Chapter 1
Vascular Endothelium in Health and Disease

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Key Learning Points

- All vascular endothelial cells have a common embryonic origin but show clear bed-specific heterogeneity in morphology, function, gene and protein expression, determined by both environmental stimuli and epigenetic features acquired during development.
- Endothelial heterogeneity is also demonstrated by regional differences in the release of vasoactive and inflammatory mediators in response to stimuli such as changes in shear stress and hypoxia, and in expression of pro- and anti-coagulant molecules.
- The first identified endothelium-derived relaxing factor (nitric oxide; NO) is a short-lived free radical synthesised from L-arginine by endothelial NO synthase (eNOS) and destroyed by reactive oxygen species (ROS).
- Carbon monoxide (CO) and hydrogen sulphide (H_2S) are two other gaseous mediators contributing to endothelium-dependent modulation of vascular tone.
- Endothelium-dependent hyperpolarisation (EDH) is an important regulator of blood flow and blood pressure and plays a predominant role in smaller vessels.
- Arachidonic acid is released from endothelial cell membrane phospholipids and metabolised into a number of vasoactive factors by cyclooxygenase (COX), lipoxygenase and cytochrome P450 monooxygenase enzymes.
- Endothelin-1 (ET-1) is a potent vasoconstrictor which is released from the vascular endothelium. NO strongly inhibits the release of ET-1 and ET-1 attenuates

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NO-mediated dilation. Therefore, ET-1 and NO are functionally interdependent and many of the cardiovascular complications associated with endothelial dysfunction may be due to an imbalance in this relationship.

- Angiogenesis is the growth of new blood vessels formed by endothelial cell tubes sprouting from existing vessels. This process involves the following sequence of events:
  - Activation of endothelial cells
  - Degradation of extracellular matrix by matrix metalloproteinases
  - Proliferation and directional migration of endothelial cells
  - Formation of endothelial tubes
  - Maturation of new vessels by recruitment of pericytes and smooth muscle cells to stabilise endothelial sprouts and secrete extracellular matrix molecules to form the vascular basement membrane
- Angiogenesis in response to ischaemia is largely controlled by hypoxia-inducible factor-1 (HIF-1). Recruitment and proliferation of bone marrow-derived endothelial progenitor cells to form new vessels (vasculogenesis) is a separate but complimentary process which occurs simultaneously in ischaemic and wound tissue to augment perfusion.
- The endothelium is one of the few surfaces that can maintain blood in a liquid state during prolonged contact. The endothelium also prevents thrombin formation by expressing tissue factor pathway inhibitor that binds to clotting factor Xa and thrombomoduli, which in turn bind to and inactivate thrombin, thus blocking its pro-coagulant activity. Several other haemostatic factors are also expressed by the endothelium, in particular von Willebrand factor (vWF) and plasminogen activator inhibitor-1 (PAI-1).
- Vascular endothelium has potent anti-platelet aggregation properties which are mediated by the synthesis of prostacyclin (PGI_2) and NO.
- The endothelial response to inflammation and infection involves the production of inflammatory cytokines (such as interleukin-8) and the expression of surface adhesion molecules (selectins), which facilitate leukocyte adhesion and migration to the site of inflammation/infection.

1.1 Introduction

The endothelium, first described over 100 years ago as an inert anatomical barrier between the blood and cells of the vessel wall, is now recognized as a dynamic organ with secretory, synthetic, metabolic, and immunologic functions. Endothelial cells play an obligatory role in modulating vascular tone and permeability, angiogenesis, and in mediating haemostatic, inflammatory and reparative responses to local injury. To fulfil these roles, the endothelium is continuously responding to spatial and temporal changes in mechanical and biochemical stimuli. Such responsiveness is affected through receptors for growth factors, lipoproteins, platelet

products, and circulating hormones, which regulate changes in RNA and protein expression, cell proliferation, and migration or the release of vasoactive and inflammatory mediators [1].

All vascular endothelial cells have a common embryonic origin but show clear bed-specific heterogeneity in morphology, function, and gene and protein expression, determined by both environmental stimuli and epigenetic features acquired during development. Thus, the endothelium should not be regarded as an homogenous tissue but rather a conglomerate of distinct populations of cells sharing many common functions but also adapted to meet regional demands [2]. In most blood vessels, continuous endothelium provides an uninterrupted barrier between the blood and tissues and ensures tight control of permeability at the blood-brain barrier. In regions of increased trans-endothelial transport such as capillaries of endocrine glands and the kidney, the presence of fenestrae, transcellular pores approximately 70 nm in diameter with a thin fenestral diaphragm across their opening, facilitate the selective permeability required for efficient absorption, secretion, and filtering. In hepatic sinuses, the presence of a discontinuous endothelium with large fenestrations (0.1–1 mm in diameter) lacking a fenestral diaphragm, provides a highly permeable and poorly selective sieve essential for transfer of lipoproteins from blood to hepatocytes [1, 3].

Beyond these structural variations, endothelial heterogeneity is also manifest in regional differences in the release of vasoactive and inflammatory mediators, in responsiveness to stimuli such as changes in shear stress and hypoxia, and in expression of pro- and anti-coagulant molecules. For example, the contribution of nitric oxide (NO) to endothelium-dependent vasodilation appears to be greater in large conduit arteries compared to small resistance vessels [4]. Expression of von Willebrand factor (vWF), a circulating glycoprotein that mediates platelet adhesion to the subendothelial surface of injured blood vessels, displays a mosaic pattern in the aorta and in selected capillary beds [5]. These regional differences between endothelial cells extend to their susceptibility to injury in the face of cardiovascular risk factors such as hypercholesterolemia, diabetes and smoking, and thus impact the function of the vasculature both in health and disease.

This chapter provides an overview of how the endothelium regulates four key aspects of cardiovascular homeostasis; vascular tone, angiogenesis, haemostasis and inflammation.

1.2 Endothelium-Dependent Regulation of Vascular Tone

Since the first report of endothelium-dependent modulation of the contractile state of smooth muscle cells in the artery wall [6], it has become apparent that endothelial cells release a plethora of vasoactive factors in response to a wide range of mechanical and chemical stimuli. Many of these factors also modulate processes such as inflammation, cell adhesion, and coagulation, highlighting the crucial physiological role of the endothelium, and why endothelial dysfunction is pivotal in the

development of cardiovascular diseases such as atherosclerosis and hypertension. Here we will focus on the four major pathways underlying endothelium-dependent modulation of vascular tone; the gaseous mediators NO, carbon monoxide (CO) and hydrogen sulfide (H_2S), endothelium-dependent hyperpolarisation (EDH), metabolites of arachidonic acid, and endothelin.

1.2.1 Gaseous Mediators

The first endothelium-derived relaxing factor described by Furchgott and Zawadski was subsequently identified as NO, a short-lived free radical synthesized from L-arginine by endothelial NO synthase (eNOS) and destroyed by reactive oxygen species (ROS). Once released from the endothelium, NO activates the haem-dependent enzyme soluble guanylyl cyclase in surrounding smooth muscle cells, leading to formation of cyclic guanosine monophosphate (cGMP). Subsequent activation of cGMP-dependent kinase leads to phosphorylation of a diverse range of target proteins such as large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels, Rho kinase, myosin light chain phosphatase and phospholamban, that mediate smooth muscle cell relaxation and hence vasodilation [7]. This signalling pathway is terminated by phosphodiesterase enzymes that degrade cGMP. These enzymes are inhibited by the drug sildenafil used for treatment of erectile dysfunction [8]. NO can also act in a cGMP-independent manner (e.g. nitrosylation of proteins), which will not be discussed here but has been reviewed by Lima et al. [9].

eNOS is a bidomain enzyme; an N-terminal oxygenase domain with binding sites for haem, tetrahydrobiopterin, oxygen and the substrate L-arginine which supports catalytic activity, and also a C-terminal reductase domain which binds the co-factors nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide and flavin adenine dinucleotide. Transfer of electrons from NADPH to flavins in the reductase domain and then to the haem in the oxygenase domain is required so that the haem iron can bind oxygen and catalyze the synthesis of NO from L-arginine. Binding of the ubiquitous Ca^{2+} regulatory protein calmodulin (CAM) facilitates transfer of electrons from the reductase to the oxygenase domain and is critical for activation of the enzyme [10].

eNOS is constitutively expressed in all endothelial cells but regulation of enzyme activity by physiological and pathophysiological stimuli occurs via a complex pattern of transcriptional and post-translational modifications. Both eNOS mRNA and protein levels are increased by fluid shear stress via activation of a pathway involving c-Src-tyrosine kinase and transcription factor nuclear factor κ-light-chain-enhancer of activated B cells (NFκB). At the post-translational level, eNOS activity is highly regulated by substrate and cofactor availability as well as by endogenous inhibitors, lipid modification, direct protein-protein interactions, phosphorylation, O-linked glycosylation, and S-nitrosylation.

Agonists at endothelial G-protein coupled receptors (GPCRs) such as bradykinin and acetylcholine elicit Ca^{2+}-CAM-dependent NO production via phospholipase

C-mediated generation of inositol 1,4,5-trisphosphate ($InsP_3$) and subsequent release of Ca^{2+} from intracellular stores. Activation of tyrosine kinase linked receptors such as the vascular endothelial growth factor (VEGF) receptor, and mechanical stimulation of the endothelium by shear stress, lead to phosphorylation of eNOS at Ser1177 to increase the Ca^{2+} sensitivity of the enzyme so that it can be activated at resting Ca^{2+} levels. Distinct kinase pathways can mediate eNOS phosphorylation; shear stress elicits phosphorylation of Ser1177 via protein kinase A, whereas insulin and VEGF cause phosphorylation of the same residue via the serine/threonine protein kinase Akt. Conversely, phosphorylation of the enzyme at Tyr657 within the flavin mononucleotide domain, or Thr495 within the CAM-binding domain, inhibits enzyme activity [11].

Within endothelial cells, eNOS is targeted to invaginations of the cell membrane called caveolae, membrane microdomains enriched in cholesterol and sphingolipids, and defined by the presence of the scaffolding protein caveolin-1. Caveolae sequester diverse receptors and signalling proteins including GPCRs, growth factor receptors, and Ca^{2+} regulatory proteins such as CAM. Thus, targeting of eNOS to this region facilitates communication with upstream and downstream pathways. Within caveolae, caveolin-1 tonically inhibits eNOS activity, thereby limiting the production of NO. The binding of Ca^{2+}-CAM leads to disruption of the caveolin-1/eNOS interaction and increases eNOS activity [12]. Other associated proteins such as platelet endothelial cell adhesion molecule-1 (PECAM-1) modulate eNOS activity by virtue of their function as scaffolds for the binding of signalling molecules such as tyrosine kinases and phosphatases [13].

The release of NO by stimuli such as shear stress, circulating hormones (e.g. catecholamines, vasopressin), plasma constituents (e.g. thrombin), platelet products (e.g. serotonin), and locally-produced chemical mediators (e.g. bradykinin) plays a critical role in mediating acute changes in local blood flow and tissue perfusion. Shear stress-stimulated NO production is central to exercise-induced increases in blood flow in skeletal muscle [14]. Production of NO in response to serotonin released from aggregating platelets, dilates coronary arteries to prevent clots from occluding vessels [15]. Mice lacking eNOS are hypertensive and infusion of L-arginine analogues, competitive inhibitors of eNOS, cause alterations in local blood flow and in systemic blood pressure, demonstrating the importance of endothelium-derived NO in long-term cardiovascular control in vivo [11]. In humans, elevated levels of an endogenous inhibitor of eNOS, asymmetric dimethylarginine, are associated with hypertension and increased cardiovascular risk [16].

In addition to its vasodilator actions, NO is now recognized as playing other protective roles in the vasculature as a regulator of inflammation and vessel repair. Loss of NO-mediated vasodilation, due to reduced expression or activity of eNOS and/or oxidative stress-mediated reductions in NO bioavailability, is a hallmark of endothelial dysfunction associated with cardiovascular risk factors such as hypercholesterolemia, smoking, diabetes and obesity. Loss of endothelium-derived NO tips the homeostatic balance in favour of vasoconstriction, proliferation, activation of platelets and blood clot formation, and inflammation.

Therefore loss of NO contributes to clinical manifestations such as high blood pressure, atherosclerosis, and thrombosis, which are associated with significant morbidity and mortality [17].

Although they have received much less attention than NO, two other gaseous mediators, CO and H_2S, also contribute to endothelium-dependent modulation of vascular tone. CO is generated within endothelial cells during catabolism of heme by the enzyme heme oxygenase (HO). Of the two known isoforms, HO1 is an inducible form associated with increased oxidative stress while HO2 is constitutively expressed in endothelial cells and, like eNOS, activated by Ca^{2+}-CAM. The strongest evidence for a physiological role of endothelium-derived CO in regulation of vascular tone has come from studies of the cerebral circulation in which GPCR agonists and hypoxia cause Ca^{2+}-CAM- and HO-dependent vasodilation. CO-mediated smooth muscle relaxation is due to activation of soluble guanylyl cyclase which increases cGMP levels, leading to cGMP-dependent kinase-mediated activation of BK_{Ca} channels. In addition, CO can bind directly to the heme moiety bound to BK_{Ca} channels to directly elevate Ca^{2+} sensitivity of the channels [18]. To date, little is known about the mechanisms regulating CO production or alterations in HO/CO signalling in disease states.

Release of endothelium-derived H_2S, synthesized from cysteine by cystathionine γ-lase (CSE), is stimulated by many factors including acetylcholine, increases in shear stress, oestrogens, and plant flavonoids. Evidence from mice lacking CSE suggests an important role for H_2S in the physiological maintenance of blood pressure [19]. Like eNOS, CSE activity is regulated by a complex integration of transcriptional, post-transcriptional, and post-translational mechanisms. The rapid metabolism of H_2S via an oxygen-dependent pathway within mitochondria suggests that it may play a greater role in hypoxic rather than normoxic tissues [20].

Several signalling mechanisms have been described for H_2S such as reaction with heme-containing proteins, protein S-sulfhydration, reaction with ROS, and reduction of protein disulfide bonds to thiols [21]. The vasodilator action of H_2S has largely been attributed to hyperpolarization of the smooth muscle membrane potential mediated by opening adenosine triphosphate-sensitive K^+ (K_{ATP}) channels; H_2S sulfhydrates K_{ATP} channels at Cys43 to facilitate the binding of phosphatidylinositol(4,5)bisphosphate, a physiological activator of these channels [22]. However, the sensitivity of H_2S-evoked vasodilation to blockers of small (SK_{Ca}) and intermediate (IK_{Ca}) conductance Ca^{2+}-activated K^+ channels has also raised the possibility that H_2S may evoke relaxation via EDH [23].

Interactions between NO and H_2S signalling pathways can occur at a number of points, making for a complex relationship between the two mediators; NO inhibits CSE activity via S-nitrosation but can increase CSE expression and cellular uptake of its substrate cystine, and conversely, H_2S potentiates cGMP accumulation via the inhibition of phosphodiesterase. Alterations in H_2S production and/or signalling have been observed in animal models of endothelial dysfunction associated with pathological conditions such as diabetes and obesity, leading to H_2S donors being considered as potential therapeutic agents for the treatment of these diseases [24].

1.2.2 *Endothelium-Dependent Hyperpolarisation*

Observations of agonist-induced endothelium-dependent vasorelaxation which persisted in the presence of inhibitors of COX and eNOS and was accompanied by hyperpolarisation of the vascular smooth muscle cell membrane potential led to identification of a third endothelium-derived relaxing factor, endothelium-derived hyperpolarising factor (EDHF). Hyperpolarisation of the smooth muscle cells reduces the probability of opening the voltage-dependent Ca^{2+} channels, thus reducing Ca^{2+} influx to cause relaxation. A range of agents have been proposed to account for the actions of EDHF including K^+, epoxyeicosatrienoic acids (EETs) and C-type natriuretic peptide. However, it is now widely accepted that, rather than a diffusible factor, endothelium-dependent hyperpolarisation (EDH) of vascular smooth muscle is mediated by direct electrical coupling between endothelial and smooth muscle cells via myoendothelial gap junctions [25].

The initiating step in EDH-mediated vasorelaxation is activation of endothelial SK_{Ca} and IK_{Ca} channels. These channels, activated by increases in intracellular Ca^{2+} via CAM which is constitutively associated with the channels, are voltage-independent and thus can operate at negative membrane potentials close to the K^+ equilibrium potential. Inhibition of endothelium-dependent relaxation by a combination of SK_{Ca}/IK_{Ca} channel blockers is now regarded as the hallmark of EDH-mediated vasodilation, but the relative contribution of the two channels varies between different stimuli. This ability of endothelial cells to generate stimulus-specific responses to diverse inputs is facilitated by organization of SK_{Ca} and IK_{Ca} channels into spatially distinct microdomains that allow for differential activation by localized increases in Ca^{2+}. SK_{Ca} channels are located within caveolae at inter-endothelial junctions on the luminal surface where together with eNOS, they can respond to local Ca^{2+} increases elicited by shear stress-induced activation of transient receptor potential vanilloid type 4 (TRPV4) channels. In contrast, IK_{Ca} channels are located on the abluminal surface close to myoendothelial contact points where they are activated by localized, $InsP_3$-mediated increases in Ca^{2+} evoked by GPCR agonists (Fig. 1.1) [26].

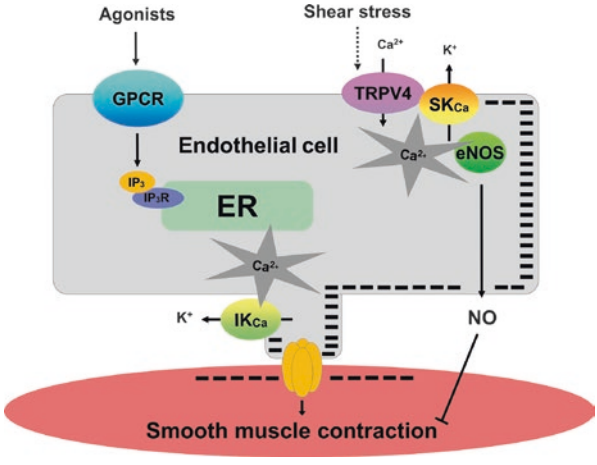

Fig. 1.1 Schematic showing differential localization and activation of endothelial SK_{Ca} and IK_{Ca} channels. SK_{Ca} channels are located on the luminal surface and respond to local Ca^{2+} increases elicited by shear stress-induced activation of TRPV4 channels. IK_{Ca} channels are located on the abluminal surface where they are activated by localized, $InsP_3$-mediated increases in Ca^{2+} evoked by GPCR agonists

The importance of EDH as a regulator of blood flow and blood pressure in vivo is demonstrated by enhanced resistance artery tone and elevated systemic blood pressure seen in mice lacking endothelial SK_{Ca} and/or IK_{Ca} channels. Loss of EDH, due to changes in expression or activity of SK_{Ca}/IK_{Ca} channels, contributes to experimental hypertension and diabetes-related erectile dysfunction. In contrast, resistance of the EDH pathway to the deleterious actions of ROS may allow EDH-mediated vasodilation to be maintained in the face of reduced bioavailability of NO in atherosclerosis and heart failure. Thus, selective activation of endothelial SK_{Ca} and IK_{Ca} channels is a potential therapeutic avenue for the future [27].

1.2.3 Metabolites of Arachidonic Acid

Within endothelial cells, arachidonic acid, released from cell membrane phospholipids by phospholipases, is metabolized by COX, lipoxygenase (LO), and cytochrome P450 monooxygenase (CYP) enzymes to yield an array of vasoactive factors.

COX enzymes metabolise arachidonic acid to endoperoxide intermediates which are then converted to a range of eicosanoids (e.g. prostacyclin (PGI_2), thromboxane A2 (TXA2)) through the actions of various synthases. Two isoforms of COX are found in the endothelium. The constitutively expressed COX-1 has long been regarded as vasculoprotective, the predominant product being PGI_2 which acts on prostanoid (IP) receptors to cause vasodilation and inhibition of platelet aggregation via activation of adenylyl cyclase to increase levels of cyclic-adenosine monophosphate (cAMP). PGI_2 also inhibits platelet and lymphocyte adhesion to endothelium, limits vascular smooth muscle cell proliferation and migration, and counteracts the production of pro-inflammatory growth factors [17]. However, evidence is now emerging that GPCR-mediated activation of endothelial COX-1 can generate other products such as TXA2 which activates thromboxane (TP) receptors on smooth muscle cells and so functions as an endothelium-derived contracting factor (EDCF). Stimulation of TP receptors elicits not only vasoconstriction but also proliferation of vascular smooth muscle cells, platelet adhesion and aggregation, and expression of adhesion molecules on endothelial cells. A shift from production of endothelium-derived relaxing factors to COX-dependent EDCFs is implicated in endothelial dysfunction associated with ageing, diabetes, and hypertension in both animal models and humans. Since activation of TP receptors is the common downstream effector, selective antagonists of this receptor may have therapeutic potential in the treatment of cardiovascular diseases [28].

COX-2 was first identified as an inducible form of the enzyme regulated at the level of gene expression and associated with inflammation. However, it is expressed in some blood vessels in the absence of overt signs of inflammation, and may be a major source of vasculoprotective PGI_2; hence the deleterious cardiovascular consequences seen in some patients treated with selective COX-2 inhibitors [29].

LO enzymes deoxygenate polyunsaturated fatty acids to hydroperoxyl metabolites. The three LO isoforms expressed in endothelial cells are 5-LO, 12-LO, and 15-LO, which correspond to the carbon position of arachidonic acid oxygenation. Each LO oxygenates arachidonic acid to form a stereospecific hydroperoxyeicosatetraenoic

acid (HPETE) which are unstable and rapidly reduced to the corresponding hydroxyeicosatetraenoic acid (HETE). 5-LO is the initial enzyme in the synthesis of pro-inflammatory leukotrienes but 5-LO products do not seem to be involved in regulation of vascular tone. In contrast, products from the 12-LO and 15-LO pathways are vasoactive but show species and vessel variation in the responses they elicit. 12-HETE causes relaxation of a number of peripheral arteries including human coronary arteries but causes vasoconstriction in dog renal arteries. In the same vessels, 15-HPETE and 15-HETE cause slight vasorelaxation at lower concentrations but contractions at higher concentrations mediated by activation of TP receptors.

Cytochrome P450 (CYP) enzymes add oxygen across the double bonds of arachidonic acid to produce four cisepoxides, 14,15-, 11,12-, 8,9-, and 5,6-epoxyeicosatrienoic acids (EETs). Two CYP enzymes have been cloned from human endothelium, CYP2C8/9 and CYP2J2, both of which produce mainly 14,15-EET with lesser amounts of 11,12-EET. The latter are also the major EETs released from endothelial cells in response to GPCR agonists (e.g. acetylcholine, bradykinin) and physical stimuli such as cyclic stretch and shear stress. EETs are rapidly metabolized by esterification into phospholipids or hydration to dihydroxyeicosatrienoic acids by soluble epoxide hydrolase. EETs can cause vasodilation via a number of different pathways. They can stimulate endothelial TRPV4 channels, which are non-selective cation channels that mediate Ca^{2+} influx, to activate eNOS or IK_{Ca}/SK_{Ca} channels to cause EDH. In contrast, endothelium-dependent flow-induced dilation is linked to release of 5,6-EET to activate smooth muscle TRPV4 channels which form a complex with BK_{Ca} channels, thus coupling local increases in Ca^{2+} to membrane hyperpolarisation and vasorelaxation [30]. Development of 14,15-EET analogues such as 14,15-epoxyeicosa-5Z-enoic acid revealed strict structural and stereoisomeric requirements for relaxations suggesting a specific binding site or receptor mediating EETs actions. G protein coupled receptor 40 (GPR40), a member of a family of GPCRs that have fatty acids as ligands, was recently detected in endothelial and smooth muscle cells, and suggested to be a low-affinity EET receptor. 11,12-EET stimulation of GPR40 increased expression of COX-2 and connexin 43, a key component of myoendothelial gap junctions, but a role for this receptor in EET-mediated changes in vascular tone remains to be established [31].

In some models of endothelial dysfunction reduced bioavailability of NO is counteracted by increased production of EETs which can maintain endothelium-dependent vasodilator responses. Thus, strategies aimed at enhancing production of endothelium-derived EETs or inhibiting their degradation, may represent a new therapeutic approach to endothelial dysfunction [32].

1.2.4 Endothelins

Endothelins are a family of 21 amino acid peptides of which there are three members (ET-1, ET-2, ET-3) with a high level of homology and similar structure [33]. Endothelial cells produce only ET-1; endothelin ET-2 is produced in the kidney and

intestine, while ET-3 has been detected in the brain, gastrointestinal tract, lung and kidney. ET-1 is a potent vasoconstrictor inducing long-lasting vasoconstriction at a half maximum effective concentration in the nano-molar range, at least one order of magnitude lower than values reported for other vasoconstrictor peptides such as angiotensin II [34].

ET-1 is not stored by endothelial cells. Production is regulated at the level of gene expression with the rate of transcription being responsive to stimulants and inhibitors to allow rapid changes in the amounts released. Pro-inflammatory factors such as transforming growth factor-β and tumour necrosis factor–α (TNFα), insulin, and angiotensin II up-regulate ET-1 mRNA whereas NO, PGI_2 and shear stress cause down-regulation. ET-1 is synthesized as a larger protein, the pre-proET-1 (203 amino acids) that is cleaved to pro-ET-1 (38 amino acids) and then to ET-1 (21 amino acids) by endothelin-converting enzymes. The half-life of ET-1 protein and mRNA is 4–7 min and 15–20 min, respectively, and most plasma ET-1 (90%) is cleared by the lungs during first passage.

The biological effects of ET-1 are mediated by two GPCR subtypes, ETA and ETB which have opposing effects on vascular tone. ETA receptors on vascular smooth muscle cells are responsible for the majority of ET-1 induced vasoconstriction; activation of phospholipase C increases formation of $InsP_3$ and diacylglycerol, and the resultant increase in intracellular Ca^{2+} and activation of protein kinase C cause vasoconstriction. ETB receptors are mainly present on endothelial cells and play an important role in clearing ET-1 from the plasma by internalising the receptor complex once ET-1 has bound. Activation of endothelial ETB receptors induces vasodilatation by stimulating the release of PGI_2 and NO. Inhibition of ETB increases circulating ET-1 levels and blood pressure in healthy subjects demonstrating that although ET-1 is regarded as primarily a vasoconstrictor, ETB-mediated vasodilation is also physiologically important [34].

ET-1 is not only a vasoactive factor. Acting via ETB receptors, ET-1 modulates the formation and degradation of extracellular matrix (ECM) and thus plays a role in vascular remodelling. Acting via ETA, ET-1 promotes smooth muscle proliferation contributing to neointima formation following vascular injury and to thickening of the arterial wall in pathological conditions such as pulmonary arterial hypertension, atherosclerosis and venous graft occlusion. As NO strongly inhibits the release of ET-1 from the endothelium, and ET-1 attenuates NO-mediated dilation, ET-1 and NO are functionally interdependent and many of the cardiovascular complications associated with endothelial dysfunction may be due to an imbalance in this relationship [35].

1.3 Angiogenesis

Angiogenesis is the growth of new blood vessels formed by endothelial cells sprouting from existing vessels. In adults it is a protective mechanism initiated in response to tissue hypoxia, ischemia or injury. It is also a key process in pathological

conditions such as proliferative diabetic retinopathy and neovascularization of tumours and as such, inhibitors of angiogenesis have received considerable interest as a potential therapeutic strategy. The angiogenic process depends on a complex transcriptional network coordinating production and release of numerous cytokines and growth factors [36].

Angiogenesis requires a sequence of individual processes:

1. Activation of endothelial cells,
2. Degradation of ECM by metalloproteinase enzymes,
3. Proliferation and directional migration of endothelial cells,
4. Formation of endothelial tubes,
5. Maturation of new vessels by recruitment of pericytes and smooth muscle cells to stabilize endothelial sprouts and secrete ECM molecules to form the vascular basement membrane (Fig. 1.2).

The endothelial cells that sprout from the parent vessel (tip cells) possess long and motile filopodia that extend towards the source of pro-angiogenic growth factors and respond to other guidance cues to enable directional vessel growth [37].

Endothelial cell migration requires the dynamic regulation of interactions between integrins and the surrounding ECM. Integrins are cell surface receptors which provide adhesive and signalling functions and link the actin cytoskeleton of the cell to the ECM at areas called focal adhesions. Phosphorylation of focal adhesion kinase, a cytoplasmic non-receptor tyrosine kinase, in response to pro-angiogenic signal molecules stimulates cell contraction thus allowing cell movement

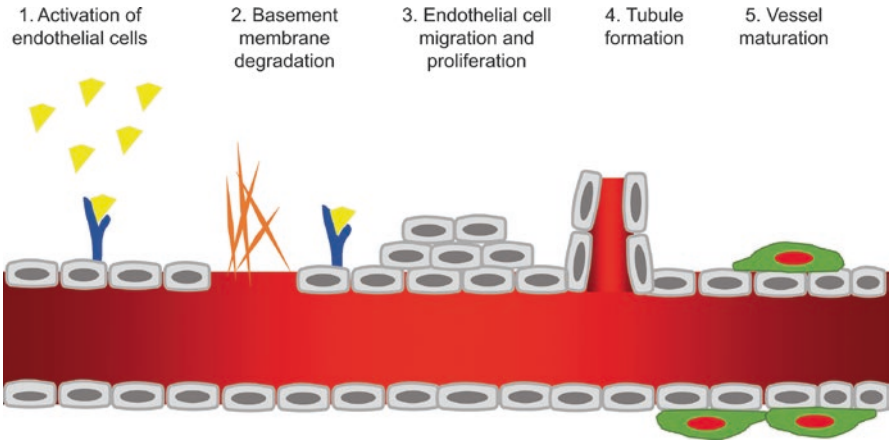

Fig. 1.2 Schematic of process of angiogenesis. Angiogenesis involves the following complex sequence of events: (1) Activation of endothelial cells. (2) Degradation of extracellular matrix by matrix metalloproteinases. (3) Proliferation and directional migration of endothelial cells. (4) Formation of endothelial tubes. (5) Maturation of new vessels by recruitment of pericytes and smooth muscle cells to stabilise endothelial sprouts and secrete extracellular matrix molecules to form the vascular basement membrane

on adhesive contacts. Subsequent integrin inactivation destroys the adhesive complex and allows detachment of the cell in its new location [38].

Cell-cell contacts between endothelial cells, essential for development of patent vessels, are mediated by cell surface receptors such as PECAM-1, a 130 kDa member of the immunoglobulin superfamily, which acts like a docking molecule to allow other proteins to provide further strength to vascular structures. Cadherins such as vascular endothelial cadherin are transmembrane proteins which provide weak adhesive cell-cell forces, further stabilized by catenins, intracellular proteins linking the cadherin cell surface molecule to the actin cytoskeleton.

Angiogenesis in response to hypoxia and ischaemia is largely controlled by the transcription factor hypoxia-inducible factor-1 [39]. HIF-1 has multiple subunits; HIF-1α which is produced continuously but rapidly degraded in the presence of oxygen, and HIF-1β which is constitutively expressed. Under hypoxic conditions, HIF-1α degradation is inhibited, and the stabilized protein translocates to the nucleus, where it dimerizes with HIF-1β. The dimer binds to hypoxia response elements on more than 60 HIF–responsive genes that function to enhance oxygen delivery and increase metabolism. Central angiogenic signals driven by increased HIF-1 activity include VEGF, fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and angiopoietins [40].

FGF, VEGF and PDGF stimulate endothelial cell proliferation and migration. Their high affinity for heparan sulfate glycosaminoglycans on the endothelial cell surface facilitates binding to receptors and provides a reservoir of these factors in the ECM, which can be released during wounding or inflammation. FGF binds to the receptor tyrosine kinase FGFR-1 to increase endothelial migration and promote capillary formation. FGF also enhances PDGF expression via a VEGF-dependent mechanism illustrating the cross talk and synergism that occurs within these growth factor pathways. In addition, FGF-mediated proteolysis of ECM components and induction of the synthesis of collagen, fibronectin, and proteoglycans by endothelial cells contribute to ECM remodelling.

VEGF stimulates endothelial replication and migration and increases vessel permeability, facilitating extravasation of plasma proteins to form a provisional ECM to support cell migration. mRNA for VEGF and VEGF-receptors has been detected in the tips of invasive angiogenic sprouts, and antibody blockade of VEGF signalling significantly decreases microvessel outgrowth. PDGF produced by angiogenic endothelial cells is required for the recruitment, proliferation, and survival of pericytes for vessel stabilization and maturation. PDGF acts on two transmembrane receptor tyrosine kinases, PDGF-α and -β. PDGF-β expressed on pericytes is critical to their recruitment. Disruption of signalling at these kinases is associated with vascular abnormalities in physiological and pathological angiogenesis.

Angiopoietins are ligands of endothelial-specific Tie receptors that have multiple effects on the angiogenic process, particularly interactions between endothelial cells, pericytes, and the basement membrane. For example, angiopoietin-1 acts on Tie-2 to stimulate secretion of growth factors from endothelial cells, which in turn stimulate differentiation of surrounding pericytes into smooth muscle cells.

Conversely, angiopoietin-2 is an antagonist of the actions of angiopoietin-1 and so acts as a naturally occurring inhibitor of angiogenesis [40].

Recruitment and proliferation of bone marrow–derived endothelial progenitor cells (EPCs) to form new vessels (vasculogenesis) is a distinct but complimentary process which occurs simultaneously in ischaemic and wounded tissue to augment perfusion [41]. First described in 1997, classification of EPCs is still controversial, although the most well-accepted definition is that they are circulating endothelial cells expressing CD45, CD34 and CD133 surface antigens [42]. EPCs express proteins such as L-selectin and mucosal vascular cell adhesion molecule 1 (VCAM-1) which facilitate adhesion to mature endothelial cells. Incorporation of EPCs into the endothelial cell layer induces the release of proangiogenic factors such as VEGF, resulting in further recruitment of pro-angiogenic cells and enhanced angiogenesis. EPCs have been proposed as a potential cell-therapy to promote neovascularization in ischaemic tissues. This idea is supported by many studies demonstrating the role of EPCs in improved tissue perfusion in animal models of ischaemia, and clinical data showing that administration of EPCs to patients with myocardial infarction or chronic angina is associated with positive trends in perfusion [43].

1.4 Haemostasis

Endothelial cells play a pivotal role in regulating blood flow by exerting effects on the coagulation system, platelets, and fibrinolysis. Under normal physiological conditions, the endothelium provides one of the few surfaces which can maintain blood in a liquid state during prolonged contact [3]. A key factor in blood clot formation is activation of the serine protease thrombin which cleaves fibrinogen, producing fragments that polymerise to form strands of fibrin. Thrombin also activates factor XIII, a fibrinoligase, which strengthens fibrin-to-fibrin links, thereby stabilising the clot and stimulating platelet aggregation. Heparan sulfate proteoglycan molecules provide an anti-thrombotic endothelial cell surface by serving as co-factors for antithrombin III, causing a conformational change that allows this inhibitor to bind to, and inactivate, thrombin and other serine proteases involved in the clotting cascade. The endothelium also prevents thrombin formation by expressing tissue factor pathway inhibitor (TFPI) which binds to clotting factor Xa. TFPI and antithrombin III both contribute to physiological haemostasis, and both show impairment in acquired thrombotic states. A third endothelial anti-coagulation mechanism is expression of thrombomodulin. Binding of thrombin to cell surface thrombomodulin removes its pro-coagulant activity, and the thrombin-thrombomodulin complex activates protein C, a vitamin K-dependent anticoagulant. Activated protein C, helped by its cofactor protein S, inactivates clotting factors Va and VIIa [44].

The anti-platelet aggregation properties of the endothelium are largely mediated by release of PGI_2 and NO. As with smooth muscle relaxation, PGI_2 inhibits platelet aggregation through the activation of IP receptors and activation of adenylyl cyclase, whereas NO inhibits platelet adhesion, activation, secretion, and aggregation

through a cGMP-dependent mechanism. NO inhibits agonist-dependent increases in intra-platelet Ca^{2+} to suppress the Ca^{2+}-sensitive conformational change in the heterodimeric integrin glycoprotein IIb–IIIa required for fibrinogen binding. NO also promotes platelet disaggregation by impairing the activity of phosphoinositide 3-kinase, which normally supports conformational changes in glycoprotein IIb–IIIa, rendering its association with fibrinogen irreversible. Should a blood clot form, fibrinolysis depends primarily on the action of plasmin, an active protease formed from its precursor, plasminogen, upon stimulation by tissue-type plasminogen activator [44].

Under physiological conditions there is a haemostatic balance, and in addition to these anti-thrombotic mechanisms, the endothelium also synthesises several key haemostatic components, with vWF and plasminogen activator inhibitor-1 (PAI-1) being particularly important. PAI-1 is secreted in response to angiotensin IV, providing a link between the renin-angiotensin system and thrombosis. In addition to anti-coagulant activity, binding of thrombin to thrombomodulin accelerates its capacity to activate thrombin-activatable fibrinolysis inhibitor which cleaves fibrin and other proteins, resulting in the loss of plasminogen/plasmin and tissue plasminogen activator binding sites and thus retarding fibrinolysis. Perturbations such as those that may occur at sites of injury, inflammation, or high shear stress tip this haemostatic balance in favour of a pro-thrombotic and anti-fibrinolytic microenvironment. Critical steps include loss of cell surface heparan proteoglycan molecules and increased expression of the transmembrane glycoprotein tissue factor which initiates coagulation by stimulating the activation of clotting factors IX and X, and pro-thrombinase, with subsequent fibrin formation. Tissue factor accumulates in experimentally injured vessels and accumulation in some atherosclerotic plaques is likely to account for their high thrombogenicity [45].

1.5 Inflammation

The development of inflammatory reactions by the endothelium in response to injury or infection is critical for the maintenance and/or repair of the normal structure and function of the vessel wall. However, excessive inflammation can lead to severe tissue damage and contribute to the development of atherosclerosis. The interaction between endothelial cells and inflammatory cells such as leukocytes depends on the production of inflammatory cytokines (e.g. interleukin 8; IL-8) to attract leukocytes, and expression of adhesion molecules (e.g. selectins) to facilitate their adhesion and migration towards the site of infection. Loosely tethered leukocytes first roll over the endothelial surface, then arrest, spread, and finally migrate between endothelial cells to attach onto underlying ECM components [46] (Fig. 1.3).

Leukocyte rolling involves endothelial adhesion molecules which transiently bind to carbohydrate ligands on leukocytes to slow passage through the blood vessel. E- and P-selectin are expressed only on the surface of activated endothelial cells

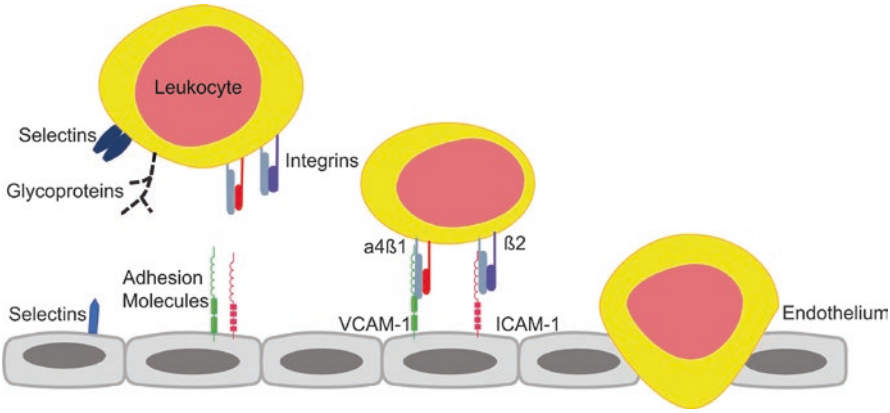

Fig. 1.3 Schematic of adhesion and migration of leukocytes. Inflammatory cytokines attract leukocytes and increase expression of adhesion molecules (e.g. selectins) to facilitate their adhesion and migration towards the site of infection. Loosely tethered leukocytes first roll over the endothelial surface, then arrest and migrate between endothelial cells to attach onto underlying ECM

whereas L-selectin is constitutively expressed on leukocytes and binds to ligands induced on the endothelium at sites of inflammation or on other leukocytes. The role of individual types of selectins in leukocyte rolling shows stimulus- and time-dependent variation. Immediate stimulation of leukocyte rolling induced by histamine or thrombin depends on rapid expression of P-selectin. Surface levels of this adhesion molecule decline after only 30 min. In contrast, TNFα stimulates delayed leukocyte rolling and adhesion to endothelial cells through the induction of E-selectin, surface levels of which peak after 12 h and decline after 24 h. Both E- and P-selectin are expressed on the surface of endothelial cells overlying atherosclerotic plaques, affirming the importance of these molecules in the development of atherosclerosis.

Firm adhesion of leukocytes is promoted by binding of cytokines to leukocyte GPCRs resulting in rapid activation of β1 and β2 integrins to increase their affinity for adhesion molecules of the immunoglobulin superfamily, intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1). ICAM-1 is constitutively expressed on endothelial cells, but levels are increased by stimuli such as TNFα peaking at 6 h and remaining elevated for 72 h. ICAM-1 mediates firm adhesion of blood cells by acting as a ligand for leucocyte β2 integrins. VCAM, a ligand for integrins α4β1 and α4β7, principally mediates the adhesion of monocytes, lymphocytes, eosinophils, and basophils to the endothelial surface. Expression of VCAM-1 is induced by cytokines, oxidized low-density lipoproteins, and ROS acting, as with induction of ICAM-1, primarily via NF-κB.

The migration of leukocytes through the endothelium requires the transient disassembly of endothelial cell junctions. Firm adhesion of leukocytes to the endothelium induces clustering of adhesion molecules like ICAM-1 and VCAM-1, triggering activation of intracellular signalling pathways which induce endothelial cell actin cytoskeleton and cell junction remodelling. The remodelling process

involves numerous pathways including Rho GTPase signalling, protein phosphorylation, and ROS generation, but a key event is reorganization of PECAM-1 dimers. PECAM-1 localizes to intercellular junctions of endothelial cells, forming homodimers linking two cells. Leukocytes also express PECAM-1 and the dissociation of PECAM-1 dimers between endothelial cells to form dimers between emigrating leukocytes and endothelial cells is critical for leukocyte migration [47].

1.6 Conclusions

The endothelium, once viewed as an inert physical barrier, is a dynamic secretory organ fulfilling numerous roles in maintenance of cardiovascular homeostasis. Endothelial cells from different parts of the vasculature show highly differentiated functions. Advances in defining endothelial functions at the molecular level may lead to targeted therapies to alleviate chronic endothelial dysfunction associated with the progression of cardiovascular disease.

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Further Reading

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