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Endothelial cell biology

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Abstract

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

Keywords

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

1.1 Introduction

In 1661, Marcello Malpighi described for the first time the existence of capillaries in the mesenterium and the lung of a frog. The anatomical research of blood vessels was greatly advanced and stimulated by contributions of the pioneers in the development of microscopy, Antonie van Leeuwenhoek (1632–1723) and Jan Swammerdam (1637–1680), who developed with Friedrich Ruysch (1638–1731) the injection techniques for coloured solutions into the vessel lumen. Friedrich Gustav Jacob Henle introduced the expression ‘epithelium’ in 1837. Between 1841 and 1859, Henle, von Koelliker and Frey showed that the capillaries have their own wall, like a structureless skin with nuclei. A forceful discussion started about the origin, development and functions of endothelial cells, lasting until around
For many years the endothelium was thought of as an inert single layer of cells that passively allowed the passage of water and small molecules across the vessel wall. In the 1920s and 1930s a new area began when Lewis and Shibuya published their first results on cultivation of endothelial cells. Between 1884 and 1950, 135 papers were published dealing with various cultivation techniques for endothelial cells (Thilo-Korner and Heinrich, 1983).

1.2 Morphology of the endothelium

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

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

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

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

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

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

Arteries and veins

A well-known anatomical and physiological distinction between vessels is that of arteries and veins (Carmeliet, 2003). Arterial vessels carry afferent circulation and are exposed to the highest pressure and flow and are characteristically surrounded by a thick medial layer consisting mostly of vascular smooth muscle cells. In contrast, venous vessels carry efferent circulation with low pressure, have less surrounding smooth muscle, and possess specialized structures, such as valves, to ensure blood flow in a single direction. Although differences in fluid dynamics within the circulation play an important role in determining the characteristic structure of an artery or vein, recent evidence suggests that the identity of endothelial cells lining these vessels is established before the onset of circulation by genetic mechanisms during embryonic development (Lawson et al., 2002; Wang et al., 1998). Several breakthrough discoveries have led to our current understanding of the molecular difference between arterial and venous endothelial
cells. In 1998, the group of Anderson showed that EphrinB2 and EphB4 were specific markers for arterial and venous endothelial cells, respectively (Sato, 2003), which showed for the first time that the arterial–venous distinction had a genetic basis. Consequently, in the cardiovascular system, EphrinB2 expression is restricted to the arteries, smooth muscle cells, pericytes and mesenchyme that surround sites of vascular remodelling. EphB4 is expressed predominantly on venous and lymphatic endothelial cells (Harvey and Oliver, 2004). The difference between arteries and veins is also guided by gridlock (grl), an artery-restricted gene that is expressed in the lateral posterior mesoderm and acts downstream of the notch signalling pathway (see below). The gridlock gene was first described by Weinstein and Fishman in 1995 (Weinstein et al., 1995; Zhong et al., 2000). In 2001, the same researchers observed that the Notch signalling pathway is regulated by the earlier described gridlock gene. In mammals, four different Notch receptors (Notch 1–4) have been cloned and characterized and these receptors bind to five ligands (Jagged 1 and 2 and Delta-like 1, 3 and 4). The Notch pathway is activated when endothelial cells adopt a venous phenotype but when this pathway is inhibited by the gridlock gene, endothelial cells assume the arterial fate. Among the potential molecules that may act upstream of the Notch pathway to induce arterial differentiation is vascular endothelial growth factor (VEGF). Most recently, three independent groups discovered that VEGF act as an inducer of the arterial fate of endothelial cells (Harvey and Oliver, 2004). In zebrafish it was discovered that the sonic Hedgehog pathway, which lies upstream of VEGF, also functions in regulating the arterial fate of endothelial cells (Sato, 2003). Since their isolation in the early 1990s, members of the Hedgehog family of intercellular signalling proteins have been recognized as key mediators of many fundamental processes of embryonic development. Several studies suggest an important role for sonic Hedgehog, in particular, during blood vessel development. Recent work has shown that sonic Hedgehog can promote angiogenic blood vessel growth in part by inducing the expression of vascular endothelial growth factor, and as well as angiopoietin-1 and -2. These observations suggest that sonic Hedgehog may cooperate with vascular-specific growth factors during the development of the embryonic vasculature.

1.3 Endothelial cell adhesion and interactions

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

Endothelial cell–cell interactions

Cell–cell-interactions are important for the regulation of tissue integrity, and the generation of barriers between different tissues and body compartments. Individual
cells are anchored together through adhesion junctions, organized in three categories: tight junctions, adherens junctions and gap junctions (Bazzoni and Dejana, 2004; Dejana, 2004). The adhesion molecules that function in these structures as well as several other molecules important in cell–cell adhesion will be discussed. The intercellular interactions, mediated by these adhesion receptors, are important in the regulation of intracellular signalling.

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

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

![Figure 1.1](image)

**Figure 1.1** Endothelial cell–cell junctions transmembrane adhesive proteins between endothelial cells are organized in three classes. Members of the tight junctions are claudins, occludin, junctional adhesion molecules (JAMs) and endothelial cell selective adhesion molecule (ESAM). The adherens junctions are represented with adhesion molecules like vascular endothelial cadherin (VE-cadherin), which, through its extracellular domain, is associated with vascular endothelial protein tyrosine phosphatase (VE-PTP). Nectin has a role in the organization of both tight junctions and adherens junctions. Outside these junctional zipper-like molecules, platelet endothelial cell adhesion molecule (PECAM) participates to endothelial cell–cell adhesion. In endothelial cells, neuronal cadherin (N-cadherin) is connecting endothelial cells to pericytes and smooth muscle cells. Gap junctions are composed of arrays of small channels that permit small molecules to shuttle from one cell to another and thus directly link the interior of adjacent cells. Adapted from a figure by Dejana (2004). (A colour reproduction of this figure can be viewed in the colour section towards the centre of the book).
apical and a basolateral side. These structures are located at the boundary between apical and basolateral domains. The main function of tight junctions is their barrier function. The adhesion molecules that form these structures have a molecular architecture that is highly complex. Zonula-occludens-1 (ZO-1) was first discovered in 1986 and is perhaps the most extensively studied tight junction molecule (Dejana, 2004). Other important tight junction proteins are occludins, claudins (Schneeberger and Lynch, 2004), junctional adhesion molecules (JAMs; Keiper et al., 2005) and endothelial cell selective adhesion molecule (ESAM; Hirata et al., 2001).

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

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

Endothelial cells have also other cell-specific homophilic adhesion proteins at the intercellular contacts. Two of the most studied are platelet endothelial cell adhesion molecule-1 (PECAM-1) and S-endo-1, both members of the immunoglobulin superfamily. The amino-terminal immunoglobulin-like domain of PECAM-1 is involved in homophilic binding on adjacent cells. Other domains of this molecule are involved in heterophilic adhesive interactions with several ligands such as αβ3, CD38 and several proteoglycans (Jackson, 2003). S-endo-1 (also termed CD146, Mel-CAM, MCAM, MUC18 or A32 antigen) is a membrane glycoprotein involved in homophilic cell–cell interactions, but its binding partner is still unknown (Bardin et al., 2001).

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

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

These interactions with the extracellular matrix occur mainly through integrins and heparan sulphate proteoglycans. Integrins are heterodimeric transmembrane proteins that consist of an α and a β subunit. There are 18 known α and eight known β subunits which form at least 24 different heterodimers in mammals. These molecules recognize ECM components and are expressed by all adhesive cells (Iivanainen et al., 2003). Integrin-mediated cellular adhesion to ECM leads to intracellular signalling and modulates endothelial cell adhesion by targeting matrix degrading enzymes to the site of sprouting. For example, integrin αvβ3, the integrin that is the best characterized for its role in angiogenesis, interacts directly with MMP-2 (Brooks et al., 1996). Another function of integrins is the regulation of the activity of a number of angiogenic and antiangiogenic factors, for example, αvβ3 directly associates with, and regulates the signalling of, vascular endothelial growth factor (VEGF) receptor 2. In addition, αvβ3 is induced in endothelial cells by angiogenic growth factors such as VEGF and bFGF. Other antiangiogenic molecules, such as endostatin, angiostatin and thrombospondin, that are natural components of the ECM, can also bind to αvβ3 and disrupt the endothelial cell–extracellular matrix interactions. Finally, it is known that many signalling pathways activated by integrins are also directly or indirectly activated by growth factors (Li et al., 2003; Stupack and Cheresh, 2004).

A second group of endothelial receptors are the cell surface heparan sulphate proteoglycans (HSPGs) (Iivanainen et al., 2003). Many matrix components have
heparin binding motifs that mediate the interaction with cell surface HSPGs. This group of cell surface adhesion molecules consist of a core protein that is covalently linked to heparin sulphate-type glycosaminoglycan side-chains. There are two main HSPG gene families that are present in the membrane of cells: the syndecans and glypicans. Syndecans are transmembrane molecules that signal through various pathways by their cytoplasmic tail. Glypicans do not have a hydrophobic transmembrane or cytoplasmic domain and are anchored to the cell surface at the extracellular site by a glycosyl-phosphatidyl-inositol (GPI) anchor. This anchor gives glypicans the potential to participate in intracellular signalling. HSPGs can also contribute to signalling by interaction with other matrix receptors that anchor directly to the cytoskeleton and serve as an integrin co-receptor. Endothelial cells express syndecan-1, syndecan-4, glypican-1 and glypican-4. Other membrane glycoproteins, which carry heparan sulphate side-chains and are present on endothelial cells, are betaglycan and CD44. Syndecan-1 and 4 are known to be induced during neovascularization during wound repair (Gallo et al., 1996).

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

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

Endothelial cells are also known to be of critical importance to the selection of white blood cells forming an inflammatory infiltrate. At the time of inflammation, due to either tissue injury or infection, several processes occur to facilitate the infiltration of leukocytes into the tissue. Among these are vasodilatation, increased blood flow and release of histamine and inflammatory cytokines. Due to these processes the endothelial cells become activated and interactions with leukocytes occur. The different steps in leukocyte sequestration into the surrounding tissue are tethering, rolling of the leukocyte along the vessel wall, firm adhesion to the endothelial cells, and transmigration though the vascular wall. All these sequential steps in the adhesion cascade are mediated through the intricately regulated expression of adhesion molecules (Figure 1.2) (Yadav et al., 2003).

A group of adhesion molecules, important in tethering and rolling of leukocytes along the venular wall, are the selectins (Bevilacqua, 1993; Elangbam et al., 1997; Gonlugur and Efeoglu, 2004; Kaila and Thomas, 2002). The term selectin was proposed to highlight the amino terminal lectin domain and the selective function and expression of these molecules. The first member of this family is E-selectin

![Figure 1.2](image)

**Figure 1.2** Leukocyte vessel wall interactions during vascular inflammation at the initial stages of inflammation endothelial cells become activated and the expression of selectin is upregulated. Cytokines at the site of inflammation activate leukocytes and the rolling of leukocytes occurs via interactions with endothelial cell P-selectin and E-selectin. Leukocyte VLA-4 and LFA-1 interact in the stage of firm adhesion with ICAM-1. The transmigration of leukocytes is mediated by endothelial cell PECAM-1. Adapted from a figure by Kakkar and Lefer (2004). (A colour reproduction of this figure can be viewed in the colour section towards the centre of the book).
which is only present on activated endothelium and inducible, over several hours, by interleukin-1, tumour necrosis factor and endotoxin (bacterial lipopolysaccharides). E-selectin participates in rolling of leukocytes along the endothelial surface. P-selectin is found in \( \alpha \)-granules of platelets and Weibel-Palade bodies of endothelial cells. It is activated by thrombin, histamine and platelet-activating factor and can be rapidly distributed to the cell surface by exocytosis. It plays a role, like E-selectin, in rolling of leukocytes during immune surveillance. The last member, L-selectin, is constitutively expressed mainly on the surface of leukocytes, such as monocytes and neutrophils. E-, P- and L-selectin bind to one or more types of carbohydrate, such as sialyl Lewis, P-selectin glycoprotein ligand-1 and CD34, sulphated polysaccharides and phosphated mono- and polysaccharides (Elangbam et al., 1997).

After a leukocyte has rolled along the venular surface, it adheres firmly to it. This process occurs through immunoglobulin-like cell adhesion molecules on the endothelial surface (Bevilacqua, 1993; Elangbam et al., 1997; Gonlugur and Efeoglu, 2004). Intercellular adhesion molecule-1 and -2 (ICAM-1 and ICAM-2) and vascular cell adhesion molecule-1 (VCAM-1) are the key players in adhesion of leukocytes. Like E-selectin, ICAM-1 can be upregulated quickly by interleukin-1 and tumour necrosis factor over a period of several hours. ICAM-1 is expressed on a variety of cell types and may contribute to adhesion in several events. ICAM-2 is mainly expressed by endothelial cells and is not dependent on cytokine regulation. Endothelial cells respond to interleukin-1 and tumour necrosis factor also by upregulating the expression of VCAM-1. These three adhesion molecules interact with neutrophils, monocytes, lymphocytes and natural killer cells through interaction with the \( \beta2 \)-family of integrins such as leukocyte function associated antigen-1 (LFA-1), membrane-activated complex-1 (Mac-1) and very late antigen-4 (VLA-4).

Mucosal vascular addressin cell adhesion molecule-1 (MAdCAM-1) is preferentially expressed on endothelial cells in intestinal mucosa, submucosa and Peyer’s patches and plays an important role in leukocyte homing to the mucosal immune compartment. MAdCAM-1 contributes to both rolling and firm adhesion by binding L-selectin and \( \alpha 4\beta7 \) integrin present on lymphocytes. MAdCAM-1 expression is upregulated on human intestinal microvascular endothelial cells by stimulation with tumour necrosis factor-\( \alpha \), interleukin 1-\( \beta \), or lipopolysaccharides.

Once captured and activated, adherent leukocytes are stimulated to de-adhere, by RhoA-mediated signalling delivered by the cytoplasmatic tail of ICAM-1, and migrate through the vessel wall (Nourshargh and Marelli-Berg, 2005; van Buul and Hordijk, 2004; Yadav et al., 2003). From there they traverse the endothelial cell layer and the basement membrane. Leukocyte endothelial cell extravasation mostly takes place through small gaps at intercellular endothelial junctions, the paracellular route, and sometimes through the endothelial cells themselves, the transcellular pathway, a mechanism which is still obscure (Engelhardt and Wolburg, 2004). In the paracellular route, leukocytes prefer to migrate through sites where the junctional complexes are less tight. Molecules like VE-cadherin, platelet-endothelial cell adhesion molecule-1, CD99, ICAM-2 and members of the
junctional adhesion molecule (JAM) family (Ebnet et al., 2004; Muller, 2003), of which the precise involvement is still unknown, guide the leukocytes through the endothelial barrier. The process of transmigration seems to prepare the leukocytes for migration in the extravascular tissue (Yadav et al., 2003).

1.4 Coagulation and haemostasis

Since endothelial cells are in direct contact with the blood, they play an important role in haemostasis and blood coagulation (Guyton, 2005). Antithrombotic, prothrombotic and fibrinolytic activities of endothelial cells are discussed here.

Prothrombotic activities of endothelial cells

The initial event in wound healing after vascular injury is the attraction, adherence and aggregation of circulating platelets. Fibrinogen and vWF are the major adhesive components that form bridges between different platelets. P-selectin, stored in the α-granules of platelets and the Weible-Palade bodies of endothelial cells, is rapidly expressed, by exocytosis, on the cell surface of platelets and endothelial cells and will contribute to adhesion of platelets to endothelial cells and leukocytes (Geng, 2003). Once aggregated in the platelet plug, platelets release stored growth factors such as VEGF, PDGF, TGF-β and such cytokines as PF4 and CD40L that induce proliferation, differentiation and migration of endothelial cells and smooth muscle cells (Anitua et al., 2004; Rhee et al., 2004). The contribution of platelets to angiogenesis in vitro is due to VEGF and not due to bFGF (Verheul et al., 2000).

Tissue factor (TF), expressed by endothelial cells, is known as the primary cellular initiator of blood coagulation (Figure 1.3). It can upregulate the balance between proangiogenic and antiangiogenic factors by playing a key role in the regulation of VEGF and thrombospondin (Zhang et al., 1994). The expression of TF can be upregulated by bFGF (Kaneko et al., 2003).

Antithrombotic activities of endothelial cells

Thrombomodulin (TM) is a high affinity thrombin receptor that is produced by endothelial cells and is the key player in initiating processes of anticoagulation (Figure 1.3). TM converts thrombin from a procoagulant protease into an anticoagulant and slows down the clotting process. The thrombin/TM complex plays a role as a cofactor in the activation of the zymogen protein C (PC) into activated protein C (APC) (Sadler, 1997). This leads to inactivation of factor Va and thrombin, and results in fibrinolysis induced by plasmin. TM is also expressed by other cells types such as keratinocytes, osteoblasts and macrophages (Boffa and Karmochkine, 1998).
The inhibitor of TF, tissue factor pathway inhibitor, exists in two forms in humans, TFPI-1 and TFPI-2. These are expressed by endothelial cells and have a major physiological inhibitor function of the coagulant pathway (Price et al., 2004).

**Fibrinolytic activity of endothelial cells**

A fibrin matrix is formed after wounding of a blood vessel and after leakage of plasma from blood vessels in areas of inflammation and tumourigenesis. The fibrin matrix acts as a barrier preventing further blood leakage and it provides a structure for new microvessels to infiltrate in damaged or activated tissue (Dvorak, 2000). Whenever a blood clot is formed, a large amount of plasminogen is trapped in the clot along with other plasma proteins (Guyton, 2005). Endothelial cells excrete certain serine proteases, plasminogen activators, which convert the zymogen plasminogen into an active protease plasmin, which in turn degrades fibrin into soluble fibrin degradation products. Endothelial cells are able to produce and control two different types of plasminogen activators, tissue-type plasminogen...
activator (t-PA) and urokinase-type plaminogen activator (u-PA). Both serine proteases are involved in the conversion of the inactive plasminogen to the active plasmin. Plasminogen activators do not only contribute to the formation of new microvessels by degradation of fibrin, but also have a crucial role in the prevention of thrombosis. To prevent fibrin clots in the lumen of a blood vessel, and subsequently ischaemia and eventually death in surrounding tissue, endothelial cells produce anticoagulant molecules. Inhibition of the fibrinolytic system may occur either at the level of the PA, by specific plasminogen activator inhibitors (PAI-1 and PAI-2), or at the level of plasmin by α2-antiplasmin. Inhibition of platelet aggregation and stimulation of fibrinolysis, however, should be area and time limited, to prevent recurrent bleeding. Molecular interactions between the fibrinolytic system and matrix metalloproteinases (MMPs) systems have been recognized at different levels, suggesting that both systems play a crucial role in extracellular matrix degradation or remodelling.

1.5 Transport

Endothelial cells play an important role in the transport of molecules across the vascular wall. Several pathways for molecular transport are described, among which are transcellular transport (through cells) via caveolae and their generated channels and paracellular transport (between cells) via intercellular (inter-endothelial cell) junctions (Simionescu et al., 2002).

Caveolae

Endothelial cell vesicles were first described by Palade in 1953 in the capillaries of the heart, after which they have been identified in a variety of cells and tissues. Endothelial cells are among the richest in caveolae. There are clear variations in their surface density between the known morphological types of endothelium; the largest population of caveolae are present in the continuous endothelium, while their numbers are much lower in the fenestrated endothelium and sporadic in discontinuous endothelium (Stan, 2002). These plasmalemmal vesicles take part in a number of functions; the bulk transport of large (and even small) solutes across the endothelial barrier by transcytosis, endocytosis, potocytosis, signal transduction and control of cholesterol trafficking. The specific marker and major component of caveolae is caveolin-1, an integral membrane protein (20–22 kDa). It is a member of a multigene family of caveolin-related proteins, which show similarities in structure but differ in properties and distribution. In addition, single caveolae from opposite fronts could fuse and form occasional transendothelial channels that allow the passage of small molecules across the cell (Simionescu et al., 1975).

It was postulated that caveolae could also fuse and form complex clusters that are open at both fronts of the endothelial cell, the so-called vesiculo-vacuolar organelles or VVOs (Feng et al., 1996; Dvorak and Feng, 2001; Feng et al., 2002).
VVOs span the entire thickness of vascular endothelium thereby providing a potential trans-endothelial connection between the vascular lumen and the extra-vascular space. They are large collections of vesicles and vacuoles focally expressed in the microvasculature that accompanies tumours, in venules associated with allergic inflammation and in the endothelia of normal venules, often in a parajunctional location. Structures of endothelial junctions vary along the vascular segments. Intercellular junctions are specialized regions of the plasma membrane that are organized when two cells come into contact. In the endothelium, junctional complexes comprise tight junctions, adherens junctions and gap junctions (Bazzoni and Dejana, 2004). Gap functions are communications structures, which allow the passage of small molecular weight solutes between neighbouring cells (Bazzoni and Dejana, 2004).

It has been postulated that vesicles, although morphologically identical, characterize at least two functionally distinct entities: endocytic vesicles and transcytotic vesicles. Endocytosis is the uptake of plasma proteins and molecules by the endothelial cell for its own use and transcytosis refers to the transport of plasma proteins to the subjacent cells and tissues.

Endocytosis in endothelial cells occurs either by a specific mechanism (receptor-mediated) or by a non-specific process (fluid phase or adsorptive). Fluid phase endocytosis is a process in which molecules ingested in bulk by caveolae are delivered to endosomes, multivesicular bodies and lysosomes where the degradation occurs. Adsorptive endocytosis is specific for molecules that bind by electrostatic forces to the cell surface and their microdomains. Receptor mediated endocytosis involves internalization by specific binding sites localized in coated pits and coated vesicles that direct the molecules to endosomes, to be further degraded in lysosomes or to reach multivesicular bodies.

The transcellular transfer of molecules between compartments is named transcytosis and is a basic process shared by epithelial cells and endothelial cells (Simionescu et al., 1975). Transcytosis can also occur in two kind of processes; non-specific (fluid phase and non-specific adsorptive transcytosis) and specific (receptor-mediated). In fluid phase transcytosis, a fraction of plasmalemmal vesicles take up plasma and then transfer through the cytoplasm to reach the abluminal front where they apparently discharge their content. Non-specific adsorptive transcytosis implies an electrostatic attachment of permeant molecule to a vesicle carrier, which translocates the probe across the endothelial cell. For this kind of transcytosis, the number and affinity of binding sites is the deciding factor. Some molecules make use of the fluid phase and adsorptive transcytosis in one process (Simionescu et al., 2002).

1.6 Angiogenesis

Angiogenesis is the process of new blood vessel formation from pre-existing vasculature. Growth of blood vessels during embryogenesis occurs mainly through
vasculogenesis and angiogenesis (Carmeliet, 2003). Vasculogenesis involves the \textit{de novo} differentiation of endothelial cells from stem cells, called angioblasts, which assemble into a primary capillary plexus. This primitive early network differentiates by angiogenesis, where new capillaries arise through sprouting, branching and intussusceptive growth from pre-existing capillaries. Arteriogenesis refers to subsequent stabilization of these sprouts by mural cells and remodelling into larger blood vessels.

In adults, angiogenesis is stimulated during many physiological processes, such as wound healing, tissue remodelling/regeneration and the female reproductive cycle. Angiogenesis is also of critical importance in a large variety of pathologies such as rheumatoid arthritis, atherosclerosis and cancer. The field of angiogenesis started to gain real attention when it was hypothesized that 1) tumours are most vulnerable at the level of their blood supply and 2) because tumours are dependent on angiogenesis, blocking angiogenesis would be a tool for therapeutic intervention (Folkman, 1971).

The process of angiogenesis is tightly regulated by positive and negative regulators. The balance between both determines the level of ongoing angiogenesis. A number of angiogenic growth factors and inhibitors are discussed below.

\section*{Pro-angiogenic factors}

The first growth factor, important in angiogenesis, was discovered in the 1980s. The discovery of basic fibroblast growth factor (bFGF), also known as FGF-2, confirmed Folkman’s theory that tumours are dependent on angiogenesis (Folkman and Shing, 1992; Kerbel, 2000). It is a pleiotropic mitogen for growth and differentiation, affecting various mammalian cells, among which are endothelial cells, and organ systems. bFGF stimulates all major steps in the angiogenesis cascade and is involved in wound healing, differentiation, proliferation, haematopoiesis, motility and apoptosis, neurothropic activity, vasculogenesis, migration, adhesion, metastasis and tumour angiogenesis. bFGF belongs to a larger family of 23 members and is one of the main stimulators in angiogenesis (Botta \textit{et al.}, 2000; Okada-Ban \textit{et al.}, 2000; Reuss and von Bohlen, 2003). bFGF signalling occurs through four high affinity tyrosine kinase transmembrane receptors (FGFR1-4). It is produced by many cells, including macrophages and tumour cells, and subsequently secreted into the extracellular matrix. At the start of angiogenesis, it can be released from the matrix. bFGF expression can be downregulated by interferon-\textgreek{a} and -\textgreek{b}. There is an intricate interaction with other growth factors, such as VEGF, that results in a synergistic action in many endothelial cell functions.

VEGF is another important player in the stimulation of angiogenesis. VEGF, or vascular permeability factor, induces vasodilatation of the existing vessels and increases permeability of the vessel wall. VEGF is also a general activator of endothelial cell proliferation and mobility (Ferrara \textit{et al.}, 2003). The main member of the VEGF family of growth factors is VEGF-A. Exon splicing of the human
VEGF-A gene results in seven isoforms that have different properties. The three major isoforms, named after their molecular size, are VEGF121, VEGF165 and VEGF189.

The VEGF family also includes VEGF-B, VEGF-C, VEGF-D and placental growth factor (PIGF). VEGF-B is an angiogenic protein related to VEGF-A, although it acts on a different set of tyrosine kinase receptors. VEGF-C and VEGF-D have been shown to act as lymphangiogenic growth factors. PIGF stimulates angiogenesis and collateral growth in ischaemic heart and limb with a comparable efficiency to VEGF (Autiero et al., 2003). VEGF signals through two main tyrosine kinase receptors, VEGFR1 or Flt-1, and VEGFR2 or KDR (McColl et al., 2004). It has been demonstrated that stimulation of angiogenesis is mainly mediated by signalling through VEGFR2. Endothelial cells also express cell surface VEGF binding sites that can mediate angiogenesis, neuropilin 1 and neuropilin 2, different from the two tyrosine kinase receptors. Like VEGF, PIGF has a role in angiogenic switch by interacting with VEGFR1 and in that way synergizes with VEGF (Autiero et al., 2003).

The angiopoietin (Ang) family includes four members that all bind to the endothelial tyrosine kinase receptor Tie-2 (Jones, 2003). The most remarkable characteristic of this family is the opposing effect of the different ligands binding to the receptor. Ang-1 and Ang-4 can activate the Tie-2 receptor while Ang-2 inhibits the Ang-1-induced Tie-2 phosphorylation. Ang-1, via phosphorylation of Tie-2, is chemotactic for endothelial cells and is involved in embryonic vascular remodelling and promotes cell survival, sprouting and tube formation and reduces inflammation. In adults, it is involved in maturation and stabilization of mature vessels. Ang-2 is involved in postnatal angiogenic and vascular remodelling events and in detaching smooth muscle cells and loosening underlying matrix, thereby allowing endothelial cells to migrate as inter-endothelial cell contacts are relaxed (Thurston, 2003). Ang-2, in concert with VEGF, is also angiogenic, although in the absence of VEGF, Ang-2 may actually induce vessel regression.

Transforming growth factor (TGF) is a multifunctional cytokine involved in proliferation, differentiation, migration and survival of many cell types (Sun, 2004). Three isoforms exist with overlapping and distinct functions. Signalling occurs via one type II receptor and two type I receptors (TGF receptors). Overexpression of TGF-β results in fibrotic conditions, since TGF-β promotes accumulation of extracellular matrix molecules. At the initial stage of tumour growth, TGF-β has a tumour suppressor function due to its inhibition of cell growth. In later stages, it enhances tumour growth by suppressing the immune system and enhancing the cell cycle thereby promoting angiogenesis.

The Eph-B receptors and their ligands are another example of pro-angiogenic molecules. Ephrins can be divided into two subclasses: EphrinA ligands (EphrinA1-A5) are tethered to the cell surface via a GPI-anchor, whereas EphrinB ligands (ephrinB1-B3) are inserted into the plasma membrane via a transmembrane region followed by a conserved cytoplasmic domain. These ligands and their receptors (Eph tyrosine kinase receptors) regulate axon guidance and bundling in
the development of the brain, control cell migration and adhesion. The Ephrin-Eph system functions in cell-to-cell rather than long range communications, because Eph receptors and all known Ephrin ligands are attached to the plasma membrane. As described earlier, the Ephrin-B2 ligand was located strictly on arterial endothelial cells, while the corresponding Eph-B4 receptor marked only venous endothelial cells (Patan, 2000).

Different interactions between VEGF, angiopoietin and Eph/ephrin have been described. The most recently identified genes that contribute to these processes are retinaldehyde dehydrogenase 2 (Raldh2), Norrin, Frizzled-4 (Fz4) and Noge-B (Harvey and Oliver, 2004). Generation of Raldh2-null mice indicate that retinaldehyde (RA) plays a crucial role in mammalian vascular development and that it is required to control endothelial cell proliferation and vascular remodelling during vasculogenesis. The Raldh-2 null mice were unable to produce active RA in the embryo, failed to remodel the primary vascular plexus and did not recruit supporting mural cells to developing vessels (Bohnsack et al., 2004; Lai et al., 2003). The Nogo isoforms A, B and C are members of the reticulon family of proteins. Nogo-A and Nogo C are highly expressed in the central nervous system, whereas Nogo B is found in most tissues. Sessa and co-workers described Nogo-B as a regulator of vascular function. Their study shows that NogoB protein is highly enriched in blood vessels, and that the genetic loss of Nogo-A/B expression markedly augments the injury response in femoral arteries (Acevedo et al., 2004). Xu and colleagues defined a Norrin and Fz4 signalling system that plays a central role in vascular development in the eye and ear (Xu et al., 2004).

**Inhibitors of angiogenesis**

Angiogenesis is also regulated by endogenous inhibitors. Without trying to be exhaustive, several of these molecules are reviewed here. Platelet factor-4 and interferon-α are among the first endogenous molecules described to display inhibitory activity on endothelial cells (Kolber et al., 1995; Gupta et al., 1995). In later years, more endogenous molecules with angiostatic activity were described. Among these were thrombospondin-1 (Rastinejad et al., 1989; Good et al., 1990; Grossfeld et al., 1997), interferon-γ inducible protein-10 (Luster et al., 1995). Other members of this class of endogenously produced anti-angiogenic proteins are angiostatin (O’Reilly et al., 1994), endostatin (O’Reilly et al., 1997), and Bactericidal Permeability Increasing protein (van der Schaft et al., 2002). It is interesting to note that many of these molecules are proteolytic fragments of collagens and other macromolecules. The scientific interest in these molecules is enormous since it is recognized that these molecules may have application in the treatment of diseases that are dependent on angiogenesis. Although receptors have been described for several angiogenesis inhibitors, detailed mechanisms of action have not yet been elucidated.
Angiogenesis in different physiological and pathological processes

The most highly investigated physiological processes in which angiogenesis is crucial are wound healing and the female reproductive cycle. The dynamic mechanism of angiogenesis, in response to tissue injury and subsequent wound healing, is regulated by serum and extracellular matrix components. The most important angiogenic cytokines in wound repair are VEGF, angiopoietins, bFGF and TFG-β (Li et al., 2003). The role of angiogenesis in the reproductive cycle is extensively reviewed in a special issue of Angiogenesis (volume 8, issue 2, 2005).

In diabetes mellitus altered regulation of angiogenesis is the cause of many pathologies (Martin et al., 2003). Vasculopathies in the retina and kidney are found, and situations like impaired wound healing, increased risk of rejection of transplanted organs and impaired formation of coronary collaterals can be observed in these patients. VEGF is the main player in diabetic retinopathy and its expression can be increased by several factors including bFGF, PIGF, TNF, TGF-β, IL1. In diabetic nephropathy with aberrant angiogenesis, abnormal local levels of VEGF were detected and angiotensin II expression was increased.

In rheumatoid arthritis and other inflammatory disease, the formation of new vessels plays an important role (Bodolay et al., 2002; Koch, 2003). The synovium of rheumatoid arthritis is known to have a highly vascularized phenotype. At the site of inflammation, leukocytes will undergo extravasation and attack the synovium of various joints. The delivery of an excess of angiogenic factors will cause synovitis. As in the previously described pathologies, the same angiogenic factors (VEGF, bFGF, TGF-β, TNF-α, IL1, angiopoetins) have been described to play an important role in this pathology.

Another research field that has attracted interest is the therapeutic neovascularization in cardiovascular ischaemic regions (Cao et al., 2005). During hypoxia or ischaemia cascade starts with the activation of the HIF-pathway that in turn will increase erythropoietin and VEGF expression which will lead, finally, to an increased blood flow and oxygen delivery.

Tumour angiogenesis

Although many pathologies are associated with changes in angiogenesis regulation (in diabetes, Martin et al., 2003; rheumatoid arthritis, Bodolay et al., 2002; Koch, 2003; and cardiovascular ischaemic conditions, Cao et al., 2005), the field of angiogenesis really developed from the oncological arena. It was realized that tumours can only grow to a volume of 1–2 mm³ without the attraction/formation of new blood vessels (Folkman, 1971; Folkman, 1995). Tumours of 1–2 mm³ subsequently switch to an angiogenic phenotype and recruit blood vessels from the surrounding stroma. Angiogenesis is therefore pivotal to tumour formation, and can serve as a prognostic factor for cancer.
Several mechanisms of neovascularization have been identified. Among these are sprouting angiogenesis which involves the proliferation and migration of endothelial cells from pre-existing blood vessels and the organization into tubular vascular structures. Intussusceptive angiogenesis (Burri et al., 2004), the formation of transvascular tissue pillars into the lumen of a pre-existing vessel and subsequent ‘splitting’ of the vessels in two new vessels, was first observed in postnatal remodelling of capillaries in lungs. New vessels can also grow through the use of circulating endothelial progenitor cells (EPCs). Extensive data now support the existence of these cells and their contribution to the formation of new blood vessels (Hristov and Weber, 2004; Ribatti, 2004). Currently, work is also focusing on an alternative tumour blood supply known as vasculogenic mimicry. Vasculogenic mimicry is the process in which tumour cells provide themselves a secondary circulation system of vasculogenic structures lined by the tumour cells themselves. This process is independent of angiogenesis. It is hypothesized that tumour cells acquire the capacity to form a tubular circulatory system by de-differentiation and acquisition of endothelial characteristics. The concept of vasculogenic mimicry was first described in melanomas (Maniotis et al., 1999). The critics on this first publication were abundant (Bissell, 1999; McDonald and Foss, 2000; McDonald et al., 2000; Shubik and Warren, 2000) because evidence that these patterned networks contributed to tumour blood flow appeared to be lacking. Nowadays several research groups are seeking the molecular mechanisms behind vasculogenic mimicry and proof of its contribution to blood flow.

Endothelial cells also contribute to the formation of lymph vessels. Lymphatic vasculature develops shortly after blood vessels during embryogenesis and might have the same origin (Al-Rawi et al., 2005; Alitalo and Carmeliet, 2002). It is hypothesized that venous endothelial cells become responsive to lymphatic signals, differentiate and make lymphatic sprouts. However, the existence of lymphangioblasts or precursors has also been proven (Schneider et al., 1999). Lymphangiogenesis is regulated by VEGF-C and VEGF-D and its receptor VEGFR3. Since the availability of antibodies specifically recognizing lymph vessels, such as antibodies to Prox-1 (Wigle et al., 2002; Wilting et al., 2002), VEGFR-3 (Makinen et al., 2001; Jeltsch et al., 1997; Saharinen and Petrova, 2004), podoplanin (Breiteneder-Geleff et al., 1999) and LYVE-1 (Banerji et al., 1999), this area of research has been accelerated. The involvement of lymphangiogenesis in tumour growth and metastasis formation (Alitalo and Carmeliet, 2002; Saharinen et al., 2004) is currently under investigation.

1.7 Isolation and culture of endothelial cells

Endothelial cells are extensively used in culture to study diverse aspects of endothelial cell biology and angiogenesis. Among the most extensively used endothelial cells of human origin are human umbilical vein endothelial cells (HUVEC) that can easily be isolated by perfusion of the umbilical vein with
trypsin and have been successfully cultured since 1973 (Jaffe et al., 1973). However, a major drawback of primary endothelial cells is their limited lifespan in culture before they enter senescence. By immortalization, a number of endothelial cell lines have been obtained that can be cultured for prolonged periods of time, such as HMEC-1 (reviewed by Bouis et al., 2001). It has been shown that endothelial cells isolated from fresh tissues retain their phenotype in culture for some time (Bussolati et al., 2003; Folkman et al., 1979; Griffioen, 1997; Kallmann et al., 2002), allowing for detailed analysis of their functional properties. However, over time their phenotype may change as a consequence of the altered microenvironment in culture (Favre et al., 2003). Therefore, depending on the research goal, it may be desirable to use endothelial cells isolated directly from the relevant tissue. For example, gene expression profiles of specific subsets of endothelial cells, such as tumour endothelial cells, were obtained from pure populations of endothelial cells (Madden et al., 2004; van Beijnum and Griffioen, 2005).

It has been recognized that there is considerable heterogeneity in endothelial cells derived from different locations in the body, which is related to the microenvironmental factors acting in each different organ and the specialized function for the endothelium therein (McCarthy et al., 1991; Zetter, 1988). Therefore, the use of capillary endothelial cells derived from the condition under study is preferred over the use of more distant endothelial cell types. Isolating capillary endothelial cells from tissues generates challenges related to the relative limited percentage of endothelial cells present in tissues and the impossibility of cannulation of the vessels, unlike larger vessels.

A number of different techniques can be employed to isolate endothelial cells from tissues for subsequent culture or analysis. In general, the isolation of capillary endothelial cells starts with the mechanical disruption of the tissue, usually followed by enzymatic digestion to detach cells from each other and the matrix. Depending on the type of tissue, enzyme concentrations and incubation times may vary (Manconi et al., 2000; Scott and Bicknell, 1993). The most commonly used enzymes are collagenase (Bussolati et al., 2003; Fawcett et al., 1991; Folkman et al., 1979), trypsin and dispase or combinations thereof (Marelli-Berg et al., 2000). Merely plating the thus created single-cell suspensions will yield many impurities that in time may overgrow the endothelial cells. Hence, purification of the endothelial cells is generally required. First, this can be performed manually by weeding the culture to remove contaminant colonies, e.g. using Pasteur pipettes or cell scrapers (Chung-Welch et al., 1988; Folkman et al., 1979). In addition, nylon mesh filters can be applied to minimize the number of contaminant cells seeded initially (Richard et al., 1998). Furthermore, gradient density centrifugation may be applied to separate microvascular segments from non-endothelial cells such as fibroblasts and epithelial cells (Grimwood et al., 1995).

Based on the expression of specific markers, endothelial cells can be separated from contaminating cell types by using immunomagnetic separation techniques. This technique uses antibodies coupled to paramagnetic particles or beads that are incubated with the heterogeneous cell samples to capture the endothelial cells. By
placing the samples in a magnetic field, the captured endothelial cells can be separated from contaminating cells. Alternatively, endothelial cells can be purified by using fluorescence activated cell sorting (FACS) following antibody or ligand binding. The most commonly used antigens are CD31 (Demeule et al., 2001; McGinn et al., 2004), factor VIII-related antigen (Patel et al., 2003), CD36 (Giordano and Mitola, 2000), CD105 (Bussolati et al., 2003), CD133 (Gussin et al., 2004), VCAM-1 (Gerritsen et al., 1995) and CD146 (St Croix et al., 2000). Also frequently used are beads coated with the lectin *Ulex europaeus* agglutinin-1 (UEA-1) (Hewett and Murray, 1993) that binds specific glycoproteins on endothelial cells.

A number of properties can be used to characterize the endothelial cells after isolation and purification. The expression of endothelial markers such as CD31, CD34, factor VIII-related antigen, angiotensin-converting enzyme (ACE), as well as binding to UEA-1 can be examined. Furthermore, the response of endothelial cells to various cytokines, such as tumour necrosis factor-α, can be assessed to validate the culture (Hewett and Murray, 1993). Another commonly used method is monitoring the ability of endothelial cells to take up a fluorescent derivative of acetylated low density lipoprotein, Dil-Ac-LDL by scavenger receptor (Fawcett et al., 1991).

Establishing a long-term culture of endothelial cells isolated from tissues remains a challenge. Cells derived from different tissues may require different culture conditions and supplementary growth factors (Relou et al., 1998; Scott and Bicknell, 1993). These endothelial cells usually require the presence of human serum for survival. In addition, an artificial matrix, such as collagen, fibronectin or gelatin, needs to be provided in the culture vessel to which the endothelial cells may adhere (Terramani et al., 2000; Relou et al., 1998; Folkman et al., 1979). To date, diverse cultures of endothelial cells have been established that can be used to study endothelial cell biology related to, for example, angiogenesis, thrombosis and haemostasis.

### 1.8 Endothelial cell heterogeneity and organ specificity

The endothelium exhibits a remarkable diversity of cellular properties that are adapted to the needs of the underlying tissue (Aird, 2003). Even within one organ, endothelial cells display heterogeneity.

At the structural level, endothelial cells at different locations in the vasculature exhibit differences in size, shape, thickness and endothelial cell junctions (Aird, 2003). Between tissues, there is also considerable difference in continuity of the endothelial lining as described above in the adhesion section. Endothelial cell heterogeneity may be explained by variations in the microenvironment (‘nurture’) as well as by genetic predisposition (‘nature’) (Aird, 2004). Interactions between endothelial cells and their precise microenvironments are thought to play an important role in determining the phenotype of the endothelial cell. Depending on the tissue or organ, different soluble mediators may be present that act on the endothelial cells and endothelial cells may be in contact with different cell types or matrix components. In addition, haemodynamic factors may also govern changes in endothelium.
Embryonic endothelial cells are very ‘plastic’ and most of the highly specialized characteristics of endothelial cells are induced during development, whereas adult endothelium is more stable, though still capable of responding to extrinsic factors.

Endothelial cells isolated from particular sites in the body that are subsequently cultured may lose some of their tissue-specific characteristics (Ribatti et al., 2002). For example, blood–brain barrier endothelial cells that specifically express P-glycoprotein lose this expression in vitro. When cultured in the presence of brain matrix or astrocytes, the expression is restored. However, matrix derived from other organs is not capable of stimulating this expression (Aird, 2003). Aortic endothelial cells, when cultured on lung extracellular matrix components, express lung-specific endothelial adhesion molecule (Zhu et al., 1991).

Though highly influenced by its surroundings, the endothelium may retain some of its original properties in vitro, in the absence of its normal microenvironment (Kallmann et al., 2002). Endothelial markers exist that show constitutive expression throughout the body, such as CD31 and von Willebrand factor.

The molecular diversity of the endothelium has been explored by using phage display technology to identify organ- and disease-specific protein expression on the surface of endothelial cells. This approach has provided clues of a vascular address system that can be used for organ-specific targeting of normal blood vessels or disease related targeting of blood vessels, such as in tumours (Pasqualini et al., 2002). Differences exist between endothelial cells present in tumours compared with endothelial cells in healthy tissue counterparts (Parker et al., 2004). Furthermore, additional heterogeneity may exist between different tumours and even within tumours (Pasqualini et al., 2002).

In vivo phage display offers an unbiased approach to identify surface markers on endothelial cells. Random peptides displayed on filamentous phage have been injected in mice and recovered from different organs and malignancies where they interacted with endothelial surface molecules. Peptides specific for brain vasculature have been identified as well as peptides that home to lung, pancreas, skin, intestine, uterus, adrenal gland, retina and prostate (Rajotte et al., 1998) and tumour vasculature (Pasqualini et al., 2002).

The identification of the molecule targeted by the peptide displayed on phage remains a challenging task, however. The famous RGD sequence targets αV integrins in tumour vessels (Pasqualini et al., 1997) and the NGR sequence was shown to target aminopeptidase N or CD13 (Pasqualini et al., 2000). Diverse preclinical studies have shown the targeting potential of these peptides. Using these peptides as targeting moieties shows promise for therapeutic applications, in particular in vascular targeting of tumours. Coupling of drugs to tumour vasculature specific peptides can increase the selective effect of the drug and reduce side effects. Even without conjugation the peptide motifs may be active in vivo. A peptide inhibitor of hyaluronan inhibited inflammation-induced leukocyte migration in mice (Mummert et al., 2000). Similarly, selective peptide inhibitors of MMP-2 and MMP-9 localized to mouse tumours and prevented tumour growth (Koivunen et al., 1999).
Overall, mapping the zip codes of the vasculature is a daunting task, but will offer great potential, both for therapeutic applications and for a more general understanding of the endothelial heterogeneity. In addition, it may shed light on preferred metastasis of tumour cells to specific organs.

1.9 Gene expression in endothelial cells

Endothelial cells in a particular microenvironment interact with various types of cells, such as fibroblasts, pericytes, immune cells, tumour cells or organ cells. In addition, endothelial cells can interact with various ECM components and are exposed to different growth factors and cytokines released by its neighbours (Jung et al., 2002). As a result, endothelial cells lining different vessels in different tissues exhibit morphological and functional specializations that are reflected in their global gene expression profiles (Ho et al., 2003; Chi et al., 2003). A gene expression comparison of cerebral endothelial cells (HCEC), part of the blood–brain barrier, with HUVEC revealed that HCEC are characterized by the expression of genes associated with neuroprotection and growth support. Marked differences in growth factor protein release between HCEC and HUVEC in culture were observed that supported the gene expression data (Kallmann et al., 2002).

Endothelial specific gene expression

It has long been recognized that specific growth factors and cytokines act on endothelial cells by interaction with specific receptors on the endothelium. There are different vascular endothelial growth factor (VEGF) isoforms as well receptor isoforms (reviewed by Ferrara, 2004), that are expressed in endothelial cells. More recently, a tissue-specific angiogenic growth factor, endocrine gland derived vascular endothelial growth factor (EG-VEGF), was identified that is selectively expressed in steroidogenic glands and promotes growth of the endocrine gland endothelium (LeCouter et al., 2001).

Large-scale gene expression surveys have shed light on transcriptional differences between diverse types of cells. In addition, public data sets are available to mine expression data generated by various methods such as SAGE and microarray analysis. Using a combination of these approaches, endothelial cell specific genes were identified (Ho et al., 2003). A total of 64 genes were identified that were differentially expressed in endothelial cells compared with non-endothelial cells with a three- to 55-fold difference in expression level, of which 44 were identified by ‘virtual subtraction’. Among these were the known endothelial markers CD31, von Willebrand factor, thrombospondin 1, KDR and VE-cadherin. In addition, other novel expressed sequence tags (ESTs) were identified to be differentially expressed in endothelial cells compared with non-endothelial cells.
By using an in silico approach, Huminiecki and colleagues (Huminiecki and Bicknell, 2000) identified four different endothelial specific transcripts. One of the sequences, magic roundabout, showed homology to ROBO1, an axonal guidance molecule, and was termed ROBO4 (Huminiecki and Bicknell, 2000). Later the protein was associated with sites of active angiogenesis, most notably tumour angiogenesis, and was shown to be induced by hypoxia (Huminiecki et al., 2002). Three additional genes were identified, endothelial cell specific molecule-1, 2 and 3 (ECSM1-3). ECSM2 has a predicted transmembrane domain and is possibly an adhesion molecule and ECSM3 has putative functions in extracellular matrix remodelling (Huminiecki and Bicknell, 2000).

Protein disulfide isomerase (PDI) is a ubiquitously expressed protein with important functions in protein folding (Freedman et al., 1994). A novel PDI-like protein was identified to be highly expressed in endothelial cells by comparison with SAGE maps, and named EndoPDI (Sullivan et al., 2003). In contrast to PDI, which is required for endothelial cell survival under resting as well as under stress conditions, EndoPDI exerts its protective role only under conditions of stress (Sullivan et al., 2003).

Gene expression in in vitro models of angiogenesis

Gene expression profiling has been applied to endothelial cells treated with different pro-angiogenic cytokines in various models to elucidate genes involved in angiogenesis. Different studies describing gene expression analysis of endothelial cells stimulated with various cytokines in vitro identified components of the MAPK signalling pathway (Jun, myc, MAP2K3) and cytokine signalling components (Flt1, IL-8, IL6ST, CCL2, TEK), as well as cell cycle mediators (MCM2, CDC20, CCND2) (Abe and Sato, 2001; Gerritsen et al., 2003b; Jih et al., 2001). However, few genes emerge that are consequently regulated under the influence of, for example, VEGF (Abe and Sato, 2001; Gerritsen et al., 2003b; Jih et al., 2001). Variations in experimental set-ups will have contributed to the observed differences. An interesting observation comes from Gerritsen and colleagues (2003b), who studied the effects of HGF and VEGF on gene expression in endothelial cell culture. Only limited overlap was observed between the gene expression changes induced by HGF or VEGF, although the combination of HGF and VEGF showed moderate overlap with that of VEGF alone, suggesting an additive effect of HGF on gene expression changes. In all treatment groups however, a clear ‘cell cycle signature’ was observed, with the upregulation of several cyclins, CDCs and mitosis regulators being dominant. In general, most studies describing gene expression analysis of endothelial cells stimulated with growth factors and cytokines show a predominant upregulation of genes involved in cell cycle, apoptosis and metabolism, suggestive of early events that mediate the activation of endothelial cells (Figure 1.4a) (van Beijnum and Griffioen, 2005).
Figure 1.4  Functional classification of annotated genes associated with (Tumour) angiogenesis
(a) Genes upregulated by growth factor stimulation of endothelial cells in vitro were classified
according to their functions \( (N = 464) \) (Abe and Sato, 2001; Gerritsen et al., 2003b; Jih et al.,
2001; van Beijnum and Griffioen, 2005; Wang et al., 2000; Zhang et al., 1999). (b) Genes
associated with capillary tube formation of endothelial cells in vitro \( (N = 385) \) (Aitkenhead et al.,
2002; Bell et al., 2001; Gerritsen et al., 2003a; Kahn et al., 2000; Wary et al., 2003). (c) Putative
tumour endothelial markers overexpressed in endothelial cells isolated from tumour tissue and
compared with non-tumour endothelial cells \( (N = 69) \) (Madden et al., 2004; Parker et al., 2004;
St Croix et al., 2000). Gene function was associated with signal transduction (A, squares),
transcription (B, light grey), cell cycle regulation or apoptosis (C, diagonal lines), cytoskeleton
(D, dotted), extracellular matrix remodelling (E, black), growth factor, hormone or cytokine action
(F, dotted grey), metabolism (G, dark grey), protein turnover, modification and transport
(H, dotted lines), cell surface molecules, antigens, receptors and adhesion molecules (I, white), or
unknown function (J, checkerboard) (van Beijnum and Griffioen, 2005).
Alternative angiogenesis models use three-dimensional matrices such as Matrigel or type I collagen for tube formation and endothelial differentiation in vitro. Integrins and adhesion molecules, e.g. NRCAM and VCAM, are commonly upregulated during tube formation of endothelial cells in vitro (Bell et al., 2001; Gerritsen et al., 2003a; Glienke et al., 2000; Aitkenhead et al., 2002). Genes involved in matrix remodelling, such as matrix metalloproteinases, are also induced during this process (Figure 1.4b) (Glienke et al., 2000; van Beijnum and Griffioen, 2005).

It is apparent that different models of angiogenesis give rise to different gene expression signatures. Depending on the applied culture conditions, specific responses are induced in the cells. However, stimuli applied in vitro might be incomplete for a perfect mimic of a microenvironment in vivo containing additional cells such as pericytes, stromal cells and epithelial cells that strongly influence the phenotype of endothelial cells and hence the gene expression profile.

**Gene expression in endothelial cells in vivo**

Although models of angiogenesis are valuable for initial analysis of endothelial cell biology and its associated gene expression, they will never reach the full potential of using endothelial cells directly isolated from the relevant sources. One of the advantages of using cell culture models that mimic an in vivo situation in vitro is the virtually unlimited supply of cells that can be obtained for these purposes. Furthermore, assay conditions can be carefully monitored and can be kept constant ensuring a certain degree of reproducibility. However, in cell culture systems, cells are no longer in their natural environment and might respond aberrantly to certain stimuli, giving a false representation of the in vivo situation. Also, the culture process itself may induce changes (Favre et al., 2003). When regarding tumour angiogenesis, endothelial cells have generally resided in a tumour microenvironment for months to years, whereas culture systems only cover a time period of days. This discrepancy between in vitro and in vivo conditions might translate in an incomplete or hampered mimic of in vivo conditions.

Relatively few studies have used freshly isolated tumour endothelial cells for gene expression analysis. Pioneering work from St Croix and co-workers (2000) resulted in the description of several tumour endothelial markers (TEMs), genes that showed overexpression in endothelial cells isolated from a fresh colon tumour tissue sample compared with endothelial cells isolated from a normal colon mucosa sample. Serial analysis of gene expression (SAGE) was applied to RNA isolated from endothelial cells that had been selected from the tissues using P1H12 antibody-coupled magnetic beads after enzymatic digestion. Interestingly, TEMs showed a strong bias towards genes functioning in extracellular matrix turnover such as collagens Iα1, Iα2, IIIα1, IVα1, VIα3 and XIIα1, and matrix metalloproteinases MMP-11 and MMP-2, stressing the importance of ECM remodelling during angiogenesis in vivo. Furthermore, many TEMs were involved in adhesion
and cytoskeletal remodelling, already shown to be involved in tube formation. Genes thought to play a role in the initiation of angiogenesis, e.g. nuclear signalling molecules, and genes involved in cell cycle regulation, metabolism and proliferation were almost completely absent (St Croix et al., 2000).

More recently, SAGE profiles have been published representing malignant brain endothelium and invasive breast carcinoma endothelium (Parker et al., 2004; Madden et al., 2004). Collagens Iα1, IVα1, IVα2 and VIα1 were shown to be overexpressed in glioma endothelial cells, as were a number of matrix metalloproteinases (Madden et al., 2004). A similar profile is apparent in breast tumour endothelial cells compared with normal breast endothelial cells (Parker et al., 2004). Combining the information in these three SAGE data sets, it is readily apparent that genes involved in extracellular matrix remodelling and cell–cell or cell–matrix contact represent the majority of upregulated genes during tumour angiogenesis in different types of tumours (Figure 1.4c). Furthermore, it has emerged that global gene expression in endothelial cells in in vitro models of angiogenesis differs considerably from that of endothelial cells in vivo (van Beijnum and Griffioen, 2005).

Gene expression in endothelial cells of different origins

Gene expression is tightly controlled during vascular development. Eph receptors and their ligands play an important role in both angiogenesis and blood vessel development (Wang et al., 1998). During vessel development, guidance of endothelial cells is tightly regulated, similar to neuronal guidance. Diverse receptor–ligand pairs direct the growth of endothelial cells. Interestingly, many molecules originally identified to play roles during neurogenesis have been associated with angiogenesis, such as the semaphorin receptors neuropilin-1 and neuropilin-2 (Yuan et al., 2002), plexin D1 (Torres-Vazquez et al., 2004), ephrinB2 and its receptor EphB4 (Wang et al., 1998). Very recently, Unc5b, a netrin receptor, has been related to vascular development and guidance of endothelial cells (Lu et al., 2004).

Differences in gene expression have been observed between arterial and venous endothelium. These differences are established even before circulation begins (Lawson and Weinstein, 2002). Venous-specific genes, identified by large-scale gene expression analysis of endothelial cells of diverse origin include EphB4, and a number of genes critically involved in left–right asymmetry such as smoothened, lefty-1, lefty-2 and growth differentiation factor (Chi et al., 2003). Interestingly, defects in left–right symmetry are frequently accompanied by vascular malformations (Meno et al., 1998). Among the arterial endothelial specific genes are the cell surface proteins Notch4, EVA1, CD44 and Ephrin-B1, metabolic enzymes such as alcohol dehydrogenase A1 and endothelial lipase, keratin 7 and the basic helix-loop-helix transcription factor Hey2 that is induced by Notch signalling. Additional evidence for the involvement of Hey2 in arterial differentiation was provided by transduction of HUVEC with a retroviral construct containing the
Hey2 gene (Chi et al., 2003). Hey2 transduction induces the upregulation of different artery-specific genes such as EVA1 and keratin 7. Hey2 induced follistatin expression may act to antagonize venous endothelial cell expressed genes such as lefty-1 and lefty-2 (Chi et al., 2003).

Clear differential expression is also evident between large vessel derived endothelium and microvascular endothelium. Genes involved in biosynthesis and remodelling of extracellular matrix, such as fibronectin, osteonectin and collagens Vα1 and Vα2 are overexpressed in large vessel endothelium. Basement membrane proteins such as collagens IVα1 and IVα2, and its interaction partners such as integrins α4, α1, α9 and β4 are overexpressed in microvascular endothelial cells. In addition, large vessel endothelial cells express genes associated with neuronal guidance such as those discussed above. In contrast microvascular endothelial cells express receptors for paracrine signals of neuroglial cells such as IL-1 receptor, IL-6 receptor, PDGFR and endothelin receptor 1. Furthermore, microvascular endothelial cells express secreted factors that promote survival and differentiation of neuroglial cells (Chi et al., 2003).

Tissue-specific endothelial gene expression is likely to reflect the specialization of the particular vascular bed. Peptide phage display has previously identified a number of peptides specifically reacting with proteins on the endothelial surface in defined locations in the body (Pasqualini et al., 2002). Additional tissue-specific genes were identified by Chi et al, who showed SIX3 to be specific for nasal endothelium. Osteoglycin, sFRP and PLA2G12 were specific for lung endothelium, whereas galanin and the calcitonin receptor emerged as uterus specific. Skin endothelium expressed many genes involved in cholesterol biosynthesis, such as stearoyl-CoA desaturase (SCD), fatty acid desaturase (FADS2) and 3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMGCS1), pointing towards active roles played by the endothelium in the organ physiology (Chi et al., 2003).

1.10 Conclusions

In conclusion, endothelial cells from different blood vessels and originating in different tissues have diverse expression profiles that relate to tissue physiology and may actively be involved in disease-related processes. However, data generated using cultured endothelial cells may be biased due to phenotypic drift of endothelial cells in culture. In this regard, gene expression data generated directly from isolated endothelial cells or by using in vivo approaches may prove superior.

References

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