Chapter 5
Pathophysiology of Angiogenesis and Its Role in Vascular Disease

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Key Learning Points

- Angiogenesis is the process of new blood vessel formation from pre-existing vessels that is critical for growth and development and in response to tissue ischaemia such as that seen during a myocardial infarction or in peripheral artery disease.
- Uncontrolled angiogenesis is a key contributor to the development and progression of malignant cancers and atherosclerotic plaque formation. Angiogenesis-associated diseases are the leading causes of mortality and morbidity worldwide. Impaired angiogenic responses underpin the mechanisms associated with diabetes- and age-related vascular complications.
- The intricate balance between desirable physiological angiogenesis and unwanted pathological angiogenesis involves the regulation of a suite of signalling

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pathways, regulatory factors and cell-to-cell interactions. Physiological angiogenesis is primarily mediated by the hypoxia transcription factor HIF-1α while pathological angiogenesis is driven by the inflammatory transcription factor NFκB. Numerous angiogenic mediators can be driven by both hypoxia and inflammation.

5.1 Introduction

The human circulatory system is comprised of a complex branching network of blood vessels designed to transport oxygen and nutrients to cells, remove waste products and facilitate immune surveillance. Given its diverse functions, this vascular network must be responsive and capable of adapting to a range of tissue microenvironments, stressors and changing metabolic demands. In the physiologic state, this is achieved through dynamic yet highly coordinated processes of blood vessel growth and remodelling, which are under the balanced control of both stimulating and inhibiting factors. In disease states, however, these processes are frequently dysregulated, with inadequate, excessive or abnormal vessel growth potentially leading to a broad range of clinical pathologies [1].

Angiogenesis refers to the process in which new blood vessels, in particular capillaries, are formed from the pre-existing vascular network. Angiogenesis is distinguished from vasculogenesis, which refers to the assembly of a primary vascular plexus, typically in the developing embryo, that arises *de novo* from the differentiation of mesoderm-derived precursors called angioblasts [2]. Recent decades of research have begun to reveal some of the cellular and molecular mechanisms that underpin the contribution of angiogenesis to the pathophysiology of vascular diseases. Pro-angiogenic stimuli activate endothelial cells (ECs) to detach from their basement membranes, then migrate and proliferate to form branching tubular structures, driving the sprouting of new capillaries from the primary plexus [2, 3]. Further remodelling involves the recruitment of mural cells such as vascular smooth muscle cells (VSMCs) and pericytes, as well as the laying down of extracellular matrix (ECM) to provide structural stability and facilitate vessel maturation [3]. This process continues until pro-angiogenic cues subside or are inhibited, at which point vessel growth becomes quiescent and anti-angiogenic factors predominate.

It is well recognised that angiogenesis is a critical process in normal postnatal growth and development. It is crucial in providing nourishment to granulation tissue during wound healing, as well as in the formation of collateral vessels as part of an adaptive response to vascular occlusion and ischaemia [4]. The failure of adequate angiogenesis plays an important role in conditions such as ischaemic heart disease, peripheral arterial disease (PAD), delayed wound healing and ischaemic stroke. Conversely, excessive pathological angiogenesis driven by inflammation is a key contributor to the development and progression of malignant cancers, atherosclerotic plaques, proliferative retinal disease and inflammatory arthritides, as well as many other pathologies [5]. A detailed and holistic understanding of the factors

which promote or suppress angiogenesis in these contexts could prove therapeutically useful. Indeed, several pharmacological, gene- and cell-based approaches for modulating angiogenesis have been trialled for various vascular conditions, though with mixed success to date [6].

In this chapter, we outline a basic mechanistic framework of the process of angiogenesis and summarise the key molecular factors that regulate it, particularly those that have current or potential clinical relevance. We will highlight the importance of angiogenesis in health, as well as in the pathophysiology of various ischaemic and inflammatory vascular diseases. In addition, we review the current range of therapeutic strategies that are designed to target angiogenesis and examine the barriers and pitfalls that have limited more successful clinical translation so far. Finally, we explore several emerging therapies that are showing great promise in pre-clinical models and offer some insights on future directions of research inquiry.

5.2 Basic Mechanisms of Angiogenesis

We will first overview the cellular mechanisms that underlie blood vessel formation and the key molecular factors that are known to regulate it. Primitive blood vessels are first formed through vasculogenesis, a process that occurs most prominently during embryonic development. Vasculogenesis involves the differentiation of mesoderm-derived angioblasts into ECs, which then establish a primary vascular plexus [2, 7]. Animal models suggest that this process requires a threshold level of vascular endothelial growth factor (VEGF)A expression from the embryonic endoderm [2]. VEGFA is the most well-characterised of all known vascular growth factors. It binds to and activates tyrosine kinase VEGF receptors (VEGFR)1 and VEGFR2, stimulating them to dimerise and become autophosphorylated to initiate intracellular signalling transduction [8]. During vasculogenesis, VEGFA acts in a paracrine manner on VEGFR2 on the surface of angioblasts to induce their differentiation [2]. The resulting ECs proliferate and assemble into primitive vascular cords that undergo differentiation to form arteries or veins [3]. Following vasculogenesis, new blood vessels arise from the pre-existing network through two major mechanisms: sprouting and intussusceptive (or non-sprouting) angiogenesis (Fig. 5.1).

5.2.1 Sprouting Angiogenesis

Vessel sprouting is the better characterised “classical” form of angiogenesis that typically occurs in the early stages of development, though intussusceptive angiogenesis can occur concurrently [2]. In response to VEGFA and other pro-angiogenic factors which are predominantly triggered by hypoxia, ECs are activated to release a range of proteolytic enzymes, particularly matrix metalloproteases (MMPs), that initiate breakdown of the underlying basement membrane [2, 3, 8]. These facilitate

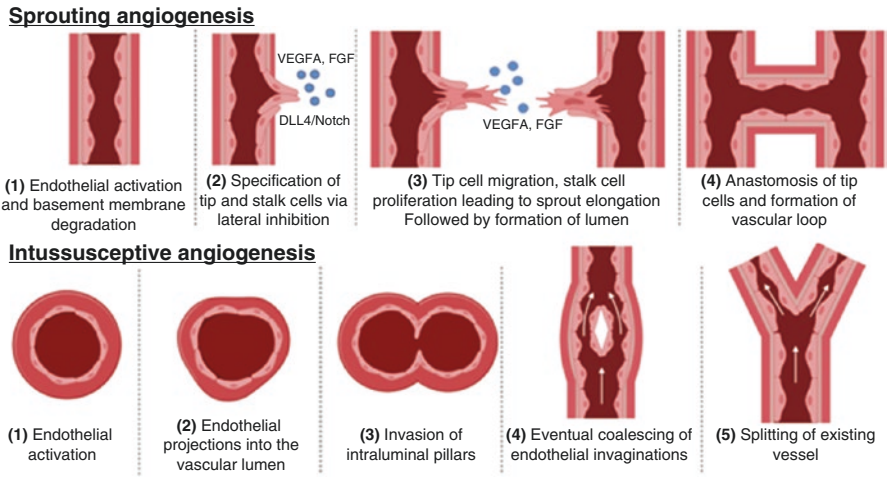

Fig. 5.1 Sprouting and intussusceptive angiogenesis. New blood vessels can form from pre-existing vessels by either sprouting angiogenesis or intussusceptive angiogenesis. Sprouting angiogenesis occurs in response to VEGFA and other pro-angiogenic factors, triggering the activation of endothelial cells (ECs) to release a range of proteolytic enzymes, that initiate basement membrane degradation. Migrating ECs then become either tip cells, which continue to direct sprout outgrowth or stalk cells which proliferate behind tip cells to support sprout elongation. Vascular loops are then formed when the tip cells anastomose. Intussusceptive angiogenesis involves the splitting of existing perfused blood vessels by intraluminal tissue pillars. These are formed as ECs undergo morphological rearrangement of their cellular junctions resulting in endothelial invaginations that extend into the vascular lumen. These protrusions usually develop on opposite sides of the vessel wall and eventually coalesce. Expansion of the intraluminal pillar ultimately results in vessel duplication as the parent vessel is split into two new capillaries

the detachment and chemotactic migration of ECs, pericytes and VSMCs towards angiogenic cues. Migrating ECs are then specified to become either "tip cells", which spearhead new sprouts via motile invasive filopodia, or "stalk cells" which proliferate behind tip cells to support sprout elongation [3, 7]. The specification of tip cells and stalk cells is dynamic and occurs via competitive lateral inhibition. Activation of VEGFR2 by VEGFA induces the expression of delta-like ligand 4 (DLL4) and the ECs that express this most efficiently are specified as tip cells [3]. In adjacent ECs, DLL4 acts on Notch receptors, leading to downregulation of VEGFR2 and upregulation of VEGFR1, which inhibits tip cell-like behaviour and instead promotes a stalk cell phenotype [3]. Tip cells continue to direct sprout outgrowth by probing for both attractive and repulsive guidance cues; several classes of ligand-receptor interactions are implicated in this such as ephrins which bind Eph receptors and semaphorins which activate neuropilin receptors [3, 6, 7]. Meanwhile, stalk cells undergo proliferation to extend the sprouts, eventually forming a lumen. This commonly occurs as cords of ECs become morphologically modified and flatten out to open up a lumen (cord hollowing), or via the development of pinocytic intracellular vacuoles which coalesce in adjacent ECs to form a lumen (cell hollowing) [3, 7].

The sprouting process continues through a dynamic balance of VEGFA/VEGFR2 and DLL4/Notch signalling. Eventually, the filopodia of adjacent tip cells interact and anastomose with each other to form a vascular loop, strengthened by intercellular junctions comprised of vascular endothelial (VE)-cadherins [3]. These proteins provide structural stability but also have a crucial role in maintaining EC survival [8]. Stalk cells also gradually transform into "phalanx cells", a monolayer of ECs with reduced proliferative capacity, that re-establish the basement membrane and form tight junctions [3]. To develop functional vessels, VSMCs and pericytes must also be recruited to the vessel wall to facilitate vessel stabilisation, maturation and deposition of ECM. This process is regulated by transforming growth factor (TGF)-β signalling as well as platelet-derived growth factor (PDGF)B released from ECs [3]. PDGFB activates PDGF receptor (PDGFR)β on VSMCs and pericytes, stimulating their migration, proliferation and incorporation into the vascular wall. These mural cells also produce angiopoietins (Ang), Ang-1 and Ang-2, which bind to tyrosine kinase with immunoglobulin-like and epidermal growth factor-like domain (TIE)-2 receptors on ECs [3, 7, 8]. In mouse models, deficiency of TIE receptors is embryonically lethal [2]. Ang-1 promotes pericyte adhesion and the maintenance of vascular barrier function by stabilising inter-endothelial junctions, while Ang-2 partially antagonises these effects leading to relative vascular permeability [3, 7, 8].

As angiogenic cues subside, further vascular remodelling is regulated by mechanical and metabolic influences. The initiation of blood flow in these new vessels exposes ECs to shear stress, activating PDGFB/PDGFRβ signalling pathways and transcription factors such as Krüppel-like factor (KLF)2 that promote luminal patency and contribute to adaptive remodelling [3]. ECs that line well-perfused vessels are maintained and become quiescent, while hypoperfused vessels conversely undergo regression. The delivery of adequate oxygen and nutrients to ECs also helps to establish quiescence by reducing both glycolytic and oxidative metabolism [9]. The prolyl hydroxylase domain (PHD) proteins use available oxygen to suppress hypoxia-inducible factors (HIFs), leading to reduced expression of VEGFA and other pro-angiogenic factors [4]. Vascular integrity is further maintained by strengthening cell-cell junctions and cell-ECM adhesion, as promoted by fibroblast growth factors (FGF) including FGF1 and FGF2, Ang-1, and receptors for various ECM components called integrins [3, 8]. EC survival is also maintained by intracrine (internal) VEGFA signalling, which induces the expression of the anti-apoptotic factor B-cell lymphoma (BCL)-2 [3].

5.2.2 Intussusceptive Angiogenesis

Non-sprouting or intussusceptive angiogenesis (IA) is also an important, yet often under-recognised, mode of vessel growth. Its primary function appears to be remodelling and pruning of vascular networks that were previously formed by vasculogenesis or sprouting angiogenesis [10, 11]. As opposed to the outgrowth of blind-ended capillary sprouts that are not initially perfused, the defining feature of IA is the

splitting of existing perfused blood vessels by intraluminal tissue pillars. These are formed as ECs undergo morphological rearrangement of their cellular junctions resulting in endothelial invaginations that extend into the vascular lumen [10]. These protrusions usually develop on opposite sides of the vessel wall and eventually coalesce as they are invaded by pericytes and myofibroblasts which deposit collagen-rich ECM. Expansion of the intraluminal pillar ultimately results in vessel duplication as the parent vessel is split into two new capillaries or, if it occurs at a point of bifurcation, the branching angle of the vessel may be altered [11]. Asymmetric pillar growth can also lead to pruning and regression of redundant vessels [11]. In contrast to sprout growth, the basement membrane remains intact during IA and ECs do not undergo significant migration or proliferation [10]. IA can therefore occur more rapidly and expends less metabolic energy compared to sprouting.

Although intussusception contributes substantially to angiogenesis in a variety of tissues under both physiological and pathological settings, the exact molecular factors that initiate and maintain it are less well understood due to a lack of appropriate experimental models. Unlike sprouting angiogenesis, the importance of VEGFA and VEGFR2 is ambiguous, as some studies suggest that VEGFA has a necessary role in IA, while others show that VEGFA is in fact downregulated and selective blockade of VEGFR2 does not significantly affect the occurrence of IA [10, 12, 13]. It may be that other VEGF isoforms are responsible, or VEGFA may be binding to alternative receptors such as VEGFR1. Other angiogenic factors, however, may also be important such as Ang-1 and their TIE-2 receptors. Targeted TIE-2 deletion in mice has been found to compromise pillar formation [14], while Ang-1 over-expression produces enlarged vessels with numerous small invaginations, a phenotype that resembles IA [10, 15]. FGF2 is also thought to stimulate PDGFB and PDGFRβ expression leading to the recruitment of pericytes into the intraluminal pillars during IA [10]. Beyond these factors, haemodynamic forces such as shear stress and cyclic stretch are also implicated in the remodelling of vascular networks through IA. This is suggested by observations that the extent of IA in chick chorioallantoic membranes *in vivo* was enhanced in response to increased blood flow induced experimentally by clamping off side branches [16].

A more detailed understanding of the occurrence, mechanisms and regulation of IA is clearly necessary to provide insight into its role in health and disease. This may inform the development of therapies directed specifically at this common, yet often overlooked, form of angiogenesis.

5.3 Angiogenesis in Health and Disease

Having reviewed the basic cellular and molecular mechanisms underlying sprouting and non-sprouting vessel growth, we will now overview pertinent aspects of angiogenesis and its regulation in various physiological and pathological contexts. The principal drivers of angiogenesis include hypoxia and inflammatory stimuli, and these can interact significantly through overlapping

pathways. Inhibitors of angiogenesis provide crucial counter-balances to prevent excessive angiogenesis, and these may be lost in pathological conditions.

5.3.1 *Physiological Angiogenesis*

In health, angiogenesis is an important contributor to normal homeostatic processes, such as growth and development, the menstrual cycle, wound healing and the adaptive response to ischaemia. Hypoxia is a potent trigger for angiogenesis and typically may occur due to vascular occlusion or when an expanding tissue mass outgrows its blood supply. Under such conditions, the key transcription factor HIF-1α is activated and serves as the master regulator of downstream signalling, upregulating the expression of numerous pro-angiogenic genes with promoter regions containing hypoxia-response elements (Fig. 5.2) [4]. These include VEGFA,

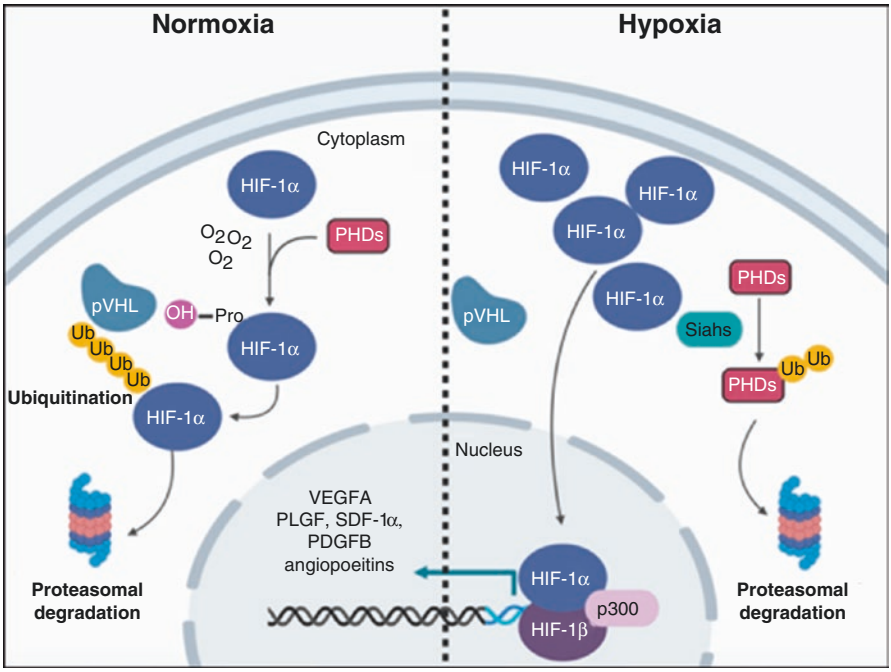

Fig. 5.2 Key hypoxia-driven cellular mechanisms that drive physiological angiogenesis. The hypoxia-driven transcription factor, HIF-1α is the master regulator of physiological angiogenesis. In normoxia, HIF-1α is inhibited post-translationally by the PHD proteins, which use oxygen as a co-substrate to hydroxylate proline residues on HIF-1α, targeting HIF-1α for ubiquitination and proteasomal degradation by the von Hippel-Lindau (VHL) complex. In hypoxia, transcriptional activation of the ubiquitin ligases Siahs target and degrade the PHDs. This prevents HIF-1α from being degraded, allowing it to accumulate and translocate into the nucleus where it binds and activates numerous pro-angiogenic genes including VEGFA, PLGF, SDF-1α, PDGFB and angiopoietins

the angiopoietins, a homologue of VEGFA called placental growth factor (PLGF), stromal-derived growth factor (SDF)-1α and PDGFB [4, 17]. In normoxia, the activity of HIF-1α is inhibited post-translationally by the PHD proteins, which use oxygen as a co-substrate to hydroxylate proline residues on HIF-1α [4]. This enables HIF-1α to be recognised by the von Hippel-Lindau ubiquitin ligase complex, which then targets it for ubiquitination and proteasomal degradation [17]. A drop in intracellular oxygen conversely leads to transcriptional activation of Siah1 and Siah2, ubiquitin ligases which target and degrade the PHDs, thereby relieving the inhibition of HIF-1α and promoting its stability [18]. HIF-1α is then able to promote the expression of proteins, particularly VEGFA and SDF-1α, which induce angiogenesis locally and/or stimulate postnatal vasculogenesis via the mobilisation of precursor cells including mesenchymal stem cells and endothelial progenitor cells (EPCs) from bone marrow [4, 17]. These mechanisms are crucial in supporting the adaptive hypertrophic response of skeletal muscle to exercise training. They also underpin the development of a collateral circulation that can help to salvage ischaemic tissue, maintain its function and delay symptom onset and progression. This is particularly pertinent in the context of angina due to ischaemic myocardium in coronary artery disease (CAD), claudication from the compromised vascular supply to skeletal muscle in PAD, and neurological deficits from ischaemic neuronal or glial cells in stroke and vascular dementia.

Wound healing represents yet another critical function of angiogenesis in health. The rapid expansion of a dense capillary network provides the necessary substrates to support tissue repair and regeneration. Once wound closure is complete, angiogenesis is inhibited, and these vessels must be pruned back and remodelled into a mature network [19]. Though traditionally viewed as a process occurring along with granulation tissue formation during the proliferative phase, angiogenesis is in fact closely linked with all stages of wound healing [20]. A number of pro-angiogenic factors are stimulated in the initial haemostatic phase. Thrombin directly upregulates receptors for VEGFA on ECs while also inducing the release of MMPs that initiate basement membrane degradation [20]. Tissue injury also liberates factors such as FGF2 sequestered within the ECM and activated platelets release various mediators including VEGFA, Ang-1, PDGFB and TGF-β [20, 21]. The hypoxic gradient between healthy and injured tissue also enhances HIF-1α signalling to promote angiogenesis [4]. In the inflammatory phase, vascular permeability is increased in response to cyclooxygenase (COX)2 [20]. This facilitates the recruitment of peripheral blood monocytes into damaged tissue and their differentiation to macrophages, which further amplifies the release of pro-angiogenic factors. During the proliferative phase from about 3–5 days post injury, the neovascular network starts to form in earnest through sprouting and non-sprouting angiogenesis [21]. These new vessels support the migration of macrophages and fibroblasts into the wound space, forming granulation tissue through the deposition of ECM rich in fibrin, fibronectin and vitronectin as regulated by TGF-β signalling [21]. In turn, endothelial tip cells interact with these ECM components by increasing their surface expression of $\alpha_v\beta_3$ integrins, helping to direct EC invasion through the ECM and expansion of the capillary network [21]. As normoxic conditions return and the acute

inflammation subsides, inhibitors of angiogenesis predominate, and the wound matures as fibrinous ECM is gradually replaced by collagen [21]. ECM components also give rise to many endogenous inhibitors of angiogenesis such as thrombospondin-1, the tissue inhibitors of MMPs, angiostatin, tumstatin and endostatin, the latter two being cleavage products of collagen types IV and XVIII respectively [6, 20]. These factors ensure that angiogenesis does not persist unnecessarily, allowing for excessive redundant vessels to be pruned through EC apoptosis as the wound undergoes continuous remodelling [21].

5.3.2 *Pathological Angiogenesis*

In contrast to the critical role of angiogenesis in development, ischaemia and wound healing, maladaptive and excessive angiogenesis are hallmarks of various pathological chronic inflammatory states. The molecular signalling pathways involved in inflammation are known to be intimately linked with the activation of angiogenesis (Fig. 5.3). In turn, these new vessels help to sustain the inflammatory response as they provide additional conduits for the delivery of immune cells and cytokines. In

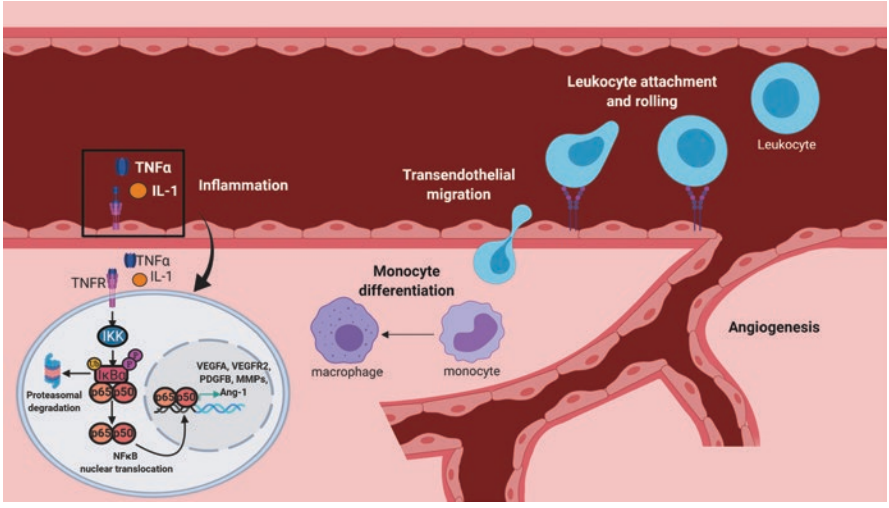

Fig. 5.3 Key inflammatory-driven cellular mechanisms that drive pathological angiogenesis. In response to local inflammatory stimuli such as TNFα and IL-1, the key inflammatory-driven transcription factor NFκB is activated in ECs which upregulate the expression of cell surface adhesion molecules that facilitate the tethering and rolling of leukocytes along the vascular endothelium. Within inflamed tissue, circulating monocytes differentiate into macrophages and produce a myriad of mediators that can directly and indirectly promote angiogenesis. Central to this is the activation of nuclear factor (NF)κB, a key transcription factor that not only switches on genes involved in orchestrating the inflammatory response, but also stimulates angiogenesis through the upregulation of VEGFA, VEGFR2, MMPs, PDGFB and Ang-1

response to local inflammatory stimuli such as various interleukins (IL-), tumour necrosis factor (TNF)α and interferon (IFN)γ, activated ECs upregulate the expression of several transmembrane glycoproteins including the P- and E-selectins, intercellular cell adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 [5]. These surface molecules facilitate the tethering and rolling of leukocytes along the vascular endothelium. Coupled with factors that increase vascular permeability such as VEGFA, COX-2 induced prostaglandins, histamine from mast cells, and nitric oxide (NO) produced by inducible NO synthase (iNOS), these molecules promote the transmigration of leukocytes across the endothelium [5]. Within inflamed tissue, circulating monocytes differentiate into macrophages and produce a myriad of mediators that can directly and indirectly promote angiogenesis. Central to this is the activation of nuclear factor (NF)κB [22], a key transcription factor that not only switches on genes involved in orchestrating the inflammatory response, but also stimulates angiogenesis through the upregulation of VEGFA, VEGFR2, MMPs, PDGFB and Ang-1, among others [5]. Various chemokines including monocyte chemoattractant protein (MCP)-1 are also released leading to the further recruitment of monocytes that propagate and sustain the inflammatory and angiogenic response [5]. These factors drive ECs to detach from their basement membrane and undergo migration and proliferation. Proteolysis of the ECM also releases sequestered factors such as FGF2 that can further potentiate angiogenesis [5].

The maintenance of an unbalanced pro-angiogenic state appears to be a common pathological feature in a range of chronic inflammatory conditions. Rheumatoid arthritis (RA), for instance, is characterised by progressive and destructive inflammation of the joint synovium driven by a network of cytokines such as IL-1, IL-6 and TNFα [23]. These mediators, along with relative tissue hypoxia, strongly induce pro-angiogenic factors to establish new vessels that augment the inflammatory response. Compared to healthy controls, patients with RA have markedly elevated levels of VEGFA in the serum and synovial fluid [24], and their synovial fibroblasts demonstrate increased expression of Ang-1 and Ang-2 [25]. A similarly pathological interaction of hypoxic and inflammatory signalling triggers neovascularisation in proliferative ocular diseases such as wet age-related macular degeneration (AMD), retinal vessel occlusion and diabetic retinopathy. A central feature of these conditions is the over-expression of VEGFA leading to rapid formation of new vessels in the choroid and retina [5]. These vessels are typically fragile and leaky, predisposing to haemorrhage, oedema and the deposition of exudates that can obscure central light perception. The upregulation of adhesion molecules and chemotactic factors further allows the infiltration of inflammatory cells which propagate the vicious angiogenic cycle [5].

Similar mechanisms are involved in the pathogenesis of several vasculitic syndromes. Dysregulated angiogenesis has been linked to granulomatosis with polyangiitis, Takayasu's arteritis, Kawasaki disease, Giant Cell Arteritis and thromboangiitis obliterans, among many others [26]. Regardless of the initiating factors, these vasculitides are characterised by vigorous inflammation occurring in the vessel wall leading to intimal VSMC proliferation, fibrosis and luminal thrombosis [26]. This is driven by an array of cytokines and chemokines produced by activated macrophages

and T-lymphocytes, in particular various interleukins, TNFα, IFNγ, MCP-1, VEGFA and MMPs [26]. These factors also potently enhance angiogenesis, which in turn facilitates recruitment of more inflammatory cells and is integral to sustaining the metabolic demands of the inflamed tissue. As these vessels are progressively occluded by vascular wall thickening, scarring and thrombosis, resulting in tissue ischaemia which further promotes an angiogenic response that can drive inflammation [26].

In malignant neoplasms, activation of a highly dysregulated "angiogenic switch" is also a well-known contributor to tumour growth, survival and metastasis [1]. As tumour cell clones acquire oncogenic mutations and the expanding tumour mass outgrows its blood supply, local hypoxia potently stimulates angiogenesis through HIF-1α signalling [6]. This occurs on a background of tumour-associated inflammation mediated via TNFα and NFκB pathways in ECs, myeloid cells, fibroblasts and other stromal cells [5]. There is therefore uncontrolled amplification of pro-angiogenic factors such as VEGFA, FGF2, PDGFB, hepatocyte growth factor (HGF) and Ang-2, resulting in a highly disorganised vascular network characterised by leaky, irregular vessels with limited basement membrane coverage and mural support from pericytes [1, 3, 6]. The lack of reliable perfusion through these vessels also impairs oxygen and nutrient delivery, in turn triggering further pathological angiogenesis and providing optimal conditions for tumour intravasation into the vasculature and subsequent dissemination. In addition, factors such as PLGF are thought to recruit bone-marrow derived precursors to facilitate tumour-associated vasculogenesis by acting on VEGFR1 [27]. Besides sprouting and non-sprouting angiogenesis, it is now known that tumour cells also have the ability to establish alternative aberrant forms of vascularisation. These include vascular mimicry, a mechanism whereby tumour cells can form pseudo-vessels by adopting an EC-like morphology [1, 3], as well as vessel co-option, where tumours bypass angiogenesis by hijacking and utilising pre-existing vessels within adjacent non-malignant tissue [28]. Although the mechanisms underlying these processes are less well understood, they are recognised to be poor prognostic factors and confer resistance to current anti-angiogenic therapies [6].

5.3.2.1 Angiogenesis and Atherosclerosis

Given the central importance of atherosclerosis in vascular diseases, an overview of the angiogenic mechanisms that drive the development of atherosclerotic plaque deserves specific attention. Atherosclerosis is a progressive inflammatory process that typically spans decades. It is initiated by a range of factors including haemodynamic stress that can cause endothelial injury and dysfunction, often first manifest as thickening of the arterial intima. This disruption allows atherogenic lipids, particularly cholesterol delivered by low-density lipoprotein (LDL) particles, to be deposited into, and accumulate within, the sub-endothelial space, where they become oxidised by reactive oxygen species (ROS) [5, 29]. Oxidised LDLs are potent stimulants of the inflammatory response, in which circulating monocytes are

recruited into the damaged intima and are activated to become macrophages. As these macrophages attempt to scavenge oxidised LDLs, they become lipid-laden foam cells, in turn releasing numerous pro-inflammatory cytokines and chemokines, which promote plaque growth by stimulating the proliferation and migration of VSMCs from the arterial media into the intimal layer [29]. The VSMCs produce a poorly developed ECM and form a fibrous cap that encapsulates the expanding lipid- and debris-filled necrotic core. These plaques become clinically evident as they cause progressive stenosis of the arterial lumen or are acutely occluded by thrombus following erosion or rupture of the fibrous cap, crucially leading to end-organ ischaemia, typified by myocardial infarction (MI), PAD and ischaemic stroke syndromes.

There is now a well-recognised relationship between plaque growth, plaque instability and the extent of plaque angiogenesis. Indeed, in advanced atherosclerotic plaques, vulnerable regions are associated with greater degrees of inflammation and neovascularisation [30]. In the early stages of plaque growth, there is angiogenic expansion of the vasa vasorum, a network of microvessels in the adventitial and outer medial layer of medium and large arteries whose function is to provide oxygen and nutrients to the vessel itself (Fig. 5.4) [30, 31]. This occurs in

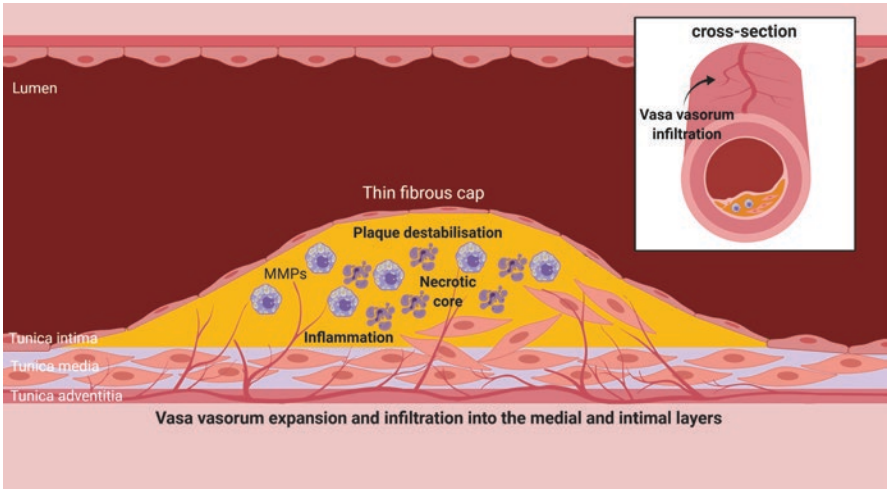

Fig. 5.4 Angiogenesis in the atherosclerotic plaque. In the early stages of plaque growth, there is angiogenic expansion of the vasa vasorum, a network of microvessels in the adventitial and outer medial layer of medium and large arteries which provide oxygen and nutrients to the vessel itself. As the plaque advances, progressive intimal thickening, lipid deposition and medial hyperplasia contribute to relative hypoxia, strongly driving vasa vasorum angiogenesis and the ectopic extension of adventitial neovessels into the intima to support ongoing plaque growth. The highly inflammatory milieu created by plaque macrophages further stimulates angiogenesis. These new vessels accentuate plaque inflammation by providing additional routes for the infiltration of inflammatory cells and cytokines. The release of MMPs and other proteases that facilitate invasion of ECs through the ECM also contribute to plaque instability through breakdown and thinning of the fibrous cap

response to several synergistic factors. Arterial hypertension leads to increased cyclical compression of the vasa vasorum especially during systole. As the plaque advances, progressive intimal thickening, lipid deposition and medial hyperplasia contribute to relative hypoxia, strongly driving vasa vasorum angiogenesis and the ectopic extension of adventitial neovessels into the intima to support ongoing plaque growth [30, 31]. The highly inflammatory milieu created by plaque macrophages further stimulates angiogenesis through numerous factors as previously outlined, particularly VEGFA, the TNFα and NFκB pathways, PDGF, FGF1 and FGF2, and the angiopoietins [29, 30]. Oxidised LDL and phospholipids themselves can also directly promote pathological angiogenesis [29]. New vessels are also thought to arise from the arterial lumen, though to a much lesser extent than via the vasa vasorum [31]. Regardless of the source, these new vessels accentuate plaque inflammation by providing additional routes for the infiltration of inflammatory cells and cytokines. The release of MMPs and other proteases that facilitate invasion of ECs through the ECM also contribute to plaque instability through breakdown and thinning of the fibrous cap [30, 31]. In a similar way to angiogenesis occurring in malignant tumours, plaque neovessels also have a paucity of tight junctions, along with discontinuous basement membrane and pericyte support. Such fragile intimal neovessels predispose to intraplaque haemorrhage [31]. This is a well-established marker of plaque instability as the extravasation of erythrocytes with cholesterol-rich membranes adds to the growing lipid load, and the abundance of free haemoglobin containing iron further activates macrophages and enhances oxidative stress within the plaque [29, 31].

Vascular calcification (VC), particularly occurring within the intima, is also recognised as a prominent feature of advanced atherosclerotic plaque, though the role and mechanisms of calcification remain rather poorly defined. In both CAD and PAD settings, calcium scores of atherosclerotic lesions are known to be associated with a higher risk of adverse cardiovascular events [32, 33]. VC in the form of spotty patchy intimal microcalcifications is typical of atherosclerosis [34]. It is thought to arise from macrophages and VSMCs that are stimulated to differentiate into osteoblast-like cells, acquiring an osteogenic phenotype by increasing the expression of bone-forming factors such as bone morphogenetic protein-2 and osteoprotegerin [34]. This is likely driven by a combination of factors including endothelial injury, increased generation of ROS, the accumulation of oxidised lipids and apoptosis of VSMCs providing nucleation sites for calcium and phosphate deposition [32, 34]. The role of angiogenesis in this process is still unclear, though it has been proposed that new vessels born out of the atherosclerotic inflammatory milieu may serve as highways for the delivery of osteogenic cytokines and the migration of osteogenic precursors into the plaque body [22, 35]. A clear relationship between angiogenesis and VC, however, has not been demonstrated *in vivo*, and the role of VC as a stabilising or precipitating factor for plaque rupture remains controversial.

Tobacco smoking, a strong risk factor and causative agent for atherosclerosis and tumorigenesis, is also known to enhance pathological angiogenesis. Nicotine, the principal addictive agent in tobacco, has potent mitogenic, pro-migratory and

tubulogenic effects on ECs at clinically relevant concentrations [36]. Nicotine acts on nicotinic acetylcholine receptors on ECs and VSMCs to promote the expression of key pro-angiogenic factors such as VEGFA and the FGFs [36]. Nicotine also stimulates the release of catecholamines which contribute to arterial hypertension by increasing heart rate and peripheral vascular resistance, contributing to endothelial dysfunction as an initiating step to atherosclerosis [36].

Despite the currently available repertoire of procedural interventions for atherosclerosis such as angioplasty, endovascular stenting and vein grafting, angiogenesis can still pose significant challenges. Indeed, angiogenesis has been implicated in the development of neointimal hyperplasia and neoatherosclerosis, which can lead to complications such as in-stent restenosis and thrombosis [37]. While it is clear that angiogenesis has a critical role in the pathophysiology of atherosclerosis, efforts to modulate it for therapeutic benefit have been largely unsuccessful, due in part to its highly complex and still incompletely understood biology.

5.3.2.2 Angiogenesis in Aneurysmal Disease

Angiogenesis is also believed to contribute to the pathogenesis of aneurysmal disease, which is of obvious interest in vascular surgery. Although the precise aetiology of aneurysms remains elusive, they are typically characterised by an inflammation-driven degeneration and weakening of the vascular wall, resulting in abnormal dilatation and an elevated risk of vessel rupture or thromboembolic complications [38]. There is increased degradation and turnover of ECM components such as elastin and collagen, which appear to be related to the increased expression of MMPs in the setting of a dense inflammatory infiltrate [38]. Much of the evidence implicating angiogenesis comes from the study of aortic aneurysms, though it is conceivable that similar mechanisms may be at play in the development and progression of aneurysms at other sites such as the cerebral, popliteal and renal arteries. In the healthy aorta, the vasa vasorum is usually rather sparse, but several histopathological studies of both thoracic and abdominal aortic aneurysms (AAA) have demonstrated angiogenic expansion of the vasa vasorum extending from the adventitia into the medial layer [39–41]. This occurs in close association with the presence of activated inflammatory cells such as macrophages and mast cells, which produce a host of cytokines including VEGFA, TNFα, FGF2 and various ILs that have pro-angiogenic actions [38, 39]. In thoracic aortic aneurysms, Ang-1, Ang-2 and FGF1 have also been found to be enriched within the medial layer [40]. During the process of vessel sprouting and EC migration, the ECM is continually degraded by MMPs, collagenases and plasminogen activators, contributing to structural weakening of the vascular wall [38]. The upregulation of adhesion molecules and chemokines in these immature and highly permeable neovessels also recruits more inflammatory cells, further promoting pathological angiogenesis [38, 39]. Hypoxia also has a postulated role. In the normal aortic wall, particularly infra-renally, the relative lack of vasa vasora means much of the nourishment is dependent on the diffusion of oxygen and nutrients from the lumen across the intimal layer [38]. However,

this is impaired as the aneurysmal wall expands and may be exacerbated by the presence of intraluminal thrombus, which is relatively common in AAA. As such, hypoxia-driven HIF-1α/VEGFA-dependent signalling drives further neovascularisation within developing AAAs [38], though this mechanism appears not to be as relevant for thoracic aortic aneurysms [40].

Importantly, the extent of aneurysmal neovascularisation is not only correlated with medial degeneration, elastin destruction and greater inflammatory infiltration, but studies have also demonstrated increased angiogenesis at sites of aneurysmal rupture concomitant with elevated expression of pro-angiogenic factors [42, 43]. Indeed, it has been suggested that anti-angiogenic therapies may be able to prevent aneurysmal progression and complications. Such agents are yet to be realised in human patients despite some encouraging results from rodent models of AAA, which have investigated the use of a soluble decoy VEGFA receptor, and an inhibitor of mast cell degranulation, tranilast, showing reduced aneurysm formation associated with decreased angiogenesis and inflammation [44, 45].

5.3.2.3 Diabetes- and Age-Impaired Angiogenesis

Diabetes mellitus [20] is a rapidly expanding global health epidemic known to be associated with a range of vascular complications. The pathophysiological hallmarks of these include dysregulated angiogenesis and accelerated atherosclerosis driven by inflammation, hyperglycaemia, insulin resistance and dyslipidaemia [46]. In patients with type 1 or type 2 DM, CAD represents the major cause of death and there is an estimated two- to four-fold increase in CAD mortality compared to non-diabetic individuals [47]. In addition, an extensive association exists between DM and PAD, with significantly higher incidence rates of claudication, critical limb ischaemia, lower extremity ulcers and major limb amputations [48]. These are likely due to a combination of factors including impaired immune function, reduced vascular collateral formation, diffuse atherosclerosis and peripheral neuropathy in those with DM. This is further compounded by microvascular dysfunction and inflammation, which is important in chronic kidney disease (CKD) and proliferative retinopathy.

Ample evidence indicates that diabetes-related vascular complications are related to significant impairment of physiological angiogenesis. Exposing cultured ECs to high glucose conditions *in vitro* reduces their capacity to migrate and form a capillary tubule network [49]. In mouse models of DM, hyperglycaemia also results in significantly delayed wound healing, as well as impaired recovery of blood flow in the setting of hindlimb ischaemia [49]. The mechanisms underlying these effects are complex and multi-faceted. Chronic hyperglycaemia leads to the generation of ROS and advanced glycation end-products (AGEs) [50]. These contribute to endothelial dysfunction by antagonising the protective effects of NO signalling, which usually helps to regulate vascular tone via VSMC relaxation, but also prevents platelet aggregation and thrombosis, abates excessive immune responses, and enhances endothelial growth and repair [50]. Diabetic hyperglycaemia is also linked

to diminished ischaemia-induced stability of HIF-1α and reduced production of NO via impaired endothelial NO synthase (eNOS) activity [49]. These pathways culminate in decreased production of angiogenic growth factors such as VEGFA, FGF2 and TGF-β, while AGEs can also downregulate or inactivate VEGFR2 leading to defective VEGF signalling [50]. Expression of PDGFB and the activation of Ang-1/Tie2 signalling in ischaemia is also reduced in various models of DM [50]. From a vasculogenic perspective, increased oxidative stress and disrupted NO signalling in DM has been linked to reduced numbers of circulating EPCs, which in turn produces fewer ECs with impaired proliferative and migratory capacity [50, 51]. This decrease in the mobilisation, homing and delivery of EPCs from the bone marrow to sites of injury is due to inhibited VEGFA and SDF-1α signalling in DM [50]. These changes ultimately lead to a defective neovascularisation response to ischaemia.

Additionally, impairment of wound healing is a key contributor to diabetic morbidity and mortality. This is most commonly manifest as ulceration in the distal limbs and is often complicated by concurrent peripheral neuropathy, which severely compromises the sensing of pressure and pain such that cuts and developing blisters go unnoticed by patients for prolonged periods. Peripheral neuropathy arises from the additive effects of oxidative stress, enhanced non-enzymatic glycation and dysfunction of neural proteins, reduced angiogenic and neurotrophic growth factors, and structural microangiopathic changes to neural blood vessels [52]. Once formed, diabetic wounds are also significantly predisposed to infectious complications, particularly cellulitis and osteomyelitis, as a result of diminished immune defences. Indeed, monocytes and granulocytes from individuals with DM characteristically exhibit defective phagocytic function and responses to chemotactic signals [50]. Moreover, the wound perfusion and oxygenation required to support the metabolic demands of immune cells is often severely reduced by the presence of diffuse atherosclerotic stenoses in the major arteries of patients with DM, leading to necrotic cell death. As a result, the ability to mount an angiogenic and vasculogenic response to wound hypoxia is strongly attenuated in DM, leading to delayed formation of wound granulation tissue and disrupted ECM remodelling [50].

Abnormal angiogenic processes also play a role in the development and progression of CKD, for which DM is one of the leading causes, along with hypertension. In the early stages of diabetic nephropathy, hyperglycaemia is thought to induce the over-expression of VEGFA, Ang-2 and TGF-β signalling, leading to glomerular EC proliferation and the formation of immature vessels with deficient basement membrane and aberrantly high permeability [53]. Combined with the presence of glomerular hypertension and impaired eNOS signalling, this promotes extravasation of plasma proteins resulting in albuminuria, arteriolar hyalinosis and nodular deposits in the mesangial matrix which are characteristic of diabetic CKD [53]. As the nephropathy advances, ongoing ROS and AGE formation causes progressive destruction to the glomerular filtration apparatus accompanied by widespread fibrotic changes [53]. Further injury to the endothelium, podocytes and the renal tubular epithelium eventually leads to frank proteinuria and reduced VEGFA expression in the late stages of diabetic nephropathy [53].

Excessive pathological angiogenesis is also a hallmark of diabetic retinopathy. In the ocular vasculature, chronic hyperglycaemia induces the production of ROS and AGEs, while microvascular occlusions promote HIF-1α signalling, both leading to over-stimulation of VEGFA [52–54]. This initiates a dysregulated angiogenic response characterised by leaky vessels that allow the extravasation of fluid, lipid and protein exudates, often clinically manifest as diabetic macular oedema [5]. There is also upregulation of pro-inflammatory cytokines such as TNFα, IL-1β and MCP-1 along with various adhesion molecules and integrins, which together recruit inflammatory cells, contribute to ECM breakdown via MMPs, and potentiate the VEGFA-mediated angiogenic response [53]. Concurrently, there is activation of the polyol metabolic pathway in which excess glucose is reduced to sorbitol. Accumulation of intracellular sorbitol is toxic to retinal neurons and pericytes as a result of increased osmotic and oxidative stress [55]. The combined mechanisms of progressive retinal injury and proliferative angiogenesis in DM ultimately lead to vision loss perpetuated by oedema, exudates and haemorrhage.

Beyond the impact of DM, there is emerging evidence that angiogenic processes are linked to obesity and can be substantially altered with ageing. These are issues with increasing prominence particularly in developed countries. Obesity and metabolic syndrome are now conceptualised as states of chronic low-grade inflammation, accompanied by elevated serum levels of pro-inflammatory factors such as TNFα, IL-6, resistin and C-reactive protein [5]. These are secreted in response to signalling from adipocytes and associated macrophages in visceral adipose tissue and likely facilitate inflammatory angiogenesis in other tissues [5]. Yet, adipose tissue itself, with its ability to rapidly expand and regress, is also highly angiogenic. Relevant mediators include VEGFA and FGF2, which can be induced by insulin, as well as leptin, a key regulator of energy balance that is elevated in obesity and has demonstrated pro-angiogenic actions [1]. It is not yet known whether an anti-angiogenic therapeutic approach would prove useful for obesity.

Conversely, ageing is related to significant impairment of physiological angiogenesis. Poorer wound healing and a blunted angiogenic response to ischaemia likely account for the higher risk of vascular complications with increasing age. Indeed, diminished ischaemia-driven expression of VEGFA, VEGFR2 and reduced HIF-1α stability has been reported in aged patients and animal models compared to younger controls [56, 57]. Similar to DM, ageing is also associated with senescence of ECs and EPCs, resulting in reduced production of angiogenic growth factors and impaired capacity for proliferation, migration and vasculogenesis. Cellular senescence is thought to be related to the accumulation of ROS with ageing, leading to inhibition of cyclin-dependent kinases that regulate the cell cycle, as well as decreased telomerase activity and telomere shortening in ECs. Age-dependent loss of sex hormones, particularly oestrogens, may also contribute as these are well-known to be protective against senescence and have pro-angiogenic actions. Conversely, increased ROS and attenuated NO signalling with age likely contribute to endothelial dysfunction, atherosclerosis and exacerbation of pathological angiogenesis [58].

5.4 Targeting Angiogenesis in a Clinical Setting

Given the ubiquity of angiogenic dysfunction in vascular disease, there has been long-standing interest in developing therapies that are able to appropriately suppress pathological angiogenesis or augment physiological angiogenesis. To date, only anti-angiogenic therapies have demonstrated sufficient benefits in randomised trials to justify their routine clinical use, particularly in the setting of cancer chemotherapy and proliferative eye disease. Therapeutic stimulation of angiogenesis by way of genetic manipulation, cell transfer or recombinant agents remains an attractive possibility for the management of ischaemic conditions, though no such therapies have yet achieved widespread clinical translation. Promisingly, various alternatives are also emerging from pre-clinical models that are capable of exerting both pro- and anti-angiogenic effects depending on the conditional context.

5.4.1 Pharmacological Inhibition of Angiogenesis

Anti-angiogenic therapies that primarily target VEGF signalling pathways are currently being used clinically in the therapeutic inhibition of tumour angiogenesis for several malignancies. These therapies primarily target VEGF signalling pathways and include monoclonal antibodies directed against VEGFA or VEGF receptors and various small molecule tyrosine kinase inhibitors (TKIs) that inhibit ligand-activated autophosphorylation of multiple VEGF and PDGF receptors [3, 6, 58]. Bevacizumab, a humanised monoclonal antibody against VEGFA, is the first-line therapy for metastatic colorectal cancer (CRC) [6, 58] and is also effective in the treatment of metastatic non-squamous non-small cell lung cancer (NSCLC), renal cell carcinoma (RCC) and epithelial ovarian cancer when combined with standard chemotherapy regimens [58, 59]. Several TKIs including sunitinib, sorafenib and pazopanib have shown efficacy as monotherapy for metastatic RCC, a tumour type known to express high levels of VEGFA, which renders them rather sensitive to anti-VEGF approaches [6]. Other notable anti-angiogenic agents include aflibercept, a soluble VEGF receptor [58] that has been successfully trialled in addition to standard chemotherapy as second-line therapy for patients with metastatic CRC [60]. Ramucirurab, a monoclonal VEGFR2 antibody, is effective in the treatment of advanced gastric and gastro-oesophageal junction adenocarcinomas, and as second-line therapy for metastatic NSCLC [58, 61].

Beyond cancer therapeutics, anti-angiogenic agents have also been used extensively to treat ocular neovascularisation diseases. In conditions such as wet age-related macular degeneration and diabetic retinopathy, aberrant VEGF signalling plays a central role in stimulating excessive growth of new vessels that are fragile and abnormally permeable [5]. Locally-directed intravitreal anti-VEGFA therapies, such as pegaptanib and ranibizumab, have been effective in slowing the progression of these retinal diseases, and in many cases, leading to

improvements in visual acuity [6]. Intravitreal forms of bevacizumab and aflibercept are also routinely used in this setting [3].

Several non-VEGF-directed anti-angiogenic therapies are also undergoing early phase clinical trials, again predominantly in the cancer setting. These therapies target other key regulators including Ang-2 [58], HGF and its receptor c-MET [58] and the $\alpha_v\beta_3$ integrins [62]. Beyond these, a recombinant form of endostatin, one of the endogenous inhibitors of angiogenesis, as well as several anti-angiogenic gene transfer approaches are also currently being tested as cancer therapies [63].

5.4.2 Limitations of Current Anti-angiogenic Therapies

Despite the successful uptake of anti-angiogenic therapies in cancer therapeutics and proliferative eye diseases, the currently available agents do have several limitations. Anti-VEGF therapies have been associated with a range of adverse effects including hypertension [61, 64], proteinuria [64], higher incidences of haemorrhagic events [64], increased rates of impaired wound healing [64] and a significantly increased risk of both venous and arterial thromboembolic events [65]. This likely reflects inhibition of the physiological role of VEGF in reducing endothelial activation and the attenuation of the adaptive angiogenic response to ischaemia. Beyond these adverse effects, many patients are refractory to current anti-angiogenic therapies. Several cancer types are unresponsive to anti-VEGF treatments altogether, or can develop resistance to VEGF signalling leading to progressive disease [6] while some tumours are able to switch to non-sprouting modes of vascularisation or develop VEGF-independent vessel growth [3, 6]. Tumour-associated fibroblasts and macrophages release chemokines and factors such as SDF-1α, FGF2 and HGF that recruit bone marrow-derived cells to facilitate tumour neovascularisation, bypassing the reliance on VEGF signalling [6]. Moreover, the extended use of anti-angiogenic therapies is postulated to promote the selection of tumour cell clones that are more resistant to hypoxia and less dependent on angiogenesis [3].

For these reasons, there are now renewed research efforts into developing more effective therapies that target multiple angiogenic pathways rather than VEGF alone. Multi-functional agents capable of both promoting and suppressing angiogenesis under specific conditions are also being explored with the hope of avoiding the adverse effects of non-selective anti-angiogenic therapies.

5.4.3 Therapeutic Stimulation of Angiogenesis

While anti-angiogenic therapies have enjoyed relative translational success so far, therapies designed to stimulate angiogenesis in ischaemic cardiovascular conditions are still in their relative infancy. By enhancing vessel growth, these therapies have the potential to improve tissue perfusion and facilitate tissue repair and recovery.

The unmet demand for such therapies is immense. There are many patients who may be refractory to pharmacological risk factor-based therapies or are unsuitable for either surgical or catheter-based revascularisation procedures.

Several approaches have been tested particularly in the setting of myocardial or peripheral limb ischaemia. These include the administration of recombinant angiogenic proteins such as FGF1, FGF2 and various isoforms of VEGFA [66, 67]. Early studies in animal models of MI demonstrated augmentation of collateral blood flow and functionally significant myocardial angiogenesis [66]. The findings in placebo-controlled human trials, however, have been more ambivalent. While intra-arterial delivery of recombinant FGF2 in patients with intermittent claudication modestly improves peak walking time [68], similar intra-coronary infusions of FGF2 in patients with CAD did not produce lasting improvements in myocardial perfusion or exercise tolerance [69]. Intracoronary and intravenous delivery of recombinant VEGFA also failed to significantly alter symptom- and perfusion-based outcome measures in patients with CAD [70].

With the recognition that such recombinant proteins have a relatively short tissue half-life and are generally less targeted therapies, there has been keen interest in developing gene-based approaches instead. These involve transgenes encoding various pro-angiogenic proteins that are delivered via plasmids or adenoviral vectors, the latter allowing for higher transduction efficiency at the expense of potentially greater immunogenicity [66]. In the setting of PAD, intramuscular administration of transgene constructs containing various VEGFA isoforms as well as FGF1, FGF2, HGF and HIF-1α have yielded largely disappointing results in randomised trials [71]. A possible exception to this may be HGF plasmids, which have shown improvements in tissue oxygenation, ulcer healing rates and pain at rest compared to placebo in patients with chronic limb-threatening ischaemia (CLTI) [72, 73]. For patients with CAD, the landscape for angiogenic gene therapy is similar. Numerous trials have been conducted with plasmid and adenoviral-delivered VEGFA isoforms, VEGFC, FGF4, HGF and HIF-1α, but these have not generated consistently successful outcomes [74, 75]. It is at least encouraging that gene therapies have not produced significant adverse safety signals to date, and this has supported the resolve for ongoing development. Proposed ideas for more successful translation include more judicious patient selection, more specific anatomical targeting of ischaemic tissues, the use of adeno-associated or retroviral vectors that may promote more durable transgene expression, and the repurposing of gene therapies as adjuncts following revascularisation procedures [71, 74, 75].

Cell-based therapies have also been keenly investigated as strategies for stimulating angiogenesis. These involve the transplantation of precursor cells with vasculogenic potential, which can then home to sites of ischaemic injury where they differentiate into ECs that can form vessels *de novo* [76]. A number of randomised placebo-controlled trials have been conducted in patients with CLI using autologous bone marrow-derived mononuclear cells injected either intra-muscularly or intra-arterially [71]. These have had mixed success, as earlier studies demonstrated promising improvements in ankle-brachial index, tissue oxygenation, rest pain and maximal pain-free walking time [77, 78], while the largest and most recent of these

trials was negative [79]. Trials of cell therapies have also been undertaken in patients following acute MI or during elective percutaneous coronary intervention. In these settings, intra-coronary transplantation of precursor cells have led to significant improvements in left ventricular ejection fraction and myocardial perfusion in various trials [66]. However, some larger clinical trials have struggled to replicate these findings [76].

At present, the utility of stimulating therapeutic angiogenesis in ischaemic vascular diseases is limited by relative inefficacy and discrepancies in trial findings. There also remains theoretical concern that repeated use of these pro-angiogenic therapies may unintentionally stimulate pathological inflammatory or tumour-associated angiogenesis, though fortunately these effects have not been borne out in clinical trials to date, likely because these therapies are only administered on a short-term basis. Nevertheless, these recombinant proteins, gene and cell-based therapies hold great promise. Further refinement and testing of these agents in large-scale trials will be required before they can be successfully translated to the clinic.

5.4.4 Emerging Angiogenesis-Modulating Therapies

In light of the issues encountered with targeted pro- and anti-angiogenic therapies, attention has shifted towards identifying agents that might have differential effects on angiogenesis depending on the context. Indeed, there is evidence from *in vitro* and *in vivo* studies that some of the currently available lipid-lowering therapies have such effects. Statins, in particular, are mainstays of therapy for cardiovascular disease and are known to have pleiotropic effects independent of their lipid-lowering functions. They have been shown to modulate angiogenesis in a dose-dependent manner, with low doses promoting EC migration, proliferation and capillary tubule formation, while high doses conversely have anti-angiogenic actions in models of atherosclerotic plaque development and tumour growth [80, 81]. Fenofibrate is commonly used to treat patients with dyslipidaemia but has also demonstrated conditional angiogenic effects; inhibiting tumour-associated angiogenesis [82] but can conversely rescue diabetes-related impairment of angiogenesis in murine models of peripheral limb ischaemia [83]. These findings suggest that there may be considerable scope to repurpose currently available pharmacological therapies to take advantage of their beneficial modulation of angiogenesis for a range of vascular diseases.

A wealth of epidemiological evidence has established that serum HDL concentrations are inversely correlated with the rates of cardiovascular disease [84] and cancer [85] while elevated HDL levels are associated with improved survival and prognosis following MI [86, 87]. Recent studies have shown that HDL conditionally regulates angiogenesis; promoting physiological vessel growth in ischaemia but suppressing it in pathological inflammatory contexts [88, 89]. Despite this, therapies designed to increase endogenous serum HDL levels have been largely disappointing in clinical trials with respect to reducing the risk of major adverse

cardiovascular events. This is thought to be related to endogenous HDL particles that have been rendered dysfunctional via chemical modifications such as oxidation or glycation in the setting of atherosclerosis or diabetes [90]. There is now keen interest, therefore, in the use of purified rHDL infusions and analogues of apoA-I instead. Embedding of apoA-I or rHDL onto endovascular stent platforms is also being investigated, as these moieties may improve stent biocompatibility by supporting rapid re-endothelialisation while inhibiting neointimal hyperplasia, stent thrombosis and neoatherosclerosis [91, 92].

microRNAs (miRNAs), small non-coding RNAs that post-transcriptionally regulate gene expression, are the leading next generation biopharmaceuticals for the treatment of complex multi-faceted diseases [93]. The importance of miRNAs in vascular development and angiogenesis was first observed when the critical miRNA processing enzyme Dicer was inhibited with embryonic lethality observed due to an underdeveloped vascular system [94]. miRNAs that regulate angiogenesis by targeting angiogenic genes include miR-34a, miR-124, miR-29, miR-126, miR-150, miR-221/222 and miR-17–92 cluster [95]. miRNAs that are modulated by pro- or anti-angiogenic factors include miR-483-3p, miR-21, miR-210, miR-296, miR-93, miR-206, miR-26, miR-155, miR-424, miR-27b and miR-130a [95]. It is therefore likely that the pleiotropic action of targeting specific pro- or anti-angiogenic miRNAs will give significant therapeutic advantages over single gene-targeted therapies currently in clinical use. Indeed, miRNA targeting drugs are already showing promise in Phase I and II clinical trials in a wide range of diseases [96]. Furthermore, miRNAs can be released from the cell into the bloodstream and are extremely stable in the extracellular environment, where they can be taken up within tissues [97]. Circulating miRNAs have emerged as a new class of disease biomarkers [98]. Patients with CAD had reduced levels of angiogenesis-associated miRNAs including miR-126, miR-17 and miR-92a and miR-155 [99]. In individuals with diabetes-associated PAD, lower circulating miR-126 levels were found to be associated with lower ankle-brachial index while levels of the anti-angiogenic miRNAs miR-15a and miR-16 were elevated and predicted the occurrence of amputation in CLI patients [100]. It is likely that the role of miRNAs as both potential therapeutic targets and clinical diagnostic markers will provide an alternate approach that can either complement current therapies or open new avenues for better informed clinical diagnosis and targeted personalised therapies.

5.5 Conclusion

Significant advances have been made in our understanding of angiogenesis and the cellular and molecular factors that modulate it under both physiological and pathological conditions. While angiogenesis is a fundamental and adaptive process in development, wound healing and ischaemic conditions, dysregulated angiogenesis is also a pathological hallmark of cancer, atherosclerosis, proliferative eye disease and many other inflammatory conditions. Targeting angiogenesis as a therapeutic

approach for vascular disease has so far been fraught with issues, not least of which is the risk of inadvertently exacerbating pathological angiogenesis while trying to augment physiological angiogenesis, and vice versa. Indeed, therapeutic angiogenic stimulation is not yet a clinical reality, and anti-angiogenic therapies, though more common, still have numerous limitations. New strategies therefore demand consideration. These include more detailed characterisation of the molecular factors and agents capable of differentially modulating angiogenesis in different contexts, refining novel methods to deliver existing therapies in a more targeted fashion, and the discovery of new gene- and cell-based technologies. Appropriately harnessing these will be crucial for the ongoing battle against a range of vascular diseases which contribute immensely to morbidity and mortality worldwide.

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