial endothelium. According to this revisionist view of the role of endothelium in atherogenesis, desquamative injury occurred in later phases of atherogenesis rather than as an initial step. While the macrovascular endothelial cell held the centre stage of endothelial biology in atherosclerosis for several decades, microvascular endothelial cells probably participate importantly in atherogenesis as well.

This chapter will review current concepts of homeostatic properties of the normal endothelium, with a special view towards maintaining arterial health. We will then consider the major alterations in endothelial homeostatic mechanisms that contribute to atherogenesis, the progression of this disease and its complications. In particular, we will highlight some recent advances in the molecular mechanisms that regulate the atheroprotective functions of normal endothelial cells and go awry during atherogenesis. Finally, we will consider how therapeutic strategies that alter the course of atherosclerosis may act by combating endothelial dysfunction.

2
Homeostatic Functions of the Normal Endothelium

The normal endothelial cell possesses a panoply of properties that actively maintain vascular health on a moment-to-moment basis. These homeostatic functions of normal endothelium include haemocompatibility, appropriate regulation of vascular tone, and anti-inflammatory actions.

2.1 Haemocompatibility

The endothelial cells that line the arterial intima maintain constant contact with blood without triggering thrombosis. The blood compartment contains not only platelets, poised to form thrombi, but also the proteins of the coagulation cascade, ready to form fibrin at the slightest provocation. The normal endothelial cell holds these pro-thrombotic and pro-coagulant capacities of blood in check. The endothelial lining provides a physical barrier between the platelet and sub-endothelial adhesive proteins such as collagen that can activate platelets through glycoprotein VI and von Willebrand factor (vWF), which promotes platelet adhesion through glycoprotein Ib. Moreover, nitric oxide, produced by arterial endothelial cells that experience laminar shear stress can combat platelet aggregation (see S. Moncada and E.A. Higgs; R. Busse and I. Fleming, volume II). By expressing thrombomodulin, endothelial cells actually can render low concentrations of thrombin anti-coagulant and pro-fibrinolytic. Heparan sulphate proteoglycans that line the endothelial surface further serve to limit activation of the coagulation cascade in normal arteries. Should a stray thrombus form at some site in the arterial intima, robust fibrinolytic mechanisms associated with the healthy endothelial surface and normal blood stand at the ready to degrade the thrombus. Endothelial cells produce both urokinase and tissue type plasminogen activators (see J. Arnout et al., volume II). Thus, under normal circumstances the endothelial cells resist thrombosis, express anti-coagulant properties and possess potent fibrinolytic properties that protect against thrombus accumulation.

Recent work has identified a transcriptional regulatory pathway that contributes to endothelial haemocompatibility under homeostatic conditions. Exposure of endothelial cells to laminar shear stress increases the transcription factor Kruppel-like factor 2 (KLF2) (Dekker et al. 2002; SenBanerjee et al. 2004; Parmar et al. 2006), which promotes expression of thrombomodulin and endothelial nitric oxide synthase (eNOS) and also inhibits the cytokine-mediated induction of tissue factor and plasminogen activator inhibitor-1 (PAI-1), the endogenous inhibitor of fibrinolysis (Lin et al. 2005). Consistent with these effects, sustained elevation in KLF2 levels increase blood clotting time (Lin et al. 2005).

2.2 The Role of the Endothelium in Maintaining Normal Arterial Tone

Most of the regulation of blood flow occurs at the level of arterioles (see G. García-Cardeña and M.A. Gimbrone Jr., volume II). However, the normal endothelial cell of macrovessels can combat arterial spasm by endogenous production of vasodilator substances. The endothelium produces abundant prostacyclin, a potent vasodilator and anti-thrombotic mediator (see K. Egan and G.A. FitzGerald, volume I). These vasodilator stimuli oppose macrovas-

cular as well as microvascular constriction. Excepting atypical circumstances, contemporary concepts of the pathogenesis of the acute coronary syndromes accord a minor role to vasospasm as a *primary* cause of unstable angina or acute myocardial infarction. However, mediators associated with thrombosis, including serotonin (5-hydroxytryptamine) and even thrombin itself, may evoke substantial *secondary* regional vasoconstrictor responses at the level of the muscular artery, producing local vasoconstriction and aggravating ischaemia distal to sites of subtotal thrombosis, e.g. epicardial coronary arteries.

2.3 Anti-Inflammatory Properties of the Normal Endothelium

Under usual circumstances, i.e. when conditions of laminar shear stress prevail, endothelial cells exhibit a number of anti-inflammatory properties. For example, blood leucocytes of all classes—major mediators of inflammatory responses—adhere poorly to arterial endothelial cells that experience laminar shear stress. The endogenous vasodilator, nitric oxide, possesses anti-inflammatory properties. In particular, the relatively low levels of production of nitric oxide that result from the action of eNOS can limit leucocyte adhesion to endothelial cells. Furthermore, nitric oxide can inhibit cytokine-mediated exocytosis of Weibel–Palade bodies—an endothelial organelle that contains prothrombotic substances such as vWF and P-selectin (Matsushita et al. 2003). In addition, nitric oxide may limit the activation of nuclear factor kappa B (NF-κB), a central hub of transcriptional control in inflammatory signalling during atherosclerosis (Peng et al. 1995). Finally, recent studies also implicate KLF2 as conferring potent anti-inflammatory properties to endothelial cells (SenBanerjee et al. 2004). This may occur, at least in part, via the ability of KLF2 to induce eNOS expression and activity. In addition, KLF2 directly inhibits NF-κB function by recruiting away key co-factors required for optimal NF-κB transcriptional activity (SenBanerjee et al. 2004), consequently inhibiting adhesion molecule expression, e.g. vascular cell adhesion molecule-1 (VCAM-1), and strongly reducing immune cell attachment to endothelial cells. Thus, under usual circumstances in healthy arteries in vivo, the endothelium exerts an anti-inflammatory influence over the local environment.

3 Alterations in Endothelial Homeostasis Promote Atherosclerosis and its Complications

3.1 Activated Endothelial Cells Promote Coagulation and Thrombosis and Inhibit Fibrinolysis

Although resting endothelial cells resist the formation and accumulation of blood clots, the provoked endothelial cell can exhibit a spectrum of actions

that promote coagulation, thrombosis and clot stability. Reciprocally, mediators generated during thrombosis can activate endothelial and smooth muscle cells, and perpetuate aspects of the pathophysiology of atherosclerosis. When endothelial cells encounter pro-inflammatory cytokines or certain bacterial products such as lipopolysaccharide, they express the potent pro-coagulant tissue factor. Tissue factor binds activated coagulation factor VIIa, which further captures activated factor X and accelerates its action many-fold. In the context of atherosclerosis, the bulk of tissue factor probably arises from lesional macrophages and smooth muscle cells. In sepsis, however, endothelial tissue factor expression probably promotes disseminated intravascular coagulation and other thrombotic complications. Certainly in patients with preexisting atherosclerotic lesions, systemic infections may render the plaques pro-coagulant. This mechanism probably provides a pathophysiological explanation for associations between acute infectious illnesses and myocardial infarction (Libby et al. 1997).

The endothelial cell that encounters stimuli implicated in atherogenesis alters its usual anti-coagulant, anti-thrombotic and pro-fibrinolytic palette of functions, tipping the balance towards clot formation and stability. Overproduction of PAI-1, a major secretory product of endothelial cells exposed to pro-inflammatory cytokines, plays a pivotal role in impaired fibrinolytic activity by endothelial cells in the context of atherosclerosis (Vaughan 2005). In addition, smooth muscle cells associated with atherosclerotic lesions can overproduce PAI-1, limiting endothelial cell fibrinolytic capacity from within the lesion. Adipose tissue constitutes a major source of plasma PAI-1. Individuals with diabetes and the metabolic syndrome, as well as central obesity, have elevated circulating levels of PAI-1. Thus, both "solid state" sources of PAI-1 from within the plaque and "fluid phase" PAI-1 conspire to overcome the usual fibrinolytic capacity of endothelial cells that ordinarily limit the stability of arterial thrombi (Fig. 2).

3.2
Endothelial Cell Mediation of Inflammation in Atherogenesis

The early light microscopic studies of experimental atherosclerosis revealed adhesion of blood leucocytes to the arterial intimal endothelial cells as a very early morphological alteration during atherogenesis (Poole and Florey 1958). Subsequent electron microscopic observations buttressed the seminal work performed in Oxford during the 1950s (Joris et al. 1983; Faggiotto et al. 1984). The unravelling of endothelial adhesion biology in the 1980s led to the identification of a number of selective leucocyte adhesion molecules expressed on the surface of vascular endothelial cells that mediate this capture of blood leucocytes (Fig. 3). A combination of in vitro and in vivo studies identified the immunoglobulin superfamily member VCAM-1 as a candidate receptor for leucocyte integrins that mediate firm attachment of mononu-

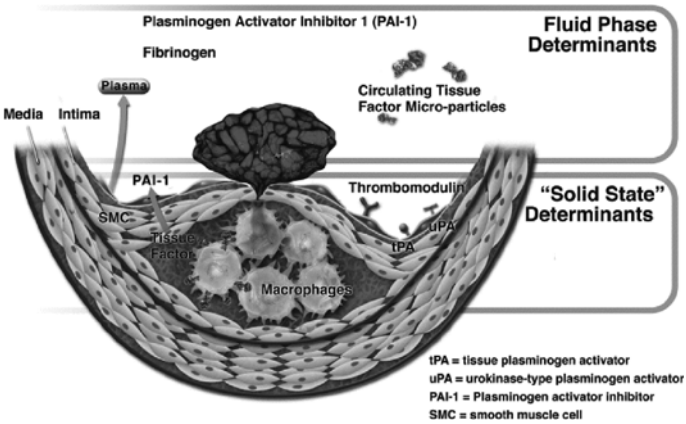

Fig. 2 Both solid state and fluid phase factors regulate the haemocompatibility of the endothelium. The normal endothelial surface has the remarkable property of maintaining blood in a liquid state during prolonged contact. The regulation of local haemocompatibility depends on both solid state and fluid phase determinants as depicted here. In the normal endothelium, the solid state determinants include the expression of thrombomodulin and plasminogen activator on the surface of the endothelial cell. In addition, heparan sulphate proteoglycans on the endothelial surface can have anti-coagulant properties due to interaction with anti-thrombin III. Prominent fluid phase determinants of haemocompatibility include the production of prostacyclin and of plasminogen activator inhibitor-1 (PAI-1). The normal endothelial cell does not express appreciable levels of pro-coagulant tissue factor. Under normal homeostatic circumstances, the anti-coagulant and pro-fibrinolytic properties of the endothelial monolayer prevail. However, as described in the text, the injured or inflamed endothelial cell tips the haemostatic balance towards coagulation and away from fibrinolysis. The fluid phase of blood also undergoes dynamic regulation. For example, extra-endothelial sources of PAI-1, fibrinogen and other coagulation factors change according to risk factor profile and in response to injury or infection. In the abnormal artery, the "solid state" compartment becomes more complex, e.g. production of tissue factor by smooth muscle cells and macrophages and their production of PAI-1 as well. In addition, the endothelial cell, when activated, can release von Willebrand factor from its intracellular storage vesicle, the Weibel–Palade body, and express pro-coagulant tissue factor. At any moment the regional propensity for a thrombus to form and evade fibrinolysis and propagate depends on the balance between these local solid state and systemic fluid phase determinants. From Libby (2006)

clear cells to activated endothelium (Cybulsky and Gimbrone 1991; Li et al. 1993). Normal arterial endothelial cells in vivo and resting endothelial cells in culture express little or no VCAM-1. However, arterial endothelial cells readily express VCAM-1 in response to pro-inflammatory cytokines in vitro or atherogenic conditions such as hypercholesterolaemia in vivo (Fig. 4). Endothelial cells both in vitro and in vivo express low levels of intracellular adhesion molecule-1 (ICAM-1) in the resting state. Like VCAM-1, cytokines and atherogenic stimuli enhance ICAM-1 expression in vitro and in vivo.

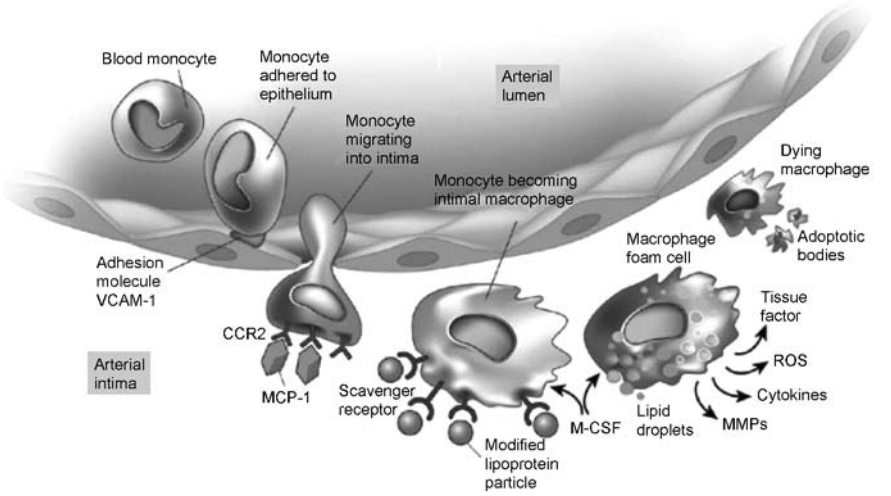

Fig. 3 The role of the endothelium in initiating the chronic inflammatory response during atherogenesis. The normal endothelial monolayer resists prolonged contact with blood leucocytes. When appropriately activated, the endothelial cell expresses adhesion molecules that promote the capture of blood monocytes. VCAM-1 certainly contributes to this process, although other adhesion molecules doubtless contribute to monocyte recruitment as well. Once adherent, the monocytes receive chemoattractant signals that stimulate their directed migration into the intima so that a chemoattractant stimulus, e.g. MCP-1, interacts with a chemokine receptor, e.g. CCR2. Other chemokines and chemokine receptors are likely to contribute to monocyte migration during atherogenesis as well. Once resident in the intima, endothelial cell factors may modulate behaviour of the mononuclear phagocytes. For example, the endothelial cell can express M-CSF and GM-CSF, survival factors, co-mitogens, and stimulators of pro-inflammatory functions of macrophages. In this manner, endothelial cells play a pivotal role in initiating atherogenesis through the recruitment of leucocytes. Although this example depicts the recruitment of mononuclear phagocytes, T cells, mast cells, and B cells all can localise in human atherosclerotic plaques and probably involve formally similar mechanisms of recruitment that use overlapping patterns of adhesion molecular and chemokine expression. From Libby (2002)

Kinetic experiments indicated that VCAM-1 expression precedes monocyte accumulation in the artery wall of hypercholesterolaemic rabbits. Ultimately, loss-of-function experiments in genetically altered mice established a role for VCAM-1 in murine atherosclerosis. Loss of ICAM-1 function appeared not to modulate the atherogenic process in mice in parallel experiments (Cybulsky et al. 2001). The selectins mediate saltatory or rolling interaction of blood leucocytes with endothelial cells. Among the selectins, P-selectin (CD62P) may participate in leucocyte recruitment during experimental atherogenesis (Wagner 2005).

Once attached to the intimal endothelium, adherent leucocytes receive chemoattractant signals that promote their penetration into the intima (Fig. 3). Chemokines of various classes participate in stimulating this directed migra-

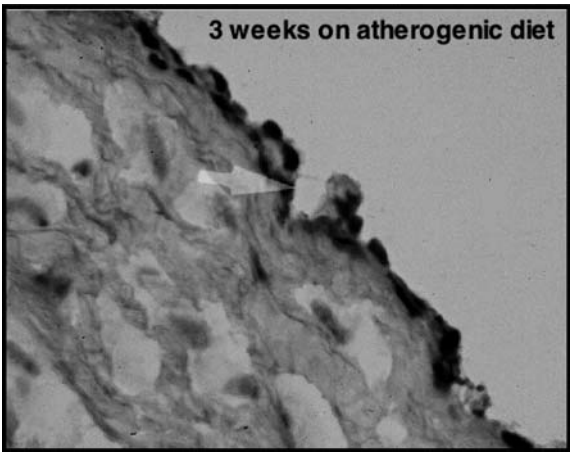

Fig. 4 An atherogenic diet rapidly induces VCAM-1 expression by endothelial cells in vivo. This figure depicts a section of the aorta from a rabbit that consumed an atherogenic diet for 3 weeks. The *arrowhead* points to an endothelial cell that expresses VCAM-1, as shown by immunohistochemical staining with a monoclonal antibody. Note the adherent leucocytes. The normal rabbit aortic endothelial cell expresses little or no VCAM-1. The atherogenic diet can induce VCAM-1 expression as early as 7 days following initiation. After Li et al. (1993)

tion of leucocytes to take up residence within the arterial intima. Chemokines implicated by in vitro and in vivo studies in regulation of murine atherosclerosis include macrophage chemoattractant protein-1 (MCP-1), which interacts with the chemokine receptor 2 (CCR2) (Gu et al. 1998; Peters and Charo 2001). Interleukin-8, eotaxin, and fractalkine may also participate in chemoattraction of leucocytes during atherogenesis (Boisvert et al. 2000; Haley et al. 2000; Lesnik et al. 2003).

Thus, cytokines play a key role in recruiting inflammatory cells into nascent atherosclerotic plaques by regulating adhesion molecule expression and chemoattraction. Other cytokines activate the leucocytes that accumulate within lesions (Fig. 3). For example, macrophage-colony stimulating factor (M-CSF) and granulocyte M-CSF localise in lesions and can perpetuate macrophage maturation and activation during atherogenesis (Smith et al. 1995; Rajavashisth et al. 1998). Endothelial cells can express the genes that encode these regulators of leucocyte differentiation and function.

Curiously, initial concepts postulated that endothelial cells responded to protein mediators of inflammation such as the cytokines. However, endothelial cells themselves can produce many of the pro-inflammatory proteins implicated in atherogenesis. The endothelium not only responds to but also can elaborate these pro-inflammatory stimuli, indicating a potential regulatory function of endothelial cells as gatekeepers of inflammation (Libby 1990). Strategically located at the interface of the blood compartment and tissues,

endothelial cells are perfectly placed for sensing the fluid phase environment and transducing signals to the subjacent smooth muscle cells as well as recruiting inflammatory cells. From this perspective, the endothelial cell could play a pivotal role in the initiation of atherosclerosis by regulating the local inflammatory response. A number of fluid phase stimuli and those associated with thrombosis can elicit pro-inflammatory functions of endothelium. For example, constituents of modified lipoproteins including certain oxidised phospholipids can exert pro-inflammatory actions on endothelial cells (Berliner and Watson 2005). Thrombin also can promote inflammatory effects, both by altering endothelial gene expression and inducing exocytosis of Weibel–Palade bodies that contain prothrombotic substances such as vWF and P-selectin (Matsushita et al. 2003). CD40 ligand, a cell surface-associated cytokine expressed by cells within atheromata as well as platelets, can engage the receptor CD40 on endothelial cells and evoke a pro-inflammatory programme from the endothelium. As activated platelets exteriorise CD40 ligand, this pathway illustrates the link between thrombosis and endothelial inflammation (Henn et al. 1998; Libby and Simon 2001).

Oxidative stress and inflammation go hand-in-hand during atherosclerosis. Oxidative stress, as indicated by overproduction of reactive oxygen species such as superoxide anion (O_2^-), can aggravate inflammatory aspects of atherosclerosis. By enhancing the activity of NAD(P)H oxidases, protein mediators such as angiotensin II can regulate the production of reactive oxygen species by endothelial and other vascular cells (Griendling and Harrison 2001; Heinecke 2003). Indeed, modulation of oxidative stress may represent another pathway by which physiological flow can alter endothelial inflammation. Recent work has shown that laminar shear stress can reduce the expression of a thioredoxin-interacting protein (TXNIP) that inhibits the activity of thioredoxin (Yamawaki et al. 2005; Fig. 5). A decrement in TXNIP expression in response to laminar shear stress can activate thioredoxin, which in turn can bind to apoptosis signal-regulating kinase 1 (ASK1), a stimulator of Jun kinase (JNK) and p38. This inhibitory limb can limit JNK and p38 activation by pro-inflammatory stimuli such as tumour necrosis factor. Ultimately, this anti-inflammatory pathway limits the expression of VCAM-1 in response to pro-inflammatory cytokines. As thioredoxin can mitigate oxidative stress and inflammation, this recently unravelled pathway supports the tight interconnections between inflammation and oxidative stress.

Disturbed flow characteristic of sites predilected for atheroma formation disrupts the usual atheroprotective functions of the vascular endothelium (see R. Busse, I. Fleming, volume II). Disturbed flow will interrupt the basal expression of endogenous anti-oxidants such as superoxide dismutase in endothelial cells, and also can alter the activity of transcriptional mediators that regulate endothelial function. For example, disturbed flow can activate NF-κB, the key pro-inflammatory transcriptional mediator (Nagel et al. 1999; Tzima et al. 2005), thereby increasing pro-inflammatory functions such as VCAM-1.

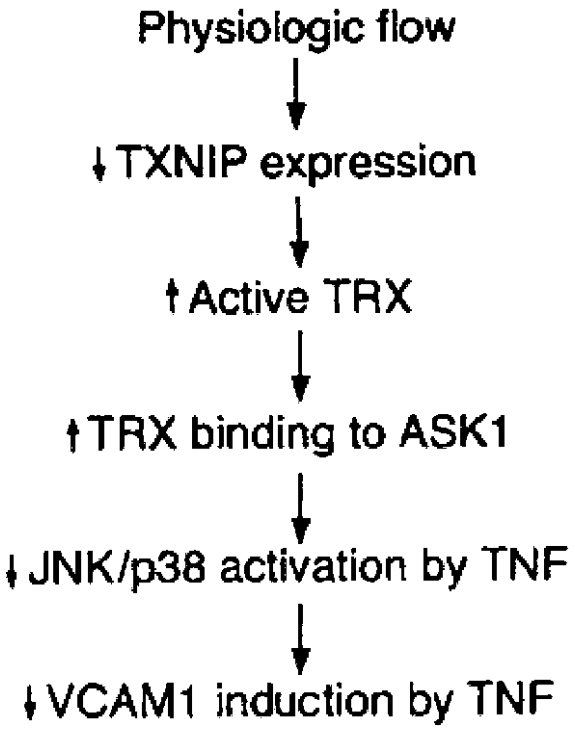

Fig. 5 Flow regulates endothelial inflammation by modulating the expression of thioredoxin-interacting protein (TXNIP). Laminar flow decreases expression of TXNIP, which activates the major anti-oxidant protein thioredoxin (TRX). In addition to its anti-oxidant properties, TRX can bind to apoptosis-signal regulating kinase 1 (ASK1), an activator of Jun kinase (JNK) and p38 kinase. The interaction of TRX and ASK1 limits activation of JNK and p38 by tumour necrosis factor (TNF), and ultimately inhibits the ability of pro-inflammatory mediators such as TNF to induce VCAM-1 on the endothelial surface. From Yamawaki et al. (2005)

Notably, NF-κB can also inhibit the expression of anti-inflammatory factors such as KLF2, thereby reducing cellular levels of molecules that may oppose its pro-inflammatory effects (Kumar et al. 2005; Fig. 6).

3.3 Dysregulation of Microvascular Function in Atheromata

Traditionally, studies of endothelial dysfunction have focussed on macrovascular endothelial cells. However, accumulating evidence supports a role for microvascular dysfunction in atherosclerosis as well. Pathologists described microvascularisation of atherosclerotic plaques generations ago. Periodically "rediscovered" (Barger et al. 1984), neovessel formation within atherosclerotic plaques has garnered considerable interest recently (Moulton et al. 1999).

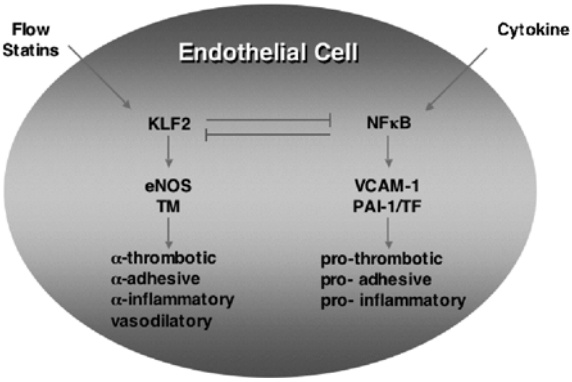

Fig. 6 The role of Kruppel-like factor 2 (KLF2) in transcriptional control of the endothelial cell inflammatory balance. Laminar shear stress and the statin class of drugs can activate KLF2, which in turn augments the homeostatic and anti-inflammatory properties of endothelial cells (*left*). In contrast, pro-inflammatory cytokines activate nuclear factor-kappa B (NF-κB), which mediates the expression of a cassette of injury response and pro-inflammatory properties associated with activated endothelial cells (*right*). Competition for shared co-activators mediates cross-talk between these two pathways of transcriptional regulation of endothelial cell activity. See text for details

Poorly delineated without special morphological techniques, we now appreciate that atheromata contain abundant microvessels. Such neovascular channels most often arise by angiogenesis from vasa vasorum. However, some morphological studies suggest that invaginations of macrovascular lumenal endothelium may also give rise to microvascular channels within atheromata. Regardless of their origin, these microvessels may contribute importantly to the progression and complication of atherosclerotic plaques. First, microvascular channels may provide a portal for trafficking of leucocytes. Indeed, in the established human atherosclerotic lesion, VCAM expression occurs most abundantly on microvascular endothelial cells, as opposed to the macrovascular endothelium (O'Brien et al. 1993). The large surface area of the multiple plexi of microvessels that abound within atheromata furnish ample surface for leucocyte recruitment and potential egress from atheromata.

In addition to leucocyte trafficking, neovascular channels within atheromata may participate in plaque progression by promoting intraplaque haemorrhage (Brogi et al. 1993; Kolodgie et al. 2003). Like new vessels that form in the diabetic retina, neovascular channels within the atherosclerotic plaque may be friable, hyperpermeable and prone to cause microhaemorrhage within plaques. Haemosiderin deposits co-localise with vWF in the extravascular space that surrounds neovascular plexi in atheromata, providing evidence for in situ haemorrhage and thrombosis (Fig. 7). As noted already, products associated with thrombosis can stimulate inflammation and enhance the migration and proliferation of smooth muscle cells as well as their elaboration of extra-

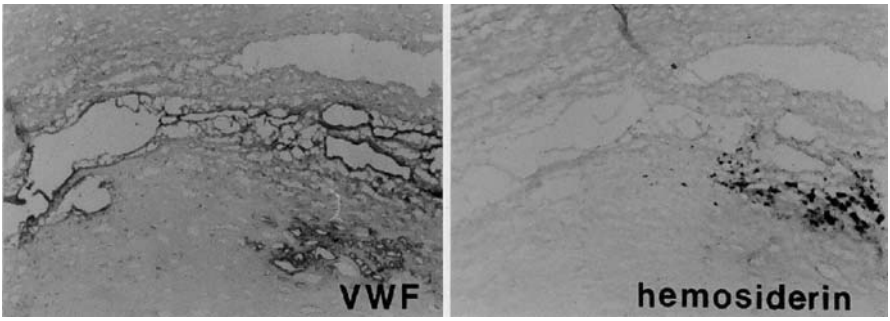

Fig. 7 Microvessels within the plaque can promote thrombosis in situ and oxidative stress. Adjacent sections from a human carotid endarterectomy specimen stained for von Willebrand factor (vWF) (*left panel*) and Prussian blue staining for hemosiderin (*right panel*) display a rich plexus of microvessels, including vascular lakes deep in the atherosclerotic intima. The vWF staining highlights the endothelial cells that line microvascular channels, often unapparent on conventionally stained histological preparations. Note also the extravasation of vWF (*left panel*, *lower right quadrant*). This finding probably indicates thrombosis in situ. suggesting an important role for microvascular endothelial cells in the pathobiology of plaque progression and complication. Note also co-localisation of hemosiderin with the microvascular plexus. As iron can promote oxidative stress through the Fenton reaction, intraplaque haemorrhage originating from disrupted microvessels may promote oxidative stress as well. After Brogi et al. (1993)

cellular matrix proteins. Therefore, microvascular haemorrhage may represent not only a mechanism of rapid plaque expansion but also, on a chronic and often sub-clinical basis, a pathway for plaque progression.

Further, the microvessels within plaques may provide a platform for progression by supplying blood and nutrients to the atheroma. Analogous to tumour angiogenesis, inhibitors of angiogenesis can limit experimental atherosclerosis in mice, presumably by inhibiting nutrition and oxygen supply required for plaque growth (Moulton et al. 1999). Thus, microvascular endothelial dysfunction as well as altered homeostatic properties of macrovascular endothelium can promote atherosclerosis.

3.4 Endothelial Erosion and the Complication of Atherosclerotic Plaques

Early formulations of the response-to-injury hypothesis postulated a denuding endothelial injury as an initial step in atherogenesis (see Sect. 1 above). Curiously, we now have come full circle to revisit the question of endothelial desquamation in atherogenesis. Current thinking, however, accords a more prominent role for desquamative endothelial injury in the late stage of atherosclerosis, characterised by thrombotic complications, than the initial phase of this disease. Abundant pathological evidence suggests that a disruption of atherosclerotic plaques precipitates most acute fatal coronary thrombi (Davies 1996).

Similar mechanisms may apply to other arterial beds as well. A fracture of the plaque's fibrous cap accounts for the majority of these fatal plaque disruptions. However, a substantial minority of events, ranging from one fifth to one quarter of fatal coronary thrombi, result not from a through-and-through rupture of the plaque's fibrous cap but rather from a patch of endothelial denudation referred to as superficial erosion (Libby and Theroux 2006). While some observers describe inflammation as a concomitant of sites of superficial erosion and thrombosis (van der Wal et al. 1994), others report a bland, proteoglycan-rich context for sites of superficial erosion that cause fatal coronary thrombosis (Virmani et al. 2002).

Little direct evidence supports specific mechanisms of endothelial erosion in advanced atherosclerotic plaques. However, a number of experimental studies suggest mechanisms of endothelial desquamation that may pertain to this type of plaque disruption. Abundant evidence supports over-expression of matrix-degrading proteinases in atherosclerotic plaques (Dollery and Libby 2005). Pro-inflammatory mediators regulate the activity of these matrix-degrading proteases associated with atherosclerotic lesions. Much interest has focussed on the role of interstitial collagenases in weakening the protective fibrous cap, which fails during plaque rupture and thrombosis. However, endothelial cells can express matrix-degrading proteinases in response to inflammatory stimuli that may promote lesion complication. Interstitial collagenases localise to microvascular but not macrovascular endothelial cells in human atheromata, and may function importantly in neovessel formation, a phenomenon relevant to plaque complication (see the previous section). Endothelial cells, however, also can express non-fibrillar collagenases, including matrix metalloproteinases specialised in degrading basement membrane types of collagen (collagen type IV) as well as activators of these non-fibrillar collagenases. Oxidised lipoproteins can promote the expression and activation of type IV collagenase by vascular endothelial cells (Rajavashisth et al. 1999). In this manner, inflammation and risk factors associated with atherosclerotic events may endanger the tethering of endothelial cells to their subjacent basement membrane rich in type IV collagen. The endothelial cells may become literally "unglued" from their subjacent matrix and thus predisposed to desquamation and subsequent thrombosis (Libby et al. 2000).

Death of endothelial cells may also contribute to endothelial cell denudation in the context of superficial erosion and thrombotic complications of atherosclerosis (Libby et al. 2000). Multiple links exist between inflammation and endothelial apoptosis (see K. Ley and J. Reutershan; L.E. Spieker et al., volume II). Recent studies have identified a novel link between inflammation and endothelial cell death, which is of potential relevance to superficial erosion. The enzyme myeloperoxidase produces hypochlorous acid (HOCl), a potent pro-oxidant and chlorinating species. Elegant chemical studies have established the presence of the products of HOCl modification in human atherosclerotic plaques (Heinecke 2003). Traditionally, cardiovascular biologists have used

myeloperoxidase as a marker of the granulocyte, a cell type generally considered to arrive at the site of atherosclerotic plaques after, not before, their disruption. A sub-population of mononuclear phagocytes within lesions and in blood can contain myeloperoxidase as well (Hazen et al. 1999; Sugiyama et al. 2001). HOCl can induce endothelial apoptosis (Sugiyama et al. 2004). Moreover, sub-populations of mononuclear phagocytes enriched from those that contain myeloperoxidase and produce HOCl can promote endothelial apoptosis in vitro and produce lacunae in endothelial monolayers in vitro. In this manner, HOCl derived from myeloperoxidase may drive endothelial apoptosis and contribute to desquamative endothelial injury and thrombosis in advanced atherosclerotic plaques. Interestingly, a sub-population of human mononuclear phagocytes also expresses neutrophil elastase (Dollery et al. 2003), an enzyme that attacks elastin, another constituent of the sub-endothelial extracellular matrix. Induced by pro-inflammatory stimuli, neutrophil elastase also may contribute to endothelial desquamation and ulceration of atherosclerotic plaques.

4
Endothelial Dysfunction as a Therapeutic Target in Atherosclerosis

The foregoing discussion and other chapters in this book have highlighted the role of the endothelial cell in endogenous protection against atherosclerosis and as a central cell in the formation of atherosclerotic plaques and their complications. The endothelium relates to atherosclerosis in other ways as well, notably as a target of anti-atherosclerotic interventions and therapies.

Multiple contributions to this compendium have illustrated how laminar shear stress elicits homeostatic, atheroprotective functions from vascular endothelial cells. Many observational studies have linked increased physical activity to reduced rates of atherosclerotic events. The beneficial effects of regular physical activity may derive in part from increased shear stress and nitric oxide production from endothelial cells.

Pharmacological agents that mitigate atherosclerosis and its complications also may act at the level of the endothelial cell. For example, aspirin reduces cardiovascular events in many at-risk populations, a protective effect that may derive in part from an interplay in arachidonic acid metabolism between platelets and endothelial cells. Aspirin irreversibly acetylates both major isoforms of cyclooxygenase, the enzyme responsible for prostanoid production. The anucleate platelet cannot re-synthesise these enzymes, yet the endothelial cell can renew the enzyme. Thus, the production of prostacyclin, the anti-aggregatory and vasodilatory prostanoid produced by endothelial cells, can persist while platelet cyclooxygenase that produces thromboxane, a vasoconstrictor and pro-aggregatory prostanoid, continues. Interestingly, the COX-2 isoform of cyclooxygenase appears to produce most of the prostacyclin in hu-

mans. The apparent thrombotic hazard of the selective COX-2 inhibitors may result from interruption of endothelial production of prostacyclin while leaving platelet thromboxane production intact (see K. Egan and G.A. FitzGerald, volume I).

Long-term interruption of the renin–angiotensin–aldosterone pathway consistently reduces atherosclerotic complications in higher risk populations. Angiotensin II activates the oxidases in endothelial and smooth muscle cells that heighten oxidative stress, a constant companion of inflammation during atherogenesis (Griendling and Harrison 2001). Therefore, the endothelium may constitute one target of inhibitors of renin–angiotensin–aldosterone signalling and furnish a mechanism of reduced atherosclerotic events in individuals receiving such agents.

Oxidised phospholipids associated with modified lipoproteins can elicit pro-inflammatory and pro-atherogenic programmes directly from vascular endothelial cells (see above). Thus, lipid-lowering therapy might reduce atherosclerotic events by limiting exposure of endothelial cells to these pro-inflammatory lipids. Experimental lipid lowering by diet can reduce oxidised low-density lipoprotein (LDL) accumulation and superoxide production, and concomitantly suppress VCAM-1 expression and microvascularisation in lesions (Aikawa et al. 2002; Aikawa and Libby 2004; Fig. 8). The same study also demonstrated that lipid lowering increased expression of eNOS, an anti-inflammatory factor (Aikawa et al. 2002). Importantly, multiple clinical studies have shown that dietary lipid lowering or diets enriched in polyunsaturated or monounsaturated fats can reduce atherosclerotic events (Krauss 2005). Certain polyunsaturated fatty acids, notably docosahexaenoic acid, can limit cytokine-induced endothelial activation (De Caterina et al. 1995). In this manner, reduced inflammatory activation of endothelial cells may explain the apparent beneficial effects of dietary regimens associated with lipid lowering or enriched in biologically active unsaturated fatty acids.

The statin class of drugs not only lowers levels of LDL and modestly raises the atheroprotective high-density lipoprotein (HDL) particles, but also may act directly at the level of the endothelial cell (see R. Busse and I. Fleming, volume II; Libby and Aikawa 2002). Statins may increase endothelial production of nitric oxide by stabilising eNOS messenger RNA. Additionally, statins can inhibit the prenylation of small G proteins, providing an additional mechanism of potential anti-atherosclerotic actions distinct from alterations in blood lipoproteins (Schonbeck and Libby 2004; Rikitake and Liao 2005). Finally, recent work implicated KLF2 as a mediator of statin-induced atheroprotective effects on cultured endothelial cells (Jain and Ridker 2005; Parmar et al. 2005; Sen-Banerjee et al. 2005; Fig. 6).

Fibric acid derivatives have reduced atherosclerotic complications in some but not all populations studied. Fibrates potently lower triglycerides and modestly raise HDL levels, thus accounting for some of their putative cardiovascular benefits. Like the statins, however, fibric acid derivatives may have

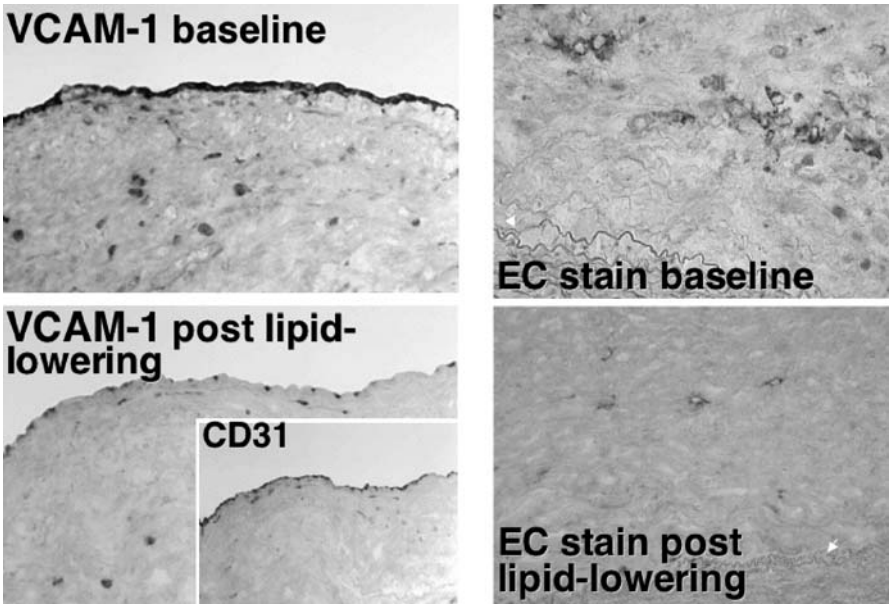

Fig. 8 Lipid lowering reduces VCAM-1 expression and microvessels in experimental atherosclerosis. These immunostained sections derive from rabbits with diet-induced atherosclerosis (*top panels* denote baseline) or from animals from the same cohort subjected to lipid lowering by dietary intervention for a 16-month period following the baseline observations (denoted as *lipid-lowering*). The *left panels* show staining for VCAM-1. Note the robust expression of VCAM-1 in the baseline atherosclerotic lesions and the marked decrease in VCAM-1 after lipid lowering, despite persistence of CD31-positive endothelial cells (*inset*). The baseline lesions on the *right* contain abundant microvascular channels shown here in profile by staining with *Ulex europaeus* lectin. After lipid lowering (*bottom right panel*), microvascular channels are much less evident. After Aikawa et al (2002); Aikawa and Libby (2004)

"off-target" actions that contribute to their putative atheroprotective effects. In particular, fibrates can activate peroxisome proliferation activated receptor-α(PPAR-α). We showed expression of PPAR-α by human endothelial cells in atheromatous plaques (Marx et al. 1999). Several laboratories have shown that PPAR-α activation can limit endothelial activation in vitro, as exemplified by VCAM-1 and tissue factor expression (Delerive et al. 1999; Marx et al. 2001).

Taken together, accumulating evidence suggests that many of the lifestyle modifications and pharmacological interventions that reduce atherosclerotic risk may involve direct effects on endothelial cells by mechanisms described in other contributions to this volume.

5 Conclusion

From the very earliest stages of atheroma initiation through to its ultimate thrombotic complications, the endothelial cell occupies a central position. A healthy endothelium, characterised by expression of the atheroprotective functions defined above, can forestall atherosclerosis formation. The complication of existing atherosclerotic lesions depends in no small measure on functions of endothelial cells, notably those that regulate coagulation, thrombosis and fibrinolysis. Moreover, endothelial cells are likely to regulate the progression of atherosclerosis. Notably, microvascular endothelial cells may provide a prominent pathway for leucocyte trafficking during lesion progression. As normal endothelial function protects against atherosclerosis, lifestyle and pharmacological interventions that target the endothelium may limit atherogenesis and improve prognosis of individuals with established disease. The common thread that unites endothelial function, both homeostatic and dysregulated, involves inflammation. Inflammatory pathways and tightly linked oxidative stress orchestrate many of the adverse functions of endothelial cells that critically regulate the disease and its clinical manifestations. At mid-twentieth century, Altschul remarked that "we are as old as our endothelium" (Altschul 1954), mimicking Osler's aphorism that we are as old as our arteries. Bernard Shaw famously urged that we "stimulate our phagocytes" to overcome disease, often caused in his day by infectious organisms that required a robust immune system for host defence (Shaw 1911). Our twenty-first century motto may well be "douse the flame of endothelial inflammation to maintain vascular health and promote longevity".

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Endothelial Cells and Cancer

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Abstract Endothelial cells play a key role in the development and function of blood and lymph vessels. Excessive proliferation and transformation of endothelial cells lead to pathological angiogenesis/lymphangiogenesis or vascular malfunctions which are hallmarks of malignant disorders. There is emerging evidence that circulating endothelial progenitor cells (EPCs) also contribute significantly to these processes. Major progress has been achieved over the past few years in the identification of key molecules involved, and in targeting tumour angiogenesis for human therapy. Current research efforts are concentrated on deciphering the origin and functional properties of endothelium in various tumours, as well as endothelial neoplasms themselves. The aim of these studies is to investigate the molecular mechanisms regulating mobilisation of EPCs from bone marrow, and their homing and differentiation into mature endothelium in situ at sites of neovascularisation, as well as the role of viral oncogenes in regulating the plasticity and extending the life span of endothelial cells. Integrated understanding of the mechanisms regulating the properties and function of endothelial cells during tumourigenesis is resulting in the development of a number of exciting and bold approaches for the treatment of cancer.

Keywords Endothelium · Cancer · Neoplasm · Angiogenesis

1 Introduction

1.1 Endothelial Cells: Role in Vascular Development and Function, and Impact on Health

The growth of new blood and lymph vessels is required for embryonic development and stimulates the healing of injured tissues; however, it also promotes tumour growth and inflammatory diseases (Carmeliet 2003). Endothelial cells play a key role in neovascularisation. Blood and lymphatic capillaries consist of thin-walled vessels composed of a single layer of endothelial cells (microvascular endothelium), and larger vessels are surrounded by mural cells (pericytes in medium-sized vessels, or smooth muscle cells in large vessels). New endothelial cells originate through differentiation from endothelial progenitor cells (EPCs) or proliferation of mature endothelium in pre-existing vessels. In a normal adult, most vasculature is quiescent, with only 0.01% of endothelial

Table 1 Angiogenesis and lymphangiogenesis in endothelial and non-endothelial neoplasms

System/organ	Processes characterised by abnormal angiogenesis/lymphangiogenesis*
Reproductive system (uterus, ovary, breast)	Neoplasms[a,b]
Bone, joints	Neoplasms[a,b]
Liver, kidney, lung and other epithelia	Neoplasms[a,b]
Brain, nerves, eye	Neoplasms[a,b]
Skin	Neoplasms[a]
	Kaposi's sarcoma[a,d]
Blood vessels	Atherosclerosis[a]
	Haemangioma[c]
	Haemangioendothelioma[c]
Lymph vessels	Tumour metastasis[b]
	Lymphoproliferative disorders[b,c]
	(e.g. lymphangioma, lymphangiosarcoma)
Haematopoiesis	Kaposi's sarcoma[a,d]
	Haematological malignancies[d]

*List of selected examples [a]Increased vascularisation due to abnormal angiogenesis [b]Increased lymphangiogenesis or derailed growth of lymphatic endothelium [c]Endothelial cell hyperplasia [d]Abnormal differentiation of progenitor cells or reprogramming of endothelial cells

cells undergoing division. Quiescent vascular and lymphatic endothelial cells are activated by (lymph)angiogenic factors such as various members of the vascular endothelial growth factor (VEGF) family, which stimulate endothelial cell proliferation and migration, thereby promoting new vessel formation (Alitalo et al. 2005; Ferrara 2004). Recent evidence suggests that EPCs also contribute significantly to de novo vessel formation during wound healing, limb ischaemia, post-myocardial infarction and endothelialisation of vascular grafts. However, excessive proliferation of endothelial progenitor or mature differentiated endothelial cells contributes to numerous neoplastic and non-neoplastic disorders (Table 1). For example, the growth of the majority of human tumours is accompanied by an increase in the number of proliferating endothelial cells and in vascularity. The transformation of endothelial cells is also implicated in the pathogenesis of Kaposi's sarcoma and other neoplasms of blood and lymph vessels. Current research is aimed at identifying the molecular mechanisms regulating these processes to enable effective targeting of endothelium for human cancer therapy.

2
Endothelial Progenitor Cells and Cancer

For many years, the prevailing dogma stated that vessels in the embryo developed from endothelial progenitors, whereas sprouting of vessels in the adult resulted only from division of differentiated endothelial cells (Fig. 1). Recent evidence, however, indicates that EPCs also contribute to vessel growth in ischaemic, inflamed or malignant tissues in the adult (Asahara and Isner 2002; Luttun and Carmeliet 2003; Rafii et al. 2002).

2.1
Endothelial Progenitor Cell Plasticity and Differentiation into Mature Endothelial Cell Sub-lineages

Endothelial and haematopoietic cells share a common progenitor, putatively termed the*"haemangioblast"* (Fig. 2). Both share certain antigenic determinants, including Flk-1 (vascular endothelial growth factor receptor 2, VEGFR-2), Tie-2, c-Kit (CD117), AC133 (CD133) and CD34.

During embryonic development, haemangioblasts in the yolk sac, in the arterial wall of the aorta and in the aorta-gonadal-mesonephros region form aggregates (blood islands) in which the inner cells develop into haematopoietic precursors and the outer population into endothelial cells. Angioblasts migrate extensively before in situ differentiation and plexus formation. VEGF, VEGFR-2 and basic fibroblast growth factor (bFGF) influence angioblast differentiation (Carmeliet et al. 1996; Carmeliet and Collen 1999; Ferrara et al. 1996; Shalaby

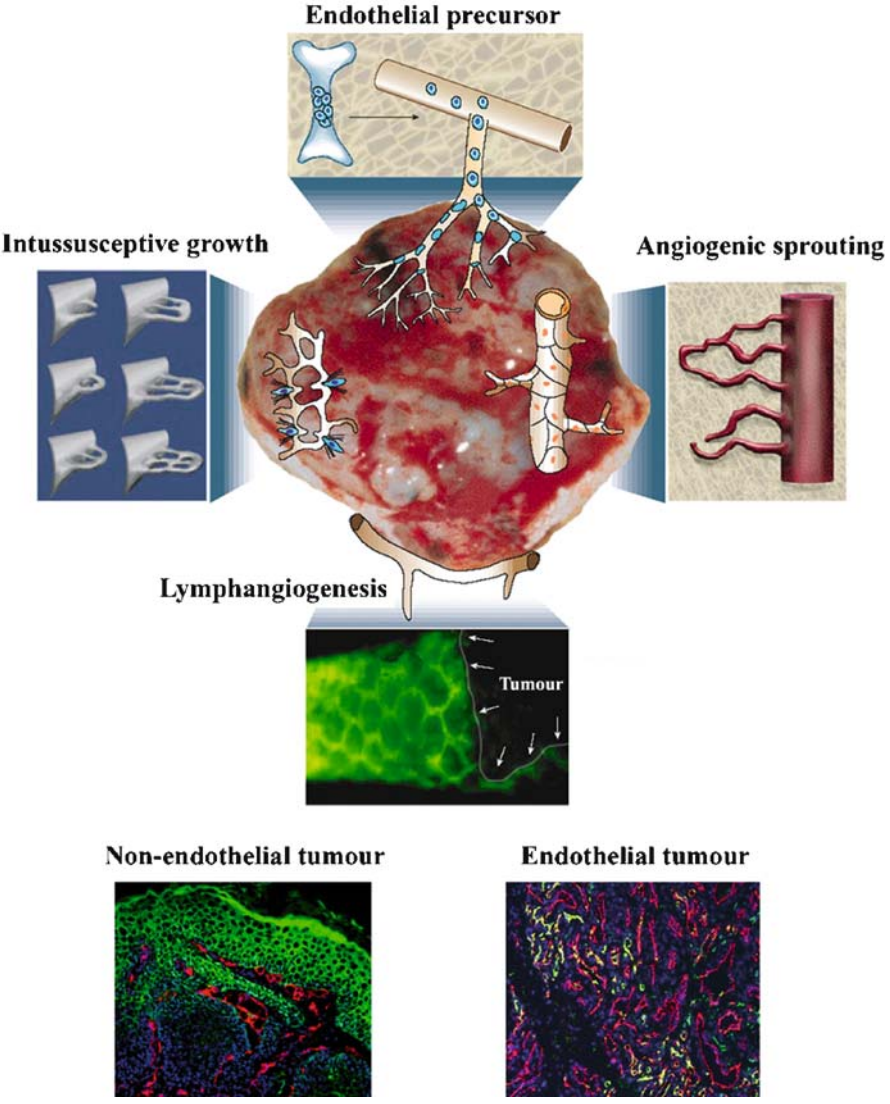
et al. 1997), whereas VEGFR-1 suppresses haemangioblast commitment (Fong et al. 1999). Molecules mediating interactions between endothelial cells and matrix macromolecules, fibronectin or matrix receptors (integrins) also affect vasculogenesis (Koch et al. 1995; Lyden et al. 1999; Table 2).

In the adult, EPCs are a unique cell population existing in peripheral blood mononuclear cells (PBMNCs), being derived from the bone marrow (BM) (Iwami et al. 2004). These cells incorporate into sites of active tumour neovascularisation, but also after ischaemic events in limb and myocardium, and thus contribute to de novo vessel formation (Asahara and Isner 2002). More recent studies have introduced the concept that the origin of EPCs may not be limited to the BM, e.g. tissue-specific stem/progenitor cells possibly provide in situ cells as another source of EPCs (Tamaki et al. 2002). Irrespective of origin, EPCs are mobilised and capable of homing to sites of neovascularisation under the influence of appropriate cytokines and growth factors, including VEGF as a critical molecule for vasculogenesis and angiogenesis (Asahara et al. 1999). Increased circulating VEGF promotes the mobilisation of EPCs from BM, resulting in increased numbers of circulating EPCs (Asahara et al. 1999; Hattori et al. 2001). Similar modulation of EPC kinetics resulting in their mobilisation to sites of neovascularisation has been observed in response to other haematopoietic stimulators, such as granulocyte-macrophage colony stimulating factor (GM-CSF) (Takahashi et al. 1999), angiopoietin (Ang-1; Hattori et al. 2001), stroma-derived factor (SDF)-1 (Yamaguchi et al. 2003), and erythropoietin (Heeschen et al. 2003). Rafii and colleagues proposed that mobilisation of EPCs from the BM requires angiogenic factor-mediated activation of matrix metalloproteinase (MMP)-9, which leads to the release of the soluble Kit ligand (Heissig et al. 2002). This ligand would in turn pro-

Fig. 1 Cellular mechanisms of tumour (lymph)angiogenesis. Tumour vessels grow by various mechanisms: (1) the host vascular network expands by budding of endothelial sprouts or formation of bridges (angiogenesis); (2) tumour vessels remodel and expand by the insertion of interstitial tissue columns into the lumen of pre-existing vessels (intussusception); and (3) endothelial cell precursors (angioblasts) home from the bone marrow or peripheral blood into tumours and contribute to the endothelial lining of tumour vessels (vasculogenesis). Lymphatic vessels around tumours drain the interstitial fluid and provide a gateway for metastasising tumour cells. Newly developed blood and lymphatic vasculature contribute to the development of both endothelial (e.g. angiomas and lymphangiomas) and non-endothelial (e.g. epithelial tumours) neoplasms. In non-endothelial tumours, endothelial cells (immunostained with panvascular endothelial marker CD31; *red*) contribute to skin cancer tumour angiogenesis [epithelial tumour cells express green fluorescent protein (GFP) construct under the control of keratin 14 promoter; *green*]. Endothelial tumours are mainly formed of blood or lymph vessels (i.e. endothelium) with a minor proportion of supporting tissue [endothelial origin of cells is revealed by double immunofluorescence with panvascular endothelial marker CD31 (*red*) and lymphatic endothelium-specific marker LYVE-1]. (Adapted from Carmeliet and Jain 2000; Hirakawa et al. 2005; Dadras et al. 2004)

mote proliferation and motility of EPCs within the BM microenvironment, thus creating permissive conditions for their mobilisation into the peripheral circulation.

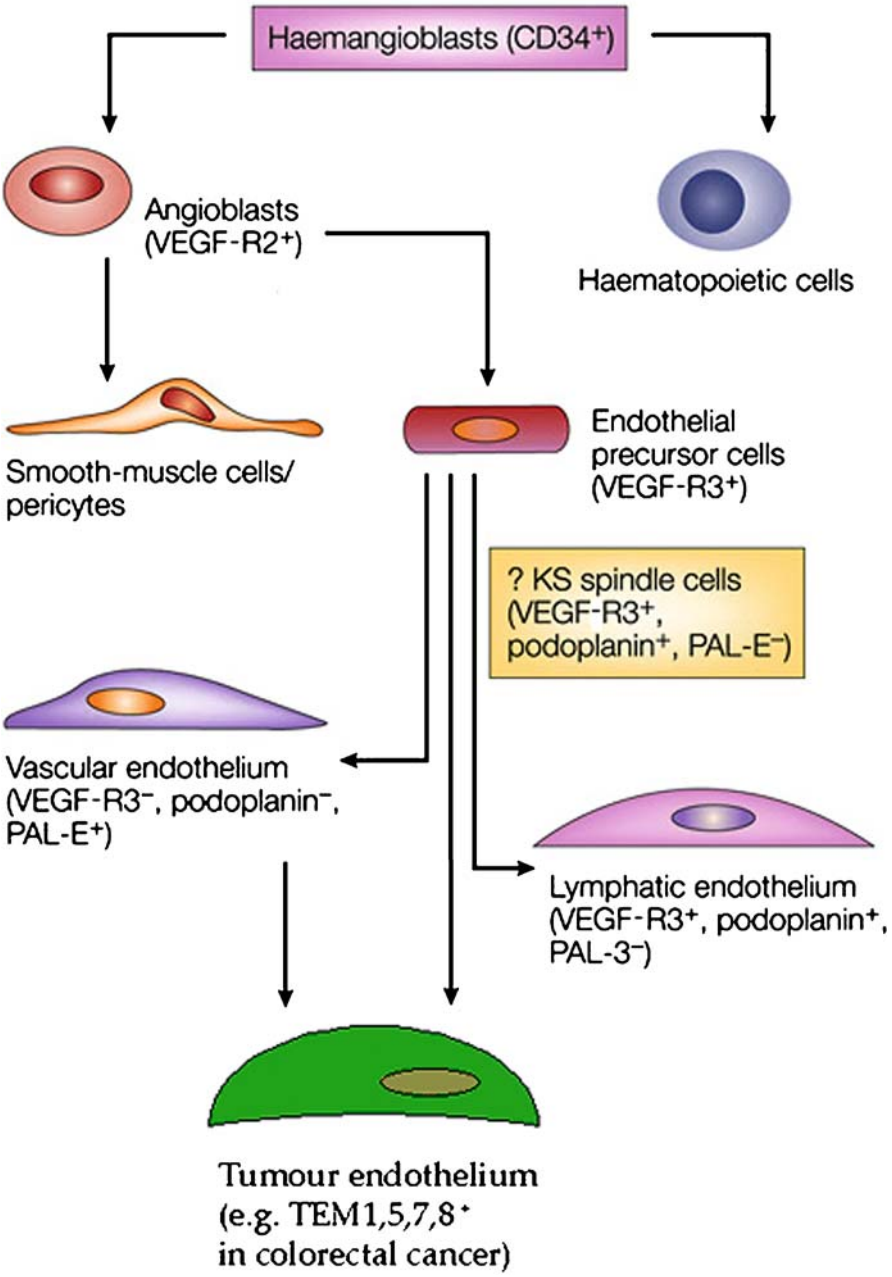
In situ (at the sites of neovascularisation), EPCs differentiate to endothelial lineage cells (mature endothelial cells), but not all endothelial cells are alike. Heterogeneity of differentiated endothelial cells depends on commitment of EPCs to produce arterial, venous or lymphatic endothelium, indicating that EC progenitors have remarkable *phenotypic plasticity* (Fig. 2). Recent genetic



studies offer insight into the signals controlling arterial and venous identities of endothelial cells, but little is known about the various pathways specifying them. For examples, the Notch pathway, with its ligands (Delta-like-4, Jagged-1 and Jagged-2) and receptors (Notch-1, Notch-2 and Notch-3), promotes the arterial fate of endothelial cells by repressing venous differentiation (Lawson et al. 2001; Zhong et al. 2001). Sonic Hedgehog and VEGF act upstream, whereas the bHLH (basic helix-loop-helix) transcription factor Gridlock probably acts downstream of Notch to determine arterial fate, even before the onset of flow (Lawson et al. 2001; Zhong et al. 2001). Ephrin-B2 and its receptor EphB4 are involved in establishing arterial versus venous identity, perhaps by fusing arterial and venous vessels at their junctions (Yancopoulos et al. 2000; Table 2). Furthermore, the molecular profile (transcriptome and proteome) of microvascular

Fig. 2 Putative pathways of normal and tumour endothelial cell differentiation. Endothelial cells in non-endothelial and endothelial tumours derive from either pre-existing normal vessels or from endothelial progenitors (see also Fig. 1). Normal endothelial cells are derived from progenitor cells (haemangioblasts) that can give rise to both endothelial and blood cells. Vascular endothelial growth factor (VEGF) family members stimulate these cells to develop into endothelial cells. The VEGF receptor-2 (VEGFR-2 or Flk-1) can be used to identify progenitors of the endothelial lineage (angioblasts). Angioblasts can give rise to smooth-muscle cells and pericytes, which form the outer walls of most blood vessels, or to endothelial precursor cells. These are committed to becoming either vascular endothelial cells (endothelioblasts) or lymphangioblasts (committed to becoming lymphatic endothelium). Lymphatic endothelial cells express VEGFR-3. VEGF-C and VEGF-D signal through VEGFR-3 to induce the proliferation, activation and migration of lymphatic endothelial cells (lymphangiogenesis). In the adult, VEGFR-3 has been shown to be expressed only on lymphatic and new vascular (neoangiogenic) endothelial cells. Another specific marker for lymphatic endothelial cells is the podocyte cell-surface mucoprotein podoplanin. The origin of the endothelial tumours is usually confirmed by the expression of blood and lymphatic endothelial-specific markers in neoplastic cells. For example, spindle cells which constitute Kaposi's sarcoma (KS) tumours have always been thought to belong to the endothelial lineage. The fact that KS spindle cells express both VEGFR-3 and podoplanin, but not the capillary/venous PAL-E cell-surface marker (as vascular endothelial cells do), indicates that these cells represent mature lymphatic or lymphatic precursor cells. It is possible that Kaposi's-sarcoma-associated herpesvirus (KSHV) exploits the endothelial-cell developmental process to promote its own replication, similar to the manner in which Epstein–Barr virus (EBV) and human papillomavirus (HPV) exploit normal B cell and epithelial-cell differentiation pathways, respectively. Like other viral-induced cancer cells, it is unlikely that these spindle cells represent terminally differentiated cells. Neoplastic cells in many other endothelial tumours (e.g. angiomas and lymphangiomas) express markers of both lymphatic and blood vessel endothelium. Therefore the origin of the majority of these tumours remains enigmatic. Tumour endothelial cells in non-endothelial tumours (see also Fig. 1) derive from either normal endothelium or from endothelial progenitors that acquire specific properties and express specific markers [e.g. tumour endothelial markers (TEM) 1,5,7 and 8] that are not expressed in normal endothelium. (Adapted from Boshoff and Weiss 2002, and further modified)

endothelial cells isolated from various vascular beds is often organ-specific (Chi et al. 2003). Finally, tumour endothelium is also different from normal endothelium within the same organ (St Croix et al. 2000). To accommodate local



physiological requirements, endothelial cells acquire specialised characteristics and functions that are determined in part by the host tissue (Risau 1998).

The tumour microenvironment in particular has a profound effect on the transcriptome of endothelial cells. Cytokines and angiogenic molecules secreted by cancer and immune cells modulate the expression of cellular adhesion molecules and other surface markers on the tumour endothelium. For example, VEGF and tumour-necrosis factor (TNF)-α up-regulate, whereas transforming growth factor (TGF)-β1 down-regulates, adhesion molecules (Jain et al. 1996). Because the expression and activity of general angiogenic factors such as VEGF and Ang-1 vary greatly in different tumours, these determine EC heterogeneity and the possibility of the organ-specific mechanisms of angiogenesis (LeCouter et al. 2002; St Croix et al. 2000). Tumour vessels change their phenotype and express surface proteins that are absent or barely detectable in quiescent vessels, with the "molecular signature" of tumour endothelium largely depending on the tumour type (Eliceiri and Cheresh 1999; Huang et al. 1997; Lal et al. 2001; St Croix et al. 2000). Epitopes specific for tumour endothelial cells represent attractive targets for anti-angiogenic therapy (Fig. 2; described in more details in Sect. 5).

2.2 Endothelial Progenitor Cells in Tumour Neovascularisation

Gene expression analysis comparing EPCs to endothelial cells has revealed that human endothelial cell precursors resemble freshly isolated endothelial cells from tumours, rather than cultured endothelial cells (Bagley et al. 2003). Recent studies demonstrate that angiogenic factors and chemokines direct EPCs to tumour neovessels (Spring et al. 2005). During tumour angiogenesis (described in more details in Sect. 5) the levels of circulating VEGF have been shown to rise (Connolly et al. 1989a, b; Leung et al. 1989; Senger et al. 1986). Increased circulating VEGF promotes the mobilisation of EPC from the BM, resulting in increased numbers of circulating endothelial progenitors in various pathologies including neoplasms (Asahara et al. 1999; Hattori et al. 2001). Human BM-derived cells have been shown to infiltrate human tumours (Peters et al. 2005) and to give rise to up to 16% of the tumour neovasculature in normal mice, complementing resident endothelial cells in sprouting new vessels (Ruzinova et al. 2003). Peters et al. analysed the tumour endothelial cells in six individuals who developed cancers after bone-marrow transplantation with donor cells derived from individuals of the opposite sex and found that an average of 4.9% of cells of the total endothelial cell population were derived from the BM (Peters et al. 2005). In contrast to these studies, De Palma et al. suggested that the percentage of EPCs that are truly incorporated into a growing vessel wall is very low and that the majority of BM-derived cells homing in on the tumour vasculature are adherent perivascular mononuclear cells, which may contain angiogenic factors (De Palma et al. 2003). Further-

Table 2 Factors affecting endothelial cells differentiation and function during vasculogenesis, angiogenesis and lymphangiogenesis

Factor	Receptor	Function
VEGF family members (VEGF-A, -B, -C, -D; PlGF) (secreted factors)	VEGFR-1, VEGFR-2, VEGFR-3 (receptor tyrosine kinases)	Stimulate angio/vasculogenesis (differentiation of haemangioblasts and proliferation and migration of mature endothelial cells), permeability
VEGF-C; VEGF-D (secreted factors)	VEGFR-3 (receptor tyrosine kinase)	Lymphangiogenic factors (differentiation, growth and remodelling of lymphatic vessels)
Ang-1 (secreted factor)	Tie-2 (receptor tyrosine kinase)	Stabilise vessels, inhibit permeability and EPC release from BM
Ang-2 (secreted factor)	Tie-2 (receptor tyrosine kinase)	Antagonist of Ang-1, destabilises blood vessels; plays role in lymphangiogenesis
	Integrins $\alpha_V\beta_3$, $\alpha_V\beta_5$, $\alpha_5\beta_1$ (receptors for matrix macromolecules and proteinases)	Regulate endothelial cells attachment to extracellular matrix molecules, migration and survival, as well as vasculogenesis
bFGF	FGFR	Stimulate angio/arteriogenesis and angioblast differentiation
VE-cadherin; PECAM (CD31) (endothelial junctional molecules)		Stabilisevessels, inhibit permeability
SDF-1 (secreted factor)		Stimulate mobilisation and differentiation of BM-derived progenitor cells, vasculogenesis

Table 2 (continued)

Factor	Receptor	Function
Ephrins (ligands tethered to membrane)	Eph receptors (receptor tyrosine kinases)	Regulate arterial/venous specification during EPC differentiation
TGF-β1, endoglin	TGF-β receptors	Stimulate extracellular matrix production
AC133 (glycoprotein)		Regulate angioblast differentiation
PDGF-A and -C	PDGFR-α and -β	Recruitment of angiogenic stroma
PDGF-B	PDGFR-β	Vessel maturation through recruitment of smooth muscle cells
Plasminogen activators, MMPs		Remodel matrix, release and activate growth factors such as bFGF
EG-VEGF	EG-VEGFR-1 and EG-VEGFR-2 (GPCRs)	Organ-specific angiogenesis
Adrenomedullin	Heterodimeric complex formed by GPCR CL and one of the RAMPs (1,2 or 3)	Stimulate vasculo/angiogenesis (differentiation of EPC; proliferation, migration and network formation of mature endothelial cells) and vasodilatation; regulate permeability
Chemokines	Various	Pleiotropic role in angiogenesis
Prox-1 (homeobox transcription factor)		Growth and differentiation of lymphatic endothelium
	Integrin $\alpha_9\beta_1$(receptor for matrix macromolecules and proteinases)	Lymphangiogenesis

Table 2 (continued)

Factor	Receptor	Function
Foxc2 (transcription factor)		Lymphangiogenesis (lymphatic patterning, negative regulator of lymphatic vessel hyperplasia)
	Soluble VEGFR-1 Soluble NRP-1	Sink for VEGF, VEGF-B, PlGF
Angiostatin and related plasminogen kringles		Suppress tumour angiogenesis
Endostatin (collagen XVIII fragment)		Inhibit endothelial growth
Vasostatin; calreticulin		Inhibit endothelial growth
TIMPs, MMP inhibitors		Suppress pathological angiogenesis

Abbreviations: EPC, endothelial progenitor cells; BM, bone marrow; VEGFR, VEGF receptor; PlGF, placental growth factor; NRP-1, neuropilin-1; MMP, matrix metalloproteinase; TIMPs, tissue inhibitors of MMP; SDF-1, stroma-derived factor; HGF, hepatocyte growth factor; CL, calcitonin-receptor-like receptor; RAMP, receptor activity modifying protein; EG-VEGF, endocrine gland-derived VEGF; GPCR, G protein-coupled receptor; Based on: Carmeliet and Jain (2000); Alitalo et al. (2005); Ferrara and Kerbel (2005); Yancopoulos et al. (2000); additional selected references are in the text

more, EPCs deriving from other tissue sites might also contribute to tumour angiogenesis.

Thus, BM-derived cells appear to contribute to tumour angiogenesis, of which a small and variable proportion is probably true EPCs. The contribution (as well as precise nature) of EPCs to angiogenesis is still an intensively debated issue. However, it is clear that enumeration of circulating EPCs in individuals with cancer may be useful in predicting the outcome of therapy or disease course (Bertolini et al. 2003; Schmidt-Lucke et al. 2005). Studies in mice indicate that the number of circulating EPCs is affected by systematic exposure to angiogenic regulators such as VEGF and can decline in response to anti-angiogenic treatments such as anti-VEGFR-2 antibody therapy (Shaked et al. 2005). Chemotherapeutic drug responses can be measured in part by the level of EPCs in the circulation (Bertolini et al. 2003). Thus, measurement of circulating EPCs may provide a useful assessment of disease susceptibility or response to therapies. However, controversies and investigation concerning the phenotype of circulating EPCs have not completely resolved which circulating cells give rise to endothelial cells during in vivo angiogenesis and lymphangiogenesis in tumours (Oliver 2004).

The observation that VEGFR-1^+ (VEGF receptor 1, described in Sect. 3.1) BM-derived haematopoietic cells provide a niche for tumour metastases (Kaplan et al. 2005) further opens the debate on the relationships between haematopoietic and endothelial precursors and their role in tumour angiogenesis and growth.

3
Angiogenesis and Lymphangiogenesis in Cancer

Vessels can grow in several ways. *Vasculogenesis* refers to the formation of blood vessels by endothelial progenitors (as described in Sect. 2), while *angiogenesis* and *arteriogenesis* refer to the sprouting of pre-existing vessels and subsequent stabilisation of these sprouts by mural cells. Collateral growth denotes the expansive growth of pre-existing vessels, forming collateral bridges between arterial networks (Carmeliet 2003). When vessel growth is deregulated, it has a major impact on our health and contributes to the pathogenesis of many disorders. Excessive angiogenesis or lymphangiogenesis occurs in cancer, psoriasis, arthritis and blindness. In cancer, the blood vessel network is expanded to meet the demand of a growing tumour mass, and lymphatic vasculature provides the path for metastasising cells. Other common disorders associated with abnormal blood vessel growth include obesity, atherosclerosis and inflammatory diseases (Table 1; Fig. 1). In addition, abnormal angiogenesis or lymphangiogenesis and endothelial transformation are hallmarks of infectious diseases and several *endothelial neoplasms* (e.g. Kaposi's sarcoma, haemangioma and lymphangioma; Table 1; Fig. 1).

3.1 Tumour Angiogenesis

Blood vessels in the embryo form through vasculogenesis; that is, through in situ differentiation of undifferentiated precursor cells (angioblasts) into endothelial cells which assemble into a vascular labyrinth (Risau 1997). Subsequently, this primitive network expands by angiogenesis (sprouting or intussusception from pre-existing vessels) (Patan et al. 1996). In the adult, physiological neovascularisation occurs in the female reproductive tract (e.g. during ovulation) and during wound healing. Pathological angiogenesis is a hallmark of cancer and various ischaemic and inflammatory diseases (Table 1). Vascular density often correlates with tumour grade (differentiation) and prognosis in various malignancies (Fox and Harris 2004).

Tumour vessels develop by angiogenesis (sprouting or intussusception from pre-existing vessels) or by co-option of normal vasculature by tumour cells (Carmeliet and Jain 2000). Circulating endothelial precursors, shed from the vessel wall or mobilised from BM, also contribute to tumour angiogenesis (Asahara et al. 2000; Rafii 2000; Fig. 1). Tumour angiogenesis requires the coordinated action of a variety of growth factors and cell-adhesion molecules in tumour, endothelial and mural cells (Coultas et al. 2005; Table 2). Amongst these, members of the VEGF and Ang family play a predominant role (Carmeliet and Jain 2000; Yancopoulos et al. 2000; Table 2).

VEGF-A is central in tumour angiogenesis (Ferrara 2004; Table 2). VEGF-A binds to two receptor tyrosine kinases (RTK), VEGFR-1 (Flt-1) and VEGFR-2 (KDR, Flk-1). Of the two, it is now generally agreed that VEGFR-2 is the major mediator of the mitogenic, angiogenic and permeability-enhancing effects of VEGF-A. Mice engineered to lack VEGFR-2 fail to develop a vasculature and have few endothelial cells (Shalaby et al. 1995).

The significance of VEGFR-1 in the regulation of angiogenesis is more complex. Under some circumstances, VEGFR-1 may function as a "decoy" receptor that sequesters VEGF-A and prevents its interaction with VEGFR-2 (Ferrara and Kerbel 2005). However, there is growing evidence that VEGFR-1 has significant roles in haematopoiesis and in the recruitment of monocytes and other BM-derived cells that home in on the tumour vasculature and promote angiogenesis (Gerber et al. 2002; Hattori et al. 2002; Luttun et al. 2002). During sprouting angiogenesis, vessels initially dilate and become leaky in response to VEGF-A. Members of the angiopoietin family, such as Ang-1 and Ang-2, are required for further remodelling and maturation of the vasculature through recruitment of mural cells (Yancopoulos et al. 2000). Ang-2 might also play a direct role in tumour angiogenesis and lymphangiogenesis (Alitalo et al. 2005; Oliner et al. 2004). A number of other activators, as well as inhibitors, of tumour angiogenesis have been identified and these molecules have an established role in the development and differentiation of the vessel wall (Table 2). Thus, platelet-derived growth factor (PDGF)-B is required for recruitment of

pericytes and maturation of the microvasculature (Lindahl et al. 1997). Furthermore, recent studies have emphasised the significance of tumour-derived PDGF-A (and potentially PDGF-C) and PDGFR-α signalling in the recruitment of an angiogenic stroma (heterogeneous compartment comprising fibroblastic, inflammatory and immune cells) which produces VEGF-A and other angiogenic factors (Dong et al. 2004). Finally, negative regulators of angiogenesis, including thrombospondin, vasohibin and several fragments of larger proteins including angiostatin, tumstatin and vasostatin have been identified (Table 2). The precise role of these proteins during tumour angiogenesis remains to be clearly defined, although several hypotheses have been proposed, including that they bind to specific integrins and affect endothelial cell migration and survival in the case of endostatin and tumstatin (Ferrara and Kerbel 2005).

Maintenance of new vessels depends largely on the survival of endothelial cells. Endothelial apoptosis in neovasculature is induced through deprivation of nutrients or survival signals (Carmeliet and Collen 1999; Gerber et al. 1999; Jain et al. 1998). VEGF [through its interaction with vascular endothelial (VE)-cadherin] (Carmeliet and Collen 1999) and Ang-1 are vital survival factors for tumour neovasculature. Their depletion causes tumour vessels to regress, especially when vessels have only been recently assembled and are still immature. This can occur as a result of insufficient maturation of newly formed vessels because cross-talk between endothelial and mural cells is essential in maintenance of a functional vasculature. In contrast, most angiogenesis inhibitors cause endothelial apoptosis directly through interaction with cell-surface molecules in endothelial cells (Table 2). Endothelial apoptosis can also be induced by nitric oxide, reactive oxygen species, interferon-ɣ and VEGF pathway inhibitors (described in Sect. 5).

Hypoxia is a strong stimulus for angiogenesis in numerous disorders including cancer (Harris 2002). Hypoxia is a frequent feature of the microenvironment of solid tumours and constitutes one of the driving forces of cancer growth and progression. Cells in tumours become hypoxic when too distant from nearby vessels. Hypoxia activates hypoxia-inducible transcription factors (HIFs), which function as master switches to induce the expression of several angiogenic factors, including VEGF, nitric oxide synthase (NOS), placenta-derived growth factor (PlGF), Ang-2, adrenomedullin and others produced by tumour and inflammatory cells (Table 2; Harris 2002). HIF operates in concert with the product of the von Hippel–Lindau (VHL) tumour suppressor gene. Under normoxic conditions, the VHL protein targets HIF for ubiquitination and degradation (Safran and Kaelin 2003). Inactivating VHL mutations occur in about 50% of renal cell carcinomas, where particularly high levels of VEGF-A expression have been found (Seizinger et al. 1988).

The up-regulation of VEGF-A and other angiogenic factors (Table 2) is not only linked to hypoxia or VHL mutations. A very broad and diverse spectrum of oncogenes is associated with up-regulation of angiogenic factors, including mutant ras, erbB-2/Her2, activated EGFR and bcl-abl in tumour cells (Ker-

bel et al. 2002; Rak et al. 1995). Viral oncogenes are also implicated in the up-regulation of members of VEGF and Ang families of angiogenic factors in endothelial cells in Kaposi's sarcoma (described in Sect. 4.4) (Wang et al. 2004). Besides VHL, inactivation/mutation of various other suppressor genes also result in the upregulation of VEGF expression in tumour and stroma cells (Brugarolas and Kaelin 2004). In other endothelial cell neoplasms, such as haemangioma and lymphangioma, pathological angiogenesis relies on expression of known growth factors, but the mechanisms regulating their up-regulation remain unknown (Ritter et al. 2002; described in Sect. 5).

3.2 Lymphangiogenesis and Cancer Progression

The lymphatic vasculature forms a vessel network that drains extravasated interstitial fluid proteins and cells from tissues and returns them back to the venous blood circulation. Lymphatic vessels are also an essential part of the body's immune defence. Deregulated lymphangiogenesis has an important role in the pathogenesis of several diseases such as lymphoedema, various inflammatory conditions and cancer. Malignant tumours expressing lymphangiogenic factors can directly activate lymphangiogenesis and lymphatic metastasis (Karpanen et al. 2001; Mandriota et al. 2001; Skobe et al. 2001; Stacker et al. 2001).

3.2.1 Differentiation of Lymphatic Endothelium in the Embryo

Lymphatic endothelial cells arise by sprouting from embryonic veins in the jugular and perimesonephric areas (Sabin 1909). From here they migrate to form primary lymph sacs and the primary lymphatic plexus, which is composed of capillary-like vessels (Alitalo et al. 2005). The homeobox transcription factor Prox1 is essential for these initial developmental events. The immature lymphatic endothelial cells (LECs) that are in a process of terminal differentiation express specific markers such as LYVE-1 (lymphatic vessel hyaluronan receptor-1). As differentiation and lymphangiogenesis progress, additional lymphatic markers are expressed during formation of lymph vessels and capillaries (Fig. 2).

Studies over the past 10 years revealed a signal transduction system for LEC differentiation, growth, migration and survival. This system is formed by VEGF-C and VEGF-D and their receptor VEGFR-3 (Achen et al. 1998; Joukov et al. 1996; Kaipainen et al. 1995; Makinen et al. 2001). These molecules play a significant role in lymphangiogenesis (Oliver 2004). The discoveries of specific markers of lymphatic endothelium and key lymphangiogenic factors have enabled the study of the lymphatic vasculature in tumours.

3.2.2
Molecular Regulation of Lymphangiogenesis in Cancer

Tumour cells activate peri-tumoural and intra-tumoural lymphangiogenesis as demonstrated by the use of lymphatic-specific molecular markers (Beasley et al. 2002; Karpanen et al. 2001; Mandriota et al. 2001; Skobe et al. 2001; Stacker et al. 2001; Wigle et al. 2002; Fig. 1). Proliferating intra-tumoural lymphatic vessels are present in certain human cancers, such as melanomas, head and neck carcinomas and xenograft tumour models overexpressing lymphangiogenic factors (Dadras et al. 2003; Maula et al. 2003). VEGF-C- and VEGF-D-induced lymphangiogenesis mediates tumour cell dissemination (metastasis). In mouse models, the induction of tumour lymphangiogenesis by VEGF-C promotes breast cancer metastasis (Skobe et al. 2001). Levels of VEGF-C expression by some types of primary tumours seem to correlate with the degree of lymph-node metastasis (Karpanen et al. 2001; Mandriota et al. 2001). VEGF-D also induces the formation of intra-tumoural lymphatic vessels in a mouse tumour model, and its expression by tumour cells facilitates the spreading of the tumour to regional lymph nodes (Stacker et al. 2001). A direct link between VEGF-C or VEGF-D expression and metastasis was established with the use of a soluble VEGFR-3-immunoglobulin fusion protein (VEGF-C/D trap) or blocking anti-VEGF-D antibodies (He et al. 2002; Karpanen et al. 2001; Stacker et al. 2001).

Although these studies provide support for the contribution of VEGF-C, VEGF-D and their receptor, VEGFR-3, in lymphatic metastasis, the mechanisms of these effects and the manner by which tumour cells enter the lymphatic system have only recently been addressed. For example, some evidence indicates that the lymphatic endothelium actively participates in metastasis formation by secreting chemokines such as CCL21 (SLC, 6Ckine and Exodus), whose receptor (CCR7) is expressed on some tumour cells (Zlotnik 2004). Finally, the presence of functional lymphatics around tumours appears to be sufficient for lymphatic metastasis, suggesting that intra-tumoural lymphatics are not always required (Padera et al. 2002; Fig. 1).

VEGFR-3 might also play a role in the infection of endothelial cells by KSHV (or human herpesvirus-8, HHV8) (Zhang et al. 2005) which then leads to transformation and reprogramming of the endothelium (Hong et al. 2004; Wang et al. 2004; described in Sect. 4.4). KSHV envelope glycoprotein gB interacts with VEGFR-3 and $\alpha_3\beta_1$ integrin and activates both, resulting in endothelial cell growth and migration (Zhang et al. 2005). VEGF-C and VEGFR-3 are co-expressed in endothelial cells in lymphangiomas (neoplasms of lymphatic vessels; described in Sect. 4), suggesting that these molecules may take an active part in the formation of these endothelial neoplasms by autocrine or paracrine regulation (Huang et al. 2001).

Although previous and current studies enabled progress towards understanding the biology of the lymphatic system, important questions remain unanswered. Are new lymphatic vessels and capillaries formed only by the

proliferation of endothelial cells from pre-existing lymphatics, or can they also be formed from endothelial precursors, lymphangioblast cells or by endothelial budding from veins? One study has shown that lymphatic endothelial progenitor cells derived from the circulation contribute to inflammation-associated lymphangiogenesis in human renal transplants, but not to tumour lymphangiogenesis (Kerjaschki et al. 2006).

4 Neoplasms of Endothelial Cells

Unrestricted proliferation of endothelial cells contributes not only to the development of non-endothelial tumours (e.g. epithelial cancers) but also to the pathogenesis of several endothelial neoplasms (Fig. 1; Table 1). These "*endothelial tumours*" are heterogeneous in their clinical description and behaviour, as well as in their aetiology. They are mainly formed of blood or lymph vessels (i.e. endothelium), with supporting tissues, and represent developmental defects or true neoplasms.

Haemangiomas are benign tumours of the vascular endothelium and are the most common tumours of infancy (Bell 2003; Ritter et al. 2002). These neoplasms are incapacitating, but little is known about their aetiology (Vikkula et al. 1998). Haemangiomas are characterised by an initial phase of rapid proliferation, which is followed, in most cases, by spontaneous involution. Although most lesions resolve without complication, there are circumstances in which these tumours become vision- or life-threatening, e.g. occurring near the eye, airway or other vital structures. VEGF and bFGF are implicated in their progression (Bielenberg et al. 1999). Interferon-β was identified as a potential endogenous inhibitor of this tumour, because it was found to be expressed in the epidermis underlying involuted, but not proliferating, haemangiomas. Therefore it was suggested that an imbalance in positive and negative regulators of angiogenesis is associated with the development of this tumour. Although these studies have described biological characteristics of haemangiomas in different phases and identified pathways which could be involved, the causative factors in haemangioma growth and involution remain to be identified (Ritter et al. 2002).

Lymphangiomas are benign neoplasms of lymphatic vessels. Derailed growth of VEGFR-3/podoplanin-positive lymphatic vessels results in lymphangioma, with subsequent formation of secondary lymphoedema due to impaired lymph fluid drainage. Lymphangiomas affect most organs, although they are most commonly found in the soft tissues of the head, neck and axilla, where they consist of a benign multi-cystic mass of dilated lymphatic channel networks (Alitalo and Carmeliet 2002).

Angiosarcomas and *lymphangiosarcomas* are malignant endothelial tumours of blood and lymphatic vessels respectively. In these malignancies, the neoplastic endothelial cells autonomously produce (lymph)angiogenic growth factors

and VEGF, as well as angiopoietins and Tie2 receptors (Brown et al. 2000; Zietz et al. 1998). Increased expression of the p53 tumour suppressor and mdm-2 proto-oncogene proteins leads to a loss of regulation of VEGF expression through decreased thrombospondin-1 regulation in angiosarcomas (Zietz et al. 1998).

Recent studies provide insight into the transcriptional profile and possibly an origin of some of these endothelial tumours (Hong et al. 2004; Ritter et al. 2002; Wang et al. 2004). These neoplasms may arise from genetic alterations or viral infections. For instance, products of KSHV and human immunodeficiency virus type-1 (HIV-1) have been implicated in the pathogenesis of Kaposi's sarcoma, found in approximately 30% of AIDS patients (Albini et al. 1996; Boshoff et al. 1995; Chang et al. 1996; Flore et al. 1998). HIV-1 Tat protein activates VEGFR-2, binds endothelial $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins and retrieves bFGF from the extracellular matrix (Albini et al. 1996). In general, Kaposi's sarcoma remains the most studied amongst endothelial tumours, and recent studies provide further insight into the pathogenesis of this neoplasia.

Kaposi's sarcoma is a neoplasm characterised by vascular nodules in the skin, mucous membranes and internal organs. It is endemic in sub-Saharan regions in Africa and is frequently encountered in HIV-1-infected individuals (Boshoff and Weiss 2002). The nodules are composed of clusters of spindle-shaped tumour cells and characterised by a prominent vasculature. The finding that spindle cells and cells lining the irregular vascular spaces in Kaposi's sarcoma express both blood and lymphatic endothelial cell markers suggests their endothelial origin (Fig. 3). Development of Kaposi's sarcoma is associated with infection by KSHV (Chang et al. 1994). The transcriptional profile of Kaposi's sarcoma is akin to LECs (Hong et al. 2004; Wang et al. 2004; Fig. 3). Furthermore, in vitro infection of blood vascular endothelial cells with KSHV resulted in the expression of several lymphatic endothelial cell-specific genes, although KSHV-infected lymphatic endothelial cells also showed some infidelity of phenotypic gene expression (Hong et al. 2004; Wang et al. 2004). It is still unclear whether KSHV primarily infects LECs to precipitate Kaposi's sarcoma, or whether the virus infects EPCs and steers their differentiation towards a LEC genotype.

KSHV encodes for a number of proteins that could directly play a role in endothelial proliferation. These included a viral cyclin (Chang et al. 1996), a viral FLICE inhibitor (vFLIP), and a latency-associated nuclear antigen (LANA). LANA interacts with both p53 and pRb, and can transform rodent cells (Radkov et al. 2000). KSHV also encodes for a number of cellular homologues, which could play a direct role in promoting angiogenesis. These include a viral encoded G protein-coupled receptor (vGPCR) (Bais et al. 2003), and a number of chemokine homologues such as macrophage inflammatory proteins (vMIPs) (Boshoff et al. 1997).

Most spindle cells in Kaposi's sarcoma lesions only express KSHV latent genes (including v-cyclin, vFLIP and LANA), but a fraction of cells also ex-

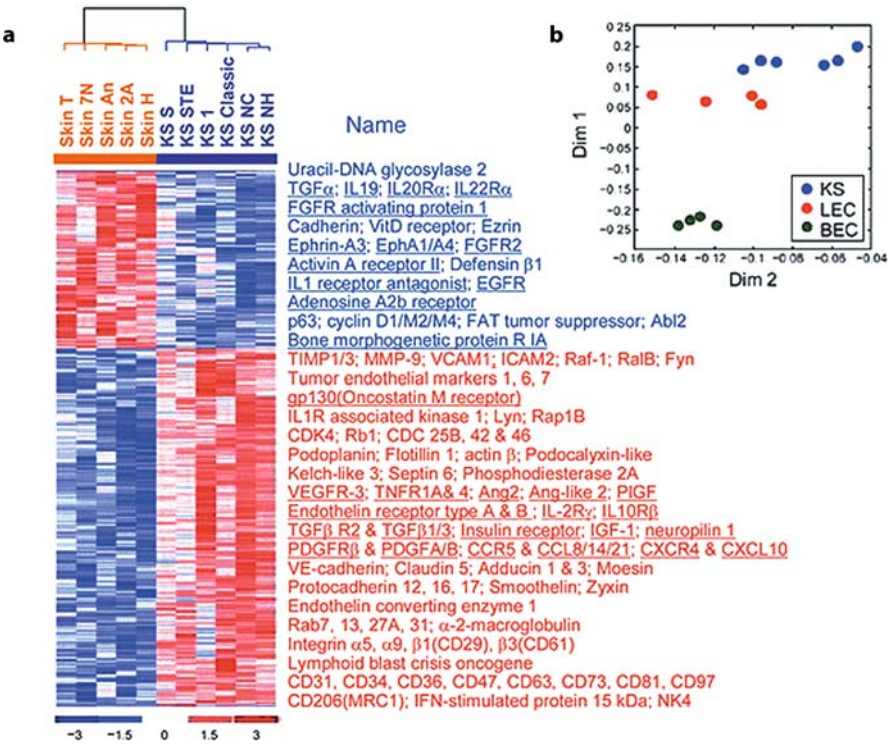


Fig. 3 a,b Relationship between the expression signature of Kaposi's sarcoma and blood and lymphatic endothelial cells. **a** Gene expression microarray (GEM) data for Kaposi's sarcoma is shown through a heat map of 1,482 genes that differentiate sarcoma (KS) and skin sample groups ($p \leq 0.05$). Selected down-regulated genes are shown in *blue* and upregulated genes in *red*. Cytokines and chemokines and their receptors are *underlined*. *Colour scale* indicates units of standard deviation (SD) from the mean expression of each gene. **b** Multi-dimensional scaling (MDS) plot using the lymphatic and blood vessel endothelial cell (LEC-BEC) discriminatory gene signature $n=114$; $p \leq 0.2$). (Adapted from Wang et al. 2004)

press lytic proteins. These lytic proteins (including vGPCR and vMIPs) might attract EPCs to lesions, where they are infected by the virus. Latent and lytic infection therefore appears to be important in the maintenance of this endothelial tumour.

5 Targeting Endothelium

Inhibiting angiogenesis and lymphangiogenesis is a promising strategy for the treatment of cancer. Major progress has been achieved over the past few years, and there is now proof that an anti-angiogenic approach, when combined

with chemotherapy, results in increased survival in patients with advanced malignancies (Ferrara and Kerbel 2005). The majority of these approaches are focussed on targeting tumour endothelium and mechanisms regulating its survival, proliferation and migration, as well as inhibition of EPC recruitment to sites of neovascularisation in tumours.

Many angiogenesis inhibitors are currently in phase I, II or III clinical trials. The inhibitors tested include agents with diverse mechanisms of action (several of which are not known). At present, inhibitors of the VEGF pathway are the most clinically advanced. Several strategies exist to inhibit VEGF signalling. These include monoclonal antibodies targeting VEGF-A (e.g. bevacizumab) or VEGF receptors. Bevacizumab, a humanised variant of a murine anti-VEGF-A monoclonal antibody used in early proof-of-concept studies (Kim et al. 1993), is the only current FDA-approved specific anti-angiogenic treatment for cancer (Ferrara 2004). Chimaeric soluble receptors such as "VEGF-trap" (domain 2 of VEGFR-1 and domain 3 of VEGFR-2 fused to the Fc fragment of an antibody) are also undergoing clinical development. Additional extracellular inhibitors are aptamers that bind to the heparin-binding domain of VEGF165 (pegaptanib). A variety of small-molecule VEGF RTK inhibitors that inhibit ligand-dependent receptor autophosphorylation of VEGFR-1 and VEGFR-2 are being tested. Additional strategies to inhibit VEGF signalling include antisense and siRNA targeting VEGF-A and its receptors.

Cell surface molecules that are preferentially expressed in tumour, but not normal, endothelium are attractive targets for anti-angiogenic therapies. The tumour vascular bed-specific expression of a variety of proteins, including cell-surface antigens, has been evaluated (Aird et al. 1997; Arap et al. 1998; St Croix et al. 2000). In vivo selection of phage display libraries has yielded peptides (for example, amino acid sequences RGD and NGR) that preferentially recognise vessels in subcutaneous tumours in mice (Arap et al. 1998). These peptides can be used to target therapeutic agents to tumours. The serial analysis of gene expression (SAGE) identified molecules preferentially expressed on endothelium in blood vessels in malignant colorectal tissues (St Croix et al. 2000). These molecules are conserved tumour endothelial markers in mice and human and therefore could also present attractive targets for the development of anti-angiogenic therapies (Carson-Walter et al. 2001). The challenge now is to discern how specific these "vascular zip codes" are, as targeting drugs to the tumour vasculature has the potential to change the paradigm for cancer treatment.

The recent discovery of lymphangiogenic factors VEGF-C and VEGF-D and their receptor VEGFR-3 (see Sect. 3.2.1) has opened novel diagnostic and therapeutic avenues for anti-lymphangiogenic therapy, and for the treatment of lymphoedema and metastasis in particular. Blocking monoclonal antibodies that target these factors or their receptor(s) and small molecules that inhibit the tyrosine kinase catalytic domain of these receptors could be used for the inhibition of tumour metastasis. For example, lymphatic spreads induced by VEGF-D

were blocked with an antibody specific for VEGF-D (Stacker et al. 2001). Also, VEGF-C-induced tumour growth, lymphangiogenesis and intralymphatic tumour growth were inhibited by adenoviral expression of the soluble VEGFR-3 receptor, which "traps" available VEGF-C and VEGF-D (Karpanen et al. 2001). However, caution is warranted, since destruction of lymphatic vessels could further elevate the already increased interstitial fluid pressure inside the tumours, thereby further impairing the delivery of other anti-cancer drugs.

Inhibition of EPC mobilisation from the BM also has tremendous therapeutic potential, as evidenced by the inability of tumours to grow in animals that lack functional EPCs (Lyden et al. 1999, 2001). Preliminary work in animal models suggests that agents that inhibit EPC mobilisation may comprise an effective cancer therapy (Capillo et al. 2003), but further work is needed before this strategy can be applied to human neoplasms. This includes endothelial neoplasms such as Kaposi's sarcoma, where the contribution of KSHV-infected EPCs as well as resident differentiated endothelium has been suggested (Pyakurel et al. 2006).

As the cellular and molecular mechanisms of angiogenesis differ in various tissues (Carmeliet 2000, 2003), the therapeutic inhibition of angiogenesis should be adjusted to the target tissue. The recent discovery of tissue-/organ-specific regulators of angiogenesis, such as endocrine gland-derived VEGF, suggests a potentially novel approach to this problem (LeCouter et al. 2002). A principal benefit of tissue-specific angiogenic therapeutics could be the elimination of systemic, undesired effects associated with broad-spectrum angiogenic molecules. Furthermore, in endothelial neoplasms, where mechanisms of angiogenesis are not well-characterised, alternative therapies could be considered, depending on the outcome of studies investigating the cellular and molecular mechanisms involved.

6 Conclusions

Both the blood and lymphatic endothelial systems are central in the pathogenesis of human malignancies. Lymphatics are often the first port of call for tumour metastases, and blood vessels provide essential nutrients and oxygen supply to promote tumour cell proliferation. The secretion of pro-angiogenic and lymphangiogenic molecules by tumour cells initiates the growth of these vessels. Recent evidence also indicates that circulating endothelial cells and endothelial precursor cells are mobilised by the growing tumour mass, further contributing to angiogenesis and the cytokine environment enabling tumour cell proliferation. Understanding the molecular mechanisms underlying the proliferation of blood and lymphatic endothelial cells should lead to novel ways to prevent and treat cancer.

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Gene Therapy: Role in Myocardial Protection

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Abstract Heart failure associated with coronary artery disease is a major cause of morbidity and mortality. Recent developments in the understanding of the molecular mechanisms of heart failure have led to the identification of novel therapeutic targets which, combined with the availability of efficient gene delivery vectors, offer the opportunity for the design of gene therapies for protection of the myocardium. Viral-based therapies have been developed to treat polygenic and complex diseases such as myocardial ischaemia, hypertension, atherosclerosis and restenosis. Some of these experimental therapies are now undergoing clinical evaluation in patients with cardiovascular diseases. In this review we will focus on the latest advances in the field of gene therapy for treatment of heart failure and their clinical application.

1 Introduction

Despite significant advances in the clinical management of cardiovascular disease (CVD), acute myocardial infarction (MI) and heart failure (HF) due to coronary artery disease (CAD), cardiomyopathy and systemic vascular disease remain the prevalent causes of premature death across all age and racial groups (Kannel and Belanger 1991). The complexity of the pathological processes leading to heart disease and the lack of specific predictive markers have been major impediments to the development of effective preventive therapies, despite the identification of various risk factors and sensitive risk assessment technologies (Wilson et al. 1998; D'Agostino et al. 2000; Stein 2002). Consequently, the focus has been on the design of "rescue" treatments for overt symptoms of the disease, such as hyperlipidaemia, myocardial ischaemia, left ventricular pump failure and haemodynamic overload (McMurray and Pfeffer 2002). Although these therapies have improved the clinical outlook for patients affected by MI and HF, morbidity and mortality associated with these diseases remain high, indicating the need for more effective treatments.

The current availability of efficient vector systems such as adeno-associated virus (AAV) (Robbins and Ghivizzani 1998; Monahan and Samulski 2000), and the recent identification of several gene targets associated with heart disease (Colucci 1997; Givertz and Colucci 1998), offer opportunities for the design of gene therapies for myocardial protection and rescue. The ability of AAVs to confer long-term and stable protein expression with a single administration of the therapeutic gene (Kaplitt et al. 2000) renders them ideally suited for delivery of therapeutic genes.

In this chapter we review the major advances in gene-based therapies for heart disease, with emphasis on strategies for protection and rescue of the failing heart, their clinical feasibility and a perspective on future developments in the field. We will highlight the breakthroughs, the challenges in making the transition from pre-clinical studies to clinical application, and the opportunities ahead in this exciting and growing field.

2 Tools and Strategies for Genetic Manipulation of the Cardiovascular System

The major hindrance to the development of effective gene therapies for CVD has been the unavailability of efficient vectors and delivery tools for genetic manipulation of the heart and blood vessels. The main types of vectors used in cardiovascular gene therapy are summarised in Table 1. Most of the current vectors lack tissue specificity and express transgenes only transiently (Mah et al. 2002; Niidome and Huang 2002), rendering them unsuitable for use in chronic CVD. Non-viral vectors usually yield low and transient gene transfer

efficiency due to lack of genomic integration and rapid degradation (Niidome and Huang 2002). Therefore, recombinant viruses have become the preferred vectors for cardiovascular gene transfer (Table 1). These replication-deficient viral particles deliver genetic material with higher efficiency than non-viral vectors. Some viral vectors, such as AAV and lentivirus, are capable of sustained expression of the therapeutic gene (Mah et al. 2002), rendering them suitable for use in chronic myocardial and vascular diseases.

The most common somatic gene therapy strategy for cardiovascular disease involves the exogenous overexpression of a full-length or partial complementary DNA (cDNA) encoding a gene whose endogenous activity may be absent or attenuated as a result of disease. The goal is to restore normal function or reverse disease progression (Fig. 2; Melo et al. 2004). The therapeutic gene may encode an intracellular protein, in which case the therapeutic effect is predominantly autocrine. Alternatively, the therapeutic protein may be secreted and exert physiological effects in a paracrine or endocrine fashion. Such "gain-of-function" strategies have been employed for the overexpression of cytoprotective and pro-angiogenic genes in animal models and in patients with vascular and myocardial disease (Isner 2002; Melo et al. 2004). In other instances, the short-term silencing of (loss-of-function) pathogenic genes may be desirable and sufficient to halt disease progression. Towards this goal, acute inhibition of transcription and translation can be achieved by treatment with short single-stranded antisense oligodeoxynucleotides, ribozymes and, more recently, using RNA interference technology (Fig. 2; Mann and Dzau 2000; Stein 2001). These molecules inhibit translation by hybridising in a sequence-specific manner to the target mRNA. As an alternative strategy, double-stranded "decoy" oligonucleotides bearing DNA consensus binding sequences (*cis*-elements) have been used to inhibit the transactivating activity of target transcription factors (Fig. 2; Mann and Dzau 2000). The decoy is usually delivered in molar excess, effectively sequestering the target transcription factor and rendering it incapable of binding to the promoter region of the target gene(s).

3
Indications

3.1
Pathogenesis of Heart Disease and Targets for Gene Therapy

Myocardial ischaemia associated with CAD is the primary cause of myocardial failure (Funk and Krumholz 1996). Acute ischaemic events, if sufficiently prolonged, will lead to irreversible damage and infarction, underlined by alterations in membrane fluidity, intracellular hydrogen ion concentration and metabolic activity and eventual cell death, resulting in arrhythmia and im-

Table 1 Vectors used for transfer and manipulation of genetic material in cardiovascular tissues

Vector	Transfer efficiency in vivo	Sustainability of therapeutic effect	Level of expression	Target cells	Potential risks	Host immune response
Non-viral						
Cationic liposomes	+	Short	+	Quiescent and dividing	Cytotoxicity	+
HVJ-liposomes	+++	Short	++	Quiescent and dividing	Cytotoxicity	+
Naked plasmid	+	Short	+	Quiescent and dividing	Cytotoxicity	+
Viral						
Retrovirus	++	Life-long	++	Dividing	Cytotoxicity oncogenesis	+
Lentivirus	+++	Life-long	+++	Quiescent and dividing	Cytotoxicity, viral mutation	+
Adenovirus	+++++	Moderate	+++++	Quiescent and dividing	Cytotoxicity,inflammation	++++
Adeno-associated virus	+++	Life-long	+++	Quiescent and dividing	Oncogenesis, viral mutation	+
Herpes simplex virus	+++	Long	+++	Quiescent and dividing	Cytotoxicity, viral mutation	+++

paired pump function (Carden and Granger 2000). Paradoxically, reoxygenation of the ischaemic myocardium induces a robust increase in reactive oxygen species (ROS), which triggers a profound inflammatory response and may exacerbate the damage initiated during ischaemia (Carden and Granger 2000; Yellon and Baxter 2000). In time, the left ventricle undergoes a process of remodelling characterised by myocyte hypertrophy, interstitial fibrosis, chamber dilatation and increased propensity for contractile dysfunction that ultimately leads to ventricular failure (Pfeffer et al. 1991). The remodelling process is complex and highly dependent on the activity of matrix metalloproteinases (MMPs), a group of zinc-dependent proteases that are involved in extracellular matrix degradation (Peterson et al. 2000). Chronic ischaemic heart disease is also characterised by a heightened inflammatory state and oxidative stress (Mehta and Li 1999). The increased levels of pro-inflammatory cytokines suppress myocardial contractility and activate neurohormonal systems, such as the renin–angiotensin system, which promote ventricular fibrosis and remodelling. Taken together, the pathophysiology of ischaemic heart disease offers several exciting targets for intervention with gene therapy which may provide benefits to HF patients.

3.2 Protection Against Myocardial Ischaemia

The vascular endothelium usually remains in a quiescent, non-proliferative state, and with the exception of the female reproductive tract and neoplastic disease, post-natal neovascularisation is rare (Carmeliet 2000). However, injury, inflammation and oxidative stress activate the endothelium, resulting in cell proliferation, migration and formation of new vascular networks by angiogenesis (Carmeliet 2000). In patients and animal models with ischaemic heart disease, the progressive occlusion of the coronary artery leads to a chronic imbalance in myocardial oxygen supply and demand, which stimulates the development of collateral vessels thus maintaining tissue perfusion and oxygenation (Ware and Simons 1997). This native adaptive response of the myocardium, however, does not provide adequate compensation in the face of severe ischaemia, and depression of cardiac function ensues, which in time leads to heart failure.

Evidence of enhanced neovascularisation and functional recovery of ischaemic myocardium has been reported in animal and human studies after exogenous supplementation of proangiogenic cytokines by gene transfer (Giordano et al. 1996; Ueno et al. 1997; Mack et al. 1998; Symes et al. 1999; Tio et al 1999; Ueda et al. 1999). This novel strategy, commonly known as therapeutic angiogenesis, offers a potentially efficacious method for treatment of coronary artery disease in clinical cases where percutaneous angioplasty or surgical revascularisation has been excluded. In all cases, improvement in tissue perfusion was accompanied by morphological and angiographic evidence of new

vessel formation, thus establishing a relationship between improved tissue viability and neovascularisation. For example, Mack et al. (1998) demonstrated improvement in regional myocardial perfusion and left ventricular function in response to stress in an ameroid constrictor model of chronic myocardial ischaemia in pigs following intramyocardial delivery of vascular endothelial growth factor $(VEGF)_{121}$ by adenovirus. Using intracoronary injection of an adenovirus vector encoding human fibroblast growth factor (FGF)-5, Giordano et al. (1996) also showed a significant improvement in blood flow and a reduction in stress-induced functional abnormalities as early as 2 weeks after ameroid placement around the proximal left circumflex coronary artery in pigs, in association with an increase in capillary-to-fibre ratios.

3.3 Protection from Ischaemia and Reperfusion Injury

The continuum of myocardial injury that is initiated by a coronary ischaemic event and perpetuated by reperfusion (I/R injury) may be clinically manifested in patients undergoing thrombolytic therapy following an acute coronary episode. The increase in ROS formation during reperfusion of the ischaemic myocardium may eventually deplete the buffering capabilities of endogenous anti-oxidant systems, thereby exacerbating the cytotoxic effects of these reactive species (Park and Lucchesi 1999). The development of gene therapies for acute MI has been difficult because the time required for transcription and translation of therapeutic genes with the current generation of vectors exceeds the time window for successful intervention. An alternative gene therapy for myocardial protection is to "prevent" I/R by the transfer of cytoprotective genes into the myocardium of high-risk patients prior to ischaemia, using a gene delivery method that could confer long-term therapeutic gene expression. This novel concept of "preventive" gene therapy would protect the heart from future I/R injury, thereby minimising the need for acute intervention. Given the prominent role of oxidative stress in I/R injury, a therapeutic approach aimed at increasing endogenous anti-oxidant reserves should, in principle, be a useful strategy for prevention/protection in patients at risk of acute myocardial infarction. This strategy would potentiate the native protective response of the myocardium (Williams and Benjamin 2000), rendering it resistant to future ischaemic insults.

We have evaluated the feasibility of anti-oxidant enzyme gene transfer as a long-term first line of defence against I/R-induced oxidative injury, using an rAAV vector for intramyocardial delivery of haem oxygenase (HO)-1 gene in a rat model of myocardial I/R injury (Melo et al. 2002). Our findings show that HO-1 gene delivery to the left ventricular risk area several weeks in advance of MI results in approximately 80% reduction in infarct size. The reduction in myocardial injury in the treated animals is accompanied by decreases in oxidative stress, inflammation and interstitial fibrosis. Consistent with the histopathol-

ogy, echocardiographic assessment showed post-infarction recovery of left ventricular function in the HO-1-treated animals, whereas the untreated control animals presented evidence of ventricular enlargement and significantly depressed fractional shortening and ejection fraction. Thus, these findings suggest that AAV-mediated delivery of HO-1 may be a viable therapeutic option for long-term myocardial protection from I/R injury in patients with CAD. Comparable findings were found with extracellular superoxide dismutase (ecSOD) gene transfer (Chen et al. 1998; Li et al. 2001). This secreted metalloenzyme plays an essential role in maintenance of redox homeostasis by dismutating the oxygen free radical superoxide.

The inhibition of pro-inflammatory genes involved in the pathogenesis of I/R injury offers another option for cardioprotection. Morishita et al. (1997) showed that pretreatment with a decoy oligonucleotide capable of inhibiting the trans-activating activity of the pro-inflammatory transcription factor nuclear factor (NF)-κB reduces myocardial infarct after coronary artery ligation in rats. Similarly, intravenous administration of antisense oligonucleotide against angiotensin-converting enzyme messenger (m)RNA (Chen et al. 2001) significantly reduces myocardial dysfunction and injury following ischaemia and reperfusion. Although the rapid in vivo degradation of oligonucleotides would preclude their use in long-term myocardial protection, they may be useful in the treatment of acute myocardial ischaemia and cardiac transplantation (Stepkowski 2000) by providing a tool for inhibiting pro-oxidant, pro-inflammatory and immunomodulatory genes activated by ischaemia and reperfusion. For example, treatment with antisense oligonucleotide directed against intercellular adhesion molecule (ICAM)-1 was shown to prolong cardiac allograft tolerance and long-term survival when administered ex vivo prior to transplantation into the host (Poston et al. 1999). Such an approach could be beneficial in the preparation of donor hearts for transplantation. For example, oligonucleotide-mediated inhibition of anti-inflammatory genes and adhesion molecules in donor organs in advance of transplantation could be used to suppress the acute inflammatory response that ensues upon reperfusion of the transplanted organ in the recipient.

3.4 Myocardial Hypertrophy and Remodelling

The progression of heart failure due to haemodynamic overload, chronic myocardial ischaemia or acute myocardial infarction is invariably accompanied by hypertrophy and remodelling of the left ventricle (Sutton and Sharpe 2000). This process, which usually begins as an adaptive physiological mechanism aimed at normalising wall stress in response to the increased load or myocyte death from infarction, eventually becomes maladaptive, resulting in alteration in ventricular geometry, mechanical decompensation and contractile failure. Following MI, the left ventricle undergoes an early healing phase during which

the infarcted area expands, resulting in wall thinning and ventricular dilation that leads to increased wall stress. This is followed by long-term dilation of the non-infarcted region and myocyte hypertrophy and interstitial fibrosis, leading to ventricular chamber distortion and enlargement (Sutton and Sharpe 2000).

Inhibition of ventricular remodelling is a prime target in the treatment of heart failure, and the long-term survival benefits of therapies such as angiotensin-converting enzyme (ACE) inhibition and β-blockade in patients suffering from MI or heart failure are attributed, at least in part, to a decrease in left ventricular remodelling. Pharmacological inhibition of these pathways attenuates the hypertrophic and remodelling process and delays the progression of disease (McMurray and Pfeffer 2002). More recently, treatment with MMP inhibitors was shown to attenuate post-infarction left ventricular dilation effectively (Asakura et al. 2002), indicating this could be a potential therapeutic strategy for the treatment of heart failure. Genetic manipulation of these targets may prove to be an effective alternative therapy to current pharmacological approaches for treatment of heart failure.

Gene therapies aimed at inhibiting hypertrophic and pro-fibrotic pathways should be useful in limiting the extent of remodelling. For example, inhibition of AT_1-R signalling by antisense reduces cardiac hypertrophy in a renin-overexpressing transgenic rat, independently of systemic effects (Pachori et al. 2002), suggesting a role of local angiotensin II in inducing the hypertrophic phenotype. A similar approach could be used for inhibition of cardiotrophic factors such as calcineurin and protein kinases (Taigen et al. 2000). Antisense inhibition of myocardial transforming growth factor-β1 factor signalling and metalloproteinase activity could be employed as strategies to reduce fibrosis and remodelling. Conversely, myocardial overexpression of anti-hypertrophic factors may be used as a strategy to reverse hypertrophy in failing hearts. Li et al. (1997) demonstrated that cardiac-specific overexpression of insulin-like growth factor-1 in mice prevented myocyte death in the viable myocardium and attenuated ventricular dilation and hypertrophy after MI. Similarly, cardiac overexpression of glycogen synthase-3β, an endogenous antagonist of calcineurin action, was reported to inhibit hypertrophy in response to chronic β-adrenergic stimulation and pressure overload (Antos et al. 2002).

4 Complications

One disadvantage of using viral vectors for gene therapy is that some viral proteins may trigger a robust immune reaction which may reduce the duration of transgene expression (Mah et al. 2002); however, recent developments have led to the production of vectors with attenuated immunogenicity (Chirmule et al. 1999). Furthermore, there is a risk, albeit remote, that these vectors may revert to their wild-type phenotype, raising concerns about biological hazards

such as oncogenesis and insertional mutagenesis (Mah et al. 2002). The impetus at the current time is to develop vectors with enhanced tissue specificity that are capable of directing expression of the therapeutic transgene in response to pathophysiological stimuli such as hypoxia and oxidative stress.

Vector delivery to cardiovascular tissues is problematic. The selectivity of the endothelium and the presence of the basement membrane restrict the diffusion of some vectors. A variety of specialised balloon catheters have been developed for intravascular delivery, but the efficiency of vector delivery is moderate, at best (Feldman and Steg 1997). A novel approach for local vascular gene delivery uses stents coated with genetically engineered cells or with plasmid or adenoviral vectors expressing therapeutic genes (for review see Sharif et al. 2004). Specific modifications of the vector backbone or capsid proteins have also been reported to increase the efficiency of vector uptake by the endothelium. Catheters have also been used for intracoronary gene delivery to the myocardium, but efficiency is low (Isner 2002). Intramyocardial injection is routinely used as a strategy for local transgene delivery in the myocardium, but transgene expression is restricted to the vicinity of the injection site (Isner 2002). New catheters are now available which allow more precise intramyocardial gene injection with the assistance of trans-oesophageal echocardiographic and mapping techniques (Sylven et al. 2002).

As discussed above, gene therapy can be used to target transcription factors which control downstream signalling systems. However, this strategy lacks specificity because several genes may be under the control of the targeted transcription factor, and the target gene may be under the influence of multiple transcription factors (Mann and Dzau 2000). New strategies are currently being developed to improve the specificity of gene knock-down. For example, nucleic acid and peptide aptamers have been used to inhibit protein function without altering the genetic complement of the host (White et al. 2000).

5
Combination and Synergistic Use of Gene Therapy

As discussed above, induction of blood vessel formation or "angiogenesis" has been a popular target of gene therapy, as several studies have used agents like VEGF and FGF to enhance collateral blood vessel formation following myocardial ischaemia (Asahara et al. 1995). Recent studies have demonstrated the therapeutic potential of administering various angiogenic growth factors to augment revascularisation in the ischaemic limb (Asahara et al. 1995) as well as myocardium (Giordano et al. 1996; Mack et al. 1998). However, it has been demonstrated that although VEGF is a strong angiogenic factor, it also increases the permeability of capillaries. Indeed, the presence of severe oedema has been reported following VEGF delivery (Masaki et al. 2002; Vajanto et al. 2002). Therefore, several studies have explored the possibility of a "synergistic"

therapy, with the combined use of VEGF and FGF for induction of functional capillary formation (Asahara et al. 1995). It has been demonstrated that such a combination may be a better approach than using a single factor alone. The mechanisms of such an effect are not clear. However, it can be speculated that since leakage of the fluid component and worsening of oedema might decrease the efficiency of tissue perfusion, there is a possibility that the net blood flow conduction in capillaries formed by VEGF would be considerably less than the expected blood flow matching to the gross volume of the capillary bed. In contrast, bFGF not only promotes the proliferation of endothelial cells, but also induces development of the medial layer and adventitia (Klagsbrun 1989), which could sustain and support the endothelial layer from the outside. Therefore, bFGF possibly increases the function of VEGF-induced capillaries by preventing leakage of fluid, resulting in synergistic angiogenic effects after combined gene delivery of VEGF and bFGF.

6
Clinical Gene Therapy: Applications to Interventional Cardiology

Despite the compelling pre-clinical evidence about the feasibility and efficacy of gene therapy in the treatment of cardiovascular diseases, only a few small-scale trials have been carried out (Edelstein et al. 2004). Of the 918 trials that have been finished or are currently under way worldwide, only 8.3% are in cardiovascular disease. The majority of these trials evaluated the therapeutic efficacy of angiogenic gene transfer in the treatment of coronary and peripheral ischaemia (for reviews see Isner 2002; Yla-Herttuala and Alitalo 2003). Although the trials generally support the feasibility and safety of angiogenic gene transfer, the clinical findings have been inconclusive with regard to the efficacy of angiogenesis gene therapy. In a phase I study in 5 male patients aged 53–71 years of age with CAD that did not respond to conventional anti-angina therapy, intramyocardial delivery of naked plasmid encoding $VEGF_{165}$ into the ischaemic myocardium led to reduction of anginal symptoms and improvement, albeit modest, in left ventricular function concomitant with reduced ischaemia (Losordo et al. 1998). Vale and colleagues (2001) reported significant reductions in weekly anginal attacks for as long as 1 year after catheter-based delivery of naked VEGF-2 (VEGF-C) assisted by electromechanical NOGA mapping of the left ventricle in patients with chronic myocardial ischaemia. The recently published results of the Angiogenic GENe Therapy (AGENT) double-blinded, randomised, placebo-controlled trial using dose-escalating adenovirus-mediated intracoronary delivery of FGF-4 in 79 patients with angina showed a general trend towards an increase in exercise tolerance and improved stress echocardiograms at 4 and 12 weeks after gene transfer compared to the patients receiving placebo, in association with angiographic evidence of neovascularisation (Grines et al. 2002). However, the trial was not

sufficiently powered to detect statistically significant differences between the treated and placebo groups in the treadmill exercise time to fatigue.

Some early-phase trials have also been undertaken to evaluate the effect of cell cycle inhibition on neointima proliferation and vein graft failure. We carried out a phase I prospective, randomised double-blind trial of human saphenous vein graft treatment with E2F decoy (Project In Ex-Vivo Vein Graft Engineering Via Transfection, PREVENT-1) in high-risk patients suffering from peripheral arterial occlusive disease (Mann et al. 1999). Using non-distending pressure to deliver the E2F decoy oligonucleotide ex vivo prior to arterial interpositional grafting, we demonstrated that E2F decoy treatment was safe and feasible. Although the results were preliminary, the study provided evidence that cytostatic gene therapy is feasible for clinical application. More recently the PREVENT II has largely confirmed the finding of the PREVENT I trial. The PREVENT II is a randomised double-blinded, placebo-controlled phase II trial designed to evaluate the effect of E2F decoy treatment on coronary artery bypass grafting failure (unpublished findings, Grube et al., American Heart Association meeting, November 2001; for commentary see McCarthy 2001). The interim results confirmed the feasibility and safety of using E2F-1 decoy. Analysis of the secondary end-points using quantitative coronary angiography and three-dimensional intravascular ultrasound demonstrated increased patency and adaptive vessel remodelling characterised by reduction in neointimal size and volume in the treated group 1 year after treatment, leading to a 40% reduction in critical stenosis. These results will now need to be confirmed in adequately sampled and powered phase III studies in patients with coronary and peripheral vessel disease in order to validate further the therapeutic value of this approach. Another phase I trial (Restenosis Gene Therapy Trial, REGENT-I) is currently underway to evaluate the efficacy of catheter-based inducible nitric oxide synthase (iNOS) gene delivery to prevent restenosis of coronary arteries treated by percutaneous transluminal coronary angioplasty.

The success of clinical gene therapy will ultimately be determined by our ability to resolve the outstanding issues regarding safety and efficacy. Larger and more adequately controlled multi-centre trials are warranted. Stringent criteria need to be applied in the selection of patients. For example, candidates for therapeutic angiogenesis often have an impaired angiogenic response because of underlying endothelial dysfunction (Yla-Herttuala and Alitalo 2003). In addition, objective end-points for assessing efficacy need to be standardised and implemented, as well as measures to assess and overcome potential short- and long-term complications, such as oedema, hypotension, retinopathy and neovascularisation of occult neoplasms. The use of gene therapy for vasculoproliferative diseases also has to overcome efficacy issues. The complexity of the pathological process involved in restenosis suggests that genetic manipulation of multiple targets may be necessary for effective and sustained therapeutic benefit.

Strategies to accelerate endothelial recovery should also be considered, since endothelial damage plays a pivotal role in the subsequent development of restenosis and graft atherosclerosis.

7 Perspectives and Future Directions

Several molecular mechanisms underlying many of the most common cardiovascular diseases have recently been identified. This has led to the development of an array of gene-based strategies with potential therapeutic value for the treatment of these diseases. Some of these strategies have already made the transition from the pre-clinical phase into clinical trial and are now being considered for use in human patients, while several others are currently undergoing safety and feasibility evaluation in early-phase trials. Notwithstanding these significant advances, there is still need for further developments in several aspects of cardiovascular gene therapy. Progress in vector and delivery technologies have not kept pace with the identification of novel therapeutic targets. All vectors currently in use for the transfer of genetic material do not meet the criteria of the "ideal" vector. Emphasis needs to be put on the development of vectors that are amenable to endogenous regulation, and with the capability of conferring tissue specificity of transgene expression. Such a degree of spatial and temporal control over transgene expression will enhance the safety of human gene therapy protocols and potentially overcome many of the ethical issues that can arise because of non-specific transgene expression, such as germ cell line transmission. Much of this development can be carried out using current vector platforms. Rigorous systematic evaluation of the safety and efficacy of delivery strategies and improvement of delivery devices are also essential prerequisites for human gene therapy protocols.

The optimal genetic therapy for complex diseases such as CAD and MI may require a combination of cell transplantation and pro-angiogenic gene therapy for long-term sustenance of the regenerated myocardium. Such potentially synergistic combinatorial approaches have seldom been considered in the design of cardiovascular gene therapy strategies, which have traditionally been developed around a single therapeutic target. Genomic profiling and screening is being employed for molecular phenotyping of patients and will permit the detection of disease-causing polymorphisms and the design of individualised therapies. The convergence of gene transfer technology and genomic technology will facilitate the elucidation of novel genes and may help uncover new roles for previously known genes, thereby leading to the discovery of novel therapeutic targets and approaches for myocardial protection.

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