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Jawahar L. Mehta
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Biochemical Basis and Therapeutic Implications of Angiogenesis

Second Edition

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Preface

The previous edition of the book *Biochemical Basis and Therapeutic Implications of Angiogenesis* was published in 2013. It included several chapters on the pathophysiology of angiogenesis and the clinical application of the information derived from several laboratories around the world. The book included several chapters devoted to the therapeutic implications of growth factors, endothelial progenitor cells, microRNA, and angiogenesis inhibitors derived from natural sources.

The editors were told by the publisher that the book was immensely successful and received over 35,000 downloads since its publication. The publishers asked us to develop a second edition of the book wherein we would have updated information on the pathogenesis of atherosclerosis, underlying mechanism, and therapeutic applications of this information.

It was a major task to ask some of the authors of previous edition to update their chapters to include state-of-the-art information, and to ask new authors to contribute novel information.

In the last several years, a number of investigators have described various signals and pathways leading to the evolution and persistence of angiogenesis. These include a number of growth factors, receptors, redox state, and pro-inflammatory state. Based on microarray technology, a host of known pathways leading to angiogenesis have been confirmed and novel pathways postulated.

As our understanding of the role of various triggers and inhibitors of angiogenesis has expanded, several novel therapies for a myriad of disease states have been proposed. For example, angiogenesis inhibitors have been approved and are used to arrest the growth of some tumors. On the other hand, a number of trials have been conducted to test the value of pro-angiogenic factors in myocardial ischemia. The results of these studies are not consistent and not as revealing as once thought.

We thought it timely to get several world-class experts on different aspects of angiogenesis to present their work again in one book. While this book does not cover each and every aspect of angiogenesis, it covers most relevant issues in the biology of this interesting phenomenon that plays a critical role in physiology and pathology. We have organized it in five sections. The chapters in the first part of this book mainly deal with the role of growth factors, neuropeptides, and signal

transduction mechanisms as well as cellular regulation by mast cells, integrins, and stem cells. The second part of this book deals with the role of angiogenesis in cancer. The third part addresses the therapeutic implications of angiogenesis in eye disorders. The fourth part deals with the therapeutic implications of angiogenesis in cardiovascular disorders and peripheral vascular disease. The last part deals with therapeutic implications of angiogenesis in miscellaneous disease states such as diabetes and stroke.

We are grateful to Mr. Sheik Mohideen and Ms. Merry Stuber at Springer for their continuous advice and understanding during the editorial process. Finally, we thank the authors for contributing the best of their work for inclusion in this book. We hope the readers will find this compilation of work from several laboratories useful in understanding the pathobiology of angiogenesis and designing new therapies. Dr. Mehta would like to thank his wife, Paulette, and children, Asha and Jason, for their eternal support, and his colleagues for inspiration and all their help over the years.

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Molecular Mechanisms in Angiogenesis

Chapter 1
Endothelial Growth Factor Receptors in Angiogenesis

David J. Bruce and Peng H. Tan

Abstract It is hard to underestimate the role of endothelial growth factor receptors in the generation of new blood vessels. This axis is involved in vascular development in embryos and angiogenesis in adults. As the signaling of these tyrosine kinase receptors has been elucidated, we have gained an appreciation of the complex interactions with other receptors, co-receptors, and downstream pathways.

Its involvement in pathology makes it a particularly tempting therapeutic target with its manipulation offering several theoretical benefits. The most intensely studied is the role of anti-VEGFR drugs in cancer chemotherapy. Initial trials were disappointing but a decade ago the first drug targeting the vascular endothelial growth factor (VEGF) axis was approved, providing a vital proof of concept. Therapies specifically targeting the receptor are in early development for prevention of neovascular diseases of the eye. Conversely, promotion of revascularization following vascular occlusion is another possible application being studied.

While these therapies show promise, the manipulation of VEGF receptors themselves remains a relatively small niche in the therapeutic armory. A deeper understanding of the receptor, its co-receptors, and the downstream web of signaling is required to complete the pieces of the puzzle and unlock the potential of this receptor pathway.

Keywords Angiogenesis • VEGFR • Neovascularization • Tumorigenesis • Growth factor • Signaling interactions • Tyrosine kinase inhibitor • Receptor

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Table 1.1 Growth factors, receptors, and co-receptors involved in angiogenesis [4, 5]

Receptor	Role
VEGFR	Receptor for VEGF, VEGF, PlGF
Tie-1, Tie-2	Receptor for angiopoietin (Ang)
FGFR	Receptor for fibroblast growth factor (FGF)
PDGFR	Receptor for platelet-derived growth factor (PDGF)
NRP	Co-receptor for VEGFR
HSPG	Co-receptor for VEGFR

1 Introduction: The Signaling Axes Behind Angiogenesis

Angiogenesis begins with the remodeling of the initial lattice of vascular endothelial cell precursors, which results in differential growth of the vessels to form branches and sprout new vessels. This requires coordinated communication within the preliminary lattice of homogenous vessels. Intercellular signaling continues to play a part in the recruitment of supporting cells including smooth muscle, pericytes, and fibroblasts [1] and in the breakdown and deposition of the extracellular matrix [2]. This development and remodeling of the vascular network is controlled by the interaction of angiogenic growth factors with their receptors and the subsequent downstream signaling networks [3] (Table 1.1).

One of the most important and closely studied of these is the vascular endothelial growth factor (VEGF) pathway which plays a central role. This signaling cascade influences numerous cellular events involved in angiogenesis including endothelial cell proliferation and migration, remodeling of the extracellular matrix, increased vascular permeability, and survival of new blood vessels [6]. In addition, the angiopoietin- Tie pathway has been identified as a second vascular tyrosine kinase system that is essential during vasculogenesis and adult vascular homeostasis [7].

This chapter will aim to cover some of the evidence for the role of the endothelial growth factor receptors in angiogenesis and the interactions of these receptors with complementary signaling pathways. The therapeutic potential of VEGF receptor manipulation will be discussed, with particular emphasis on antitumor therapies where most work has been focused.

2 The VEGF Axis

The VEGF family of signaling molecules was originally identified as a potent mediator of vascular permeability [8] but is now known to stimulate vasculogenesis in embryos and angiogenesis in adults [9]. The downstream pathways form a complex network involving cross talk with other signaling axes [10]. The end result of these

pathways is an increase in vascular permeability and the stimulation of cell survival, proliferation, and migration, ultimately leading to angiogenesis or lymphangiogenesis [3, 9, 11, 12].

2.1 VEGF Structure and Function

VEGF is part of the cysteine knot growth factor superfamily [13] and is found in mammals as a family of structurally homologous secreted glycoproteins: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF). VEGF is usually secreted as a dimeric glycoprotein [9].

VEGF-A is the best characterized of this family. The *VEGF-A* gene is found on chromosome 6 (locus 6p21.1) [14], and alternative exon splicing produces six isoforms (Table 1.2). All of them contain binding domains for the receptors VEGFR-1 and VEGFR-2 [15], but it is the splicing of exons encoding different C-terminal domains that gives each isoform their own unique biochemical properties [14]. Proteolytic processing and other posttranslational modifications act to further refine VEGF activity [17].

The different isoforms of VEGF have different solubility and bioavailability. This is due to the ability of VEGF to tether to the extracellular matrix to varying degrees, allowing it to act in a paracrine fashion [18]. The effect of the localization of VEGF is reflected in embryos expressing a VEGF splice variant lacking heparin- binding and ECM interaction domains. The disruption of VEGF-A signaling, due to failure to generate concentration gradients, leads to endothelial cells failing to form additional branches and impaired filopodia function [19]. Matrix metalloproteinases (MMP) cleave VEGF-A releasing the receptor binding domain. Blocking this activity can arrest the angiogenic switch associated with carcinogenesis [20].

Table 1.2 Isoforms of human VEGF-A [15, 16]

Isoform	Receptors and co-receptors	
VEGF 121	VEGFR1, VEGFR2	Predominant in humans. Secreted into systemic circulation
VEGF 145	VEGFR1, VEGFR2, HSPG	
VEGF 165	VEGFR1, VEGFR2, NRP, HSPG	Predominant in humans
VEGF 183	VEGFR1, HSPG	
VEGF 189	VEGFR1, HSPG	Bound to ECM proteoglycans
VEGF 206	VEGFR1, HSPG	Bound to ECM proteoglycans

Table 1.3 The VEGFR receptors and their ligands [16]

Receptor	Gene	Ligands	Localization
VEGFR1	*FLT-1*	VEGF-A, VEGF-B, PlGF	Vascular endothelium
VEGFR2	*KDR*	VEGF-A, VEGF-C, VEGF-D, VEGF-E	Vascular and lymphatic endothelium
VEGFR-3	*FLT-4*	VEGF-C, VEGF-D	Lymphatic endothelium

2.1.1 The VEGF Receptors

VEGF binds to a family of receptor tyrosine kinases, known as the VEGF receptors (VEGFRs). There are three characterized VEGFRs (Table 1.3) which are structurally similar, with an extracellular ligand binding domain, a transmembrane helix, and a cytoplasmic region containing a kinase domain (Fig. 1.1) [22]. When the ligand has bound the extracellular domain, the intracellular domain autophosphorylates, and the receptors dimerize. VEGFRs activate various downstream signaling pathways (Fig. 1.2) [9]. The VEGFRs are expressed by endothelial cells with each member of the family showing distinct expression patterns, as revealed by in situ hybridization and northern blot [23].

VEGFR-1

VEGFR-1 binds VEGF-A and is the only known cell surface receptor for VEGF-B and PlGF [21]. While VEGF-A binds VEGFR-1 with higher affinity than VEGFR-2, the tyrosine kinase activity of VEGFR-1 is weaker than VEGFR-2. It has been suggested that VEGFR-1 acts either as a "decoy" receptor to reduce the bioavailability of VEGF-A by forming inert complexes or as a dominant antagonist of the axis [24]. Additionally, PlGF may act by displacing VEGF-A from VEGFR-1, thus increasing VEGF-A bioavailability [25]. Both in vitro fluorescence studies and in vivo experiments showed that cells which lack VEGFR-1 had decreased sprout formation and reduced migration. However, a soluble isoform of VEGFR-1 (sVEGFR-1) rescued angiogenesis indicating that VEGFR-1 may have a positive regulatory role, possibly related to its effect on the localization of VEGF [12]. This effect has been noted in $FLT^{-/-}$ mutant vessels, where both membrane-bound and soluble forms of VEGFR-1 rescued aberrant endothelial proliferation but only soluble VEGFR-1 rescued vessel branching. Hence, it may be that heterogeneous sVEGFR-1 expression underlies the spatial variation of VEGFR-2 signaling which are required for correct branching development [26].

Heterodimers form between VEGFR-1 and VEGFR-2, which may augment certain signaling pathways. In cell lines expressing both receptors, signaling pathways leading to PLCγ activation were enhanced, and these cells showed more efficient migration towards VEGF-A compared to cells expressing VEGFR-1 alone [27].

VEGFR-2

VEGFR-2 is vital to angiogenesis. In murine models, knockout of VEGFR-2 leads to aberrant vascular development and embryonic lethality [10]. VEGFR-2 binds VEGFR-A, VEGF-C, VEGF-D, and VEGF-E. It is found on both vascular and

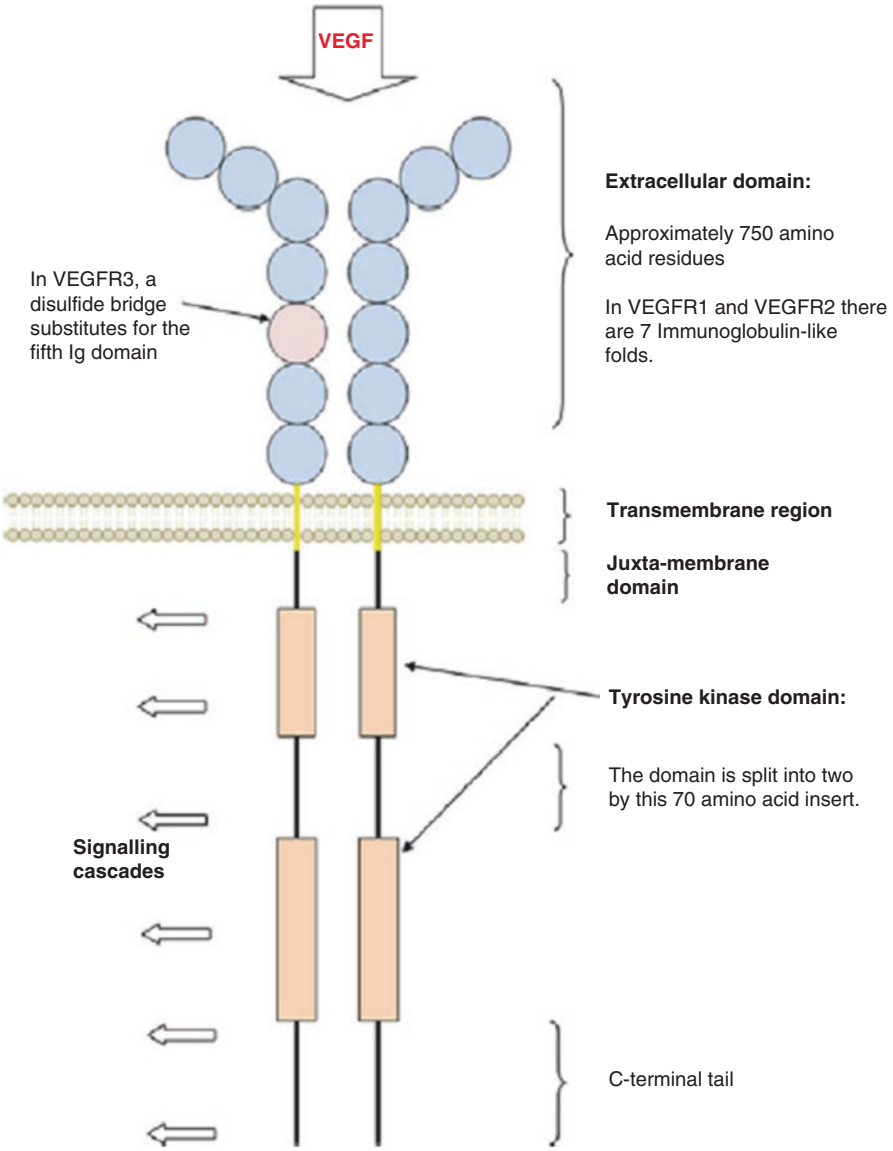

Fig. 1.1 The structure of VEGF receptor (*VEGFR*). The VEGFR has an extracellular domain that consists of seven immunoglobulin (Ig)-like domains, except in VEGFR3 where the fifth domain is replaced by a disulfide bridge. The intracellular domain consists of a tyrosine kinase domain, interrupted by a 70 amino acid insert, a juxta-membrane region, and a C-terminal tail. The binding of signaling molecules to phosphorylation sites present on the intracellular domain initiates cell signaling (see Fig. 1.2). Studies of the crystal structure of VEGFR1 show that the second Ig domain is the ligand binding site. The third Ig domain determines ligand binding specificity in VEGFR2 [11]

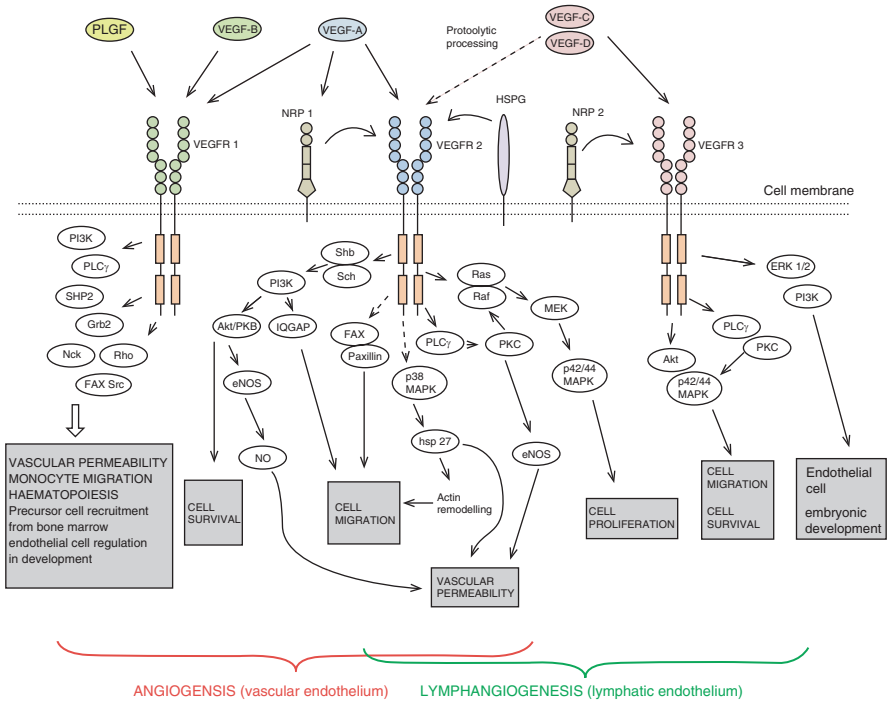

Fig. 1.2 VEGFR signaling pathways. VEGF ligands bind to particular VEGFR homodimers and heterodimers. Co-receptors may modify this interaction, such as the neuropilins and heparan sulfate proteoglycans. VEGFR1 is found on vascular endothelium, VEGFR3 is found on lymphatic endothelium, and VEGFR2 is found on both. These signaling pathways stimulate cell survival, proliferation, and migration and increase vascular permeability, ultimately leading to angiogenesis or lymphangiogenesis (Adapted from Refs. [11, 21])

lymphatic endothelium [16]. VEGFR-2 has at least four autophosphorylation sites. The tyrosine residues Tyr1054 and Tyr1059 are required for maximal kinase activity [28], and Tyr1175 is known to be crucial for PLCγ/PKC/MAPK signaling which is necessary for endothelial cell proliferation [29]. Tyr1175 is also bound by the adaptor protein Shb, and inhibition of Shb reduces PI3K activation and subsequent VEGF-induced cell migration [30].

A number of pathways are known to be necessary for VEGFR-2-mediated cell migration. Two complementary pathways mediate VEGFR-2 activation of FAK; one involves the Src kinase while the other involves RhoA and ROCK. This in turn initiates paxillin and vinculin recruitment [31].

Studies on human umbilical vein endothelial (HUVEC) cells indicate that VEGFR-2 is involved in the regulation of the actin cytoskeleton via Ras activation. Inhibition of this signaling pathway inhibits branching morphogenesis. The Ras pathway mediates ERK activation which is required for VEGF-mediated cell proliferation [32]. ERK signaling is also involved in promoting cell survival and proliferation but is inhibited by p38MAPK suggesting cross talk between these pathways influences

the cellular response to angiogenic stimuli [33]. VEGF-mediated activation of cdc42 and p38MAPK regulates actin polymerization and stress fiber reorganization leading to endothelial cell migration via heat shock protein 27 activation [33].

In HUVEC cells, VEGFR-2 co-localizes with the scaffolding protein IQGAP1 to the leading edge of migrating cells. IQGAP1 interacts with the cell cytoskeleton to manipulate cell motility and morphogenesis [34].

In summary then, VEGFR-2 plays a vital role in endothelial cell survival, proliferation, and migration and stimulates vascular permeability and invasion. These processes are crucial for angiogenesis [16].

VEGFR-3

VEGFR-3 in adults is associated with the lymphatic endothelium. It is found to be expressed exclusively on lymphatic endothelial and some high endothelial venules [35].

There are two splice variants in humans and both have a high affinity for VEGF-C and VEGF-D [36]. VEGFR-3 forms homodimers or heterodimers. The binding of VEGF-C induces VEGFR-2/VEGFR-3 heterodimer formation, and the function of the regulatory tyrosine phosphorylation sites differs in the various ligand-induced dimerized complexes [37]. VEGFR-3 is responsible for inducing cell migration and prevents apoptosis via Akt and p42/p44MAPK [38]. Pathways involving PKC, ERK1/2, PI3K, PLCγ, SHP2, and the transcription factors STAT3 and STAT5 are all influenced by VEGFR-3 signaling [9].

Murine studies have shown lethal defects in vasculogenesis, severe anemia, and cardiac effusion in VEGFR-3-deficient embryos. This may be due to a direct result of reduced VEGFR-3-mediated signaling. However, it is also possible that these effects are a result of cross talk with VEGFR-2, perhaps by the reduction of VEGF-C bioavailability to VEGFR-2 due to binding of VEGF-C to VEGFR-3 [39]. It is thought that VEGFR-3 may negatively regulate VEGFR-2 signaling [40]. VEGFR plays an important role during angiogenic sprouting, where endothelial cells develop as either tip or stalk cells. VEGFR-3 has also been implicated in reinforcing Notch signaling in tip cells of angiogenic sprouts and thus influencing tip to stalk cell conversion [41].

3 Co-Receptors

VEGFR activity may be modulated by co-receptors. Neuropilin (NRP) is a transmembrane glycoprotein involved in neuronal axon guidance but is also known to act as a co-receptor for VEGFR. However, the mechanism underlying the enhanced signaling remains uncertain. It may be that VEGFR2/NRP1 complex formation increases VEGFR affinity for VEGF, or that the intrinsic catalytic activity of the kinase domain is enhanced, or the signaling complex may be stabilized with a prolonged half-life. While there is still some disagreement in the literature, it is likely that these receptor complexes are induced by VEGF binding [9, 42]. Furthermore, VEGF may directly bind NRP, and while NRPs lack intrinsic catalytic activity, they may associate with other transmembrane proteins to stimulate signal transduction [9]. This potential

alternative signaling pathway may be a mechanism for resistance to anti-angiogenic therapies, as supported by the augmentation of the effects of Bevacizumab when used in combination with anti-NRP antibodies [43].

Heparan sulfate proteoglycans (HSPGs) have been identified as co-receptors to VEGFR during angiogenesis and vasculogenesis, where it acts to augment the duration and magnitude of the response and influences localization of VEGF/VEGFR complexes. In situ studies showed a direct interaction between HSPG and VEGFR, and blockade of HSPG interactions inhibited in vivo hyperpermeability. This supports the possibility of targeting HSPGs in ischemic disease [44]. Chondroitin sulfate proteoglycans bind angiogenic growth factors, and their production is increased in cells lacking HSPGs in vitro. It appears that there may be functional overlap with HSPGs during sprouting angiogenesis but the in vivo implications of this interaction remain unclear [45].

4 The Role of the Endothelial Growth Factor Receptors in Angiogenesis and Neovascularization

4.1 The Role of the Endothelial Growth Factor Receptors in Tumorigenesis

Tumor growth is restricted by the availability of a suitable vascular supply, and angiogenesis is required for a tumor to develop beyond a few millimeters in diameter. In vivo anti-VEGF antibody administration inhibits tumor growth. The VEGF-VEGFR pathway may also exert a direct effect on the proliferation and growth of tumor cells themselves [16]. However, immunohistochemical analyses of several human cancers have indicated that VEGFR-2 and VEGFR-3 are not expressed by tumor cells. Hence, animal models and in vitro experiments should be interpreted with caution [46].

4.1.1 VEGFR-1

VEGFR-1 is known to be upregulated in several tumors. In pancreatic cancer cells, VEGFR-1 stimulated upregulation of transcription factors associated with motility and invasion [47], and a similar effect is seen in colonic cancers [48]. In vitro multiple myeloma cells were found to express only VEGFR-1, and ablation of VEGFR-1 signaling was sufficient to inhibit cell proliferation and motility [49]. Other tumors in which VEGFR-1 is upregulated include prostate, glioblastoma, and malignant melanoma [16].

sVEGFR-1 has been identified in breast, pancreatic, lung, and ovarian cancers and leukemias [16]. sVEGFR-1 shows antitumor effects when administered in vitro and in vivo. This is likely due to its interception of VEGF-A. In a rat hepatocellular carcinoma model, tumor weight was decreased 19-fold in cells transduced with sVEGFR-1 [50]. Consequently, it may be that the soluble isoform of VEGFR-1 could be developed for cancer treatment.

4.1.2 VEGFR-2

A rat anti-VEGFR-2 antibody, known as DC101, inhibits the spread and growth of metastases in several models via apoptosis of vascular endothelial cells [51]. In vitro, DC101 inhibits neovascularization, while in vivo models showed inhibited growth of several cancers including breast, melanoma, and both primary and secondary lung tumors. Growth was also repressed in human xenografts of epidermoid, glioblastoma, pancreatic, and renal cancers [52]. In a murine lymphoma model, inhibition of either VEGFR-1 or VEGFR-2 led to altered vessel growth and development. However, inhibition of both was required for tumor regression [53] supporting the use of multi-targeted inhibitors in antitumor therapies.

4.1.3 VEGFR-3

VEGFR-3 is a key receptor in lymphangiogenesis and is strongly expressed in human tumours including lung, cervical, breast, prostate and colorectal cancers. Higher levels of both VEGF-C and VEGFR-3 correlate with increased metastases and shorter survival [54]. VEGFR-3 is upregulated on the tumor vasculature but poorly expressed in cells from a number of human tumors. Therefore, it is unlikely to affect the tumor cells directly [46]. Overexpression of VEGF-C induced hyperplasia in peri-tumor lymphatics and led to an increased lymph flow rate, an effect which was suppressed by anti-VEGFR-3 antibody administration [55].

4.1.4 Prognosis

VEGFR-1 and VEGF expressions were found to correlate closely to microvessel density and were important predictors of poor prognosis and clinical progression in nephroblastoma [56]. Similarly, VEGFR-1 and VEGFR-2 were upregulated in glioblastoma vascular cells, but not in low-grade blastoma [57]. In non-small cell lung cancer patients, co-expression of VEGFR-1 and VEGF was associated with a shorter survival time. Co-expression of VEGFR-1 and VEGFR-2 was an independent prognostic factor [58]. Thus, VEGF/VEGFR signaling could potentially provide useful prognostic information in the clinical setting.

4.1.5 Metastasis

Tumor angiogenesis is preceded by recruitment of endothelial precursor and hematopoietic cells which may act to “prepare” microenvironments for metastatic spread [59]. VEGFR-1 induces MMP9 which degrades ECM to allow remodeling. The upregulation of MMP9 in healthy lung appears to promote lung metastasis, but this effect was abolished in VEGFR-1 knockout mice [60]. However, the rate of metastases formation in murine models was not affected by VEGFR-1 blockade, which

may be due to alternate signaling pathways for inducing the "pre-metastatic niche" [61]. In human gastric cancer patients, the rate of metastasis was increased in those patients expressing high levels of VEGFR-1 in bone marrow and blood [62]. Further work is needed to elucidate the precise role of VEGFR-1 in metastatic spread.

4.1.6 Autocrine Signaling

VEGF may act in an autocrine or paracrine manner to amplify the malignant potential of cells co-expressing VEGFR-1 and VEGFR-2 [58]. In a study of myelodysplastic patients, monocyte and myeloid precursor cells were found to co-express VEGFR-1 or VEGFR-2 in a majority of patients. It is speculated that VEGF may act in an autocrine fashion to promote leukemia cell survival [63]. Autocrine signaling has also been suggested to occur with VEGF/VEGFR-1 signaling present on breast cancer cells to increase cell invasion [64]. Autocrine signaling via the VEGF-VEGFR2-NRP1 axis has been suggested as a mechanism of resistance to anti-angiogenic therapies, associated with VEGFR2-NRP1 recycling and a pool of VEGFR2 present in the cytosolic compartment of glioblastoma multiforme (GBM) cells. Bevacizumab transiently reduces GBM tumor growth, but in vitro inhibition of VEGFR2 attenuates glioma stem-like cell viability, thus suggesting a possible role of VEGF receptor inhibition to augment current anticancer therapies [65].

4.1.7 Therapeutic Applications

Tumor cells are inherently heterogeneous and genetically unstable. The high mutation rate leads to the evolution of resistant cell lines. Endothelial cells are more stable with a lower mutation rate, although there is data to suggest that tumor endothelial cells may be derived from tumor stem cells. In a murine in vivo model, repeated administration and discontinuation of an angiogenesis inhibitor did not lead to drug resistance, and tumors remained dormant for longer following repeated treatment. However, there has been a lack of reproducibility of these findings [66]. Nevertheless, inhibitors of angiogenesis provide an intriguing method of restricting tumorigenesis.

Murine models of VEGFR-2 and VEGFR-3 inhibition show a reduction in metastases in lymph nodes and lung. However, simultaneous inhibition of both has a more potent effect [67]. In another experiment, gene therapy-mediated inhibition of VEGFR-1, VEGFR-3, Tie-1, and Tie-2 led to significantly decreased ovarian tumor mass [4]. Thus, targeting multiple VEGF receptors and interacting pathways, such as the angiopoietin network, may increase the efficacy of treatment.

Inhibition of VEGFR in tumors may help to normalize the vasculature and allow effective delivery of drugs and decrease resistance to radiotherapy. Blockade of VEGFR-2 creates a "normalization window" during which period combined treatment led to the most marked tumor regression in murine brain tumors [68]. However, excessive destruction of tumor vessels may hinder drug delivery and generate undue hypoxia. The inhibition of VEGFR2 leads to angiopoietin-1 upregulation, pericyte

proliferation, and MMP activation and the breakdown of pathologically thick vascular basement membrane. Measurement of the levels of hypoxia could allow optimization of the schedules of anti-VEGFR and radiotherapy combination therapy [69].

Several small-molecule tyrosine kinase inhibitors (TKIs) have entered clinical development. These inhibitors were originally identified by screening large peptide libraries, but more recently the use of molecular modeling analysis using X-ray crystallographic data has provided a more targeted approach. The TKIs commonly compete for the ATP-binding site within the tyrosine kinase, thus ablating phosphorylation and consequent downstream signaling. The ATP-binding region within the kinase domain is well conserved between several receptor tyrosine kinase families; thus, several VEGFR TKIs have been found to also inhibit other receptor pathways [70].

Initial trials of inhibitors were disappointing with several drugs failing in clinical trials. However, the field has been reinvigorated following the successful licensing of the VEGF inhibitor Bevacizumab which provided the proof of concept for the field. Several inhibitors of the VEGF receptor are in development and a number of licenses have been granted for their use (Table 1.4) [70]. A TKI known as SU11248 (Sunitinib) which targets class II/V RTKs, including VEGFR, PDGFR, c-kit, and FLT3, has been licensed for gastrointestinal stromal tumors [71] and renal cell carcinoma [72] following successful Phase III trials. Further trials are underway looking at its potential use in breast, non-small cell lung, and hepatocellular cancer [70].

Table 1.4 Some antitumor VEGFR inhibitors in clinical development [70]

Drug	Trade name	Target receptors	Trial phase
AG-013736	Axitinib	VEGFR, PDGFR, c-kit	III
AMG-706	Motesanib	VEGFR, PDGFR, c-kit	III
ZD2171	Cediranib/Recentin	VEGFR, PDGFR, c-kit	III
PTK787	Vatalanib	VEGFR	III
BMS582664	Brivanib	VEGFR, FGFR	III
SU11248	Sutent/Sunitinib	VEGFR1, VEGFR2, FLT3, PDGFR, c-kit, cRET	IV licensed for use in gastrointestinal stromal tumors and renal cell carcinoma
ZD6474	Vandetanib	VEGFR1, VEGFR2, EGFR1	III
TKI 258	Dovitinib	VEGFR1, VEGFR2, PDGFR, FGFR, c-kit	III
BAY43-9006	Sorafenib/Nexavar	VEGFR2, VEGFR3, PDGFR, c-kit, FGFR1, B-raf	IV licensed for use in hepatocellular carcinoma and renal cell carcinoma

The multi-targeted nature of SU11248 is a key factor in its efficacy. This is demonstrated by the selective VEGFR inhibitor called SU10944 in combination with Gleevec (a PDGF receptor inhibitor) which showed similar in vitro and in vivo activity compared to SU11248. However, when only one of SU10944 or Gleevec was given, the efficacy was significantly inferior [73]. Another successful VEGFR inhibitor is BAY 43-9006 (Sorafenib) which was originally developed to inhibit Raf-1 and the RAF/MEK/ERK pathway. Again, BAY 43-9006 may owe its efficacy to the inhibition of several RTKs. BAY 43-9006 prolonged patient survival in Phase III trials in hepatocellular carcinoma and renal cell carcinoma [70], but at present its prohibitive cost has limited its use [74]. In orthotopic murine models of human non-small cell lung cancer, MEK inhibition in combination with the VEGFR inhibitor Cediranib led to superior anti-angiogenic and antitumor effects [75].

More specific targeting of VEGFRs can be achieved by the use of monoclonal antibodies. The use of monoclonal antibodies to target VEGF/VEGFR signaling has seen success in Bevacizumab, a monoclonal antibody to VEGF-A, which is licensed for use in several cancers [76]. The anti-VEGFR antibody DC101 has been shown to reduce tumor growth in murine models of colon cancer [77]. However, results of VEGFR-targeted antibodies have been less successful in humans. One of the most advanced is IMC-1121B (Ramucirumab) which entered Phase II trials for gastric cancers, breast cancer, hepatocellular carcinoma, and non-small cell lung cancer [70]. The use of small interfering RNA (siRNA) to depress VEGFR-2 expression has been shown to reduce invasion in vitro, but the use of this strategy remains a distant prospect [78].

One advantage of VEGFR inhibition is the anticipation of a relatively favorable toxicity profile compared to conventional chemotherapy. Life-threatening adverse events associated with cytotoxic agents have been rarely seen with angiogenesis inhibitors [79]. Side effects may be due to the inhibition of VEGF signaling or off-target effects, for example inhibition of other kinases. Off-target effects are more dependent on patient- or treatment-specific factors, such as comorbidities and disease stage, whereas the on-target effects tend to be seen with all TKIs [11]. While multi-targeted TKIs have shown more efficacy in the treatment of tumors, this is associated with an increase in off-target adverse events [80]. Therefore, a careful balance must be met between improving efficacy and minimizing toxicity.

4.2 The Eye

VEGF-A is produced by retinal pigment epithelium (RPE) in humans. There is limited knowledge of the role of VEGF in the maintenance of the adult ocular vasculature, but VEGF-A may act in a paracrine fashion between the RPE and choriocapillaries. Currently, inhibition of VEGF does not appear to have adverse effects on the ocular vasculature [81]. However, intraocular angiogenesis and changes in vascular permeability underlie the development of retinal vascular disorders including retinal vein occlusion, diabetic retinopathy, and age-related macular degeneration

(AMD) [82]. Disease activity reflects the balance between pro-angiogenic and anti-angiogenic factors. There is impairment of vascular autoregulation, macular edema, and aberrant development of retinal vessels [83]. Levels of VEGF-A are high in these conditions. In animal models, specific isoforms of VEGF-A are increased in certain disorders. Tissue displaying choroidal neovascularization in patients with AMD expresses both $VEGF_{121}$ and $VEGF_{165}$ isoforms of VEGF-A [81]. $VEGF_{164}$ (the murine orthologue of $VEGF_{165}$) is a potent in vivo inducer of angiogenesis and inflammation in the eye. It stimulates ICAM-1 expression on endothelial cells via VEGFR2 and chemotaxis of monocytes via VEGFR1. In vitro $VEGF_{165}$ induces activation of human VEGFR1 more efficiently than other isoforms [84].

Soluble VEGFRs are found in the vitreous in patients with vitreoretinal disease. sVEGFR-1 levels were found to increase with age, and lower levels were associated with more active proliferative diabetic retinopathy. This suggests that sVEGFR may be a useful tool in tipping the balance to hinder neovascularization [82]. However, it has also been suggested that increased levels of sVEGFR-2 in the vitreous may contribute to increased vascular permeability in macular edema. Further work is required to elucidate the role of sVEGFR in the eye and evaluate its potential for therapeutic use [83].

4.2.1 Therapeutic Applications

VEGF-inhibition is known to be effective for treatment of ocular diseases involving neovascularization, and anti-VEGF treatments have been licensed for use. Pegaptanib (Macugen) is a ribonucleic aptamer which selectively binds $VEGF_{165}$ and thus attenuates VEGFR2 activation. The VISION trials validated the use of Pegaptanib in neovascular AMD with significantly reduced loss of vision compared to placebo. Subsequently, the VEGF inhibitors Ranibizumab (Lucentis) and Bevacizumab (Avastin) were developed, although only Ranibizumab is FDA approved. Both agents are derived from the same murine anti-VEGF mAb, but Ranibizumab consists of the Fab fragment and has a binding affinity 20-fold greater than Bevacizumab. The MARINA trial of Ranibizumab not only showed a reduction of vision loss but an improvement in acuity in 1 in 3 patients. Ranibizumab is superior to the previous benchmark, photodynamic therapy, and it has shown a favorable safety profile in Phase IV trials. Bevacizumab has shown similar efficacy but is not FDA approved, largely due to financial implications for the pharmaceutical industry [85].

A number of other therapies targeting the VEGF/VEGFR pathway in the eye are under investigation. VEGF trap is a fusion protein containing the VEGFR1 and VEGFR2 binding sites and thus inhibits VEGF-A and PlGF [81]. A Phase I/II study of intravitreal administration to 21 patients showed improvements in vision, and Phase III trials are currently underway [85].

Inhibitors of the VEGFRs themselves are also in development, and small-molecule TKIs have shown some promise in preclinical models of ocular neovascular disease. PTK/ZK is a TKI which acts on all the VEGFRs. A murine model of ischemia-induced

Table 1.5 Tyrosine kinase inhibitors (TKIs) in development for eye disease

TKI	Route	Sponsor
Pazopanib	Topical	GlaxoSmithKline, PA, USA
TG100801	Topical	TargeGen, CA, USA
TG101095	Topical	TargeGen, CA, USA
AG013958	Sub-Tenon	Allergan, CA, USA
AL39324	Intravitreal	Alcon, TX, USA
PTK787 (Vatalanib)	Oral	Novartis, CH

retinopathy showed that intravitreal injection of this TKI significantly reduced angioproliferative retinopathy [86]. Another TKI named SU5416, which selectively inhibits VEGFR2, also inhibits neovascularization in mouse cornea [87]. Subsequently, several TKIs have entered early clinical trials (Table 1.5). Unfortunately, a Phase I/II study of the TKI AG-013958 for the treatment of choroidal neovascularization was recently terminated due to a lack of efficacy [88]. siRNA has been used to target both VEGF and VEGFR. SIRNA-027 targets the VEGFR, and while initial trials are encouraging, the overall clinical benefit is still uncertain [85].

These are still in the early stages of development with trials in Phase II. These TKIs target VEGFR but some, such as Pazopanib, also have activity against other tyrosine kinases such as PDGFR and c-kit [81, 89, 93].

Anti-VEGF therapy is undoubtedly beneficial in the treatment of neovascular eye disease, but there is scope for development. Other approaches to targeting this pathway have not been as successful as anti-VEGF mAb therapy, but many of these alternative drugs have shown early promise. The development of drugs which can be delivered topically or orally would be a major step forward in increasing the availability of anti-VEGF/VEGFR therapies to patients.

4.3 Pro-angiogenic Therapies

VEGF/VEGFR signaling is known to be involved in the maintenance of vascular function and homeostasis in the adult. VEGF stimulates endothelial production of nitric oxide and prostacyclin which act as vasodilators and inhibit platelet aggregation and smooth muscle cell proliferation within the vasculature [42]. Genetic deletion of endothelial VEGF in a murine model caused endothelial degeneration and apoptosis, which led to vascular pathology including hemorrhage, perforation, infarcts, and sudden death. Exogenous VEGF could not rescue these defects suggesting involvement of autocrine VEGF signaling [90]. Consequently, anti-angiogenic therapies targeting the VEGF/VEGFR axis have significant cardiovascular side effects which must be monitored [42].

PlGF, a member of the VEGF family, is produced during angiopoiesis in the placenta. Additionally it is produced by infarcted myocardium and its expression is also significantly higher in brain microvascular endothelial cells following oxygen

and glucose deprivation in vitro. PlGF administration led to significantly higher VEGFR-2 expression and it may be that VEGFR2 plays a role in PlGF-mediated neuroprotection [91]. In vivo models have shown a beneficial effect of simvastatin, which may be due to VEGFR2 activation of Akt and nitric oxide synthase [92].

Pro-angiogenic therapies may provide an important treatment modality for those patients with vascular disease unsuitable for invasive revascularization procedures. The premise would be to induce the formation of collateral blood vessels in order to revascularize ischemic tissue. At present most research is focused on the use of angiogenic growth factors such as VEGF-A. Preclinical animal models have provided the evidence for the development of these therapies, but so far there has been limited success in translating this success in clinical trials [42].

5 Conclusion

VEGF-mediated signaling influences a range of cellular events underpinning blood vessel growth and has a firmly established role in vasculogenesis and angiogenesis. While this axis has been intensely dissected, many of the subtleties are yet to be understood. The activity of the VEGF receptor is modulated by co-receptors such as NRP [43] and HSPG [44] and downstream there is a web of interacting signaling pathways, such as the angiopoietin-Tie pathway [1].

The advent of successful novel therapies targeting the VEGF/VEGFR axis gave impetus to the research in this field. Currently, clinical trials are revealing the potential of inhibiting the VEGF receptor to combat cancer [70] and neovascular eye disease [85]. Conversely, its use as a pro-angiogenic treatment following infarction has also been explored [91]. Furthermore, due to the targeted nature of VEGFR inhibitors, the side effect burden is reduced compared to standard cytotoxic chemotherapy [79].

The VEGF receptor itself provides a tempting target for novel therapeutic strategies, but despite this it has only limited clinical applications at present. The most promising drugs appear to be useful as adjuvants to chemotherapy regimens [70]. Elucidating the signaling mechanisms based around VEGF and its receptor will not only enable us to better understand the fundamental underpinnings of vascular development and regrowth; it will provide us with the tools required to manipulate this axis.

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Chapter 2
The Role of Integrins in Angiogenesis

Ghazaleh Tabatabai

Abstract Angiogenesis critically depends on environmental factors. In particular, cellular adhesion and migration events play a critical role in the formation of new blood vessels from pre-existing cells in multiple pathological conditions. Integrins are a large family of cell surface receptors that transfer signals from the extracellular microenvironment into the intracellular compartment of endothelial cells or tumor cells. In this chapter, we review the role of integrins in inducing and maintaining angiogenesis by regulating the survival, proliferation and migration of endothelial cells as well as of tumor cells. Furthermore, we summarize some pharmacological approaches for modulating integrin signaling in tumor angiogenesis.

Keywords Integrins • Angiogenesis • Cancer • Glioma • Integrin inhibition

1 Introduction

The growth of new vessels from preexisting vessels depends on the migration and invasion of endothelial cells through the extracellular matrix (ECM). During this process, cellular adhesion to the ECM plays a crucial role. Endothelial cells are connected to the ECM via adhesion molecules that are critical for their survival, growth and migration [1].

Integrins constitute a large family of cell surface transmembrane molecules and are the main cell surface receptors mediating adhesion to the ECM. They are composed of α and β subunits. Eighteen α subunits, 10 β subunits and 24 different heterodimeric integrin molecules have been identified. Alpha and β subunits can form heterodimers in multiple ways, e.g. αv can associate with many different β-subunits. Other α-subunits, however, only form heterodimers with one specific β-subunit partner (Fig. 2.1). Of note, the α-subunit seems to be important for determining the

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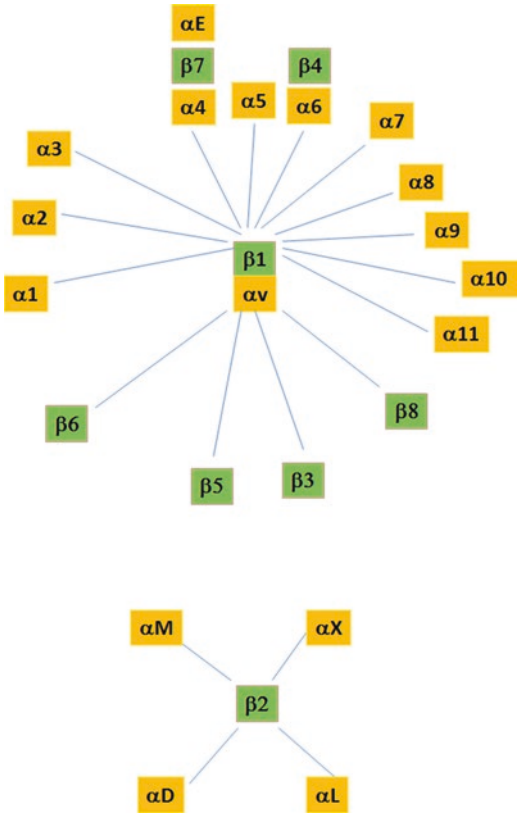

Fig. 2.1 The integrin family is a diverse family of heterodimeric proteins each containing an α and a β subunit (Modified from Tabatabai et al. [50])

ligand-binding properties of the integrin heterodimer. Each heterodimer is capable of binding to a subset of ligands. In turn, a single ECM ligand can bind to several different integrins. In addition to ECM ligands, integrins mediate binding to matrix metalloproteinases (MMPs) and cell surface immunoglobulin-type receptors such as vascular adhesion molecule (VCAM). Taken together, integrin-induced endothelial cell migration is a key regulator of physiological and pathological angiogenesis. In tumors, integrins enable the crosstalk between tumor cells and the surrounding stromal components [2, 3].

2 Bidirectional Signaling by Integrins Regulates Cellular Fate

Many integrins are not constitutively activated, i.e. the ligand binding site of the integrins is closed, and the affinity for ligand binding is low. Activation occurs by binding of extracellular ligands, i.e. laminins or vitronectins. Engagement and clustering of integrin receptors lead to the formation of focal contact sites where cells

adhere to ECM. These contact sites are called focal adhesions. They function as a nexus for attracting elements of the cytoskeleton, signaling molecules and adaptor proteins. The transmembrane helices and short cytoplasmic tails of the integrin α-subunit and β-subunit are important for initiating and coordinating cellular responses. Integrin cytoplasmic tails are between 15 and 78 amino acids in length for α-subunits and between 46 and 68 amino acids in length for β-subunits. These cytoplasmic tails of integrins do not contain enzymatic activity. Therefore, interaction with cytoplasmic adaptor proteins is crucial for mediating intracellular signaling [3]. These adaptor proteins can associate with the cytoplasmic tails of integrins and form a bridge between the cell surface and the cytoplasm.

Classical focal adhesions contain signaling complexes composed of growth factor receptors, focal adhesion kinase, integrin-linked kinase, Src, phosphoinositide 3-kinase (PI3K), and actin-associated cytoskeletal proteins [4]. Often, the activation of the Rho family of GTPases occurs by integrins upon the formation of focal adhesions. This activation induces immediate cellular migration events including formation of lamellipodia at the invading front of the cell as well as releasing of ECM adhesion contacts at other parts of the cell to enable directed migration and invasion. Not only migration and invasion events are the results of integrin-induced signaling that occur in these focal adhesions. The induction of the Ras GTPase family, for example, mediates signals to the PI3K/Akt and the Ras/mitogen activated protein (MAP) kinase signaling pathway leading to the activation of several transcription factors, including NF-kB, HoxD3 or Id proteins [5, 6] that are key players in cell cycle regulation and cell survival. These examples illustrate that integrin-mediated signaling from the ECM to the intracellular compartment, so called outside-in signaling, regulates migration, invasion, cytoskeletal organization, cell survival and cell cycle progression. These are critical events during angiogenesis. In reverse direction, integrin-mediated binding can be modulated from the intracellular to the extracellular compartment, so called inside-out signaling. Certain changes from the intracellular domain of the integrin heterodimers can regulate the binding affinity of the extracellular integrin receptor to the ECM components [7]. This bidirectional signaling leads to the formation of a complex crosstalk network regulating the activation status of integrins. Thus, depending on the activation status, even opposite effects might occur, e.g. either enhancement of cell survival or induction of apoptosis, indicating that integrin signaling is among the key regulators for determining cell fate [8, 9].

3 Integrin Binding to Proteolytic Protein Fragments

Migration and invasion of endothelial cells is facilitated by MMP-mediated degradation of ECM. However, these proteolysis events also lead to the formation of proteolyzed protein fragments including tumstatin [10], endostatin [11] and PEX [12], that have antiangiogenic activity by antagonizing integrin signaling in endothelial cells.

4 Crosstalk of Integrins with Other Signaling Pathways

Since angiongesis is a highly regulated process involving several signaling pathways, a coordinated crosstalk between the key signaling pathways is a necessity. This holds also true for integrin signaling in angiogenesis. Crosstalks with several other signaling pathways exist, e.g. with Notch, VEGF or TGF-β signaling.

4.1 Notch Signaling

Notch signaling is an evolutionary highly conserved pathway that plays crucial roles, e.g. for cellular fates during embryogenesis. A role of Notch signaling in angiogenesis has recently been established including vascular development, vessel patterning and vascular maturation [13]. Notch signaling leads to the recruitment of vascular smooth muscle cells to newly formed vessels. This leads to perivascular coverage and stabilization of the newly formed vessels. In this process of vascular maturation, vascular smooth muscle cells interact with the Notch ligand Jagged 1 on endothelial cells. This interaction leads to upregulation and activation of αvβ3. Thus, αvβ3 acts downstream upon Notch activation and allows vascular smooth muscle cells to adhere to endothelial basement membrane [14].

4.2 VEGF Signaling

The VEGF family has several members and mediates multiple functions including vascular permeability, angiogenesis, lymphangiogenesis and tumorigenesis. In angiogenesis, VEGF-A is the predominant VEGF family member. Due to several gene splincing events, diverse mature VEGF-A isoforms exist. The best studied isoform so far is VEGF-A165. It binds to VEGF-R2 for mediating downstream signaling. The small GTPase Rap1 activates and promotes VEGF-A signaling in endothelial cells. This activation occurs partly via αvβ3 activation [15].

Recent evidence suggests that VEGF-A can directly bind to integrins, specifically to integrin α9β1 or αvβ3. The direct interaction of VEGF-A with α9β1 occurs via a three-amino acid sequence, EYP, that is encoded by the exon 3 of VEGF-A. Upon binding of VEGF-A to α9β1, endothelial cell migration is induced [16]. Direct interaction of VEGF-A with αvβ3 contribute to the adhesion, migration and survival of human umbilical arterial endothelial cells [17, 18].

4.3 TGF-β Signaling

TGF-β is a key molecule controlling a variety of cellular processes, including proliferation, differentiation, apoptosis and migration. The TGF-β isoforms and the corresponding receptors are expressed by many different cell types. TGF-β is released as an inactive cytokine. The latent complex must be activated. The regulation of this activation step provides a site-specific control of TGF-β function and might explain the diversity of TGF-β effects depending on the composition of the respective microenvironment. For this activation step, integrins seem to play an important role. Six integrins can bind latent TGF-β including avb1, avb3, avb5, avb6 and avb8 and a8b1. The binding of integrins is mediated by an RGD motif that is present in the latency-associated peptide region of the latent complex. Of note, the latent forms of TGF-β 1 and TGF-β 3, but not TGF-β 2, contain the RGD motif. TGF-β 1 activity is highly dependent on the activation step by integrins: Transgenic mice were generated carrying a single point mutation in the RGD integrin binding motif of latent TGF-β 1 that changed RGD to RGE. TGF-β $1^{RGE/RGE}$ mice expressed latent TGF-β 1 in a form that cannot bind integrins. TGF-β $1^{RGE/RGE}$ mice developed defects identical with those seen in mice that are TGF-β 1 deficient, i.e. vasculogenesis defects during embryonic development and multi-organ inflammation at the age of 2–3 weeks postnatal. These data strongly indicated that integrin-mediated activation of latent TGF-β 1 is absolutely required for TGF-β 1 functions in vivo [19]. Inhibition of β8 integrin in glioma cells leads to reduced activation of latent TGF-β [20]. Moreover, treatment of glioma cells with the integrin inhibitor cilengitide (see below) results in detachment and decreased TGF-β_1 and TGF-β_2 mRNA and protein expression, reduced phosphorylation of Smad2 and reduced TGF-β-mediated reporter gene activity [21].

5 Integrin Inhibition in Tumor Angiogenesis

Angiogenesis is necessary for tumor growth, dissemination and metastasis. Integrins are key regulatory proteins for tumor angiogenesis. In view of current clinical applications of integrin inhibitors, we will focus on the role of integrins and integrin inhibition in brain tumor models.

In malignant gliomas, the integrins αvβ3 and αvβ5 are predominantly expressed. They are detected on angiogenic tumor endothelial cells and on tumor cells [22, 23]. Interestingly, αvβ3 is selectively expressed in gliomas and is absent in normal brain as demonstrated by positron emission tomography (PET) imaging studies using the tracer [18F] Galacto-RGD. The positive PET signals have been confirmed by biopsy and histological analysis of the tumor tissue [24]. These findings support αvβ3 as a rational candidate for specific therapeutic targeting in glioblastomas. Currently, the standard therapeutic strategies for patients with glioblastoma include surgical resection as feasible or biopsy, and radiotherapy plus concomitant and adjuvant temozolomide chemotherapy

[25, 26]. Even with this multimodality approach, the median survival of these patients does not exceed 15 months. Therefore, new therapeutic targets are needed to improve the prognosis of patients with glioblastoma. In this regard, integrins have become attractive candidate molecules for therapeutic intervention and have been explored in preclinical and clinical studies.

Preclinical studies have suggested important biological functions of integrins in malignant gliomas via tumor cell migration, invasion and adhesion, and angiogenesis [27–29]. The correlation of expression levels and activities of MMP-2 and -9, of Bcl-2 family members and of αvβ3 integrin in 12 human glioma cell lines to glioma cell migration and invasion revealed that αvβ3 integrins alone did not predict a migratory or invasive phenotype. A neutralizing αvβ3 integrin antibody, however, inhibited migration and invasion selectively in cell lines with αvβ3 integrin expression. This indicates that αvβ3 integrin has a key role in migration and invasion of malignant glioma cells.

Most glioma cell lines detach from the cell culture dish, but do not die when exposed to integrin αvβ3/5 antagonists, either antibodies or the RGD-mimetic peptide cilengitide. These observations suggest that the potential anti-glioma activity of integrin antagonists (see below) are unlikely to be related to direct cytolytic activity against glioma cells. However, others have reported that cilengitide leads to detachment of glioma cells from the ECM with subsequent apoptosis [28].

Treatment of animals with combined treatment of experimental U87MG gliomas with the RGD peptide mimetic S247 and fractionated radiation therapy has been more effective than either treatment alone, suggesting antiangiogenic activity with reduced microvessel densitiy resulting in improved survival. Tumor cell proliferation was also significantly reduced after S247 or irradiation of U87 tumors as assessed by Ki67 staining of histological sections. Importantly, the combination of S247 and radiotherapy resulted in better results. CD31 staining showed reduced vessels after combined treatment as compared to S247 treatment or irradiation alone. Further, histological sections from S247-treated and irradiated xenografts showed pronounced reduction of Akt phosphorylation and increased TUNEL immunostaining indicating endothelial cell apoptosis. This suggests that αvβ3 antagonism might confer sensitization towards radiotherapy. It remains to be clarified whether the combination of S247 and radiation therapy results in synergistic or additive effects.

Concurrent treatment of orthotopic U251 gliomas with cilengitide and radiotherapy in vivo increased the rate of apoptotic cell death and the survival of animals compared with either treatment modality alone. Interestingly, a critical parameter for a successful combination of integrin antagonism and radiotherapy seems to be the time-line of treatment modalities. For example, single dose of cilengitide led to synergistic effects when administered 4–8 h before radiation, but not when administered 2 h before or after radiation [30]. Combined treatment with cilengitide and radiation therapy significantly increased the rate of apoptotic and autophagic cells. It remains unclear, however, whether the observed treatment-induced apoptosis in vivo in this study was occurring primarily in the U251 glioma cells or in the endothelial cell compartment, and whether the induction of apopto-

sis may be considered a valid surrogate marker for prolonged survival of the animals. In vitro, the treatment of glioma cell lines with cilengitide induces detachment in a concentration-dependent manner without significantly affecting proliferation and survival [28]. The effect of integrin inhibition prior to radiation might indicate that cilengitide induces changes in the vascular architecture. Of note, tumor vessels that are formed by highly proliferating angiogenic endothelial cells are often functionally inefficient leading to reduced perfusion, hypoxia and resistance to irradiation. Thus inhibition of tumor angiogenesis by αvβ3 antagonism may restore normal perfusion and reduce tumor hypoxia by normalizing the vasculature. A recent MRI-based study by Muldoon and colleagues [31], however, demonstrated that inhibition of αv integrins actually increased vascular permeability questioning the value of vascular normalization. Another explanation might be the interference with hypoxia-regulated pathways. Indeed, hypoxia increases αvβ3 and αvβ5 expression. Depletion of αvβ3 and αvβ5 integrins by siRNA decreased the transcriptional activity of hypoxia-inducible factor 1 α (HIF-1α) and reduced hypoxia [32]. Thus targeting αvβ3 and αvβ5 might sensitize tissues to radiation by reduction of hypoxia. Taken together, the "correct" scheduling of radiation therapy and cilengitide might reduce hypoxia by induction of vascular changes and enhances the beneficial effects of radiation. This, however, remains to be proven by imaging studies such as refined MRI modalities or vascular-specific PET imaging in humans. Indeed, a clinical trial using MRI and PET imaging studies during Cilengitide treatment has just been launched in patients with glioblastoma to address the question of vascular changes in patients. The above-mentioned observations, nonetheless, demonstrate important role of integrins for glioma angiogenesis. Integrin inhibition is, therefore, considered a promising target for the treatment of malignant glioma because both endothelial and tumor cells express this target. The following paragraphs will briefly review clinical experience with integrin inhibitors against malignant glioma.

6 Integrin Inhibitors in Clinical Development

6.1 GLPG0187

GLPG0187 (Galapagos SASU, Romainville, France) is a Arg-Gly-Asp (RGD) antagonist targeting integrin receptors avb1, avb3, avb5, avb6, avb8 and a5b1. Thus, compared with cilengitide (see paragraph below), the range of targeted integrin receptors is broader. Preclinical studies suggested anti-tumor activity including recuced tumor growth and metastasis [51, 52]. A dose-escalating phase 1 clinical trial recently evaluated dose-limiting toxicity, safety and tolerability in patients with advanced or metastatic tumors that did not have any other options for standard therapies. While the drug showed dose-propotional pharmacokinetic profile, no maximal tolerated dose was established [53].

6.2 Cilengitide

Cilengitide (Merck Serono, Darmstadt, Germany) is a synthetic Arg-Gly-Asp (RGD) pentapeptide with a molecular weight of 588.7 mass units. In general, maximal plasma concentrations are reached within 1 h after the injection and the half-life is approximately 3–5 h. Cilengitide binds to the RGD ligand-binding motif (ligand binding site) on the integrin receptors αvβ3 and αvβ5 and has no effect on adhesions mediated by α1β1, α2β1, α5β1 integrins [33]. Cilengitide reduces vascular endothelial growth factor (VEGF)-induced angiogenesis in chorioallantoic membranes [34] and the proliferation of human umbilical vein endothelial cells [35].

Phase I studies with cilengitide demonstrated that the drug is well tolerated when given intravenously twice a week. No dose limiting toxicity was observed up to a dose of 2400 mg/m^2. Cilengitide was also well tolerated in adult and pediatric patients with recurrent glioma or other brain tumors. Tumor responses were observed both at lower and higher doses. Enzyme-inducing anticonvulsants did not interfere with the pharmacokinetics of cilengitide. In a study by Gilbert and colleagues [36], cilengitide was given for 2 weeks at two doses before surgical intervention. Drug concentrations were significantly higher in the tumor than in the corresponding plasma. Of note, cerebrospinal fluid concentrations were approximately in the range of 1% of the plasma concentrations and were reached within 3 h after injection. This study supports the concept that cilengitide penetrates blood brain barrier and is enriched in the tumor tissue of patients with glioblastoma [36].

In a randomized phase II trial enrolling 81 patients with glioblastoma, utility of a lower and a higher dose of cilengitide (500 and 2000 mg, flat dosing) were explored. Objective responses were observed in 5% (500 mg) and 13% (2000 mg) of the patients. Progression-free survival at 6 months was 10% and 15%, and overall survival was 6.5 versus 9.9 months in the low-does and high-dose groups, respectively [37]. Four-year survival rate was 2.4% in patients treated with 500 mg and 10% in patients treated with 2000 mg [38].

A European phase II trial in 52 newly diagnosed patients with glioblastoma evaluated the addition of cilengitide (500 mg) to standard radiation and chemotherapy with temozolomide. Treatment was well tolerated. Progression-free survival at 6 months was 69%, median survival 16.1 months, with a 2-year survival rate of 35%. Compared with historical controls, the 23 patients with a methylated O^6-methylguanine–DNA methyltransferase (*MGMT*) promoter appeared to benefit most from the addition of cilengitide. Overall survival at 15 months was 75% in patients with a methylated and 47% in patients with an unmethylated *MGMT* promoter [39]. The relation between *MGMT* methylation status and benefit from cilengitide is, however, controversial. Glioma cell response in in vitro studies was unaffected by cilengitide alone or cilengitide in combination with temozolomide after modulation of *MGMT* expression levels, i.e. by ectopic expression of *MGMT* in *MGMT*-negative or by shRNA-mediated *MGMT* silencing in *MGMT*-positive glioma cells [28]. It seems that vascular normalization and improved tumor perfusion might be a key mechanism of action of cilengitide in this setting. Enhanced perfusion allows better "delivery" of temozolomide chemotherapy to which tumor cells with a methylated *MGMT* promoter are particularly sensitive [40].

Nabors and colleagues [41] reported on a safety run-in and randomized phase II study of cilengitide and standard radio and temozolomide chemotherapy in newly diagnosed glioblastomas comparing the addition of 500 mg vs 2000 mg cilengitide. The primary endpoint, median overall survival was compared with historical controls. The combination of cilengitide with radiation and temozolomide chemotherapy was well tolerated. The median overall survival was 19.7 months for all patients, 17.4 months for those who received 500 mg of cilengitide, and 20.8 months for those who received 2000 mg cilengitide. For future trials, the authors suggested use of 2000 mg cilengitide. However, these data from uncontrolled phase II clinical trials need to be interpreted with caution as there was no control arm without the new agent. Larger randomized trials are required to confirm the efficacy of these novel treatment approaches.

Based on the benefit observed in particular in patients with a methylated *MGMT* promoter in the tumor, a phase III trial was conducted in patients with glioblastoma with methylated *MGMT* promoter. Patients were screened upfront for *MGMT* promoter methylation by methylation-specific PCR. Eligible patients were randomized either to standard therapy consisting of temozolomide and radiation, or to 2000 mg cilengitide twice weekly in addition to standard therapy. Maintenance cilengitide therapy was continued for up to 18 months. The trial was performed in 25 countries including 146 study sites. The primary endpoint was overall survival. In the cilengitide group, median overall survival was 26.3 months (95% CI 23.8–28.8), in the control group 26.3 months (95% CI 23.9–34.7), hazard ratio was 1.02. The 2-year survival rate did not differ between treatment groups, either and was 56% in both groups. Taken together, the trail did not show a benefit from the addition of cilengitide to standard therapy in MGMT-methlyted newly diagnosed glioblastoma patients. The authors discussed potential reasons for this negative result despite promising phase 1 and phase 2 trial results. Probably, the twice weekly administration was not appropriate for a drug with a serum half-life in the range of 2–4 h [54]. Other reasons might include the lack of a predictive biomarker that would have allowed to guide patient selection for this integrin-targeted compound.

In parallel, a randomized phase II study in patients with newly diagnosed glioblastomas with an unmethylated *MGMT* promoter was conducted ongoing. The CORE trial evaluated safety, feasibility and efficacy of intensified daily cilengitide 2000 mg with radiation and temozolomide. Thereafter, all patients received 6 cycles of standard 5-day out of 28 days temozolomide plus cilengitide dosed at 2000 mg twice weekly. The treatment was continued until progression or toxicity. Patients were randomized into the following 3 arms: first arm, cilengitide 2000 mg twice weekly with standard radiation and temozolomide chemotherapy and with 6 adjuvant cycles of temozolomide followed by cilengitide maintenance; second arm, cilengitide 2000 mg from Monday through Friday with radiation and concomitant temozolomide chemotherapy, followed by cilengitide twice weekly with 6 adjuvant temozolomide cycles followed by cilengitide maintenance; third arm, standard radiation and temozolomide chemotherapy with 6 cycles of temozolomide. In total, 265 patients were randomized. Median overall survival was 16.3 months in arm 1, 14.5 months in arm B and 13.4 months in the control arm. Median progression-free

survival was 5.6 months in arm 1, 5.9 months in arm 2 and 4.1 months in the control arm. Both cilengitide dosages were well tolerated. However, the trial does not provide evidence for clinical efficacy of cilengitide in newly diagnosed *MGMT*-unmethylated glioblastoma patients [55].

Taken together, CENTRIC and CORE failed to show convincing efficacy of cilengitide in newly diagnosed glioblastoma. Immunohistochemistry studies in the biomarker cohort of both trials, i.e. cohort of patients with tumor tissues for further translational studies, included correlations of integrin stainings with clinical outcome. Interestingly, higher avb3 expression correlated with improved progression-free and overall survival in the CORE biomarker cohort, but not in the CENTRIC biomarker cohort. Limitations of this study might include the fact that integrin expression does not necessarily reflect actual integrin activity [56].

6.3 ATN-161

ATN-161 (Tactic Pharma, Evanston, IL) is a α5β1 integrin antagonist and decreased the phosphorylation of mitogen-activiated protein kinase [42, 43]. Treatment with ATN-161 in a breast cancer model induced a significant dose-dependent decrease in tumor volume and metastasis to bone and soft tissues. Histological analysis revealed reduced microvessel density and cell proliferation. In a phase I study, ATN-161 was administered to patients with advanced solid tumors excluding brain tumors. Treatment was well tolerated at all dose levels from 0.1 through 16 mg/kg [44]. The clinical development, however, seems not to be further continued.

6.4 DI17E6

DI17E6 (Merck Serono, Darmstadt, Germany) is a pan anti-αV antibody. In a preclinical melanoma study, DI17E6 was covalently coupled to doxorubicin-loaded human serum albumin nanoparticles. These tailored nanoparticles specifically targeted αvβ3-positive melanoma cells and displayed significantly higher cytotoxic activity than the free drug alone [45]. Phase I evaluation has recently been completed, and it will be further investigated in combination with cetuximab in colorectal cancer.

7 Conclusions

Integrins play a crucial role in physiological and pathological angiogenesis. Endothelial cell invasion events critically depend on integrin-mediated signaling. Bidirectional integrin signaling regulates cell proliferation, cell cycle progression, migration, invasion of tissues and cell survival. Integrin antagonists occur naturally

and are derived from proteolytic degradation of ECM. Integrin inhibition, particularly in combination with cytotoxic chemotherapy and/or radiation therapy is a promising strategy for targeting angiogenesis and tumor cells in certain tumor entities.

Indeed, preclinical evidence suggests that inhibition of integrins is an attractive approach for anti-glioma therapies. It seems crucial to design treatment algorithms while paying attention to the schedule, e.g. time point of injection when combined with radiation therapy or choosing the right dose. Of note, administration of low nanomolar concentrations of RGD mimetics led to a pro-angiogenic and pro-invasive tumor phenotype in melanoma and lung cancer models. This was mainly explained by an activation of the Rab4 pathway by nanomolar RGD mimetics. This activation promotes the recycling of internalized VEGF-R2. This, in turn, inhibits the degradation of VEGF-R2 and allows relocalization of VEGF-R2 to the cell surface and thus promotes cellular responses to VEGF [46]. Yet, the plasma concentrations achieved with the doses of cilengitide used in the ongoing clinical trials exceed the potentially proangiogenic concentrations by orders of magnitude [47].

Taken together, integrins are among key mediators of angiogenesis, and thus attractive targets for therapy of diseases with pathological angiogenesis. For ensuring therapeutic success, however, it will be crucial to carefully define which integrins to selectively target in which disease at which dosage and which timepoints [48, 49]. Furthermore, it seems necessary to identify rational combination therapies with further compounds to exploit potential synergies. The involvement of molecular imaging tools for diagnosing and monitoring integrin activation, e.g. RGD PET [57] seems crucial. An integrative profile of these parameters might allow the establishment of biomarkers for predicting clinical benefit from integrin inhibition as an important prerequisiste for accurate patient stratification in further clinical investigations of integrin-targeted approaches.

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Chapter 3
Toll-Like Receptors in Angiogenesis

Karsten Grote, Jutta Schuett, Harald Schuett, and Bernhard Schieffer

Abstract Mammalian Toll-like receptors (TLRs) represent pattern recognition receptors of the immune system and are related to the Toll protein of *Drosophila*. Pathogen-associated molecular patterns (PAMPs) of microbial and viral origin bind to TLRs and initiate the innate and adaptive immune response. However, TLRs are not solely found on cells of the immune system but also on non-myeloid cells in various tissues, e.g., on vascular cells. In addition to PAMPs, there is increasing evidence that TLRs also recognize endogenous ligands. Recent studies demonstrate the contribution of distinct TLRs in different inflammatory disorders such as cardiovascular diseases, rheumatoid arthritis, systemic lupus erythematosus, and cancer. Many of these disorders are characterized by enhanced angiogenesis which is mainly trigged by inflammation. However, this inflammation-induced angiogenesis is not only important for pathogen defense during acute infection or chronic inflammatory disorders but as well involved in regenerative processes during wound healing and tissue repair. There is cumulative evidence that TLR activation by exogenous as well as endogenous ligands especially contributes to the angiogenic process in this scenario. The present chapter will summarize the current understanding of TLR-linked signal transduction in angiogenesis during inflammatory processes with future prospects for pro- or antiangiogenic therapy.

Keywords Angiogenesis • Toll-like receptors • Pathogen-associated pattern • Damage-associated molecular patterns • Inflammation

1 Introduction: Toll! Everything Started in *Drosophila*

A group of maternal effect genes are necessary for the embryo patterning of the fruit fly *Drosophila melanogaster* including the Toll gene. Lack of function experiments revealed that the Toll gene product provides the source for a morphogen gradient in

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the dorsal-ventral axis of the *Drosophila* embryo [1, 2]. Mutants in this gene were originally identified in 1985 by the group of Christiane Nüsslein-Volhard at the Max-Planck-Institute in Tübingen/Germany. The name of the gene derives from her exclamation "Das ist ja toll!" which translates as "That's amazing!" during microscopic observation of the drosophila mutants. Three years later, the Toll gene of *Drosophila* was cloned in the lab of Kathryn Anderson, the first author of the initial studies [3]. In 1992, Christiane Nüsslein-Volhard was awarded with the Nobel Prize for her groundbreaking research. Later on, Toll was found to play an important role in the fly's immune response by the group of Jules Hoffmann [4, 5]. In total, nine Toll receptors are encoded in the *Drosophila* genome, including the Toll pathway receptor Toll. The induction of the Toll pathway by fungi or by gram-positive bacteria leads to the activation of antimicrobial peptides. After proteolytical cleavage, binding of the extracellular ligand Spaetzle to the Toll receptor controls the expression of the antifungal peptide gene drosomycin. Mutations in the Toll signaling pathway dramatically reduce survival after fungal infection demonstrating the importance of this pathway for immune response.

2 Toll-Like Receptors in Mammalians

The identification of the *Drosophila* Toll pathway and the subsequent characterization of Toll-like receptor (TLR) function have reshaped the current understanding of the immune system. Mammalian homologues of the *Drosophila* Toll protein have been discovered 10 years later in the mid-1990s of last century which were consequently named TLRs [6, 7]. A scientific highlight in mammalian TLR discovery was the identification of TLR4 as the functional receptor for bacterial lipopolysaccharide (LPS) in mice carrying a mutation in the TLR4 gene by Bruce Beutler [8]. Based on this important discovery, Bruce Beutler was awarded with the Nobel Prize in 2011 which he shared with Jules Hoffmann for his findings in *Drosophila* and Ralph Steinmann who discovered dendritic cells. The discoveries of Hoffmann and Beutler triggered an explosion of research in innate immunity. Around a dozen different TLRs have now been identified in humans and mice comprising an entire receptor protein family [9]. All of them have initially been described as guardians of the innate immunity recognizing invading pathogens in the front line on the plasma membrane or after phagocytosis and processing on endosomal membranes, respectively. TLRs represent cognate pattern recognition receptors (PRRs) of the innate immunity and recognizing a high diversity of molecules common in pathogens of bacterial and viral origin referred to as pathogen-associated molecular patterns (PAMPs). The specificity of TLRs for their ligands was mainly investigated in mice with functional mutations carrying an increased risk of infection. TLR ligation induces the activation of inflammatory pathways such as the mitogen-activated protein kinase (MAPK) cascade or nuclear factor κB (NFκB) and finally leads to the expression of cytokines and co-stimulatory molecules [10]. Thus, TLRs activate a potent immunostimulatory response and the signal that is transmitted from TLRs

must therefore be tightly controlled. Structurally, all TLRs are type I integral membrane proteins consisting of an ectodomain comprised of leucine-rich repeats (LRRs) and a cytoplasmic domain containing a Toll/interleukin-1 receptor homology domain (TIR), which is required for signaling. TLRs occur as dimers; different receptor assemblies as mono- or heterodimers are known [11–13]. TLR2 builds heterodimers; in this regard TLR2/1 dimers sense bacterial triacylated lipopeptides were as TLR2/6 dimers sense bacterial diacylated lipopeptides. The LPS receptor TLR4 and TLR9, the receptor for unmethylated CpG-motifs in bacterial and viral DNA homodimerize and TLR4 may additional forms heterodimers with TLR2 in microglial cells in response to ethanol [14]. Homodimerization is presumed to be the case for TLR3 which senses synthetic and double-stranded RNA of viral origin (dsRNA) as well as for TLR5 which detects flagellin from bacteria. TLR7 and TLR8 recognize synthetic imidazoquinolines components and single-stranded RNA (ssRNA) and TLR8 has been shown to dimerize with TLR7 and TLR9. TLR10 is the only pattern-recognition receptor without known ligand specificity and biological function and maybe is a modulatory receptor with mainly inhibitory effects [15]. It has been demonstrated that TLR10 can heterodimerize with TLR1 or TLR2 [16]. More recently, TLR11, 12 and 13 have been identified in mouse. Interestingly, an eukaryotic ligand have been described for TLR11, namely a profilin-like molecule from the obligate intracellular protozoan parasite Toxoplasma gondii [17], which is recognized in cooperation with TLR12 [18]. Finally, it has been shown that TLR13 is the functional receptor for a conserved sequence in the 23S ribosomal RNA (rRNA) from bacteria [19]. The number of putative TLR interaction partners and identified PAMPs that bind to TLRs is already large and diverse and is still growing (Fig. 3.1, Table 3.1) [20].

A fundamental basis of TLR signaling is dependent upon the recruitment and association of adaptor molecules that contain the structurally conserved TIR domain. Signaling by TLRs involves five so far identified adaptor proteins known as myeloid differentiation primary response gene 88 (MyD88), MyD88-adaptor-like (MAL, also known as TIRAP), TIR-domain-containing adaptor protein inducing interferon-β

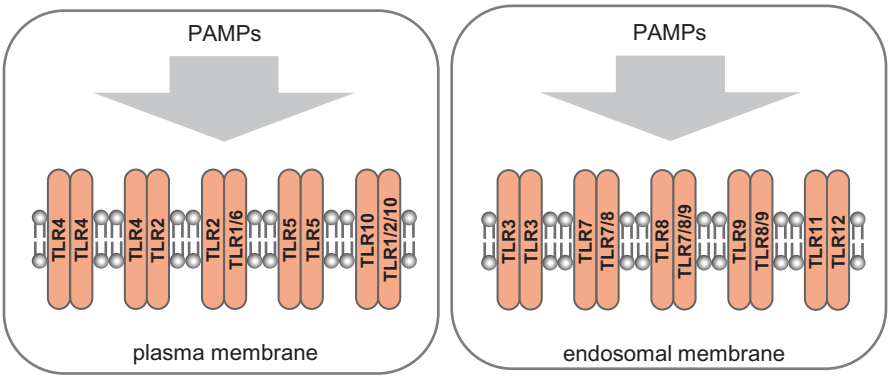

Fig. 3.1 TLRs form homo- and heterodimers

Table 3.1 TLRs and their PAMPs

TLR	PAMP	Pathogen
TLR1	Triacylated lipopeptides	Bacteria
TLR2	Diacylated lipopeptides Triacylated lipopeptides Peptidoglycan Lipoteichoic acid	Bacteria Bacteria Bacteria Gram-positive bacteria
TLR3	Double-stranded RNA	Viruses
TLR4	Lipopolysaccharide Heat shock proteins Viral proteins	Gram-negative bacteria Bacteria Viruses
TLR5	Flagellin	Bacteria
TLR6	Diacylated lipopeptides	Bacteria
TLR7	Single-stranded RNA	Viruses
TLR8	Single-stranded RNA	Viruses
TLR9	Unmethylated CpG DNA	Bacteria
TLR10	Unknown	
TLR11	Profilin-like molecule	Protozoa (Toxoplasma gondii)
TLR12	Profilin-like molecule	Protozoa (Toxoplasma gondii)
TLR13	23S rRNA	Bacteria

(TRIF; also known as TICAM1), TRIF-related adaptor molecule (TRAM; also known as TICAM2), and sterile α- and armadillo-motif-containing protein (SARM). These adaptor molecules provide the necessary framework to recruit and activate downstream kinases and transcription factors that regulate the host inflammatory response. The canonical TIR pathway is dependent on MyD88, the immediate adapter molecule that is common to all TLRs, except TLR3. An alternative MyD88-independent pathway is controlled by TRIF, the only TLR3 adaptor, whereas TLR4 binds both MyD88 and TRIF. The remaining three adaptor proteins serve as co-adaptors (MAL, TRAM) or even as a negative regulator (SARM). MAL and TRAM are just used by few TLRs. MAL recruits MyD88 to TLR2 and TLR4, whereas TRAM recruits TRIF to TLR4 [13]. After ligand binding to the specific TLR and assembly of the adaptor proteins, the activated membrane receptor complex induces the interleukin-1 receptor-associated kinase (IRAK) and tumor necrosis factor receptor-associated factor (TRAF) family members. The IRAK family – with their four members: IRAK1, IRAK2, IRAK4, and IRAKM – plays a pivotal role in mediating almost all TLR-mediated functions. All IRAK family members contain an amino-terminal death domain and a serine/threonine kinase domain. IRAK4 is known to be essential for TLR-mediated cellular responses. After TLR ligation, IRAK4 phosphorylates IRAK1 [21]. IRAK activation results in the recruitment/activation of TRAF family members such as TRAF3 and TRAF6, along with other E2 ubiquitin protein ligases which activate a complex containing transforming growth factor-β-activated kinase 1 (TAK1), TAK1-binding protein 1 (TAB1), TAB2, and TAB3 [22]. MyD88dependent TAK1 activation induces the NFκB pathway and MAPK members such as the extracellular signal-regulated kinase (ERK)1/2, p38,

and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) to initiate the expression of inflammatory cytokines [9–11]. The TLR3 pathway is MyD88-indendent but TRIF-dependent that activates TRAF6 and NFκB, resulting in the expression of inflammatory cytokines [23]. But TLR3 engagement also induces the expression of type I interferons (IFNs) via interferon regulatory transcription factor (IRF) 3 [24]. TLR7 and TLR9 engagement induces the secretion of inflammatory cytokines through the activation of NFκB via MyD88. However, TLR7 and TLR9 can also induce the expression of type I IFNs through the activation of IRF7 [25]. Taken together, PAMP ligation to TLRs leads to the expression of effector molecules which finally organize the body's immune responds to pathogens (Fig. 3.2).

There is accumulating evidence from recent research that TLRs have distinct different functions beyond simple pathogen recognition. In a more complex immunologic view, an important role in dendritic cell maturation and T cell activation established TLRs as a link between innate and adaptive immunity [26]. Furthermore, the detection of several TLR members in multiple tissues and cell types – besides cells of the immune system – led to a more wide-ranging view on TLRs. Especially inflammatory disorders such as ischemic coronary artery disease [27] and liver disease [28] but also autoimmune diseases [29] are critically influenced by TLRs. Moreover, an involvement of TLRs in allograft acceptance/rejection during transplantation [30] or contact allergy to nickel [31] has been shown. Of interest, an

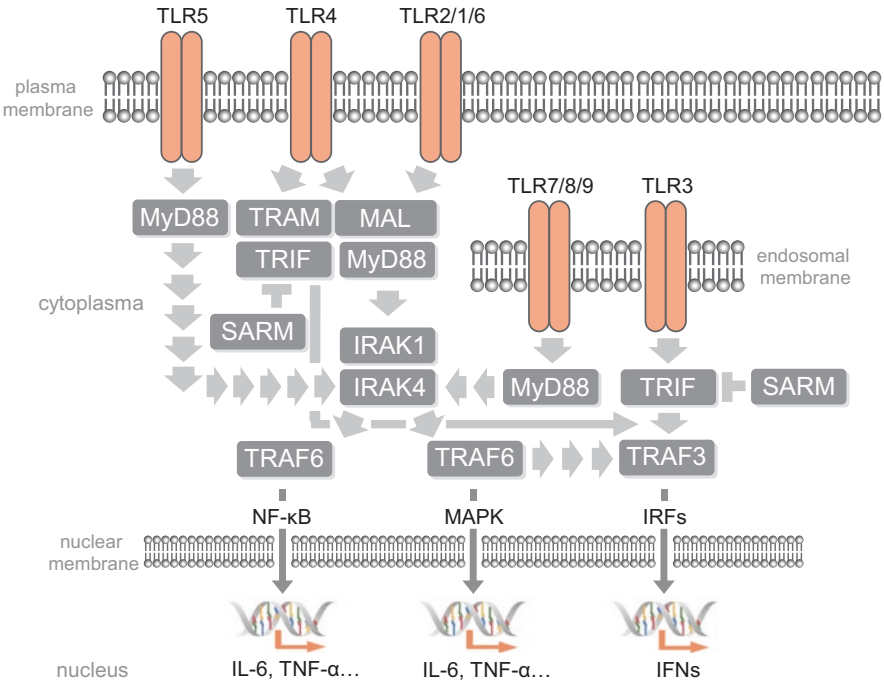

Fig. 3.2 TLR adaptor molecules and signaling pathways

Table 3.2 Endogenous TLR ligands

TLR	Endogenous Ligand
TLR2	Hyaluoran Biglycan Heat shock proteins High-mobility-group-protein B1
TLR3	RNA
TLR4	Fibronectin Fibrinogen Hyaluoran Biglycan Heparin sulfate Heat shock proteins High-mobility-group-protein B1 Oxidized low density lipoprotein
TLR9	Mitochondrial DNA High-mobility-group-protein B1

interaction of TLRs with endogenous ligands released during tissue damage and fibrosis or from apoptotic cells has recently been discovered and seems to regulate many sterile inflammatory processes [32]. In this regard the term danger or damage-associated molecular patterns (DAMPs) has been introduced. These ligands include proteins and peptides, polysaccharides and proteoglycans, nucleic acids, and phospholipids, which are cellular components or extracellular matrix (ECM) degradation products (Table 3.2). Recent studies provided clear evidence that endogenous ligand-mediated TLR signaling is involved in pathological conditions such as tissue injury, autoimmune diseases, and tumorigenesis. The ability of TLRs to recognize endogenous ligands appears to be essential for their function in regulating noninfectious inflammation. Furthermore, a novel role for TLRs in wound healing [33, 34] and liver regeneration [35] also in response to endogenous ligands [32] has been reported, suggesting even a regenerative aspect in TLR biology.

3 Angiogenesis: General Remarks

Physiological tissue function depends on adequate supply of nutrients and oxygen through blood vessels. Consequently, the cardiovascular system is the first organ system that develops during embryogenesis. Blood vessels in the embryo form the hemangioblast by differentiation of common mesodermal progenitor cells. The hemangioblast forms aggregates that evolve into hematopoietic precursor cells and angioblasts which further assemble the primary capillary plexus as differentiated endothelial cells. The formation of this primitive network on the basis of progenitor cells is called vasculogenesis. On the contrary, angiogenesis describes the enlargement of capillaries which sprout or become divided by pillars of periendothelial cells (intussusception) or by transendothelial cell bridges followed by remodeling

and maturation processes that transform the primary capillary plexus into a complex network of functional vessels [36]. Further covering and stabilization of vessels by smooth muscle cells as well as the enlargement of preexisting collateral arterioles is summarized as arteriogenesis. Although in the adult most vessels arise through angiogenesis, vasculogenesis may also be involved to some extent. Therefore, both processes are summarized in the hypernym neovascularization, which is involved in organ growth and wound healing but also contributes to pathological processes in malignant and inflammatory disorders [37–39].

Angiogenesis through sprouting and subsequent remodeling of capillaries into larger vessels has been extensively studied, and several essential steps have been described. Vascular endothelial growth factor (VEGF) and its receptors (VEGFR) have been identified as central regulators of both vasculogenesis and angiogenesis [40]. Until now, five VEGF ligands have been identified which occur in different spliced and processed variants and all of them represent secreted dimeric glycoproteins of ~40 kDa. In addition to VEGF A–D, also placenta growth factor (PLGF) belongs to the VEGF family as well. These ligands bind to the three receptor tyrosine kinases VEGFR1–3 with an overlapping pattern and co-receptors such as heparan sulfate, proteoglycans, and neuropilins. Different VEGFRs have distinct different functions; VEGFR1 is involved in the recruitment of hematopoietic progenitor cells and migration of monocytic cells whereas VEGFR2 and 3 are essential for the function of endothelial cells, especially during angiogenesis. Initially, VEGF was described to increases vascular permeability [41], thereby permitting extravasation of plasma proteins that establish a preliminary scaffold for migrating endothelial cells. For the emigration of endothelial cells from their resident site, interendothelial cell contacts and periendothelial cell support have to be dissolved, leading to destabilization of the mature vessel. Angiopoietin (Ang) 2, an inhibitor for tyrosine kinase with Ig and epidermal growth factor (EGF) homology domains (Tie) 2 signaling are involved in detaching smooth muscle cells and breaking up the ECM [42, 43]. Especially during angiogenesis, the interaction of the Ang-Tie system with the VEGF system becomes apparent. Capillaries sprout and subsequently grow alongside a VEGF gradient. Endothelial cells at the leading edge of the migration front, so-called tip cells, exhibit numerous filopodia and express members of the VEGFR family. Subjacent endothelial cells could be subdivided in highly proliferative and differentiating stalk cells and resting phalanx cells which both express components of the Ang/Tie system [43]. Furthermore, proteinases of the plasminogen activator, matrix metalloproteinases (MMPs), and chymase families influence angiogenesis by degrading ECM and by liberating growth factors, e.g., VEGF, basic fibroblast growth factor (bFGF), and insulin-like growth factor (IGF)-1, sequestered within the ECM. When the path has been cleared, endothelial cells can proliferate and migrate to remote sites [39].

Angiogenic sprouting is controlled by a tightly regulated balance of activators and inhibitors. In addition to VEGF, Tie2 phosphorylation by Ang1 is chemotactic for endothelial cells and stabilizes VEGF-initiated endothelial networks by stimulating the interaction between endothelial cells and periendothelial cells [43]. Members of the FGF and platelet-derived growth factor (PDGF) family support

angiogenesis presumably by recruitment of mesenchymal or inflammatory cells. Another key component of sprouting angiogenesis by regulating tip cell vs. stalk cell communication is the highly conserved Delta/Notch signaling pathway. Mammalians possess four different notch receptors, referred to as Notch1–4. Notch receptors are single-pass transmembrane receptors and capable of binding the membrane-bound ligands Delta-like (Dll) 1–4 and Jagged. Notch signaling in the stalk cells induces a quiescent and non-sprouting phenotype in endothelial cells whereas adjacent tip cells express Dll4, therefore promoting sprouting activity [44]. In addition, molecules are involved which mediate cell-cell or cell-matrix interactions, e.g., $\alpha_v\beta_3$ which localizes MMP-2 at the endothelial cell surface and promotes endothelial cell spreading. Moreover, a continuously number of molecules are discovered which are proangiogenic upon exogenous administration, including erythropoietin, leptin, hepatocyte growth factor (HGF), EGF, IGF-1, tissue factor (TF), and several other cytokines, chemokines, and growth factors [39, 45]. Even hematopoietic growth factors such as granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) have been shown to exhibit proangiogenic potential [46]. On the contrary, angiogenesis inhibitors suppress endothelial cell proliferation and migration, e.g., angiostatin, endostatin, antithrombin III, IFN-β, leukemia inhibitory factor (LIF), and platelet factor 4 [39, 47]. Thus, various pro- and antiangiogenic factors cooperate to regulate the angiogenic process.

Finally, vessel maturation finalizes the angiogenic process. Proliferating endothelial cells initially assemble as solid cords which acquire additional lumen formation. Lumen formation is accomplished by thinning of endothelial cells or fusion of preexisting vessels, mediated by VEGF, Ang1, and integrins such as $\alpha_v\beta_3$ or α_5 and controlled by the inhibitory effects of thrombospondin-1. Additional important steps after lumen establishment involve the differentiation of endothelial cells according to the environmental demands, maturation into a functional three-dimensional endothelial network, and the protection of quiescent endothelial cells against apoptosis [39, 45]. Periendothelial cells are essential for vascular maturation and completion of angiogenesis. Nascent vessels are stabilized by pericytes in case of capillaries. In case of arteries, arterioles, veins, and venules, smooth muscle cell recruitment and growth mediated by VEGF or PDGF are crucial for vessel stabilization. They thereby provide hemostatic control and protect the new endothelium-lined vessel against rupture and regression [39, 47].

Blood vessel formation in the adult includes vasculogenesis, angiogenesis, and arteriogenesis. Impaired neovascularization represents a therapeutic target in several pathologies associated with insufficient blood supply, e.g., acute myocardial infarction or chronic peripheral artery disease. Formation of new vessels and remodeling of the preexisting vasculature are essential for a successful therapy. Therefore, different treatment strategies involving administration of growth factors, cytokines, or progenitor cells are considered [48]. Important for the understanding of angiogenic mechanisms in these pathologies is the knowledge of variations from physiologic angiogenesis. In contrast to the physiological processes, pathologic angiogenesis is often promoted by inflammation. Monocytes, platelets, mast cells,

and other leukocytes are recruited to sites of inflammation or wound healing, partly by proangiogenic factors such as VEGF [37, 39]. Moreover, development of solid tumors strictly depends on a growing capillary network – termed as tumor angiogenesis – ensuring sufficient supply with oxygen and nutrients. Accordingly, antiangiogenic concepts aim at the inhibition of tumor angiogenesis and thereby tumor nutrient supply [49]. In this regard, the first antitumor therapy with a VEGF-neutralizing monoclonal antibody for the treatment of metastasizing bowel cancer was approved by the US Food and Drug Administration in 2004.

4 Inflammation-Induced Angiogenesis

Disorders associated with perpetuated angiogenesis are considered to be angiogenic inflammatory diseases. Inflammation plays not only a key role in pathogen defense during infection; it also plays a key role in repair mechanisms, e.g., wound healing and subsequent tissue regeneration. Physiological wound healing requires the integration of complex cellular and molecular events. The repair process is tightly controlled involving different cell types during the phases of initial inflammation as well as the successive cell migration, cell proliferation, and angiogenesis. Several angiogenic mediators, including growth factors, cytokines, MMPs, matrix macromolecules, cell adhesion receptors, chemokines, and chemokine receptors, have been implicated in the process of capillary formation [50]. Of note, cytokines and growth factors released at the site of injury are essential for the repair process [51]. In this regard, angiogenesis, the reestablishment of a capillary network by endothelial cells, is mainly initiated and maintained by the major proangiogenic factor VEGF. Besides endothelial cells, the angiogenic process involves also other cell types including inflammatory cells which represent a major source of growth factors and critically contribute to angiogenesis [52]. Platelets, mast cells, primarily monocytes/macrophages, neutrophils, and other leukocytes are recruited to sites of wound healing, partly by the action of the proangiogenic factors such as VEGF. All these cells in turn release proangiogenic factors such as VEGF, bFGF, TGF-β, PDGF, tumor necrosis factor (TNF)-α, insulin-like growth factor (IGF)-1, monocyte chemotactic protein (MCP)-1, interleukin (IL)-6, IL-8, and many more. All these factors finally attract endothelial cells, smooth muscle cells, pericytes, and fibroblasts to accomplish vessel growth in order to restore sufficient blood supply [39]. Newly formed blood vessels again enhance inflammatory cell recruitment setting up a stimulating forward loop. In this regard, inflammation often promotes angiogenesis establishing the term inflammation-induced angiogenesis.

In inflamed tissues a regulatory network is involved in the control of angiogenesis. Accumulating evidence suggests an association between angiogenesis and inflammation in pathological situations. Therefore, angiogenesis and inflammation seem to be intimately involved in many chronic inflammatory disorders with distinct etiopathogenic origin, including rheumatoid arthritis, diabetes, cancer, and many more. For example, there is considerable evidence of an interrelationship

between the mechanisms of angiogenesis and chronic inflammation in inflammatory bowel disease (IBD). The increased expression of endothelial junction adhesion molecules found in IBD patients indicates the presence of active angiogenesis. Evidence that angiogenesis is involved in IBD was also obtained from animal models of colitis, most notably from studies of angiogenesis inhibition. Moreover, serum levels of VEGF correlate with disease activity in human IBD [53]. This concept has been further supported by the finding that several previously established non inflammatory disorders, such as obesity, display both inflammation and angiogenesis in an exacerbated manner [54]. In addition, the interplay between recruited inflammatory cells and local endothelial cells and fibroblasts at sites of chronic inflammation, together with the fact that inflammation and angiogenesis can actually be triggered by the same molecular events, further strengthen this association. Angiogenesis might be targeted by several specific approaches that could be therapeutically used to control inflammatory diseases.

5 Toll-Like Receptors in Inflammation-Induced Angiogenesis

It is experimentally well established that angiogenesis and inflammation represent two prominent processes involved in normal physiologic responses and pathological states. Emerging evidence also suggests that TLRs have an important role in maintaining tissue homeostasis by regulating the inflammatory and tissue repair responses to injury. Infectious disorders result in inflammation which in turn promotes angiogenesis mainly by the action of growth factors released by different leucocytes. Even though the association of inflammation and angiogenesis has been established for a while the knowledge about the role of TLRs in this context is still limited [55]. However, a significant number of publications demonstrate that several TLR agonists are able to induce the expression and secretion of angiogenic factors from different cell types in vitro. The majority of these studies remain rather descriptive in this context and are very much focused on LPS and VEGF. Up to know, only few data document a direct involvement of TLRs in angiogenesis, both in physiological and in pathophysiological settings.

6 Toll-Like Receptors in Infection-Induced Angiogenesis

Accumulating evidence points to a direct contribution of TLRs to the angiogenic process following bacterial infections, also referred to as infection-induced angiogenesis. In this regard, PAMPs from various bacterial species – super abound in an infection setting – are known to act via different TLRs. A possible influence of TLRs on angiogenic processes was first discovered in the context of adenosine and its A_{2A} receptor ($A_{2A}R$). The nucleoside adenosine was found to stimulate angiogenesis through upregulation of VEGF, thereby participating in tissue protection following

ischemic events. In 2002 Leibovich et al. described a synergistic interaction of $A_{2A}R$ agonists with LPS through the TLR4 pathway. This interaction resulted in a strong upregulation of VEGF and downregulation of TNF-α in macrophages [56] and could also be demonstrated for TLR2, 7, and 9 [57], representing an angiogenic switch. This synergy observed in vitro seems to play an important role in vivo, too. Given the fact that MyD88-deficient mice showed markedly slower wound healing and reduced generation of new capillaries in response to an $A_{2A}R$ agonist [34]. In terms of TLR4, it is very likely that LPS induces adenosine which in turn promotes angiogenesis through $A_{2A}R$ by the upregulation of VEGF expression in macrophages [58].

Independent of the $A_{2A}R$ system, Pollet et al. showed that the TLR4 ligand LPS directly stimulates endothelial sprouting in vitro via a TRAF6-, NFκB-, and JNK-dependent mechanism. However, the responsible angiogenic growth factors remained elusive in this context [59]. Furthermore, a so far unidentified TLR ligand seems to be involved in the formation of angiogenic lesions resulting from infection with the facultative intracellular bacterium *Bartonella henselae*. This bacterial infection leads to the activation of hypoxia-inducible factor-1 (HIF-1) and thus to an enhanced MCP-1 production in endothelial cells which in turn induces chemotaxis of monocytes in order to initiate angiogenesis by VEGF production. Interestingly, MCP-1 production was independent of LPS/TLR4 but dependent on NFκB [60, 61]. A serious problem of severe ocular infection is pathological corneal neovascularization which could finally lead to visual disorders. In this regard, it has been shown that VEGF and TLR4 expression are upregulated in response to LPS and that VEGF expression is TLR4-dependent [62].

But angiogenesis also contributes to the regeneration process during liver fibrosis which is associated with increased endotoxin levels in the gut and portal circulation. Jagavelu et al. recently demonstrated a key role for the TLR4/MyD88 axis during VEGF production and the subsequent angiogenic process in liver endothelial cells following LPS stimulation [63]. Likewise, mycoplasma infections could be accompanied by enhanced angiogenesis and microvascular remodeling which are features of the chronic inflammation as elicited by *Mycoplasma pulmonis* infections of the respiratory tract [64]. In this regard, we recently investigated the highly angiogenic properties of the specific TLR2/6 agonist macrophage-activating lipopeptide of 2 kDa (MALP-2), a diacylated lipopeptide which occurs in *Mycoplasma* species and gram-positive bacteria. Interestingly, this process seems to be independent of VEGF. We discovered a TLR2/6-dependent induction of the MAPK cascade and NFκB and a strong secretion of GM-CSF in particular from endothelial cells and to a lesser degree from monocytes. Accordingly, MALP-2-induced angiogenesis in vitro and in vivo could be suppressed by inhibition of GM-CSF [65]. Similarly, human bone marrow mesenchymal stem cells (MSCs) secreted growth factors in response to a TLR2/6-dependent stimulation by MALP-2. This process in turn promoted proangiogenic properties of endothelial cells such as migration, proliferation, and tube formation in vitro in a paracrine manner. MSCs isolated from the bone marrow of sheep and co-cultivated with MALP-2 ex vivo significantly enhanced capillary density of skeletal muscle after autogenic implantation of these MSCs [66]. This renders MALP-2 potentially eligible for therapeutic angiogenesis or cell therapy.

In addition to an acute infection upon injury, there are different acute or chronic inflammatory disorders which are also associated with bacterial infection independent of an initial injury. Arthritis is characterized by inflammatory cell infiltration into the concerned joint. Progression of the disease includes self-perpetuating destruction of articular cartilage and extensive angiogenesis in the synovial membrane. Especially TLR2 ligands of gram-positive bacteria such as peptidoglycan (PGN) seem to be responsible for this angiogenic phenotype characterized by the induction of VEGF in chondrocytes [67] and accordingly VEGF and IL-8 in fibroblasts [68]. In light of immune defense, infection-induced angiogenesis might represent a general mechanism to restore blood flow in order to recruit immune cells for pathogen clearance and tissue regeneration with implication for future angiogenic therapy.

7 Toll-Like Receptors in Tumor Angiogenesis

The development of cancer has been associated with microbial infection, injury, inflammation, angiogenesis, and tissue repair. The role of TLRs in tumor angiogenesis is quite diverse just as cancer itself. Tumor inflammation could promote tumor angiogenesis, immunosuppression, and finally tumor growth. However, the mechanism controlling inflammatory cell recruitment to the tumor is not well understood. Cyclooxygenase (COX)-2 is known to play a crucial role in *Helicobacter pylori*-associated gastric cancer. In this regard, Chang et al. demonstrated that *H. pylori* acts through TLR2 and TLR9 to activate the MAPK cascade leading to COX-2-dependent prostaglandin E_2 (PGE_2) release and thereby contributing to cancer cell invasion and angiogenesis [69]. Furthermore, extracellular HSP70 peptide complexes are able to promote the proliferation of hepatocellular carcinoma cells in a TLR2/4 dependent manner [70]. Besides exogenous ligands that contribute to TLR-mediated tumor angiogenesis, also parts of the extracellular matrix (ECM) which are implicated in a variety of human cancers can induce VEGF expression in endothelial cells. Biglycan as one component of the ECM increases the interaction of NFκB and the HIF-1α promotor in a TLR2- and TLR4-dependent manner resulting in VEGF secretion, enhanced cell proliferation and tube formation. VEGF released by endothelial cells in turn promotes cancer cell migration and metastasis [71]. On the other hand, stimulation of TLRs with particular agonists can also cause antitumor activity, interfering with cancer proliferation and angiogenesis by mechanism still incompletely understood. For instance, the immunomodulatory TLR9 agonist IMO inhibited microvessel formation and tumor growth [72]. Likewise, TLR3 agonists not only affect tumor microenvironment by suppressing angiogenesis but also directly induce tumor cell apoptosis and inhibit tumor cell migration [73]. Interestingly, siRNAs may produce therapeutic effects in a target-independent manner through the stimulation of the TLR3/interferon pathway and suppression of angiogenesis. Injection of siRNAs against different targets led to a comparable reduction in liver tumors and to an inhibition of tumor vasculature

remodeling. In addition, polyI:C treatment reduced liver tumors and decreased hepatic arterial blood flow, indicating that TLR3 may mediate antiangiogenic and antitumor properties [74].

In all likelihood, there are two different possibilities for TLR agonists to limit tumor growth. First, by altering the tumor microenvironment and inhibiting angiogenesis and second, by clearing tumor cells due to enhanced activity of natural killer and tumor-reactive T cells. In this regard, the TLR7 agonist imidazoquinoline and the TLR9 agonist unmethylated CpG oligonucleotides were shown to exhibit strong local activity against leukemia, and respective phase I trials are currently in progress at different centers [75]. We recently identified proangiogenic properties for the TLR2/6 ligand MALP-2 [65]. Interestingly, there are also antitumor activities reported for MALP-2 [76–78]. However, whether MALP-2 affects tumor angiogenesis is currently unknown. Concanavalin-A (ConA) is another TLR2/6 agonist that promotes endothelial cell proliferation trough a JAK/STAT3-mediated increase in the expression of colony-stimulating factor-(CSF) 2 and -3 in human mesenchymal stromal cells [79]. TLR4 expression in the tumor microenvironment was found to be associated with adenocarcinoma in human samples and in the murine model. Adenocarcinoma patients with higher TLR4 expression in stromal compartment had a significantly increased risk in disease progression. These data suggest that high TLR4 expression in the tumor microenvironment represents a possible marker for disease progression in colon cancer [80]. So far, there are different polymorphisms in several TLR gene clusters known which may shift balance between pro- and anti-inflammatory cytokines, modulating the risk of infection, chronic inflammation, and cancer. This may offer the possibility for improved diagnostics in patients. Future studies in large populations should shed light on the significance of TLR polymorphisms for cancer prevention [81].

8 Endogenous Toll-Like Receptor Ligands in Angiogenesis

Sustained pro-inflammatory responses in diseases such as rheumatoid arthritis, atherosclerosis, diabetic retinopathy, and cancer are often associated with increased angiogenesis that contributes to tissue disruption and disease progression. In recent years, there was accumulating evidence that also endogenous ligands which are released during ECM breakdown or by apoptotic cells could bind to different TLRs (Table 3.2). In this context, the high-mobility group B1 (HMGB1) which is released by necrotic cells has been recognized to signal through the receptor for advanced glycation end products (RAGE) and via TLR2 and TRL4. Activation of these receptors resulted in the activation of NFκB and the upregulation of angiogenic factors like VEGF in both hematopoietic and endothelial cells [82]. HMGB1 released at wound sites initiates TLR4-dependent responses that contribute to angiogenesis by regulating endothelial permeability and vascular growth [83, 84]. The interaction of HMGB1 and TLR4 also mediates the recruitment of endothelial progenitor cells to the sites of neovascularization by upregulation of stromal cell-derived factor-1

(SDF-1) [85]. Recent data by van Beijnum et al. identified HMGB1 even as an important modulator of tumor angiogenesis [86]. Thus, targeting the HMGB1 signaling cascade may constitute a novel therapeutic approach to angiogenesis-related diseases. Following this line, inflammation-induced oxidative stress and angiogenesis is emerging as an important mechanism underlying numerous processes from tissue regeneration and remodeling to cancer progression. Interestingly, West et al. recently reported that end products of lipid oxidation such as ω-(2-carboxyethyl) pyrrole (CEP) are generated and accumulate during inflammation, wound healing, and in tumors. CEP is specifically recognized by TLR2 but not TLR4 or scavenger receptors in endothelial cells, leading to a MyD88-dependent angiogenic response that is independent of VEGF [87]. In this regard, stress-sensing by TLR2 seems to be a major driver of angiogenesis [88, 89]. Apparently, also endogenous ligands, which accumulate during inflammatory tissue disruption and enhanced oxidative stress conditions, are capable of promoting angiogenesis via a TLR-dependent pathway. Thus, TLRs are activated not only in response to tissue-invading pathogens but also pathogen-independent. In both cases TLRs have important functions in the recruitment of immune cells in order to initiate a regenerative program: in the first case mainly to eliminate invading pathogens and in the second case to clear the affected tissue from apoptotic cells and cellular debris. Obviously, angiogenic processes are involved in both scenarios (Fig. 3.3).

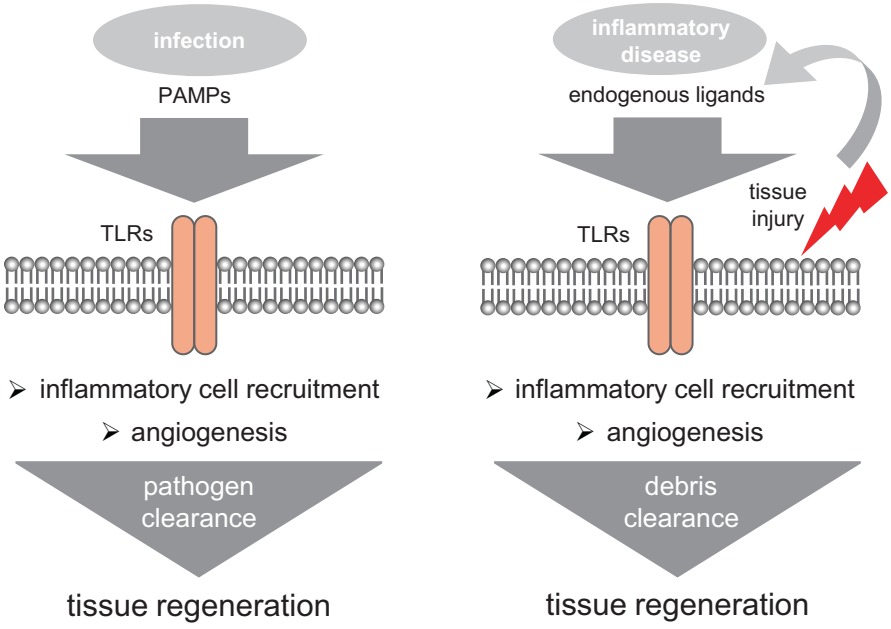

Fig. 3.3 Tissue regeneration through TLRs

9 Oxidative Stress and Toll-Like Receptor-Dependent Angiogenesis

Increased oxidative stress is closely related to many disease pattern, e.g. to the pathology of cardiovascular diseases like atherosclerosis, myocardial infarction and stroke. If the well-balanced homeostasis of oxidative and anti-oxidative processes is shifted towards increased formation of reactive oxygen species (ROS), the resulting oxidative stress leads to the onset of various inflammatory processes. However, there is growing evidence of a potential regenerative crosstalk between oxidative stress and TLRs in angiogenesis in recent years. In this regard, Chen et al. reported that decreased NADPH oxidase (NOX)1 and 4 expression, ROS formation as well as increased vascularization in fat grafts after enrichment with adipose-derived stem cells is TLR4-dependent [90]. In addition, Menden and colleagues described a new mechanism which could be involved in microvascular remodeling after sepsis in the lung. They reported that NOX2 inhibition attenuated LPS-mediated Ang2 signaling and capillary network formation in human pulmonary endothelial cells in vitro [91]. This signaling axis involves the NFκB and MAPK pathways and suggests a tied cooperation of TLR- and NADPH oxidase-dependent signaling to coordinate endothelial regeneration after infection. Interestingly, we observed a related cooperation of TLRs and the NADPH oxidase. In an ongoing project, we identified NOX2-derived superoxide anions as important regulators for GM-CSF release from endothelial cells in response to TLR2/6 stimulation (unpublished data). In this regard, we have already shown that TLR2/6-induced GM-CSF release mediates endothelial proliferation, migration and angiogenesis [65]. The already in the last section mentioned study by West et al. additionally suggested that endogenous end products of augmented oxidative stress could represent a new class of TLR ligands [87]. Since ROS do not only accelerate pathological processes, but are also important for many signaling transduction pathways, an interaction with TLRs in a regenerative aspect seems logical and opens up new possibilities for future research. Particularly because the number of studies in the field is still small.

10 A Side Glance on NOD Receptors and Angiogenesis

Although this chapter is focused on the role of TLRs in angiogenesis we want to take a brief look at a subfamiliy of the nucleotide-binding oligomerization domain (NOD)-like receptors at this point. The NOD receptors NOD1 and NOD2 are intracellular receptors, which sense conserved motifs of bacterial peptidoglycan and are the founders of the entire NOD-like receptor family. Just as TLRs, they belong to the class of PRRs with important functions in immune defense [92]. They were identified at the turn of the millennium by sequence homolog searches. Receptor ligation leads to recruitment of the receptor-interacting protein kinase 2 (RIPK2) followed by the activation of the MAPK and NFκB pathway and subsequently to the

induction of many well-known inflammatory genes such as IL-6 and TNF-α. Besides immune defense, recent work showed the involvement of NOD1 and NOD2 in many inflammatory diseases, e.g. IBD, cardiovascular disease and metabolic disease [93]. Campbell and colleagues reported, that NOD2-deficient mice displayed a substantial delay in acute wound repair [94] pointing to an even regenerative role of NOD receptors. However, reports on angiogenesis are very limited so far and to our knowledge there is up to now only one single study existing. Interestingly, Schirbel et al. demonstrated that NOD1 and NOD2 agonist – just like TLR agonists – are capable of inducing proliferation, migration, transmigration and tube formation of human intestinal microvascular endothelial cells in vitro as well as angiogenesis in a mouse model in vivo [95]. These processes were consequently mediated via RIPK2, MAPK and NFκB signaling. Different to TLRs, no endogenous NOD ligands have been identified so far which might play a role in angiogenesis. This new research field as well offers many opportunities to shed more light on the overall picture of PRRs in angiogenic processes.

11 Summary and Therapeutic Perspectives

Accumulating evidence points to a crucial role of TLRs in angiogenesis. However, the mode of action of TLRs in this context is quite diverse. TLR activation consistently promotes angiogenesis in various inflammatory settings in response to both exogenous and endogenous ligands. In regard to an acute local infectious scenario, the angiogenic process seems to be important for sufficient blood supply and the recruitment of immune competent cells for pathogen clearance and subsequent tissue regeneration. In contrast, chronic local infection or prolonged pathogen-independent inflammation leads to excessive angiogenesis with eventually pathological consequences. It should not be left unmentioned here that TLRs may also prevent angiogenesis. In endothelial progenitor cells isolated from umbilical cord blood, TLR3 activation specifically inhibits their proangiogenic properties [96]. Similar, blocking TLR2 in endothelial cells has been shown to promote angiogenesis by a crosstalk between TLR2 and CXCR4 and the activation of proangiogenic kinases downstream of CXCR4 [97]. Moreover, TLR2-deficient mice undergoing hindlimb-ischemia exhibit an augmented capacity to stimulate angiogenesis. A process, that seems to be mediated by immune cells rather than endothelial cells [98]. However, specific ligands for these antiangiogenic effects have not been described yet. Pro- and antiangiogenic properties of TLRs are likewise reported in tumor angiogenesis.

In the future, modulation of TLR signaling could provide the basis for the development of novel therapeutic approaches in diverse settings. Stimulation of TLRs with specific ligands could be used for future therapeutic angiogenesis. However, beneficial effects of therapeutic angiogenesis may be negatively impacted by side effects of pharmacological substances such as statins or non-pharmacologic hormones such as erythropoietin. Moreover, certain requirements for this therapeutic

process are warranted. First, as simple as it may sound but no harm should be induced especially tumor induction or tumor growth should be avoided. Second, in order to promote a sustained recovery, endogenous mechanisms of angiogenesis should be induced rather using an excessive administration of exogenous factors which may also act as antigens or inducing tolerance when applied over a long period of time. Finally, organ-specific requirements for recovery should be considered, e.g., for cerebral reconstitution angiogenesis, neurogenesis, synaptogenesis, and neuronal and synaptic plasticity should be induced in parallel [99].

Thus, therapeutic modulation of TLR signaling is a very attractive and novel but also sophisticated therapeutic approach to promote angiogenesis. In order to induce long-term organ repair and restoration after ischemic events, for example, detrimental TLR signaling should be inhibited and in parallel beneficial TLR signaling should be induced. From this point of view, inhibitory strategies targeting TLR signaling seem to be plausible in chronic and persistent infectious situations such as rheumatoid arthritis. Small molecules or siRNA against specific TLRs or their downstream targets may provide novel tools to combat local inflammation via inhibition of angiogenesis. Especially advanced tissue penetration properties of those engineered molecules render them applicable and superior for the use in tissues which are inaccessible for antibiotics. Likewise, inhibitory strategies targeting TLRs could be used to inhibit pathological tumor angiogenesis in order to limit tumor growth. In particular, modulation of TLR3, TLR7, and TLR9 activity seems to be a potential future therapeutic target [72–74]. However, great caution is required since pro- and antiangiogenic properties with subsequent pro- or antitumorigenic properties of different agonists recognized by the same TLR are reported.

The potentially most promising future therapeutic approach is the application of specific TLR agonists in damaged ischemic or hypoxic tissues in order to promote angiogenesis and subsequent tissue regeneration, especially when the tissue damage is not initiated or accompanied by severe infection, e.g., in peripheral arterial occlusive disease. In such settings, a single application of TLR agonists mimics an infectious scenario without prolonged local pathogen presence. Such an initial therapeutic boost of the immune system with specific TLR agonist aims to launch a defined regenerative program including enhanced angiogenesis. Of note, the application of single proangiogenic growth factors has already been tested in clinical trials. However, in the case of VEGF monotherapy, large-scale trials have not yet yielded consistent beneficial results [100, 101]. This may be related to recent observations that several other potent proangiogenic factors act in concert with VEGF for proper vessel formation and maturation [43, 44, 47]. In this regard, stimulation of specific TLRs (e.g., TLR2/6) may provide an opportunity to induce a specific pattern of proangiogenic growth factors for sufficient vessel growth and tissue regeneration. Thus, we raised our hope on biologicals such as the lipopeptide and TLR2/6 agonist MALP-2. Recent results from our group indicated that the proangiogenic properties of MALP-2 critically depended on the induction of the growth factor GM-CSF in endothelial cells and monocytes [65]. Additional experiments in a vascular endothelial denudation model in mice revealed promising effects of MALP-2 on endothelial regeneration after vascular injury [102]. Those experimental data are the basis for

studies in larger experimental animals and future applications using MALP-2 or related agonists in patients, who suffer from peripheral vascular damage or occlusion in diabetes or post percutaneous vascular interventions or even following stroke. Nevertheless, the question remains how to apply such substances since local delivery is preferred in order to avoid side effects and promote endogenous proangiogenic restoration effects downstream of the site of application. Therefore, we aim to test coating procedures on traditional devices such as drug-eluting stents or coated balloons widely used in interventional cardiovascular medicine. However more innovative devices/treatment approaches such as nanofibers, polymer biodegradable soaked stents with TLR ligands, or endovascular patches placed in the occluded vessel or as seal on the balloon-disrupted vascular segment are in the focus of our interest.

In summary, modulation of TLR activity may offer the possibility for different future therapeutic concepts. Inhibition of TLRs is maybe favorable in settings of prolonged infection/inflammation to rescue the inflamed tissue or to inhibit pathological tumor angiogenesis to limit tumor growth. The contrary concept, TLR stimulation, offers a promising option to promote therapeutic angiogenesis for tissue regeneration.

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Chapter 4
Vascular Stem Cells in Regulation of Angiogenesis

Jingwei Lu, Vincent J. Pompili, and Hiranmoy Das

Abstract Angiogenesis is the process by which new vessels are generated from the preexisting blood vessels, which is the major contributor of postnatal neovascularization process. Disruption or dysregulation of angiogenesis is involved in various pathological conditions, such as ischemia and tumor progression. Stimulation of angiogenesis was proposed to be able to restore the blood flow and contribute to the tissue recovery in ischemia, while inhibition of angiogenesis can impede tumor progression. The importance of angiogenesis has generated tremendous interest in studying the mechanisms and to find out major contributors of the process. The current stem cell research has significantly improved our understanding of angiogenesis and its possible therapeutic application. Hypoxia is the most important driving force of angiogenesis, while other factors, such as chemokines and cytokines, haptotaxis, and mechanotaxis, are also important in regulating neovascularization process. In this chapter, we will focus on the progenitor cells that contribute to the angiogenesis and the underlining mechanisms involved in this process.

Keywords Angiogenesis • Stem cells • Hypoxia • Cytokines • Hypotaxis • Mechanotaxis • Signaling molecules • MicroRNA

1 Introduction

Galen, the second-century physician, speculated that the vascular system served to carry blood and provide nutrition to the human body [1]. It is now well established that the vascular system provides the main network of channels for nutrients (such as amino acids, electrolytes, oxygen, and hormones) to all the body tissues. Disturbances in the vascular system, mainly blocking the blood supply to the

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tissues, cause a variety of circulatory diseases, from peripheral artery disease to peripheral venous disease, and include among them vascular diseases like aneurysms, renal artery stenoses, and Buerger's disease [2]. Disruptions of angiogenesis play a critical role in the pathological progression of various ischemic diseases, such as stroke, ischemic heart disease, and the multiple peripheral vascular disease syndromes, resulting in a shortage of blood supply and which eventually induce apoptosis and necrosis of cells and the tissues of the vascular system. Angiogenesis, however, plays an important role in the regeneration of such ischemic tissues. In a seemingly contradictory role to that in the ischemic diseases, angiogenesis contributes to damage caused by the progressive growth of malignant tumors. Targeting tumor growth by targeting tumor angiogenesis, as in using various drugs to reduce blood supply to the tumor, is one of the major therapeutic considerations for effective control. Rapid proliferation of tumor cells, with lack of blood supply and lack of oxygen, triggers upregulation of vascular endothelial growth factor (VEGF) secretion, which promotes the angiogenesis process. The importance of angiogenesis in pathological conditions has generated interest in studying the mechanisms and signaling pathways for angiogenesis. Various stem cells were proposed to be important for initiation of the angiogenesis process. Mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs), which were shown to repair ischemic tissues, have great ability to promote angiogenesis via neovascularization and thereby to reduce the amount of ischemic tissue damage [3, 4]. Other cell types such as smooth muscle stem cells and vascular pericytes were also shown to be beneficial for the process of angiogenesis. In this chapter, we will focus on the role of various stem cells on the angiogenesis process and on the molecular mechanisms that promote these stem cells to form new blood vessels.

2 Angiogenesis and Stem Cells

The wall of blood vessels contain endothelial cells, mural cells, and extracellular matrix (ECM). The inner lining of blood vessel is the endothelium, which is a thin layer of endothelial cells. Mural cells are specified as determined by the location of the vessel; they could be pericytes, smooth muscle cells, and fibroblasts. The mural cells are embedded in the extracellular matrices [5]. The various types of cells forming blood vessels could be derived from multiple stem/progenitor cells. Circulating endothelial progenitor cells (EPCs) and HSCs could differentiate into endothelial cells thus directly contributing to the angiogenesis process. MSCs, though they may not be able to directly differentiate into endothelial cells, can secrete factors, such as VEGF, and promote the neovascularization process. Other progenitor cells, such as vascular pericytes and smooth muscle progenitor cells, can also contribute to angiogenesis (Fig. 4.1).

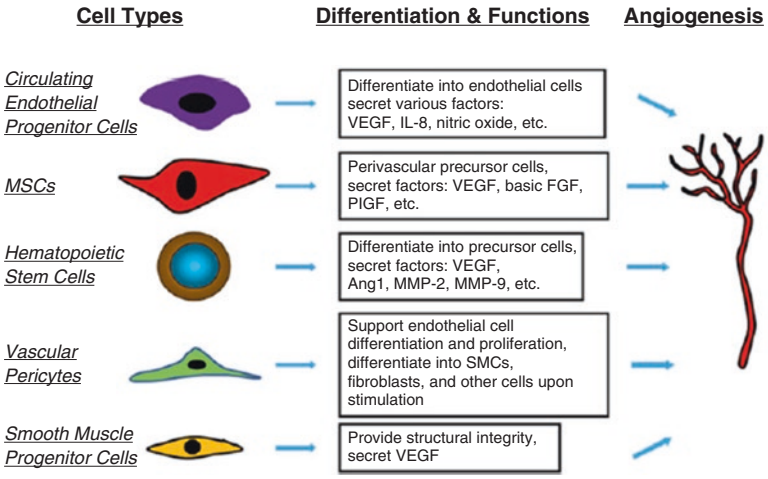

Fig. 4.1 Contribution of various stem/progenitor cells and their secretory molecules in the angiogenesis process (*MSCs* mesenchymal stem cells, *SMCs* smooth muscle cells, *VEGF* vascular endothelial growth factor, *IL-8* interleukin-8, *FGF* fibroblast growth factor, *PlGF* placental growth factor, *Ang1* angiopoietin 1, *MMP* matrix metalloproteinases)

2.1 Circulating Endothelial Progenitor Cells

The first study on putative EPCs was based on isolation of CD34+ mononuclear blood cells. The isolated cells were adhered to plastic and differentiated into endothelial cells upon culture [6]. Since the discovery of EPCs, various markers have been proposed to identify EPCs, such as CD34, CD133, expression of both CD133 and vascular endothelial growth factor receptor (VEGFR) 2, and expression of monocyte/macrophage-related molecule CD14 with minimal CD34 molecule [7]. The functional role of circulating EPCs has been actively investigated during the past few years. It was shown that higher level of VEGF may induce a rapid mobilization of HSCs and bone marrow-derived circulating endothelial precursor cells, which contribute to postnatal angiogenesis and hematopoiesis [8]. However, further study has shown that bone marrow-derived cells do not significantly contribute to tumor- or cytokine-induced angiogenesis rather tumor- or VEGF-induced angiogenesis is involved [9]. Based on their proliferation properties, two different categories of EPCs were identified in the peripheral blood, early EPCs and late EPCs. Early EPCs secrete more angiogenic cytokines, such as VEGF and interleukin (IL)-8 than do late EPCs; however, late EPCs produce more nitric oxide and incorporate more readily into human umbilical vein endothelial cell monolayers and form capillary tubes as compared to early EPCs [10].

2.2 Hematopoietic Stem Cells

HSCs and EPCs develop in close proximity to each other within the embryo. HSCs share the same ancestor with EPCs, called the hemangioblast. The existence of hemangioblasts was supported by various experimental observations, but its role during development is still controversial. Even though evidence has shown that single cell-derived colonies could produce both hematopoietic and endothelial cells in vitro, only a small portion of hematopoietic and endothelial cells were derived from hemangioblasts during development, which indicated that hemangioblasts might not be as significant as originally expected [11]. However, these studies illustrated the relationship of hematopoietic and endothelial lineage and indicated the possibility that HSCs might facilitate the angiogenesis during embryonic development and postnatal development. Indeed in acute myeloid leukemia (AML)-1-deficient embryos, which lack definitive hematopoiesis, defective angiogenesis in the head and in the pericardium was observed. The disruption in angiogenesis of para-aortic splanchnopleural (P-Sp) explant culture was rescued by addition of HSCs [12]. The recruitment of myeloid cells was found to be associated with formation of new blood vessel during pathological angiogenesis, and depletion of circulating myeloid cells significantly reduced the density of microvessels in a bioengineered human vascular implant [13]. The functional role of HSCs during angiogenesis may come from expression of proangiogenic factors such as VEGF and Ang1 and remodeling factors such as matrix metalloproteinase (MMP)-2 and MMP-9, which promote angiogenesis and guide the migration of endothelial cells [12]. It was found that hematopoietic cytokines SDF-1, induced by soluble Kit ligand, thrombopoietin, erythropoietin, and granulocyte-macrophage colony-stimulating factor (GM-CSF) released from platelets, enhanced neovascularization through mobilization of chemokine receptor (CXCR)-4+ VEGFR1+ hemangiocytes [14]. The important role of hematopoietic cells in angiogenesis has received great attention and proposed to be important target for anti-angiogenesis therapy following radiotherapy during treatment of tumor progression [15].

2.3 Mesenchymal Stem Cells

MSCs are present in many organs and function to maintain and regenerate connective tissues and replace damaged tissues following injury or inflammation. MSCs could efficiently stabilize nascent blood vessels in vivo acting as perivascular precursor cells, although differentiation of MSCs into endothelial cells was not detectable [16]. Co-implant human primary endothelial cells with human bone marrow MSCs showed enhanced formation of a network of functional, mature blood vessels accessed by in vivo whole body bioluminescence imaging in immunodeficient mice

[17]. Transplantation of MSCs was shown to be able to decrease fibrosis and myocardial scarring and improve myocardial regeneration in infarct-damaged hearts, through paracrine effects, via secretion of VEGF, basic fibroblast growth factor (bFGF), and placental growth factor (PlGF), even though MSC differentiation into ECs was not clearly demonstrated [18].

2.4 Smooth Muscle Progenitor Cells

Smooth muscle cells in the vascular system provide the structural integrity of the vessel wall. Recent study has shown that smooth muscle progenitor cells may have a potential role in angiogenesis. In a murine stroke model, it was shown that co-injection of smooth muscle progenitor cells with EPCs gave better results than administration of EPCs alone for vascular remodeling, cell proliferation, and neuroblast migration [19]. Perturbation in the signaling of transforming growth factor (TGF)-β, which is a multifunctional cytokine and plays an important role in carcinogenesis, was reported to affect endothelial and smooth muscle cell function and to contribute to tumor angiogenesis and tumor progression [20]. Smooth muscle cells can also contribute to angiogenesis by secreting mitogens, such as VEGF upon response to the hypoxia [21].

2.5 Vascular Pericytes

Pericytes are located surrounding the endothelial cells of the capillaries. Clonally isolated cells expressing pericyte markers were shown to be myogenic in culture in vivo [22]. It was proposed that pericytes derived from MSCs retain nascent stem cell properties, were recruited to the nascent microvascular wall during development and postnatal growth, and remained in a growth-arrested state until triggered to resume proliferation and differentiation later [23].

3 External Factors Regulate Angiogenesis Process

There are three distinct mechanisms, which promote cell migration during angiogenesis, chemotaxis, haptotaxis, and mechanotaxis. Chemotaxis directs cell migration toward a gradient of soluble chemoattractants, such as VEGF and bFGF. Haptotaxis attracts cells toward a gradient of immobilized ligands such as integrins binding to ECM components. Mechanotaxis promotes cell migration by mechanical forces, such as fluid shear stress [24]. Other factors including hypoxia will also be discussed here (Fig. 4.2).

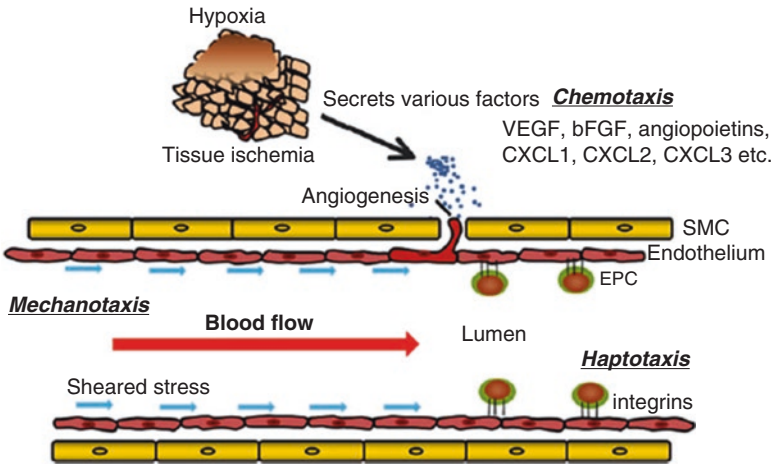

Fig. 4.2 Factors regulating angiogenesis process. Hypoxia, chemokines and cytokines, hypotaxis, and mechanotaxis are the major factors induce and regulate angiogenesis process (*EPCs* endothelial progenitor cells, *SMC* smooth muscle cells, *VEGF* vascular endothelial growth factor, *bFGF* basic fibroblast growth factor, *CXCL* chemokine (CXC-motif) ligand, *MMP* matrix metalloproteinases)

3.1 Chemotaxis: Cytokines, Chemokines, and Growth Factors

Various cytokines and soluble proteins, such as VEGF, bFGF, angiopoietins, FGF-2, hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), TGF-β, interleukins, and tumor necrosis factor (TNF)-α, promote the migration of endothelial cells during angiogenesis. VEGF is a major factor that regulates angiogenesis. Various factors can induce the production of VEGF, and hypoxia was reported to be one of them. Hypoxia is able to enhance the production of VEGF and its receptors [25]. Production of reactive oxygen species (ROS), for example, hydrogen peroxide (H_2O_2), also upregulates the gene expression of VEGF in endothelial cells [26]. VEGF was also found to be expressed by almost all solid tumor as an angiogenic mitogen and so is now targeted for anti-angiogenesis therapy for tumor metastasis [27]. VEGF and its family members stimulate cellular responses by binding to the tyrosine kinase receptors called VEGFRs. VEGFR1 (Flt-1) is required for the recruitment of hematopoietic precursors and migration of monocytes and macrophages [28]. VEGFR1-deficient mice die in utero between 8.5 and 9.5 days post-coitum due to early defects in the development of hematopoietic and endothelial cells [29]. The functional role of VEGFR2 (KDR/Flk-1) has been linked with proliferation, migration survival, and increased permeability, all of which contributes to the angiogenesis process [30].

VEGF plays critical roles in endothelial differentiation, in acquisition of arterial endothelial cell identity, and in the vascular patterning of vertebrate embryos. VEGF ligands and receptors such as VEGF-A, the prototype of VEGF ligand, VEGFR1, VEGFR2, and VEGFR3 regulate vasculogenesis and angiogenesis during various

stages of growth [31]. By studying a series of nerve-specific Cre lines, it was shown that peripheral nerve-derived VEGF promotes arterial differentiation through the $VEGF^{164}$-NRP1 positive-feedback loop [32]. It was further demonstrated that VEGF acted downstream of sonic hedgehog (Shh) and upstream of Notch pathway in the differentiation of endothelial cells to arterial fate [33].

Other factors also play important roles in promoting angiogenesis including bFGF, angiopoietins, HGF, PDGF, EGF, TGF-β, TNF-α, etc. Slow release of bFGF (using gelatin hydrogels) can promote new blood vessel formation compared with a control group in a murine limb ischemia model [34]. Angiopoietin was required for endothelial development from progenitors circulating in human cord blood. More specifically, endogenous angiopoietin-1 regulates initial endothelial cell commitment, while angiopoietin-2 improves expansion of the endothelial cell progeny [35]. Angiopoietin-1 and angiopoiein-2 may also play important role in regulating recruitment of mural cells during angiogenesis [36]. It was shown that overexpression of HGF in smooth muscle cells can be beneficial in EPC differentiation, proliferation, and migration [37]. Further study has shown that HGF stimulates migration and tube formation of human umbilical vein endothelial cells in a Nox2-dependent manner [38]. However, transplantation of bone-derived MSCs showed no significant differences in promoting angiogenesis with or without HGF, which indicated that further study is needed to investigate the interplay between HGF and MSCs [39].

Chemokines are a family of small chemotactic cytokines and are classified by the presence of four cysteine residues in conserved locations. Members of the chemokine family are divided into four groups CC chemokines, CXC chemokines, C chemokines, and CX_3C chemokines. Many chemokines were proven to be angiogenic such as CXCL1, CXCL2, and CXCL3. These chemokines activate endothelial cells upon binding with their receptors. It was reported that functional differences among endothelial cells is dependent on the level of expression of CXC chemokine receptors [40]. It was also proposed that CXC chemokine IL-8; growth-related oncogenes alpha, beta, and gamma; granulocyte chemotactic protein 2; and epithelial neutrophil-activating protein-78 mediate angiogenesis in the absence of preceding inflammation partially through interaction with CXC chemokine receptor 2 (CXCR2) [41]. CXCR2 is a member of the G-protein-coupled receptor family and is expressed in endothelial cells. CXCR2 knockout mice exhibited defective neutrophil recruitment, an altered temporal pattern of monocyte recruitment, significant delay in epithelialization, and decreased neovascularization in wound-healing processes [42]. It was shown that upon binding to IL-8, CXCR2 activates the Rac pathway, which leads to cell retraction and formation of gaps between neighboring cells. Translocation of Rac into the plasma membrane eventually results in endothelial activation [43]. These experiments suggest that CXCR2 plays an important role in the recruitment of cells and promoting angiogenesis. Other than CXCR2, VEGF- and bFGF-activated angiogeneses were also partially mediated through CXCR4. Stimulation of human umbilical vein endothelial cells with VEGF or bFGF was shown to be able to induce upregulation of CXCR4. It was further shown that chemokine SDF-1α, which specifically bind CXCR4, is a potent chemoattractant for endothelial cells and participates in angiogenesis stimulated by VEGF and bFGF [44].

3.2 Haptotaxis

Haptotaxis is the directional motility of cells by the ligands typically presented in the ECM. Exposure of ECM and binding to integrin help homing and recruitment of the immune cells during the angiogenesis process. These ECM and integrin molecules are also critical for homing of transplanted HSCs to the bone marrow and the recruitment of inflammatory cells to the sites of inflammation [45]. It was shown that hematopoietic progenitor cells of β_2 integrin-deficient mice are less capable of homing to the ischemic site and that improving neovascularization and preactivation of the β_2 integrins expressed on EPCs augmented the EPC-induced neovascularization [46]. Antagonists of integrin $\alpha_4\beta_1$ were shown to be able to block the adhesion of monocytes to endothelium and prevented monocyte stimulation during angiogenesis [47]. It was further shown that administration of α_4 integrin antibody resulted in increased numbers of circulating EPCs in vivo and systemic administration of anti-α_4 integrin antibody increased recruitment and the incorporation of bone marrow EPCs in newly formed vasculature of hind-limb ischemia and myocardial infarction models [48]. Integrin-dependent homing of progenitor cells can be enhanced by various factors. It was reported that high-mobility group box 1 (HMGB1) activated EPC migration in a RAGE (HMGB1 receptor expressed on EPCs)-dependent manner and was inhibited by β_1 and β_2 integrin inhibition. HMGB1 could rapidly increase the affinity of integrin and induce polarization of integrin, which might be related to the corresponding enhanced adhesion capability of EPCs [49]. Pharmacologic activation of Epac1, a nucleotide-exchange protein for Rap1, could increase Rap1 activity and stimulate the adhesion of various human progenitor cells. EPCs, CD34+ hematopoietic progenitor cells, and MSCs are activated through increased β_2 and β_1 integrin-dependent adhesion and activated progenitor cells home to the ischemic muscles in an increased amount as a result, neovascularization occurs [50].

3.3 Mechanotaxis

Mechanotaxis is the directed movement of cells by mechanical cues, such as fluidic shear stress and stiffness of substrate. Endothelial cells which make up the inner lining of blood vessels are constantly under fluid-mediated shear stress in vivo, and it was shown that this mechanical stress-mediated signaling contributes to each step of endothelial migration, cell-ECM adhesion, and cell–cell adhesion processes [51]. Shear stresses were reported able to induce changes in the shape of endothelial cells and partial disassembly of adherent junctions [52]. It was shown that endothelial cells, cultured on type I collagen-coated coverslip and wounded later, enhanced wound healing under higher shear stress [53]. The endothelial cell alignments induced by fluid shear stress were proposed to act through the p38/mitogen-activated protein (MAP) kinase-activated protein kinase 2 (MAPKAP kinase 2)/heat shock

protein (HSP) 25/27 pathway due to its critical role in actin dynamics. It was shown that by inhibiting p38 signaling, endothelial elongation and alignment were blocked in the direction of flow, elicited by shear stress [54]. Other mechanisms involving G protein have also been studied. It was shown that shear stress-induced cytoskeletal reorientation was abolished in cells overexpressing dominant negative Rac 1. This indicated that the Rac GTPase might play a role in regulating endothelial cytoskeleton by shear stress [55]. The endothelial cell reorientation in response to shear stress was further studied and was proposed to follow a two-step process involving Rho-induced depolarization, followed by Rho−/Rac-mediated polarization and migration in the direction of flow [56].

3.4 Hypoxia

Hypoxia plays a critical role in neovascularization, both in embryonic development and in postnatal development. During embryonic development, the vascular system is stimulated by an inadequate supply of oxygen, which is caused by rapid expansion of embryonic tissues. In adult tissues, the blood vessels do not undergo significant growth, and the oxygen concentrations remain relatively constant between 30 and 50 mm of Hg. In pathological conditions, however, as in ischemia, hypoxia is created by the lack of blood, which is the main carrier of oxygen, and reduction of the oxygen level triggers angiogenesis. Important molecules involved in the hypoxia response include prolyl hydroxylase domain-containing proteins (PHDs) and hypoxia-inducible factors (HIFs). PHDs play an important role in oxygen sensing by inhibiting HIFs expression and by promoting HIFs degradation. HIF is a key transcription factor governing a large set of gene expressions for hypoxia adaptation, for example, the inhibition of PHD suppressed lipopolysaccharide-induced TNF-α expression. Reducing oxygen will lead to poor hydroxylation activities by PHDs and thus lead to accumulation of HIF-α. Hundreds of proteins were regulated by HIFs in response to hypoxia. It was shown that hypoxia, by regulating HIF, stimulates the production of various angiogenic cytokines such as VEGF and angiopoietin-1 and promotes proliferation of embryonic hemangioblasts [57]. Hypoxia can also promote recruitment of bone marrow-derived vascular modulatory cells through HIF-1α, which enhances the synthesis and secretion of endothelial molecules on vascular progenitor cells, such as CD31, VEGFR2, and endothelial NO synthase (eNOS) [58]. Even though hypoxia has been demonstrated to be useful in maintaining undifferentiated stem cells, researchers have found that hypoxia can also stimulate differentiation of stem cells in certain condition [59]. Hypoxia may stimulate adipose stromal cells (ASCs) into endothelial-like cells. It was shown that secretion of VEGF correlates inversely with oxygen concentration, and ASCs assumed an endothelial phenotype characterized by their ability to form tubes when seeded with differentiated endothelial cells on Matrigel assays [60]. ASCs were reported to be able to express endothelial markers when cultured with VEGF and

differentiated in response to local cues into endothelial cells, which contributed to neoangiogenesis in a hind-limb ischemic model [61]. HIF-α, in response to hypoxia, regulates a variety of genes such as uPAR, collagen prolyl 4-hydroxylases, matrix metalloproteinases, and tissue inhibitors of matrix metalloproteinases, which were proposed to facilitate endothelial transition from a stable growth-arrested state to a plastic proliferative phenotype [62].

4 Signaling Molecules Involved in Angiogenesis

Several complex signaling pathways are involved in angiogenesis. However, two major signaling pathways play critical roles in angiogenesis, the Notch-signaling pathway and the hedgehog-signaling pathway, and these will be discussed here. We shall also discuss miRNAs, which are involved in the angiogenesis process (Fig. 4.3).

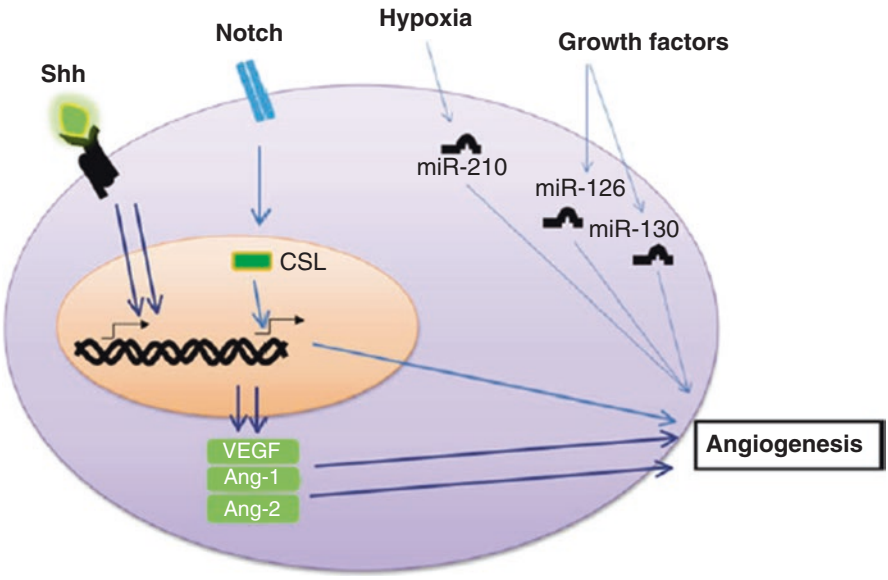

Fig. 4.3 Signaling molecules involved in cellular angiogenesis. Various cellular signaling molecules are involved in the angiogenesis process includes notch pathway, hedgehog pathway, hypoxia, and growth factors. MicroRNAs are also participating in the regulation of angiogenesis process (*Shh* sonic hedgehog, *CSL* combination of three proteins CBF1, Su (H), and Lag-2, *miR* microRNA, *VEGF* vascular endothelial growth factor, *Ang* angiopoietin)

4.1 Notch and Delta Signaling

Notch-signaling pathway is highly conserved with four different Notch receptors, NOTCH1, NOTCH2, NOTCH3, and NOTCH4, and five ligands from the jagged (Jagged-1 and Jagged-2) and Delta (Delta-like 1, Delta-like 3, and Delta-like 4) families plus modifier proteins from the Fringe family (lunatic, manic, and radical fringe) [63]. Notch proteins play critical role throughout embryonic development, such as cell survival, self-renewal for stem cells, and lineage determination for developing cells. Upon ligand activation, the cytoplasmic domain of Notch is proteolytically released, translocates into the nucleus, activates CSL [CBF1, Su (H), Lag-2], and converts them to transcriptional activators. The Notch/CSL-dependent signaling directly targets HERP families of transcriptional repressors, which are involved in multiple aspects of vascular development including muscle differentiation, angiogenic processes, arterial-venous cell fate determination, and vascular morphogenesis in mice [64]. The Delta-Notch-signaling pathway also targets members of the Hey family, the loss of which led to global lack of vascular remodeling and massive hemorrhage [65]. It was also shown that the differentiation-associated growth arrest in endothelial cells activated by Notch pathway was mediated by mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt pathway [66].

4.2 Hedgehog Signaling

Hedgehogs interact with heparin on the cell surface through N-terminal basic domains. The molecular weight of this class is around 19 kDa. The role of hedgehog signaling in angiogenesis was brought to attention a decade ago. It was shown that Shh, a hedgehog homolog in mammals, can induce expression of two families of angiogenic cytokines, including all three VEGF-1 isoforms and angiopoietins-1 and -2 in interstitial mesenchymal cells. Shh was able to induce robust angiogenesis and augment blood flow recovery and limb salvage in an induced hind-limb ischemia model of aged mice [67]. By studying murine brain capillary endothelial cells (IBE cells) and human umbilical endothelial cells, it was shown that Shh-induced capillary morphogenesis through stimulating PI3-kinase activity [68]. During development, it was demonstrated that hedgehog proteins participate in the embryonic endothelial and fibroblast cell migration and play a role in the angiogenesis process [69]. In a diabetic wound-healing murine model, gene therapy of Shh together with bone marrow transplantation resulted in accelerated wound recovery partially by enhanced recruitment of bone marrow-derived progenitor cells and promoting production of angiogenic cytokines [70].

4.3 MicroRNA

In recent years it was found that microRNAs play an important role in regulating endothelial differentiation and in promoting angiogenesis. By studying zebra fish embryos, it was found that mechano-sensitive zinc finger transcription factor klf2 activates the VEGF-signaling pathway by inducing expression of endothelial-specific microRNA mir-126 [71]. Dicer is key enzyme, which contributes to the maturation of microRNA. Specific silencing of Dicer using siRNA has led to altered expression of key regulators of angiogenesis such as TEK/Tie-2, KDR/VEGFR2, Tie-1, endothelial nitric oxide synthase, and IL-8 in endothelial cells [72]. Furthermore, reduction of endothelial microRNAs by inactivation of Dicer reduces postnatal angiogenic response to exogenous VEGF, tumors, limb ischemia, and wound-healing models [73]. These findings indicate that microRNAs play important roles in regulating endothelial cells during the angiogenesis process. Multiple microRNAs have been found to influence the angiogenesis process including microRNA-17, 92, 23, 27, 24, 130a, 181a, and 210. Till recently, few microRNAs have been identified to regulated endothelial differentiation, and microRNA-mediated control of endothelial differentiation remains to be explored [74].

5 Conclusions and Future Directions

Major efforts were given in studying the mechanisms of angiogenesis in various pathological conditions. These efforts will significantly improve our understanding of therapeutic angiogenesis. Various regulating factors including microRNAs were found to be important during angiogenesis. Numerous treatments are under development targeting appropriate regulatory factors of angiogenesis in the context of pathological condition of the disease. Results are now available from many clinical trials using various stem cells for the treatment of ischemia [3, 75, 76]. It was shown that HSCs and MSCs were indeed able to improve the vascularization process in ischemic tissues and to improve clinical outcomes in both animal model and in clinical use [77]. Nevertheless, the future role of stem cell treatment compared to current pharmacologic treatment remains undetermined. Moreover, the best timing for the possible administration of stem cells is still unknown. As we learn more about the molecular mechanisms of angiogenesis, we are likely to find an effective window for future stem cell therapy to improve the outlook for the recovery of ischemic tissues.

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Chapter 5
Role of Transforming Growth Factor Beta Family in Angiogenesis

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Abstract Transforming growth factor-beta (TGFβ) is a pleiotropic factor that plays pivotal roles in both vasculogenesis and angiogenesis, and thus is indispensable for development and homeostasis of the vascular system. TGFβ drives vascular responses via its binding to a TGFβ receptor complex formed by type I and type II receptors, as well a type III co-receptors present on both endothelial and mural cells. Signaling by these receptors is context dependent and tightly regulated, particularly on cultured endothelial cells, where TGFβ can either promote or suppress endothelial migration, proliferation, permeability and sprouting. These, together with evidence obtained from knock-out animals for different TGFβ receptor types, and genetic studies in humans linking mutations in TGFβ signaling components to cardiovascular syndromes, suggest that TGFβ is a central mediator of angiogenesis, where it may play contrasting roles depending on the stage of the process. This review presents an overview of knowledge accumulated to date on TGFβ's role in angiogenesis as well as vascular biology and vascular disease, and discusses potential applications of this knowledge to the treatment of angiogenesis-dependent diseases such as cancer.

Keywords TGFβ • ALK • Endoglin • SMAD • Endothelial cell • Pericyte • Angiogenesis • HHT • Fibrosis • Cancer

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1 TGFβ Molecule Family- Sources, Activation and Regulation of Transcription

1.1 The TGFβ family

The transforming growth factor beta (TGFβ) superfamily consists of 33 members, most of which are dimeric, secreted polypeptides that regulate proliferation, survival/apoptosis, migration, adhesion, invasiveness and self-renewal properties in responsive cells [49, 107]. Depending on the cell and tissue type, modulation of these cellular properties by TGFβ superfamily members will regulate different processes ranging from gastrulation to formation of a functional vascular system during embryonic development, as well as organ morphogenesis and homeostasis at various post-natal stages [107].

The TGFβ superfamily is conserved through metazoan evolution and includes TGFβs (1–3), bone morphogenic proteins (BMPs 1–20), growth and differentiation factors (GDFs including myostatin), activins (A and B), inhibins (A an B), nodal, leftys (1 and 2), and Mullerian inhibiting substances (MIS) [49]. The TGFβ family, the focus of this review, comprises 3 different isoforms encoded by separate genes: TGFβ1, 2 and 3. Human TGFβ2 and TGFβ3 have a 70% homology with TGFβ1 [86]. TGFβ1 is the predominant and most ubiquitous isoform, while the other two are expressed in a more limited spectrum of cells and tissues. The three isoforms have overlapping functions in vitro; however, mice deficient in individual isoforms show non-overlaping phenotypes suggesting that each TGFβ isoform has distinct functions in vivo [30].

1.2 TGFβ Sources

TGFβ ligands are not specific to a particular cell type, as their secretion has been observed in a variety of normal and malignant cells. In addition, almost every cell in the body expresses TGFβ receptors (TGFβRs) and so is capable of responding to TGFβ [157]. TGFβ was initially isolated from platelets, due to their high content of the ligand. However, bone cells, particularly osteoblasts, are the highest producers of TGFβ currently known. Strong intracellular TGFβ staining has also been reported in adrenal cortex, megakaryocytes, cardiac myocytes, chondrocytes, renal distal tubules, ovarian glandular cells and chorionic cells of mouse placenta, among others (reviewed in [120]). In carcinomas, as well as in sites of wound healing, TGFβ is expressed by epithelial cells, associated fibroblasts and myofibroblasts, and infiltrating immune cells, such as macrophages and T lymphocytes [148].

1.3 Synthesis and Activation of TGFβ

All TGFβ ligands are synthesized as precursor polypeptides, containing a longer 25 kDa N-terminal pro-peptide, followed by a C-terminal, 12.5 kDa mature polypeptide. Two of these precursors form a dimer via disulfide bonds. The pro-peptide and mature peptide are cleaved by furin-like proteases while trafficking via the exocytic pathway, but remain associated by the disulfide bonds. Once cleaved, the pro-peptide becomes the "latency associated peptide" (LAP), which acts as a chaperone during exocytosis of the complex. LAP also aids in TGFβ deposition into the extracellular matrix (ECM) and keeps TGFβ inactive within its core once the complex is secreted [107]. LAP-TGFβ is known as the small latent complex (SLC) that often exists in association with the latent TGFβ binding protein (LTBP), which, together with the SLC, forms the large latent complex (LLC) [157]. LAP's direct interaction with LTBP as well as with ECM components such as fibronectin and fibrillin, among others, mediates TGFβ's deposition into the ECM [107]. Cleavage-dependent activation of the mature C-terminal dimeric TGFβ ligand from its ECM-deposited form is mediated by a number of proteases including, thrombospondin 1 (TSP-1), plasmin, cathepsin D, matrix metalloprotease (MMP) 2 and 9, calpain, chymase, elastase, endoglycosidase F, and kallikrein. In addition, acidic environment, reactive oxygen species (ROS), heat and sheer stress have also been shown to activate TGFβ (reviewed in [10, 49, 157]). However, in many physiological situations, integrins have been shown to be the critical players in TGFβ activation. An RGD sequence present in TGFβ's LAP mediates its binding to all αv integrins, and αvβ3, αvβ5, αvβ6 and αvβ8 have all been shown to release active TGFβ via both proteolysis-independent and dependent mechanisms [157].

1.4 Regulation of Transcription by TGFβ

Once activated, TGFβ initiates signalling by inducing the activity of specific serine/threonine kinase type I and type II receptor heterotetrameric complexes. These in turn phosphorylate specific effector proteins called SMADs (**s**mall **m**others **a**gainst **d**ecapentaplegic), which then translocate to the nucleus (signalling events discussed in more detail below). Nuclear SMAD complexes bind to chromatin, and, together with other transcription factors regulate gene expression. A list of SMAD target genes have been published elsewhere [107]. Among these, the inhibitors of differentiation (Id) family of transcription factors, vascular endothelial growth factor (VEGF), and thrombospondin-1 (TSP-1) are important modulators of angiogenesis. TGFβ also signals in a non-canonical manner to modulate the level and function of effector proteins in the absence of changes in gene transcription [147]. Misregulation of TGFβ signalling plays roles in a number of pathologies, including autoimmune and cardiovascular disorders, and cancer [99]. Cardiovascular disorders resulting from abnormal TGFβ signalling include hereditary hemorrhagic telangiectasia (HHT), cardiac remodelling/fibrosis and pulmonary arterial hypertension, among others [53].

1.5 TGFβ's Role in Angiogenesis

Genetic studies in mouse and human have provided evidence for the importance of components of the TGFβ signalling pathway in vascular morphogenesis, including formation of the primitive vascular plexus, and the recruitment of pericytes/smooth muscle cells necessary for vessel wall integrity [53]. Deletion of TGFβ1 in the mouse results in embryonic lethality because of defective yolk sac vasculogenesis. Targeted deletion of ALK1, ALK5, TGβRII and endoglin results in similar phenotypes. All of these knockout embryos die during mid-gestation due to hyper-dilated, impaired, leaky vessels [119]. These vascular abnormalities are similar to those described in patients with HHT [53]. Endothelial and smooth muscle cell-specific targeting of TGFβRII and ALK5 suggests that TGFβ signalling in both compartments is required for proper vessel development, but likely at different stages [53, 118].

In the next sections, we present a detailed overview of current knowledge on TGFβ signalling in endothelial and associated vascular cells, such as pericytes and smooth muscle cells, and the role of this signalling at the various stages of the angiogenesis process. We also provide some evidence of differential TGFβ signalling in physiologic versus pathologic angiogenesis and discuss potential applicability in therapeutic intervention.

2 TGFβ Receptors and Signalling

TGFβ members signal through type I and type II serine/threonine kinase receptors. There are 7 members of the type I receptor family, also known as Activin-receptor like kinases (ALK) 1–7, and 5 members of the type II receptor family (TGFβRII, BMPRII, ActRIIA, ActRIIB, and MISRII) [132]. TGFβ also signals via accessory, type III, TGFβ receptors: endoglin and betaglycan (discussed in a later section). Reflective of its role in signalling in a multitude of cell types, there are relatively fewer studies devoted specifically to TGFβ signalling in vascular endothelium. In the sections below, except where explicitly stated, the signalling events and outcomes described have not yet been validated in endothelial cells.

In most cell types TGFβ 1–3 isoforms signal through an ALK5-TGFβRII complex, however endothelial cells also express, and signal through an ALK1-TGFβRII complex [112]. The balance between activation of these two signalling pathways regulates endothelial cell functions such as proliferation and migration, and this balance is believed to regulate the switch of endothelial cells from quiescent mature vessels into activate angiogenic sprouts. In fact, genetic mutants of TGFβ receptors, ALK5 and endoglin, inhibit angiogenesis in vitro, and result in embryonic lethality in mice due to vascular defects [119].

In the absence of ligand, type I and II receptors form homodimers with themselves, which upon ligand binding complex with each other to form a heterotetramer [162]. Formation of this tetrameric complex brings together the constitutively active

type II receptor with the type I receptor, resulting in auto- and trans-phosphorylation at various serine residues in the receptors' GS domain. Once activated by these phosphorylation events the TGFβ receptors become functional serine/threonine kinases, and subsequently phosphorylate and activate several intracellular signalling molecules [94].

Although TGFβ receptors are classically referred to as serine/threonine kinases, upon ligand binding TGFβRII also becomes auto-phosphorylated at multiple tyrosine residues [78]. These phosphorylated tyrosines then act as docking sites for various Src homology 2 (SH2) and phospho-tyrosine binding (PTB) domain containing molecules, such as: **S**rc **h**omoly and **c**ollagen homology (Shc), and **g**rowth factor **r**eceptor **b**ound protein **2** (Grb2) [123]. These adaptor molecules function as scaffolding proteins, bringing together the TGFβ receptor's tyrosine kinase function with various protein substrates [44]. TGFβ receptors, through their action as both serine/threonine and tyrosine kinases, are able to activate several intracellular signalling cascades, including the canonical Smad signalling pathway, as well as several non-canonical signalling pathways, such as: PI3K/AKT, RhoA dependent, and JNK, p38 and ERK MAPK pathways (reviewed in [169]).

2.1 Canonical SMAD Signalling

The SMAD family of proteins is composed of three classes: receptor SMADs (R-Smads 1, 2, 3, 5 and 8), inhibitory SMADs (I-Smads 6 and 7), and the common SMAD (Co-SMAD4) [132]. R-SMADs are recruited to TGFβRI following receptor activation, and interact indirectly via auxillary proteins such as **s**mad **a**nchor for **r**eceptor **a**ctivation (SARA) [144]. Once recruited R-SMADs become phosphorylated by TGFβRI in their C-terminal SSXS domain; R-SMADs 1, 5 and 8 are activated by ALKs 1–3 and 6, whereas R-SMADs 2–3 are activated by ALKs 4, 5 and 7 [103].

Once activated, R-SMADs dissociate from TGFβRI and form heterodimers with Co-SMAD4, or heterotrimers containing two R-SMADs and one SMAD4, which then translocate to the nucleus [61]. This translocation is guided by **n**uclear **l**ocalization **s**equences (NLSs) in the MH1 domains of SMAD3 and SMAD4, which mediate their interaction with importin proteins β1 and α, respectively [73, 159]. Other mediators of SMAD nuclear translocation and retention include components of the nuclear pore complex, and the Hippo signalling transcriptional co-activators TAZ (**t**ranscriptional co-**a**ctivator with PD**Z** binding domain) and YAP1 (**Y**es-**a**ssociated **p**rotein **1)** (reviewed in [48]). Interestingly YAP1 was recently identified as a key mediator of angiogenesis in the developing retina [21]. In this study, active YAP1 promoted endothelial sprouting, which was mediated by Angiopoietin 2 (ANG-2), a transcriptional target of YAP1 [21]. Whether YAP1's role in angiogenesis relates to its function in TGFβ signalling remains unclear.

Once in the nucleus, SMAD complexes function as transcription factors and regulate transcription through their direct interaction with DNA containing **SMAD-B**inding **E**lements (SBEs), as well as co-repressors, co-activators (CBP and p300),

and other transcription factors [134]. The diversity of these transcriptional complexes directs the tissue and dose-dependent regulation of transcription by the SMAD proteins.

TGFβ stimulation affects the transcription of several hundred genes [68]. Targets of R-SMAD and Co-SMAD transcriptional regulation include proteins involved in regulating cellular proliferation, apoptosis and the **e**pithelial-to-**m**esenchymal **t**ransition (EMT) or its endothelial equivalent, the **e**ndothelial-to-**m**esenchymal **t**ransition (EndoMT) [155]. Additionally, TGFβ signalling results in expression of I-SMADs 6 and 7, whose promoters contain SBEs [138]. The I-SMADs then establish a negative feedback circuit on TGFβ signalling through their ability to negatively regulate signalling pathway activation on multiple levels; with SMAD7 expressed in response to, and antagonizing all TGFβ signalling, and SMAD6 expressed specifically in response to, and antagonizing the SMAD1, 5, and 8 signalling pathways [164].

I-SMADs contain various functional domains that enable their inhibitory function. Through their MH2 domain SMADs 6 and 7 are able to compete with R-SMADs for TGFβRI binding; thus inhibiting R-SMAD phosphorylation and subsequent Co-SMAD4 complex formation [104]. I-SMADs are also capable of recruiting E3 ubiquitin ligases, Smurf1 and Smurf2 (**S**mad **u**biquitination-**r**elated **f**actor 1/2), to activated TGFβRI leading to its polyubiquitination and subsequent proteasomal degradation [66]. Smad7 can additionally interfere with TGFβ signalling at the level of receptor activation via its ability to recruit the phosphatase GADD34-PP1c to the activated receptor complex [133].

In addition to their functioning in the cytoplasm, at the level of receptor and R-SMADs inhibition, I-SMADs also function in the nucleus at the level of transcriptional repression. Through its MH2 domain SMAD7 is able to bind directly to DNA to prevent SMAD2, 3 and 4 binding [134]. Finally, once bound to DNA, I-SMADs recruit histone deacetylases (HDACs) to the promoter regions of SMAD target genes, leading to chromatin compaction and transcriptional inhibition [59].

2.2 Non-canonical Signalling Pathways

MAPK: TGFβ signalling can also lead to activation of MAPK (**m**itogen **a**ctivated **p**rotein **k**inase) signalling pathways, including ERK (**e**xtracellular signal-**r**egulated **k**inase), p38 and JNK (**J**un **N**-terminal **k**inase) signalling. This activation is likely independent of SMAD-dependent transcription, due to the rapid onset of MAPK phosphorylation (5–15 minutes) [114], and the ability of cells genetically deficient in Smad activation, to maintain their ability to activate MAPK signalling in response to TGFβ [35]. ERK MAPK becomes activated through a receptor tyrosine kinase (RTK)/RAS/ERK pathway. Following TGFβ ligand binding to TGFβRII, type I and II receptors becomes phosphorylated on 3 tyrosine residues, Y259, Y336 and Y424, in the receptors' cytoplasmic domain [78]. These phosphorylated tyrosine residues are then bound by SH2 and PTB domain containing adaptor

molecules. Grb2 is a SH2 domain containing protein that complexes with SOS in the cytoplasm and upon RTK phosphorylation is recruited to the receptor. Once localized to the RTK, the GRB2/SOS complex is able to activate membrane-localized RAS, bridging TGFβ receptor activation with the MAPK signalling pathway. In its activated, GTP-bound state, RAS is able to phosphorylate and activate the RAF-MEK-ERK MAPK cascade [44, 123].

ERK, through its functioning as a serine/threonine kinase, regulates intracellular mitogen signalling in the cytoplasm, but also translocates to the nucleus where it regulates the activity of various transcriptional regulators [168]. Through its modulation of gene transcription ERK mediates TGFβ-induced disassembly of adherens junctions and enhanced migration, two key events in TGFβ-induced EMT [114]. As such, ERK activation is required, however insufficient (must cooperate with Smad signalling), for TGFβ-induced EMT [28]. In endothelial cells, TGFβ-mediated activation of ERK was found to promote migration in a context-dependent manner, whereby co-expression and interaction of TGFβ co-receptor endoglin and the scaffolding protein β-Arrestin2 antagonizes TGFβ-dependent ERK signalling and endothelial cell migration [82]. In addition, endoglin targets ERK signalling and its downstream effectors c-Myc and cyclin D1 in a TGFβ-independent manner. This mechanism possibly cooperates with SMAD-dependent downregulation of c-Myc by TGFβ, and its overall growth inhibitory effect in endothelial cells [117].

JNK and p38 MAPK signalling pathways are also activated in response to TGFβ signalling. This activation is dependent on the scaffolding protein TRAF6 (**T**NF **r**eceptor **a**ssociated **f**actor **6**), which associates with activated TGFβRII through its C-terminal TRAF domain. TGFβRII-bound TRAF6 undergoes auto-polyubiquitination, leading to its association with the MAP3K (MAP kinase kinase kinase), TAK1 (**T**GFβ-**a**ctivated **k**inase **1**) [150]. TAK1 is required for activation of both the JNK and p38 MAPK pathways, via activation of MKK4-JNK and MKK3/6-p38 cascades, respectively [135]. In fact, TAK1 is indispensable for JNK and p38 MAPK activation and embryos deficient in TAK1 suffer from vascular defects whose phenotype is similar to ALK1 and endoglin mutants [60]. The activation of TGFβ-TAK1-JNK/p38 MAPK pathways is independent of SMAD-mediated transcription, however these signalling pathways cooperate with SMAD signalling in order to regulate TGFβ-induced cellular functions such as apoptosis [89] and EMT [163]. Recent studies revealed that different isoforms of p38 MAPK are responsible for the differential effects of VEGF and TGFβ on endothelial cells. In particular, TGFβ is able to shift the outcome of VEGF signalling by directing VEGF-dependent activation of p38 isoforms, specifically from p38β (pro-survival) to p38α (pro-apoptotic). Thus, in the absence of TGFβ, VEGF supports endothelial proliferation but when TGFβ is also present, endothelial cell death can occur [40].

Rho-GTPase: TGFβ also rapidly activates RhoA (**R**as **ho**molog A) signalling in a SMAD-independent manner [33]. However, TGFβ signalling has also been shown to lead to localized down regulation of RhoA protein in response to TGFβ activation of the Par6 polarity pathway [116]. Par6 (**Par**titioning-defective member **6**) is a scaffolding protein that complexes with TGFβRI at tight junctions. Following ligand binding to TGFβRII, it travels to the tight junction where it complexes with TGFβRI, and

phosphorylates Par6 at serine 345. Activated Par6 recruits the E3 ubiquitin ligase, Smurf1, to tight junctions where it ubiquitinates and targets RhoA for degradation; leading to localized RhoA down regulation at tight junctions [116]. This localized degradation is responsible for the dissolution of tight junctions, reorganization of the actin cytoskeleton and extension of filopodia [151], all of which are essential for EMT [116]. The potential role of the Par6 pathway in vascular biology has been recently highlighted by in vitro studies on EndoMT (discussed in detail in later sections), a process essential for heart valve formation in the developing embryo. It was observed that blockade of Par6 activation abrogated EndoMT in response to TGFβ2, and this was dependent on the presence of both ALK-5 and type III TGFβ receptor betaglycan [143]. We have recently demonstrated Par6 activation in response to TGFβ1 in bovine aortic endothelial cells, particularly at low (0.5 ng/mL and lower) TGFβ concentrations (Richard et al., manuscript in preparation). Since low TGFβ concentrations have been previously observed to be pro-angiogenic [121] our results suggest that Par6 activation might mediate angiogenesis in response to TGFβ.

PI3K/AKT: The phosphatidylinositol-3-kinase (PI3K)/ v-AKR mouse thymoma homolog (AKT) signalling pathway is also activated downstream of TGFβ through TGFβRI-dependent phosphorylation of PI3K, an upstream kinase of AKT. PI3K interacts with TGFβRII independent of receptor activation and upon ligand stimulation is brought in contact with TGFβRI, where it is phosphorylated [165]. Downstream activation of the AKT signalling pathway is required for TGFβ-induced EMT, and does so by two proposed mechanisms; first, through its ability to mediate TGFβ-induced actin filament reorganization and enhanced cellular migration, and secondly, through AKT's activation of downstream mTOR (**m**ammalian **t**arget **o**f **r**apamycin) [7]. The mTOR signalling pathway regulates cellular translation levels, and Akt-mTOR activation is believed to facilitate Smad-mediated transcriptional programs. The signalling crosstalk between TGFβ and PI3K in endothelial cells was rather unexplored until the past 5 years. Lee et al. [83] reported an indirect interaction of endoglin with both the p85 and p110α subunits of PI3K, which facilitates modulation of PI3K activity by TGFβ ligands, whereby TGFβ1 inhibits while BMP9 promotes PI3K/Akt activity in an endoglin-dependent manner. A more recent study identified PI3K class II α-isoform (PI3K-C2α) as a key mediator of TGFβ-dependent angiogenesis, via its pivotal role in TGFβ receptor endocytosis, a critical process in SMAD signalling activation [3].

3 TGFβ and Endothelial Sprouting, Proliferation and Permeability

Extensive evidence suggests that TGFβ plays a role during the activation phase of angiogenic sprouting by promoting vascular permeability, proliferation and migration of endothelial cells. TGFβ also mediates the reverse events that occur during the resolution phase of angiogenesis, including inhibition of endothelial cell migration and proliferation and decreased permeability, which are necessary for vessel stabilization [80].

3.1 Endothelial Sprouting

Endothelial sprouting involves two distinct endothelial cell phenotypes: the tip cells which lead the newly forming vessel sprout, and the stalk cells, which proliferate and form the lumen of the new vessel [46]. These cells are initially part of a mature vessel. An increase in permeability and migratory characteristics allows these cells to delaminate from the endothelium and become involved in the newly forming vessel sprout. Proliferation must be suppressed in the tip cells and enhanced in the stalk cells to ensure their respective functions. Finally, when the new vessel is in place there must be a reversion back to characteristics of cells in a quiescent endothelium, which includes a decrease in permeability, proliferation and migratory characteristics [46].

One of the key regulators of sprouting during angiogenesis is vascular endothelial growth factor (VEGF)-A, since tip cell migration largely depends on a gradient of VEGF-A, which binds to VEGF receptor 2 on endothelial cells [47]. Studies in mice and zebrafish have contributed to our understanding of the role of VEGF and Notch signalling in vessel sprouting. VEGF binding to VEGFR2 on a tip cell activates VEGFR signalling leading to increased expression of the Notch ligand Dll4 (**D**elta-**l**ike **l**igand **4**), which in turn binds to Notch1 receptor on adjacent stalk cells. Notch signalling in the latter reduces VEGR2 and VEGR3 expression, making them insensitive to VEGF stimulation, thereby suppressing the tip cell phenotype [72].

TGFβ effects on in vitro endothelial cell sprouting are variable, including induction, repression or no effect depending on the concentration of TGFβ, the type of endothelial cells employed and the source of signalling activation; *i.e*. whether constitutively activated receptors or exogenously added ligand were used [58]. The nature of the angiogenic response to TGFβ depends on the balance of ALK1 versus ALK5 signalling input, with ALK1 predominantly promoting sprouting and ALK5 favoring the resolution/stabilization phase of angiogenesis [58]. The inhibitory effect of an ALK1 antibody on endothelial cell sprouting in vitro and on angiogenesis in two different tumor models supports this concept [102]. However, recent studies on developmental angiogenesis in mice suggest that, rather than promoting sprouting, ALK1 signalling cooperates with Notch signalling to repress VEGF responsiveness, tip cell formation and sprouting [77]. Whether these discrepancies represent differences in developmental versus pathologic angiogenesis remains to be determined. Interestingly, we observed that TGFβ1 decreases endothelial VEGFR2 [71] expression via ALK5 signalling. Thus, ALK5 signalling may potentially contribute to endothelial cell insensitivity to VEGF stimulation that might be necessary for both maintenance of the stalk cell fate during sprouting and the resolution phase of angiogenesis.

Finally, EndoMT has been hypothesized to be one of the mechanisms mediating angiogenic sprouting in response to TGFβ. EndoMT is the process whereby cells from a quiescent, stable endothelium delaminate from this cell layer and take on a fibroblastoid phenotype. During this process endothelial cells experience loss of adherens and tight junctions and their associated markers including: vascular endothelial (VE)-cadherin, zona occludens (ZO)-1 and claudin-5. The cells transition

towards a mesenchymal phenotype is associated with the gain of mesenchymal markers such as α-smooth muscle actin and fibroblast specific protein-1, as well as motility [101, 166]. As previously mentioned, EndoMT mediates cardiac development and is also responsible for pathologic tissue fibrosis [166]. However, it was not until very recently that the involvement of EndoMT in angiogenesis was demonstrated, by the finding that both SLUG and SNAIL, two prototypical EMT/EndoMT-associated transcription factors, are required for endothelial cell sprouting [154]. Complementary observations of enhanced SLUG levels in colorectal cancer blood vessels suggest that EndoMT is an important component of pathologic angiogenesis [154]. Our group's assessment of various markers of EndoMT in response to TGFβ isoforms 1 and 2 in bovine aortic endothelial cells indicates that EndoMT-like changes, such as an increase in expression and nuclear translocation of SNAIL, SLUG and ZEB1, and reduction of VE-cadherin expression, occur in response to TGFβ1 and/or TGFβ2 as early as 6 hours after stimulation and might be enhanced by hypoxia in an isoform-specific manner. Further, hypoxia enhances canonical TGFβ signalling via SMAD2, and appears to be a key determinant of SNAIL's differential involvement in endothelial cell sprouting in response to TGFβ2 but not to TGFβ1 [31].

3.2 Endothelial Permeability

Signalling via the ALK5 TGFβ receptor has been shown to both promote and inhibit vascular permeability, depending on cell context. TGFβ can induce permeability in pulmonary endothelial cell monolayers, which is attenuated by treatment with SB-431542, an ALK5 kinase inhibitor [12]. Specifically, SB-431542 up-regulates the expression of the endothelial specific tight junction component, claudin-5 [153]. In contrast, in vivo blockade of TGFβ signalling in mouse retinal endothelial cells leads to increased permeability and decreases vessel barrier function. Both in vivo and in vitro analyses demonstrated that TGFβ blockade resulted in increased endothelial permeability characterized by decreased interaction between the tight junction proteins occludin and ZO-1 [149].

As mentioned above, TGFβ-mediated EndoMT may contribute to an increase in endothelial permeability that is necessary for angiogenic sprouting. During EndoMT there is a decrease in expression of the adherens junction protein VE-cadherin, as well as tight junction proteins ZO-1 and claudin-5. TGFβ has been shown to down-regulate claudin-5 at the transcriptional level, and VE-cadherin has been observed to upregulate expression of claudin-5 [115, 139]. Thus, TGFβ-mediated downregulation of VE-cadherin during EndoMT [31] can indirectly decrease expression of claudin-5, resulting in the loss of both adherens junctions and tight junctions with a concomitant increase in endothelial permeability.

Along with TGFβ, VEGF has also been shown to be an important mediator of endothelial permeability during angiogenesis [56]. VEGF has a demonstrated function in modulating VE-cadherin at the adherens junctions through tyrosine phosphorylation, which leads to an increase in permeability [37]. Since TGFβ induces

VEGF expression in vascular endothelial cells, this relationship may provide an alternative mechanism whereby TGFβ can modulate VE-cadherin expression and therefore increase endothelial cell permeability [39]. TGFβ's ability to downregulate VEGF receptor 2 expression can also provide an additional means by which TGFβ can regulate and perhaps reverse vascular permeability during the resolution phase of angiogenesis. Finally, vascular permeability is also modulated by interactions between endothelial cells and the smooth muscle cells/pericytes that invest blood vessels. TGFβ's role in these interactions is discussed in more detail below.

3.3 Endothelial Proliferation and Migration

TGFβ can enhance cell proliferation at low doses and suppress proliferation at high doses. The presence of both type I receptors ALK1 and ALK5 may provide a means by which TGFβ's dual role in proliferation is regulated [51]. Activation of ALK1 has been primarily shown to stimulate proliferation and migration of endothelial cells during the activation phase of angiogenesis [51, 52]. The downstream effector of ALK1 responsible for this process is ID1, an inhibitor of differentiation that is required for proliferation and migration. When ALK1 is active, both endothelial cells and fibroblasts are induced to express ID1 [25, 51]. However, it is important to mention that a constitutively active ALK1 in combination with ALK5 is a potential negative regulator of endothelial cell migration and proliferation. This effect was mediated by inhibition of JNK and ERK activation by ALK1, which may thus cooperate with ALK5 in the resolution phase of angiogenesis [27, 76, 98].

In contrast to ALK1, ALK5 seems to have more defined anti-proliferative roles during both the activation and the resolution phases of angiogenesis [51]. It is believed that activated SMAD2/3 proteins cooperate with nuclear co-repressors to repress the transcription of c-*Myc* and cyclin-dependent kinase (*Cdk*) genes, and with nuclear co-activators to activate transcription of p15 and p21, two major inhibitors of the cell cycle, collectively inhibiting proliferation [29, 108]. ALK5 has been specifically shown to prevent proliferation and migration in endothelial cell spheroid assays and embryonic stem cell derived endothelial cells, whereas the ALK5 kinase inhibitor, SB-431542, has opposite effects [91, 153]. Furthermore, in vitro studies have found that ALK5-induced blood vessel maturation is mediated by the induction of plasminogen activator inhibitor (PAI)-1 in endothelial cells. PAI-1 prevents degradation of the provisional extracellular matrix that surrounds the nascent vessel, hence promoting vessel maturation during the resolution phase [51]. Thus, ALK5 likely plays roles in both inhibiting proliferation of the tip cells during the activation phase of angiogenesis, and in modulating both tip and stalk cell phenotypes during the resolution phase of angiogenesis. While a balance between ALK1 and ALK5 may be important to mediate the effects of TGFβ on the endothelium, their actions are not mutually exclusive and they may serve as regulators of one another. This is further controlled by their differential interactions with endoglin and is discussed in more detail below. Thus, the variation in roles played

by ALK1 and ALK5 as well as the balance between these two type I TGFβ receptors is likely dependent on cellular context, with the cross-talk between them providing a mechanism whereby TGFβ can strategically regulate proliferation of the tip and stalk cells during angiogenesis.

Finally, it should also be noted that VEGF promotes proliferation of endothelial cells during angiogenesis in a concentration-dependent manner [47]. Since VEGF is a positive regulator of proliferation and TGFβ has been shown to be an inducer of VEGF expression in endothelial cells, this interaction provides yet another regulatory mechanism for TGFβ to control proliferation [39, 47].

4 TGFβ Co-receptors in Angiogenesis

The human type III TGFβ co-receptors endoglin and betaglycan are type I integral membrane glycoproteins [50, 105]. Betaglycan is universally expressed on nearly all cell types and is the most highly expressed of the TGFβ superfamily receptors [156]. However, the expression of betaglycan in some cell types, specifically vascular endothelial cells with the exception of those forming the endocardium [15], appears to be weak or absent, and instead endothelial cells predominantly express the related TGFβ co-receptor, endoglin [156]. Both endoglin and betaglycan are generally expressed on the cell surface as homodimers, with endoglin homodimers being linked by disulfide bridges; however, endoglin and betaglycan are capable of forming heteromeric complexes in microvascular endothelial cells [105, 156]. Both type III co-receptors also exist as soluble forms. Betaglycan shedding is mediated in part by membrane-type metallo proteinase 1/matrix metalloproteinase 14 (MT1-MMP/MMP14) and plasmin [75], while soluble endoglin is produced by cleavage of the membrane-bound endoglin at close proximity to the transmembrane domain by MMP14 [65].

Endoglin expression is potently stimulated by hypoxia, BMP9, and TGFβ via ALK1, while TNFα exerts an inhibitory effect on endoglin expression in endothelial cells [86, 87, 129]. Both betaglycan and endoglin cytoplasmic domains can be phosphorylated by serine/threonine kinases [14, 69]. ALK5 is responsible for the phosphorylation of endoglin's cytoplasmic tail, which has been shown to be necessary for the activation of TGFβ-dependent ALK1 signalling [124]. Thus, ALK5 is indirectly responsible for ALK1 activation via endoglin, which in turn is necessary for endothelial cell proliferation. The phosphorylation of endoglin has been shown to influence its subcellular localization, by modulating its interaction with adhesive proteins such as zyxin and **z**yxin-**r**elated **p**rotein 1 (ZRP-1), hence modifying the adhesive properties of endoglin expressing cells [69, 167].

It is not fully understood how endoglin regulates TGFβ dependent responses. Endothelial cells that lack endoglin experience decreased proliferation due to diminished ALK1 activity and increased ALK5 activity [79]. The increase in ALK5 activity and subsequent TGFβ-induced growth inhibition, even at low concentrations of TGFβ which normally promote proliferation [51], may also be due in part to decreased inhibition of ALK5 by ALK1 [79]. ALK1 has been shown to interrupt

ALK5 signalling, likely acting downstream of SMAD2/3 phosphorylation [52]. Thus ALK1 may be involved in a negative regulatory mechanism that is able to mediate the anti-proliferative effects of ALK5 in endothelial cells. However, endoglin association with TβRII results in alteration of its phosphorylated status, thus ensuing loss of ALK5 from the TGFβ receptor complex, possibly explaining endoglin's inhibitory effect on ALK5 signalling [13]. Furthermore, studies conducted on human umbilical vein endothelial cells demonstrate that ALK1-dependent inhibition of cell adhesion is counteracted by endoglin phosphorylation [13, 152]. These results suggest that endoglin interaction with TGFβ signalling receptors via both its extracellular and cytoplasmic domains might affect TGFβ cell responses.

4.1 Regulation of TGFβ Ligand Access to Co-Receptors

Betaglycan binds multiple members of the TGFβ family, including TGFβ1, TGFβ2, TGFβ3, Activin A, BMP2, BMP4, and BMP7 [36, 38, 67]. Betaglycan also plays a role in presenting the ligand to TβRII, leading to either enhanced or inhibited signalling while interacting with the TβRII [125]. Unlike betaglycan, endoglin binds TGFβ1and TGFβ3 but not TGFβ2 [20]. Other endoglin ligands include activins and BMPs, and endoglin can also interact with activin type II receptors [8]. Therefore, functional differences and similarities found between betaglycan and endoglin could be due to differences between these two proteins' ligand binding profiles.

In the case of the type III co-receptor betaglycan, its function as a co-receptor to specific members of the TGFβ superfamily is carried out through its ectodomain, which consists of two independent ligand-binding domains. The residual carboxy-terminal half of the protein is necessary for protein anchoring to the cell membrane [36]. Comparative studies between endoglin and betaglycan intracellular responses to TGFβ signalling found a distinctive role for the extracellular domains [84]. Exchanging the extracellular domain between these two co-receptors did not alter endoglin ligand binding potential, however, in contrast to betaglycan, TβRII is essential for endoglin binding of TGFβ1, activin A, BMP2 and BMP7 [8, 84]. The soluble form of endoglin reduced binding of TGFβ1 by interfering with its interaction with TGFβ receptor type II, and soluble endoglin suppressed TGFβ1 signalling in endothelial cells [146].

As previously mentioned, TGFβ can signal in endothelial cells through either ALK1 or ALK5, resulting in the stimulation of endothelial cell proliferation and migration (ALK1) or inhibition of these responses (ALK5) [79]. Forced expression of endoglin led to inhibition of TGFβ/ALK5 signalling and subsequent blockade of TGFβ induced growth inhibitory effect on endothelial cells [55, 79, 84, 130]. In mouse embryonic endothelial cells ALK1 and TGFβRII are directly bound to endoglin, but ALK5 can only bind to this complex via interaction with TGFβRII [122]. In the presence of ligand, this leads to dual phosphorylation of endoglin, first by ALK5 then by ALK1; thus signalling via SMAD2/3 is endoglin independent while downstream activation of SMAD1/5/8 is enhanced by endoglin in these cells [122]. Moreover, endoglin can block apoptosis in response to hypoxia and TGFβ. When endoglin-expressing

and endoglin-deficient endothelial cells were both exposed to TGFβ1 under hypoxic stress, the presence of endoglin was sufficient to block the synergistic pro-apoptotic effect of TGFβ1 and hypoxia [87]. Additionally, and as mentioned above, in endothelial cells the endoglin cytoplasmic tail interacts with β-arrestin2, leading to endoglin-mediated inhibitory effects on TGFβ induced ERK activation and migration [82]. Finally, endoglin is able to inhibit cell migration through its interaction with LIM domain containing focal adhesion proteins such as zyxin, possible in a TGFβ independent fashion [23]. Endoglin deficient endothelial cells also have impaired localization of zyxin to their focal adhesions in response to BMP9, which may also involve mechanotransduction mediated cross-talk with the Hippo pathway leading to altered cell adhesion [167].

During embryogenesis, inflammation, and wound healing modifications in vascular structure occur and endoglin expression is elevated during these modifications [64, 142]. The importance of endoglin function in maintaining normal vascular structure is underlined by the relationship between mutations in the endoglin gene and hereditary hemorrhagic telangiectasia (HHT), which is a disorder characterized by the formation of small dilated blood vessels and arteriovenous malformations (AVMs) in the vasculature of lung, liver, and brain [1, 85]. Studies done to elucidate the role endoglin plays in the enhancement of the TGFβ/ALK1 signalling pathway suggest that endothelial cell response to TGFβ is critically dependent on endoglin functional association with ALK1 [13]. The results from these studies agree with what is seen in cases of HHT where the predominant mutations are in either human endoglin (*ENG*) or ALK1 (*ACVRL1*) genes [62, 100].

4.2 TGFβ and Vascular Mural Cells

The structure of microvessels varies between different tissue beds, and one of the major alterations is in the nature and prevalence of mural cells. Pericytes are found in capillaries, venules and small arterioles, while true vascular smooth muscle cells are associated with larger arterioles and the macrocirculation [43]. In addition, there are significant differences in pericyte coverage and phenotype between vascular beds [126] and the ratio of pericytes to endothelium can vary from an almost 1:2 ratio in retina [2] to less than one pericyte for every ten endothelial cells. Pericytes can also be additionally specialized for tissue specific vascular function, becoming glomerular mesangial cells (kidney) or Ito/stellate cells (liver), for example [43].

Mural cells play significant roles in the stabilization, functionality and phenotype determination of the microcirculation, and recruitment of these cells is an essential part of the so-called ‘resolution’ stage of sprouting angiogenesis [141]. During development, platelet derived growth factors (PDGFs) act as potent chemoattractants for mural cell precursors and are produced by endothelial cells during vasculogenesis in the embryo and during sprouting angiogenesis in adult tissue. There is evidence from in vitro studies that PDGF-B can induce TGFβ production via the MAPK/ERK pathway, and angiopoietin 1 (ANG-1) production via the PKC

and PI3K pathways during vascular smooth muscle differentiation of 10 T1/2 cells [111]. Furthermore, TGFβ can downregulate this PDGF-B induction of ANG-1, and both TGFβ and ANG-1 synergistically reduce PDGF production by vascular endothelial cells, suggesting that cross talk between endothelium and mural cell precursors is essential for maturation of the microvascular bed [111].

Recently, the TGFβ co-receptor endoglin was implicated in integrin-mediated mural cell adhesion of vascular endothelium, and loss of endoglin can lead to increased vascular permeability modulated by pericytes [128]. TGFβ signaling in pericytes triggers basal lamina hypertrophy implicated in the loss of blood-neural barrier [136] and retinopathy [145] under diabetic conditions. There is also evidence that monocyte chemoattractant protein 1 (MCP-1) is also a chemoattractant for vascular smooth muscle and 10 T1/2 cells [96]. MCP-1 is upregulated in ischemic regions of brain associated with endoglin positive microcirculation, and in human brain microvessel endothelial cells exposed to ischemia in vitro, highlighting the potential role of TGFβ mediated pathways in angiogenic recovery of reperfused brain after stroke [137]. Neuropilin-1 expressing mononuclear cells are recruited to semaphorin3A expressing endothelial cells, where they respond by releasing TGFβ leading to endothelial SMAD2/3 activation [54]. This establishes a TGFβ-mediated feedback loop for enhanced semaphorin3A expression and further stabilization of nascent vessels, with no alteration in pericyte coverage. VEGF enhanced this pathway at low concentrations and inhibited it at high concentrations, providing a possible model for the biphasic action of this angiogenic factor [54]. Importantly, this work emphasizes that TGFβ-dependent stabilization of angiogenic vessels can also occur in a mural cell-independent fashion.

Culture of 10 T1/2 cells with vascular endothelial cells leads to activation of latent TGFβ (similar to what is seen with endothelial/smooth muscle cell co-culture [4]), and subsequent TGFβ driven 10 T1/2 cell differentiation into pericyte like cells [26]. Endothelial-mural cell precursor contact is required for this TGFβ activation, and co-culture of endothelial cells with mesenchymal precursors from mutant mouse embryos demonstrates that cell coupling via gap junction protein connexin 43 is essential for this activity [57]. Co-culture of endothelial cells and 10 T1/2 cells enhances the survival of both cell types; ECs require active ALK5 signalling for this, while 10 T1/2 cells in co-culture employ other pathways for survival [149]. This TGFβ mediated reciprocal interaction between vascular components is relevant for both vascular functionality (such as permeability/barrier function) and neural retinal cell survival in adult mice [149]. Proper pericyte/endothelial cell interactions are also essential for maintaining blood-brain barrier characteristics in cerebral vessels. This is mediated via endothelial cell SMAD4 signalling, which in cooperation with Notch signalling leads to increased N-cadherin expression and stable endothelial-mural cell adhesion [88]. In mesenchymal stem cells, TGFβ induces production of the Notch ligand Jagged1 and subsequent vascular smooth muscle cell specific gene expression via SMAD3 and Rho kinase pathways [74].

TGFβ signalling via endoglin or ALK1 is able to reduce endothelial activation via TGFβ/ALK5, and therefore tends to promote vessel destabilization and proliferation/sprouting [79]. Endoglin in cooperation with αv integrin leads to TGFβ activation

and also signals for subsequent reduced pericyte migration. The matricellular protein 'secreted protein acidic and rich in cysteine' (SPARC) is able to interfere with TGFβ mediated inhibition of pericyte migration via its ability to prevent endoglin from incorporating into pericyte focal contacts and associating with αv integrin [127]. Interestingly, endoglin is able to associate with αv integrin independent of the formation of focal adhesions, and endoglin may interfere with pericyte focal adhesion formation or maturation, partially accounting for its ability to reduce mural cell migration upon TGFβ stimulation [127]. Rivera et al. propose a model whereby, as pericytes come into contact with endothelial cells, SPARC is degraded or removed from the integrin complex, leading to TGFβRII/αv integrin/TGFβ interactions and subsequent signalling [127]. Recently, SMOC1(**S**PARC-related **mo**dular **c**alcium-binding protein **1**), a related protein of the matricellular family, was shown to regulate the balance of ALK5 vs ALK1 mediated TGFβ signalling in retinal endothelial cells [6]. These changes occurred via interactions with endoglin, and downregulation of SMOC1 lead to reduced expression of the TGFβ/ALK5 target α2 integrin, and subsequent alterations in angiogenic phenotype [6].

Mice null for endothelial expression of the tumor suppressor LKB1 (**l**iver **k**inase B1) display early embryonic death associated with defective yolk sac vessel recruitment of mesenchymal precursors of vascular smooth muscle cells, similar to what is seen in endoglin knockout murine embryos [17, 93]. Heterozygous deletion of LKB1 in endothelial cells (as driven by a Tie2-Cre system) resulted in normal microcirculation, but revascularization was impaired in an ischemic limb model [113]. LKB1 null endothelial cells were defective in TGFβ production, implicating this kinase in regulation of TGFβ synthesis [93]. LKB1 in complex with LIP1 is able to block SMAD4 binding to DNA thus further negatively regulating TGFβ signalling [106], and activation of AMPK (**AM**P-activated **p**rotein **k**inase), a downstream effector of LKB1 inhibited TGFβ induced SMAD2/3 gene expression [90]. These latter two studies were not performed in endothelial cells, however, so the exact role of the LKB1/AMPK/ TGFβ pathway in angiogenesis remains to be clarified.

5 Pathological Angiogenesis

Angiogenesis plays key roles in reproduction, development, growth and wound healing, and can drive so-called angiogenesis dependent diseases such as diabetic retinopathy, chronic inflammation and cancer [41, 42]. There is growing evidence that, despite underlying fundamental similarities, the angiogenesis occurring under such pathological settings displays significant alterations in pathways and processes. While such differences complicate our understanding of the angiogenic process, they can also provide opportunities for therapeutic intervention specifically targeting pathological neovascularization [19, 24, 42]. In this section, we describe in more detail some examples of 'pathological angiogenesis' where TGFβ plays a significant role.

5.1 HHT

Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant syndrome associated with epistaxis, arteriovenous malformations in multiple organs, and dilated regions of high capillary density or telangiectases [1]. Disruption of endothelial TGFβ/BMP9 signalling leading to impaired mural cell attachment/function seems to be a major pathobiological event underlying this disease (as reviewed by [140]). There are two commonly identified genetic defects in this condition accounting for two types of HHTs; HHT1 arises due to mutation of the TGFβ type III receptor endoglin, and HHT2 from mutation in the TGFβ type I receptor ALK1 [1]. Additionally, SMAD4 mutation has also been identified in patients with a syndrome characterized by both juvenile polyposis and HHT [45]. There are also at least two additional gene loci associated with familial HHT: HHT3 and HHT4 [9, 22]. More recently, interactome mapping identified the beta subunit of the protein phosphatase PP2A as complexing with endoglin, ALK1 and TGFβRII [161]. Interaction of PP2A with endoglin regulates nitric oxide synthase 3 (NOS3) activity, leading to endothelial stabilization. Loss of PP2A functionality could thus lead to the endothelial dysfunction seen in HHT, and PP2A beta subunit is a candidate gene for HHT3 [161].

Pulmonary circulation is especially affected in all HHT cases, leading to potential for life threatening hemorrhage. Studies have found a general loss of pulmonary capillaries and gain of AVM with primarily venous identity endothelium, perhaps due to excessive endothelial proliferation. Thus, loss of TGFβ regulation of endothelial quiescence and endothelial differentiation may be an underlying molecular defect in these individuals [97]. This is highlighted by a mutation in *PTPN14* associated with HHT, especially the pulmonary manifestations [11]. *PTPN14* (**p**rotein **t**yrosine **p**hosphatase **n**on-receptor type **14**) codes for a protein tyrosine phosphatase; its expression is modulated by both ALK1 and EphrinB2, and Ptpn14 knockdown leads to increased angiogenesis in vitro due to enhanced number of tip cells [11]. In support of this, anti-angiogenic approaches are effective in murine models of HHT and show some clinical utility in human patients [5], perhaps by 'normalizing' vascular defects through enhanced recruitment of mural cells [81].

5.2 Organ Fibrosis

Due to the known association between TGFβ signalling and fibrosis in many systems, it is perhaps not surprising that vascular manifestations of this situation arise. Both the mural cell and endothelial cell components of the microcirculation are documented targets of TFGβ mediated fibrosis in several organs. For instance, during the development of liver cirrhosis, hepatic stellate cells or Ito cells (the vascular sinusoidal mural cells) express excessive collagen upon TGFβ signalling, and in a neuropilin-1 dependent fashion [16]. TGFβ-mediated fibrosis in hepatic stellate cells is prevented by the activity of IQ motif containing GTPase activating protein 1

(IQGAP1), which acts to recruit SMURF1 to TGFβRII, thus regulating TGFβRII degradation [92]. In the kidney, the renal glomerulus is prone to mesangial cell proliferative glomerulonephritis. Mesangial cells are modified and specialized pericytes of the glomerular filtration capillaries, and their proliferation is driven in part by excessive TGFβ production [160]. Interestingly, the bioactive lipid mediator sphingosine-1 phosphate1 (S1P1) is able to cross activate TGFβ signalling in renal mesangial cells via Smad1, 2, and 3 [160], indicating possible transactivation of TGFβ signalling pathways. Partial deletion of TGFβRII in renal endothelial cells reduced EndoMT and concomitant fibrosis in a murine model of chronic kidney disease [158]. This was accompanied by reduced Smad2 mediated signalling with sparing of 'proangiogenic' Smad1/5 pathways in this model [158].

In addition to targeting vascular mural cells to promote fibrosis, TGFβ can also promote fibrosis via EndoMT (discussed above in the context of angiogenesis). This phenomenon is well documented in heart and kidney fibrosis, and is mediated by the SNAIL family of transcriptional repressors. Both canonical and non-canonical TGFβ signalling, including SMAD, MEK, PI3K, p38 MAPK, c-Abl and PKC-δ signalling have been reported to mediate an EndoMT response to TGFβ (reviewed in [102]). Apart from its role in somatic tissue fibrosis, TGFβ-induced EndoMT has been linked to cancer fibrosis in a variety of tumor types, including retinoblastoma [95] and esophageal adenocarcinoma [110]. Additional observations indicate that TGFβ-mediated fibrosis can contribute to cancer progression by enhancing metastasis [92].

5.3 Cancer

Perhaps the best-studied examples of pathological angiogenesis where TGFβ plays a significant role is in the neovascularization of solid tumors, and numerous clinical trials of anti-angiogenic approaches targeting this pathway are underway (as reviewed by [63]). TGFβ orchestrates a switch from vascular inhibition to pro-angiogenic activity, likely indirectly via stimulation of cancer cell production of pro-inflammatory and immune suppressive gene products (as reviewed by [141]). Proteomic comparison of angiogenesis in glioblastoma to physiological angiogenesis (endometrial tissue) found numerous TGFβ target genes overexpressed in glioma vessels compared to endometrium, in particular TGFβ induced protein ig-h3, periostin, integrin-αv, and tenascin C [109]. We found that TGFβ was able to down-regulate the expression of VEGFR2 in colorectal tumor vasculature in an Alk5/SMAD2 dependent fashion [71]. VEGFR2 expression on glioma blood vessels increased with tumor progression, and the proportion of phospho-SMAD2-positive endothelial cells was significantly higher in tumor vessels compared to normal brain vasculature [70]. There is evidence that ALK1 signalling in cancer angiogenesis may modulate cross talk between EC and pericytes, and inhibition of ALK1 may be especially effective in VEGF refractory tumors [24]. Our results support the possibility that ALK5 activation in endothelial cells may be, at least in part, responsible for development of tumor vessel refractoriness to VEGF inhibition.

Finally, endoglin, an essential modulator of TGFβ signalling in endothelial cells has been shown to be significantly upregulated in tumor-associated endothelium and its expression correlated with poor prognosis in patients with various tumor types including breast, lung, colorectal, prostate, gastric, endometrial, hepatocellular, ovarian, cervical and head and neck cancers, as well as glioblastoma (reviewed in [131]). Tumor growth and vascularization is reduced in *ENG*- heterozygous mice [32], and both endoglin-neutralizing antibodies [131] and soluble endoglin [18] target the tumor vasculature and inhibit tumor growth in experimental models, suggesting endoglin as another potential therapeutic target in cancer. Recently, studies using a murine model of pancreatic neuroendocrine cancer reported complex outcomes when TGFβ family signalling in angiogenesis is manipulated. In particular, while knockout of both endoglin and ALK1 reduced primary tumour growth synergistically, ablation of the ALK1 ligand BMP-9 resulted in enhanced metastasis to liver, perhaps modulated by increased EndoMT [34]. Further, in vitro modelling of retinoblastoma angiogenesis suggested that retinal pericytes were an important source of angiogenesis suppression, mediated via TGFβ signalling [95]. These findings suggest caution is required when targeting TGFβ family induced angiogenesis in neoplasia.

In summary, this review highlights the central actions of TGFβ on the vascular components undergoing angiogenesis, which are broad ranging and context dependent. In particular, the pleomorphic responses to TGFβ occurring in pathological versus physiological angiogenesis provide avenues for improved understanding and therapeutic control of these events.

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Chapter 6
Angiogenesis-Based Strategy by Hepatocyte Growth Factor for the Treatment of Ischemic Organ Diseases: From Biology to Clinical Trials

Shinya Mizuno

Abstract Hepatocyte growth factor (HGF) was originally identified as a potent mitogen of hepatocytes in 1980s. HGF induces mitogenic, motogenic and morphogenic activities in epithelial cells through tyrosine phosphorylation of its receptor, c-Met. HGF-c-Met axis is necessary for embryogenesis, organogenesis and tissue repair of almost epithelial organs. Indeed, a loss in HGF-c-Met signaling pathways leads to organ damage and dysfunction during acute and chronic diseases. In the early 1990s', HGF was shown to be an angiogenic regulator via direct effects on endothelial cells (ECs). HGF plays an important part for vascular branching tubular formation via mitogenic, motogenic and morphogenic activities. HGF stabilizes endothelial barrier function via Rac1-dependent cascades. HGF is an anti-inflammatory ligand through inhibiting NF-κB activation in ECs. HGF protects ECs from injurious stresses. However, local HGF production is impaired due to cylic AMP depletion under an ischemic condition. In contrast, c-Met expression is up-regulated, in response to a local hypoxia. When HGF is exogenously injected to hypoxic regions, angiogenic regeneration is induced, associated with an increase in blood flow. This is a rationale why HGF supplemental therapy improves ischemic organ diseases, such as peripheral arterial disease (PAD) and cardial arterial disease (CAD) at least in animal models. Now, clinical trials are ongoing worldwide to determine an optimal condition of HGF supplemental therapy for the treatments of PAD and CAD. In this review, a therapeutic potential of HGF will be discussed, with a focus on biological mechanisms and preclinical or clinical outcomes during ischemic organ diseases.

Keywords Angiogenesis, CAD, c-Met, HGF, Hypoxia, PAD

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1 Introduction

Organogenesis and tissue regeneration depend on, more or less, angiogenesis (*i.e.*, formation of new blood vessels) to supply nutrients and oxygen. The major blood vessels are lined by endothelial cells (ECs), are surrounded by mural cells such as pericytes and vascular smooth muscle cells (VSMCs). The proper interplay between ECs and VSMCs are required for the formation and physiological function of blood vessels [2]. Thus, sequential evens, such as growth, migration and morphogenesis of the vascular cells, should be tightly regulated during angiogenesis. In contrast, abnormal angiogenesis is an increased risk for vascular disorder, most of which are related to pre-existing diseases including hypercholesterolemia, diabetes and hypertension. The end-stage vascular deterioration leads to onset of a life-threatening disease, such as peripheral arterial disease (PAD) and coronary arterial disease (CAD) [88]. For the medical control of these diseases, it is important to elucidate the molecular mechanism whereby angiogenesis is induced, maintained or deteriorated under pathological conditions.

HGF is discovered as a mitogen for rat hepatocytes in primary culture. HGF cDNA was cloned, based on the purified HGF protein [53, 62]. On the other hand, c-Met was identified as an oncogenic protein that induces malignant formation of normal cells [11]. c-Met was identified as a functional receptor for HGF [6]. HGF is a multi-functional growth factor that exerts mitogenic, motogenic and morphogenic functions in various cells via c-Met signaling (Fig. 6.1), hence contributing to embryogenesis and organ regeneration. Using animal models, numerous scientists demonstrated that organ failures become evident, due to an insufficient production of HGF, while HGF supplemental therapy improve these pathological conditions [20, 56, 89]. HGF exerts regenerative and protective effects on parenchymal cells, such as epithelial cells, cardiomyocytes and neuron [22, 26, 63]. Thus, an initial attention was paid to a direct effect(s) of HGF on functional cells, to explain the possible therapeutic outcomes in vivo.

HGF targets not only parenchymal cells but also ECs to induce angiogenic actions at least in vitro [7]. Morishita and his co-workers accumulated evidence that HGF administration is useful for the attenuation of CAD, PAD and other ischemic diseases [58]. This review describes a new concept that loss of HGF-c-Met signaling causes CAD or PAD along with decreased angiogenesis, while gain of HGF-c-Met function leads to improvement in ischemia, in part, via the enhancement of angiogenesis [3, 78, 85]. There is now ample evidence to show the therapeutic effects of HGF on ischemic diseases in animals, and more importantly in human. Prior to discussion of in vivo effect, biological functions of HGF, required for vessel formation without edema, should be described in the following two sections.

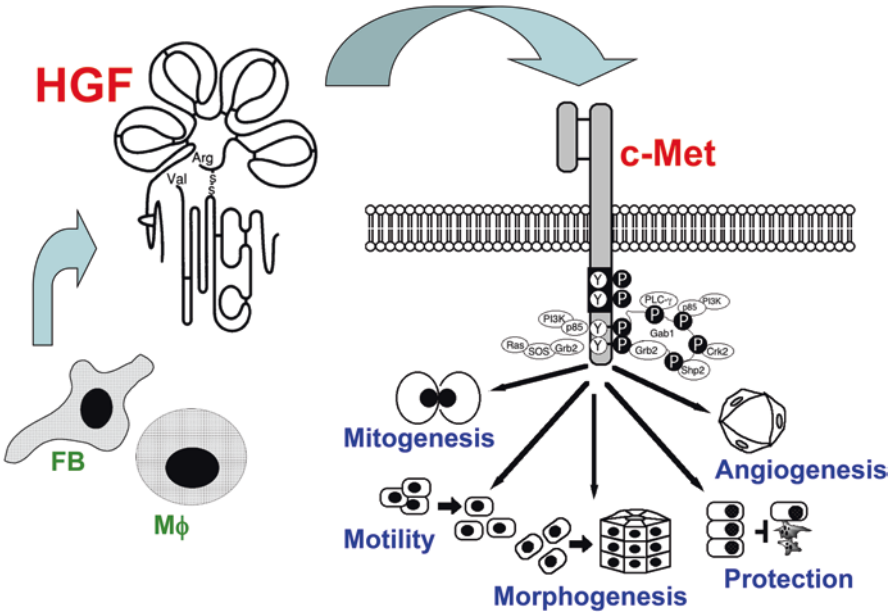

Fig. 6.1 Structure and biological functions of HGF. HGF is produced and secreted as pro-HGF by stroma cells such as fibroblasts (*FB*), macrophages (*Mφ*) and so on. Secreted pro-HGF is cleaved at Arg^{494} and Val^{495} by HGF-activators, such as urokinase-type plasminogen activator. The accurate binding of HGF to c-Met triggers signaling transduction. The ATP-dependent phosphorylation at three residues in the c-Met active loop kinase domain, Tyr-1230/34/35 is an initial step for activating c-Met. Phosphorylation at Tyr-1349/56 in the C-terminal docking site is required for various bio-functions via recruiting down-stream adaptors. For example, phospho-Tyr1349/56-dependent recruitment of Grb2-SOS activates Ras-ERK cascades, leading to cellular proliferation. Association and tyrosine phosphorylation of Gab-1, a docking protein that couples c-Met with multiple signaling proteins such as PI-3kinase, PLC-γ, Shp-2, and Crk-2, plays definite roles in HGF-induced morphogenesis and motility [64]

2 Biological Aspect for Angiogenic Roles of HGF

In the early 1990s, basic scientists suggested that HGF is a crucial regulator for sustaining homeostasis of endothelial morphology and its function. Rosen and his colleagues found for the first time that "scatter factor (SF)" stimulates motility and migration of ECs in vitro [75, 76]. In addition, "tumor cytotoxic factor (TCF)" enhances mitogenic activity of human ECs (*i.e.*, HUVEC) in the culture [83]. Of interest, SF or TCF was identical to HGF following its cDNA cloning. The angiogenic actions by HGF were reproducible in the rabbit cornea [7], or in mouse sub-skins [15]. The sequential effects of HGF, such as mitogen, motogen and morphogen (*i.e.*, 3 M activities) are necessary for HGF to acquire the angiogenic phenotypes. Here, molecular basis of HGF-mediated angiogenesis is discussed, mainly focusing on the physiological roles of HGF in endothelial growth, migration and tubular formation.

2.1 Molecular Basis for Mitogenic Actions

The mitogenic activity of HGF was well conserved in various types of ECs [7]. VEGF and FGF2 are also known to induce proliferation of ECs, but HGF is the most potent mitogen among three growth factors [65]. The MAPK downstream cascades are important for HGF-c-Met axis to elicit mitogenic actions. Actually, c-Met tyrosine phosphorylation by HGF leads to a rapid activation of MAPKp42/44 (i.e., ERK1/2), while ERK1/2-inhibitors diminished the angiogenic roles of c-HGF. Next, activated ERK1/2 causes STAT3 phosphorylation and subsequent c-Jun promoter activation [60]. In the culture of ECs, NOS inhibitor (*i.e.*, LAME) attenuated the HGF-primed ERK1/2 activation. Of note, K^+-channel blocker attenuated the eNOS activation and HGF-induced mitogenesis. These results suggest that an initial enhancement of K^+ influx by HGF triggers eNOS activation, MAPK activation, and eventually, mitogenic phenotypes are induced in HUVEC cells [38]. In contrast to other cytokines such as FGF2, HGF does not stimulate proliferation of vascular smooth muscle cells, although these cells express c-Met on the cell surface [45, 65].

2.2 Mechanisms of HGF-Induced EC Motility or Migration

Sequential events for endothelial migration include: (i) attachment of ECs to extracellular matrix protein (ECM) such as collagens in an initial phase; and (ii) cyto skeletal re-arrangement and cell motility post-lamellipodia formation; and (iii) ECM degradation for invasion across the basement membrane in a late phase. In the initial or middle step, sphingoshine1-phosphate (S1P) and its catalyzing enzyme, ShpK1 are involved in HGF-induced lamellipodia formation [12]. Phosphorylation of ShpK1 by HGF-c-Met-ERK1/2 pathway leads to an increase in S1P levels, assembly of p-ShpK1 to actin-cortaclin and subsequent migration. Gab1 is a docking protein of c-Met and governs c-Met downstream signaling via changing adaptor molecule partners. For example, phosphorylation of Gab1 by HGF-c-Met leads to Gab1-SHP2 complex, and then its downstream pathway of ERK1/2-Erg (and of ERK5/KLF2) contributes to endothelial migration and stabilization, respectively [81]. In the late step of migration, HGF reduces the expression of an adhesion molecule (*i.e.*, VE-cadherin) to facilitate cell motility via the loss in cell-cell contact [48]. ECM degradation is required for ECs to invade in neighboring tissues across basement membranes. For this purpose, HGF activates ECM-degrading enzymes, such as MT1-MMP and MMP2 in ECs [15, 99]. Activation of iNOS by HGF is also involved in EC motility [72]. Each molecular event in “each phase” is required for EC migration.

2.3 Molecular Basis for Morphogenesis

HGF has a unique morphogenetic activity, which forms polarized, tubular and branching structure of epithelial cells in collagen gels. HGF also induces in vitro and in vivo capillary tube formation of ECs [15]. In matrigel plug assay, HGF induces blood vessel formation that contains vasculature with surrounding VSMCs [15]. The adaptor protein Gab1 is required for HGF-induced morphogenesis of ECs. EC-specific Gab1-knockout mice (Gab1-ecKO mice) showed no abnormality of vascular development, but showed little angiogenic response in a mouse model of limb ischemia. Indeed, HGF did not induce the migration, morphogenesis and sprouting in a culture of Gab1-null ECs [109].

Src signaling pathway is required for HGF-mediated morphogenic actions, because Src gene deletion led to the loss in HGF-mediated vessel formation [30]. In HGF-induced vascular lumen and cord formation, sprouting of vessels was also inhibited by the several inhibiters that target Rho kinase and MMPs, which is a key player for migration or ECM degradation [87]. Overall, HGF-c-Met-Gab1-induced downstream responses (such as Rho activation and MMP induction) are required for vessel formation and functional maturation.

Recently, Arf6, a small GPTase, was shown to be a key mediator for HGF-mediated endothelial tubular formation in a collagen-gel based 3-D culture [23]. Briefly, activation of Arf6 by HGF leads to up-regulation of integrin-β on surface, and this is critical for tight contact of ECs-ECM, focal adhesion formation and morphogenesis. However, it is still unclear whether Arf6 activation is linked with Gab1-SHP2 complex, and future studies would shed more light on this notion.

Overall, activation of c-Met by HGF is critical for ECs to induce its morphology and function through the alteration of downstream signaling molecules (Table 6.1). HGF is also important for "lymphatic" vessel formation via 3 M–based biological activities in vitro, and more importantly, in vivo [14, 31, 77]. Angiogenic actions by HGF are inducible in animal models, in response to hypoxic stresses, as discussed later (see, Sects. 5 and 6).

3 Anti-edematous Mechanisms by HGF

Peri-vascular edematous lesions, occasionally associated with local inflammation, produce pathological events, such as pain, swelling and so on. Thus, it is important to stabilize the barrier function of ECs during therapeutic angiogenesis, especially in capillary tubular formation. Notably, VEGF gene therapy often induces edematous changes even if it may be effective in patients [74]. In contrast, HGF has multiple roles, such as anti-edematous, anti-inflammatory and anti-apoptotic outcomes (Table 6.1) to prevent peri-vascular edema, as described below.

Table 6.1 Biological functions and pathways of HGF-c-Met signalings for neovascularization

1. Mitogenesis		4. Barrier stabilization	
Discovery in HUVEC model	Shima et al. [83]	TER up via GSK3β	Liu et al. [44]
MAPK p42/44 (ERK1/2)	Nakagami et al. [60]	Tiam1→Rac1 pathway	Birukova et al. [4]
Stat3 – c-Jun	Nakagami et al. [60]	Met-CD44v10 interaction	Singleton et al. [79]
K^+ influx	Kuhlmann et al. [38]	Arf→Rac1 pathway	Tian et al. [95]
eNOS pathway	Kuhlmann et al. [38]	Arf-Rac1 activation on IQGAP	Tian et al. [96]
2. Motility		Inhibition of VEGF-Rho path	Birukova et al. [5]
Discovery in EC culture	Rosen et al. [75]	5. Anti-inflammation	
ERK1/2→S1p-ShpK1 pathway	Fu et al. [12]	Inhibition of VEGF-NFkB path	Min et al. [52]
Gab1-SHP2→ERK-Erg pathway	Shioyama et al. [81]	EC growth without inflammation	Kaga et al. [28]
ECM degradation by MMPs	Wang and Keiser [99]	Inhibition of NF-κB and ICAM1	Mizuno and Nakamura [54]
iNOS-NO pathway	Purdie et al. [72]	Inhibition of LPS→NFκB path	Meng et al. [51]
3. Morphogenesis		6. Anti-apotosis	
In vivo cornea assay	Bussolino et al. [7]	PI3K-AKT path	Nakagami et al. [60]
In vivo angiogenesis	Grant et al. [15]	(stimuli: TNF-α, AGE, LDL)	Zhou et al, [110], Yu et al. [106]
Gab1 pathway	Zhao et al. [109]	ERK1/2 path (stimuli: Ang-II)	Lee et al. [40]
Src dependency	Kanda et al. [30]	Bcl-2 induction	Yamamoto et al. [101]
Rho, MMP pathways	Somlyo et al. [87]	FOX1/3 phosphoryration	Zhang et al. [108]
Arf→integrin-β for focal adhesion	Hongu et al. [23]	7. Pericyte recruitment	
Lymph vessel formation (in vitro)	Kajiya et al. [31]	PI3K-AKT path	Taher et al. [90]
Lymph vessel formation (in vivo)	Saito et al. [77]	FAK or Pyk2 path	Ma et al. [46]
		Angiopontin-1 induction	Kobayashi et al. [36]

For abbreviations see text

3.1 Molecular Basis for Barrier Stabilization

Transendothelial electrical resistance (TER) is a physiological indicator of the permeability in a monolayer culture of ECs. VEGF causes a decrease in TER as its original name (*i.e.*, vascular permeability factor) indicates. In contrast, HGF enhance the TER, associated with cortical actin thickening and GSK-3β phosphorylation [44]. Such an effect of HGF on barrier stabilization is mediated via Tiam1, a guanine nucleotide exchange factor (GEF) of Rac-GTPase. HGF inhibits thrombin induced loss of endothelial barriers via Tiam1-activated Rac1-GTPase pathway [4]. CD44v10 is an isoform of cellular surface CD44 and is necessary for c-Met phosphorylation and internalization by HGF, possibly during an initial phase of caveolin-based endocytosis [79].

Birukova and his co-workers have accumulated evidence that another GEF, Arf is also critical for HGF-induced stabilization of barrier function: Arf is necessary for a rapid activation of Rac1-GPTase by HGF, possibly via microtubule-dependent pathway [95, 96]. In this process, a downstream adapter, IQGAP1 acts as a platform anchorage for recruiting actin-filament or microtubules. Of note, Asef active-form, primed by HGF, targets and activates Rac1 on IQGAP1 platform, and then cytoskeletal molecules (such as cortactin and Arp2/3) are recruited in periphery for cortical actin ring formation. Such a molecular hierarchy likely contributes to the enhancement of endothelial barrier function by HGF. Indeed, endogenous Arf is necessary for HGF administration to prevent pulmonary edema in a mouse model of acute lung injury [51].

VEGF-induced Rho-GPTase activation is a key event for a loss in endothelial barrier. Indeed, VEGF randomizes focal adhesions via a Rho pathway. In contrast, activation of Rac1 by HGF leads to redistribution of focal adhesions to cell periphery. Of note, VEGF-induced Rho-dominant events (including randomized focal adhesions) are counteracted by HGF-Rac1 pathway [5]. As a result, HGF can block EC barrier dysfunction and VEGF-mediated vascular permeability.

3.2 Anti-inflammation

Previous studies revealed the unexpected inflammatory role of angiogenic factors. For example, VEGF enhances leukocyte adhesion to ECs by up-regulating the production of adhesion molecules such as ICAM-1 in an NF-κB-dependent manner, contributing to the inflammation [34]. In addition, FGF2 evokes inflammatory response by activating NF-κB and increasing levels of inflammatory cytokines (such as IL-8 and MCP-1) in VSMCs [28].

In contrast to these growth factors, HGF exerts anti-inflammatory effect on ECs. Adhesion molecules (such as ICAM-1 and E-selectin) are induced via NF-κB pathway, and this is necessary for transendothelial migration of leukocytes. In culture of

ECs, HGF counteracted the TNF-α-mediated induction of ICAM-1 through the inactivation of NF-κB. As a result, ICAM-1 induction was blocked by HGF in a model of HUVEC [54]. Such an inhibitory effect of HGF was also seen in a culture model of E-selectin induction in ECs [47]. As mentioned, VEGF induces endothelial inflammation, while HGF prohibits the VEGF-induced NF-κB activation and ICAM1 induction in ECs [52].

Of interest, HGF directly targets ECs or macrophages to suppress the production of pro-inflammatory cytokines, such as IL-6 and IL-8, in part, via inactivation of NF-κB pathway [29, 51]. These anti-inflammatory but not pro-inflammatory actions of HGF have a beneficial effect on the protection of vascular and organ cells.

3.3 Anti-apoptosis

Protection of resident ECs is also important for HGF to block veri-vascular edema. Indeed, HGF protects ECs from various types of injury via activating anti-apoptotic signaling cascades. For example, ECs become apoptotic, in response to TNF-α exposure, while HGF blocks apoptosis via a PI3K-AKT pathway [60]. Likewise, HGF protects ECs from apoptosis, caused by advanced glycation end products (AGE) and low-density lipoprotein (LDL) cholesterol [106, 110], possibly in a PI3K-AKT-dependent manner. Angiotensin-II (Ang-II) is crucial for not only hypertension but also tissue fibrosis during chronic organ failure. Ang-II also induces apoptosis in ECs, while HGF inhibits Ang-II-mediated apoptotic events via activating ERK1/2 pathways [40]. Moreover, induction of Bcl-2, an anti-apoptotic molecule by HGF contributes to protection of ECs from apoptosis, caused by hypoxia, glucose and AGE [61, 101, 111]. Under hypoxia and reperfusion injuries, reactive oxygen species (ROS) causes apoptosis in ECs via activating xanthine oxidase (XO). Of note, HGF inhibits XO-induced ROS production through XO inactivation and Ca^{2+} influx [108]. As a result, ROS-induced apoptosis is largely blocked by HGF. Additionally, HGF inhibits superoxide-induced apoptosis via a rapid phosphorylation of FOXO1/3, a member of FOXO family [41]. Such anti-apoptotic effects of HGF on ECs will contribute to prohibit or reverse numerous ischemic diseases through the maintenance of local blood flow.

3.4 Pericyte Recruitment

Recruitment of pericytes in small vessels plays a critical role in the maintenance of vascular homeostasis (including avoidance of vascular leak syndrome). Indeed, HGF contributes to motility of VSMCs. In response to HGF, VSMCs rapidly form lamellipodia to acquire migratory phenotypes via PI3K-AKT pathway [90]. In addition, HGF-mediated focal adhesion re-distribution and activation of FAK or of Pyk2 depends on MAPK-ERK1/2 cascade [46].

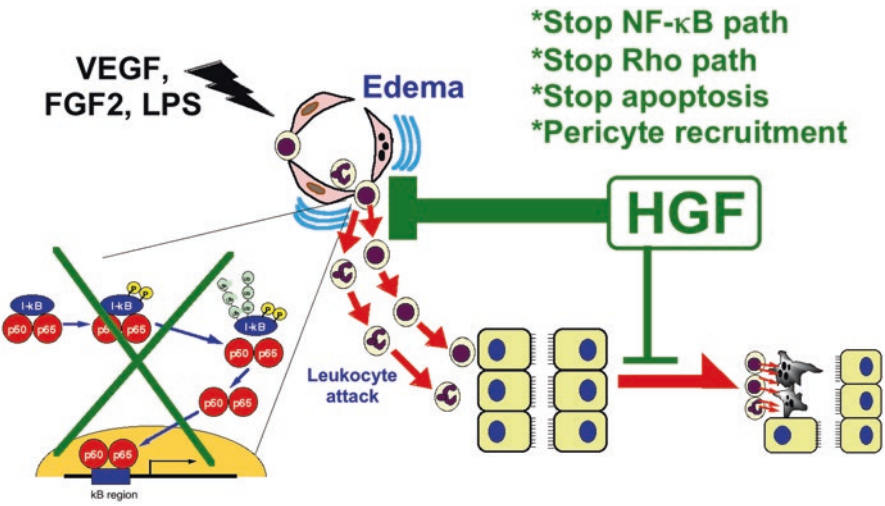

Fig. 6.2 Anti-edematous effects of HGF on vascular cells. Under ischemic or septic conditions, VEGF, FGF2 or LPS can activate NF-κB via degradation of inhibitory anchor, I-κB. In contrast, HGF counteracts these inflammatory signaling pathways, leading to the reduced ICAM-1 expression and suppressed leukocyte infiltration. HGF also inhibits VEGF-mediated Rho pathway to prevent a loss in endothelial barrier function. Furthermore, HGF inhibits vascular cell apoptosis via PI3K-AKT pathways, along with Bcl2 up-regulation. These direct effects of HGF on ECs lead to prevention of edematous lesions during ischemic organ. Recruitment of pericytes by HGF is also contributable for anti-edematous outcomes, possibly as a compensated reaction against local inflammation

Angiopoietin-1 (Angp1) is a key molecule for the recruitment of VSMCs to EC wall. Notably, Angp1 up-regulates the HGF production, while Angp1-induced VSMC migration is largely diminished by anti-HGF antibody, hence suggesting HGF as a key mediator for Angp1 to recruit pericytes in pre-vascular regions [36]. Endothelium-derived HGF recruits pericytes or VSMCs in microvessels, and this event contributes to the inhibition of vascular permeability and inflammation, possibly through the paracrine mechanism.

As a result, HGF was shown to be a physiological regulator to block edematous lesion, via a GEF-Rac1 pathway(s) in ECs. Anti-inflammatory and anti-apoptotic effects of HGF also participate in anti-edematous mechanisms. In addition, pericytes, recruited by Angp1→HGF cascade, supports the endothelial integrity. All of them are contributable for HGF to induce neovascularization without edema (Fig. 6.2).

4 Preclinical Evaluation of HGF During PAD

PAD is a representative disease that develops when the inner wall of arteries, frequently in the legs, are occluded by the plaques made of fat, cholesterol and calcium. Diabetes, hypertension and obesity often cause this disease. Drugs with

anti-platelet or vasodilator effect, and vascular surgical manipulation with a balloon catheter or metal stent are used for the treatment of PAD. Neovascularization induced by growth factors are considered as a promissing therapy for PAD. During the past 20 years, Morishita et al. have accumulated evidence to provide POC for HGF treatment in PAD [78]. In this section, the regulation of HGF production under PAD-related conditions and the therapeutic effects of HGF on PAD are discussed.

4.1 Loss of Local HGF Production During Experimental PAD

Production of HGF in stroma cells in injured organs is ordinarily increased in response to tissue damages [64]. In the animal models of PAD, however, HGF production in vascular tissues is decreased, due to hypoxia. Indeed, cyclic-AMP (cAMP) is known as a transcriptional inducer of HGF mRNA, whereas cAMP is depleted under hypoxia [20]. Persistent hypoxia enhances expression of TGF-β, an inhibitor of HGF production. Overall, local HGF production is impaired in the rabbit model of PAD. In patients with arterioscrelosis obliterans (ASO), HGF transcription and protein production are also decreased in hypoxic legs than in normal tissues [56]. Thus, it is likely that the local self-defensive system (*i.e.*, paracrine system) is impaired due to hypoxia.

Oppositely, circulating levels of HGF is elevated in patients of PAD, particularly with collateral formation (*i.e.*, enhancement of endocrine system) [105]. High serum concentration of HGF is positively correlated with collateral vessel formation. Thus, serum HGF could be a clinical marker for PAD and/or collateral formation. The increase in serum HGF levels might be a compensation for the loss of 'local' HGF production by distant organs, but this is not sufficient for the complete inhibition of skin ulcer, as seen in diabetic mice [103].

4.2 Pre-clinical POC of Recombinant HGF for Treating PAD

Hypoxia down-regulates HGF production in vascular tissues, while its receptor, c-Met is up-regulated in response to hypoxia-inducible transcriptional factor, HIF1. This reciprocal effect by local hypoxia prompted researchers to examine whether HGF supplemental therapy is reasonable for treating PAD under HGF-deficient and c-Met-sufficient conditions. When recombinant HGF protein was injected into the ischemic hindlimb of rabbits via a femoral artery during PAD, angiogenesis was successfully induced. As a result, muscular necrosis, due to ischemia, was improved in HGF-treatment group [20]. Such an effect by the local application was reproducible when HGF was intravenously administered [56], along with the collateral vessel formation. The neovascular density is higher in HGF group than in VEGF group

Table 6.2 Preclinical proof-of-concept of HGF-induced therapeutic effects in animal models of PAD

Diseases, strategy, route	Animal models	Therapeutic outcomes	References
1. HGF alone			
Recombinant HGF, lintra-artery injection	CLI rabbit	Angiogenesis, Inhibited muscular necrosis	Hayashi et al. [20]
Recombinant HGF, lintra-artery injection	CLI rabbit	Angiogenic collateral growth	Morishita et al. [56]
Recombinant HGF, local application	Diabetic mouse	Angiogenesis, reduced skin ulcer	Yoshida et al. [103]
Naked HGF-plasmid, im	CLI rat and rabbit	Increased blood flow	Taniyama et al. [92]
HVJ-HGF cDNA, im	Diabetic rat	Increased blood flow	Taniyama et al. [93]
Naked HGF-plasmid, im	Lipo-A TG mouse	Increased blood flow	Morishita et al. [57]
Naked HGF-plasmid (VM202), im	CLI rabbit	Collateral vessel growth, Improved hypoxia	Pyun et al. [73]
2. Combination therapy			
HGF + prostacyclin synthase	Diabetic rats	Additive effects on neuropathy	Koike et al. [37]
HGF + FGF2 in collagen microsphere	CLI mouse	Additive effect via enhanced angiogenesis	Marui et al. [49]
Naked HGF-plasmid im + GCSF s.c.	CLI mouse	Synergic effect via BM cell recruitment	Ieda et al. [25]
BM-MN cells + Ad-HGF vector, local	CLI mouse	Enhanced angiogenesis via anti-apoptosis	Yamamoto et al. [102]
3. HGF-inducible therapy			
Wnt1-transfected EPCs, systemic	CLI mouse	HGF induction, angiohgenesis	Gherghe et al. [13]
HVJ-Ets1 cDNA, local application	CLI rat	HGF up, VEGF up, enhanced angiogenesis	Hayashi et al. [18]

Abreviattions: *HVJ-HGF* HVJ liposome containing HGF cDNA, *Lipo-A TG* lopoprotein-A transgenic, *BM-MN* bone marrow mononuclear, *EPCs* endothelial progenitor cells. For other keys see text.

in vivo [98]. The initial studies in the late 1990s provide a proof-of-concept (POC) in the potential use of HGF for treating PAD (Table 6.2), followed by clinical trials, as discussed later.

4.3 HGF Naked Plasmid Therapy

The loss of HGF production in hypoxic tissues provides a rationale why HGF supplemental therapy is useful for reducing PAD-associated pathological conditions, such as ulcer, massive necrosis and infection. Given that a half-life of HGF

protein is very short (<5 min) in vivo, it is important to develop a method for stable production and/or retention of HGF, specifically in the injured tissues. HGF cDNA-containing 'naked' plasmid vector is now available for this purpose.

Morishita's group found that naked plasmid-based HGF supplement improved the PAD-related conditions in an animal model [92]. They for the first time demonstrated the angiogenic outcomes (such as an increase in collateral vessel density) in the ischemic hind limbs of rats post-HGF gene transfection. Consistently, local blood flow was increased in the HGF-transfected rats, and this was associated with the local detection of human (*i.e.*, exogenous) HGF. Such a beneficial effect by HGF-expressing naked plasmid was also reproducible in a rabbit model of PAD. Overall, intra-muscular injection of HGF-plasmid around ischemic areas was effectivey for minimizing PAD. Such a simple method using 'naked' plasmid is also useful for improving hind-limb ischemia in diabetic mice [93], or in lipoprotein-A-transgenic mice [57].

In this method, subjective genes (such as HGF) are not incorporated into host genome. Furthermore, HGF cDNA-containing plasmid does not stimulate malignant metastasis in a tumor-bearing mouse model [50], hence sporting its safety even in the long-term application in muscular tissues. In addition, Korean group newly constructed an HGF-expressing plasmid vector (*i.e.*, genomic-cDNA hybrid). This construct, pCK-HGF-X7 vector (VM202), produces full from HGF (*i.e.*, HGF_{728aa}) and 5 amino acid-deleted HGF (*i.e.*, HGF_{723aa}) to acquire the possible additive effect. As expected, this vector efficiently improved the local blood flow via an increase in collateral vessels in a rabbit model of hind limb ischemia [73]. Both vectors, developed in Japan and Korea, are now in the process of clinical trials for treating human PADs, as described later.

4.4 Alternative Strategy for HGF-Based Angiogenesis in PAD

Prostaglandins (such as PG-E1/E2 or PG-I1/I2) are known to enhance HGF production [64]. FGF2 has an additive effect on HGF-induced mitogenesis of ECs [65]. Moreover, FGF2 or Wnt1 also induces HGF production in ECs. Growing evidence indicates that these HGF-inducing cytokines are also available for attenuating PAD-related conditions, as summarized.

Prostacyclin (PGI) synthase enhances HGF-mediated biological actions via PGI1/2 generation. Indeed, co-transfection of HGF plasmid with PGI synthase gene markedly promoted the neoangiogenesis in a mouse model of hind-limb ischemia [37]. A slow release of FGF2 did not significantly improve the hind-limb ischemia in mice. However, when FGF2 was administered together with HGF in the slow release system, a decrease in local blood flow was restored, as evidenced by a laser Doppler [49]. GCSF plays a critical role for the recruitment of bone marrow-derived progenitor cells into regenerating vessels. Combination of HGF with GCSF synergistically improved the hind-limb hypoxia in mice, along with the increase in bone marrow-derived cells in neovessels [25]. Such a synergistic effect was also

reproduced: an adenovirus-based HGF gene therapy was performed in a mouse model of ischemia, together with an injection of autologous bone marrow-derived cells [102]. GCSF and HGF are effective for bone marrow cell- and resident endothelium-based regeneration, respectively.

Wnt1 is a ligand to activate β-catenin signaling. Local injection of Wnt1 protein leads to an increase in vessel density in ischemic muscles of mice, and this was associated with up-regulation of HGF in the local cites [13]. Ets1 is a common transcriptional regulator to initiate HGF and VEGF mRNA transcription. Forced induction of Ets1-expressing cDNA via an HVJ-liposome method leads to local up-regulation of HGF (and VEGF) [18]. Under such an HGF-sufficient condition, hind-limb hypoxia was improved, along with an increased vessel density in rats. Thus, combined and/or alternative methods may be helpful for promoting therapeutic potentials of HGF during the PAD treatment.

PAD is a representative model for rationalizing HGF supplemental therapy as a pathogenesis-based strategy (Fig. 6.3). Morishita and his co-workers provided this principle in 1999, using animal models. Now, this effort leads to a practice of HGF plasmid therapy in clinical trials of PAD, as described latter (see, Sect. 6).

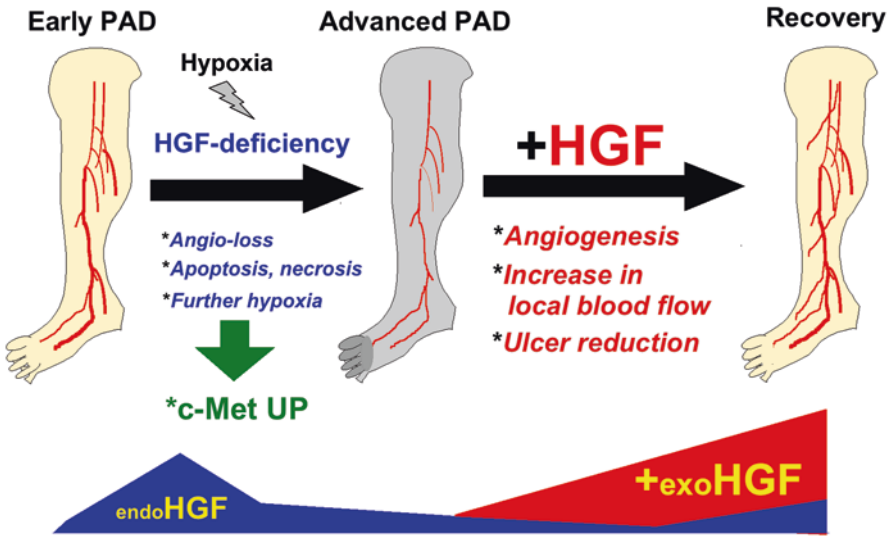

Fig. 6.3 HGF-based therapeutic angiogenesis during PAD progression. In an early phase of PAD, local HGF production is up-regulated in response to inflammatory cytokines, such as TNF-α. However, persistent hypoxia down-regulates endogenous HGF (endoHGF) production, in part, via a loss in local levels of cAMP that is necessary for HGF transcription. Under such a hypoxic condition, vascular structure and function are damaged via apoptotic cell death. In reciprocal to a loss in ligand HGF, c-Met is up-regulated via a HIF1-dependent transcriptional pathway. When exogenous HGF (exoHGF) or its gene is injected into damaged areas, angiogenic reaction is induced via HGF-mediated functions, such as mitogen, motogen and morphogen. In addition, a rapid phosphorylation of nitric oxide synthase (NOS) by HGF contributes to an increase in blood flow via NO-dependent vessel tone relaxation. Overall, skin ulcer is repaired via HGF-mediated proliferation and migration of keratinocytes under such an aerobic condition

5 Therapeutic Angiogenesis in Other Ischemic Organs

The direct angiogenic effects of HGF contribute to the improvement in ischemia in other organs. Coronary artery disease (CAD) is the most common type of heart disease and is now the leading cause of death worldwide. The decrease in coronary artery flow causes local hypoxia and loss in cardiomyocytes, leading to myocardial infarction (MI), sometimes associated with cardiac dysfunction and fibrosis. Forced induction of angiogenesis by HGF leads to the attenuation of other ischemic organs, such as brain, lung, kidney and so on. The preclinical POC of HGF has been accumulated in animal models, as followed.

5.1 HGF-Based Angiogenic Treatment for Heart Diseases

Circulating HGF is markedly increased in the patients of acute MI [86]. In the late phase of MI-manifesting rats, the expression of local HGF becomes faint [3]. Inversely, the expression of c-Met is augmented in the capillary ECs after heart ischemia [68], suggesting the role of HGF during CAD. The angiogenic actions of HGF in the heart were shown in the model of acute MI [3]. HGF gene transfection into rat hearts by HVJ-liposome method increased the number of vessels in the ischemic hearts, especially near the infarcted myocardium. This effect was associated with the increase in cardiac blood flow and in the attenuation of cardiac functions, as measured by the left ventricular (LV) ejection fraction [3]. Such an HGF-mediated angiogenic activity was also reproducible in a rat model of chronic MI: an adenoviral vector containing human HGF cDNA was injected into the limb muscles 3 days after heart ischemia, resulting in an increase in plasma human HGF in treated mice, followed by an increase in the number of coronary vessels [43]. Overall, LV remodeling and dysfunction were improved in the HGF-treated mice compared with controls, as indicated by the greater % of fractional shortening and LV+/−dP/dt. Similar angiogenic effect by HGF was seen in a hamstor model of dilative cardiomyopathy [94]. Of note, HGF gene therapy is useful for suppressing neointimal formation post-PTCA. When HGF cDNA-containing HVJ-liposome was injected into the carotid artery of rabbits soon after the balloon injury, neointimal hyperplasia was suppressed via the induction of re-endothelialization [19], hence indicating that a rapid restoration of EC integrity by HGF is a reasonable strategy to suppress re-stenosis after angioplasty. Overall, HGF-induced angiogenesis should be considered as a new option to for treating cardiovascular diseases [58].

5.2 Cerebrovascular Diseases

Cerebrovascular disease is characterized by a pathological condition that affects the arteries supplying the brain, frequently caused by a clot deposition in the brain (thrombosis or embolism). These vascular disorders reduce local nutrients and oxygen tension, followed by the ischemia, stroke with hemorrhage or embolization. Sustained disease leads to massive neuronal cell death, cerebreal edema and increase in intracranial pressure, which is a risk for attendant complications such as lethal brain herniation occasionally resulting in brain death. Thus, therapeutic strategies adopted for the treatment of cerebrovascular disease are to prevent clot deposition by using thrombolytic or anti-platelet agents, to inhibit neuronal cell death and to recover the cerebral blood flow while decreasing vascular permeability.

In a cerebral ischemia model, intra-ventricular administration of HGF stimulated angiogenesis and increased vascular lumens [97]. Pre-ischemic transfection of HGF gene using HVJ-liposome method into subarachnoid space also resulted in the increase in vessel number and blood flow after occlusion of carotid artery [104]. HGF gene transfer immediately after occlusion also stimulated angiogenesis on the brain surface and improved cerebral blood flow [104]. Importantly, angiogenic action of HGF on the brain is obtained without edematous formation and disruption of the brain-blood barrier [84]. Rather, HGF protects ECs from apoptotic cell death and inhibits the leakage of brain-blood barrier [10]. Such an angiogenic effect of HGF was also observed in the mouse model of Alzheimer's disease, followed by the prevention of behavior dysfunction [91].

5.3 Lung Emphysema and Other Pulmonary Diseases

Lung emphysema, mostly caused by a long-term smoking, is a type of chronic pulmonary disease in which alveolar architecture is destructed and overinflated, along with the impairment of gas exchange (*i.e.,* hypoventilation). Transfection of HGF gene into normal lung increased capillary density and blood flow, suggesting that HGF administration assists the recovery of respiratory diseases through the induction of angiogenesis [69]. In a rat model of elastase-induced lung emphysema, endogenous HGF levels were transiently elevated, but decreased along with the disease progression, while transfection of HGF gene into the rats increased vascular density, resulting in improvement in lung ischemia and exercise intolerance in vivo [80]. Interestingly, HGF also increased the fraction of circulating EC progenitor cells (Sca-1$^+$, c-kit$^+$, Flk$^+$) and their engraftment into the lung capillary endothelium [27]. Thus, HGF promotes angiogenesis by enhancing the *in situ* proliferation of resident ECs, and by inducing engraftment of bone marrow-derived progenitor cells in vessels. Anyway, such an angiogenic response was well conserved during HGF-mediated recovery from other pulmonary diseases, such as acute lung injury or pulmonary hypertension in rodent models [51, 70].

Table 6.3 Angiogenesis-based preclinical studies on HGF supplemental therapy in various ischemic organs

Diseases, strategy, route	Animal models	Therapeutic outcomes	References
1. Heart			
HVJ-HGF cDNA, intra-myocardium	MI, rat (acute)	EC proliferation, reduced infarction	Aoki et al. [3]
Adeno-HGF, intra-myocardium	MI mouse (chronic)	Angiogenesis, anti-fibrosis, improved LV function	Li et al. [43]
HVJ-HGF cDNA, intra-anterium	DM, hamster	Angiogenesis, anti-fibrosis, improved LV function	Taniyama et al. [94]
HVJ-HGF cDNA, coronary arterial	BA injury, rabbit	Re-endothelialization, inhibited neointimal hyperplasia	Hayashi et al. [19]
2. Brain			
Recombinant HGF, ventricular injection	Brain ischemia, rat	Increased vessels, Bcl2 induction in neurons	Tsuzuki et al. [97]
HVJ-HGF cDNA, subarachoid space	Brain ischemia	Increased blood flow, neoangiogenesis	Yoshimura et al. [104]
Recombinant HGF, local injection	Brain ischemia, mouse	Anti-apotosis in EC, improved larning memory	Date et al. [10]
HVJ-HGF cDNA, via cisterna magna	Brain ischemia, rat	Engiogenesis, inhibition of neurological defect	Shimamura et al. [84]
Naked HGF-plasmid, ventricular injection	Amyroid-β, mouse	Better congnityve function, angiogenesis, BDNF up	Takeuchi et al. [91]
3. Lung			
HVJ-HGF cDNA, testis iv	Emphysema, rat	Angiogenesis, Increased blood flow, Alveolar repair	Shigemura et al. [80]
Recombinant HGF, systemic injection	Emphysema, mouse	Increase in BM-EPCs, BM-based angiogenesis	Ishizawa et al. [27]
HVJ-HGF cDNA, testis iv	PH, rat	Angiogenesis, Anti-stenosis, Angio-protection	Ono et al. [70]
Recombinant HGF, iv	LPS-ALI, mouse	Synergic effect via BM cell recruitment	Meng et al. [51]
4. Kidney, liver and skin			
Recombinant HGF, ip	Glomerulonephritis, rat	Increased vessels, better renal function	Mori et al. [55]
Naked HGF plasmid, intra-kidney	GM-renal fibrosis, rat	Increased vessel, anti-fibrosis, better renal function	Chen et al. [8]

(continued)

Table 6.3 (continued)

Diseases, strategy, route	Animal models	Therapeutic outcomes	References
Naked HGF (w/o VEGF) plasmid, iv	Cirrhosis+70%PHx, rat	EC proliferation, Enhanced liver growth	Oe et al. [67]
HVJ-HGF cDNA, local application	Skin wound, rat	Neovascularization, re-epithelization, anti-scar	Nakanishi et al. [66]

Abbreviations: *LV* left ventricular, *DM* dilated cardiomyopathy, *BA injury* balloon catheter-mediated arterial injury; *PH* pulmonary hypertension, *LPS-ALI* lipopolysaccharide-induced acute lung injury, *GM* gentamicin, *70%PHx* 70% partial hepatectomy. For other keys see text or table/figure legends

Angiogenic action is also involved in HGF-mediated regenerative outcomes in renal fibrosis [8, 55], liver cirrhosis [67] and scleroderma [66]. In summary, HGF supplement therapy was found to be effective in various types of ischemic diseases, such as CAD, PAD, brain ischemia, lung emphysema, renal fibrosis, etc., in part, through angiogenesis without edema (Table 6.3). These experimental studies also encourage clinical practice of HGF gene therapy, especially with a focus on chronic CAD, as followed.

6 Clinical Trials of HGF for the Treatment of Ischemic Diseases

HGF is now one of the most potent angiogenic ligands among growth factors. HGF causes an increase in local blood flow, possibly via a nitric oxide-dependent pathway. HGF production is impaired due to a local loss in cAMP under an ischemic condition, while c-Met is up-regulated via HIF1-dependent pathway [64]. Of importance, compensation for a loss in endogenous HGF by adding exogenous HGF leads to improvement in the ischemic conditions, in part, through the induction of angiogenesis with enhanced blood flow in preclinical studies. Several lines of clinical trials suggest a potential use of HGF for the treatment of ischemic diseases in humans, without significant adverse effects.

6.1 Naked Plasmid Containing HGF cDNA (Collategene)

During the past 20 years, Morishita *et al*. accumulated POC in animal models. They found that local application of a naked plasmid of HGF cDNA produces an angiogenic effect on CAD and PAD in animals [3, 92, 93]. Based on this background, they first designed a clinical trial (*i.e.*, as an open-labeled study) for the evaluation of safety and effectiveness of naked HGF-plasmid therapy, with a focus on critical limb

ischemia (CLI). The HGF plasmid consists of cDNA fragment of human HGF inserted into pVAX1 vector (3.0 kb), called "Collategene". This HGF-plasmid DNA was injected in ischemic limbs of 6 CLI patients (arteriosclerosis obliterans [n = 3]; Buerger disease [n = 3]), with the primary endopoint of 12 weeks [59]. This therapy improved the local blood flow, as evidenced by an increase in ankle pressure index in 5 of 5 patients. The size of 8 of 11 ischemic ulcers in 4 patients was reduced >25%. Apparent edema was not observed in any patient throughout the trial. As a result, there was a reduction of pain scale in 5 of 6 patients, suggesting HGF's effect.

This group further evaluated the potentials of Collategene in CLI patients via "multicenter, double-blind, placebo-controlled" study [82]. Placebo or plasmid was injected on days 0 and 28, followed for 12 weeks. The overall improvement rate of the primary end point was 70.4% (19/27) in HGF group and 30.8% (4/13) in placebo group, with a significant value. In Rutherford 5 patients, HGF achieved a significantly higher improvement rate (100% [11/11]) than placebo (40% [2/5]). HGF-plasmid improved QOL. There were no major safety problems.

Such an effect of HGF-plasmid was also confirmed in a clinical trial in the U.S [71]. In this study, HGF-plasmid was injected in the ischemic muscles of CLI patients (*i.e.*, 0, 0.4 and 4 mg with 2 or 4 weeks interval). As a result, HGF-plasmid dose-dependently improved the hypoxia, as checked by oxygen tension. No significant adverse effects were seen among all groups.

6.2 VM202

Korean group newly constructed pCK-HGF-X7 (VM202), a naked cDNA plasmid designed to express two isoforms of HGF (HGF_{723aa} and HGF_{728aa}) under the control of human CMV promoter. Previous studies suggested the effectiveness of VM202 for treating CAD or PAD in animals [17, 73]. Based on this POC, some groups attempted to evaluate the potential of VM202 in clinical trials.

Phase-I clinical study of VM202 was performed in Seoul National University for evaluating its safety in patients with severe ischemic heart disease [35]. Intra-cardial transfer of VM202 (0.5 or 2.0 mg) was performed in a right coronary artery (RCA) territory following the coronary artery bypass grafting (CABG). No serious complications were seen throughout the 6-month follow-up period. This therapy improved global myocardial function, such as wall motion score. In the RCA region, there was a significant increase in the stress perfusion or wall thickness of the diastolic and systolic phases. Overall, intra-cardial injection of VM202 was shown to be promising during CAGG, with a tolerable dose of 2 mg.

The usefulness of VM202 was also evaluated in diabetic neuropathy through a double-blind, placebo-controlled study of HGF gene therapy in the US [32]. In this study, patients were randomized to receive 8 mg or 16 mg VM202 per leg (or placebo) via the intra-muscular injections on day 0 and 14. This naked plasmid therapy improved the pain scores at least for 3 months, without a significant adverse effect. This clinical study suggests that 2 days of treatment may be sufficient to provide symptomatic relief with improvement in QOL for several months.

Such a beneficial effect of VM202 was also confirmed in PADs (including CLI), by other studies, with a statistical significance in clinical score, in Korea or the U.S [16, 21, 33]. Another naked type of HGF-plasmid (*i.e.*, pUDK-HGF) may be available for symptomatic relief of CLI, especially of pain, tested in a small-sized clinical study in China [9].

6.3 Ad-HGF Vector

Adenovirus vector is one of the most popular vectors used in human gene therapy. Preclinical studies imply that an adenovirus vector carrying HGF-plasmid (Ad-HGF) produces therapeutic effects, including chronic MI in rats [43]. Chinese group developed the method of Ad-HGF for the induction of collateral arterial growth and improvement of post-infarct heart function in a pig model [100]. This group designed a phase-I clinical trial for evaluating safety and usefulness of Ad-HGF. The 18 patients were randomized to receive 3 doses of Ad-HGF (5×10^9 pfu, 1×10^{10} pfu and 2×10^{10} pfu), followed by an arterial transfer of Ad-HGF via a coronary catheter. No serious complication was seen up to 35 days in an acute phase and 11–14 months in follow-up. They next evaluated the effect of intra-cardial Ad-HGF transfer during CABG surgery as an open-labeled clinical trial [107]. Myocardial hypoxia in the Ad-HGF-injected area was improved in 3 cases of the low-dose group, 5 cases of the middle-dose group, and all of the high-dose group, hence suggesting a therapeutic potential of Ad-HGF during cardiac surgery such as CABG.

Clinical results of HGF are still hopeful, especially for the treatment of PAD or CAD. Indeed, there is now emerging evidence to show the promising results of HGF in various ischemic diseases, tested in humans (Table 6.4). Further studies are necessary for the establishment of an optimal condition for use of recombinant HGF (or HGF-plasmid) therapy in the treatment of PAD or CAD.

7 Summary and Perspective

HGF was, as its name indicated, originally identified as a potent mitogen for hepatocytes [64]. But now, HGF is an essential and sufficient regulator to elicit embryogenesis, organogenesis and tissue repair in numerous organs. The initial studies in the early 1990s delineated HGF as an angiogenic factor (see, Sect. 3). HGF is necessary for neovasculization through mitogenic, motogenic and morphogenic effects on ECs. A rapid activation of Rac1 by GEFs (such as Arf) is involved in HGF-mediated stabilization of endothelial barrier. HGF inhibits inflammatory actions via the repression of NF-κB-mediated cascade. However, persistent ischemia leads to HGF down-regulation via cAMP depletion, and then organ damage or dysfunction is further accelerated, due to a loss in the intrinsic repair mechanism. Notably, hypoxia up-regulates c-Met expression (*i.e.*, SOS sign), in reciprocal to a decrease in HGF. Thus, HGF supplemental therapy is reasonable to overcome the hypoxia-induced pathological status (Fig. 6.4).

Table 6.4 Clinical trials of HGF gene teraphy in patients with PAD, CAD or diabetic neuropathy

HGF gene, route, contry	Subjects	Therapeutic outcomes	References
1. PAD			
Naked HGF cDNA (Collategene), im (open-labeled, n = 6), im, Japan	ASO or Buerger disease	No edema, Reduced pain, Reduced ulcer Increase in ankle pressure index	Morishita et al. [59]
Naked HGF cDNA (Collategene), im (double-blind, placebo-controlled study n = 40), Japan	CLI	Improved QOL ($p = 0.014$, significant)	Shigematsu et al. [82]
Naked HGF cDNA (Collategene), im (HGF-STAT trial, n = 93), USA	CLI	Improved $TcPO_2$	Powell et al. [71]
Naked HGF cDNA (VM202), im (Phase-I, 2 mg vs. 16 mg, n = 12), USA	CLI	Improved ankle brachial index (12 months follow-up), Improved toe brachial index (12 months follow-up)	Henry et al. [21]
Naked HGF cDNA (VM202), im (Phase-I, 4, 8, 12, 16 mg, n = 21), China	CLI	Improved pain score, Increased $TcPO_2$, Improved wound healing (66.7%)	Gu et al. [16]
Naked HGF cDNA (pUDK-HGF), im (Phase-I, 4-16 mg, n = 21), China	CLI	No adverse effect, decreased pain score, Ulcer healing, improved $TcPO_2$	Cui et al. [9]
Naked HGF cDNA (VM202), im	CLI	Ulcer healing rate ($p < 0.005$, high dose vs. placebo)	Kibbe et al. [33]
(Phase-II, placebo, low, high, n=52), USA		Improved $TcPO_2$ ($p<0.05$, high dose vs. placebo)	
2. CAD			
Naked HGF cDNA (VM202), intra-myocardium during CABG (Phase-I, 0.5 and 2.0 mg), Korea	Chronic MI	Improved wall motion score ($p = 0.0084$, significant), Improved stress perfusion ($p = 0.024$, significant)	Kim et al. [35]
Ad-HGF, intra-myocardium during CABG (low, middle, high doses), China	Chronic MI	Improved hypoxia (dose-dependent) No significant adverse effect	Yuan et al. [107]
3. Diabetic neuropathy			
Naked HGF cDNA (VM202), im (placebo, 8 or 16 mg, double-blind), USA	Diabetic neuropathy	Improved pain score ($p = 0.03$, significant at 3 months)	Kesseler et al. [32]

Abbreviations: *ASO* arteriosclerosis obliterans, *TcPO2* trans-cutaneous oxygen tension. For other keys see text or table/figure legends

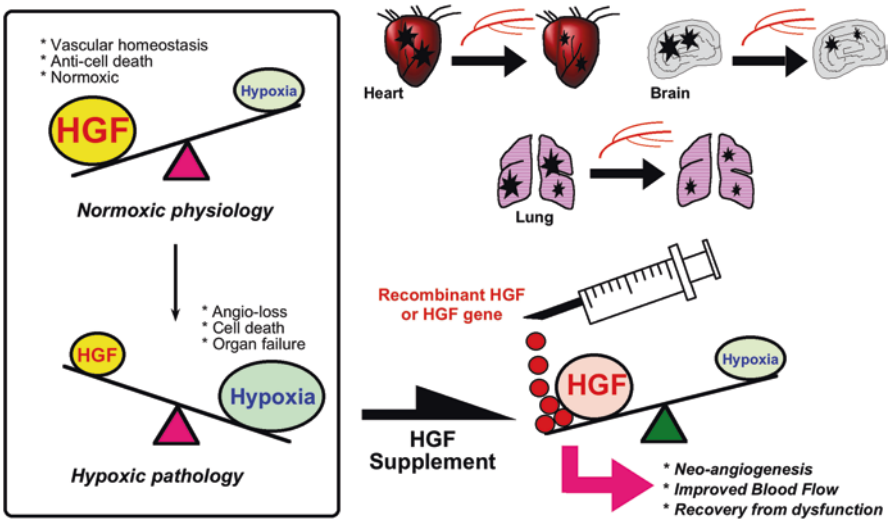

Fig. 6.4 Pathogenesis-based HGF therapy for curing ischemic diseases. A balance between HGF and hypoxia is involved in determining the prognosis of ischemic organ diseases, such as CAD, brain ischemia and so on. In the early stages of ischemic disorders, HGF production is transiently enhanced to suppress local hypoxia. When HGF dominates this aerobic balance, regenerative, protective and anti-fibrotic events occur as a compensatory response. However, local hypoxia is gradually enhanced during the advanced stage to prohibit HGF production, due to a decrease in cyclic AMP level. Under such a hypoxia-dominant condition, impairment in vascular network leads to a rapid progression of organ damage and/or dysfunction. To reverse the pathogenic balance, HGF supplementation therapy should be considered as a promising strategy for the induction of neo-angiogenesis, a common pathway leading to recovery from organ failure in numerous organs, such as heart, lung, liver, kidney and possibly the nervous system as well

How exogenous HGF is efficiently and specifically accumulated in injured areas? Some medical "devices" supports the efficient method of HGF gene transfection. Japanese group developed a unique method using a spring-powered Jet injector for this purpose [39]. Indeed, local gene expression ratio in the skin was 100-fold higher in Shima-Jet group than in non-Jet group, leading to a rapid angiogenesis and wound healing in animal models. Ultrasound-targeted micro-bubble technique enhanced the transfection of HGF-plasmid in the infracted hearts in rats, along with the enhanced angiogenesis [42]. A laser-induced stress wave-based gene transfer of HGF is also considered as a promising strategy for local transfection, as evidenced in a rat model of free-skin graft [1].

Tissue engineering also supports HGF-based regenerative therapy. Tabata and his coworkers in Kyoto University developed a slow release method using a biomaterial anchorage. For example, collagen microshere is useful as a sustained release carrier, which contributed to the HGF-enhanced angiogenesis in an animal model of PAD [49]. Drug-eluting stent (DES) is used as a slow release carrier of drug to prevent *in*-stent restenosis of coronary arteries post-angioplasty. HGF-treated DES may be available for reducing *in*-stent neointima formation [24], possibly via a rapid restoration of endothelial integrity.

Now, several lines of clinical trials are ongoing worldwide to determine an optimal condition of HGF therapy for curing PAD or CAD. A naked HGF-plasmid (such as Collategen or VM202) is one of the most practical drugs for an angiogenic therapy, because of its simple preparation. Some medical devices or biomaterials facilitate the efficient production or retention of HGF in the injured areas under organ ischemia. HGF is a physiological ligand to drive an intrinsic repair system under an aerobic condition. Thus, HGF supplemental therapy may provide a new avenue for the development of self-repair therapy, because angiogenesis is a common event for producing aerobic condition. Therapeutic angiogenesis will be a key card for future development of cytokine-based regenerative medicine.

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Chapter 7
Functions of MicroRNAs in Angiogenesis

Xiao Li, Yuqiao Chang, Zufeng Ding, Zhikun Guo, Jawahar L. Mehta, and Xianwei Wang

Abstract Angiogenesis is defined as formation and growth of new blood vessels that sprout from existing vascular network. Angiogenesis plays a very important role in the physiological and pathological situations such as development, ischemia, atherosclerosis, wound healing, and cancer growth and metastasis. MicroRNAs (miRNAs or miRs) are endogenous, short, noncoding RNAs found in eukaryotic cells. MiRs are major posttranscriptional regulators that negatively regulate gene expression by binding to their target messenger RNAs for degradation and/or translational repression. The main function of miRs is gene regulation. MiRs have been found to modulate many pathophysiological process including cell differentiation, contraction, migration, proliferation, apoptosis, and tissue inflammation. There are more than 1, 000 miRs in human genome, some of them are involved in angiogenesis. In this review, we will summarize the recent progress on function of miRs in angiogenesis.

Keywords MicroRNAs • Angiogenesis • Endothelial cells • Vascular endothelial growth factor

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Abbreviations

Ang	Angiotensin
bFGF	Basic fibroblast growth factor
CTGF	Connective tissue growth factor
ECs	Endothelial cells
EGF	Endothelial growth factor
EGFL7	Epidermal growth factor-like domain 7
eNOS	Endothelial nitric oxide synthase
FLT1	FMS-related tyrosine kinase 1
Fus-1	tumor suppression candidate 2
HGS	Hepatocyte growth factor-regulated tyrosine kinase substrate
HIF	Hypoxia-inducible factor
IFN-γ	Interferon γ
IGF-1	Insulin-like growth factor 1
IRS1	Insulin receptor substrate 1
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MMPs	Matrix metalloproteinases
MRE	MicroRNA responsive element
PDGF	Platelet-derived growth factor
PE	preeclamptic
PGF	Placental growth factor
PIK3R2	Phosphoinositol-3 kinase regulatory subunit 2
PTEN	Phosphatase and tensin homolog
RCC	Renal cell carcinoma
RL	Renilla luciferase
ROS	Reactive oxygen species
SCF	Stem cell factor
SGA	Small-for-gestational-age
Shh	Sonic hedgehog
SPRED1	Sprouty-related peotein
Sufu	Suppressor of fused
TGF-β1	Transforming growth factor β1
TIMP	Tissue inhibitor of metalloproteinase
VEGF	Vascular endothelial growth factor

1 Introduction

Angiogenesis is defined as the formation of new blood vessels that sprout from existing vascular network. It is a complex process that involves differentiation, proliferation, migration, and maturation of endothelial cells (ECs) [1]. Angiogenesis is

an important phenomenon not only in physiological situations but also in pathological conditions like myocardial, cerebrovascular, renal, and limb ischemia [2]. As described elsewhere in this book, angiogenesis plays a central role in the development of certain cancers and cancer metastasis. Basic fibroblast growth factor (bFGF) was the first growth factor identified to play an important role in angiogenesis, followed by vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), tyrosine kinase receptors Tie-1 and Tie-2, and the Tie-2 angiopoietin ligands [3, 4].

Endothelial migration and proliferation enhance the generation of primary capillaries that undergo remodeling by sprouting, branching, or intussusception [5]. There is increasing evidence to support the concept that angiogenesis participates in the progression of atherosclerotic plaque [6]. Enhanced angiogenesis is a major cause of tumor growth and progression defining an angiogenic switch [7]. The development of plaque angiogenesis in the process of atherosclerosis is regulated by multiple signals such as hypoxia, reactive oxygen species (ROS), and inflammation, which are also closely associated with the development of certain cancers [2, 4].

MicroRNAs (miRNAs or miRs) are endogenous, small, noncoding RNAs that negatively regulate gene expression by binding to their target messenger RNAs for degradation and/or translational repression [8]. miRs are critical modulators for vascular functions such as cell differentiation, contraction, migration, proliferation, and apoptosis [9]. MiRs have been associated with inflammation, oxidative stress, and angiogenesis. Several miRs are involved in vascular function, and some of them, such as miR-15, -16, -17, -21, 27a, -92, -296, -130a, -378, -210, -214, -221/-222, -467, -195/497, -424/503, Let7f, and -126, have been identified to participate in angiogenesis.

2 MiR-16 Family and Angiogenesis

Accumulating evidence indicates that miR-16 family has a close relationship with angiogenesis in physiological and pathophysiological conditions. Members of this family include miR-15a/b, miR-16, miR-195, miR-424 and miR-497. miR-103, miR-107, and miR-646 have also been included into the “extended” miR-16 family [1].

2.1 MiR-15 and miR-16 and Angiogenesis

MiR-15 and miR-16 were firstly reported to inhibit VEGF expression in human carcinoma cell line [2]. Both of miRs belong to the miR-16 family. Several studies profiling miRNA expression in ECs demonstrated that these two miRs are expressed at high levels, indicating their potential importance in angiogenesis [3]. Multiple computational programs have predicted that potential regulation of VEGF by

miR-16 through a miR responsive element located ~260 bases downstream of the translation stop in the VEGF 3′-UTR. Karaa et al. verified this regulation by using VEGF expression together with miR-16 in Hela cells, which validated the targeting of miR-16 to a predicted binding site in the VEGF 3′UTR [2].

In hypoxic conditions, miR-15b and miR-16b display anti-angiogenic activities through regulating the expression of hypoxia-inducible factor-1α (HIF-1α), Ang2, VEGF-A, VEGF kinase insert domain receptor and FGF-R1 in vitro [4]. Overexpression of miR-15/-16 decreases hypoxia-induced VEGF expression, while knockdown of them increases VEGF expression in ECs [5]. A further study shows that miR-15/-16 also regulates tumor angiogenesis, which is involved in hypoxia and the expression HIF-2α [2]. Increase of HIF-2α stabilizes c-Myc/Max heterodimer that represses the transcription of miR-15/-16 in hypoxic condition, and then promotes tumor angiogenesis and hematogenous metastasis of colorectal carcinoma [2]. It has also been shown that the enhanced miR-16 expression decreases cell growth and proliferation and induces apoptosis through reduction cyclin D1 and BCL2 levels in MCF-7 cell line [6]. Chen et al. [7] demonstrated that miR-16 is down-regulated in human glioblastoma samples in contrast to the normal brain tissues. Additionally, overexpression of miR-16 in the A172 and U87 glioblastoma cell lines represses the function of co-cultured ECs (including proliferation, migration, extension, tube formation) and angiogenesis by targeting Bmi-1.

Ischemic diseases are always characterized by hypoxia. It has been shown that miR-15/-16 cluster regulates angiogenesis in ischemic tissues. For example, Spinetti et al. showed that the circulating miR-15a and -16 were marked increased in the serum of critical limb ischemia (CLI) patients with and without T2DM in a clinical study [8]. The increase of circulating miR-15a was positively associated with increased risk of adverse events in type 2 diabetes mellitus (T2DM) patients [8]. The expression of miR-15a and miR-16 regulated functions of proangiogenic cells and further promoted the formation of new blood vessels [8]. In an animal study, anti-miR-15a/-16 treatment improved post-ischemic blood flow recovery and muscular arteriole density in an immune-deficient mouse model [8]. This findings indicate that the circulating miR-15a and miR-16 may serve as a prognostic biomarker in CLI patients undergoing revascularization.

2.2 MiR-195/-497 and Angiogenesis

A recent study shows that overexpression of miR-195 and miR-497 reduces proliferation of human primary mesenchymal stromal/stem cells (MSCs) [9]. Conditioned medium from MSCs overexpressed miR-195 or miR-497 and markedly decreased VEGF expression and reduced the formation of endothelial vessels in chicken embryo eggs [9]. Of note, the inhibitory effect of miR-195 on angiogenesis was much greater than miR-497 [9].

Expression of miR-195 was greatly increased in human endothelial progenitor cells (hEPCs), and inhibition of miR-195 expression could promote cell proliferation, autophagy, migration and angiogenesis of hEPCs under hypoxic conditions [10].

MiR-195 was also found to inhibit breast cancer growth and angiogenesis through inversely modulating the expression of insulin receptor substrate 1 (IRS1) and suppressing the function of IRS1-VEGF pathway [11].

MiR-497 expression was low in human ovarian cancer tissues, which may result the increase of angiogenesis [12]. A further study disclosed that miR-497 exerted its function of anti-angiogenesis by suppressing VEGFA expression and, in turn, impairing the VEGFR2-mediated PI3K/AKT and MAPK/ERK pathways. MiR-497 was found to be downregulated in non-small cell lung cancer, and its ectopic expression significantly inhibited tumor growth and angiogenesis [13]. Wang et al. clarified the role of miR-497 in ovarian cancer angiogenesis by using in vitro assays and clinical ovarian cancer tissues. They found that downregulation of miR-497 in ovarian cancer tissues was associated with the increased angiogenesis [12].

2.3 MiR-15/-107 Group and Angiogenesis

The miR-15/-107 group regulates gene expression involved in cell division, proliferation, metabolism, stress response, and angiogenesis [14]. The miR-15/-107 group has also been implicated in human cancers, cardiovascular diseases, and neurodegenerative diseases. Yamakuchi et al. [15] suggested that miR-107 can mediate p53 regulation of hypoxic signaling and tumor angiogenesis by targeting HIF-1β. A study by Finnerty et al. [16] provides insights into upstream regulation of miR-107 expression through specific p53-responsive promoter regions. It was shown that overexpression of miR-107 inhibited the vascular density of tumor xenografts in vivo. Further, upregulation of miR-107 in glioma cells leads to an inhibition of human brain microvascular endothelial cell proliferation, migration, and vascular tube formation via downregulation of VEGF expression in the in vitro co-culture conditions [17]. Karaa et al. [2] reported that miR-16 strongly regulated VEGF expression in the process of the formation of new blood vessels. In addition to miR-15/-107, miR-17-92 cluster, miR-210, miR-221/-222, miR-126 and -130a have also been shown to participate in blood vessel development in normal and/or tumor tissues [18, 19] (Fig. 7.1).

3 MiR-17-92 Custer and Angiogenesis

MiRs are frequently transcribed together as polycistronic primary transcripts that are processed into multiple individual mature miRs in animals. The genomic organization of these miR clusters is often highly conserved, indicating an important role for coordinated regulation and function. As a polycistronic miR gene in human genome, the miR-17-92 cluster encodes six miRs, including miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1, which are tightly grouped within an 800 base-pair region in human chromosome 13 [21, 22]. Both sequences of these mature miRs and their organization are highly conserved in all vertebrates. For example, MiR-17, miR-20a, and miR-20b have overlapping function via targeting

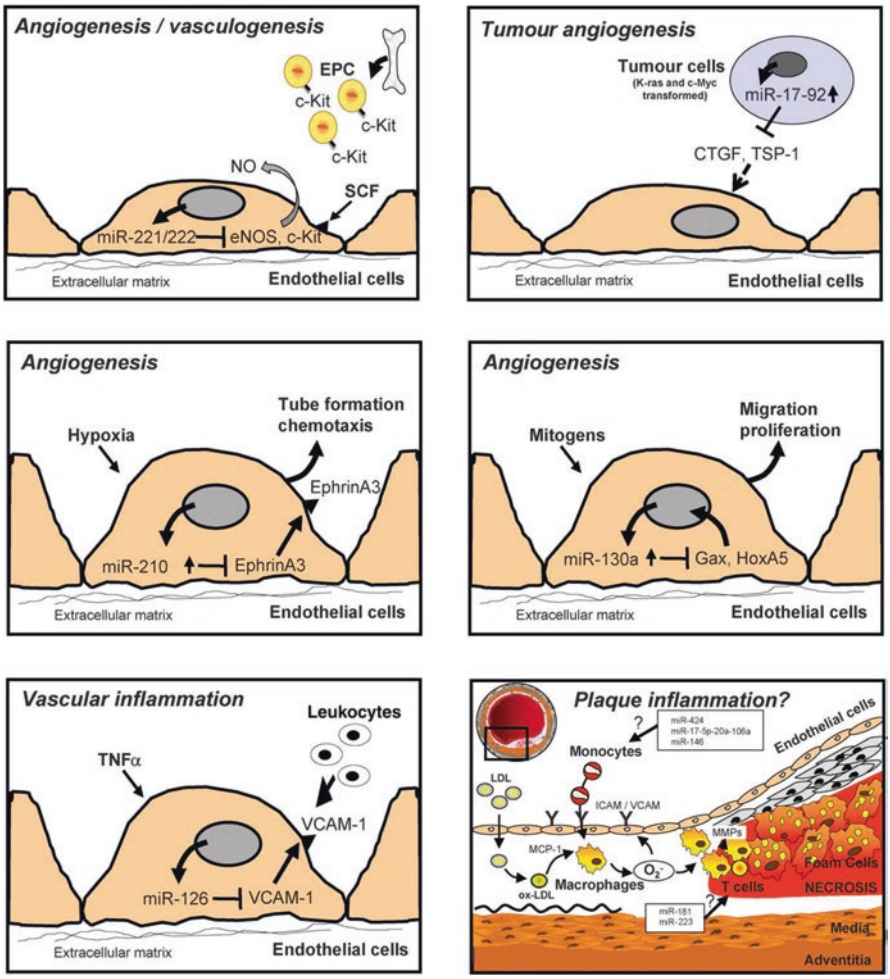

Fig. 7.1 Role of miRNAs for vascular biology. Schematic illustration of the specific functions of miR-221/miR-222, miR-17-92, miR-210, miR130a, and miR-126 for vascular biology (Urbich et al. [20])

similar sets of genes, including interleukin 8 (IL-8), HIF-1α, VEGF-A, ephring-B2, tissue inhibitor of metalloproteinases 2, matrix metallopeptidase 2 (MMP2), and Eph receptor B4 [23].

MiRs encoded by the miR-17-92 cluster and its paralogs are known to act as oncogenes. Expression of these miRs promotes cell proliferation, suppresses apoptosis of cancer cells, and induces tumor angiogenesis [24]. C13orf25 gene expression in association with genomic amplification and may play an important role in tumorigenesis and resulting poor prognosis [25]. The highly conserved human miR-17-92 cluster is located in the third intron of an approximately 7 kb primary transcript known as C13orf25 [26]. It evolved two miR-17-92 cluster paralogs in mammals

due to ancient gene duplications: (1) the miR-106b-25 cluster that is located within the thirteenth intron of the protein-coding gene MCM7 (chromosome 7) and (2) the miR-106a-363 cluster that is located on the X chromosome. MiR-17-92 and miR-106b-25 clusters are both abundantly expressed across many tissues and cell types. Of note, the miR-106a-363 cluster is undetectable or expressed at trace levels in all settings that have been examined [27].

The miR-17-92 cluster first attracted attention following a series of observations linking these miRs to cancer pathogenesis [24]. Overexpression of miR-17-92 in Ras expressing murine carcinoma cells resulted in enhanced tumor angiogenesis in vivo in a non-cell autonomous manner. The downregulation of the potent endogenous inhbitor of angiogenesis thrombospondin-1 together with several proteins containing thrombospondin type 1 repeats has been shown to be involved in this pathway [28]. Transfection of ECs with components of the miR-17-92 cluster, induced by VEGF treatment, rescued the induced expression of thrombospondin-1 and the defect in endothelial cell proliferation and morphogenesis initiated by the loss of Dicer [29]. Inhibition of miR-17 and miR-20a increased the number of blood vessels in Matrigel plugs, but antagomiRs that target miR-18a and miR-17-92 were less effective [29]. The aforementioned study support that miR-17-92 promotes tumor angiogenesis by targeting antiangiogenic proteins thrombospondin-1 and connective tissue growth factor (CTGF), therefore regulating angiogenesis in a non-cell-autonomous manner [30] (Fig. 7.2).

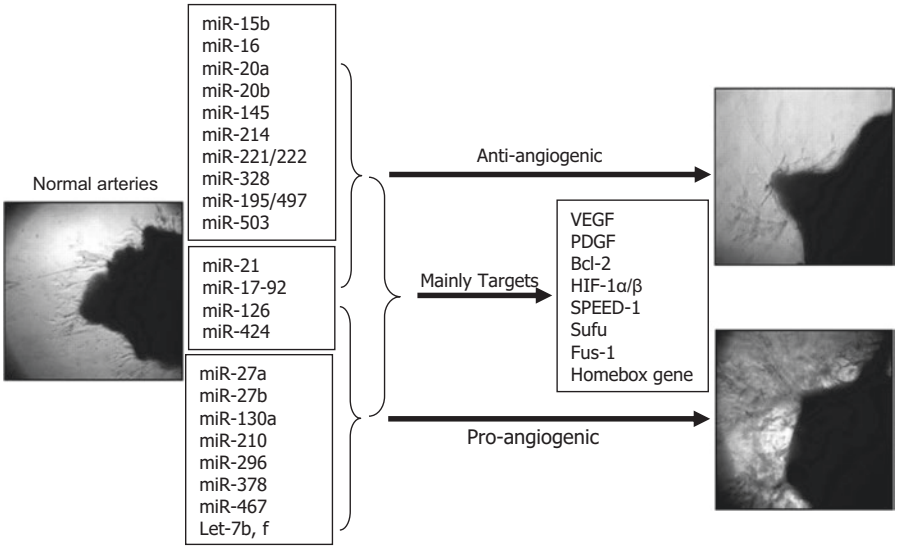

Fig. 7.2 Regulation of angiogenesis by antiangiogenic miRNAs (miR-15b, miR-16, miR-20a, miR-20b, miR-145, miR-214, miR-221/-222, miR-328, miR-195/-497, miR-503), dual-directional miRNAs (miR-21, miR-17-92, miR-126, miR-424) and pro-angiogenic miRNAs (miR-27a, miR-27b, miR-126/-126*, miR-130a, miR-210, miR-296, miR-378, miR-467, Let-7b, f). MiRNAs regulate the angiogenic responses to growth factors by targeting angiogenic factors, receptors, and signaling molecules

Bioinformatic binding prediction tools suggest that the transcriptional activator ELK-1 (member of ETS oncogene family) potentially binds to the miR-17-92 cluster promoter sequence, which is positively regulated by mitogen-activated protein kinase (MAPK) [31]. Chamorro-Jorganes, Lee et al. [32] demonstrated that VEGF-induced upregulation of miR-17-92 cluster in ECs is mediated by ERK/ELK1 activation suggesting that ELK-1 and the miR-17-92 cluster are the targets of MAPK. Recent studies also indicate that the upregulation of miR-17-92 cluster in vitro is necessary for endothelial cell proliferation and angiogenic sprouting. Additionally, genetic evidence indicates that miR-17-92 iEC-KO mice have blunted physiological retinal angiogenesis during development and diminished VEGF-induced ear angiogenesis and tumor angiogenesis via upregulation of THBS1 [32]. Shuang et al. [33] reported that miR-17a and miR-92-1 play crucial roles in argonaute 2-mediated angiogenesis by targeting angiogenesis-related gene in myeloma angiogenesis.

The miR-17-92 cluster is now considered one of the oncogenes. Whereas, Huabin Ma [34] demonstrated that miR-17-92 suppressed tumor progression in colorectal cancer mouse model by inhibiting multiple angiogenesis inducing genes, including TGF-β type II receptor, HIF-1α, and VEGF-A. It has also been reported that miR-17 and miR-19 negatively regulate the expression of pro-angiogenic Janus kinase 1 (JNK-1) and cyclin D1 [35]. Hinkel et al. [36] observed that inhibition the expression of miR-92a exerts cell-protective, pro-angiogenic, and anti-inflammatory effects. Downregulated miR-92a can significantly reduce infarct size and postischemic loss of function in pigs. According to above views, miR-17-92 cluster has a dual role in tumor development.

Landskroner-Eiger et al. [37] demonstrated that miR-17-92 EC KO mice exhibit accelerated blood flow recovery and enhanced arterial vessel density after limb ischemia. MiR-17-92 EC KO mice also have greater numbers of the hindlimb and coronary arterial vessels in the absence of ischemia, suggesting that the endogenous miR-17-92 cluster plays a critical role in developmental arteriogenesis/collateral vessel genesis. More importantly, miR-19a/b plays a central role in this process. Frizzled-4 (FZD4) and low-density lipoprotein receptor-related protein-6 (LRP6) are main targets of miR-19a/b [37]. The expression of miR-19a negatively regulates FZD4, LRP6 and WNT signaling, and antagonism of miR-19a/b can improve blood flow recovery after ischemia through reducing the repression of WNT signaling by targeting FZD4 and LRP6 in the aged mice, which is similar with that in the miR-17-92 EC-specific KO mice. These findings suggest that the miR-17-92 cluster, especially miR-19a/b, physiologically suppresses arteriogenesis.

4 MiR-126/miR-126* and Angiogenesis

Recently, a protein named epidermal growth factor-like doman 7 (EGFL7, also known as VE-statin, MEGF7, Notch4-like protein, or Zneu1) was described as novel endothelial cell-derived factor that is involved in the regulation of the spatial arrangement of cells during vascular tube assembly or blood vessel formation [38].

This protein is conserved among vertebrates but an orthologue is also found in *Drosphila melanogaster* [39]. In humans, there are three alternative isoforms containing the same open reading frame but are transcribed from separate promoters [40]. Gene analysis revealed that EGFL7 is expressed within the neurons of adult mice, indicating that EGFL7 serves diverse biological functions in various tissues and not only in the vascular system [41]. Soncin et al. [42] showed that EGFL7 inhibits human aortic smooth muscle cells' migration, but not proliferation, indicating that EGFL7 might have a role in new vessel maturation, parker et al. [43] established the role of EGFL7 as an important tubulogenic factor in the process of vasculogenesis. Recently, schmidt et al. provided another compelling clue for resolving the function of EFGL7 protein in angiogensis [44].

MiR-126 (also referred to as miR-126-3p) and its complement miR-126* (miR-126-5p or miR-123) are derived from EGFL7 gene and harbor both miRNAs within intron 7 in all vertebrates [45]. Both miR-126 and miR-126* are relevant for the development of the cardiovascular system, cardiovascular diseases, and the formation of certain cancers [46]. MiR-126 performs an abundant expressed level in highly vascularized tissues, and it is the only miRNA known to be expressed specifically in the endothelial lineage and hematopoietic progenitor cells [47].

Function of miR-126/miR-126* in angiogenesis, though it remained an enigma for quite some time, has now been clarified. MiR-126 seems to regulate endothelial cell angiogenic activity in response to angiogenic growth factors such as VEGF and bFGF, through targeting multiple proteins that modulate angiogenesis and vascular integrity [46]. Fish et al. [48] found that miR-126 regulates the response of ECs to VEGF. In addition, knockdown of miR-126 in zebrafish resulted in loss of vascular integrity and hemorrhage during embryonic development. MiR-126 functioned in part by directly repessing negetive regulators of the VEGF pathway, including the Sprouty-related, EVH1 domain-containing protein 1 (SPRED1), and phosphoinositol-3 kinase regulatory subunit 2 (PIK3R2). SPRED1 contains a predicted target sequence for miR-126, and it plays a key role in miR-126 mediated pro-angiogenic action [49]. Increased expression of SPRED1 or inhibition of VEGF signaling in zebrafish resulted in defects similar to miR-126 knockdown [45]. Both MiR-126 overexpression and PIK3R2 downregulation in endothelial progenitor cells led to similar pro-angiogenic functions [50]. In addition, ultrasound-mediated miR-126-3p delivery in chronic ischemic hindlimb muscles of the rats demonstrated that miR-126 improved tissue perfusion and vascular density by inhibiting SPRED1 and PIK3R2 and upregulating VEGF and angiopoietin-1 signaling [51].

MiR-126 promotes proliferation, migration, and angiogenic capacity of umbilical endothelial progenitor cells. In the pregnant rats, it was found that miR-126 increased vascular sprouting, placenta and fetus weights [50]. A positive correlation was found between miR-126 and VEGF expressions [52]. MiR-126 overexpression significantly upregulated VEGF expression in BeWo cells, whereas miR-126 downregulation decreased VEGF expression [53]. Similarly, deletion of miR-126 (miR-$126^{-/-}$) in the mice leads to the formation of fragile and leaky vessels, lumen collapse, aberrant endothelial tube hierarchy, hemorrhages, and impaired endothelial cell

proliferation and migration. The above information indicates a crucial role of miR-126 in embryonic and postnatal angiogenesis, post-traumatic vascular regeneration, and endothelial function.

Studies by Parker et al. [43] showed that loss of EGFL7 function in zebrafish embryos specifically blocks vascular tubulogenesis. EGFL7 is downregulated in quiescent ECs, but is upregulated in the endothelium of proliferating tissue, such as some tumors. Angiogenesis and vascular integrity can be disrupted through modulation of miR-126 expression. Since miR-126 is embedded within EGFL7, it is possible that miR-126 increases the sensitivity of these activated ECs to VEGF or other growth factors through repression of SPRED1 and/or PIK3R2 expression. Sessa et al. [54] reported that miR-126-dependent suppression of PIK3R2 increased the expression of pro-angiogenic factor angiopoietin-1, whose function is to facilitate stabilization and maturation of growing blood vessels. Work by nicoli et al. [55] demonstrated that mediated by the mechanosensitive zinc finger-containing transcription factor klf2a, miR-126 can be induced by blood flow, leading to the activation of VEGF signaling in the endothelium. Due to damage of vascular integrity and defects in endothelial cell proliferation, migration, and angiogenesis, endothelial-specific deletion of miR-126 in mice causes leaky vessels, hemorrhage, and partial embryonic lethality. Subsequent miR-126 knockdown studies in zebrafish showed that miR-126 induced hemorrhage and collapse of lumen-containing vascular structures [56]. These observations are consistent with the concept of miR-126 binding to the 3′UTR of the VEGF-A mRNA [57]. Considering the key role of miR-126 in the regulation of angiogenesis and vascular integrity, it has been proposed that miR-126 may be an important target for pro- or antiangiogenic therapies.

MiR-126 plays a crucial role in tissue repair by inducing angiogenesis and vascular tissue remodeling in the injured blood vessels. Jansen et al. [58] reported that miR-126 was transported into recipient cells by endothelial microparticles, functionally regulated the target protein SPRED1, and promoted vascular endothelial repair. It has also been reported that miR-126 maintains silencing in adult ECs and represses endothelial proliferation and migration. However, in the injured cardiac muscles, the regenerative activity of miR-126 initiates vascular repair and results in the induction of anti-apoptotic and cardioprotective effects [59, 60]. Furthermore, ECs and EPCs could also release miR-126-containing microparticles that can be taken up by VSMCs and cardiac muscle cells, and lead to miR-126-driven resident cells reprogramming to a regenerative program [58]. MiR-126 can also promote the migration of EPCs by targeting the regulator of Gprotein signaling 16 (RGS16), which is an inhibitor of the CXCL12/CXCR4 signaling [61]. It has been reported that the CXCL12/CXCR4 axis and hypoxic gradients induces EPC migration and promotes VEGF production by lymphoid precursors. MiR-126 also involves in the regulation of CXCL12/CXCR4 pathway by repressing the axis in quiescent ECs and HIF-1α-dependent activation of this pathway in hypoxic and vascular injury conditions [62].

However, several studies reported a controversial role of miR-126 in tumor progression. Evidence shows that miR-126 can suppress tumor growth and tumor angiogenesis through inhibition of VEGF signaling. The interaction of miR-126 on

the 3′UTR of VEGF mRNA in colorectal cancer and gastric cancer cells was validated by luciferase reporter assay [63, 64]. Zhang et al. [63] reported that miR-126 was markedly downregulated in the colorectal cancers, and the silence of miR-126 was induced by the promoter methylation of its host gene, EGFL7. Chen Li. et al. [64] reported that low expression of the miR-126 was observed in gastric carcinoma tissues, and the enhanced miR-126 expression obviously suppressed the expression of VEGF-A and the activity of the downstream signals such as Akt, mTOR and Erk1/2 in gastric cancer cell lines SGC-7901, MKN-28 and MKN-45. In contrast, the decreased expression of miR-126 increased the expressions of VEGF-A and its downstream signals [64]. Du et al. [65] found that the expression of miR-126-3p were significantly downregulated in the hepatocellular carcinoma tissues and cells. The expression of miR-126-3p inhibits cell migration and invasion, and suppresses the formation of capillary tubes from ECs in vitro. Overexpression of miR-126-3p reduces the size of tumor and the density of microvessels in vivo. It was also found that the under-expression of miR-126 was significantly correlated with the expression of VEGF in thyroid cancers tissues [61]. MiR-126 marginally expressed in thyroid cancer tissues and cell lines. Overexpression of miR-126 in human papillary thyroid carcinoma (K1 cells) and human normal thyroid follicular cell line could markedly reduce VEGF-A level and inhibit cell proliferation of these cells [66]. Zhou et al. [67] also revealed that miR-126 has dual function in pathological angiogenesis of retina. MiR-126$^{-/-}$ mice showed defective postnatal retinal vascular development and remodeling. However, in retinal pigment epithelial cells, miR-126-3p suppressed VEGF-A function via a novel mechanism by regulating αB-Crystallin promoter activity and by directly targeting VEGF-A 3′UTR.

5 MiR-221/-222 and Angiogenesis

MiR-221 and miR-222 belong to the same family and control common targets, which are located in close proximity on Xp11.3 chromosome and might be regulated in a coordinated manner [68]. MiR-221/-222 are highly expressed in human umbilical vein endothelial cells and are known to regulate the angiogenesis. These miRs inhibit endothelial cell migration, proliferation, and angiogenesis in vitro by targeting stem cell factor (SCF) receptor, c-kit (a receptor tyrosine kinase that binds stem cell factor and mediates VEGF expression) [69]. Antisense miR-221 oligonucleotide was shown to reduce the expression of miR-221, to restore c-kit expression in HUVECs, and to abolish the inhibitory effect of high glucose on HUVECs transmigration [70]. Interestingly, Li et al. observed that miR-221 expression was induced by high glucose while c-kit expression was reduced, indicating that miR-221-c-kit pathway may play an important role in diabetes-associated vascular dysfunction [71].

Recently, miR-221/-222 have been shown to be deregulated in gliomas, which is involved in a variety of biological processes in glioma cells such as cell proliferation, apoptosis, migration and cell cycle progression. Fan et al. found that miR-221/-222

were significantly upregulated in human glioma tissues and cell lines, and played vital roles in glioma cell invasion, migration, and angiogenesis by directly targeting TIMP2 [72]. On the other hand, TIMP-2 could interact with integrin α3β1, which negatively regulates some tyrosine kinase receptor signal transduction pathways leading to cell cycle arrest of ECs and suppressing angiogenesis [73, 74]. These studies indicate that suppression of miR-221/-222 is a potential therapeutic strategy for treatments glioma from the view of angiogenesis in the future.

More recent studies have also shown that these two miRs control different target genes: miR-222 is a main regulator for inflammatory signals including IL-3 or bFGF and participates in the inflammation-mediated vascular remodeling [75]. However, miR-221 is involved in proliferative signals, and controls EC proliferation, migration, and angiogenesis [76]. Similarly, miR-221, but not miR-222, promoted proliferation of HCC cell lines and tumor growth in a recent study on liver tumorigenesis [77]. These evidences indicate that miR-221 is the key factor for cell growth and angiogenesis in the miR-221/-222 cluster.

6 MiR-378 and Angiogenesis

MiR-378 is highly expressed in $CD34^+$ hematopoietic progenitor cells, which function as an oncogene by enhancing angiogenesis, tumor cell survival, and tumor growth. By inhibiting the expression of two tumor suppressors Sufu (suppressor of fused) and Fus-1 (tumor suppression candidate 2), miR-378 reduces caspase-3 activity and enhances cell survival, tumor growth and angiogenesis [78]. Cell survival assays showed that transfection with a construct expressing an antisense sequence against miR-378, cell survival decreased significantly [78]. Sonic hedgehog (Shh) is a prototypical morphogen known to regulate epithelial/mesenchymal interactions during embryonic development. Sufu functions as a negative regulator of Shh signaling. Shh promotes large-diameter vessel formation by inducing expression of angiogenic cytokines, including VEGF and angiopoietin-1 and -2 [79]. MiR-378 can also repress the effect of Sufu in the present of the 3′-UTR, which promotes cell survival, confirming that the Sufu 3′-UTR is a target of miR-378 [80]. These findings suggest that miRNA-378 promotes cell survival and regulates tumor angiogenesis through regulating Sufu and Fus-1.

Lee et al. revealed the role of miR-378a in tumorigenesis, tumor growth, and tumor vascularization in glioblastoma for the first time [81]. They indicated that miR-378a-5p enhanced cell survival, reduced caspase-3 activity, and promoted tumor growth and angiogenesis through repression of Sufu and Fus-1 too. MiR-378 has been shown to affect VEGF-A in two ways: miR-378 directly affects VEGF-A by competing with hsa-miR-125a for the same seed-region in the VEGF-A 3′UTR causing upregulation of VEGF-A [82]; however, miR-378a-5p indirectly regulates VEGF-A affecting Shh signaling via inhibition of Sufu that is a key inhibitory component of this signaling pathway [81]. The Shh pathway in turn positively regulates VEGF-A and other regulators for the formation of new blood vessels and expression

of Ang-1and Ang-2 [83–85]. A recent study shows that miR-378a also participates in wound healing [86]. The anti-miR-378a transgenic mice show an enhanced healing as compared to WT mice following 1-week wounding [81]. MiR-Pirate378a targets integrin beta-3 and vimentin that stimulate of VEGF expression and promote EC migration and angiogenesis.

However, no studies have been performed on the angiogenic effects of miR-378a in the physiological settings or disorders such as diabetes and myocardial infarction where angiogenesis plays important roles. So, it is necessary to perform further studies to assess the mechanisms of miR-378a functions in blood vessel formation in physiological or pathophysiological conditions.

7 MiR-21 and Angiogenesis

MiR-21 is one of the firstly identified microRNAs in mammalian. Accumulating evidence indicates that miR-21 can negatively modulate angiogenesis. Plenty of studies have shown that miR-21 is highly expressed in ECs, and ERK1/2 and bFGF are the key regulators for miR-21 expression [87]. Overexpression of miR-21 in HUVECs inhibits angiogenesis including EC proliferation, migration and vascular network formation in vitro, whereas silencing endogenous miR-21 expression in ECs increases the migration of ECs about 40%, and increases the tube formation numbers and the total tube length by 2.3 and 1.9 fold, respectively [87]. The inhibitory effect of miR-21 is due to its inhibitory role in RhoB expression and cytoskeleton organization. Silencing miR-21 leads to marked increases of RhoB expression and EC migration, and silencing RhoB leads to a reduced migration of ECs [87]. Intravitreal injection also inhibits pathological angiogenesis and wound healing in a laser-induced mouse model of choroidal neovascularization [87].

However, many other studies show that miR-21 is a potential pro-angiogenic factor in some biological systems. For example, a recent study by Hu et al. shows that the increase of miR-21 promotes survival, migration and tube formation of ECs, and inhibition of miR-21 expression by antagomir exerts an opposite effect [88]. The tissue inhibitor of metalloproteinases-3 (TIMP3) is the functional target gene of miR-21 in regulating angiogenesis. MiR-21 by targeting TIMP3 regulates expression of MMP2 and MMP9, and further affects the formation of new blood vessels [88]. The animal study shows that miR-21-deficient mice display an impaired post-ischemic angiogenesis [89]. It is also shown that miR-21 mainly plays its biological activity in adult angiogenesis-promoting cells [89].

MiR-21 is observed to be upregulated in various cancers, and miR-21 can stimulate invasion and metastasis in cancer [90]. MiR-21 inhibits phosphatase and tensin homolog (PTEN) or RhoB, leading to pro-/anti-angiogenesis [91]. In cancer, miR-21 promotes tumorigenesis through its regulation of cellular ROS levels inhibited the metabolism of superoxide to hydrogen peroxide, produced either by endogenous basal activities triggers the generation of ROS [91]. MiR-21 inhibited the metabolism of $O_2^{\bullet -}$ to H_2O_2, by directing attenuating SOD3 or by an indirect mechanism

that limited TNFα production, thereby reducing SOD2 levels. Therefore, miR-21-induced tumorigenesis is partially due to the high $O_2 \bullet^-$ level generated in the cells. MiR-21 has also been reported to induce tumor angiogenesis through targeting PTEN in human prostatic cancer cells, leading to activating AKT and ERK1/2 signaling pathways, and thereby enhance HIF-1α and VEGF expression [92]. HIF-1α is a key downstream target of miR-21 in regulating tumor angiogenesis.

Accordingly, it is generally believed that miR-21 is highly expressed in ECs, but its role in angiogenesis remains controversial in in vitro study on ECs and in in vivo study on cancers.

8 MiR-210 and Angiogenesis

MiR-210, a hypoxia-induced miRNA, is a crucial element of endthelial cell response to hypoxia, affecting cell survival, migration, and differentiation [93]. In normoxic conditions, overexpression of miR-210 in ECs was shown to stimulate VEGF-induced cell migration and formation of capillary-like structures (angiogenesis). Conversely, blockade of miR-210 by transfection of anti-miRNA inhibited tube formation stimulated by hypoxia and cell migration in response to VEGF [94]. It has also been reported that miR-210 regulates renal angiogenesis under ischemia/perfusion conditions through activating VEGF signaling pathway in vivo and in vitro [94, 95]. This information shows that miR-210 probably retains the integrity vascular vessels by enhancing angiogenesis, providing a new target for modulating vascular formation.

In hypoxic HCC [96] and CRC cells [97], vacuole membrane protein 1 was identified as the direct and functional downstream target of miR-210, which mediated cancer cell migration and invasion supported by angiogenesis. Meanwhile, miR-210 contained in exosomes released by cancer cells could be transported to ECs to induce angiogenesis. In addition, miR-210 is also located in mitochondria and specifically targets the mitochondrial components [87]. MiR-210 can modulate mitochondrial respire, metabolism, and ROS production with consequences on the regulation of cell death and survival [87].

9 MiR-503/-424 Cluster and Angiogenesis

MiR-503 is an intragenic miRNA clustered with miR-424 [1]. MiR-503 is modulated by HIF-1α in the primary tumors with a hypoxic condition and its expression is very low in these tumors. MiR-503 simultaneously decreases VEGFA and FGF2 expression in cancers, demonstrating the antiangiogenesis role in tumorigenesis [98]. The low expression of miR-503 in primary tumors is due to the epigenetic mechanism. The forced overexpression of miR-503 reduces tumor angiogenesis in vitro and in vivo [98]. Further, miR-503 has also been shown to cooperatively

work with other the miR-16 family (including miR-15a/b, miR-16, miR-195, miR-424, and miR-497) and impact angiogenesis [1].

The effect of miR-424 on angiogensis still remains controversial too. A recent study shows that miR-424 contributes to post-ischemic vascular remodeling and angiogenesis [99]. The expression of miR-424 is driven by hypoxia in the cultured ECs [99]. And, the expression of miR-424 is also found to be increased in parallel with the upregulation of HIF-1α in experimental models with myocardial ischemia [99]. The expressed miR-424 can promote angiogenesis from the cultured ECs in vitro and in the athymic nude mice transplated the Matrigel containing HUVECs [99].

On the contrary, some other studies show that miR-424 inhibits angiogenesis. For example, Naskashima reported that downregulation of miR-424 contributes to the abnormal angiogenesis via regulation of MEK1 and cyclin E1 signals in senile hemangioma [100]. Specific inhibition of miR-424 induced the cell proliferation and angiogenesis of human dermal microvascular endothelial cells (HDMECs) [100]. Liu et al. also reported that miR-424 played a negative role in regulating endothelial differentiation and growth of human dental pulp cells (hDPCs) [101]. Overexpression of miR-424 decreased the expression of the vascular endothelial growth factors and resulted in low angiogenesis, and inhibition of miR-424 contributed to dental pulp repair and regeneration [101].

10 Other MiRs Related to Angiogenesis

Endothelial nitric oxide synthase (eNOS) is one of the key factors for angiogenesis. Inhibition of eNOS production in vivo inhibits angiogenesis [102]. MiR-214 expressed highly in vascular system [103] has been demonstrated to inhibit angiogenesis via inhibit of eNOS production [104]. Recently, van Mil et al. showed that miR-214 can negatively regulate in vivo and in vitro angiogenesis by inhibiting the expression of pro-angiogenic growth factors such as VEGF and PDGF [104].

MiR-296 is also an important regulator for angiogenesis [105]. Hepatocyte growth factor-regulated tyrosine kinase substrated (HGS), which mediates the degradative sorting of PDGFR as well as VEGFR and EGFR, has been identified as a target for miR-296 that mediates angiogenic function [106]. MiR-296 is upregulated in human gliomas tumor ECs, and it seems that this miR downregulates HGS expression and upregulates VEGFR2 and PDGFRβ in glioma blood vessels. Other studies have confirmed that EGF could induce miR-296, suggesting a role for miR-296 in promoting angiogenesis in tumors [105, 106].

MiR-467 affects angiogenesis in response to high glucose. It has also been identified as a translational suppressor of TSP-1 that is implicated in the pathogenesis of several diabetic complications [107]. MiR-467 was upregulated by high glucose in microvascular ECs and in breast cancer cells, where it suppressed the production of TSP-1 by sequestering its mRNA in the nonpolysomal fraction. In in vivo angiogenesis models, miR-467 promoted the growth of blood vessels, and TSP-1 was the main mediator of this effect [107].

Let-7f and miR-27b are also involved in angiogenesis. Inhibition of let-7f significantly reduces vascular sprouting while let-7b controls EC proliferation and motility and affects tube formation by regulating antiangiogenic factor TIMP-1 [108, 109]. Downregulation of miR-27a induces ZBTB10, a repressor of specificity protein (Sp) transcription factors, and therefore causes repression of Sp and Sp-regulated gene products that can decrease expression of other proteins such as VEGF and VEGF-R1 regulating angiogenesis [110]. In turn, the expression of miR-27a can be upregulated by treatment with VEGF in breast cancer stem like cells (BCSLCs). Increase of miR-27a paralleling downregulation of ZBTB10 in BCSLCs promotes angiogenesis and tumor metastasis in vivo [111]. In addition, miR-27a also mediates angiogenesis through downregulation of angiogenic factor with G patch and FHA domains 1 (AGGF1) in high-grade bladder urothelial carcinoma cells [112]. AGGF1 is newly identified as a potent angiogenic factor that highly expresses in ECs and promotes angiogenesis via regulation of VEGF [106].

MiR-130a, a miRNA strongly upregulated after exposure to fetal bovine serum in ECs, is able to antagonize the antiangiogenic activity by regulating the expression of its target gene homeobox gene GAX (growth arrest-specific homeobox) and homeobox A5 (HOXA5) [113]. MiR-20a is a member of the miR-17-92 cluster, and miR-20b is a member of the miR-106a cluster located on X chromosome, both of them are potential antiangiomiRs by targeting VEGF for repression [114, 115].

MiR-145 inhibits tumor growth and angiogenesis by targeting N-Ras and VEGF [116]. A recent study, however, revealed a novel role and mechanism of Argonaute 2 as an enhancer of myeloma angiogenesis through miRNA dysregulation, including the upregulation of pro-angiogenic miRs such as the let-7 family members and miR-17/-92 cluster and downregulation of the anti-angiogenic miRNA miR-145 [33]. The pro-angiogenic let-7 family miRs, the miR-17/-92 cluster and the anti-angiogenic miRNA miR-145 play crucial roles in AGO2-mediated angiogenesis by targeting angiogenetic genes [33]. It has been shown that overexpression of miR-21 in prostate cancer cells (DU145 cells) induces tumor angiogenesis by targeting phosphatase and PTEN, which in turn activates Akt and extracellular-signal-regulated kinases1/2 (ERK1/2) signaling pathways and finally enhances HIF-1α and VEGF expression, two of the strongest angiogenesis inducers [92].

11 Conclusion

Although a number of studies summarized here have provided much needed information on the pathways and mechanisms of miRs involved in angiogenesis, but most of studies were performed in the cultured endothelial cells and animal models. Scarcely any studies on the regulation of miRs in angiogenesis were therapeutically used in clinic, but anyway the discovery of miRs has led to discoveries of new therapeutic targets. Obviously, we need await human trails to see if these targets are successful in ameliorating human diseases states associated with angiogenesis.

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Chapter 8
Mast Cells in Angiogenesis: The Role of Angiogenic Cytokines

Domenico Ribatti

Abstract The proximity of mast cells to blood vessels has long suggested a relationship between these cells and angiogenesis. Moreover, the role of mast cells in this process is mostly certain related to the release of a large spectrum of angiogenic cytokines, including vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), transforming growth factor beta (TGFβ), tumor necrosis factor alpha (TNFα), interleukin-8 (IL-8) and angiopoietin-1 (Ang-1). In this context, mast cells might act as a new target for the adjuvant treatment of tumors through the elective inhibition of angiogenesis. Preclinical studies in experimental models using anti-cKit antibodies, or the mast cell stabilizer disodium cromoglycate have shown promising results.

Keywords Angiogenesis • Cytokines • Inflammation • Mast cells • Tumor growth

1 Introduction

Angiogenesis is stimulated by numerous 'classic' factors and other 'non-classic' regulators . Classic stimulators mostly include growth factors and cytokines, among which vascular endothelial growth factor (VEGF), placental growth factor (PlGF), platelet derived growth factor (PDGF), fibroblast growth factor-2 (FGF-2), transforming growth factors (TGFs), angiopoietins (Angs).

Moreover, evidence has been accumulated that in addition to the "classic" factors, many other "non-classic factors", including numerous endogenous peptides, among which erythropoietin (Epo), granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukins (ILs), angiotensin II (Ang II), endothelins (ETs), adrenomedullin (AM), proadrenomedullin

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N-terminal 20 peptide (PAMP), urotensin-II (U-II), leptin, adiponectin, resistin, neuropeptide-Y, vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating poly-peptide (PACAP) and substance P, play an important role [35].

Both innate and adaptive immune cells are involved in the mechanisms of endothelial cell proliferation, migration and activation, through the production and release of a large spectrum of pro-angiogenic mediators. There is increasing evidence to support the view that angiogenesis and inflammation are mutually dependent. During inflammatory reactions, immune cells synthesize and secrete pro-angiogenic factors that promote neovascularization. On the other hand, the newly formed vascular supply contributes to the perpetuation of inflammation by promoting the migration of inflammatory cells to the site of inflammation [37].

Pathological angiogenesis is linked to a switch in the balance between positive and negative regulators, and mainly depends on the release by inflammatory cells of specific growth factors for endothelial cells, that stimulate the growth of the host's blood vessels or the down-regulation of natural angiogenesis inhibitors [36].

The link between chronic inflammation and tumorigenesis was first proposed by Rudolf Virchow in 1863 after the observation that infiltrating leukocytes are a hallmark of cancer. Virchow was the first to establish a causative connection between the lymphoreticular infiltrate at sites of chronic inflammation and the development of cancer (Table 8.1).

In neoplastic tissues, inflammatory cells act in concert with tumor cells, stromal cells and endothelial cells to create a microenvironment that is critical for the survival, development and dissemination of the neoplastic mass . These interactions within the tumor microenvironment may represent important mechanisms for tumor development and metastasis by providing an efficient vascular supply and an easy escape pathway [43]. Among inflammatory cells that have been identified as modifiers of tumor microenvironment, mast cells play a crucial role [40]. Tables 8.2 and 8.3 summarize mast cell mediators and their possible effects on tumor biology.

Table 8.1 Different types of cancers and associated with chronic inflammatory disorders

Colorectal cancer, ulcerative colitis and Chron's disease
Cholangiocarcinoma, primary sclerosing cholangitis
Gastric cancer, chronic gastritis (*Helicobacter pylori*)
Lung cancer, inflammation caused by asbestsos, smoking, and sylica
Prostate cancer, *Escherichia coli* infection of prostate
Hepatocellular carcinoma, infection casued by hepatitis virus B and virus C
Melanoma, UV irradiation and associated skin inflammation
Endometrial carcinoma, endometriosis
Gallbladder carcinoma, gallbladder stone-associated chronic cholecystitis
Esophageal cancer, Barrett's esophagitis

Table 8.2 Pro-tumorigenic mediators contained in mast cell granules

Mediator	Effects and function
Histamine	Increase of vascular permeability, angiogenesis, immunosuppression
Heparin	Increase of vascular permeability, angiogenesis, matrix reorganization
Tryptase	Tissue remodeling, neovascularization, facilitate metastases
Chymase	Facilitate metastases, tissue damage and remodeling
NGF, SCF	Promote tumor growth, mast cell chemoattractant
PDGF	Promote tumor growth
VEGF	Promote angiogenesis, mitogenesis of endothelial cells
FGF-2	Promote tumor growth, neovascularization, matrix reorganization and degradation
TGF-β	Promote tumor growth, mitogenesis of endothelial cells, angiogenesis
CCL2, CCL5	Chemoattractants for mast cells and other immune cells
CXCL8	Promote neovascularization, matrix reorganization and degradation
TNF-α	Promote immunosuppression neovascularization
IL-3, 4	Promote matrix reorganization and degradation
Ang-1	Promote angiogenesis

Legend: *NGF* nerve growth factor, *SCF* stem cell factor, *PDGF* platelet derived growth factor, *VEGF* vascular endothelial growth factor, *FGF-2* fibroblast growth factor-2, *TGF-β* transforming growth factor, *CCL, C-C* motif chemokine ligand, *CXCL, C-X-C* motif chemokine ligand, *TNF-α* tumor necrosis factor alpha, *Il*, interleukin, *Ang* angiopoietin

Table 8.3 Anti-tumorigenic mediators contained in mast cell granules

Mediator	Effects and function
IL-1, 6, 9, 10	Inflammation, leukocyte migration
IL-3	Mast cell proliferation, eosinophil activation
IL-4	Tumor cell apoptosis, TH2 differentiation
IL-5	Leukocyte migration, eosinophil activation
TNF-α	Inflammation, tumor cell death
IFN-ϒ	Inflammation, leukocyte proliferation and activation
GM-CSF	Inflammatory cell proliferation, eosinophil activation
TGF-β	Inflammatory cell proliferation
PAF	Platelet activation, leukocyte chemotaxis
PGD2, PGE2	Vasodilation, neutrophil chemotaxis
LTB4, LTC4	Leukocyte chemotaxis, increase vascular permeability
MIP-α	Chemoattractant for monocytes, macrophages and neutrophils
MCP-3/4	Chemoattractant for leukocytes
Tryptase	Inflammation
NO	Vasodilation

Legend: *IL* interleukin, *TNF-α* tumor necrosis factor alpha, *INF-γ* interferon gamma, *GM-CSF* granulocyte macrophage coolly stimulating factor, *TGF-β* transforming growth factor beta, *PAF* platelet activating factor, *PG* prostaglandin, *LT* leukotriene, *MIP* macrophate inflammatory protein, *MCP* monocyte chemotactic protein, *NO* nitric oxide

2 Mast Cells and Angiogenesis

Mast cells originate from progenitor cells in the bone marrow, which move through the circulation and become mature mast cells after homing to different organs under the influence of the local microenvironment [44]. Mast cell progenitors enter the blood and exit into tissues by transendothelial migration and are undetectable in the blood. Indeed, mast cells are found in human mucosal and epithelial tissues throughout the body, in all vascularized tissues except for the central nervous system and the retina [9].

Mast cells are localized in connective tissues and are more numerous near the boundaries between the external environment and the internal milieu [15] including the skin [14], the respiratory tract [47], the gastrointestinal tract [13] and the conjunctiva [7].

It is believed that the role of mast cells to physiologic and pathological processes extends far beyond the allergic disease; they are involved in wound healing, in chronic inflammation, tumor growth, and angiogenesis, and may be considered as a component of the immune system [3, 38] .

Mast cells contain inside their secretory granules powerful biologically active molecules including cytokines, histamine, proteases and proteoglycans, which are released when mast cells are activated, exert sometimes opposing biological effects and affecting the functional profile of different resident tissue cells, like fibroblasts, smooth muscle cells, endothelial cells, epithelial cells and nerve fibers. Moreover, mast cells synthesize several pro-angiogenic molecules (Table 8.4).

Mast cells play a role in tumor growth and tumor-related angiogenesis, by releasing in the tumor stroma cytokines and growth factors, which have detrimental effects to the host by stimulating tumor cell expansion.

Table 8.4 Angiogenic factors stored in mast cells

Adrenomedullin
Chymase
Fibroblast growth factor-2 (FGF-2)
Heparin
Histamine
Interleukin-8 (IL-8)
Nerve growth factor (NGF)
Transforming growth factor beta (TGF-β)
Tryptase
Tumor necrosis factor alpha (TNF-α)
Vascular endothelial growth factor (VEGF)

3 Angiogenic Cytokines Involved in Mast Cell Angiogenesis

Mast cells migrate in vivo and in vitro in response to VEGF, PDGF, FGF-2, and PlGF-1 [10, 11, 18]. Human lung mast cells express VEGF-A, VEGF-B, VEGF-C and VEGF-D (Fig. 8.1), VEGF receptors-1 and -2 (VEGFR-1 and VEGFR-2). Supernatants of prostaglandin E2 (PGE2)- and 5′-N-ethylcarboxamido- adenosine (NECA)-activated lung mast cells induced angiogenic response in the chick embryo chorioallantoic membrane (CAM) assay that was inhibited by an anti-VEGF-A antibody (Fig. 8.2) [33].

Granulated murine mast cells and their granules are able to stimulate an intense angiogenic reaction in the chick embryo CAM assay, partly inhibited by anti-FGF-2 and anti-VEGF antibodies [33]. Certain mast cell populations released pre-formed stores of VEGF after IgE-dependent upregulation of FCεRI expression [4]. Human mast cells are a potent source of VEGF in the absence of degranulation through the activation of the EP(2) receptor by PGE2 [1], and selective release of VEGF by human mast cells is regulated by corticotropin releasing hormone (CRH) [6].

Qu et al. [32] demonstrated that FGF-2 is localized in the cytoplasmic granules of mast cells, and Gruzkau et al. [19] showed the expression of VEGF in human mast cell line HCM-1 and in human skin mast cells.

An association between VEGF, mast cells and angiogenesis has been demonstrated in laryngeal carcinoma [46], in non-small cell lung cancer, in which intratumoral mast cells express VEGF ([22, 51]). In non-small cell lung cancer a high correlation was observed between intratumoral mast cells and microvessel counts and double staining showed that most intratumoral mast cells express VEGF [22]. In melanoma, mast cells express both VEGF [52] and FGF-2 (Fig. 8.3) [34]. In prostate cancer, peritumoral mast cells express high levels of FGF-2 [23]. Moreover, after the injection of tumor cells in a rat orthotopic model, FGF-2 expressing mast cells were recruited [23].

Prevete et al. [31] demonstrated that the Ang receptor Tie2 is highly expressed on human lung mast cells and that Ang1 is a potent stimulus for mast cell chemotaxis, and Tie2 overexpression in mice induces dermal infiltration of mast cells [55]. Primary murine mast cells express Ang-1 and mast cells promote marked neovascularization, which was prevented by neutralization of VEGF-A and Ang-1 [27]. Guo et al. [20] reported that Ang-1 may be involved in tryptase-positive mast cell induced angiogenesis in pancreatic cancer, and demonstrated an increased expression of Ang-1 in tryptase-positive-treated mice.

4 Therapeutic Approach

Mast cells might act as a new target for the adjuvant treatment of tumors through the selective inhibition of angiogenesis, tissue remodeling and tumor promoting molecules, allowing the secretion of cytotoxic cytokines and preventing mast cell mediated immune-suppression. Pre-clinical studies in experimental models, using anti-cKIT

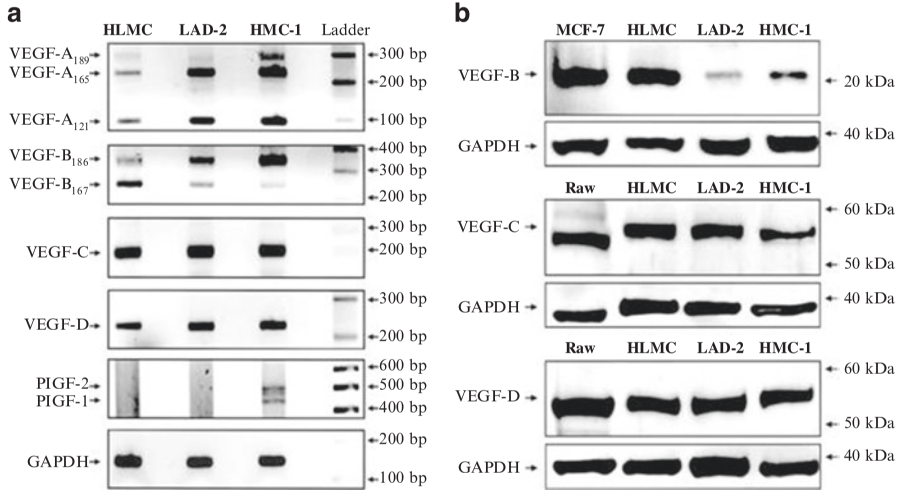

Fig. 8.1 Expression of VEGFs in human mast cells. (**a**) VEGFA, VEGFB, VEGFC, VEGFD, and PlGF RT-PCR amplification products from HLMCs, LAD-2 cells, and HMC-1 cells. (**b**) Immunoblot with anti–VEGF-B, anti–VEGF-C, anti–VEGF-D, and anti–GAPDH antibodies of HLMC, LAD-2, and HMC-1 lysates. MCF-7 and RAW 264.7 (Raw) cells were used as positive controls. Data are representative of four experiments (Reproduced from Detoraki et al. [11])

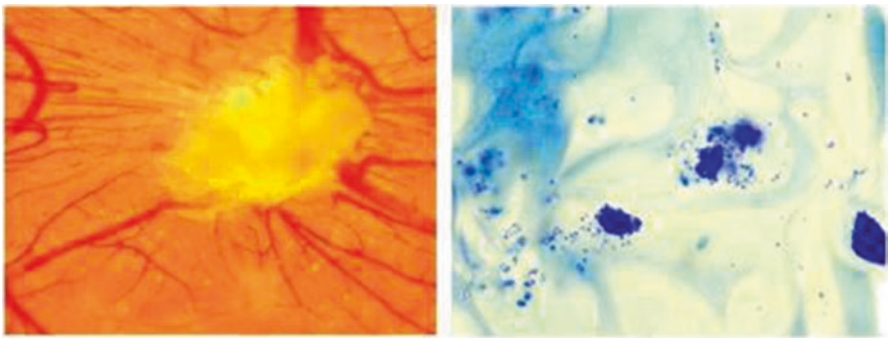

Fig. 8.2 A mast cell suspension has been delivered on the top of the chick embryo chorioallantoic membrane. Macroscopic observation shows the sponge surrounded by numerous allantoic vessels that develop radially towards the implant in a 'spoked-wheel' pattern. The histological analysis shows among the sponge trabeculae metachromatic mast cells and their secretory granules (Reproduced from Ribatti et al. [33])

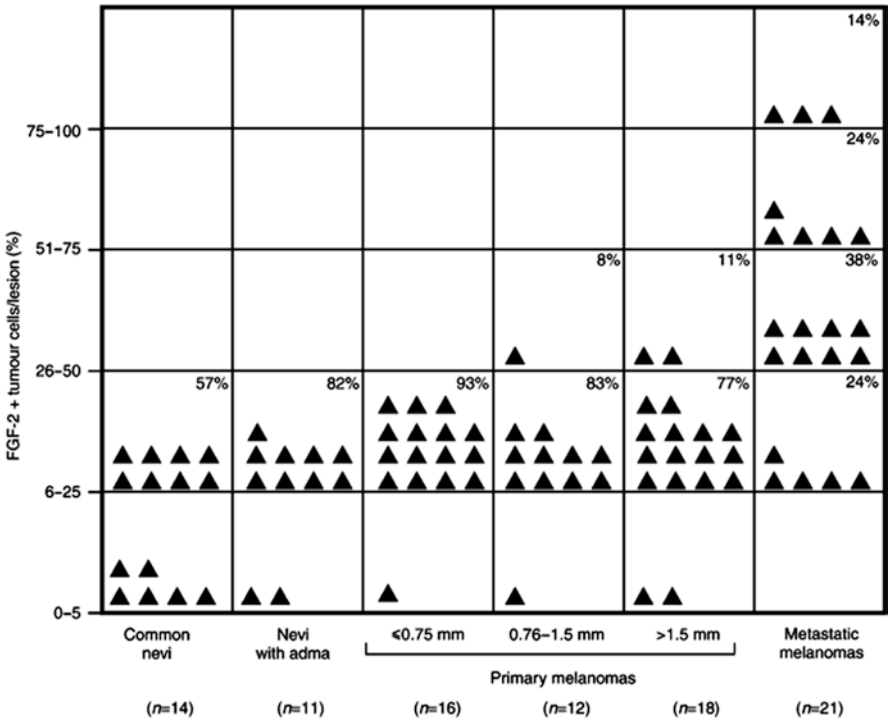

Fig. 8.3 Percentages of tumor cells expressing FGF-2 in various melanocytic lesions at distinct steps in tumor progression. Each triangle represents a single lesion. The percentages of cells stained per lesion were divided into five intensity groups (0–5, 6–25, 26–50, 51–75 and 76–100%). The percentages of lesions of each intensity group are reported (Reproduced from Ribatti et al. [34])

antibodies [21, 28], anti-TNFα antibodies [17], or the mast cell stabilizer disodium cromoglycate (cromolyn) [50] have shown promising results.

The tyrosine kinase receptor Kit (CD117) is upregulated in tumor cells and mutations in c-kit are associated to the development of gastrointestinal stromal tumor (GIST), in various forms of mastocytosis and mast cell leukemia [29]. Mast cells express high levels of c-kit and stem cell factor (SCF), the ligand for kit, is involved in mast cell development, survival, migration, and function [41]. SCF enhances tumor growth through increased production of VEGF, IL-6, IL-10, and TNFα [21], and inhibition of the SCF/Kit axis in vivo inhibits the migration of mouse bone marrow–derived cultured mast cells to tumors in a transplanted tumor model in mice [21].

Systemic mastocytosis is a myeloid disorder characterized by abnormal growth and accumulation of neoplastic mast cells in internal organs [25]. The first tyrosine kinase inhibitor introduced into the clinic STI571 (Imatinib mesylate, Gleevec) has been used for some varieties of mastocytosis, although some kit activating mutations involved in mastocytosis are resistant to its inhibitory activity [2]. In a murine model of breast carcinoma, depletion of mast cells with imatinib mesylate enhanced tumor growth [45].

In a majority of patients with systemic mastocytosis the kit inhibitors cannot block the mutated Kit [53]. KitD816V has been developed as a therapeutic target in mast cell tumors and several of the new tyrosine kinase inhibitors, including midostaurin (PKC412), nilotinib (AMN107), toceranib, masitinib, imatinib, and dasatinib, counteract malignant cell growth in patients with aggressive systemic mastocytosis or mast cell leukemia [16, 48, 49, 54]. Sunitinib inhibits c-kit mutations in systemic mastocytosis [30]. Masatinib is a tyrosine kinase inhibitor that targets c-kit receptors and is clinically developed and approved for treatment of recurrent or unresectable dog mast cell tumors and is the first approved anticancer drug in veterinary medicine [12]. Masatinib has been translated to human clinical trials for evaluating in GIST, mastocytosis and pancreatic cancer [24, 26].

Mast cell stabilizers, including gabexate mesilate and nafomostat mesilate, two inhibitors of trypsin-like serine protease, inhibit tryptase, an angiogenic factor stored in mast cell granules (Fig. 8.4) [42].

Bosquiazzo et al. [5] demonstrated that cromolyn inhibited mast cell degranulation in rat uterine cervix, which is correlated to expression of VEGF-mRNA and endothelial cell proliferation. In mouse models of pancreatic cancer, cromolyn treatment induced apoptosis of tumor cells due to clotting in blood vessels [50]. In prostate tumors of transgenic adenocarcinoma of the mouse prostate (TRAMP) mice expressing the SV40T oncoprotein under the prostate-specific rat probasin promoter, cromolyn chronic treatment inhibited the development of adenocarcinoma [29]. Cimpean and Raica [8] demonstrated that cromolyn inhibited VEGF and PDGF expression in A375 chick melanoma tumor cells implanted on the chick CAM.

Therapeutic strategies may include inhibition of recruitment of mast cells to the tumor microenvironment and blockade of pro-tumoral effects and pro-angiogenic functions. Chemoprevention with an anti-inflammatory approach has the potential to inhibit neovascularization before the onset of the angiogenic switch, resulting in a significant delay in tumor growth.

Fig. 8.4 Tryptase is angiogenic in vivo in the CAM assay. Note the presence of numerous blood vessels converging toward the implant (Modified from Ribatti et al. [39])

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Part II
Therapeutic Implications of Angiogenesis in Cancer

Chapter 9
Therapeutic Implications of Angiogenesis in Cancer

Issam Makhoul, Shebli Atrash, Konstantinos Arnaoutakis, Mazin Safar, Angela Pennisi, Laura Huffman, and Robert Griffin

Abstract Angiogenesis is one of the hallmarks of cancer. Many primed cells endowed with all cancer characteristics arise in our body but they cannot progress to become cancer-disease without activating angiogenesis. This chapter addresses the factors involved in tumor angiogenesis and the progress made to exploit this phenomenon. Tumors are very heterogeneous in their angiogenic pathways and drugs targeting angiogenesis are moderately effective in the metastatic setting with variable efficacy in different tumor types and within the same type. Antiangiogenic agents do not have any role in the adjuvant setting. The biggest challenge facing this discipline is the identification of biomarkers to select patients who are more likely to respond to these expensive treatments and those likely to suffer severe toxicity. Hence, the complexity of the task and the need to embed the study of these markers in the design of phase III randomized controlled trials.

Keywords Angiogenesis • Cancer • Vascular Endothelial Growth Factor • Angiogenic Switch • Angiogenic dormancy • Biomarker

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1 Introduction

Targeting angiogenesis in cancer is now a reality. This chapter reviews the biologic foundations of cancer angiogenesis and major therapeutic advancements targeting this aspect of cancer biology.

2 Tumor Angiogenesis

2.1 Tumor Initiation, Prevascular Phase and Tumor Dormancy

Incipient cancer cells acquire proliferative and/or survival advantages as a results of random or inherited genetic and epigenetic changes. These tumor-initiating cells belong either to the stem cell compartment or to the compartment of progenitor cells that have acquired stemness characteristics. In normal circumstances, most initiated cells are likely to be eliminated by internal and external control mechanisms. Other possible outcomes include the correction of the abnormality and the return of these cells to the pool of normal cells or the suppression of the growth of these cells in their initiation site until the suppressive mechanisms weaken, allowing the transformed cells to progress toward open cancer-disease. Transformed cells are detected by the immune system that may play either a host-protective role by detecting and eliminating them or a tumor-promoting role by amplifying the microenvironmental response and triggering the angiogenic switch.

Transformed cells undergo many changes under the pressure of the immune system, called immunoediting. Three phases of this interaction were described: Elimination, equilibrium and escape [1, 2]. Elimination leads to the eradication of transformed cells by a competent immune system. In this case, transformed cells are recognized via their neoantigens (resulting from mutations or translocations) by the adaptive arm of the immune system or via the distress signals expressed on their surface secondary to chromosomal changes (aneuploidy or hyperploidy) by the innate immune system [3, 4]. When the immune system prevents the progression of transformed cells without eliminating them, an equilibrium or a ***immune dormancy*** is reached. Equilibrium is marked by a balance between elimination-promoting cells and cytokines (IL-12, IFNγ, TNFα, CD4 Th1, CD8+ T cells, NK cells, γδT cells) and those that promote persistence (IL-23, IL-6, IL10, TGFβ, NKT cells, CD4 Th2, Foxp3+ Treg cells, and MDSCs) of the transformed tumor cells [5–7]. Under the influence of tumor microenvironment monocytes may differentiate into pro-inflammatory M1 or anti-inflammatory M2 types, which play a role in the ***angiogenic switch*** (see below) [8, 9].

The mechanism that tilts the balance established during the equilibrium phase toward tumor progression remains unclear. The production of new B and T lymphocytes in the bone marrow and the thymus, respectively and the functioning of mature

immune cells are all reduced with age [10]. In breast cancer, systemic inflammation associated with aging and local pro-inflammatory microenvironment promote cancerous progression of mammary cells primed by the loss of tumor suppressor genes [7, 11]. Pro-inflammatory cytokines (TNFα and IL-6) lead to COX2 and aromatase enzyme overexpression [12], which results in increased local concentrations of estrogens. Estrogens induce the expansion of Tregs and the inhibition of antigen presenting cells [13–15]. In addition to the gradual decline of the systemic and local immune system, dietary factors, commensal gut microbiota, use of antibiotics, physical activity and hormonal factors play variable roles in tilting the balance from equilibrium to escape [16–19].

2.2 Angiogenesis

Angiogenesis is the process by which an adult organism creates new blood vessels to meet the demand of growth or healing of injured tissues. This is a highly orchestrated process that requires the intervention of soluble mediators, cell-cell and cell-matrix interactions. Soluble mediators released after a tissue injury or in presence of a nascent tumor destabilize the quiescent endothelial cells (EC) and induce their proliferation, migration and arrangement in tube-like structures. These structures undergo a maturation process after recruitment of pericytes in capillary vessels or smooth muscle cells in larger blood vessels. As blood vessels mature they become less leaky and enter into quiescence. EC survival no longer depends on the presence of soluble mediators but on cell-cell direct or autocrine signaling. ECs that are not covered by pericytes regress [20, 21].

2.3 Soluble Factors (Table 9.1)

Vascular endothelial growth factors and placental growth factor (VEGF A, B, C and D; PlGF) and their receptors (VEGFR 1–3) are the most important and most studied system among these factors [22]. Alternative splicing of a single VEGF A gene generates six isoforms with variable length and biologic activity: 121, 145, 165, 183, 189, and 206 amino acids. VEGF121 and 165 are the most physiologically relevant. The expression of VEGFR1, 2 and 3 is not limited to endothelial cells but it extends to smooth muscle cells and bone marrow cells [20]. However, the mitogenic effects of VEGF A are only seen in endothelial cells. The angiogenic effects of VEGF A are mediated by its interaction with VEGFR2 to which it has lower affinity than to VEGFR1. It is possible that VEGFR1 functions as a decoy receptor modulating the amount of VEGF A available to interact with VEGFR2. However, the binding of VEGF A to VEGFR2 is facilitated by the expression of neuropilin-1 on endothelial cells. This membrane receptor does not have intracellular signaling activity but it preferentially binds VEGF A and presents it to VEGFR2 to facilitate

Table 9.1 Different pro-angiogenic factors and their effects on endothelial cells and other stromal components

Soluble mediators	EC									Pericytes	Fibroblasts	
	Pro	SF	TF	Apo	M	Per	uPA/PAI	EC stab	EC destab	Recruit.	Recruit.	Angiogenesis in-vivo/ other functions
VEGF	+			−	+	+	+	−	+	−		+
Ang1	+							+		+		
Ang2									+			
aFGF, bFGF	+		+	−	+		+					+
PDGF	+									+		
TGF-β	−		+		−		−	+		+	+	+
TNF-α	−		+									+
EGF, TGF-α	+											+
G-CSF, GM-CSF	+				+							
Angiogenin					+							+
TF												+
Factor V												+
Prostaglandin												+
Nicotinamide												+
Monobutyrin					+							+
αv β3-integrin				−	+							Required for bFGF Localizes MMP-2 to capillary sprouts
αv β 5-integrin												Required for VEGF action
α5 β1-integrin												Required for non-VEGF action
VE-cadherin				−		+						+

Eph-4B/ Ephrin-B2												+/ Colocalize at venous/arterial interfaces
Ephrin-A1												/Required for TNF- α
Eph-2A												/Required for endothelial cell tube formation
Blood flow/shear stress												Unperfused BV disappear

EC endothelial cell, *Pro* proliferation, *SF* sprout formation, *TF* tube formation, *M* migration, *Per* permeability, *PA/PAI* plasminogen activator/plasminogen activator inhibitor, *EC stab* endothelial cell stabilization, *EC destab* endothelial cell destabilization, *Pericyt* pericytes, *Fibrobl* fibroblasts, *Recruit* recruitment

its interaction with the receptor [23]. VEGFR3 does not interact with VEGF A but with VEGF-C and VEGF-D and is involved in lymphangiogenesis [24]. VEGF-B binds and activates VEGFR-1 as well as neuropilin-1.

VEGF A increases endothelial cell permeability by loosening adherence junctions between ECs or by enhancing the activity of vesicular-vacuolar organelles that facilitate transport of metabolites between luminal and abluminal plasma membranes; the net result is to increase extravasation of plasma proteins and formation of extracellular matrix favorable to endothelial and stromal cell migration. Furthermore, the production of plasminogen activators (uPA) plasminogen activator inhibitor-1 (PAI-1) and interstitial collagenase by ECs enhances stromal proteolysis, which helps with extracellular matrix remodeling necessary for angiogenesis [25, 26]. Furthermore, VEGF A induces proliferation and migration of destabilized endothelial cells and inhibits EC apoptosis. The production of VEGF A is induced by hypoxia through the hypoxia inducible factor (HIF) pathway [27]. It is also produced by most tumors as a result of their activated oncogenes and by several cell types including fibroblasts and inflammatory cells attracted to the tumor site [28, 29].

Angiopoietins (Ang 1–4) *and TIE receptors* (Tie1 and 2) are the other system that is involved in angiogenesis and is responsible for the integrity and survival of ECs once blood vessels are formed [30]. Four angiopoietins were identified; Ang1 binds to Tie-2, antagonizes the effects of VEGF A and increases the girth and stability of endothelium in newly formed angiogenic sprouts [31]. Ang2 binds to Tie2 and works as Ang1 antagonist. ECs activated by VEGF A increase their production of Ang-2, which acts by autocrine mechanism on the same cells. Overexpression of Ang2 in many cancers portends more aggressive phenotype and poor prognosis [32, 33].

Fibroblast growth factors (acidic-FGF and basic-FGF) belong to a large family that consists of 23 members, 18 function as ligands that interact with four receptors (FGFR1–4) [34]. Like VEGFs, they stimulate EC proliferation, migration, tube formation and production of uPA/PAI and they inhibit apoptosis [20, 34]. There is no proven role for FGFs in physiologic angiogenesis. However, it is possible that these factors play a role in local reparative angiogenesis following tissue injury where they are deposited in the extracellular matrix. Different genetic hits result in their overexpression in many cancers (gain of function mutations, gene amplification, translocations, gene fusions and altered gene splicing) [35]. Their upregulation may occur after exposure to antiangiogenic treatment and may drive resistance to endocrine therapy in breast cancer [36].

Platelet-derived growth factors (PDGFs) exist as homodimers (PDGF-AA or -BB) or heterodimers (PDGF-AB) and they bind to dimeric PDGF receptors (αα, ββ, or αβ) [37, 38]. The role of PDGFs is in inducing the maturation of blood vessels by stabilizing pericytes that are initially recruited by mechanisms dependent or independent of PDGFs.

Transforming growth factor-β is produced by ECs, pericytes and other cells. It inhibits the proliferation and migration of ECs but favor development of tube-like structures through the modulation of the extracellular matrix and the establishment of a scaffold favorable to vessel tubes formation and induces pericyte differentiation [20].

Other soluble factors with angiogenic activity include *tumor necrosis factor-α* (TNF-α), *epidermal growth factor* (EGF), *transforming growth factor*-α (TGF-α), *colony-stimulating factors* (CSFs), *angiogenin*, *angiotropin*, *tissue factor*, *factor V*, *prostaglandins*, *nicotinamide*, and *monobutyrin* [20] (Table 9.1).

2.4 Membrane-Bound Factors

Integrins, especially *αvβ3,* may regulate localized degradation of the extracellular matrix by localizing proteases to the advancing end of the blood vessel and then mediate endothelial cell migration by adhering to the modulated matrix. Integrins play a major role in tumor cell migration, invasion, proliferation and survival [39]. *VE-cadherin* localizes to adherens junctions and mediates contact inhibition of endothelial cell growth [40]. *Eph-B4/ephrin-B2* localize at the arterio-venous interface and play a major role in remodeling established primary capillary plexus [41].

2.5 Biomechanical Forces

Non-perfused vessels eventually regress while blood perfusion stimulates the growth and maturation of capillary ECs. Hence, in addition to soluble and membrane bound factors mechanical forces contribute to the pruning and remodeling of normal angiogenesis [42].

2.6 Endogenous Inhibitors of Angiogenesis

Various inhibitors of angiogenesis, including *angiostatin*, *endostatin* and *thrombospondins* and others are found in the body. The reasons for having so many inhibitors is not clear nor is fully known how these inhibitors overcome the effects of angiogenesis stimulators. Table 9.2 summarizes the effects of the most important endogenous angiogenic inhibitors [43].

2.7 The Angiogenic Switch (AS) (Fig. 9.1)

The local balance between endogenous angiogenic stimulators and inhibitors controls regulation of angiogenesis. In cancer, the balance between stimulator and inhibitor levels tilts toward stimulators leading to an “angiogenic switch – AS”. Cancer precursor lesions (such as carcinoma in situ of the breast) are usually

Table 9.2 Endogenous angiogenic inhibiting factors and their effects on endothelial cells [43]

Soluble mediators	EC									Pericyt Recruit.	Fibrobl Recruit.	
	Pro	SF	TF	Apo	M	Per	uPA/PAI/ MMP	EC stab	EC destab			Angiogenesis in-vivo other functions
Matrix derived												
Arresten	–				–							Depends on α1-integrin
Canstatin	–		–	+	–							–
Endorepellin		–	–		–							–
Endostatin	–			+	–		–	+	–			–
Fibronectin fragment (Anastellin)												–
Targeting fibronectin-binding integrins												–
Fibulin	–	–	–	+	–							–Up-regulates TSP-1
Thrombospondin (TSP)-1 and –2	–			+								–
Tumstatin	–		–	+								–
Non-matrix derived												
Interferons	–			+								
Interleukins	–											
Pigment epithelium derived factor (PEDF)	–											
Angiostatin	–											
Antithrombin III	–											
Prothrombin kringle 2	–											
Platelet factor-4	–				–							

Tissue inhibitors of metalloproteinases (TIMPs)	–											
Chondromodulin	–		–									
2-Methoxyestradiol	?											
Prolactin fragments	–											
PEX	–											
Soluble Fms-like tyrosine kinase-1 (S-Flt-1)	–											
Troponin I	–											
Vasostatin	–											

EC endothelial cell, *Pro* proliferation, *SF* sprout formation, *TF* tube formation, *M* migration, *Per* permeability, *PA/PAI* plasminogen activator/plasminogen activator inhibitor, *EC stab* endothelial cell stabilization, *EC destab* endothelial cell destabilization, *Pericyt* pericytes, *Fibobl* fibroblasts, *Recruit* recruitment

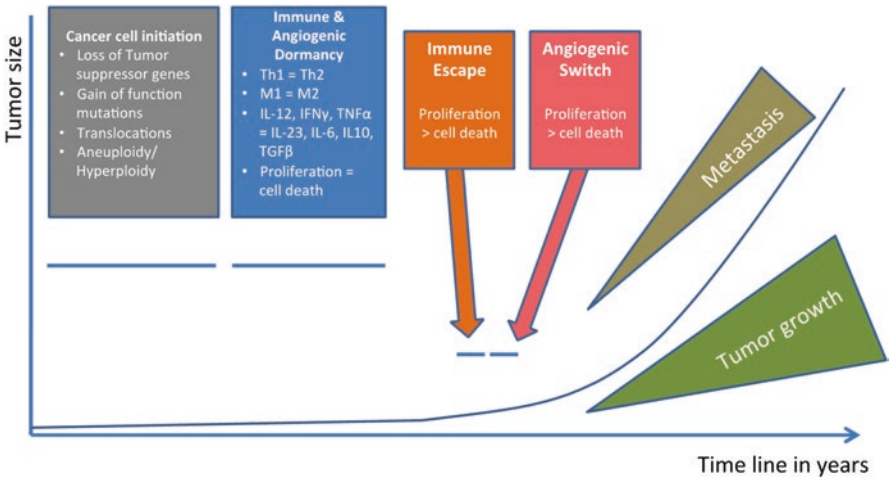

Fig. 9.1 Time line for progression from incipient cancer cells to immune escape and the angiogenic switch. Once the angiogenic switch is triggered the tumor may grow locally and cancer cells may access the blood vessels and spawn metastases to distant organs

microscopic and prevascular and a basement membrane separates their cells from the host microvessels. Experimental studies demonstrated that prevascular lesions exist in a stable state due to balanced proliferation and tumor cell death [44] and may remain in this state for months to years. The onset of neovascularization, or AS, often associated with the emergence of the invasive phenotype, can be relatively sudden [45, 46]. The AS can also start at the preinvasive phase in certain cases [47]. AS can be preceded or associated with immune escape and is understood as a shift in the net balance between positive regulators (e.g., bFGF, VEGF) and negative regulators of angiogenesis (e.g., thrombospondin-1, 16-kD prolactin, interferon (IFN)-a, IFN-b, platelet factor 4, angiostatin, endostatin, and others such as interleukin IL-12) [48]. The importance of the immune/inflammatory mechanisms is illustrated by the role of tumor-associated macrophages and other cells (Myeloid derived suppressor cells – MDSC – fibroblasts and adipocytes) in triggering the AS. Once the macrophages are polarized to the M2 phenotype, they secrete proangiogenic factors that participate in the AS. This permissive environment remains local allowing the nascent cancer to grow, invade and release cancer cells in the lymphatic or vascular systems. However, disseminated cancer cells land in distant organs and have to negotiate with their destination environment a new "license to grow" that would vary depending on the new environment.

Four mechanisms for neovascularization or AS were identified. First, direct recruitment of blood supply from neighboring blood vessels by prevascular tumors. Invasive cells secrete proteinases that breach the basement membrane and allow cancer cells to enter the stroma and induce ECs to proliferate following a concentration gradient toward the source of proangiogenic factors. Cancer cells form multiple layers (the radius of which is restricted by the oxygen diffusion limit between 80

and 150 μm) that surround every new capillary vessel [49]. Second, recruitment of endothelial progenitor cells (EPC). Vascular endothelial growth factor receptor −1 (VEGFR-1) positive bone marrow cells are recruited to the invasive focus [50, 51]. The contribution of EPCs varies in different malignancies; in lymphomas, their role is predominant (>90%) while in solid tumors (example breast cancer) they play a more limited role [52]. Third, vessel cooption where cancer cells grow and cluster around preexisting blood vessels [53]. And the last mechanism to provide tumors with blood supply is vascular mimicry; tumor form tube-like structures by dedifferentiation of epithelial cells into an endothelial phenotype [54].

2.8 Consequences of the Angiogenic Switch

New blood vessels enrich the local tumor environment with oxygen and important nutrients needed for growth and remove waste products. Furthermore, endothelial cells can provide paracrine growth and survival signaling to the tumor even before the blood starts flowing [55, 56]. The AS leads to decreased apoptosis of cancer cells by up to seven folds while their proliferation rate remains similar to the prevascular stage, leading to a rapid expansion of the tumor mass [57]. Once angiogenesis is launched, the tumor may grow locally and shed isolated cells or clusters of cells (disseminated tumor cells – DTCs) into the blood stream. Most DTCs die by anoikis or as a result of the attack of the immune system. A few DTCs may land in distant organs and go to another phase of dormancy then awaken at some point to form cancer metastases [58, 59]. Metastases are responsible for 90% of mortality in breast cancer and other solid tumors [60, 61].

Angiogenic dormancy is the phase that extends from the formation of the prevascular tumors to the point of the AS and is a part of tumor dormancy where immune and local factors play an important role to maintain the tumor at the prevascular stage. Tumor dormancy is common in most primary solid tumors and in DTCs and may extend to several years or decades. Local inflammation and age-related deterioration of the immune system in addition to the acquisition of genetic/epigenetic abnormalities lead eventually to a break of the equilibrium that maintains the dormant state and allows the tumor to start growing.

It was believed by some investigators and the lay public that surgical removal of the primary tumor might allow the micrometastases or DCTs to start growing as a result of removing a putative angiogenesis suppressive factor released by the primary (angiostatin) [62]. To date, this scenario is considered very uncommon and the search for this putative inhibitor in humans has been unsuccessful. When rapid progression of metastatic disease happens after surgery, it is very likely the result of dumping large amounts of cytokines and growth factors in the blood rather than the removal of a putative angiogenesis suppressive substance with the surgical resection of the tumor [63].

A few patients present with metastases at the same time the primary tumor is discovered. However, the most common pattern of cancer behavior is the long

interval between the radical removal of the primary tumor and the diagnosis of metastatic disease. This pattern is observed in most solid tumors such as breast cancer, colon cancer, kidney cancer, melanoma, Ewing's sarcoma and many other tumor types. Continuous growth model does not explain this pattern. Recent reports indicate that once metastases become clinically detectable, they display similar growth rate to the primary tumor that is independent of the number of years of dormancy and suggest that cancer cells undergo different dynamic during this phase [64]. The dormancy phase depends on the suppressive role of the immune system that succeed in overcoming the proliferative and angiogenic drive of cancer cells for many years.

A good example of the interaction between cancer cells, the microenvironment and the immune system is breast cancer. Breast cancer DTCs locate preferentially to the bone [65–67]. The bone marrow microenvironment offers DTCs two nurturing niches: the osteoblastic (for dormant hematopoietic stem cells -HSCs) and the vascular (for actively dividing HSCs) niches [68]. Using CXCR4, breast cancer DTCs interact with fibroblasts that express stromal derived factor-1 (SDF-1) or CXCL12, CXCR4 ligand [68]. Osteoblasts secrete many cytokines such as angiopoietin-1 and stromal cell factor that help retain HSCs and cancer cells in the niche and by activating the Notch pathway they promote cancer cell stemness and block their differentiation, which sustain tumor dormancy [69]. Post-menopausal estrogen deprivation increases bone turn-over and allows the release of many growth factors embedded in the bone (IGFs, TGFb), which may rescue estrogen deprived cancer cells (IGFs) or suppress the immune cells infiltrating and surrounding the tumor (TGFb). Finally, age-associated immune deterioration may create the conditions for the emergence and growth of metastases.

Pericyte recruitment is an important step in blood vessel maturation. This step is largely imperfect or missing in tumor angiogenesis leaving blood vessels immature and leaky [70]. The recruitment of these cells is considered essential to provide survival signaling to ECs of nascent blood vessels and to control blood flow to the tumor and fluid shifts between the blood vessels and the tumor interstitial compartment [70].

2.9 Physiologic Competence of Tumor Blood Vessels

Tumor blood vessels have been extensively characterized in a variety of animal models and in many imaging and histological studies in human tumors [71–74]. The basic understanding is that the vasculature supporting a human tumor is comprised of many different components, including co-opted vasculature that existed in the tissue of origin before the tumor grew, new blood vessels formed from the natural process of angiogenesis as a tissue increases its demand for nutrients and a portion of vascular structures that may be created due to the unique nature of rapid tumor cell growth (vascular channels, vascular mimicry etc.) [75–77]. As a result, there are heterogeneous regions of flow, nutrient diffusion and hypoxia/necrosis in most solid tumors. The mixture of vascular supply routes that a solid tumor employs to continue to grow and progress result in a variety of abnormal features to be present in the solid tumor that rarely, if ever, occur in normal tissues. The major features of

tissue physiology that are found to be at levels above or below that found in normal tissue are what are commonly referred to as the '5 Ps' or, Perfusion, Pressure, Partial pressure of oxygen, Permeability and PH. Any one of these microenvironment readouts can drive altered stress response and expression of genes thought to be involved in tumor progression, involving an angiogenic response [78, 79].

2.10 Evaluation of Angiogenesis and Prediction of Response to Angiogenic Therapy

The true angiogenesis that occurs in the tumor as the nascent blood supply is outgrown and microenvironmental cues are rapidly produced by cells that become hypoxic or acidic can be monitored and even quantified by a variety of methods. One of the hallmarks has been to measure the levels of VEGF in the circulation in a patient. Other proteins involved in the growth and maintenance of blood vessels have also shown to have promise. An example is the family of receptors and ligands related to VEGF function. For example, we observed that circulating tie2 receptor was implicated in response to anti-angiogenic therapy with bevacizumab in a clinical trial of breast cancer patients [80]. Additionally, germline variants that are present in varying levels in different patients of various proteins involved in angiogenic activity can be shown to track with treatment outcome [81]. Classical methods to assess angiogenesis in preclinical models include live tissue monitoring with intravital microscopy, histological evaluation of vessel density and a variety of in vitro endothelial cell assays that can assess growth rate, formation of tubules or other vascular-like structures, migration and branching of cell structures. Examples of these are as follows:

In vitro The most basic assessment of an anti-angiogenic effect in vitro is to evaluate the ability of a drug or other agent to inhibit EC proliferation. Therefore, a number of cytotoxic or cytostatic agents have been suggested to be anti-angiogenic. However, to truly be anti-angiogenic and not simply cytotoxic, the agent should also have more specific and selective action against cellular hallmarks of blood vessel development such as motility, migration and ability to form microtubules suggestive of a lumen in an actual blood vessel. These approaches have been well tested and studied for a host of therapeutic approaches thought to have anti-angiogenic properties [82–84].

In vivo The available assays to assess ongoing or completed angiogenesis in a tumor tissue are mostly limited to various imaging approaches that measure aspects of the circulation (volume, velocity, total flow) and histological tests that look for the amount, size and density of vascular structures in a tissue [85–87]. There are also several 'ex vivo' tissue based assays that can be used to assess various aspects of angiogenesis potential and completion. These include the chick choriollantoic membrane (CAM) assay and the aortic ring assay. In the CAM, the agent of interest is added to a developing chick embryo and the qualitative changes in amount and normal appearance of vessels in the egg are imaged [88]. The aortic ring assay involved the preparation of a cross section of aortic tissue from either rat, mouse or other small animal. The cross section (appearing like a ring) is bathed in a maintenance medium

and the therapeutic agent is added as desired. Over several weeks, the normal response of the ring is to sprout new blood vessels which grow outward from the ring. The amount and length of these 'angiogenic' protrusions can be measured and used as a guide to understand the potential of the approach to inhibit natural angiogenesis [89]. We and others have also measured the angiogenic capacity of ECs in soft agar or matrigel by assessing the degree by which 'vessels' or tubes form in the medium [89, 90] and the extent to which ECs inoculated into matrigel plugs can grow into functional vascular networks when the plug is implanted into experimental animals.

2.11 Clinical Evaluation of Prognostic and Predictive Markers of Angiogenesis

To date, no validated marker (or markers) is available to predict response of cancer to anti-angiogenic therapy, to assess escape or resistance mechanisms or predict toxicity from these agents [91]. Anti-angiogenic agents were introduced to clinical practice more than 10 years ago (first anti-angiogenic drug was approved in 2004, bevacizumab in mCRC) without clear understanding of their mechanism of action and without biomarkers for patient selection. These markers are needed to help select the patients who are more likely to respond to these expensive and potentially toxic treatments and to help evaluate mechanisms of resistance, so appropriate actions can be undertaken [91]. Considering that cancer is a dynamic and heterogeneous process in space and time and due to the complexity and redundancy of angiogenesis one marker may not be appropriate for all cancers, all the time and with all anti-angiogenic drugs. Hence the difficulty that this field has encountered from its inception.

Several methods have been used to assess angiogenesis in the clinical setting. (1) Systemic markers. One of the findings of clinical trials using angiogenic inhibitors was the possible correlation between the development of hypertension and benefit from these treatments. However, this marker develops while the patients are on treatment and cannot be used for pre-treatment selection. (2) Circulating blood markers are very interesting because of easy accessibility to blood or urine samples and the possibility to repeat the tests as needed. However, no marker (or group of markers) has shown to date a strong predictive or prognostic value to be used routinely in the clinic. (3) Tumor tissue-based markers are difficult to adapt to routine practice but may inform our understanding of the biology of angiogenesis and the effects of different interventions on the system. For example, microvessel density is now accepted as a prognostic marker but it was unable to predict for response to treatment. (4) Imaging methods need to be refined and validated. Their role may become crucial in the future as we discover new imaging modalities with better predictive and prognostic value [92]. Most, if not all, of these markers were discovered from the retrospective analysis of prospectively conducted phase III clinical trials or from single arm phase II trials. Based on the experience with different cancers and agents, it is unlikely that we will be able to find one markers that would fit all [91]. Table 9.3 summarizes the available makers.

Table 9.3 Prognostic and predictive markers of angiogenesis

	Agent	Value	Tumor type	Strength of the evidence
Clinical				
Hypertension	Bevacizumab	Predictive	CRC, breast, RCC	Moderate
Blood and germ line SNPS				
Circulating VEGF	Bevacizumab, sunitinib	Prognostic	CRC, NSCLC, RCC	Weak
SNPs in VEGF and VEGFR2	Bevacizumab	Predictive	CRC, breast	Weak
SNP VEGF-2578AA	Bevacizumab	Prognostic	Breast	Moderate
Circulating PlGF	Anti-VEGF Tx	?	CRC, others	Weak
Blood LDH	Bevacizumab	Prognostic	CRC	Weak
Il-8A-251 T	Bevacizumab	Predictive	Ovarian	Moderate
Increase plasma IL-6 on bevacizumab	Bevacizumab	Predictive	CRC, ovarian and HCC	Moderate
High serum EGF & macrophage-derived chemokine and low IL-10, IL-6, and IL-8	Anti-VEGF Tx	Predictive	CRC	Moderate
Circulating EC and bone marrow-derived EPC	Bevacizumab	Predictive	CRC, HCC	Weak
Tumor-based methods				
Tumor expression of VEGF	Bvacizumab	Predictive	CRC, breast	Weak
Microvessel density (MVD)	Multiple angiogenic Tx	Prognostic	CRC, others	Moderate
Elevated LDH and VEGFR1 mRNA in tumors	Vatalanib	Prognostic (high = better)	CRC	Moderate
Increased HIF and VEGFR2 mRNA levels in tumor	Vatalanib	Prognostic (high = worse)	CRC	Moderate
MVD	Vatalanib	Prognostic (high = better)	CRC	Moderate
MMR-D vs. MMR-P	Multiple angiogenic Tx	Prognostic (MSI high = better)	CRC	Moderate
TAMs (CCL18)	Multiple angiogenic Tx	Prognostic (high = worse)	CRC, breast	Weak

(continued)

Table 9.3 (continued)

	Agent	Value	Tumor type	Strength of the evidence
Imaging methods				
MRI and dynamic-contrast enhanced – DCE MRI	Multiple angiogenic Tx	Prognostic	CRC, breast	Weak
DEC CT	Multiple angiogenic Tx	Prognostic	CRC, breast	Weak
PET	Multiple angiogenic Tx	Predictive	CRC	Weak
Contrast enhanced US	Multiple angiogenic Tx	Predictive	CRC, HCC	Weak

CRC colorectal cancer, *HCC* hepatocellular carcinoma, *RCC* renal cell carcinoma; strength of the evidence was defined by the type of studies used to validate the marker. *Strong* If the marker is validated in prospective RCTs, *Moderate* when the marker is validated in retrospectively in phase III clinical trial,*Weak* when the marker is derived from phase II studies or results conflict regarding the findings

3 Tumor Lymphangiogenesis

Most solid tumors are thought to have a deficit in the available lymphatic vasculature present in comparison to the blood supply vasculature, and this is why there are documented features of solid tumors that include a higher than normal interstitial pressure and a general immune suppressive environment [93–95]. However, albeit deficient and abnormal, there is a lymphatic angiogenic response as the tumor grows which may be as important as or more important to understand than the more traditional angiogenic response of blood supply vessels [96]. It is also of interest to note that a healthy/normal lymphatic vasculature could be critical for the body to control solid tumor development. Thus, while some or many anti-angiogenic agents may be able to control or slow the progression of tumors initially due to the blockage of an adequate blood supply, a later and unwanted effect of these therapies may be the destruction or inhibition of lymphatic function, ultimately leading to a residual tumor mass less able to be addressed by the immune system and more resistant to other drug-based therapies due to exacerbated interstitial fluid pressure imbalances between the mass and the surrounding normal tissue environment. Various targeting strategies against lymphangiogenesis or tumor cell trafficking in the lymphatics have been developed and are waiting for clinical testing to prove if they are feasible approaches to control metastasis and/or the primary tumor [97–99].

4 Targeting Angiogenesis

4.1 FDA Approved Drugs

Approved angiogenic inhibitors developed over the past two decades fall into two categories. (1) Small molecules that target the tyrosine kinase function of VEGFR alone or with other receptors involved in angiogenesis (Table 9.4) [100, 101]. (2)

Table 9.4 Small molecules targeting angiogenesis.

Agent	US approval indications	Targets	Side effects
Axitinib	Advanced RCC (after failure of one prior systemic therapy)	VEGFR-1 to -3, PDGFR-b, and c-KIT	Diarrhea, hypertension, fatigue, and decreased appetite
Cabozantinib	Metastatic MTC (progressive) advanced RCC in patients who have received prior anti-angiogenic therapy	VEGFR-1 to -3, c-Met, RET, c-KIT, TRKB, FLT-3, AXL, and TIE2	Gastrointestinal (GI) perforation, GI fistula, and severe hemorrhage
Lenvatinib	Radioactive iodine refractory differentiated thyroid cancer and advanced RCC	VEGFRs	Hypertension, fatigue, diarrhea, nausea/vomiting, arthralgia/myalgia, proteinuria, palmar-plantar erythrodysesthesia, and dysphonia
Nintedanib	Idiopathic pulmonary fibrosis	VEGFR-1 to -3, PDGFR-a and -b, and FGFR-1 to -3, FLT-3 and Src	Thromboembolism, myocardial infarction, GI perforation, Cardiomyopathy, stroke, Hypertensive crisis
Pazopanib	Advanced RCC, advanced STS (after prior chemotherapy)	VEGFR-1 to -3, PDGFR-a and -b, FGFR-1 and -3, and c-KIT, Itk, Lck, c-Fms	Severe and fatal hepatotoxicity has been reported
Regorafenib	Metastatic CRC (previously treated); GIST (locally advanced, unresectable or metastatic; previously treated with imatinib mesylate or sunitinib malate)	VEGFR-1 to -3, PDGFR-a and -b, FGFR-1 and -2, TIE2, c-KIT, RET, RAF-1, and $BRAF^{V600E}$, DDR2, TrkA, Eph2A, SAPK2, PTK5 and Abl	Fatal hepatotoxicity has been reported
Sorafenib	Unresectable HCC; advanced RCC; thyroid carcinoma (locally recurrent or metastatic, progressive, differentiated; refractory to radioactive iodine treatment)	c-CRAF, BRAF, mutant BRAF, c-KIT, FLT-3, RET, RET/PTC, VEGFR-1 to -3, and PDGFR-b	Lymphopenia, Hypophosphatemia, Exfoliative, dermatitis, Hypertension, MI, diarrhea, fatigue, renal failure, abdominal pain weight loss, neutropenia, thrombocytopenia, Dyspnea,
Sunitinib	GIST (after disease progression or intolerance to imatinib mesylate); advanced RCC; pNET (progressive, well differentiated; unresectable locally advanced or metastatic)	VEGFR-1 to -3, PDGFR-a and -b, FLT-3, c-KIT, CSF-1R, and RET	Fatal hepatotoxicity may occur

(continued)

Table 9.4 (continued)

Agent	US approval indications	Targets	Side effects
Vandetanib	MTC (unresectable, locally advanced or metastatic)	VEGFR-2 and VEGFR-3, EGFR, RET, BRK, TIE2, EPH receptors, and Src signaling pathways	Prolongs QT interval; restricted distribution program

Monoclonal antibodies or fusion proteins that target VEGF, VEGFR or other angiogenic pathways (Table 9.5) [101].

4.2 Mechanism of Action of Antiangiogenic Drugs

VEGF/VEGFR pathway is a typical example of angiogenic pathways (Fig. 9.2). The VEGFR is a trans-membrane protein with an extracellular domain, transmembrane domain and intracellular domain that carries the tyrosine kinase activity. Upon binding of the ligand (VEGF A) with the ligand binding site, two VEGFR monomers dimerize and undergo conformational changes that activate their tyrosine kinases leading to cross phosphorylation and activation of down stream signaling pathways and the execution of the intended functions of proliferation, increased vascular permeability, migration and survival of ECs.

Antiangiogenic small molecules are hydrophobic and diffuse easily intracellularly then interact with the tyrosine kinase function by competing with ATP of the tyrosine kinase and inhibit down stream signaling. Monoclonal antibodies interact either with the ligand, VEGF (bevacizumab, aflibercept) or the receptor, VEGFR (ramucirumab).

Inhibiting the VEGF/VEGFR pathway leads to interruption of survival and proliferative signaling of ECs. The ultimate outcome is variable depending on the context in which ECs exist. ECs that depend exclusively on VEGF will undergo apoptosis leading to "collapsed vessels," while the ones that have started maturing may be able to go on to complete their pericyte coverage and become more "normal like" ECs [102]. Due to cancer heterogeneity and the presence of variably vascularized areas of the tumor, the areas of the tumor supplied by the "collapsed blood vessel" will undergo hypoxic cell death while the areas supplied by the "normalized" blood vessels will grow faster. The ultimate outcome will depend on the ratio of "collapsed to normalized" vessels. If the tumor mass is now served by a "normalized" vascular network, interstitial fluid pressure drops and blood flow increases in the tumor. This may be associated with better delivery of chemotherapy to the center of the tumor, explaining the synergistic effect of the combinations of chemotherapy and the antiangiogenic bevacizumab [103]. Conversely, tumors served by predominantly immature vessels may respond with massive collapse of their vasculature and an abrupt necrosis of the tumor ensues. Triple negative, highly proliferative breast cancers are an example of this phenotype [104].

Table 9.5 Monoclonal antibodies and fusion proteins targeting angiogenesis

Agent	US approval indication	Targets	Side effects
Bevacizumab	Approved for metastatic colorectal cancer, renal cell cancer, non small cell lung cancer (NSCLC), cervical cancer, ovarian cancer, and malignant glioma; used in combination with chemotherapy or as a single agent	VEGF	Gastrointestinal perforation, surgery/ wound healing complications, and hemorrhage
Ramucirumab	Advanced gastric or gastro-esophageal junction adenocarcinoma, as a single agent or in combination with paclitaxel; for metastatic non-small cell lung cancer in combination with docetaxel; and for metastatic colorectal cancer in combination with FOLFIRI	VEGFR2	Increased risk of hemorrhage that may be severe and sometimes fatal Neutropenia Hypertension, asthenia Fatigue, stomatitis Bleeding GI bleeding
Olaratumab	Soft tissue sarcoma not amenable to curative treatment with radiotherapy or surgery, in combination with doxorubicin	PDGFR-alpha	Infusion-related reactions (IRR)
Ranibizumab	Age-related macular degeneration (AMD), macular edema after retinal vein occlusion, diabetic macular edema (DME), diabetic retinopathy in patients with DME, and myopic choroidal neovascularization (CNV)	VEGFs	NA
Ziv-aflibercept	Metastatic colorectal cancer in combination with FOLFIRI chemotherapy in tumors resistant to or that progressed following an oxaliplatin-containing regimen	Humanized recombinant fusion protein that binds with high affinity to VEGF-A, VEGF-B, and placental growth factors	Gastrointestinal perforation, surgery/ wound healing complications, and hemorrhage

4.3 Resistance to Antiangiogenic Drugs

The therapeutic benefits of antiangiogenic agents are real but limited in duration. When these agents work they result in improvement of progression free survival or response rate in the metastatic setting that are translated into survival benefits in some but not all cancers. In the adjuvant setting, antiangiogenic agents were not

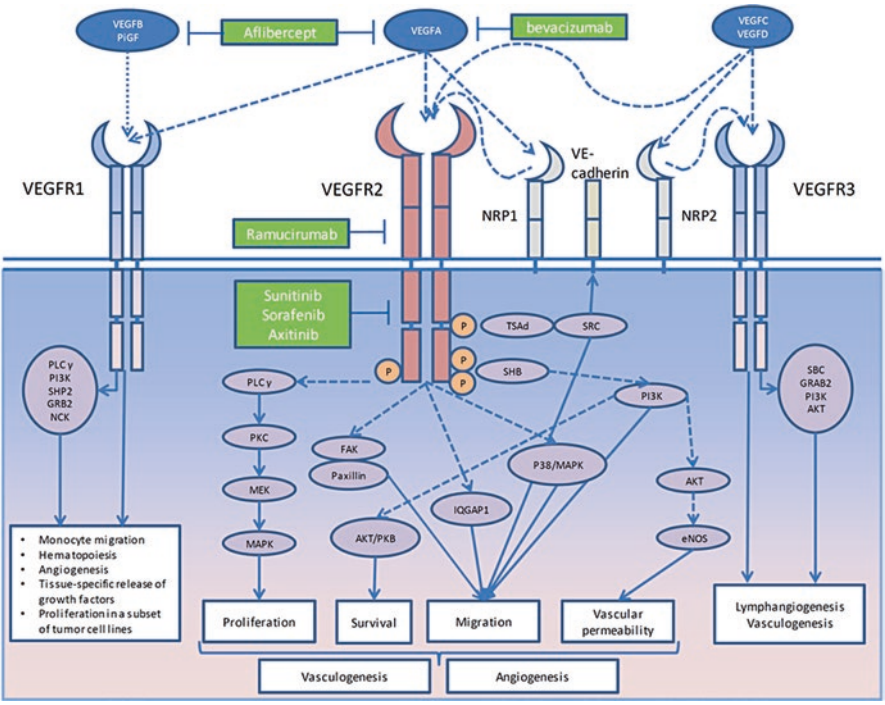

Fig. 9.2 VEGFA is the preferred ligand of VEGFR2, which is the main signaling receptor involved in angiogenesis. Binding of VEGFA to the ligand binding pocket of the VEGFR2 is followed by dimerization and trans-phosphorylation of the intracellular domain on tyrosine moieties. This is followed by the activation of cascade of downstream steps leading to the expression of genomic programs involved in proliferation, survival, migration and increased vascular permeability of the endothelial cells. The signal can be interrupted by targeting the ligand (PlGF and/or VEGF; example: aflibercept and bevacizumab), the extracellular domain of the VEGFR with antibodies (example: ramucirumab) or its intracellular domain with small molecules (tyrosine kinase inhibitors: sunitinib). VEGFR1 participate in angiogenesis but it is more involved in bone marrow cell trafficking and VEGFR3 is involved in lymphangiogenesis. NRP1, 2, neuropilin 1 and 2. NRP1 and 2 do not possess intracellular domains (From Ferrara and Adamis [105] with modifications)

able to show any benefits in colon, breast or lung cancers when they were added to standard of care in phase III RCTs.

Primary resistance to antiangiogenic therapy is defined by the absence of any benefits when these drugs are compared to best supportive care or to standard of care with and without the agent. This is reflective of the lack of a marker that preselects patients upfront before treatment administration. Secondary resistance is the progression of the cancer after an initial response. Resistance to small molecule tyrosine kinase inhibitors, usually used as single agents, can be conceived as either related to the development of mutations at the binding sites or as a result of inactivation of the drug by the tumor or as a result of activation of other angiogenic pathways that would compensate for the inhibited one. To date, there is no convincing

evidence of the presence of mutations in VEGFA or its cognate receptors [105]. Monoclonal antibodies and protein conjugates are used in combination with chemotherapy in most cases and progression can be conceived as a result of resistance to the chemotherapy, the antiangiogenic therapy or to both. The strategy of continuing the antiangiogenic agent beyond progression with a different chemotherapy is based on the assumption that progression occurs as a result of resistance to chemotherapy no to the antiangiogenic therapy. RCTs built on this premise showed real but modest clinical benefits [106].

Another model for resistance to antiangiogenic therapy assumes that alternate angiogenic pathways are activated by the tumor to circumvent the therapeutic blockade. These include FGF, PDGF and other pathways [107–110]. Alternatively, protection of the tumor vasculature may be provided by recruiting pro-angiogenic inflammatory cells [111–114] or by reinforcing the pericyte coverage, which provides contact and paracrine survival signaling to endothelial cells obviating the need for VEGF [102, 115, 116]. Other mechanisms include co-option of normal vasculature and increased metastatic seeding in lymph nodes and distant organs [107].

Some experimental models suggested that the inhibition of the VEGFA/VEGFR pathway leads to more aggressive and metastatic phenotype as a result of hypoxia and activation of EMT that takes all its magnitude at the time of interruption of the antiangiogenic treatment [117]. Pooled analysis of disease course patterns of patients randomized on bevacizumab phase III trials who discontinued the drug did not seem to confirm this hypothesis [118].

5 Therapeutic Implications in Different Cancers

5.1 Colorectal Cancer

5.1.1 Major Angiogenic Pathways

For colorectal cancer, the oncogenic hits are not clearly defined but upwards of 50% of such tumors are driven by a mutant Ras oncogene(s) (mainly K-ras and to lesser degree N-ras and H-ras). B-raf, HER-2 and activation of Akt/PTEN loss account for additional ~15%. VEGFA is a prominent player downstream of many of these oncogenic pathways, which explains the actual pharmacologic antiangiogenic agents studied and eventually approved in colorectal cancer. All approved agents either directly target the ligand—VEGFA (bevacizumab and aflibercept) or its receptors (ramucirumab). Ancillary role is possible for other pro-angiogenic factors such as other members of the VEGF family, VEGFB, VEGFC, VEGFD and VEGFE, or the placental growth factors (PlGF)-1 and -2 and their receptors, VEGFR1, VEGFR2 and VEGFR3. VEGFR2 is mainly expressed in the vasculature and is the key mediator of VEGF-induced angiogenesis and has been pharmacologically targeted with small molecule tyrosine kinase inhibitor (e.g. regorafenib) or monoclonal antibodies (e.g. ramucirumab).

5.1.2 Approved and Studied Drugs Targeting Angiogenesis in Colorectal Cancer

Bevacizumab: was first approved in colorectal cancer and is the agent that was studied more than any other antiangiogenic agents as attested by the number of trials and the bulk of the data available. Moreover, many oncologists view the other available agents as 'very similar' to bevacizumab with reasonable justification for that stand.

Historically, bevacizumab represented the inaugural product for angiogenic therapy in cancer. It was studied in combination with the then-standard regimen of bolus 5FU/leucovorin + irinotecan (IFL), a regimen used as first-line therapy for metastatic colorectal cancer [119]. In the pivotal study led by Hurwitz, the addition of bevacizumab to IFL was proven in this large prospective clinical trial to be superior to the cytotoxic regimen alone [120]. The impact on median overall survival exceeded 4 months (20 vs. 16 months) with acceptable toxicity, a finding that has very few parallels in medical oncology. The drug, and in fact the whole concept of angiogenesis as a novel approach to cancer therapy was met with sensational enthusiasm and almost considered a triumph of cancer biology. Later, far less impressive results and reports about some serious toxicities were generated but were actually insufficient to shake this initial aura of success and the drug continues to be used widely in today's oncology clinics across the US [121].

Bevacizumab has been added to the two commonly utilized chemotherapeutic regimens (FOLFOX and FOLFIRI). Moreover, the drug has been used even after progression on first line therapy with reasonable support of such use [106].

The results of individual trials tend to vary and at times seem conflicting. To objectively estimate the impact of angiogenic therapy in metastatic colorectal cancer, and realizing the substantial heterogeneity that complicates most clinical oncology datasets, pooled analysis emerge as our best tool to provide such estimate. One such pooled analysis of trials comparing chemotherapy with or without bevacizumab in untreated patients, bevacizumab was associated with 19% reduction in the risk of death, and a mere 2 months gain in the median overall survival (19.8 compared with 17.6 months) [121].

Lastly, bevacizumab has been the only anti-angiogenic drug to be tested in the adjuvant setting in colon cancer. The study—as designed was definitely negative with no benefits in disease-free or overall survival. That negative study is probably the main reason to extinguish any interest in examining this drug in the adjuvant setting. Issues that need to be reconsidered include, at a minimum, the dose (antagonizing microscopic disease as opposed to gross macroscopic metastatic cancer) and the duration of therapy (the risk of relapse post-operatively clearly extends beyond the 1 year applied in the pivotal National Surgical Adjuvant Breast/Bowel Project NSABP C-08 trial) [122]. The differences between the adjuvant and metastatic settings require special considerations and appropriate adjustments.

Aflibercept Aflibercept (VEGF Trap, Zaltrap) is a recombinant fusion protein made of VEGF binding domains of human VEGFR 1 + 2 fused to the Fc portion of human immunoglobulin G1 [123]. Though this novel pharmacologic intervention, a circulating decoy receptor is theoretically an advantage antagonizing factors that bind to the VEGFRs 1 + 2, (VEGF-A, VEGF-B, and PlGF), the clinical results are certainly no better than bevacizumab. This may be considered as an indirect confirmation of the importance of VEGFA in tumor angiogenesis.

When added to irinotecan-regimen (the widely used FOLFIRI) in patients progressing after 1st treatment with or without bevacizumab. In a prospective randomized trial, a rather modest increment in median overall survival was observed in patients treated with aflibercept (13.5 vs. 12.1 months). Given the modest impact and its considerable cost, this drug is used at markedly less frequency in the US oncology clinics [123].

Ramucirumab This is a recombinant monoclonal antibody with specificity to VEGFR-2, antagonizing its activation. The efficacy of ramucirumab has been tested in second-line treatment of metastatic colorectal cancer patients in a RCT. Results of this intervention again produced rather modest results with approximately 6-week increment in the median overall survival [124]. The FDA did grant approval in second line (as tested) but the incorporation of this drug into the US oncology clinics has been again sluggish as one can understand. Of note, the toxicities reported in ramucirumab and aflibrercept have been categorically similar to those seen with bevacizumab, lending support to the idea that antiangiogenic agents have a class effect—hypertension, proteinuria, wound healing and fistula formation.

Regorafenib This is an oral drug which was introduced as a multi-kinase inhibitor with known effects against VEGFR1–3, among other tyrosine kinases implicated in angiogenic and tumor growth-promoting pathways. The drug's activity in refractory metastatic colorectal cancer was proven against placebo with modest impact at best. Median overall survival was prolonged by 1.4 months, and the drug was approved by the FDA [125]. The drug is used as a salvage treatment in patients who have generally exhausted combination therapies and especially in patients who favor oral therapy, an opinion that attract increasing proportions of patients approaching the end of life.

5.1.3 Future Directions

The failure to identify a biomarker to preselect patients for angiogenic therapy and the use of a simplistic scheme (with the same dose regardless of tumor bulk, degree of angiogenic anomaly, or even the treatment setting—metastatic versus adjuvant) remain pressing issues that will determine future progress in the field of CRC cancer angiogenesis.

5.2 *Gastric Cancer*

5.2.1 Major Angiogenic Pathways

Although, gastric cancer is approached as one entity with minor sub-classification (Histologically, gastric adenocarcinoma can be classified into the intestinal type – cohesive neoplastic cells forming gland like tubular structures – and the diffuse type with a thickening of the stomach wall without a discrete mass) modern genomics provided more information about the underlying genetic abnormalities (expression of EGFR or VEGF and amplification of *HER2* or c-MET) [126, 127].

One can infer that VEGF signaling is a player in this disease given the activity of VEGFR-2 monoclonal antibody. Moreover, elevated serum and tumor levels of VEGF are associated with a poor prognosis in patients with resectable gastric adenocarcinoma [128, 129].

5.2.2 Anti- angiogenesis Drugs in Gastric Cancer

The impact of ramucirumab on disease outcome in this malignancy is rather modest again as seen in other indications, approved agent will need better biologic classification.

Ramucirumab was subjected to the gold standard testing in 2 large RCT s. The first trial was against placebo in 355 patients after progression on standard chemotherapy (second line). Though the benefit was statistically significant, the impact was very modest with less than a month difference in progression (2.1 versus 1.3 months), and overall survival results were equally underwhelming (5.2 versus 3.8 months) [130]. The second trial added Ramucirumab to paclitaxel chemotherapy in second-line, and was a rather large randomized trial (665 patients). Overall median survival increment of 2.2 months was reported (9.6 versus 7.4 months) providing justification for the FDA approval [131].

Multiple trials examining antiangiogenic agents were performed but many were small single arm trials (no comparator arm) that were all negative. Noteworthy among all these trials is the negative trials of bevacizumab. A large prospective randomized trial tested the addition of bevacizumab to capecitabine and cisplatin and failed to confirm significant survival advantage [132]. Other agents tested and found to be ineffective included sunitinib, regorafenib, apatinib, aflibercept, and sorafenib.

5.2.3 Future Directions

Not unlike other malignancies in the GI track or elsewhere, better classification of disease on sound biologic (as opposed to anatomic) basis and work towards biomarker development to allow clinicians and scientists to identify the appropriate patients and guide treatment in real time seems to be unavoidable for more meaningful progress in this field.

5.3 Liver Cancer

5.3.1 Major Angiogenic Pathways

Hepatocellular cancer (HCC) is notorious for having limited responsiveness to cytotoxic chemotherapy. The main stay of treatment is surgery (when possible and if the remaining remnant of the liver is normal), liver transplant, chemo- or radioembolization or directly inducing cellular damage using extreme temperatures. In the last decade or so, modest efficacy for a number of agents was reported and those systemic agents—mostly kinase inhibitors with broad list of putative targets, have been added to the armamentarium. Most of these drugs have the canonical angiogenesis receptors (e.g. VEGFR-2) among their potential targets. Whether they exert their effect – as limited as it may be – through action on tumor vasculature remains an open question even today.

5.3.2 Drugs Targeting Angiogenesis in HCC and Results of Clinical Trials

Sorafenib, a multi-kinase inhibitor is the only FDA-approved drug for HCC (approved in 2007) [133]. This is a small molecule that inhibits tumor-cell proliferation and tumor angiogenesis and increases the rate of apoptosis in a wide range of tumor models. Six hundred two HCC patients with Child-Pugh class A were randomized to receive sorafenib or placebo. Median overall survival was 10.7 months in the sorafenib group and 7.9 months in the placebo group (hazard ratio in the sorafenib group, 0.69; 95% confidence interval, 0.55–0.87; $P < 0.001$). Toxicity was manageable. Subsequently, a number of anti-angiogenic drugs were tested in HCC. Some of these agents were tested in first line and others after failure of the first line drug sorafenib and produced modest benefits at best. One unique notion in HCC might be that bevacizumab as a single agent had small impact on the disease while the general opinion in clinical oncology is that monotherapy with bevacizumab elsewhere was ineffective.

Regorafenib was compared to placebo in patients with well-preserved overall and liver health (573 patients of whom 379 were randomized to regorafenib and 194 to placebo) whose disease progressed after sorafenib first line and produced significant prolongation of median overall survival (10.6 versus 7.8 months; HR 0.63, 95% CI 0·50–0·79; one-sided $p < 0·0001$) [134].

Bevacizumab several small phase II studies using bevacizumab in combination with erlotinib or with oxaliplatin or capecitabine in second line showed variable times to progression (1.8–7.2 months) or overall survival (4.3–13.7 months). These results were not judged adequate to launch a larger phase III study especially when the side effects and quality of life were factored into the decision [135].

Sunitinib: Another orally active TKI that targets a variety of angiogenic receptors such as VEGFR, platelet-derived growth factor receptors (PDGFRs), KIT, RET, and FLT3. In a large head to head comparison between sunitinib (n = 530) and

sorafenib (n = 544) in first line treatment of HCC patients, sunitinib was not superior or equivalent but was significantly inferior to sorafenib [136].

Ramucirumab: in a large phase III trial, ramucirumab was compared to placebo in patients with HCC. Overall survival benefit was seen in patients with a Child-Pugh score of 5 (HR 0.80; 95% CI, 0.63–1.02; *P* = .06) and patients with baseline αFP levels of 400 ng/mL or more. Patients with Child-Pugh scores of 6 or 7 and 8 seem to derive no benefit from this treatment [137].

Brivanib inhibits VEGFR and FGFR [138]. A group of 395 patients with advanced HCC who received sorafenib in first line were randomly assigned (2:1) to receive brivanib or placebo plus BSC. Brivanib did not significantly improve OS.

5.3.3 Future Directions

The molecular basis of HCC remains only poorly understood. This will likely need better mechanistic classification in the future to subdivide the disease to its major subtypes—for example, the pathogenesis of cancers emerging due to Hepatitis B will differ from those arising after other viruses, or other carcinogens. In a recent sequencing effort of 81 Hepatitis B virus (HBV) positive cases, the most frequently noted oncogene was beta-catenin (~16%) followed by the Janus kinase 1 (JAK1), in ~9% of cases [139]. It follows then, that for the vast majority of cases, even within Hepatitis B positive cases remain to be determined or could vary substantially without a unifying disease mechanism. A more recent and more comprehensive study of HCC including Hep B and Hep C associated cases again revealed no dominant driver oncogene [140].

5.4 Lung Cancer

Angiogenesis plays a role in the tumor progression in general [141] and lung cancer in particular has been identified as a tumor type where angiogenesis (with VEGF pathway being the most important) is critical in tumor growth and metastasis [142]. Several clinical studies in patients with lung cancer have shown that the VEGF expression in tumor specimens is related to increased blood vessel formation, poor prognosis and decreased overall survival [143]. It is possible that certain VEGF isoforms that result from alternative splicing of the mRNA may contribute more to tumor development [144]. Different types of VEGF receptors also contribute differently to the neoplastic angiogenesis. VEGF receptor 2 appears to be more critical.

Two chemotherapy drugs that inhibit angiogenesis pathways are currently approved in the treatment of lung cancer: **bevacizumab** and **ramucirumab**. Both block the VEGF pathway in a different way and have been approved in combination to chemotherapy. Several others have shown promise and are currently being evaluated in clinical trials. The prototypic drug, which was approved in 2006 for the treatment of lung cancer, is bevacizumab.

5.4.1 Drugs Targeting Angiogenesis in Lung Cancer and Results of Clinical Trials

In a randomized phase III trial conducted by the Eastern Cooperative Oncology Group (ECOG), patients with advanced disease were randomized to bevacizumab with carboplatin and paclitaxel or carboplatin and paclitaxel alone [145]. Patients with squamous histology, hemoptysis and brain metastases were excluded. The primary end point was overall survival. Median overall survival was 12.3 months for the bevacizumab/chemotherapy arm and 10.3 months for chemotherapy alone (Hazard Ratio 0.79; $p = 0.003$). Median progression-free survival was 6.2 and 4.5 months (hazard ratio for disease progression, 0.66; $P < 0.001$) and response rates was 35% and 15% ($P < 0.001$), respectively. Rates of clinically significant bleeding were 4.4 and 0.7% ($P < 0.001$). There were 15 treatment-related deaths in the bevacizumab group, including 5 from pulmonary hemorrhage. VEGF levels before treatment did not correlate with overall survival.

In another randomized phase III trial the Avastin in Lung (AVAil), bevacizumab was tested in combination with another chemotherapy regimen (cisplatin and gemcitabine). In this trial there were 3 arms: bevacizumab at 15 mg/kg, bevacizumab at 7.5 mg/kg and placebo all in combination with chemotherapy in patients with advanced lung cancer [146]. Similarly to the ECOG study patients with squamous lung cancer, hemoptysis and brain metastases were excluded. Progression free survival was significantly improved in the bevacizumab arm compared to placebo (hazard ratio 0.75, $P = 0.0003$ and 0.85, $P = 0.0456$) both for the 7.5 and the 15 mg/kg group, respectively. Overall survival (OS) was not significantly increased with bevacizumab compared to placebo. It was thought that better or more effective second line therapies might have accounted for the lack of overall survival. A systematic review and meta-analysis [147] with data from 2194 patients from four phase II and III trials showed that bevacizumab significantly prolonged OS and PFS when added to first line platinum based chemotherapy in patients with advanced lung cancer. Bevacizumab significantly increased the risk of proteinuria, hypertension, hemorrhagic events, neutropenia, and febrile neutropenia.

Although, bevacizumab has shown efficacy in the first line setting in combination with platinum-based chemotherapy, the results of bevacizumab in combination with EGFR inhibitors have been disappointing [148]. Similarly, the recent results from a large randomized trial in the adjuvant setting where the addition of bevacizumab for up to 1 year to four cycles of standard chemotherapy did not lead to overall survival benefit [149].

In 2014, the Food and Drug Administration (FDA) approved ramucirumab for use in combination with docetaxel as a second line therapy after treatment with platinum-based chemotherapy. Ramucirumab is a fully humanized monoclonal antibody (IgG1) that binds to the extracellular domain of the VEGFR2. In a multicenter, double blind, randomized phase III trial (REVEL), patients with squamous or non-squamous NSCLC who had progressed after a first-line platinum-based chemotherapy regimen were randomized to receive docetaxel and either ramucirumab or placebo until disease progression, unacceptable toxicity, withdrawal, or death.

The primary endpoint was overall survival [150]. Median overall survival was 10·5 months for the ramucirumab arm and 9·1 months the placebo arm (hazard ratio 0·86; p = 0·023). Median progression-free survival was 4·5 months for the ramucirumab group compared with 3·0 months for the placebo group (hazard ratio 0·76; p < 0·0001). The most common grade 3 or worse adverse events in the ramucirumab were neutropenia, febrile neutropenia, fatigue and hypertension.

Given the success of monoclonal antibodies targeting angiogenesis in the treatment of lung cancer patients in the first and second line setting, a number of small molecule kinase inhibitors targeting the intracellular domain of the VEGFR (sorafenib, pazopanib, vandetanib, sunitinib, axitinib) have been studied. Results have been disappointing despite the fact that several of these agents have shown success in other tumor types.

5.4.2 Future Directions

The success of immunotherapy agents such as the PD-1, PD-L1 inhibitors and CTLA-4 inhibitors in lung cancer and other tumor types has created a lot of enthusiasm in regards to combination treatments. It appears that immunosuppression is possibly mediated by the VEEGF/VEGFR2 axis [151] and therefore the combination of one of these agents with existing or new anti-angiogenesis inhibitors should be explored. A phase I trial with ipilimumab and bevacizumab in melanoma patients showed a safe profile. Other trials with pembrolizumab and nivolumab with bevacizumab in variety of tumor types are currently ongoing.

Questions in regards to optimal drug scheduling/sequencing and potential biomarkers are currently being explored. In a study where positron emission tomography and radiolabeled docetaxel were used, bevacizumab resulted in significant reduction in the delivery of chemotherapy within 5 h and up to 4 days after the bevacizumab infusion [152]. These results are intriguing and raise the question for different drug sequencing. Similarly, there is no biomarker to improve the selection of patients who could benefit from this treatment. Ongoing efforts in other tumor types have also resulted in disappointing results [153].

A series of novel antiangiogenic agents have also emerged. These include vascular disruptive agents such as bavituximab, a monoclonal antibody against phosphatidylserine, a membrane phospholipid that is abundant in ECs. Recently, a phase III study (SUNRISE) was discontinued since the interim analysis demonstrated that the bavituximab plus docetaxel group did not show a sufficient improvement in overall survival as compared to the docetaxel group.

NGR-hTNF another vascular disruptive agent, which originates from the fusion of human tumor necrosis factor (hTNF) to the CNGRC peptide, is currently being explored. The CNGRCG peptide selectively targets tumor blood vessels by binding to an isoform of the CD13 receptor, which is present on endothelial cells of the tumor vasculature, while sparing the CD13-expressing molecules of normal tissues. An ongoing randomized phase II trial explores its role in the second line setting (NCT00994097).

In summary anti-angiogenesis in patients with lung cancer has shown a modest but true clinical benefit in a group of patients with advanced lung cancer. Most clinical questions at this point are focusing on selecting patients using a biomarker, identifying new agents and combining currently existing treatments with immunotherapy.

5.5 Breast Cancer

VEGF is the most important proangiogenic factor in breast cancer [154–156]. As breast cancer progresses, other proangiogenic factors get activated [157–159].

Animal studies using **bevacizumab** in xenograft models showed that tumors regress when the VEGF pathway is targeted [160]. However, results in humans have been disappointing. E2100 Intergroup phase III trial compared the combination of bevacizumab and paclitaxel with paclitaxel alone in first line metastatic breast cancer. The addition of bevacizumab led to a doubling of the response rate (18% vs. 36%) and progression free survival (PFS) (5.8 vs. 11.6 mo) [161]. Based on these results bevacizumab was first approved for breast cancer in first line metastatic breast cancer. The approval was contingent on the results of further studies, which ultimately did not demonstrate significant improvements in overall survival. Thus, FDA approval of bevacizumab for breast cancer was officially withdrawn in 2010. Studies of bevacizumab in the adjuvant setting were disappointing [162].

In the neoadjuvant setting, the addition of bevacizumab to chemotherapy results in increased pathologic complete response (pCR) [163]. The significance of achieving pCR with bevacizumab is not clear. While the results of neoadjuvant studies are promising, it is apparent that clinical response to bevacizumab is highly variable. For this reason, biomarkers are urgently needed to identify patients most likely to benefit.

5.6 Kidney Cancer

5.6.1 Major Angiogenic Pathways

Renal cell carcinoma (RCC) is well known to be a hypervascular tumor. Clear cell carcinoma is by the far the most common type of RCC. It appears that Hypoxia-Inducible-Factor-1 (HIF-1) activation plays a significant role in clear cell renal carcinoma progression. This activation could result from two major pathways: decrease in VHL which marks HIF-1 for degradation and, to a lesser degree, mTOR dependent pathway which is downstream from phosphatidylinositol 3-kinase (PI3K) pathway [164].

As a consequence of HIF-1 stabilization and activation, there is an increase in the expression of multiple genes contributing to the angiogenic process. HIF-1 induced

proteins to include VEGF and bFGF, which promote vascular permeability [165] and EC growth [166], respectively.

As the renal cell carcinoma grow, the immaturity of the newly formed vessels increases [167].

Drugs targeting RCC depends on the inhibition of VEGF or mTOR pathways. Two classes of medications can inhibit VEGF, tyrosine kinase inhibitors (TKI) or monoclonal antibodies.

5.6.2 Approved Drugs from Clinical Trials

The first class of drugs is TKI's targeting VEGF pathway:

Sunitinib is a TKI targeting VEGF as well as PDGF receptor. Active angiogenesis as measured by the presence of phosphorylated VEGF-receptor 2 can predict the response of RCC to sunitinib [168]. Motzer et al. compared the activity of sunitinib to interferon in a randomized clinical trial and resulted in statistically significant 5 month improvement in median overall survival in the first-line setting for metastatic good or intermediate risk RCC, despite about 7% crossover [169]. This improvement in survival established sunitinib as a standard control arm for future clinical trials. Side effects were notable for hypoglycemia and adrenal toxicity.

Pazopanib is another oral TKI. It has additional properties that include inhibition of FGFR 1& 3. This drug was compared to sunitinib in a RCT. Pazopanib showed PFS around 8.4 months with a similar overall survival benefit compared to sunitinib with probably less adverse side effects [170, 171].

The CABOSUN trial compared **cabozantinib** to sunitinib for intermediate or high-risk metastatic RCC. Cabozantinib is unique by its ability to inhibit additional target genes including MET and AXL genes; this inhibition might have contributed to its efficacy. The progression-free survival was better in the cabozantinib arm by about 3 months [172].

Axitinib is another oral TKI targeting VEGF receptors 1,2 and 3. There are no clinical trials to compare axitinib with sunitinib or pazopanib in the first line setting. The approval for this medication came from the fact that axitinib has higher efficacy with higher adverse events compared to sorafenib. However, this agent has a great clinical advantage as it allows for dose titration to increase tumor response depending on clinical tolerability. Median PFS was around 14.5 months with dose escalation [173]. Patients with severe uncontrolled hypertension should avoid axitinib.

Sorafenib is a potent small-molecule inhibitor of multiple TKIs, including VEGF receptor 2, PDGF receptor, and FGF receptor-1. Sorafenib did not show any improvement in PFS as compared to interferon. Therefore it has a limited role in the treatment of RCC.

TKI's, in general, are notable for causing hypertension and QTc prolongation. Therefore frequent EKGs are recommended (Tables 9.6 and 9.7).

The second class of drugs is monoclonal antibodies:

Bevacizumab is a monoclonal antibody to VEGF and prevents VEGF from binding and interacting with its receptor. AVOREN and CALGB 90206 trials tested

Table 9.6 clinical trials results for anti-angiogenic drugs used in RCC compared to interferon alpha

Drug	Hazard ratio (HR) for PFS with 95% confidence interval (CI)
Sunitinib [169]	HR 0.53, 95% CI, 0.45–0.64
Bevacizumab plus interferon [174]	HR 0.63, 95% CI 0.45–0.72

Table 9.7 clinical trials results for anti-angiogenic drugs used in RCC compared to sunitinib

Drug	Hazard ratio (HR) for PFS with 95% confidence interval (CI)
Pazopanib [170, 171]	HR: 1.05; 95% CI, 0.90–1.22
Cabozantinib [172]	HR: 0.65; 95% CI, 0.48–0.99
Sorafenib [175]	HR: 1.49; 95% CI, 0.92–2.38

the activity of bevacizumab when added to interferon in a randomized fashion [174, 176]. IFNa plus bevacizumab significantly increased progression-free survival in both trials.

Sequential inhibition of the VEGF pathway has been shown to be beneficial. Therefore, if a patient is suffering from tumor progression after a TKI or bevacizumab a trial of another TKI might be helpful. Examples are the use of axitinib after progression on sorafenib [177] or the use of sorafenib after procession on sunitinib [178].

The third class is mTOR inhibitors:

Previous clinical trials had shown temsirolimus to be more active than interferon as the first line for metastatic RCC [179]. Based on the results of this trial, temsirolimus was recommended for a patient with a high-risk disease. However when temsirolimus was compared with sorafenib in second line after progression on sunitinib, sorafenib was more effective [180].

mTOR inhibitors are associated with multiple side effects including hypersensitivity and pneumonitis.

Finally, lenvatinib, everolimus or the combination of lenvatinib and everolimus were compared in a 3-arm phase II study after progression on VEGF-targeted therapy [181]. The arms containing the antiangiogenic Lenvatinib results in significant PFS benefit.

5.6.3 Future Clinical Trials

Multiple new randomized trials are aiming at comparing the combination of antiangiogenic drugs with immune check point inhibitors. Below is a list of open trials:

A Phase III, Open-Label, Randomized Study Of Atezolizumab (Anti-PD-L1 Antibody) in Combination With Bevacizumab Versus Sunitinib in Patients With Untreated Advanced Renal Cell Carcinoma. NCT02420821.

A Multicenter, Open-label, Randomized, Phase 3 Trial to Compare the Efficacy and Safety of Lenvatinib in Combination With Everolimus or Pembrolizumab

Versus Sunitinib Alone in First-Line Treatment of Subjects With Advanced Renal Cell Carcinoma. NCT02811861.

A Phase III Randomized, Open-label Study to Evaluate Efficacy and Safety of Pembrolizumab (MK-3475) in Combination With Axitinib Versus Sunitinib Monotherapy as the First-line Treatment for Locally Advanced or Metastatic Renal Cell Carcinoma (mRCC) (KEYNOTE-426) NCT02853331.

A Phase 3, Multinational, Randomized, Open-label, Parallel-arm Study Of Avelumab (msb0010718c) In Combination With Axitinib (Inlyta(Registered)) Versus Sunitinib (Sutent(Registered)) Monotherapy In The First-line Treatment Of Patients With Advanced Renal Cell Carcinoma. NCT02684006.

5.7 Ovarian Cancer

Angiogenesis promotes tumor growth, ascites formation, and metastasis of epithelial ovarian cancer (EOC). Multiple clinicopathologic studies suggest that higher microvessel counts are associated with poorer outcome in both early and advanced stage EOC [182]. VEGF expression is higher in ovarian carcinoma compared to nonmalignant ovarian tumors and is associated with ascites, advanced disease, and poorer prognosis [183]. Furthermore, upregulation of VEGF and VEGF receptors in EOC has been associated with activation of signal transducers and activators of transcription suggesting that VEGF autocrine loops may stimulate growth and progression [184]. Multiple studies also suggest a role for PDGF and FGF in ovarian carcinogenesis. PDGF, FGF, and their receptors are expressed in EOC [185–190]. PDGF and receptor expression is associated with higher tumor grade and shorter survival, and receptor activation may stimulate angiogenesis through increased VEGF secretion in EOC [191, 192].

Bevacizumab is the most studied anti-angiogenesis agent in EOC in both the upfront and recurrent setting. The Gynecologic Oncology Group (GOG) protocol 218 was a 3-arm double-blind, placebo-controlled, randomized phase III trial that investigated the integration of bevacizumab in front line therapy for advanced EOC. Eligible patients included optimally and suboptimally cytoreduced stage III and any stage IV epithelial ovarian, fallopian tube, and primary peritoneal carcinoma. All arms included carboplatin and paclitaxel chemotherapy. The control arm received placebo with chemotherapy and placebo maintenance, the bevacizumab-initiation arm received bevacizumab with chemotherapy and placebo maintenance, and the bevacizumab-throughout arm received bevacizumab plus chemotherapy followed by bevacizumab maintenance. The bevacizumab-throughout arm had a longer progression free survival (PFS) compared to chemotherapy alone (median PFS 14.1 vs 10.3 months; HR 0.717; 95% CI 0.625–0.824; $P < 0.001$). There was no difference in PFS between the chemotherapy alone and the bevacizumab-initiation arms, nor was there a difference in overall survival (OS) between the three treatment arms [193]. The International Cooperative Group for Ovarian Neoplasia (ICON) 7 study was a 2 armed randomized trail comparing carboplatin and paclitaxel for 5–6 cycles to the same regimen plus bevacizumab starting cycle 2 followed by

maintenance bevacizumab. This trial included high-risk early stage (stage I or IIA with clear cell or grade 3 histology) and advanced stage (stage IIB-IV) EOC patients following primary surgery. Similar to GOG 218, ICON 7 showed that the addition of bevacizumab to standard adjuvant platinum-based chemotherapy followed by maintenance bevacizumab improved PFS compared to chemotherapy alone (median PFS 19 vs. 17.3 months; HR 0.81; 95% CI 0.7–0.94; $P = 0.004$), but did not provide a benefit in OS [194, 195].

The role of bevacizumab in the treatment of recurrent EOC has been investigated in both the platinum-sensitive and platinum-resistant setting. OCEANS, an industry sponsored trial, was a double-blind, placebo-controlled, randomized phase III trial that studied carboplatin and gemcitabine with or without bevacizumab in platinum-sensitive recurrent EOC patients. Bevacizumab or placebo were continued as maintenance following chemotherapy until disease progression or unacceptable toxicity occurred. The addition of bevacizumab improved PFS (median PFS 12.4 vs 8.4 months; HR 0.484; 95% CI 0.388–0.605, $P < 0.001$), but no significant difference in OS was detected [196, 197]. The AURELIA trial investigated single-agent chemotherapy (paclitaxel, topotecan, or liposomal doxorubicin) with or without bevacizumab in platinum-resistant EOC patients. Compared to single agent chemotherapy, the addition of bevacizumab improved PFS (6.7 vs 3.4 months; $P < 0.001$), but did not produce an overall survival benefit. The use of bevacizumab in the treatment of EOC is debated within the gynecologic oncology community due to the lack of proven benefit in overall survival [198]. Currently, bevacizumab is the only anti-angiogenesis therapy approved by the U.S. Food and Drug Administration for the treatment of EOC.

Recently other anti-angiogenic agents have been investigated in EOC including **pazopanib** and cediranib. Pazopanib maintenance following primary chemotherapy in advanced EOC patients provided improved PFS without survival benefit in a randomized phase III trial [199]. **Cediranib** has shown activity in recurrent EOC as a single agent and in combination with olaparib, a PARP inhibitor in phase II studies [200, 201]. Cediranib in combination with chemotherapy followed by maintenance therapy improved PFS in a platinum-sensitive recurrent EOC population [202]. The combination of anti-angiogenesis agents with other biologic agents represents a new treatment opportunity in EOC given its genomic complexity. There is currently an ongoing phase III trial comparing standard platinum-based chemotherapy to olaparib alone and olaparib plus cediranib in patients with platinum-sensitive recurrent ovarian cancer (NCT02446600).

5.8 Brain Tumors

5.8.1 Major Angiogenic Pathways and Related Chemotherapy/Radiotherapy Resistance

Angiogenesis plays a significant role in the development of highly aggressive glioblastoma multiforme (GBM). As a tumor grows rapidly, neo-angiogenesis is needed to supply tissues with oxygen and nutrients. Despite continuous activation of

angiogenic process, the inefficient immature neo-vasculature production results in a shortage of supply and causes necrotic tissues to form. In fact, the presence of tissue necrosis is considered one of the hallmarks of GBM's pathology. Moreover, the angiogenesis process involves increased production of hypoxia-related peptides, which seem to be related to tumor resistance to chemotherapy [203] and radiation [204]. Therefore, neo-angiogenesis is considered as a marker for worse outcomes.

Von Hippel–Lindau (VHL) is not commonly mutated in GBM. However, overexpression of hypoxia-induced factor 1 (HIF-1), typically degraded through VHL-mediated ubiquitination, might contribute to increased angiogenesis activity and chemoresistance in GBM [205]. Most aggressive GBM will have increased expression of both HIF-1 and vascular endothelial growth factor (VEGF) [206].

The other activated angiogenesis pathway is downstream from EGFR with the loss of PTEN, which opposes EGFR through AKT pathway. The loss of PTEN results in stem cell resistance to radiotherapy [207].

The concept of chemotherapy and radiotherapy resistance through those angiogenesis pathways is of particular importance in aggressive brain tumors because of those are extremely hypervascular tumors heavily dependent on angiogenesis.

5.8.2 Pseudoprogression and Angiogenesis:

De Wit et al. [208] (REF6) in 2004 described new tumor bed enhancement for GBM tumors treated with radiation therapy with or without carmustine. Recently this phenomenon was described in association with other therapies like temozolomide and radiation. Pseudoprogression happens as a result of increased tumor vessels permeability due to rapid and inefficient angiogenesis. The presence of this enhancement highlights the importance of angiogenesis and the inefficient neovascular formation in brain tumors pathology [209].

5.8.3 Approved Drugs Targeting Angiogenesis

Glioblastoma multiforme is one of the most highly vascularized of human tumors, which makes antiangiogenic therapy an attractive option for treatment.

Bevacizumab showed promising results in phase II GBM trials [210–212]. However, 2 phase III clinical trials did not translate those benefits into an overall survival benefit in the first-line setting:

AVAglio study randomized newly diagnosed GBM to radiotherapy plus temozolomide with or without bevacizumab followed by maintenance bevacizumab [213]. After completion of radiation, patients were treated with six cycles of monthly temozolomide plus bevacizumab or placebo every 2 weeks; this was followed by maintenance bevacizumab or placebo every 3 weeks until progression. Unfortunately, this trial did not show any overall survival benefit.

RTOG 0825 study had a similar design [214]. Patients were assigned to bevacizumab vs. placebo starting at week four of standard chemoradiation with temozolo-

mide, followed by 6–12 cycles of maintenance temozolomide plus bevacizumab or placebo. Overall survival did not improve with the use of bevacizumab in first-line therapy for patients with newly diagnosed glioblastoma.

5.8.4 Future Directions

Multiple new agents targeting HIF-1 are in the making.

Several drugs are being tested. These drugs may interrupt HIF-1 production like 103D5R [215] (REF13), or prevent binding to DNA like AP endonuclease 1/REF-1 E3330 [216], or inhibit HIF-1 binding to HRE like Echinomycin (NSC 13502) [217] or blocks HIF-1 mediated VEGF transcription like Geldanamycin [18].

Below is a list of clinical trials enrolling for an anti-angiogenesis approach in brain tumors:

1. A Phase II/III Study of High-dose, Intermittent Sunitinib in Patients With Recurrent Glioblastoma Multiforme: the STELLAR Study. NCT03025893.
2. A Phase 3, Randomized, Controlled, Double-Arm, Open-Label, Multi-center Study of VB-111 Combined With Bevacizumab vs. Bevacizumab Monotherapy in Patients With Recurrent Glioblastoma. NCT02511405.
3. A Phase 3, Randomized, Controlled, Double-Arm, Open-Label, Multi-center Study of VB-111 Combined With Bevacizumab vs. Bevacizumab Monotherapy in Patients With Recurrent Glioblastoma. NCT02511405.
4. A Phase III Study of Conventional Radiation Therapy Plus Thalidomide (NSC#66847) Versus Conventional Radiation Therapy for Multiple Brain Metastases. NCT00033254.

6 Conclusion

Targeting cancer angiogenesis is one of the most important achievements of cancer research in the twenty-first century culminating by the approval of bevacizumab in 2004. Since then, the FDA approved multiple drugs for patients with metastatic cancers. The first lesson is that angiogenesis, like all robust natural phenomena, is a complex process characterized by redundancy and plasticity.

However, cancer does not need angiogenesis until it starts growing beyond a certain volume. This is perhaps the reason why antiangiogenic therapy does not work in the adjuvant setting where the major targets of the treatment are disseminated tumor cells that usually enter a dormancy phase during which they do not need angiogenesis to survive. It is only when DTCs enter an active phase of proliferation that angiogenesis becomes important for them to grow and spawn metastasis again. Antiangiogenic agents add a small benefit to traditional therapies in many solid tumors and may have more substantial benefit in the most vascular ones amongst them such as liver and kidney cancer.

The most important challenge facing the field is the identification of biomarkers that would predict response of a specific tumor to a specific therapy. Until then, these expensive treatments will continue being used indiscriminately in all patients adding modest gains in survival to huge financial and morbidity burden. The only rational action will consist of conducting the appropriate prospective randomized controlled clinical trials to validate predictive biomarkers.

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Chapter 10
The Role of Angiogenesis in Non-small Cell Lung Cancer Tumor Behavior

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Abstract Currently, lung cancer is the leading cause of cancer-related death in western nations. Risk factors are usually associated with tobacco consumption, occupational exposure, radon and passive smoking. To date, many factors influence

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NSCLC behavior and, therefore, clinical response to therapeutic targets, such as epidermal growth factor (EGF) and its receptor (EGFR) and vascular endothelial growth factor (VEGF) and its receptor (VEGFR). Angiogenesis-related genetic polymorphisms are of primary interest in NSCLC research. Angiogenesis genetic polymorphisms, such as *VEGF* − 2578 C/A and *VEGF* − 1154 G/A, were correlated in previous studies with increased tumor VEGF expression, vascular density and poor survival. However, anti-angiogenic drugs did not show to be cost-effectiveness in NSCLC. This topic will address research involving angiogenesis genetic polymorphisms and NSCLC behavior.

Keywords Non-small-cell lung cancer • Vascular endothelial growth factor • Bevacizumab

Abbreviations

EGF	Epidermal growth factor
EGFR	EGF receptor
EGFR	Epidermal growth factor receptor
GWAS	Genome-wide associate study
HBO	Hyperbaric oxygen
HIF	Hypoxia inducible factor
KDR receptor	Kinase insert domain receptor
mTOR	Mammalian target of rapamycin
NSCLC	Non-small cell lung cancer
OS	Overall survival
PFS	Progression free survival
PIK3	Phosphoinositide 3-kinase
PlGF	Placental growth factor
SNP	Single nucleotide polymorphism
SOS	Guanine nucleotide exchange factor sos
TKI	Tyrosine kinase inhibitor
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor

1 Introduction

Lung cancer management has changed in the last decade [1–4]. Advances in molecular tools and targeted therapies have provided new insight for the field of oncology [5]. Targeted therapies play a major role in this field [3, 5]. Further options are also promising for lung cancer treatment, including stereotaxic radiotherapy and its use in

combination with induction chemoradiotherapy, as recently reported in a Japanese trial [6]. In light of the current knowledge, clinicians have begun selecting optimized populations that could receive improved benefits from these innovative therapeutic modalities [5, 7]. Tumor carcinogenesis pathways are now of primary interest in the field of oncology [2, 8–11]. First, angiogenesis pathways seem to be one of the key players in many cancers, including lung cancer [10]. Vascular endothelial growth factor (VEGF) and its receptor (VEGFR) are indeed important in the angiogenesis cascade [11]. Additionally, epidermal growth factor (EGF) and its receptor (EGFR) were demonstrated to be useful in the angiogenic mechanisms and clinical management of non-small cell lung cancer (NSCLC) [12]. Figure 10.1 summarizes the most important VEGF pathways. Mutations in exon 19 and exon 21 of the *EGFR* gene are a predictive factor for a positive response to EGFR tyrosine kinase inhibitors (TKIs) [13, 14]. Recent trials showed favorable outcomes regarding overall survival (OS) and progression-free survival (PFS) in selected patients treated with gefitinib [15, 16] and erlotinib [17, 18], two EGFR TKIs [19, 20]. In this framework, it is important to focus on angiogenesis research. Until recently, the role of many angiogenesis genetic polymorphisms was under investigation in NSCLC patients [12, 21, 22]. A polymorphism is a variation in a gene that leads to two or more alleles existing at a frequency of at least 1% in the general population [23–25]. A variation at a single nucleotide is referred to as a single nucleotide polymorphism (SNP) [26]. The human genome contains more than 1,000,000 SNPs, and at least 60,000 of these are in exons [25, 27]. A SNP has a functional consequence if it exists within a coding or regulatory

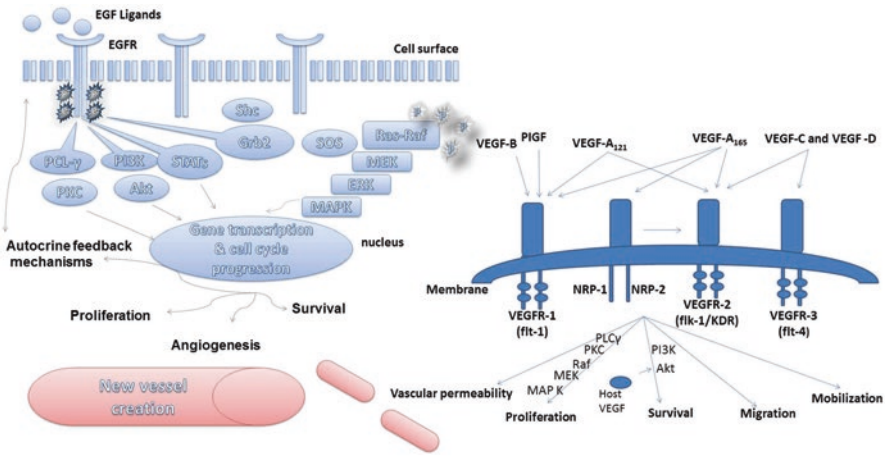

Fig. 10.1 Summary of the main VEGF and EGF pathways. *Abbreviations*: *EGF* epidermal growth factor, *EGFR* EGF receptor, *VEGF* vascular endothelial growth factor, *VGEFR* VEGF receptor, *KDR receptor* Kinase insert domain receptor, *PI3K* Phosphoinositide 3-kinase, *mTOR* Mammalian target of rapamycin, *Shc* Src homologous and collagen protein, *PCL-γ* Phospholipase Cγ, *PI3K* Phosphatidylinositol 3-kinase, *STATs* Signal transducer and activator of transcription, *PKC* Protein kinase C, *Grb2* Growth factor receptor bound protein 2, *SOS* Guanine nucleotide exchange factor sos, *MAPK* mitogen-activated protein kinase

region of a gene [27]. Otherwise, a SNP may be silent if it resides in a non-coding region of the DNA or if it results in a synonymous amino acid substitution, except in rare situations [25]. Thus, this chapter will provide a discussion regarding the state of genetic polymorphisms related to angiogenesis and non-small cell lung cancer tumor behavior, with a particular focus on risk assessment and prognosis.

2 Effects of Tumor Hypoxia and Clinical Issues

Interesting insights into the complex relationship of tumors with hypoxia can be gained by addressing the role of hypoxia as a predictor of response to different treatments. One field of active research focuses on the effect of oxygen on the response to radiotherapy (Fig. 10.2).

Tumor hypoxia influences the outcome of treatment not only in the setting of large tumors with extensive necrotic areas, but also in small tumors [26]. Hypoxia generally occurs as a result of insufficient vascularization, indicative of a long distance between the tumor cells and a functional blood vessel. Besides areas of chronic hypoxia, areas with intermittent hypoxia within tumors have also been described, thereby establishing persistent or transient resistance to radiotherapy [26, 28, 29].

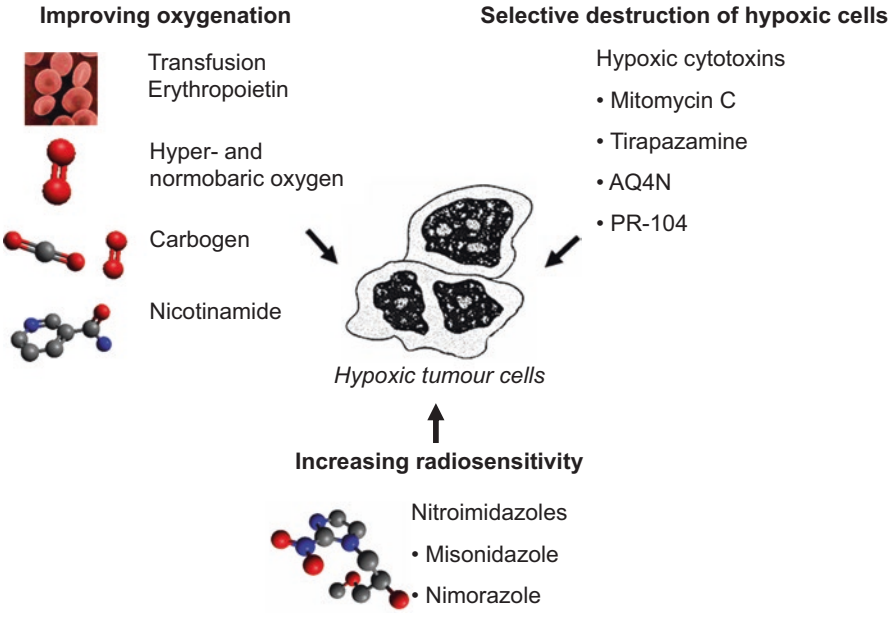

Fig. 10.2 Mechanisms of tumor hypoxia: different mechanisms which could led to influence the hypoxic tumor cells metabolism: increasing radiosensitivity, selective destruction of hypoxic cells or improving oxygenation

The role of molecular oxygen (O_2) as a critical determinant to the response of cells to radiation has been described as early as the beginning of the last century [30, 31]. The radio-sensitizing effect of O_2 is explained by the chemical properties of the molecule, whose affinity for electrons predisposes it to participate in chemical reactions that lead to DNA damage if cells are exposed to ionizing radiation. Hypoxia reduces the radiosensitivity of cells, which require approximately 3 times as much radiation to become sensitized as cells with normal oxygen tension. In addition, hypoxia has been described as an important factor for tumor aggressiveness, a fact that should be taken into account when analyzing the poor response of hypoxic tumors to radiotherapy [26, 32, 33].

To circumvent the radioprotective approaches of hypoxia, 3 main approaches of hypoxic modification have been studied: improving tumor oxygenation, increasing the radiosensitivity of hypoxic cells and selectively killing hypoxic tumor cells.

Improvement of tumor oxygenation can be achieved by pursuing hematocrit values above a certain threshold in patients. The hematocrit is an important clinical measure, considering that 40–60% of cancer patients are anemic at the beginning of radiotherapy [34]. Clinical trials examining the effects of transfusions and erythropoietin on the outcome of therapy have produced mixed results; nevertheless, hematocrit monitoring is a standard procedure in patients treated with radiotherapy [34, 35].

Hyperbaric oxygen (HBO) breathing yielded encouraging results beginning in the 1950s [36]. The physiological effects of HBO can be divided into short-term effects, such as enhanced oxygen delivery, phagocytosis activation and anti-inflammatory effects, and long-term effects, including neovascularization and stimulation of collagen production by fibroblasts [37]. Issues have been raised concerning a possible enhancement of tumor growth or metastasis by HBO, but current evidence precludes such an effect [38]. Procedural difficulties in delivering both HBO therapy and radiotherapy as well as a reduced number of patients limited the usefulness of data obtained in the first clinical trials. Meta-analyses of more recent trials showed evidence that HBO improves local tumor control and reduces patient mortality for cancers of the head and neck, local tumor recurrence in cancers of the head and neck, and cervical cancer [39, 40]. Administration of normobaric oxygen and carbogen has also been studied, with results in animal models and clinical trials showing greater variability in outcome than with HBO [41]. Carbogen breathing is often combined with other modalities that might improve response, particularly nicotinamide (a vitamin B6 analog with vasoactive properties). The accelerated radiotherapy with carbogen and nicotinamide (ARCON) protocol consists of combining accelerated therapy and nicotinamide. Phase I and II trials with this protocol reported promising results in head and neck and bladder cancer [42, 43].

Increasing the radiosensitivity of hypoxic cells has been pursued with oxygen-mimetic radiosensitizers, particularly 2-nitroimidazoles such as misonidazole and nimorazole, as shown in Fig. 10.2. Clinical trials note a possible role for these radiosensitizers as adjuncts to radiotherapy, with promising results in some tumors but little improvement in local tumor control and disease-free survival. Delayed peripheral neuropathy is an important concern with these agents, limiting their routine clinical use [41, 43].

The third approach, selectively killing hypoxic cells, involves hypoxic cytotoxins, which could play an important role in combination with radiotherapy and conventional cytotoxic chemotherapeutic agents, as these are generally more toxic to rapidly proliferating and well-perfused cells. Promising results have been reported with mitomycin C, a quinone that yielded positive results in local control and disease-free survival in head and neck and cervical carcinomas [44, 45]. Another agent, the benzotriazine tirapazamine, has a different mechanism of action on hypoxic cells from quinones and requires less pronounced hypoxia. Pre-clinical studies demonstrated the efficacy of tirapazamine in combination with radiotherapy and chemotherapy; however, phase III trials revealed issues of toxicity, and conflicting results have been reported [46]. Other agents in this group, such as the alkylaminoanthraquinone N-oxide AQ4N and the 3,5-dinitrobenzamide-2-mustard PR-104 are being actively explored [47, 48].

In a systematic review, Overgaard [41] identified 10,108 patients in 86 randomized trials designed to modify tumor hypoxia in patients treated with curative attempted primary radiation therapy alone. Overall modification of tumor hypoxia significantly improved the effect of radiotherapy on the outcome of local regional control and was associated with a significant overall survival benefit. No significant influence was found on the incidence of distant metastases or on the risk of radiation-related complications.

Importantly, the implementation of these therapeutic modalities results in a better understanding of the molecular processes involved in the relationship between hypoxia and tumor growth. An appropriate selection of patients is also important: various minimally invasive and non-invasive methods hold promise for monitoring tumor oxygen distribution before and after treatment begins, thereby allowing the clinician to tailor specific regimens for different patients [29, 43].

3 Genetic Polymorphisms Related to Angiogenesis Pathways

A brief consideration of angiogenesis in lung cancer

Angiogenesis is a crucial step for lung cancer progression [49–51]. The creation of new vessels from pre-existing vessels influences tumor growth, invasion and metastasis [52]. Recently, many studies have shown that NSCLC patients presented with higher serum VEGF than healthy controls [21, 53, 54]. Additionally, microvessel density was demonstrated to be linked with tumor aggressiveness in some studies [55, 56]. In light of this knowledge, it was hypothesized that VEGF expression could have clinical implications in disease outcome [57]. In addition, the interaction between EGF and EGFR is associated with tumor angiogenesis, as shown in Fig. 10.2. The EGF/EGFR intracellular signaling stimulates gene transcription in the nucleus and is responsible for the creation of new vessels, cell migration and metastasis [58]. Tables 10.1 and 10.2 summarize the main genetic polymorphisms studied in the lung cancer field.

Table 10.1 Summary of *VEGF* polymorphisms in lung cancer

Polymorphism	Tumor type	No. cases/controls	region	Effects on tumor risk[a]	Functional and clinical effects (VEGF levels, tumor behavior, survival)[a]	Reference
rs699947 (−2578 C/A)	NSCLC	36/0	N/A	N.Det.	CA genotype increased tumor VEGF expression and vascular density	[115]
	NSCLC	126/0	N/A	N.Det.	AA genotype associated with worse survival	[104]
	NSCLC	566/0	Asian	N.Det.	N.S.	[62]
rs1005230 (−2489 C/T)	NSCLC	566/0	Asian	N.Det.	N.S.	[62]
rs1570360 (−1154 G/A)	NSCLC	36/0	N/A	N.Det.	AA and GA genotypes increased tumor VEGF expression	[115]
	NSCLC	126/0	N/A	N.Det.	AA and GA genotypes associated with worse survival	[104]
rs833061 (−460 C/T)	Lung cancer	432/432	Asian	N.S.	N.Det.	[113]
	NSCLC	1900/1458	Caucasian	N.S.	N.Det.	[93]
	NSCLC	462/0	N/A	N.Det.	N.S.	[111]
	NSCLC	126/0	N/A	N.Det.	CC genotype associated with worse survival	[104]
rs25648 (−7 C/T)	NSCLC	568/0	Asian	N.Det.	N.S.	[62]

(continued)

Table 10.1 (continued)

Polymorphism	Tumor type	No. cases/controls	region	Effects on tumor risk[a]	Functional and clinical effects (VEGF levels, tumor behavior, survival)[a]	Reference
rs2010963 (405 G/C)	NSCLC	36/0	N/A	N.Det.	GC genotype associated with increased tumor VEGF expression and vascular density	[115]
	Lung cancer	432/432	Asian	GG genotype reduces risk of SCC	N.Det.	[62]
	NSCLC	1900/1458	Caucasian	N.S.	N.Det.	[93]
	NSCLC	462/0	N/A	N.Det.	C allele associated with improved survival	[111]
	NSCLC	462/0	N/A	N.Det.	GC and combined GC + CC genotypes associated with improved survival	[111]
	Lung cancer	88/0	N/A	N. Det.	N.S.	[112]
	NSCLC	126/0	N/A	N.Det.	N.S.	[104]
	NSCLC	566/0	Asian	N.Det.	N.S.	[62]
rs3025039 (936 C/T)	Lung cancer	432/432	Asian	CT or CT + TT genotypes reduce risk of SCC	N.Det.	[62]
	NSCLC	1900/1458	Caucasian	N.S.	N.Det.	[93]
	NSCLC	462/0	N/A	N.Det.	N.S.	[111]
	Lung cancer	88/0	N/A	N. Det.	T allele increased *VEGF* gene expression and VEGF serum levels	[112]
	NSCLC	126/0	N/A	N.Det.	N.S.	[104]
	NSCLC	568/0	Asian	N.Det.	N.S.	[62]
rs10434 (1612 G/A)	NSCLC	560/0	Asian	N.Det.	N.S.	[62]
rs833069 (intron 2 G/A)	NSCLC	560/0	Asian	N.Det.	N.S.	[62]

rs833070 (intron 2 G/A)	NSCLC	560/0	Asian	N.Det.	N.S.	[62]
rs3024994 (intron 2 C/T)	NSCLC	568/0	Asian	N.Det.	N.S.	[62]
rs3025010 (intron 5 C/T)	NSCLC	567/0	Asian	N.Det.	N.S.	[62]
rs3025035 (intron 7 C/T)	NSCLC	568/0	Asian	N.Det.	N.S.	[62]
rs3025040 (3′-UTR T/C)	NSCLC	568/0	Asian	N.Det.	N.S.	[62]
rs3025053 (3′-UTR G/A)	NSCLC	568/0	Asian	N.Det.	N.S.	[62]
rs2736100	Adenocarcinoma	13,300/19,666	USA	Increase risk adenocarcinoma	N.S.	[79]
rs31489	Lung cancer	13,300/19,666	USA	Increase risk	NS	[79]
rs4635969	Lung cancer	13,300/19,666	USA	Increase risk	NS	[79]
rs12914385	All lung cancer	13,300/19,666	USA	Increase risk	NS	[79]
rs1051730	All lung cancer	13,300/19,666	USA	Increase risk	NS	[79]
rs8034191	All lung cancer	13,300/19,666	USA	Increase risk	NS	[79]
rs12914385	Lung cancer	716/716	Japan	Increase risk	NS	[80]
rs931794	Lung cancer	716/716	Japan	Increase risk	NS	[80]
rs931794	Lung cancer	716/716	Japan	Increase risk	NS	[80]
rs12914385	Lung cancer	716/716	Japan	Increase risk	NS	[80]
rs2736100	Lung cancer	1094/1100	Korean	Increase risk	NS	[81]
rs402710	Lung cancer	1094/1100	Korean	Increase risk	NS	[81]
rs401681	Lung cancer	1094/1100	Korean	Increase risk	NS	[81]
rs31489	Lung cancer	1094/1100	Korean	Increase risk	NS	[81]
rs9295740	Lung cancer	1094/1100	Korean	Increase risk	NS	[81]

[a]Significant differences were considered when $P < 0.05$

Abbreviations: NSCLC non-small cell lung cancer, *SCC* small cell carcinoma, *N/A* not available, *N.Det* not determined, *N.S* not significant

Table 10.2 Summary of *VEGF* haplotypes in lung cancer

Haplotypes	Comparisons	Tumor type	No. cases/ controls	Ethnicity	Effects on tumor risk[a]	Functional and clinical effects (VEGF levels, tumor behavior, survival)[a]	Reference
−460, 405, 936	TCT vs all other haplotypes	Lung cancer	432/432	Asian	TCT reduces risk of lung cancer	N.Det.	[113]
	TGT vs all other haplotypes	Lung cancer	432/432	Asian	TGT increases risk of lung cancer	N.Det.	[113]
	Each haplotype vs all other haplotypes	AC	432/432	Asian	N.S.	N.Det.	[113]
	CGT vs all other haplotypes	SCC	432/432	Asian	CGT reduces risk of SCC	N.Det.	[113]
	TCC vs all other haplotypes	SCC	432/432	Asian	TCC increases risk of SCC	N.Det.	[113]
	TCC vs CGC (reference)	NSCLC	1900/1458	Caucasian	N.S.	N.Det.	[83]
	TGC vs CGC (reference)	NSCLC	1900/1458	Caucasian	N.S.	N.Det.	[83]
	CGT vs CGC (reference)	NSCLC	1900/1458	Caucasian	N.S.	N.Det.	[83]
	TCT vs CGC (reference)	NSCLC	1900/1458	Caucasian	N.S.	N.Det.	[83]
405, 936	All variant alleles vs GC (reference)	NSCLC	462/0	N/A	N.Det.	Higher number of variant alleles increased overall survival	[111]

[a]Significant differences were considered when $P < 0.05$

Abbreviations: *NSCLC* non-small cell lung cancer, *AC* adenocarcinoma, *SCC* small cell carcinoma, *N/A* not available, *N.Det.* not determined, *N.S.* not significant

VEGF and VEGFR genetic polymorphisms in cancer

The interaction between VEGF and VEGFR is one of the key regulators of angiogenesis [59–62]. Genetic variability of VEGF may play an important role in modifying cancer development and progression [57, 61, 63, 64]. The VEGF gene is located on chromosome 6p21.3 and contains 8 exons [65]. It is highly polymorphic, with more than 20 polymorphisms showing up in cancer risk studies. The polymorphisms located on regulatory regions, such as promoter regions or 5′ and 3′ untranslated regions, are of primary interest in cancer susceptibility and outcome because they may modulate VEGF expression [65]. Tables 10.1 and 10.2 summarize the main VEGF polymorphisms and their influence on cancer.

Variants in VEGFR2 and micro vessel density in lung cancer

Recent studies assessed the role of VEGFR2 genetic variants and NSCLC [21, 56]. Microvessel density (MVD) is considered a good experimental tool for evaluation of angiogenesis in NSCLC tissue. An American study [56] had defined VEGFR2 genetic variation in 3 populations (African American, Caucasian and Asian) and identified common variants that impact tumor VEGFR2 expression and vascularization. VEGFR2 re-sequencing led to the discovery of 120 genetic variants, of which 25 had not been previously reported. The Q472H point mutation increased VEGFR-2 protein phosphorylation, and also was associated with increased MVD in NSCLC tumor samples. -2854C and -2455A increased luciferase expression and were associated with higher KDR mRNA levels in NSCLC samples. -271A reduced luciferase expression and was associated with lower VEGFR-2 levels in NSCLC samples. -906C and 23408G were associated with higher KDR mRNA levels in NSCLC samples. These findings are important because they elucidate a genetic signature that may influence clinical phenotypes related to VEGFR2 function.

EGF+61 A/G genetic polymorphisms and cancer risk

The *EGF* gene is located on chromosome 4q25-q27 [66], and *EGF* polymorphisms are associated with cancer susceptibility [66–69]. In 2002, Shahbazi et al. elucidated the role of the *EGF+61* A/G polymorphism in melanoma patients [66]. They suggested that cells from individuals homozygous for the +61 A allele produce significantly less EGF than cells from homozygous +61 G ($p = 0.0004$) or heterozygous 61A/G individuals ($p = 0.001$). Furthermore, Shahbazi's study showed that elevated EGF production is important for melanoma development [66]. In 2004, Bhowmick et al. demonstrated that the *EGF* +61 A/G polymorphisms were associated with an increased frequency of glioblastomas [70]. In 2007, our group also described the role of the *EGF* +61A/G polymorphism in glioma susceptibility in a Portuguese population and showed that the +61G allele was associated with an elevated EGF expression level *in vitro* [69]. Other studies revealed that this genotype, along with the +61 G allele, is likely associated with a risk of pancreatic cancer development [71]. Furthermore, in 2009, Wu et al. also found a statistically significant association between both the *EGF* +61G/G genotype and the +61 G allele and the risk for colorectal cancer [71].

Initial studies on the role of *EGF* +61A/G polymorphisms in lung cancer risk were controversial and restricted to Asian populations [72, 73]. In 2012, a Portuguese study first showed that the cumulative influence of *EGF* + 61 A/G polymorphisms in Caucasian NSCLC patients increased susceptibility [74]. This is in agreement with a South Korean study that found a similar result in a comparative study in a schizophrenic population and healthy controls [73].

EGFR and EGFR genetic polymorphisms

EGFR is a 170 kDa transmembrane glycoprotein with an intracellular tyrosine protein kinase domain that plays critical cellular signaling roles in diverse pathways [20]. EGFR activation leads to many biological processes, including cell cycle progression, cell invasion, metastasis, angiogenesis and cell differentiation, all of which are closely related to tumor progression [5]. In transgenic mice, overexpression of EGFR initiates the formation of oligodendroglioma [75] and breast cancer [76]. In humans, EGFR is overexpressed in 50–81% of NSCLC, and its overexpression correlates with poor prognosis of NSCLC [14]. Currently, *EGFR* mutation is a noteworthy predictive biomarker in NSCLC management. In particular, in non-smoking women with adenocarcinoma histology, a mutation in exons 19 or 21 of the *EGFR* gene is predictive of an improved response to EGFR TKIs. A study conducted among 3 major hospitals of north Portugal retrospectively assessed *EGFR* status in 621 NSCLC patients [77]. This study showed that *EGFR* was mutated in 14.3% of all NSCLC patients (17.5% adenocarcinoma and 9.5% squamous cell carcinoma). Exon 19 was mutated in 43.8% of patients, and exons 20, 21, and 18 were mutated in 37.1%, 21.4%, and 6.7% of patients, respectively [77]. Given these results, *EGFR* genetic polymorphisms are also of key interest. In 2012, a Chinese study enrolled 568 NSCLC patients and examined 54 SNPs of the *EGFR* and *VEGF* genes [78]. The authors found that subjects carrying *EGFR* rs3735061 A/A and rs6958497 A/G and G/G genotypes showed significantly shorter survival times [median survival time (MST): 22.2 and 19.4 months, respectively] than those carrying rs3735061 A/G and G/G (MST: 25.1 months) and rs6958497 A/A (MST: 25.9 months) genotypes (log-rank $p = 0.015$ for rs3735061 and log-rank $p = 0.028$ for rs6958497). Nevertheless, subjects carrying *EGFR* rs759165 A/G and A/A genotypes survived significantly longer (MST: 38.7 months) than those carrying the rs759165 G/G genotype (MST: 24.7 months, log-rank $p = 0.024$). Multivariate Cox regression analyses showed that the genotypes of rs3735061 A/A and rs6958497 A/G and G/G were associated with a significantly increased risk of death from NSCLC [hazard ratio (HR) = 2.82, 95% confidence interval (CI) = 1.66–4.78 for rs3735061 A/A and HR = 1.69, 95% CI 5 1.26–2.28 for rs6958497 A/G and G/G], whereas the rs759165 A/G and A/A genotypes were associated with a significant 44% decreased risk of death from NSCLC (HR = 0.56, 95% CI 5 0.39–0.83). Furthermore, a stepwise Cox regression analysis suggested that *EGFR* rs373506, rs759165 and rs6958497 may be independent candidate biomarkers to predict NSCLC survival in this population [78].

4 Genetic Polymorphisms and Risk of Lung Cancer

GWAS variants and risk of lung cancer

Since 2009, the genome-wide association study (GWAS) group has found significant associations in chromosomes 5p15.33, 15q25, and 6p21 variants and lung cancer risk [79–82]. In the 5p15.33 locus, the most prominent were rs4635969 (OR 0.87, 95% confidence interval [CI], 0.82–0.93, $p = 9.80 \times 10^{-5}$), followed by rs31489 in CLPTM1L (also named CCR9 [MIM 612585], OR 0.90, 95% CI 0.86–0.95, $p = 2.80 \times 10^{-4}$) and rs2736100 in TERT (MIM 187270, OR 1.09, 95% CI, 1.03–1.15, p = 0.001) [79]. Additionally, the rs2736100 SNP was associated only with adenocarcinoma histology (OR 1.23, 95% CI, 1.13–1.33, $p = 3.02 \times 10^{-7}$) but not to other histologic types (OR 1.01, p = 0.84 and OR = 1.00, p = 0.93, for squamous cell carcinoma and small cell carcinoma, respectively; p = 0.001. The test for heterogeneity across histology was corrected for multiple comparisons.), as shown in Table 10.1 [79]. Thus, this study associated the rs2736100 variant in chromosome 5p15.33 with lung adenocarcinoma risk. The rs2736100 variant is located in intron 2 of the TERT gene [83]. TERT is a ribonucleoprotein that extends TTAGGG nucleotide repeats at telomeres, which progressively shorten with each cell division. Telomere shortening is associated with increased genomic instability and therefore increased risk of overall cancer development [84]. In cancer cells, reactivated TERT is linked to cellular proliferation and abnormal telomere maintenance [85]. Interestingly, TERT expression is lower in adenocarcinoma than in other histological subtypes [86]. Its re-expression may be related to the progression from bronchiolo-alveolar carcinoma to adenocarcinoma [87]. Moreover, Landi et al. also reported that other nicotinic acetylcholine receptor gene variants in chromosome 15q25 (rs4635969, CLPTM1L gene; rs12914385, CGRNA3 gene; rs1051730, CHRNA5 gene; and rs8034191, LOC 123688) were associated with an elevated overall risk of lung cancer [79]. These SNPs were also strongly associated with all major histology groups of patients who were current and former smokers [79]. In rs4324798 on chromosome 6p21.33, the association with lung cancer was weak and inconsistent throughout the studies [79, 88]. In 2012, Ito et al. reported a study in Japan with 716 lung cancer patients and 716 controls [80]. It was found that the variants of rs12914385 and rs931794 on 15q25 modified the effect of cumulative tobacco smoking on lung cancer risk, but that these two loci showed no statistically significant effects on lung cancer risk. Furthermore, association of the TERT-CLPM1L locus on 5p15 with lung cancer risk in Japanese patients was of a similar magnitude to that in Caucasians. Therefore, Ito's study confirmed the contribution of 15q25 and 5p15 to lung cancer susceptibility [82].

VEGF polymorphisms and controversies in assessing the risk of lung cancer

The VEGF gene is located on chromosome 6p21.3 and consists of 8 exons that undergo alternate splicing to form a family of proteins [89]. Several genetic polymorphisms have been described, and they are associated with variation of VEGF production and expression [8, 10, 11, 21, 65, 78, 90–93] (Tables 10.1 and 10.2).

Regarding the *VEGF* −1154 G/A polymorphism, the data are inconclusive and a recent meta-analysis conducted by Hong et al. [65] suggested that although *VEGF* -1154G/A may not be associated with cancer risk in the general population, the −1154 GG homozygote allele may confer an elevated risk of cancer in non-Caucasians compared to −1154 A carriers.

EGF+61 genetic polymorphisms and their role in lung cancer susceptibility
In 2002, *EGF*+61 A/G polymorphisms were first associated with cancer susceptibility in a melanoma model [66]. One decade later, only two Asian studies have addressed their role in lung cancer risk [72, 73]. However, in 2012, a Portuguese group showed that *EGF*+61 A/G polymorphisms are also associated with the risk of NSCLC in a population from North Portugal [74]. It was the first study in a Caucasian population and confirmed the results reported by Lim and colleagues [73] in a Korean population. Only a small number of studies addressed the role of *EGF*+61 A/G polymorphisms and lung cancer risk because *EGF*+61 A/G and *EGF*+61 G/G genotypes influence high EGF serum levels [66] and thus favor carcinogenesis in the lung [68, 94, 95]. Despite this data looks interesting, the clinical applicability is still limited and needs further larger studies for validation.

5 Genetic Polymorphisms and Non-small-cell Lung Cancer Prognosis

Current lung cancer prognostic factors
Many factors influence lung cancer prognosis [2, 4, 14, 58, 94, 96–105]. The tumor node metastasis (TNM) classification system [97], smoking status [106] and performance status [105] give physicians important clues to improve the clinical approach [4]. Currently, many platinum-based chemotherapy protocols with or without bevacizumab, EGFR TKIs, crizotinib and most recently the immune-checkpoints inhibitors (nivolumab and pembrolizumab) are interesting weapons against disease aggressiveness [5, 21]. Moreover, many efforts have been undertaken toward the genomic and molecular research to find prognostics and predictive biomarkers for lung cancer management [15, 103, 104, 106–110]. As they play a main role in angiogenesis and the overall biology of NSCLC, genetic polymorphisms in the VEGF [21, 90, 93, 111–113], VEGFR [92, 114], EGF [12, 72–74] and EGFR genes [78] will be discussed in the following paragraphs.

VEGF −2578 C/A and VEGF −1154 G/A polymorphisms and NSCLC survival
Many VEGF genetic polymorphisms have been studied alongside NSCLC survival and outcome [78, 104, 115]. In 2004, *VEGF* -2578 C/A and *VEGF*-1154 G/A played a key role in lung cancer vasculature [115]. Koukourakis et al. reported that tumors with the *VEGF* −2578 C/A genotype had significantly higher VEGF expression than those expressing the *VEGF* −2578 A/A genotype. Furthermore, the authors reported that the *VEGF*-1154 G/A polymorphism seemed to be related with poor vascularization, but the difference was not significant [115]. In 2009, a Japanese group demonstrated

that the *VEGF* −1154 AA and AG genotypes (HR, 1.482; 95% CI, 1.144–1.897; P = 0.0034) and the *VEGF* −2578AA genotype (HR, 1.797; 95% CI, 1.219–2.495; P = 0.0047) had a significant prognostic effect on survival based on univariate analysis. Based on multivariate analysis of a current and former smoker (HR, 1.407; 95% CI, 1.095–1.840; P = 0.0070), poor PS (HR, 2.249; 95% CI, 1.309–3.468; P = 0.0058) and the *VEGF* -1154AA and AG genotypes (HR, 1.419; 95% CI, 1.033–1.901; P = 0.0316) were significant independent prognostic factors for survival [104]. However, in 2010, Dong et al. studied 56 SNPs in 568 NSCLC patients [78], but the *VEGF* -2578 C/A polymorphisms did not influence survival and prognosis.

VEGF variants [−460 T / C (rs833061), 405 G/C (rs2010963), and +936 C / T (rs3025039), −2489 C/T (rs1005230)] and survival

In a previous study, VEGF serum levels were also associated with the OS of NSCLC patients treated with bevacizumab [53]. The reason for this association was that some genotypes, such as VEGF (−2578 C/A, −1498 C/T, −1154 G/A, −634 G/C, 936 C/T, and VEGF –1498 T/T), were correlated with toxicity, bevacizumab-related grades 3 and 4 hypertension, and disease outcome in patients treated for metastatic cancer [92]. In spite of controversial studies [21, 61, 90], *VEGF* – 460 T/C polymorphisms were associated with a better OS in locally advanced NSCLC after chemoradiotherapy [61]. In 2012, Naik et al. reported that the *VEGF* +405 C/G SNP, also called *VEGF -634 G/C* SNP, showed an association with age, pathological grade, and stage [90]. However, no results were consistent with survival [21]. Furthermore, *VEGF* −2489 C/T polymorphisms were not associated with OS in advanced NSCLC [78].

VEGFR2 copy number as a predictive and prognostic factor

In 2011, experimental and translational research concerning angiogenesis and NSCLC was published to assess the role of VEGFR2 in tumor behavior [56, 116]. *In vitro*, VEGFR2 copy number gains were significantly associated with resistance to platinum chemotherapy as well as increased levels of nuclear HIF-1α in both NSCLC tumor specimens and cell lines [116]. VEGFR2 genetic variation (Q472H, − 2854 C, and - 2455 A) in 3 populations had an impact on tumor VEGFR2 expression and vascularization [56]. In light of these data, it is understandable that the role of angiogenesis and NSCLC are not completely elucidated. It is true that bevacizumab did not show a satisfactory cost effectiveness relationship in advanced NSCLC treatment [21, 117]. Clinicians should expect further improved research about enhanced approaches involving anti-angiogenic therapies and NSCLC. At this moment in UK, we don't use bevacizumab to treat advanced NSCLC due to the lack of significant improvement in overall survival.

EGF+61 A/G, EGFR polymorphisms and outcome

Although the current role of *EGFR* mutations in exons 19 and 21 in advanced NSCLC treatment [5] has been elucidated, the roles of *EGF*+61 A/G polymorphisms, *EGFR* A/A (rs3735061) polymorphisms, *EGFR* A/G (rs6958497) polymorphisms and outcome still need clarification [12, 78, 118]. In 2010, a Chinese study showed that the *EGFR* genotypes rs3735061AA and rs6958497AG/GG were associated with a significantly increased risk of death for NSCLC [hazard ratio (HR) = 2.82, 95% confidence interval (CI) = 1.66–4.78 for rs3735061AA and HR = 1.69, 95% CI = 1.26–2.28 for rs6958497AG/GG],

whereas the rs759165AG/AA genotype was associated with a 44% decreased risk of death of NSCLC (HR = 0.56, 95% CI 5 0.39–0.83) [78]. Furthermore, other genetic polymorphisms in genes involved in the EGFR pathway (MAP3K1, RAF1, NRAS, and GPX7) were associated with NSCLC survival in a cohort study involving 1076 lung cancer patients [119]. In addition, a Chinese study involving 88 NSCLC patients treated with gefitinib showed that the *EGFR* rs2293347 G/G genotype was associated with the efficacy of gefitinib [120]. The response rate for the rs2293347 GG genotype was significantly higher than that of the GA or AA genotypes (71.4% versus 36.0%, $p = 0.002$). The rs2293347 GG genotype was also associated with a longer PFS compared with the GA or AA genotypes (10 months versus 3 months, $p = 0.005$). However, no significant difference was observed regarding OS, $p = 0.409$ [120]. Moreover, another Chinese study [121] with 115 NSCLC patients treated with EGFR TKIs showed a synergistic effect of the *CYP1A1***2A* and the *EGFR* intron 1 (CA) repeat polymorphisms, and this effect influenced patient clinical responses to EGFR-TKIs. Nevertheless, the *EGF*+61 A/G polymorphism was only assessed with regard to lung cancer risk [72–74] and not clinical outcome [118]. Thus, changes in the expression of EGF-EGFR pathway components may be very important in NSCLC carcinogenesis and clinical outcome. Further studies are warranted to validate this hypothesis.

6 Current and Future Developments

Lung cancer continues to be one of the major cancers around the world. Genomic profiling to improve its characterization is of particular interest in clinical practice [21, 118]. In the new era of targeted therapies, and more recently the immunotherapies, a thorough understanding of NSCLC molecular issues and immune system is crucial for clinical management [122]. It is true that angiogenic molecular mechanisms throughout the VEGF and EGFR pathways are key players of NSCLC pathogenesis. Therefore, studying genetic polymorphisms that regulate VEGF, VEGFR, EGF, and EGFR expression may be useful for improving screening strategies, therapeutic selection and patients' prognoses. However, there is no currently yet antiangiogenic effectiveness to treat NSCLC.

Competing Interest The authors declare that they do not have any competing interests.

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Chapter 11
Angiogenesis and Prostate Cancer: Friends or Foes

Sanja Stifter, Federica Patrinicola, Gianluigi Taverna, and Fabio Grizzi

Abstract A key-hallmark of cancer is the promotion of angiogenesis. While there are currently no markers of the net angiogenic activity of prostate cancer (PCa) that can help investigators to design specific anti-angiogenic strategies, it is reasonable to assume that the quantification of various aspects of tumor vasculature may provide an indication of angiogenic activity. It has been ascertained that malignant tumors can generate their vasculature in seven distinct ways, including sprouting angiogenesis, vasculogenesis, intussusceptive angiogenesis, vascular co-option, mosaic vessels, vasculogenic mimicry and trans-differentiation of cancer stem-like cells into tumor endothelial cells. Here we briefly review these ways to get blood supply for the progression of PCa, its predictive and prognostic role and the actual discrepancies in the quantitative evaluation of neovascularity.

Keywords Prostate cancer • Angiogenesis • Vascularity • Microvessel density • Biomarkers

1 Introduction

Cancer research has undergone radical changes over the last few years. The issue today is no longer the amount of basic and clinical information available, but how to handle it. However, despite this continuous progress, PCa remains a major public

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health problem throughout the world [1]. Essential for the tumor growth and progression is blood and new vascular segments are needed to supply the growing tumor mass with oxygen and nutrients [2]. Angiogenesis, the development of new branching vessels from existing vasculature, is a complex process observed in fetal growth, wound healing and endometrial hyperplasia associated with the menstrual cycle. Under these conditions, it is highly regulated: *i.e.*, "turned on" for brief periods of time and then completely inhibited [3]. However, many human diseases, including tumors, are driven by persistently up-regulated angiogenesis [4, 5]. In some non-malignant diseases, such as lobular capillary hemangioma or keloid formation, angiogenesis is self-limited, on the contrary, in tumor once begun continues indefinitely until the entire tumor is eradicated or the host dies. Angiogenesis is regulated by a balance of pro- and anti-angiogenic molecules [5], secreted from cancer cells, endothelial cells and stromal cells [6, 7]. The relative contributions of which are likely to change with tumor type and site, as well as with tumor growth, regression and relapse. Angiogenesis, defined as the formation of a new capillary network from preexisting capillaries, has been proposed for the first time in 1971 by Judah Folkman and, at now, is the most study of neovascular growth in cancer [8, 9]. In the majority of cancer, vessel growth is stimulated and they are abnormal in all aspects of their structure and function. These impede the function of immune cells in tumors and the transport of the oxygen, creating favorable environment for tumor progression and metastasis [2]. In this mechanisms are involved angiogenic regulators, such as vascular-endothelial growth factor (VEGF) family with their receptors (VEGFR1, VEGFR2 and VEGFR3), basic fibroblastic growth factor (b-FGF) and angiopoietin family (Ang-1, Ang-2 and Ang-4), leading to hypoxic and acidic tumoral regions [10]. Although it is commonly believed that the endothelial cells making-up tumor vessels are genetically stable, tumor vasculature seems to be much more unpredictable [11]. These conditions reduce the effectiveness of treatments, modulate the production of pro- and anti-angiogenic molecules, and select a subset of more aggressive cancer cells with higher metastatic potential. The significance of angiogenesis in PCa still remains controversial [12]. The complexity of angiogenesis has been one of the greatest challenges in translation research so far. Angiogenesis is an ubiquitary process and though not being the synonym for neovascularization sometimes is misused in broader sense [13]. Therefore, neovascularization is defining revascularization of particular tissue and in certain extent is significant for solid tumors among which we include PCa as well, which we will additionally address on this occasion. The process of neovascularization comprises mainly of dysfunctional endothelium, which is constantly present in a chronic inflammatory tissue milieu. Endothelial dysfunction refers among other at disruption of vascular permeability and vascular tone regulation, inflammatory and immunological reactions and cell growth [14].

2 Angiogenesis and Inflammation

In physiological settings the endothelial cells have properties necessary to metabolize, synthesize and release a variety of substances [15]. Among them growth factors and both, reactive oxygen species (ROS) and reactive nitrogen species (RNS), actively participate in reactions of oxidation and protein nitrosylation promoting cell growth [15, 16]. When activated endothelial cells gain ability to produce several inflammatory and immune mediators. They are consistent with morphological changes visible as process of adaptation upon inflammation. It has been shown that inflammation mediated by immune mediators increases the endothelial permeability and promotes processes of leucocyte adhesion, dyapedesis and migration to site of inflammation [17]. The extent of endothelial dysfunction can be measured in vivo by determination of some circulating biomarkers such as endothelin-1, E-selectin and von Willebrand factor, all of them being unspecific [18]. The factors that influence angiogenesis and neovascularization are main contributors to its pathophysiological alterations. Inflammation has been already described as process closely linked to the abnormal angiogenesis. It has been also shown that inflammatory microenvironment inevitably relies different cytokines stimulating pro-angiogenic activity by continuous vascular endothelial activation. There are numerous molecular pathways orchestrating and influencing prostate cancer progression [19].

Recently, different mechanisms have been proposed when explaining how molecules of adhesion molecular family promote angiogenesis and favorite tumor cell capacity to invade and metastasize [20, 21]. On of them is adhesion molecule member of the Ig superfamily ALCAM/DM-GRASP, first described by Burns et al. [22]. This adhesion molecule has several synonyms including DM-GRASP, SC1, BEN, ALCAM and CD166 [23–25] presumably since was recognized in wide variety of cancers. It was described *in vivo* mouse studies to be important in cancer progression [26]. Its regulation is either up-regulated or down-regulated depending on tissue of origin [21]. This makes its characterization tissue/tumor and organ functions more specific and complex [22]. Due to its expression in cancer cells it is used more and more frequently as a biomarker of cancer progression in several tumors among which is prostate cancer. Previously we analyzed CD44 and its ability to recruit and accumulate matrix metalloproteinase on the cellular surface. This way tumor cells gain pro-angiogenic immunophenotype and capacity to invade [27].

Once initiated a cascade comprises growth factors release, which additionally promote the signal transduction at cellular level. Consequently, the angiogenic switch results in increased endothelial sprouting observable at tissue architectural level. The characterization of microvascular architecture has gained increasing attention during diagnostic procedures of cancer diagnosis and is emerging target in new treatment modalities and evaluation of new drugs [28]. However, this characterization requires thorough immuno-histologic analysis on biopsy material obtained upon radical prostatectomy. It has been shown that the immunohistological analysis is comparable with well-known concept of Benoit Mandelbrot, which allows us interpretation of vascular networks as fractal objects in connection to the regional

blood flow distribution and outlined by their fractal dimension [19, 29]. This morphological observations could be useful during histological sub-classification within tumor namely, tumor pathological grade determination [20].

Inflammation has been well described in the literature as one very well linked process toward cancer evolution. In prostate the chronic inflammation additionally promotes progress of preexisting conditions such as benign prostatic hyperplasia (BPH) [30]. There is also interaction of chronic inflammatory process and proliferation. In BPH, chronic prostatic disease proliferation index is increasing, and epithelial changes can be observed progressing throughout prostatic intraepithelial neoplasia (PIN) [22]. This is considered to be the effect of sustained p38 MAP kinase pathway activity [31].

Several authors have proven that previously identified p38-MAPK is critically involved in the sensitivity of cancer cells to the anti-tumor effect of nitrogen-containing bisphosphonates (N-BPs), such as zoledronic acid (ZOL) [32–34].

Proliferation is one of the fundamental biological processes means rapid cell multiplication often referring to both pathological adaptive processes of hyperthrophia and hyperplasia depending on tissue histotype [35]. The proliferation is initiated among other triggers very often with hypoxia. The hypoxic state in PCa can be observed in microenvironment, which consists of disorganized vasculature with increased permeability [36]. The vascular mesh and intercellular leakage often present are responsible for eventual oxygen deprivation (hypoxia) and hyponutrient state. Hypoxic state in prostate microenvironment might influence the development of androgen independence in prostate cancer patients, since there is clinical evidence suggesting that after withdrawal of androgens reduction in hypoxia in tumor regions of prostate cancer occurred [37].

3 Angiogenesis and the Epithelial-Mesenchymal Transition

The prostate is an endodermal tissue that arises during late embryogenesis prostate is formed through ductal budding from the anterior urogenital sinus epithelium. Formation of the prostate is an inductive event that requires reciprocal interactions between the urogenital sinus mesenchyme and epithelium, and is dependent on testicular androgen synthesis.

Although the adult prostate lacks discernible lobular structure, the seminal papers by McNeal [38–40] defined the human prostate as having a zonal architecture, corresponding to central, periurethral transition, and peripheral zones, together with an anterior fibromuscular stroma [41].

Cunha et al. defined the fundamental parameters of these epithelial-mesenchymal interactions [42, 43]. These studies demonstrated that an AR-dependent signal from the urogenital mesenchyme is required for prostate formation, while AR is not initially required in the urogenital epithelium for prostate organogenesis, but is subsequently necessary for epithelial differentiation and secretory protein expression.

4 Prostate Cancer and Angiogenesis: More Than One Alternative

Different alternative mechanisms of tumor vascularization occur in PCa, including sprouting angiogenesis, vasculogenesis, intussusceptive angiogenesis, vascular co-option, mosaic vessels, vasculogenic mimicry and trans-differentiation of cancer stem-like cells into tumor endothelial cells [44].

Prostate cancer and the sprouting angiogenesis
Sprouting angiogenesis is a process that involves a single endothelial cell (tip cell) selected from the vasculature, overcoming its quiescent environment and forming a new vessel. This is a slow process, more than 24 h before a new capillary loop become perfused and is integrated into the vascular system. This process follows a well-defined program: endothelial tip cell migrates towards a chemoattractant angiogenic signal, such as growth factors secreted by tumor cells and their stroma. After migration, endothelial cells proliferate during the sprouting process in tumors and form a slit-like lumen that is continuous with the lumen of the "mother vessel". In the end, proliferating pericytes of "mother vessel" migrate along the basement membrane of the sprout, resulting in the maturation of the new vessel [2].

Prostate cancer and the intussusceptive angiogenesis
Intussusceptive microvascular growth (IMG), also called intussusceptive angiogenesis, is the other major angiogenic mechanism and describes the formation of a new vessel by vascular invagination, intra-luminar pillar formation and splitting [45]. This type of angiogenesis, which has been observed in a wide variety of normal and malignant tissues, is faster and more economical than sprouting and does not primarily depend on endothelial cell proliferation, basement membrane degradation, and invasion of the connective tissue [44, 46]. However, in contrast to sprouting, IMG can work only on existing vessel networks. Therefore, the most important feature of IMG seems to be its ability to increase the complexity and density of the tumor microvessel network already built by sprouting, independent of endothelial cell proliferation. In addition, IMG can provide more surface area for further sprouting. Vascular intussusception has initially been described in physiological vascular development [47] but more recently has been expanded to experimental tumors. It has been suggested that sprouting angiogenesis may switch to vascular intussusception to allow rapid development of new vessels [48]. Intussusception has been implicated in three processes of vascular growth and remodeling: *a)* IMG permits rapid expansion of the capillary plexus, furnishing a large endothelial surface for metabolic exchange; *b)* Intussusceptive arborization causes changes in the size, position, and form of preferentially perfused capillary segments, creating a hierarchical tree; *c)* Intussusceptive branching remodeling (IBR) leads to modification of the branching geometry of supplying vessels, optimizing pre- and postcapillary flow properties. The molecular mechanisms that drive vascular intussusception are currently poorly understood and whether the intussusception occurs or plays a role in PCa or tumor biology in general is currently unclear. It is now known that local stimuli, such as

intravascular shear stress, might induce a cascade of physiological or pathological reactions in endothelial cells, and new capillary development by tissue pillar formation could be one of them [49]. Furthermore, intussusception is certainly synchronized by several cytokines. Major candidates are those capable of mediating information between endothelial cells or from endothelial cells to mural cells, such as PDGF-BB, angiopoietins, and their Tie receptors, TGF-β, monocyte chemotactic protein-1, and ephrins and Eph-B receptors. In PCa, the role of IMG is still unknown.

Prostate cancer and the vasculogenesis

A major mechanism involved in the *de novo* formation of blood vasculature is called "vasculogenesis". This term describes the formation of a capillary-like network from either a dispersed or a mono-layered population of endothelial cells. Vasculogenesis has long been thought to occur only in the early phases of vascular development. However, recent studies have demonstrated that circulating bone marrow-derived endothelial progenitor cells home to sites of physiological and pathological neovascularization and differentiate into endothelial cells. Endothelial progenitor cells may be mobilized by tumor tissue derived cytokines from the bone marrow [2]. Best characterized among these cytokines is VEGF that mediates vasculogenesis through promoting endothelial cell growth, migration and mitosis, and has involvement in cancer pathogenesis, progression and metastasis. Actually, there are few evidence on the role of vasculogenesis in PCa. Recently, Wang et al. evaluate the prognostic value of VEGF in PCa, and summarize the results of related research on VEGF [50]. Although a more definitive conclusion enabling the clinical use of VEGF in PCa need more high-quality interventional original studies following agreed research approaches or standards, they found that VEGF might be regarded as a prognostic marker for PCa [50]. During tumor progression, the level of circulating VEGF has been shown to rise, and this level was found to correlate with the number of endothelial progenitor cells in the circulation. Yang et al. have shown that human bone metastatic LNCaP-derivative C4-2B PCa cell line expressed higher level of VEGF than its parental primary PCa cell line LNCaP [51]. Moreover, other studies demonstrated that PCa cells seems to modulate their microenvironment and facilitate bone-marrow-derived endothelial progenitor cells (BM-EPCs) migration and vasculogenesis by secretion of cytokines in the early stage of hypoxia [52].

Prostate cancer and the vessel co-option

Vessel co-option, also known as *mosaic vessel formation*, is a new way to obtain blood supply [2]. The use of pre-existent vessels was described first in the brain, one of the most densely vascularized organs in the body. It has been shown that vascular co-option may facilitate the infiltration of human gliomas [53]. Although in 1987 Thompson [54] had already proposed that tumors acquire their vasculature by incorporation of host tissue capillaries, the first study suggesting the existence of vessel co-option was not published until 1999 by Holash et al. [55]. In this process, vessels are surrounded, co-opted by tumor cells and no sprouts are observed. This host vascularization does not immediately undergo angiogenesis to support the tumor [2]. Co-option of pre-existing blood vessels might persist during the entire period of primary or metastatic tumor growth. In cutaneous melanoma, we found

that during tumor growth, there are no signs of directed vessel ingrowth; instead, these tumors appear to grow by co-opting the massive vascular plexus present in the peritumoral connective tissue [56]. The role of vessel co-option in tumorigenesis is still debated and, at our knowledge, there are still no studies in PCa.

Prostate cancer and the vasculogenic mimicry

Aggressive tumors may gain blood and nutrients from *de novo* vessels produced by themselves. Maniotis et al. in 1999, described the ability of highly aggressive melanoma cells to dedifferentiate into multiple cellular phenotypes, including those with endothelial-like characteristics that could form vessel-like structures to provide blood supply [57]. These channels are called vasculogenic mimicry (VM), because the channels are formed *de novo* and mimicry because the channels are not true blood vessel [58, 59]. The term VM describes the formation of fluid-conducting channels by highly invasive, genetically dysregulated, aggressive tumor cells without endothelial cell participation. As describe in melanoma, this channel is lined by thin basal lamina corresponding to the wall of the vessel, but no endothelial cells are detected [2]. Two distinctive types of VM have been described. VM of the tubular type may be confused morphologically with endothelial cell-lined blood vessels. Vasculogenic mimicry of the patterned matrix type in no way resembles blood vessels morphologically or topologically. This study is based on the principle that the walls of VM channels are similar to blood vessels in having an extracellular matrix and glycosaminoglycans that offer them PAS sustainability; however, by definition the VM channels have no endothelial lining and do not stain with endothelial markers such as CD31 or CD34. Moreover, tumor cells are aligned with external superficies of the channel [60] which was recently visualized with d2–40 immunorectivity showing concomitantly low grade PIN progression toward high grade PIN (Fig. 11.1). Blood plasma and red blood cells (RBCs) can flow through the channel, but no inflammatory cells or necrosis is found in it. VM has been found in many tumors, such as breast cancer, hepatocellular carcinoma, osteosarcoma, melanoma, ovarian carcinoma and PCa. In 2002, Sharma et al. revealed supportive evidence that VM occurs in invasive, heterogeneous PCa cell lines, and in aggressive rat and human tumors [61]. Green fluorescent protein (GFP) labeling of prostatic clonal subpopulations revealed unique cooperative interactions of epithelial- and fibroblastic-like tumor cells in the formation of perfusable vasculogenic-like networks. Furthermore, prostatic tumor cell-lined channels were also detected *in vivo* in high-grade tumors, and occurred in some cases in close proximity to conventional endothelial-lined vasculature [61]. Liu et al. investigated the role of VM in the progression of PCa and found that VM mainly exists in the high-risk PCa patients and is an independent marker of poor prognosis [62]. Recently, an *in vitro* study has shown that VM in PCa may be principally involved in bone metastasis.

Prostate cancer and the trans-differentiation of cancer stem-like cells into tumor *endothelial cells*

Recent studies have shown that cancer stem cells (CSCs) and epithelium-to-endothelium transition (EET), a subtype of epithelial-to-mesenchymal transition, accelerate VM formation by stimulating tumor cell plasticity, remodeling the extracellular matrix (ECM) and connecting VM channels with host blood vessels.

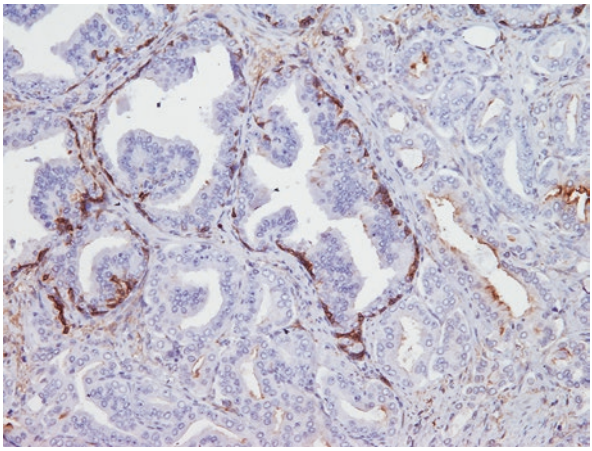

Fig. 11.1 The D2–40 immunoreactivity showing concomitantly low grade PIN progression toward high grade PIN, (x200HPF). The immunohistochemical expression is preserved in basal cell layer of glands with intraepithelial change

5 Prostate Cancer and the Microvessel Density Evaluation

While there are currently no markers of the net angiogenic activity of PCa that can help investigators to design specific anti-angiogenic treatment strategies, it is reasonable to assume that the quantification of various aspects of tumor vasculature may provide an indication of angiogenic activity. One often-quantified parameter of PCa vasculature is microvessel density (MVD), which is used to allow a histological assessment of tumor angiogenesis. In the last decade, studies have suggested the value of using MVD as a prognostic index in PCa, and it has also assumed that may reveal the degree of angiogenic activity in PCa. MVD scoring appears to be an important, simple, and applicable histologic tool for PCa evaluation in daily practice.

However, MVD has a number of limitations. The conflicting results in PCa are likely due to the differences in study designs: variability in patient population size, tumor topography, approach to selection of representative tumor areas, choice of endothelial marker, and actual counting method. The selection of the tumor area for MVD assessment has been based on two different approaches: [1] analysis of a few microscopic "hot spots" containing the maximal vascular density, and [2] selection of random representative areas of the tumor. The first approach is the most applied due to its simplicity, although there is no agreement among investigators regarding optimal microscope magnification, the number of vascular hot spots, and cut off values for low vs high MVD. The second approach of MVD assessment within larger representative areas or whole tissue may be more objective but involves more tedious examination.

Despite its importance as a prognostic indicator in untreated tumors, MVD has not been shown to be a valid measure to guide or evaluate anti-angiogenic treatment [63]. MVD does not appear to be predictive of tumor response under anti-angiogenic treatment and therefore may not be useful for stratifying patients for clinical trials [63–66]. Tumor MVD may not vary in accordance with the tissue or blood levels of any single pro-angiogenic factor. The MVD of a tumor need not to be higher, and is often lower, than that of its corresponding normal tissue, which is experiencing no

net growth. The efficacy of anti-angiogenic agents cannot be simply visualized by alterations in MVD during treatment [63, 67]. In addition, MVD is substantially limited by the complex biology characterizing tumor vasculature [68], and the highly irregular geometry that the vascular system assumes in real space, which cannot be measured using the principles of Euclidean geometry because it is only capable of interpreting regular and smooth objects that are almost impossible to find in Nature [69]. Quantitative descriptors of its geometrical complexity can be, however, abstracted from the Fractal geometry introduced by Benoit Mandelbrot in 1975 [3, 70]. The complex geometry of tumor vasculature, its structural and functional heterogeneity mean that vascular network cannot be measured on the basis of MVD estimates alone. Tretiakova et al., applying an automated image analysis to conventional and tissue microarray sections in large representative areas, demonstrated that there was no significant increase in MVD parameters in PCa *versus* matched normal peripheral zone prostatic tissue [71]. Paradoxically, several morphological indexes were higher within normal glandular prostatic tissue. Study of two-dimensional vascularity of PCa by Taverna et al. (2009) divided all cases in two groups with 56% of cases showing increase of vascular surface in PCa vs. non-tumoral areas and 44% showing a decrease of vascular surface in PCa. The second group of patients had a poorer outcome indicating that tumor progression is independent of angiogenesis [72]. These findings parallel recent data by Steiner et al. (2012) that showed no significant difference for CD31 mRNA levels from normal prostate and matched PCa ($P = 0.78$). No significant correlation for CD31 between mRNA and protein levels, show by immunohistochemistry, implies that in the typical slow growth of PCa, the angiogenesis dynamics are also quite low [73].

A non-invasive imaging technique that could reflect MVD would hold great promise in tumor detection and characterization [74]. An imaging method that could indicate an increase in MVD could have value in choosing targets for prostate biopsies [75]. This will lead to a change in biopsy strategies, bringing about a higher detection rate of PCa, and hence, a more appropriate therapeutic strategy. Preliminary data suggested that the hemodynamic indices obtained from contrast-enhanced ultrasound imaging were different between low- and high-grade PCa. Franiel et al. attempt to determine whether established histologic parameters of prognostic importance, including MVD, correlate with parameters obtained at pharmacokinetic dynamic contrast material–enhanced (DCE) dual-contrast-enhanced magnetic resonance (MR) imaging [76]. They found that blood volume and interstitial volume did not reliably correlate with the histologic parameters, mainly due to the heterogeneous vascularization of both normal prostate tissue and PCa [77]. Variability over patients is large with patients showing both increased and decreased vascularity in the tumor. Thus, determination of vascularization in a two-dimensional histological slide is not representative of the vascularity of the tissue as a whole [66]. The antibody used also seems to play a role, since it has been shown that MVD immunohistochemically determined by CD31 antibody staining was significantly lower than that obtained with CD34 antibody staining (Fig. 11.2) [78]. Moreover, correlation of histologic and MR data sets was limited by the fact that the paraffin sections are 4 μm-tick, whereas the corresponding T2-weighted images have a slice

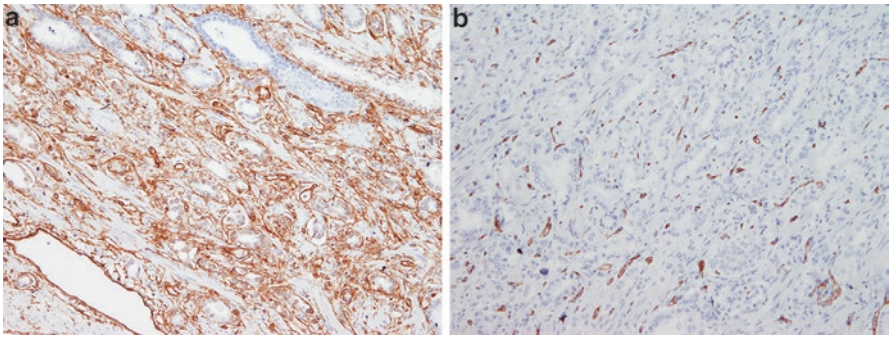

Fig. 11.2 The comparison of CD31 (**a**) antibody staining and CD34 (**b**) antibody staining, later one was of significantly lower intensity and distribution. This observation should be taken into consideration when immunohistochemically estimating and validating MVD

thickness of 3 mm and the dynamic susceptibility weighted MR DCE-MR sequence is acquired with a slice thickness of 5 mm. Computer-based 3D prostate models may in the future enable the desired detail correlation between histologic and MR imaging findings. The lack of correlation between histologic and functional parameters also raises the question of the biologic significance of functional parameters of tumor microcirculation quantified with dynamic imaging enhanced with small-molecule contrast medium. Although, Osimani et al. have recently shown that blood volume and permeability surface-area product measurements obtained with perfusion computed tomography have the highest correlation with immunohistochemical markers of angiogenesis in PCa but, before routine implementation, additional studies on larger series are needed [79].

6 Concluding Remarks

MVD as an angiogenesis predictor is inefficient per se in cancer prognosis [7]. We aimed to provide a view on novel factors that could possibly influence angiogenic switch consequently leading to progression from low grade prostatic intraepithelial neoplasia (PIN) to high grade PIN and beyond to PCa or even more aggressive, poorly differentiated, and androgen-independent histological subtypes. Angiogenic switch by its definition implies impaired angiogenesis and importance of high VEGF and VEGF receptor levels holding responsible for PCa progression has been shown. PCa is the most frequently diagnosed malignancy among men in Europe, and in the majority of countries is among the first three causes of cancer-related deaths too. If we look upon clinical prognostic parameters several of them (tumor stage, progression, metastasis and survival) strongly correlated in different studies with levels of angiogenic markers. But not only the clinical parameters have predictive value, it is also known that morphological scale of Gleason scoring system is independent prognostic marker and in the same time correlates with angiogenesis

level in PCa. This concept isn't the new one and several clinical researches are involved in this issue aiming to prove that blockage of angiogenesis could resolve the progression from an indolent to aggressive PCa. In the recent publications quantitative methodology used for angiogenesis detection gave us controversial results showing decrease of mean MVD in areas infiltrated with PCa, while relatively increased or better to consider preserved angiogenesis in cancer unaffected areas [6]. Is there rationale explanation for this observation? A plausible answer to that question can be simple as relative tissue density – meaning when you have tumor glands otherwise not present in prostatic fibrous stroma normal vascular network-will be deranged and number of vascular spaces would diminish. Yet we must admit that two structures cannot occupy the same space at the same time. This solves only one part of the problem being the tumor glands when present.

But the loose of vascular mesh is gradual also present in PIN lesions, how is this explainable? We propose the model of hypoxia inducing epithelial metabolic disruption in otherwise normal cells. Namely, in benign prostatic hyperplasia epithelial cells are proliferating, while microenvironment is preserving normal oxygenation. In the chronic prolonged inflammation due to secretory function obstruction the number of inflammatory cells and macrophages is increased leading to hypoxic state in stroma. Impaired epithelial secretory function in prostate at different levels of its utilization was previously described. This may have open potentially new therapeutic targets, which could be even broaden if we consider chemokines and chemokine receptors as markers of prostatic secretory metabolic change. Namely, hypoxic environment induces Akt activation and induction of Hypoxia inducible factor 1 (HIF 1) in his alpha conformation. Direct and consequent effect of activated HIF is activation of VEGF production and it's binding at receptor (VEGFR) sites. From that point onward the epithelial cell is promoting anti-angiogenic micro environmental state. Once the tumor cells have finished transformation from normal, benign, proliferative and hyperplasic phenotype toward abnormal, malignant, highly proliferating and dysplastic epithelia they have also gained the property of invasiveness. The malignant gland has lost basal cell layer due to hypoxic preconditioning or oncogenic mutation causing basal cell apoptosis and vanishing of the basal cell layer.

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Part III
Therapeutic Implications of Angiogenesis in Eye Disorders

Chapter 12
Angiogenesis-Based Therapies for Eye Diseases

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Abstract Age-related macular degeneration (AMD), diabetic retinopathy (DR), myopic choroidal neovascularization (mCNV) and retinal vein occlusion (RVO) taken together are leading cause of blindness worldwide. Neovascularization in these retinal disorders is induced largely by vascular endothelial growth factor A (VEGF-A) and progresses rapidly to blindness if left untreated. VEGF-A, with a central role in both normal and pathologic vascular growth within the eye, binds to VEGF-A receptors (e.g., Flt-1) on the vascular endothelium and promotes angiogenesis in response to hypoxia and other stimuli. The current standard of care in managing AMD, DR, mCNV and RVO is VEGF antibodies administered through intravitreal route to block VEGF activity, which underlies the CNV. Although this therapy improves visual acuity in a substantial proportion of patients, significant number of patients experience persistent CNV leakage, fibrotic scarring and/or

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geographic atrophy. Most patients do not achieve substantial visual improvement and a third of treated eyes progress to legal blindness. Thus, a novel therapeutic strategy, which improves outcomes while providing inhibition of angiogenesis with acceptable safety profile, is an urgent and unmet medical need. In this review, we discuss the role of VEGF and VEGF receptors in angiogenesis and the current and potential future angiogenesis based therapies for AMD, DR, mCNV and RVO.

Keywords Angiogenesis • Macular Degeneration • Diabetic Retinopathy • Retinal Vein Occlusion • Vascular Endothelial Growth Factor

1 Angiogenesis

The term angiogenesis was used for the first time in medicine in 1935 by the pathologist Arthur Herting to describe the formation of new blood vessels in the placenta. Angiogenesis plays a central role in various physiological processes, not only during fetal development but also in tissue repair after surgery or trauma. Angiogenesis is also critical during wound healing, menstrual cycle, cancer, and various ischemic and inflammatory diseases. Dysregulated angiogenesis is considered as one of the cause of many common diseases, like cancer, blindness, ischemic heart disease, psoriasis and arthritis [1–3]. Under normal physiological conditions quiescent endothelial cells are protected against insults by the autocrine action of various growth factors such as VEGF, NOTCH, angiopoietin-1 (ANG-1) and fibroblast growth factors (FGFs) in adult blood vessels. The endothelial cells are covered by pericytes, which stabilizes the endothelial cell proliferation and protects from release of cell-survival signals such as VEGF and ANG-1. However, under stress conditions such as hypoxia, endothelial cells release hypoxia inducible factor HIF-1α that activates the release of VEGF and detachment of pericytes from endothelial cells, weakening of tight junctions and increased vascular permeability and migration of endothelial cells onto this ECM surface in response to integrin signaling and release of several growth factors such as VEGF and FGF, placental growth factor (PlGF).

Endothelial cells (EC) and the mural cells (pericytes in medium-sized and smooth muscle cells (SMCs) in large vessels) are two main players in angiogenesis. Endothelial cells line the interior surface of blood vessels forming the interface between circulating blood and the rest of the vessel wall. Mural cells are contractile cells that wrap around endothelial cells keeping the homeostasis of the endothelial cells organization by communicating with the ECs both by direct physical interaction or paracrine signalling [4].

2 Molecular Mechanisms of Angiogenesis

The secretion of angiogenic factors is the triggering event that leads to vessel branching through multiple sequential steps (Fig. 12.1); from exiting the pro-quiescence program of the existing vessel, to the activation of endothelial cells and creation of a new vessel, to the consolidation and maturation of it. In adults, angiogenesis occurs when an injury or diseased tissues cause the secretion of angiogenic growth factors that diffuse in the nearby tissue, binding specific receptors situated on the surface of endothelial cells of nearby preexisting vessels activating the quiescent endothelial cells. The growth of new capillaries from preexisting blood vessels, is a complex process involving endothelial cell activation, disruption of vascular basement membranes, and migration and proliferation of endothelial cells. Migration of endothelial cells involves three major mechanisms: chemotaxis, haptotaxis and mechanotaxis (Fig. 12.2). In chemotaxis, activation of endothelial cells leads cell migration towards angiogenic growth factors (chemoattractants) such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF) followed by degradation of the basement membrane in haptotaxis where they migrate in response to integrins binding to ECM. Whereas, mechanotaxis is associated with fluid shear stress that modulates the various phases of cell migration including extension at the leading edge, adhesion to the matrix, and release of

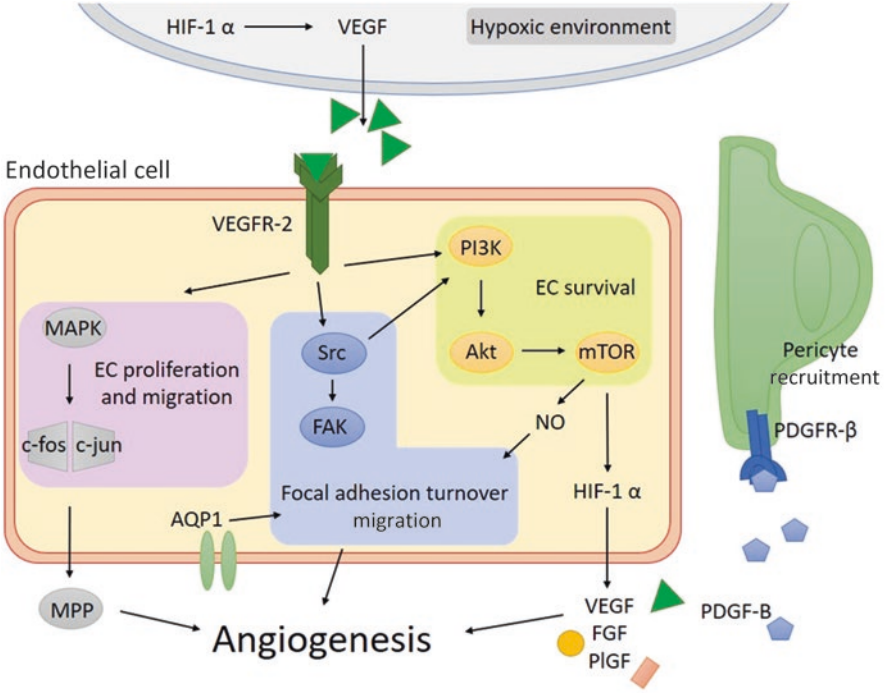

Fig. 12.1 Brief overview of angiogenesis process

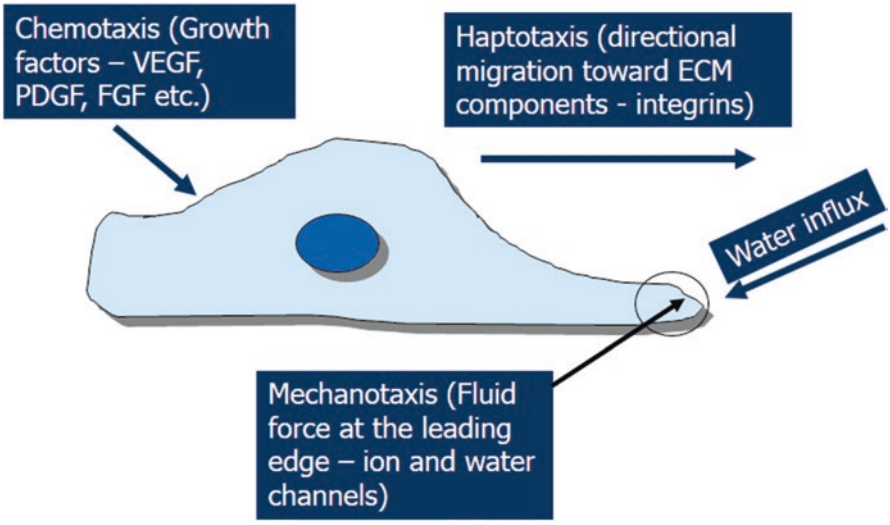

Fig. 12.2 Mechanisms involved in cell migration

adhesions at the rear edge. There are several proteins involved in the polarization and detachment steps including ENac, NHE1, NKCC1, AE2 and AQP1 to name just a few. Further, during migration cells undergo rapid changes in shape and cell volume. These changes require rapid fluid influx in order to cell polarize into frontal lobe and detach from the rear part. Finally, cells establish new basement membrane by secreting cytokines, platelet derived growth factor (PDGF) and angiopoietins to attract pericytes to stabilize the newly formed vessels [5], which can further grow or undergo remodeling to form new capillaries. In addition to endothelial cell activation, endothelial progenitor cells (EPCs) that are capable of proliferation and migration are also involved in the formation of new blood vessels in response to injury or hypoxia. However, the participation of EPCs in the formation of new blood vessels is still controversial. Prolyl hydroxylase domain 2 (PHD2) and hypoxia-inducible factor-1α (HIF-1α) modulate blood vessel shape and maintain optimal blood flow. Tight junction molecules (claudin, occluding, jam-1), adherens junction molecules (VE-cadherin), gap junction molecules (connexin-37, −40, −43), PECAM-1/CD-31, as well as a molecular crosstalk between ECs and neighboring mural cells are crucial in regulating vessel stability and permeability [6].

At rest, endothelial cells and pericytes, produce a common basement membrane. When a quiescent vessel intercept a pro-angiogenic signal, such as VEGF, ANG-2, FGFs or chemokines released by a hypoxic, inflammatory or tumor cell, pericytes will first detach from the basement membrane by proteolytic degradation mediated by secreted matrix metalloproteinases (MMPs). VEGF also stimulates the phosphorylation of β- and γ-catenins as well as p120-catenin, which are intracellular intermediary of VE-cadherin signaling. Consequently, VE-cadherin fails to cluster at the cell-cell interface and redistributes along the cell surface [7].

During the first steps of angiogenesis, Ang-2 levels increases, facilitating the destabilization of cell-cell adhesion and inhibiting Ang-1/Tie-2 pro-quiescence program, promoting endothelial cell-cell junction rearrangement and mural cells detachment. All these events together cause an increase in permeability of the pre-existing vessel and a consequent extravasation of plasma proteins, allowing deposition of a provisional matrix of fibrin that will serve as a substrate for migration of endothelial cells. This provisional matrix is loaded with pro-angiogenic factors such as VEGF and FGF that once liberated by the action of proteases, will guide the migration of ECs.

In order to create a new perfused tube from pre-existing vessels, nature has engineered a very smart process defined as sprouting (other mechanisms are known, such as intussusception, vessel co-option and vascular mimicry but the relevance of these processes is still not well understood). In this process, just one endothelial cell, known as the tip cell, equipped with filopodia to sense environmental guidance from ephrins and semaphorins, will be selected to move toward the angiogenic signal (guided by VEGF receptors, neuropilins (NRPs) and the NOTCH ligands DLL4 and JAGGED1), preventing an "en masse" disorganized movement of ECs. This tip cell will be then followed by nearby endothelial cells known as stalk cells, which will divide and elongate the growing vessel (the stalk) in response to NOTCH, NOTCH-regulated Ankyrin repeat protein (NRARP), WNTs, placental growth factor (PlGF) and FGFs. Once the stalk cells will reach the optimal number to create a new vessel they will start to organize and establish the lumen of the vessel (mediated by VE-cadherin, CD34, VEGF, and hedgehog).

The last step in angiogenesis is the consolidation of the new born vessel. Only matured and perfused vessels will survive, and to function properly, they must be covered by mural cells. Platelet-derived growth factor B (PDGF-B), ANG-1, transforming growth factor-β (TGF- β) contribute to this process [8]. To stabilize endothelial cells channels, angiogenic endothelial cells secrete PDGF-B to chemoattract PDGF receptor- β (PDGF- β) expressing pericytes [9, 10]. When the pericytes will be enough to fully cover the new vessel they will start to express ANG-1 inducing clustering of its receptor TIE-2 *in trans* at cell-cell junctions to maintain endothelial cells quiescence [11]. ANG-1 also stimulates basement membrane deposition. The basement membrane also provides signals for stabilization of the vessel, thus, inhibitors of metalloproteinases (TIMPs) and plasminogen activator inhibitor-1 (PAI-1) help the deposition of basement membrane that with act as a scaffold for stable ECs interactions.

3 VEGF Signaling

Despite the complexity of angiogenic process it is remarkable that VEGF regulates angiogenesis in such a predominant way. In mammals, there are five VEGF family members: VEGF-A, Placenta Growth Factor (PlGF), VEGF-B, VEGF-C, and VEGF-D. A numbers of VEGF-related proteins encoded by viruses (VEGF-E) and in the venom of some snakes (VEGF-F) have also been discovered [12].

VEGF-A and its receptors VEGFR-1 and VEGFR-2 play major roles in physiological as well as pathological angiogenesis. VEGF-C/D and their receptor VEGF-3 can regulate angiogenesis at early embryogenesis but mostly function as regulators of lymphangiogenesis [12, 13]. PlGF on the other hand is a multitasking cytokine that stimulates angiogenesis by direct or indirect mechanisms, however, unlike VEGF-A, PlGF is dispensable for development and is relevant only for diseases, especially for tumor cells [14, 15]. For all these reasons, in this review, we will focus our attention on the role of VEGF-A.

VEGF-A exert a variety of functions, including pro-angiogenic activity, vascular permeability activity, and stimulation of cell migration in macrophage populations and endothelial cells. In humans, through alternative splicing, VEGF-A can generate four different isoforms named upon the length of their amino acidic sequence: 121, 145, 165, and 189. VEGF-A_{165} has a weak affinity for acidic materials such as heparin/heparan sulfate and to neuropilin-1, a membrane protein involved in neuronal cell regulation and a co-receptor for VEGF-A. VEGF-A_{189} has a strong binding affinity for heparin/heparan sulfate, so, most of the VEGF-A_{189} molecules are localized on the cell surface or in the extracellular matrix.

The VEGF-A gene is unique in term of its haploid insufficiency. Heterozygotic VEGF-A knockout mice die at embryonic day 10 due to immature formation of the circulatory system [16]. This highlight the importance of fine tuning of VEGF-A levels in angiogenesis and vasculogenesis. Among the isoforms of VEGF-A, VEGF-A_{165} is the most critical, both quantitatively and qualitatively, and is sufficient and essential for angiogenesis in a VEGF-A-null genetic background in mice [17]. VEGF-A binds to and activates both VEGFR-1 and VEGFR-2, promoting angiogenesis, vascular permeability, cell migration and changes in endothelial cells transcriptional regulation via an autocrine loop of VEGF-A and its receptors contributing to endothelial functions [18].

4 Role of VEGF Receptors in Angiogenesis

Neovascularization is largely induced by vascular endothelial growth factor A (VEGF-A) [19–21]. This growth factor, with a central role in both normal and pathologic vascular growth within the eye [21–25], binds to VEGF-A receptors (e.g., Flt-1) on the vascular endothelium. Whereas, Neuropilins such as NRP1 and NRP2, VEGF co-receptors, enhance the activity of VEGF receptors and PlGF improves vessel perfusion and maturation and increases the revascularization of ischemic tissues. VEGFR-1 exists both as a membrane bound and as a soluble secreted form (also known as sFLT-1). The sFLT-1 regulates neovascularization by interacting with its ligand VEGF. VEGFR-1 signaling also promotes the growth of tumor cells in response to autocrine VEGF production.

VEGFRs are classic tyrosine kinase receptors composed of an extracellular domain for ligand binding, a transmembrane domain and a cytoplasmic domain including a tyrosine kinase domain [12]. VEGFs act through three structurally

related VEGF receptors named as VEGFR1, VEGFR2, and VEGFR3. Despite new finding that revealed a wider expression pattern of VEFGRs than initially anticipated, studies about VEFGRs expression highlighted a major role of VEGFR1 in monocytes and macrophages, VEGFR2 in vascular endothelial cells, and VEGFR3 in lymphatic endothelial cells. The binding of VEGF to its receptors induces a receptor homo- or hetero-dimerization. After dimerization, changes in the receptor conformation will lead to exposure of ATP binding site in the intracellular kinase domain with a consequent activation of the kinase domain and auto- or trans-phosphorylation of the receptor tyrosine residues as well as phosphorylation of downstream signal transducers. The cascade of tyrosine phosphorylation is tightly regulated by internalization and degradation of VEGFRs dimers or by dephosphorylation by phosphotyrosine phosphatases (DEP1, VEPTP, SHP2, PTP1B) [26]. Phosphorylated tyrosine residues act as binding sites for cytosolic signaling mediators with SH2 domains that will amplify the signal propagation eventually resulting in biological responses such as cell proliferation, migration and self-organization to form vascular perfused tubes.

Role of VEGFR1 in angiogenesis is still poorly understood and its action remains quite elusive. Its kinase activity is almost 10-fold weaker compared to VEGFR2 and it is not required for endothelial cell function. On the other hand, VEGFR1 binds VEGF-A with an affinity one order superior of that of VEGFR2. VEGFR1 null mice die at embryonic day 9 because of increased number of endothelial progenitors and formation of disorganized lumen-less vessels, while the deletion of tyrosine kinase domain from VEGFR1 results in mice with a normal vascular development. Thus, VEGFR1 appears not to be required as a signaling receptor in angiogenesis, but it may serve to capture VEGF and spatially regulates VEGFR2 signaling and vessel sprouting [27, 28]. VEGFR1 kinase activity has been shown to intervene in conditions involving inflammation such as rheumatoid arthritis [29] and early phase post stroke condition [30]. Thus, at least in part, VEGFR1 regulates endothelial function in indirect ways, through macrophagic recruitment and deposition of pro-angiogenic factors by these cells [31].

VEGFR2 is the main VEGF receptor on endothelial cells. It is essential for ECs biology during development in the adult organism, in normal or diseased angiogenesis. VEGFR2 is by far the most studied of the three VEGF receptors and it is the target of several small molecules engineered to block pathological angiogenesis in cancer. A soluble isoform of VEGFR2 is present in many tissues such as the skin, ovary, hearth, kidney, spleen and in plasma. It binds VEGFC and prevents its binding to VEGFR3 inhibiting lymphatic cells proliferation [32]. VEGFR2 knockout mice have a phenotype similar to that of VEGF-A knockout mice, resulting in death at embryonic day 8.5 by impairment of development of endothelial cells and hematopoietic cells [33]. VEGFR2 is mostly expressed in vascular endothelial cells and their embryonic precursors with the point of maximal expression occurring during the embryonic vasculogenesis or during pathological processes associated with neovascularization, as in the case of tumor angiogenesis [34]. For all these reasons VEGFR2 is believed to be the main signal transducer of VEGFA, allowing ECs proliferation, migration and differentiation during the process of sprouting of new

blood vessels from pre-existing ones. The endothelial cells proliferation is strongly induced in the case of VEGF-A/VEGFR2 binding via RAS/RAF/ERK/MAPK pathway.

VEGFR3 binds to VEGF-C and VEGF-D and it is an essential regulator of lymph endothelial function. It is strongly expressed during vasculogenesis in the developing embryo vascular endothelial cells, but later in development and in adult organisms, VEGFR3 expression is restricted to lymphatic endothelial cells. However, endothelial cells engaged in active angiogenesis, such as tumor vasculature or endothelial tip cells of sprouting vessels in the developing retina express VEGFR3 [35].

5 Anti-VEGF Therapies

Anti-VEGF stands for 'anti vascular endothelial growth factor'. Currently, three anti-VEGF drugs have been approved by the U.S. Food and Drug Administration (FDA) for the treatment of wet macular degeneration. The first breakthrough anti-VEGF therapy for the treatment of wet AMD was **pegaptanib (Macugen®)**. However, it is generally considered to be less effective than the other anti-VEGF agents and is not used in practice much anymore [36].

The current approved drugs used for anti-VEGF therapy are shown in Table 12.1 and are from the same class and work by stopping the proliferation of blood vessels in the eye. Since 2005, ophthalmologists had to face a dilemma choosing between the two closely related anti-VEGF drugs **Ranibizumab (Lucentis®)** and **Bevacizumab (Avastin®)** in treating wet AMD [37]. Bevacizumab is not a generic form of ranibizumab; ranibizumab and bevacizumab are different molecules produced in different ways. Although both are murine-derived humanized monoclonal antibodies, the active binding site of ranibizumab shows stronger binding affinity for its target. Bevacizumab (149 kDa) is larger than ranibizumab (48 kDa), but, despite the difference in size, retinal penetration studies have shown no difference in their ability to pass through the retinal tissue from the site of injection. Bevacizumab also includes the Fc portion so it may be a more potent antigenic stimulus [38]. Ranibizumab has been rigorously tested for use in neovascular AMD patients, whereas bevacizumab has been rigorously tested for intravenous use in colorectal cancer patients and used in AMD as "off-label". However, the presumed equivalent efficacy and the fact that ranibizumab is almost 40 times as costly as bevacizumab pushed doctors to engineer several head-to-head trials around the world. The comparison of AMD treatment trials (CATT) study examined the efficacy, dosing and cost-effectiveness of ranibizumab and bevacizumab for the treatment of neovascular AMD [39]. This prospective, randomized, trial revealed a significant improvement in vision with both treatments in terms of visual acuity and no significant difference between the two molecules except for the economic advantage of using bevacizumab.

The availability of the newer drug **aflibercept** has recently gained more attention. Aflibercept is a recombinant human fusion protein that acts as a soluble decoy

Table 12.1 Current approved drugs for Anti-VEGF therapy

Product name	Manufacturer	Relevance	Website
Macugen® (Pegaptanib)	Eyetech pharmaceuticals and Pfizer	VEGF antagonist approved in 2004	www.macugen.com
Lucentis® (Ranibizumab)	Genentech (Roche) and Novartis Ophthalmics	VEGF inhibitor approved in 2006	www.lucentis.com
Eylea® (Aflibercept)	Regeneron and Bayer healthcare	VEGF trap inhibitor approved in 2011	www.Eylea.US
Avastin® (Bevacizumab)	Genentech (Roche)	Off label use	www.Avastin.com

receptor for VEGF family members VEGF-A, VEGF-B and placental growth factor, preventing these ligands from binding to, and activating, their receptors. The efficacy of intravitreal aflibercept in neovascular AMD has been compared with that of intravitreal ranibizumab, the current gold standard for this indication, in two important phase III studies of virtually identical design (VIEW 1 and 2) [40]. In both trials, the recommended regimen of aflibercept [2 mg every second month (after three initial monthly doses)] was shown to be non-inferior to the recommended regimen of ranibizumab (0.5 mg every month) in terms of proportion of patients who maintained their vision after 1 year of treatment; similar results were seen when monthly dosing with aflibercept (0.5 or 2 mg) was compared with ranibizumab. During a 96 week period of observation in the VIEW studies, patients receiving the recommended regimen of aflibercept during the first year, followed by modified quarterly treatment during the second year had a similar visual acuity gain to those receiving the recommended regimen of ranibizumab, but on average required five fewer injections. So, doctors are looking forward to use this drug with the aim of reducing hospital appointments, emotional burden and treatment costs without compromising visual outcome.

In addition to demonstrating the efficacy of aflibercept, the VIEW trials provided valuable information regarding peak efficacy and durability of anti-VEGF therapy. Increasing the monthly dose of aflibercept from 0.5 mg to 2 mg did not appear to improve peak efficacy (maximum letters gained); similarly, the HARBOR trial showed that increasing the monthly dose of ranibizumab from 0.5 mg to 2 mg did not lead to further gains in vision (+10.1 letters versus +9.2 letters) [41]. These studies suggest that anti-VEGF monotherapy for neovascular AMD has reached its limit and that, in the future, combination therapy with drugs that target other biological pathways, will be necessary to improve AMD treatment.

KH-902 (Conbercept®), is another anti-vascular endothelial growth factor (VEGF) drug approved for the treatment of wet age-related macular degeneration in China. Similar to aflibercept, it is a recombinant fusion protein of key extracellular domains from human VEGF receptors 1 and 2 and IgG Fc produced in a Chinese hamster ovarian cell line. However, conbercept also contains the fourth domain of the VEGF receptor 2 [42].

Compared with aflibercept, conbercept is slightly larger, has a lower VEGF dissociation rate and higher binding affinity, exhibits decreased adhesion to the extracellular matrix, and has a lower isoelectric point that results in a longer clearance time.

6 Natural Anti-angiogenic Molecules

Many of the FDA approved anti-angiogenic factors in use are synthetic chemicals or humanized monoclonal antibodies directed against angiogenic factors or their tyrosine kinase receptors. Although these chemicals have been proved to be effective in inhibiting angiogenesis, many of them are extremely expensive, display significant toxicity, and are susceptible to resistance mechanisms. For all these reasons, the identification of natural, low toxicity, inexpensive molecules will be highly desirable. Several natural compounds have been used for centuries in different parts of the world to treat diverse disorders and nowadays, these compounds, have attracted the attention of scientists in search of natural anti-angiogenic molecules [43]. They have been tested for their potential therapeutic effects in many diseases such as cancer and inflammatory and cardiovascular diseases. In addition, several classes of natural compounds such as flavonoids, resveratrol, curcumin and genistein have been studied as antiangiogenic compounds for their potential therapeutic effects in various ocular neovascular diseases including AMD, DR and ROP using in vitro and in vivo models of angiogenesis [44].

Polyphenols, for example, are members of a large class of chemical compounds that are present in high concentration in several plants and fruits, such as tea, curcumin, grapes and berries [45]. Polyphenols exhibit anti-proliferative effects on tumor cells and ECs, inhibiting tumorigenesis through their anti-angiogenic, antioxidant and anti-proliferative properties [46–48]. Polyphenols are loosely defined as having several hydroxyl groups on aromatic rings. They are divided into classes such as phenolic acids, flavonoids, stilbenoids and lignans, according to the number of phenolic groups and the structures that connect these rings to one another [45]. The flavonoids are the most common class of polyphenolic compounds that are found ubiquitously in plants. They share a common structure of two aromatic rings that are connected together by three carbon atoms that form an oxygenated heterocycle [45]. They are divided into subclasses according to the substitutions on the heterocycle and the position and length of the linker between the cyclic moieties, and include flavonols (e.g., quercetin), flavones (e.g., luteolin and apigenin), isoflavones (e.g., genistein), flavanones (e.g., hesperetin) and homoisoflavanones (e.g., cremastranone). Many flavonoids have been studied for their beneficial roles in ocular diseases. Among the polyphenols members, resveratrol, a molecule especially present in grape and berries, can restrain tumor growth in mice by inhibiting ECs migration, proliferation and new blood vessel formation interfering with FGF2 and VEGF receptor-mediated activation of MAPK in ECs. In ovarian cancer cells, resveratrol is able to inhibit HIF-1α expression [46, 49, 50]. Catechin derivatives, pres-

ent in green tea, also inhibit VEGF and angiogenesis through suppression of protein kinase C (PKC), c-fos and c-jun in human breast cancer cells [51], or through suppression of Erk-1/2 phosphorylation in human colon cancer cells [52]. In another study, the catechin derivative epigallocatechin-3-gallate (EGCG) was shown to interfere with neutrophil-induced angiogenesis [53]. Curcumin, isolated from turmeric (*Curcuma Longa*) showed anti-angiogenesis properties and the ability to inhibit bFGF-mediated endothelial cell tube formation in vitro [54, 55].

Another important class of natural molecules that showed promising anti-angiogenesis effect is the alkaloids class. Castanospermine, an alkaloid extracted from *Castanospermum austral* is able to inhibit both migration and invasion of endothelial cells through the basement membrane, preventing new blood vessel formation [56]. Sanguinarine and brucine have been reported to suppress VEGF-mediated EC migration and sprouting both in vitro and in vivo interfering with Akt phosphorylation and Src, Erk, Akt, mTOR pathways respectively [57–59]. Other two alkaloids, 6′-debromohamacanthin A and tylophorine inhibit VEGF- and VEGFR2-mediated angiogenesis through similar mechanisms, suppressing PI3K/Akt/mTOR pathway [60, 61].

Recently, anti-angiogenic properties through inhibition of VEGFR2 phosphorylation were found in the medicinal mixture triphala churna (THL), a mixture formulated in the traditional Indian system of medicine. The tannin compounds chebulinic acid and chebulagic acid present in this mixture were the responsible for the anti-angiogenic action [62].

7 Angiogenic Retinal Diseases

7.1 Age-Related Macular Degeneration (AMD)

AMD is a leading cause of central vision loss in patients older than 65 years of age [63], and its prevalence is increasing due to increased life expectancy affecting almost 1 in 4 people by age 80 [64]. The projected number of people with AMD in 2020 is estimated ~196 million [65]. There are two main types of AMD, the dry and wet forms. Dry AMD causes some degree of visual impairment and may sometimes progress to blindness from geographic atrophy, a form of chorioretinal degeneration affecting the macula. In wet AMD, patients experience sudden loss of vision from subretinal exudation or hemorrhage caused by abnormal neovascularization, originating from the choroid (choroidal neovascularization; CNV) and less commonly, originating in the outer retina (retinal angiomatous proliferation; RAP). Left untreated, loss of photoreceptors, retinal pigment epithelial (RPE) atrophy and subretinal scarring ensue, leading eventually to irreversible blindness. CNV is induced largely by vascular endothelial growth factor A (VEGF-A) [19–21]. This growth factor, with a central role in both normal and pathologic vascular growth within the eye [21–25], binds to VEGF-A receptors (e.g., Flt-1) on the vascular endothelium.

Many growth factors are involved in the pathogenesis of neovascular AMD. Increased oxidative stress in the RPE and outer retina lead to increased levels of hypoxic inducible factor-1 (HIF-1), which up regulates several angiogenic gene products including VEGF, angiopoietin 2 (Ang2), vascular endothelial-protein tyrosine phosphatase (VE-PTP), platelet-derived growth factor (PDGF-B), stromal-derived growth factor (SDF-1) and placental growth factor (PIGF). HIF-1 also up regulates VEGF receptors (VEGFR1 and VEGFR2), PDGF receptor (PDGFRB) and chemokine receptor type 4 (CXCR4). In the presence of a compromised Bruch's membrane and RPE, these vasoactive factors contribute to CNV.

7.1.1 Current Anti-angiogenic Therapies for AMD

Macugen*, a* pegylated RNA aptamer directed specifically against the VEGF-165 isoform, and **b*evacizumab*** [66] represent the first 2 anti-VEGF agents approved for use in human. While visual acuity was maintained in patients given macugen, it has largely been replaced due to superior visual acuity gains seen in patients receiving non selective VEGF inhibitors like Ranibizumab (Lucentis), bevacizumab (Avastin), and aflibercept (Eylea) [39, 67].

In the landmark ANCHOR and MARINA trials, **Ranibizumab** was shown to deliver visual gains over 2 years of treatment with monthly intravitreal injections. In real world scenarios, however, most patients are unable to keep up with such an intensive treatment regime. Hence subsequent trials have focused on reducing the treatment burden by assessing pro-re-nata (PRN) and quarterly treatment regimens following a standard loading dose of 3 monthly injections. These trials showed that less frequent administration of ranibizumab produced inferior results compared to the monthly regime. PRN treatment regimens were comparable but still required patients to attend monthly follow up with their retina physicians. The burden of treatment is reflected in the poorer long term outcomes after patients exit the clinical trial, with most patients unable to keep up with the rigorous treatment and clinic attendance. ***Bevacizumab***, which was originally developed for treatment of colorectal cancer, has been widely used as an open label drug for CNV.

Aflibercept is the latest anti-VEGF agent to be approved for use in wet AMD. Compared to ranibizumab and bevacizumab, aflibercept has a much higher binding affinity to VEGF. It is thus able to effectively block VEGF even at low concentrations and theoretically be able to offer longer dosing intervals than the other anti-VEGF agents [68].

Although anti-VEGF-therapy (Avastin®, Lucentis®, Eylea®) has been hugely successful in treating a previously blinding disease, there is still a significant number of wet AMD patients who do not respond to treatment [69, 70]. Further, a recent 7-year follow up study (SEVEN UP; Seven-year outcomes in ranibizumab-treated patients in ANCHOR, MARINA, and HORIZON trials) observed that initial visual gains could not be sustained with anti-VEGF therapy [71]. The mean best corrected visual acuity decreased to −8.6 letters worse than baseline after 7 years. This long-term decline in the efficacy of intravitreal anti-VEGF therapy is concurrent with

substantial rates of sub retinal fibrosis (61% at 7 years) and geographic atrophy (90% at 7 years) [72, 73]. Long term follow up of patients from the CATT trial cohorts showed that 45.3% of patients developed subretinal scars at 2 years, of which more than half were classified as "fibrotic" [74]. An additional concern with repeated anti-VEGF therapy is the potential to induce RPE degeneration [74]. The SEVEN-UP study found that 98% of subjects showed RPE atrophy and other studies (CATT and IVAN studies) also noted that macular atrophy was associated with poorer visual outcome [75, 76]. Clearly, there is a need for new therapeutics to overcome these limitations with current anti-angiogenic therapy for wet AMD.

7.1.2 Novel/Experimental Agents

Anti-platelet Derived Growth Factor (PDGF) Pericytes play an important role in the maintenance of neovascular endothelial cells by secreting VEGF and other growth factors. Fovista (Ophthotech, New York, NY) is a 32-mer pegylated DNA aptamer that selectively binds to PDGF-BB and PDGF-AB receptors on pericytes. In addition, inhibition of PDGF can reduce the recruitment of non-neovascular components such as myofibroblasts, RPE, and glial cells around the neovascular endothelial cells to limit the amount of fibrovascular and fibrous tissues. In combination with an anti-VEGF agent, Fovista enhances the anti-angiogenic effect by stripping pericytes from neovascular endothelial cells. In the multi-centered randomized control phase IIb trial, combination therapy of fovista and ranibizumab achieved a 62% incremental benefit in terms of visual outcome compared to ranibizumab alone in patients with subfoveal classic CNV. The combination therapy group also had less severe subretinal fibrosis, as well as less progression of subretinal fibrosis compared to the ranibizumab monotherapy group [77]. Unfortunately, the phase III trials failed to demonstrate superiority of fovista combination therapy over ranibizumab monotherapy.

Rinucumab (Regeneron Pharmaceuticals, Inc.) is a PDGFR β inhibitor. In Phase II CAPELLA trial, rinucumab co-formulated with aflibercept did not demonstrate benefit over aflibercept alone at 12 weeks, both in terms of visual acuity and anatomical improvement. In fact, the occurrence of ocular adverse events were higher in the combination group, driven by a higher rate of conjunctival hemorrhage, eye pain and irritation.

Brolucizumab (Alcon) is a single chain humanized antibody fragment that binds to all isoforms of VEGF-A. With a molecular weight of just 26 kDa, it is the smallest anti-VEGF molecule developed for the treatment of wet AMD to date, with the potential advantage of better subretinal penetration and less risk of systemic toxicity [78]. Phase 1/2 randomized clinical trials comparing brolucizumab with ranibizumab have demonstrated non-inferiority in reduction of central retinal thickness, a longer treatment duration compared to ranibizumab, and no unexpected safety concerns [78]. Phase 3 studies are currently underway to test specifically the longer activity of brolucizumab and the possibility of a 12 weekly dosing regimen.

Abicipar Pegol (Allergan) is a designed ankyrin repeat protein (DARPin) with highly specific and high affinity binding to VEGF A isoforms. Phase 2 study of this compound for diabetic macular edema have shown encouraging results, achieving functional and anatomical results compared to monthly ranibizumab with fewer injections over a 28-week period. A Phase 3 trial (CEDAR) is currently enrolling patients and initial results are expected to be announced in 2018.

Bevasiranib (Opko Health, Inc.) is a first in class, small interfering ribonucleic acid (siRNA) designed to silence genes that produce VEGF by degrading messenger RNA (mRNA) molecules, thus preventing the translation and synthesis of VEGF. Although Phase II trials have shown promising results of bevasiranib in combination with ranibizumab [79], the Phase III trial (COBALT study) has been terminated as the treatment has been deemed to be unable to meet the primary endpoint of the study.

Tyrosine Kinase Inhibitors ***Pazopanib*** (GlaxoSmithKline, Brentford, UK) is a multi-targeted tyrosine kinase inhibitor that inhibits tumor growth factors such as stem cell growth factor (c-KIT), fibroblast growth factor receptor (FGFR), PDGFR and VEGFR1–3. Pazopanib was developed as an eye drop for the treatment of wet AMD, offering a potentially less invasive way of treating this disease. In a large Phase III clinical trial, pazopanib eye drops administered daily in conjunction with monthly or as needed ranibizumab did not provide additional therapeutic benefit compared to ranibizumab alone and did not reduce the number of as needed ranibizumab injections by the prespecified ≥50% criteria [80]. Similarly, ***Regorafenib*** (Bayer Pharma, Leverkusen, Germany) is another multi-kinase inhibitor that exerts anti-angiogenic effects by targeting the VEGFR-TIE2 tyrosine kinase and was evaluated as an eye drop therapy for wet AMD. In the DREAM study, a single arm open label Phase 2a/b study, regorafenib was unable to achieve similar visual acuity gains to established anti-VEGF therapies and was terminated after completion of phase 2a.

Multiple Growth Factor Inhibitors ***Squalamine*** is a small molecule inhibitor of multiple growth factors, including VEGF, PDGF and basic fibroblast growth factor (bFGF). In the IMPACT study, a randomized, multi-center, masked, placebo-controlled, Phase II clinical trial completed in 2015, squalamine lactate eye drops (OHR-102) demonstrated a 5.1 letter incremental benefit in visual acuity when used in combination with an anti-VEGF agent versus anti-VEGF monotherapy. The combination group also had a greater proportion of patients (42%) who achieved a ≥ 3-line gain in vision at 9 months, compared to the anti-VEGF monotherapy group (28%). ***Pan-90806*** (PanOptica, Bernardsville, NJ, USA) is a topically administered inhibitor of VEGF receptor 2, fibroblast growth factor 1–3, tyrosine kinase endothelial receptor 2, and other proangiogenic factors [81]. Initial results from the Phase1/2 study are promising, with 45–50% of treated patients with a positive response with regards to vision, lesion morphology and retinal thickness.

Integrins Integrins are a set of cell adhesion and cell signaling receptors that have been implicated in various systemic diseases from cancer to autoimmune diseases and also ocular angiogenic diseases including CNV and proliferative diabetic reti-

nopathy [82]. Targeting integrins in the treatment of CNV is promising because integrins have the advantage of targeting pathways both upstream and downstream of VEGF signaling. Anti-integrin therapy inhibits endothelial cell proliferation well before neovascular tissue can sprout, and prevents further growth of existing neovascular tissue.

ALG-1001 (Luminate, Allegro Ophthalmics) is a first in class multi-integrin receptor inhibitor designed for the treatment of neovascular AMD and diabetic macular edema [82]. In phase 1b/2a study intravitreal ALG-1001 monotherapy demonstrated visual acuity gains of 4 letters at 6 months with a prolonged treatment effect that lasted 4 months with almost complete resolution of subretinal fluid.

7.1.3 Gene Therapy

Modification of anti-angiogenic gene expression via viral vectors has the potential to provide long term sustained anti-angiogenic effect, thus overcoming the need for repeated intravitreal injections. **RetinoStat** (Biomedical, Oxford, UK) is a lentiviral Equine Infectious Anemia Virus (EIAV) vector expressing endostatin and angiostatin, angiogenic inhibitors that block endothelial cell migration and proliferation. The Phase I trial (GEM study) has shown that Retinostat has a favourable safety profile and demonstrated sustained expression after subretinal delivery [83]. ***AVA-101*** (Avalanche Biotechnologies, Inc), a subretinal gene therapy for neovascular AMD, is comprised of the adeno-associated virus serotype 2 (AAV2) vector containing the gene encoding soluble fms-like tyrosine kinase-1 (sFlt-1), which binds to and reduces the levels of VEGF and PIGF thus blunting the pro-angiogenic drive [84]. In Phase 2a study however, AVA-101 delivered a mean VA improvement of only 2.2 letters and mean retinal thickness increased by 25 mm at 12 months.

7.2 Myopic Choroidal Neovascularization

Myopic choroidal neovascularization mCNV is a sight threatening complication of pathologic myopia (PM) and is the second common form of CNV after age related macular degeneration [85, 86]. The mCNV is often bilateral and results in permanent and profound visual loss in younger individuals often during their working years [87] and shares some similarities in pathophysiology with other retinal angiogenic diseases. Over the past few years, clinical trials and real world experience have demonstrated the efficacy of intravitreal injections of anti-vascular endothelial growth factor (anti-VEGF) for treating mCNV and is now considered first line therapy [88].

Pathophysiology of mCNV The mechanism responsible for the pathogenesis of mCNV is complex and multifactorial. While there are similarities between mCNV and AMD-CNV [89, 90], additional factors specific to myopia are considered equally important. Most importantly, the development of mCNV is suggested to be

a progression of myopic maculopathy and pathologic myopia (PM) [91]. The mCNV occurs in about 5–11% of eyes with PM [92], and three theories have been proposed to explain the pathophysiology of mCNV. First theory suggests a mechanical stress on the retina from the progressive elongation of the eye [93]. This distortion of the RPE is believed to lead to an imbalance between pro and anti angiogenic factors which results in mCNV formation [94]. The second theory assumes the development of mCNV is related to a choroidal filling delay at the macular site and diffused thinning of the choroid [95]. The third theory suggests a heredo-degenerative process that results in pathologic myopia and mCNV. This theory is backed up by strong evidence for a genetic basis as suggested by twin and familial studies in pathologic myopia [96]. Further research in the pathophysiology of mCNV will likely provide insight for the optimal treatment for mCNV.

7.2.1 Current Anti-angiogenic Therapies for mCNV

Anti-vascular Endothelial Growth Factor Drugs Prior to the use of anti-VEGF therapy, treatment options for mCNV were limited to thermal laser photocoagulation, photodynamic therapy (PDT) with verteporfin and macular surgery. These treatments were largely unsatisfactory and lacked significant visual improvement and have now been superseded by anti-VEGF therapy. In mCNV, similar VEGF driven pathogenesis as AMD is postulated to occur [97]. With the success of anti-VEGF therapy in treating other retinal angiogenic diseases, it is no surprise that mCNV, has also shown good response to anti-VEGF therapy and now has become the first line treatment [88]. The strongest evidence of anti-VEGF use for mCNV comes from two large multi-centered, double-masked, randomized, controlled clinical trials [98, 99]. The RADIANCE (The Ranibizumab and PDT with verteporfin evaluation in myopic choroidal neovascularization) study was a 12-month, phase III, randomized double-masked, multicenter study comparing the efficacy and safety and efficacy of ranibizumab 0.5 mg given intravitreal versus verteporfin PDT in patients with mCNV [98]. This trial demonstrated the superiority of ranibizumab 0.5 mg over PDT with verteporfin as assessed by change in best corrected visual acuity (Ranibizumab regimens, BCVA gain 10.5–10.6 letters versus PDT with verteporfin, BCVA gain 2.2 letters up to month 3). The improvement in vision for the ranibizumab groups was maintained at 12 months with low total number of injections (3.5 to 4.0 injections) depending on retreatment regimen. In the disease activity group, patients were assessed for retreatment based on vision impairment attributable to presence of intra or subretinal fluid or active leakage secondary to mCNV. This regimen was shown to be non-inferior to retreatment according to visual acuity stabilization, at the same time with lower retreatment needs. The RADIANCE study confirmed the results of the REPAIR trial, a phase II prospective, multicenter study of intravitreal ranibizumab 0.5 mg in mCNV. The study proved the safety and efficacy ranibizumab as primary first line therapy in a cohort of treatment naïve patients with mCNV [100].

The MYRROR trial was a 48-weeks, phase III, multicenter, randomized, double-masked, sham-controlled study designed to investigate the efficacy and safety of intravitreal aflibercept 2 mg administered in patients with mCNV. 122 patients were recruited and patients in the intravitreal aflibercept group had a mean BCVA gain of 12 letters compared to a 2-letter loss in the sham group with improvement in BCVA maintained to week 48. Re-treatment was allowed in patients after the first intravitreal aflibercept at baseline based on a predefined criterion which included those who had a reduction in BCVA, increased activity clinically or on imaging, or deemed necessary by the investigators. The mean number of injections was also low in this study (4.2 injections over 48 weeks). Significant improvement in quality of life (National Eye Institute Visual Function Questionnaire 25 and EuroQol-5 Dimension score) was also demonstrated in patients treated with aflibercept [99]. These results support the early initiation of anti-VEGF treatment after diagnosis of mCNV to achieve maximal visual gains.

Although non-randomized controlled trials have been performed using bevacizumab in mCNV, many case series have also reported favorable visual outcomes. Prospective non-randomized interventional clinical studies reported significantly improved vision after treatment with bevacizumab. Gharbiya et al. reported a significant improvement in mean BCVA to 44 letters from baseline of 24.8 letters [101]. Chan et al. reported an improvement in mean BCVA logMAR (0.38 compared to 0.62 at baseline) with 72.4% achieving an improvement of 2 lines or more [102]. Iacono et al., reported improvement of mean BCVA from 54.8 letters to 59.2 letters at 24 months [103]. Ruiz-Moreno et al. reported an improvement from BCVA logMAR 0.55 to 0.38 at 12 months [104].

While anti-VEGF therapy has been used successfully in both mCNV and CNV secondary to age-related macular degeneration (AMD), key differences in the regimen has been shown in all the above studies. In CNV secondary to AMD, intensive, long term repeated injections are required. In contrast, the retreatment load is much lower in mCNV. On average, only 3–4 injections were needed over the first year in the RADIANCE and MYRROR study. Several other studies have also reported a mean number of injections of ranibizumab to be between 4–5 in the first year which tapers to 1–2 over the second and third years [105, 106]. The only time more injections were needed was when recurrence occurred with a mean of 6.9 injections for eyes with recurrence and 2.7 injections for eyes without recurrence [107]. Another difference from the treatment of AMD is that in mCNV, a 3-monthly loading phase appears to be unnecessary [108–111]. After initial treatment and stabilization, the mCNV can be monitored based on a combination of functional and morphological features. The treatment strategy suggested by the RADIANCE study used visual acuity stabilization criteria (no change in BCVA as compared to 2 preceding visits) and disease activity criteria (based on morphological features defined as intra or sub retinal fluid or active leakage secondary to mCNV as seen on OCT or leakage seen on FA) [112]. Visual acuity stabilization criteria required that vision be lost before re-treatment while disease activity guided criteria aimed to treat anatomical changes that preceded vision loss. Both strategies showed good outcomes however number of injections using disease activity (2 injections) was lower than visual acuity stabi-

lization criteria (4 injections). This suggest that the disease activity criteria provides a more sensitive approach to evaluating disease recurrence and provides earlier intervention resulting in less treatments for similar gains [98].

Data on long-term visual outcome in mCNV treated with anti-VEGF therapy are limited. Several studies have reported good visual outcomes up to 3–4 years [101, 113, 114]. Results from studies reporting follow up of 4 years and beyond are less favorable. In a retrospective cross sectional study Oishi et al. evaluated the outcome of eyes treated with intravitreal bevacizumab with follow up of at least 4 years and reported significant visual improvements at 1, 2 and 3 years, however the improvement became non-significant at 4 years after treatment. The main reason for the decline at 4 years might be related to chorioretinal atrophy (CRA), which affected 73% of eyes [106]. Sarao et al., reported similar findings in a prospective interventional study with an extension phase with mean BCVA improvement of −0.13 at 24 months in 101 eyes with mCNV treated with bevacizumab and an increase in CRA area in the same time period [115].

Strategies for Managing mCNV The optimal current management of patients with mCNV is prompt diagnosis and prompt treatment with intravitreal anti-VEGF therapy [116–118]. A possible strategy of treatment involves a single injection at diagnosis followed by a pro-re-nata regimen. Subsequent follow up should be monthly for the first 2 months [102, 119]. Monitoring for follow up can either be based on disease activity, which is defined as a drop in vision, new symptoms, or signs of mCNV activity on OCT or FA. If activity is noted anti-VEGF therapy is indicated. An alternative monitoring strategy is based on visual stability. Wong et al. suggest that, if there is no activity after an injection and 2 successive monthly visits, it is reasonable to prolong follow up to 3 monthly for the first year. Patients should be educated to return immediately if they experience any metamorphopsia or decrease in vision.

7.3 Diabetic Retinopathy (DR)

Diabetic retinopathy (DR), a specific microvascular complication of DM, remains the leading cause of acquired vision loss worldwide in middle-aged and therefore economically active people [120]. The prevalence of DR and vision-threatening DR were 34.6% and 10.2%, respectively [121]. With the increasing number of people with diabetes, the number of DR and vision-threatening DR (VTDR), which includes severe non-proliferative DR (NPDR), proliferative DR (PDR) and diabetic macular edema (DME), has been estimated to rise to 191.0 million and 56.3 million respectively by 2030 [120].

Pathophysiology of DR The pathogenesis of DR is multifactorial. Chronic hyperglycemia is thought to be the primary cause for its development [122, 123]. Development of diabetic retinopathy changes are summarized in Fig. 12.3. It compromises a retinal autoregulatory mechanism, vascular changes and retinal isch-

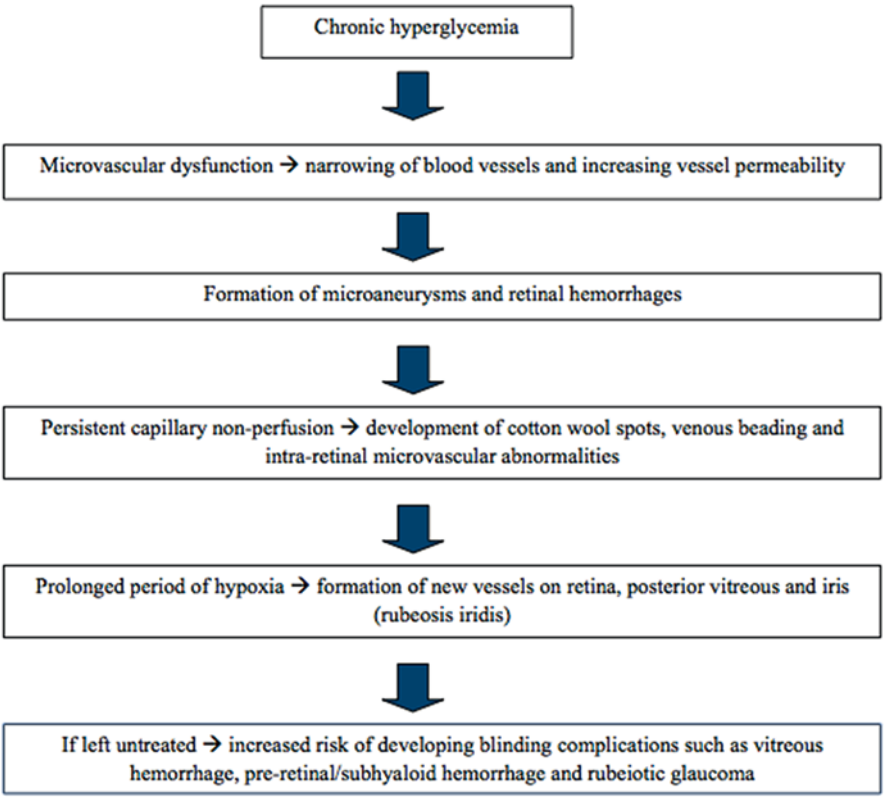

Fig. 12.3 Development of diabetic retinopathy changes

emia. Various other biochemical pathways have also been suggested to play a role in the development and progression of DR in people with diabetes—for example, the accumulation of sorbitol and advanced glycation end (AGE) products [124, 125], impaired autoregulation of retinal blood flow [126], increased level of protein kinase C [127], intraocular and serum angiotensin-converting enzyme (ACE) [128–130], plasma prekallikrein [131], erythropoietin [132] and various growth factors (e.g., vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), platelet-derived growth factors and pigment-derived factor (PEDF)) [133–135]. The accumulation of sorbitol damages vascular cells and pericytes, leading to the thickening of retinal vascular endothelial cell basement membranes and the closure of retinal capillaries.

Many angiogenic and anti-angiogenic factors had been implicated in the pathogenesis and progression of diabetic retinal disease, including vascular endothelial growth factor (VEGF) [136–143], platelet-derived growth factor (PDGF) [144, 145], increased level of nitric oxide (NO) [146], hepatocyte growth factor (HGF) [141, 142, 147], angiopoietin-2 [148, 149], cysteine-rich 61 (CYR61) [150–152] and sCD200 [153]. VEGF promotes angiogenesis and disassembly of junctions

between endothelial cells causing formation of new vessels and macular edema in patients with diabetes. Its effect is amplified by basic fibroblast growth factor (bFGF) [154, 155], which is also increased in proliferative diabetic retinopathy (PDR) [156]. Hypoxia and AGEs increase VEGF levels in the vitreous that causes neovascularization in PDR through complex signaling pathways [157]. VEGF also has the ability to induce vascular leakage in the retina by lowering the levels of 2 proteins that are critical for the maintenance of tight junctions, occludin and zonula occluden 1 (ZO-1) [158, 159]. In addition, Pigment Epithelium-Derived Factor (PEDF), a natural inhibitor of angiogenesis in the vitreous [160, 161] is decreased under hypoxic conditions in PDR eyes [147, 162–165] and decreased TGF-β levels were found in the vitreous of NPDR [147] and PDR [166] patients. TGF-β, secreted by the retinal pigment epithelial (RPE) cells [167, 168] and pericytes [169] in the retina, regulates the growth of new vessels.

7.3.1 Current Anti-angiogenic Therapies for DR

Panretinal photocoagulation (PRP) is the current gold standard treatment for patients with severe NPDR and PDR. In type 2 diabetes with severe NPDR and non-high-risk PDR, the risk of severe visual loss and vitrectomy was reduced by 50% (2.5% vs 5%, $p = -0001$) for those who received prompt PRP, compared to those who have deferred PRP until the development of high-risk PDR. More recently, the Diabetic Retinopathy Clinical Research Network (DRCR.net) showed that intravitreous Ranibizumab (IVR) could be a potential treatment for patients with PDR [130]. The IVR group showed less frequency of peripheral visual field loss (−422 dB vs −23 dB, $p < 0.001$), less need for vitrectomy (4% vs 15%, $p < 0.001$) and less incidence of DME (9% <28% $p < 0.001$) compared to the group that received PRP. For IVR, the reported risk of endophthalmitis was 0.5% (1 out of 2581).

For diabetic macular edema (DME) involving the fovea center, anti-VEGF treatment (e.g. Aflibercept, Ranibizumab and Bevacizumab) had been shown to provide a better visual acuity and anatomical outcomes, compared to focal/grid laser treatment alone. Based on DRCR.net protocol T (2-years results), both aflibercept and ranibizumab yielded more superior visual and anatomical outcomes compared to bevacizumab, with aflibercept achieving quicker recovery in the first year for those with presenting visual acuity of 20/50 or worse [131]. The addition of deferred laser treatment (>24 weeks) has also been shown to reduce the need for repeat anti-VEGF injections. RISE and RIDE studies showed patients with ranibizumab (0.3 mg and 0.5 mg) achieved approximately 10 letters more, compared than the sham group at 24 months ($p < 0.05$). The adverse events were no different between the ranibizumab groups versus sham [170]. For aflibercept, VIVID and VISTA showed aflibercept (every 4 weeks and every 8 weeks) can achieve best-corrected visual acuity improvement from baseline to week 148 of 10.4, 10.5 and 1.4 letters ($p < 0.0001$) in VISTA and 10.3, 11.7 and 1.6 letters ($p < 0.0001$) in VIVID, respectively [171].

Novel/Experimental Agents Apart from the above-mentioned therapies, research on various potential agents for DR/DME is currently underway. These include interleukin-6 inhibitor (tocilizumab) [133], Teprotumumab [134], ASP8232 [135], Abicipar Pegol [172], Luminate (ALG-1001) [173], AKB-9778 (Tie2 upregulators) [174] and anti-angiopoietin 2 therapy [175].

7.4 Retinal Vein Occlusion (RVO)

An estimated 16 million adults are affected by retinal vein occlusions (RVO) [176]. RVO is classified into branch retinal vein occlusion (BRVO) where there is occlusion at the arteriovenous intersection and central retinal vein occlusion (CRVO) where the occlusion occurs proximal or at the lamina cribrosa of the optic nerve [177]. The prevalence of BRVO is five times that of CRVO [176]. Complications that ensue include macular edema, macular ischemia and retinal or anterior segment neovascularization. Untreated BRVO can have devastating visual loss from vitreous hemorrhage and tractional retinal detachment, while non-perfused CRVO may lead to neovascular glaucoma.

Pathogenesis of RVO While the exact pathophysiology of retinal vein occlusions is not well elucidated, the associated complications are thought to be VEGF driven. Following a venous occlusion, hypoxia occurs in the retina. Hypoxia causes up regulation of growth factors, integrins and proteinases, which result in endothelial proliferation and migration [178, 179]. In ischemic retinal vein occlusion, ocular neovascularization occurs as a result of increased VEGF levels and various cytokines and growth factors such as interleukin-6 (IL-6), IL-8, interferon induced protein-10, monocyte chemotactic protein-1 and platelet derived growth factor-AA [180–182]. While macular edema was previously thought to be a result of transudation of fluid into the retina due to high intravenous pressure, current evidence point towards VEGF as a major cause of macula edema [183].

7.4.1 Current Anti-angiogenic Therapies for RVO

Anti VEGF Agents Anti VEGF agents are currently standard of care in managing retinal vein occlusions. The three main agents include ranibizumab and aflibercept which are FDA approved, and off label use of bevacizumab.

Ranibizumab Major initial randomized controlled trials assessing the efficacy of ranibizumab in RVO were the BRAVO and CRUISE studies [184–186]. In the BRAVO study, the authors investigated ranibizumab vs sham injections up till 6 months in BRVO related macular [186]. In the extension trial, the sham group had monthly ranibizumab injections till month 12 [185] where 56.0%–60.3% of the ranibizumab group and 43.9% of the sham/ranibizumab group achieved 3 lines or

more improvement. This study showed that with delayed treatment, visual improvement can still occur, but not to the level of those patients treated early.

The CRUISE study assessed the use of ranibizumab in CRVO related macular edema [184–186]. Its research methodology is similar to the BRAVO study whereby after 6 months the sham group could receive ranibizumab. At month 12 [185], the mean BCVA change was 13.9 letters in the ranibizumab group and 7.3 letters in the sham/ranibizumab group. This is despite a reduction of central foveal thickness of more than 400 μm in all groups. The authors also found that VEGF suppression not only results in resolution of macula edema, but also resolution of retinal hemorrhages. The mechanism by which retinal hemorrhages resolve is unknown, but postulated to be the effect of ranibizumab reducing the influx of RBCs, tipping the balance towards hemorrhage removal [187].

Long term data was provided by the HORIZON trial and the RETAIN study [188, 189]. The HORIZON trial found that decline in visual acuity was associated with fewer injections in CRVO patients, but BRVO patients remained stable despite fewer injections [189]. The RETAIN study confirmed that CRVO patients generally had worse prognosis than BRVO patients [188]. CRVO patients were likely to have a higher VEGF load compared to BRVO patients due to peripheral ischemia, which could account for the need for more VEGF blockade (i.e. more injections) in order to maintain vision.

The SHORE study was conducted to assess the optimal treatment regimen after RVO patients were stabilized by monthly injections for 7 months [190]. There was no difference in patients treated by PRN or monthly regimen, with over 70% achieving 20/40 or better vision at month 15. The PRN treatment group received about half the number of injections compared to monthly treatment, which strengthened the evidence for a criteria driven treatment regimen.

Aflibercept Major treatment trials with aflibercept in RVO include the VIBRANT, COPERNICUS and GALILEO study [191–194]. The VIBRANT study compared aflibercept to grid laser in treating BRVO related macular edema [191]. The grid laser group was eligible to receive intravitreal aflibercept from week 24 onwards. At week 52, 57.1% in aflibercept group and 41.1% in the laser/aflibercept group had 3 lines or more improvement in vision. Rescue intravitreal aflibercept given at week 24 onwards resulted in substantial improvements in vision and reduction in central retinal thickness. Similar to the bevacizumab trials, despite rescue with intravitreal anti-VEGF therapy the final visual outcome was still significantly worse compared to the aflibercept group (i.e. early treatment with anti-VEGF gave better visual outcome).

The COPERNICUS and GALILEO studies were phase III, randomized, double masked trials which were designed to evaluate the efficacy of aflibercept on CRVO related macular edema [192–195]. In the COPERNICUS trial, patients were randomized to receive monthly intervals of aflibercept or sham injections for 6 months [192]. In the extension trial the sham group received PRN monthly aflibercept up till month 12, and the aflibercept group received PRN treatment [193]. A 3 lines or more visual improvement was seen in 55.3% of aflibercept group and 30.1% of

sham/aflibercept PRN group [193]. In GALILEO study [194, 195], patients were initially randomized to receive 4 weekly aflibercept or sham injections up till 20 weeks. Then, from week 24 to 48, the aflibercept group received aflibercept in a PRN regimen, while the sham group continued to receive sham injections, differing in methodology from the COPERNICUS trial. At week 52, 60.2% in aflibercept group had more than 3 lines improvement compared to 32.4% in the sham group.

Bevacizumab Epstein et al. compared intravitreal bevacizumab to sham injection in a randomized trial for patients with macular edema secondary to CRVO [196, 197]. In the first part of the study, patients received either 6 monthly injections of bevacizumab or sham up to a period of 6 months [197]. In the extension trial, both groups received 6 monthly injections of bevacizumab up till month 12 (delayed treatment for the sham group). At month 12, 60% of the early treatment group achieved 3 lines or more improvement in vision, compared to 33% in the delayed treatment group. Bevacizumab was also found to be beneficial in reducing anterior segment neovascularization. After 12 months of treatment, no patients developed rubeosis in both groups [196]. This study also showed that early treatment was required in order to obtain satisfactory visual improvement. A study by Hikichi et al. [198] confirmed the long term benefit of intravitreal bevacizumab over 2 years. The percentage of patients with VA 20/40 or better was 4% at baseline and 66% at 2 years. There were no ocular adverse events and no cataract surgery was required at 2 years.

The MARVEL study compared bevacizumab to ranibizumab in the treatment of macular edema secondary to BRVO [199] where 59.4% of ranibizumab and 57.8% of bevacizumab groups had 3 or more lines improvement at 6 months. There were no significant differences in terms of reduction in central retinal thickness. Due to its small sample size (n = 75), this study failed to prove non-inferiority of bevacizumab.

There is a lack of evidence regarding the ideal treatment regimen for bevacizumab. Even though a small retrospective study showed that a treat and extend regimen may be as efficacious as a PRN regimen for BRVO related macular edema [200]. Additional studies are required to draw definite conclusions. It is also important to note that while the use of bevacizumab to treat retinal vein occlusions is efficacious and safe, it remains largely as an off-label use.

Currently, there is a lack of head to head trials comparing different anti-VEGF agents for retinal vein occlusions. Comparisons across studies are difficult due to differing patient characteristics and recruitment.

Novel/Experimental Agents Anti-VEGF therapy is currently the mainstay of treatment in retinal vein occlusions. There are newer agents being tested currently in phase I/II trials which target the angiogenesis pathway [201]. For example, AKB 9778 (Aerpio therapeutics), a competitive inhibitor of vascular endothelial protein tyrosine phosphatase (VE-PTP) is being tested in 6 patients with diabetic macula edema administered via subcutaneous injections of varying doses with no safety concerns [202]. In mice models, AKB 9778 promotes the activation of tyrosine kinase with immunoglobulin and epidermal growth factor homology domain 2 (Tie-

2), thus reducing vascular leakage and neovascularization [202]. A phase II clinical trial using conbercept for patients with macular edema secondary to RVO reported benefits for both BRVO and CRVO patients [203].

8 Future Directions in Angiogenesis Based Therapies

Currently, anti-VEGF therapy is the preferred treatment for retinal angiogenic disorders, but frequent need for intravitreal re-injections and their associated risks, treatment burden and financial costs, pose a serious problem. Also, a considerable number of the patients with wet AMD do not respond to anti-VEGF treatment. For example, intervention with ranibizumab only improves vision in one-third of patients and around 10% do not respond to the therapy. With more than 70% of wet AMD patients showing no significant vision improvements with anti-VEGF agents, high potential exists for alternative therapies. However, to compete with current treatments, new therapies will need to improve on many different areas, such as convenience, increased efficacy as well as improved safety and tolerability [204]. Some of the potential future anti-angiogenic targets are discussed below.

PPAR γ Peroxisome proliferator-activated receptor γ (PPAR γ) is a ligand activated nuclear receptor that plays an important role as a transcription factor in the regulation of gene expression linked to a variety of physiological processes. It is expressed in many different tissues such as adipose tissue, immune/inflammatory cells (monocytes, macrophages), skeletal muscle, heart, kidney, liver, lung, and the eye ball. Many of these tissues are relevant in AMD etiology, since lipid metabolism, inflammation and retinal cells are particularly involved in the progression of the disease [205]. PPAR γ is involved in many aspects of biology such as fat cell differentiation, glucose and lipid metabolism, aging, inflammation and immune response [206–208] and recent studies showed substantial anti-angiogenesis effects of activated PPAR γ in different organs [209–211]. Furthermore, PPAR γ is involved in oxidative stress mediated apoptosis and regulation of inflammatory gene expression. Since AMD often presents neo-angiogenesis, inflammation and oxidative stress mediated cell death, evidences are accumulating on the potential of PPAR γ as a pharmaceutical target for AMD treatment. PPAR γ has been reported to be highly expressed in AMD patients [212] and its ligands troglitazone and rosiglitazone, inhibit choroidal angiogenesis and RPE migration in vitro [213]. Intake of omega-3 long-chain poly unsaturated fatty acids (ω-3 LCPUFAs), agonists of PPARs, is associated with attenuation of retinal angiogenesis [214, 215]. Even phagocytosis of the photoreceptor outer segment by RPE cells, one of the most important event in retinal homeostasis, leads to activation of PPAR γ. For all these reasons PPAR γ could be a key molecule in AMD prevention and treatment, but more studies are needed to prove clinical relevance of its modulation by different natural and synthetic ligands.

Anti-immune or Anti-inflammatory Pathways Recently, comparative transcriptome analysis of AMD patients and normal human donor eyes has shed light on the molecular pathways underlying AMD's onset and progression [216]. Newman et al. [216] reported cell mediated immune responses as a pivotal feature of all AMD phenotypes. For this reason, working on the role of the immune system to treat neovascular AMD, could be a promising way to develop new treatments and discover new pharmacological targets.

mTOR Mammalian target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine kinase that plays a central role in integrating environmental cues and cues from the immune microenvironment. Sirolimus (previously known as rapamycin, Santen Pharmaceutical, Inc. and MacuSight, Inc.) was found to possess potent immunosuppressive and antiproliferative properties. Sirolimus blocks the T-lymphocyte activation and smooth muscle and endothelial cell proliferation that occurs in response to antigenic and cytokine (interleukins IL-2, IL-4, and IL-15) stimulation. Sirolimus arrests cell cycle progression by direct interaction with two intracellular proteins (immunophilin FK binding protein 12 (FKBP-12) and the mammalian target of rapamycin (mTOR)) [217]. Sirolimus markedly inhibits response of vascular endothelial cells to stimulation by VEGF [218] and inhibits hypoxia-inducible factor-1α, a major upstream regulator of VEGF [219]. In a mice model of AMD, administration of sirolimus inhibited both choroidal and retinal neovascularization [220]. A phase 1 study of 30 patients found that a single intravitreal administration of sirolimus (352 μg) was associated with improvement in visual acuity and reduction in retinal thickness. Preliminary findings suggested that subconjunctival administration (sirolimus 1320 μg) was as effective as intravitreal injection, and this feature of Sirolimus will be greatly welcomed by patients and ophthalmologists since it decrease patients discomfort for intravitreal injection.

TNF-α Tumor necrosis factor alpha (TNF-α) is a key molecule that plays a central role in inflammation, apoptosis, and immune system and the anti-TNF-α monoclonal antibody infliximab (Remicade, Centocor, Inc.) is successfully used in the treatment of many inflammatory diseases. Three wet AMD patients treated with intravenous infliximab for inflammatory arthritis showed blood vessel regression and improvement of visual acuity [221]. It has been reported that intravitreal infliximab also inhibited laser-induced CNV in rats [222]. In another study, three patients treated with intravitreal infliximab displayed an improved visual acuity and central foveal thickness [223]. A phase 1 study is currently ongoing to specifically evaluate infliximab efficacy for wet AMD.

Complement Component 3 Complement component 3 (C3), is a key activator of the complement pathway and part of the molecular program that activate innate immunity. C3, as well as others complement factors, are often found in proximity of drusen or even inside them [224] and genetic studies revealed correlation between AMD and certain C3 variants [225]. C3 induces VEGF expression *in vivo* and *in vitro*, and certain C3 gene polymorphism increases the risk of AMD [225]. Thus, inhibition of C3 has the ability to stop the complement cascade activation leading to

a decrease of local inflammation, tissue damage, and down regulation of VEGF. Thus, inhibition of immune pathways may play a therapeutic role in wet AMD. POT-4 (Potentia Pharmaceuticals) binds and inhibits C3 [226] displaying a long-lasting effect (3 to 6 months). A phase 1 study will provide safety and tolerability information on POT-4 injected into wet AMD patients.

The general idea, sustained by many different studies on the association between inflammation and neovascularization, is that, the inhibition of inflammation could prevent or slow down many of the early events in AMD pathophysiology, such as inflammatory damage to retinal tissue, drusen formation, macrophages recruitment and the establishment of a chronic inflammatory hot-spot that will establish the conditions that sustain neovascularization.

Integrin Receptor Antagonist Integrins are transmembrane proteins that mediate the attachment between a cell and its surrounding extra cellular matrix. They are composed of α and β subunits that heterodimerize to produce more than 20 different receptors. In pathologic condition such as proliferative vitreoretinopathy, altered patterns of integrin expressions are associated with RPE activation, migration, and proliferation [227] that in turn could lead to instability of the RPE monolayer and detachment from the intra-photoreceptor matrix [228]. These changes are thought to perturb RPE functions disrupting oxygen supply, nutrients uptake, and growth factors secretion, interfering with the homeostasis of the choroid/RPE/photoreceptor system [229]. It has been revealed that $\alpha v\beta 1$, $\alpha v\beta 3$, and $\alpha v\beta 5$ integrins were expressed in neovascular ocular tissue from patients with wet AMD [230]. Hammes et al. [231] showed that subcutaneous injection of $\alpha v\beta 3$ and $\alpha v\beta 5$ antagonists remarkably prevented retinal neovascularization in a mouse model of hypoxia induced proliferative retinopathy. These results indicate the possibility that $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins might be a therapeutic target for AMD [232]. Additionally, the effectiveness of $\alpha v\beta 1$ and $\alpha v\beta 5$ integrin antagonists (JNJ-26076713) against ocular neovascularization has been well documented [233]. There are two therapeutic candidates to antagonize $\alpha v\beta 1$ integrin, JSM6427 and volociximab, for the treatment of AMD.

PEDF It is a neurotrophic factor secreted by the RPE and widely expressed in central and peripheral nervous system. RPE cells are known to secrete PEDF apically to sustain photoreceptor functions and VEGF on the basal side to promote correct vascularization. PEDF is also an endogenous inhibitor of angiogenesis in the eye [234] and the correct balance between PEDF and VEGF secretion by RPE cells in the retina is extremely important. Inappropriate expression levels of PEDF and VEGF are associated with neovascularization [235]. Increases in VEGF and its receptor VEGFR2 and simultaneous decrease in PEDF were found in aged rats. These results suggest that normal aging retina is at increased risk for neovascular changes. A critical balance appears to exist between PEDF and VEGF, with PEDF counteracting the angiogenic potential of VEGF [236]. A decrease in PEDF may disrupt this balance and create a permissive environment for the formation of CNV in AMD.

ATG003 Cigarette smoking is the strongest environmental risk factor for wet AMD. Recently, it has been reported that PEDF protein expression was decreased in RPE from smoker patients with AMD compared with controls. It was also reported that nicotine, a potent angiogenic agent, increased VEGF/PEDF ratio in the RPE through nicotinic acetylcholine receptor (nAchR) [237]. In this context, a unique therapeutic eye drop for AMD treatment, ATG003, has been developed. ATG003 (CoMentis, formerly Athenagen) antagonizes nAchR pathway that mediates angiogenesis. It is the first noninvasive eye drop therapy for AMD, and phase 2 clinical trial has recently been completed. Evaluation of ATG003 therapy in combination with ranibizumab or bevacizumab are ongoing.

AdGVPEDF.11D (GenVec, Inc.) is an adenoviral vector allowing the expression of large amount of PEDF in the target tissue upon intravitreal injection. PEDF overexpression inhibits ocular neovascularization in murine AMD models [238]. A phase 1 single dose trial enrolled 28 patients with severe neovascular AMD [239]. The percentage of patients who had no change or improvement in lesion size at 6 months was 71% in the high-dose group versus 50% in the low-dose group. A clinical study suggested the possibility that anti-angiogenic activity may last for several months (up to 6 months) after a single intravitreous injection as half of the treated lesions did not change in size from baseline [239]. Although anti-VEGF therapy (intravitreal injection of ranibizumab, pegaptanib, aflibercept, and bevacizumab) is regarded as the more effective treatment for AMD now [240], the potential therapeutics of PEDF may be positive indication for future treatment of AMD.

9 Conclusions

Angiogenesis is a complex process largely induced by VEGF. Although the molecular mechanisms by which VEGF is upregulated in retinal disorders in response to hypoxia and other insults is not clear, VEGF plays a critical role in the retinal angiogenic disorders such as AMD, DR, RVO and myopic CNV. The anti-VEGF agents are the most commonly used to prevent the disease progression and provide visual improvement for these retinal diseases and they have become standard of care in treating these conditions. Bevacizumab, aflibercept, and ranibizumab suppress VEGF levels in eye when administered via intravitreal route. However, despite success with anti-VEGF-therapy, a significant number of AMD patients do not respond with appreciable clinical improvement. In fact, the mean visual outcomes actually drop below baseline visual acuity with anti-VEGF therapy observed in a 7 year follow up study. The non-responsiveness of AMD patients and insufficient efficacy of anti-VEGF drugs is not due to their inability to block VEGF pathway but rather involvement of other than VEGF angiogenic pathways in the abnormal neovascularization and therefore anti-VEGF therapies alone are not sufficient to treat neovascularization. Further, it has been demonstrated that multiple intravitreal injections of anti-VEGF antibodies induce RPE degeneration. Thus, an alternative therapeutic

strategy, which is independent of VEGF signaling pathway, is an urgent and unmet medical need. The ongoing clinical trials using non-VEGF agents such as angiopoietin 2 or vascular endothelial-protein tyrosine phosphatase and PEDF may provide benefit in suppressing the VEGF levels more effectively in AMD and other retinal disorders. In addition, sustained delivery of either existing VEGF agents or novel VEGF and/or non-VEGF agents alone or in combination may provide more effective approach to treat the angiogenesis based retinal diseases in future. Till then, frequent intravitreal injections of VEGF antibodies will continue to be the first line medical therapy for the retinal and choroidal neovascular diseases.

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Chapter 13
Anti-angiogenesis Therapy in Diabetic Retinopathy

Michael W. Stewart

Abstract Angiogenesis plays a central role in the development of diabetic retinopathy and its visually debilitating complications diabetic macular edema (DME) and proliferative diabetic retinopathy (PDR). Inflammation and ischemia, which result from hyperglycemia-induced oxidative stress, are primary drivers of diabetic retinopathy and vision loss. Several chemokines and cytokines are upregulated in eyes with diabetic retinopathy, and vascular endothelial growth factor (VEGF) is pivotal to the development of DME and PDR. Three VEGF inhibitors (aflibercept, bevacizumab, and ranibizumab) and three corticosteroids (triamcinolone, dexamethasone, and fluocinolone) are commonly used to treat the retinal complications of angiogenesis. Several phase III trials that were used to obtain regulatory approvals have produced level I evidence showing that these drugs are superior to standard therapy (laser photocoagulation or observation). Research into new anti-VEGF and corticosteroid drugs and formulations, delivery routes and devices, and molecular targets, promises to provide physicians and patients with additional treatment options in the future.

Keywords Aflibercept • Angiogenesis • Bevacizumab • Dexamethasone delivery device • Diabetic macular edema • Fluocinolone • Proliferative diabetic retinopathy • Ranibizumab • Triamcinolone • Vascular endothelial growth factor

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1 Introduction

The number of patients with diabetes mellitus (DM) throughout the world is projected to increase from 285 million in 2010 to 592 million in 2035. The increase is being driven by growth in both the overall population and the prevalence of the disease. Improving standards of living, widespread adoption of technology, and westernization of diets have increased the prevalence of metabolic syndrome, which is the major contributor to the development of DM.

Diabetes mellitus has become the leading cause of blindness in working-aged individuals in industrialized countries, with most of these cases resulting from

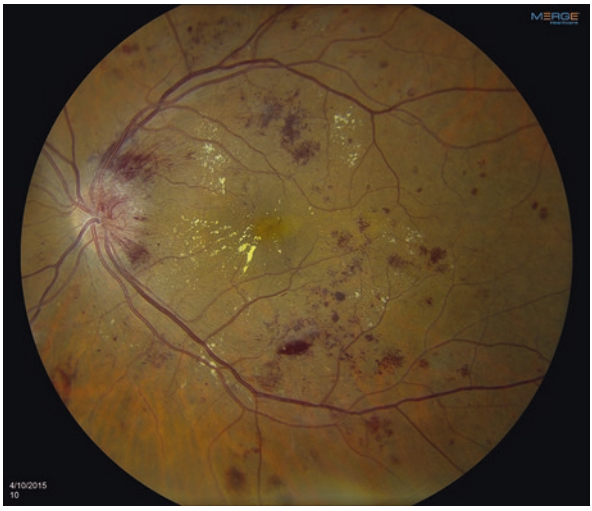

Fig. 13.1 This fundus photograph shows typical findings of diabetic retinopathy – microaneurysms, hemorrhages, and hard exudates – involving the posterior pole. Though it is difficult to determine macular thickness from a single photograph, the hard exudates near the fovea, loss of choroidal detail, and *yellow* spot in the fovea strongly support the diagnosis of diabetic macular edema

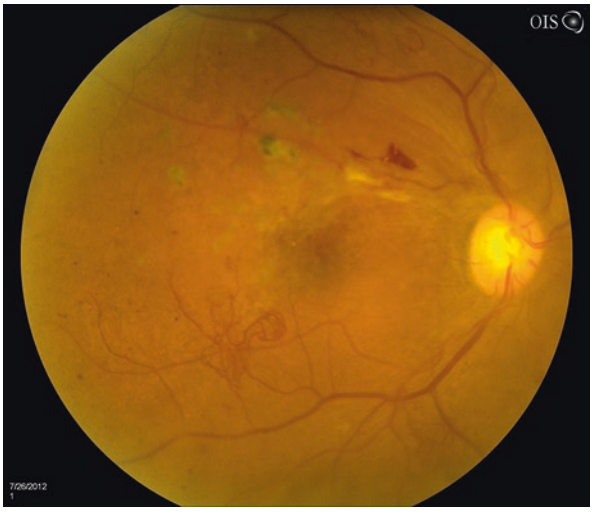

Fig. 13.2 This fundus photograph of an eye with proliferative diabetic retinopathy shows pre-retinal neovascularization inferior to the macula. Pre-retinal fibrosis extends from the superior arcade across the fovea

complications of diabetic retinopathy (Fig. 13.1). Approximately 1/3 of patients with DM have diabetic retinopathy (DR) and approximately 1/3 of these have diabetic macular edema. Seventy-five percent of diabetes-related vision loss results from diabetic macular edema and the remaining 25% arise from complications of proliferative diabetic retinopathy (PDR) (Fig. 13.2).

Neural dysfunction represents the first pathophysiologic retinal abnormality caused by diabetes but it cannot be visualized during routine ophthalmic examinations and it does not directly lead to vision loss. Diabetes induced angiogenesis, resulting from damage to retinal capillary endothelial cells, develops more slowly but closely correlates with vision loss. Manifestations of angiogenesis include breakdown of the blood-retinal barrier (BRB) and neovascularization. Anti-angiogenesis therapy aims to prevent and reverse vision loss due to these two processes.

This chapter will briefly discuss the pathophysiology of DR and will detail the recent advances in ocular pharmacotherapy that target vision loss due to angiogenesis.

2 Angiogenesis and Diabetic Retinopathy

Severe vision loss from unchecked angiogenesis has long been recognized as a major complication of DR. Michaelson (1948) postulated the contribution of a soluble "factor X" that he believed led to vitreous hemorrhage, traction retinal detachments, and iris neovascularization [43]. Vascular permeability factor (1983) was associated with breakdown of the blood-retinal barrier. Ferrara and Connolly (1989) independently discovered vascular endothelial growth factor (VEGF) and determined that it was identical to vascular permeability factor [15, 26]. These discoveries ushered in nearly three decades of prolific research that centered on the biology of VEGF and the development of drugs to block its actions.

Elevated concentrations of both VEGF and VEGFR2 have been found in animal models of diabetic retinopathy [31]. Early assays discovered that intravitreal concentrations of VEGF are elevated in patients with both DME and PDR [2], with higher concentrations generally correlating with neovascularization and not just BRB breakdown. Eyes with DME have higher aqueous VEGF concentrations than do those with nAMD and vein occlusions [28], and the concentrations correlate with the severity of the DME [29]. Vitreous VEGF concentrations in eyes with PDR fall after successful pan-retinal photocoagulation. The importance of retinal hypoxia, a major contributor to VEGF upregulation, to the development of DME was demonstrated in a proof-of-concept study in which 3 months of continuous oxygen administration significantly reduced the severity of DME [48].

VEGF is an important contributor to BRB breakdown during the early phase of DME but it may become less important when DME becomes chronic (defined as 3 years in one study). By this time chemokines and other inflammatory cytokines may drive BRB breakdown [16]. Elevated intraocular concentrations of brain-derived

neurotrophic factor, interleukin (IL)-1a, IL-6, IL-8, interferon protein-10, intercellular adhesion molecule-1, monocyte chemotactic protein-1, nerve growth factor, thymocyte growth factor-β, tumor necrosis factor-α, and placental growth factor [4, 35, 65] have been discovered in eyes with DME. Though VEGF has been the most studied cytokine in eyes with DR, these other molecules contribute to angiogenesis and emphasize the importance of inflammation in the development of DR.

3 Anti-VEGF Medications and Clinical Trials

Pre-clinical evidence suggesting that anti-VEGF therapy might be beneficial in eyes with advanced DR emerged from several sources. Injections of VEGF into monkey eyes causes retinal vascular changes – hemorrhages, increased capillary permeability, and neovascularization – that are similar to those of DR [54, 62]. The discovery that these changes could be prevented by the co-administration of VEGF-Trap A40 [52] was an important driver of drug development. Elevated concentrations of placental growth factor have been found in rats with experimental DR though its contribution to the development of DR is not yet known.

Neovascular AMD is the leading cause of blindness in developed nations and most anti-VEGF drug development initially focused on treating this condition. DME trials have generally lagged behind their nAMD counterparts by one phase, which has delayed approval by the United States Food and Drug Administration (US FDA) for the treatment of DME by 3–5 years.

Four VEGF-binding medications (aflibercept, bevacizumab, pegaptanib, and ranibizumab) have been used to treat the vascular complications of DR. Drug manufacturers pursued different development strategies, which has resulted in drugs with different structures (aptamer, antibody binding fragment, full-length antibody, and fusion protein) and pharmacokinetic behaviors, though their efficacies and adverse effects profiles are remarkably similar. Hundreds of studies reporting the efficacy of anti-VEGF therapy for DME have been published, but this chapter will concentrate on the major registration trials, from which the most reliable data has been obtained (Table 13.1).

3.1 Pegaptanib

Pegaptanib is a 50 kDa pegylated aptamer that binds to the heparin binding domain of $VEGF_{165}$ [55]. Pegaptanib was approved for the treatment of nAMD but since the mean change in BCVA was a disappointing loss of seven letters [33], pegaptanib use fell dramatically after the introduction of the other VEGF-binding drugs. Because the development of DME (compared to nAMD) appears to be more dependent on $VEGF_{165}$ than other isomers, some investigators have posited that pegaptanib may be better suited for the treatment of DME.

Table 13.1 The table lists several of the most important pharmacotherapeutic trials for diabetic macular edema and proliferative diabetic retinopathy with selected results

Important drug trials for the treatment of diabetic macular edema and proliferative diabetic retinopathy	
Bevacizumab	
DRCR.net Trial	Laser vs. Bevacizumab (1.25 mg or 2.5 mg) Bevacizumab improvement in BCVA 1 line better than laser at 12 weeks Half of bevacizumab eyes had 11% decrease in thickness at 3 weeks
BOLT Trial	2 year trial of bevacizumab vs. laser BCVA of bevacizumab vs. laser (+8.6 vs. −0.5 letters) Change in CRT (−146 μm vs. −118 μm)
Ranibizumab	
RISE/RIDE	3 year trial of ranibizumab (0.3 mg and 0.5 mg) vs. sham 24 month BCVA improvements (+13,+12; +12,+11; +3,+0.5 letters) Changes in CFT (−250,-259; −253,−270; −133,-125 μm)
RESTORE	1 year trial of ranibizumab 0.5 mg vs. ranibizumab + laser vs. laser 12 month BCVA improvements (+6.1, +5.9 and +0.8 letters) Changes in CRT (−118.7, −128.3, −61.3 μm)
DRCR.net Protocol I	5 year trial of ranibizumab + deferred laser, ranibizumab + prompt laser, triamcionlone + laser, laser + sham 12 month BCVA improvements (+9, +9, +4, +3 letters) At 5 yrs. ranibizumab + prompt vs. ranibizumab + preferred (+7.2 vs. +9.8 letters; $P = 0.09$)
DRCR.net Protocol S	1-year trial of ranibizumab 0.5 mg vs. ranibizumab + laser vs. laser ΔBCVA ranibizumab vs. laser (+2.2 vs. +0.2 letters) Less visual field loss in ranibizumab patients
Aflibercept	
VIVID/VISTA	3 year study (1 year primary endpoint) aflibercept 2 mg q4w vs. aflibercept q8w vs. laser ΔBCVA of +12.5,+10.5; +10.7,+10.7; +0.2,+1.2 letters ΔCRT of −185.9,−195.0; −183.1,−192.4; −73.3,−66.2 μm
Combination Trial	
DRCR.net Protocol T	2 year study of aflibercept vs. bevacizumab vs. ranibizumab for DME ΔBCVA in 20/40 or better eyes of +8.0, +7.5, +8.3 letters at 1 year ΔBCVA in 20/50 or worse eyes of aflibercept and ranibizumab better than bevacizumab
Triamcinolone	
DRCR.net Protocol B	2 year study of laser vs. triamcinolone 1 mg and 4 mg ΔBCVA of +1, −3, −2 letters at 2 years Elevated IOP in 13%, 16%, and 33% of eyes

(continued)

Table 13.1 (continued)

Important drug trials for the treatment of diabetic macular edema and proliferative diabetic retinopathy	
Dexamethasone	
MEAD trials	3 year study of DEX 0.7 mg, 0.35 mg vs. Sham 15-letter improvements in BCVA (22.2% vs. 18.4% vs. 12.0%; $P < 0.018$). Only 0.4% required incisional surgery for glaucoma
Fluocinolone	
FAME trials	3 year study of FA 0.2 mg, 0.5 mg vs. sham 10-letter improvements in BCVA 28.7%, 28.6%, 16.2% ($P = 0.002$ for each) Incisional surgery for glaucoma in 3.7%, 7.6%, and 0.5%

BCVA best corrected visual acuity, *CRT* central retinal thickness, *CFT* central foveal thickness, *DME* diabetic macular edema, *IOP* intraocular pressure, *DEX* dexamethasone insert, *FA* fluocinolone acetonide insert

Pegaptanib was studied in a small number of DME trials with encouraging results. In a phase II trial, 172 patients were treated every 6 weeks until the 36-week endpoint. Significantly more patients receiving 0.3 mg pegaptanib than sham had +10 letter improvements (34% vs. 10%; $P = 0.003$) and +15 letter improvements (18% vs. 7%; $P = 0.12$) in BCVA. Patients receiving 0.3 mg pegaptanib had greater mean improvements in BCVA (+4.7 vs. −0.4 letters; $P = 0.04$) and macular thickness (−68 μm vs. +4 μm; $P = 0.02$) compared to those in the sham arm [39].

In a phase II/III trial, 260 patients with center-involving DME were randomized to receive 0.3 mg pegaptanib or sham injections every 6 weeks [60]. Compared to patients randomized to sham, significantly more patients receiving 0.3 mg pegaptanib improved by +10 letters (36.8% vs. 19.7%; $P = 0.0047$) but not +15 letters (16.5% vs. 10.2%; $P = 0.2466$). At the 102-week concluding visit, patients receiving pegaptanib had greater mean gains in BCVA (+6.1 vs. +1.3 letters; $P < 0.01$). Fewer patients receiving pegaptanib required grid laser photocoagulation for persistent edema by week 54 (23.3% vs. 41.7%; $P = 0.002$) and week 102 (25.2% vs. 45.0%; $P = 0.003$).

Pegaptanib showed promise for the treatment of DME, but since it produced disappointing results in patients with nAMD, further testing of pegaptanib was halted.

3.2 Bevacizumab

Bevacizumab is a humanized, monoclonal antibody that attaches to the VEGF binding domain (amino acids 81 through 92) of all VEGF-A isoforms. Bevacizumab is approved for the intravitreal treatment of several advanced solid tumors but intraocular injections for chorioretinal vascular conditions are off-label.

The Diabetic Retinopathy Clinical Research network (DRCR.net) evaluated the short-term (12-week primary endpoint) efficacy of bevacizumab in a phase II DME

trial. One hundred twenty-one patients were randomized to receive laser photocoagulation or intravitreal injections of 1.25 or 2.5 mg bevacizumab every 6 weeks, with or without laser. Patients receiving bevacizumab experienced a one-line improvement in BCVA compared to those treated with laser. Approximately one-half of the bevacizumab treated patients experienced a decrease in macular thickness of at least 11% at week 3, but additional improvements through week 12 were not seen [17].

The prospective, single-center, 2-year Bevacizumab or Laser Therapy in the Management of Diabetic Macular Edema (BOLT) trial demonstrated the superiority of bevacizumab over laser [42, 58]. Eighty eyes were randomized to receive bevacizumab every 6 weeks as needed or laser photocoagulation every 4 months as needed. A median of 13 bevacizumab injections and 4 laser treatments were performed through 2 years. At 1 year, more patients receiving bevacizumab than laser achieved BCVA improvements of at least +15 letters (11.9% vs. 5.3%) and at least +10 letters (31% vs. 7.9%), and fewer lost more than −15 letters (2.4% vs. 26.3%) and more than −30 letters (0% vs. 5.3%). Patients receiving bevacizumab achieved greater mean improvements in BCVA compared to laser at 1 year (+8.0 vs. −0.5 letters, $P = 0.0002$) and 2 years (+8.6 vs. −0.5 letters). At 2 years, 49% of patients treated with bevacizumab improved by at least +10 letters and 32% by at least +15 letters, compared to only 7% and 4% of patients treated with laser photocoagulation. Fewer patients treated with bevacizumab lost at least −15 letters (0% vs. 14%, $P = 0.03$). Eyes receiving bevacizumab experienced greater decreases in mean macular thickness compared to those treated with laser (−146 μm vs. −118 μm).

In a *post hoc* analysis of the BOLT data, eyes with subretinal fluid at baseline were most likely to have persistent edema at 2 months [58]. The authors noted that resolution of edema by 4 months is a strong predictor of a favorable long-term response. They found that 20% of eyes with persistent edema at 12 months achieved dry retinas at 24 months and most of these eyes achieved BCVA improvements of at least +15 letters. They stated that though the 4-month response may correlate with the long-term outcome, it should not lead to withholding of therapy.

Intravitreal injections of bevacizumab have been used as surgical adjuvents to decrease intraoperative hemorrhage, facilitate fibrovascular membrane dissection [13], and reduce the incidence of postoperative vitreous bleeding [1]. Concern that pre-operative bevacizumab can worsen fibrovascular traction [5] has prompted some surgeons to recommend that bevacizumab be administered only within a few days of planned surgery. In this way, prompt intervention can be taken if a traction retinal detachment worsens or a traction-rhegmatogenous detachment develops.

A meta-analysis of randomized, controlled trials compared the safety and functional outcomes of vitrectomy for PDR that were performed with or without pre-operative intravitreal bevacizumab [68]. Eight trials (414 eyes of 394 patients) were included. The authors reported that pre-operative bevacizumab shortened the mean surgical time by 26.89 min ($P < 0.00001$) and reduced the mean number of endodiathermy applications by 3.46 ($P = 0.02$). The bevacizumab group experienced less intraoperative bleeding ($P = 0.003$) and recurrent vitreous hemorrhage within the first post-operative month ($P < 0.0001$), but the incidence of recurrent vitreous

hemorrhage after the first month was comparable between the two groups. No significant differences in other complication rates were noted except that iatrogenic retinal breaks were more likely to occur in the vitrectomy-alone group (OR 0.27; $P = 0.003$).

3.3 Ranibizumab

Ranibizumab is an antibody binding fragment with a high affinity for $VEGF_{165}$ (K_D = 46 pM – 192 pM) [27, 50]. Ranibizumab passes unaltered through the trabecular meshwork and choroid into the systemic circulation where, because of rapid excretion by the kidneys, it has a half-life of only 2 h. Ranibizumab does not suppress serum VEGF concentrations [6].

3.3.1 Pilot Studies

In the READ-1 study, 10 eyes with DME received ranibizumab injections at baseline, and 1, 2, 4, and 6 months [49]. A rapid reduction in macular edema (median of −88 μm, mean of −130 μm) was seen by day 7 and a strong correlation between macular thinning and visual improvement was noted ($r^2 = 0.78$). By the 7-month endpoint, significant decreases in mean foveal thickness (503–257 μm) and macular volume (9.22–7.47 mm^2) had occurred, and the mean BCVA improved by +12.3 letters. Patients experienced slight increases in blood pressure but no important safety signals were seen.

In a second, unrelated study, 10 patients received 0.3 or 0.5 mg ranibizumab injections at baseline, and 1 and 2 months [14]. At the 3-month primary endpoint, eyes receiving 0.3 and 0.5 mg experienced significant improvements in BCVA (+12 and +7.8 letters) and central retinal thickness (−45.3 and −197.8 μm). The BCVA decreased between 3 and 6 months.

Following these pilot studies, further ranibizumab development for DME (Fig. 13.3) progressed along 3 lines of investigation: READ/RISE/RIDE trials (United States trials sponsored by Genentech); RESOLVE/RESTORE (ex-US trials sponsored by Genentech); and Diabetic Retinopathy Clinical Research Network (National Eye Institute sponsored). The first 2 lines of investigation led to the regulatory approval of ranibizumab for the treatment of DME in the Unites States and ex-US nations respectively.

3.3.2 READ-2 and READ-3 Trials

The phase II, READ-2 trial was the first prospective, double-masked, multi-center ranibizumab DME trial [46]. One hundred twenty-six patients were randomized to receive intravitreal injections of 0.5 mg ranibizumab at baseline, and months 1, 3,

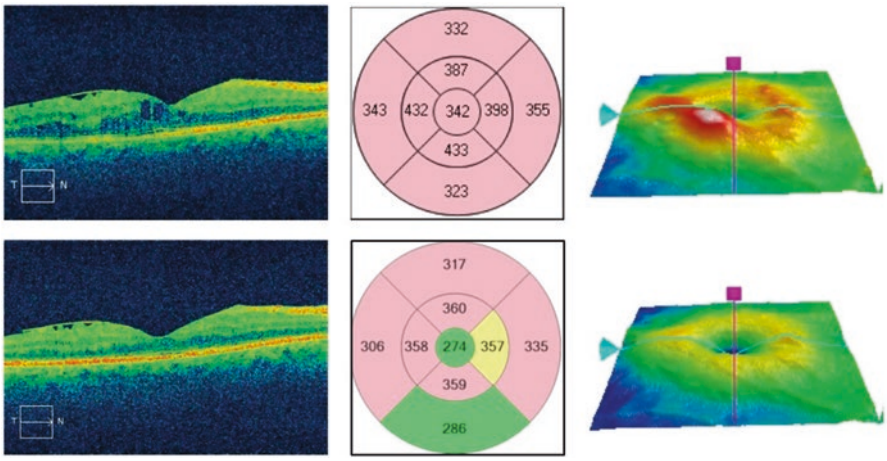

Fig. 13.3 This figure shows optical coherence tomography scans of an eye with diabetic macular edema that responded successfully to intravitreal injections of 0.3 mg ranibizumab. The *top row* (baseline) shows cystoid macular edema (central subfield thickness of 342 μm) with a mild epiretinal membrane. After 2 monthly ranibizumab injections (*bottom row*), the macular edema has completely resolved (central subfield thickness of 274 μm

and 5; focal/grid laser photocoagulation of the macula at baseline and at month 3 if needed; or intravitreal 0.5 mg ranibizumab followed by laser photocoagulation 1 week later. Mean improvements in BCVA and CMT at the 6-month primary temporal endpoint were +7, 0 and +4 letters and −95 μm, −82 μm, and −117 μm.

A protocol modification at 6 months allowed patients in the first 2 treatment arms to receive ranibizumab every 2 months PRN, whereas patients originally treated with laser and ranibizumab became eligible for additional laser and ranibizumab every 3 months if the CMT was greater than 250 μm. At 24 months, the mean improvements in BCVA and CMT were +8, +5, and +7 letters, and 340, 286, and 258 μm. Visual acuity improvements among patients in the laser group approached those in the ranibizumab monotherapy group but the excess macular thickness indicated that bimonthly injections constituted under-treatment [47].

At 24 months, a second protocol modification allowed patients to receive monthly PRN ranibizumab injections during year 3. From the 24-month to 36-month visits, patients achieved mean BCVA gains of +3, −2, and +2 letters, with each group receiving an average of 5, 2, and 3 ranibizumab injections.

In the READ-3 trial, patients with DME received either 2.0 mg or 0.5 mg ranibizumab monthly for 6 months then PRN through 12 months. The 0.5 mg dose produced greater improvements in BCVA (+10.88 letters vs. +7.39 letters) and more patients receiving the 2.0 mg dose died during the trial (3% vs. 0%).

3.3.3 RISE and RIDE

Lessons learned from READ-2, particularly regarding the consequences of under-treatment, were incorporated into the randomized, multi-center, double-masked, 3-year, phase III RISE and RIDE registration trials [45]. Seven hundred fifty-nine patients were randomized to three treatment arms: monthly 0.3 mg ranibizumab, monthly 0.5 mg ranibizumab, or sham injections. Patients were eligible for laser photocoagulation at 3 months if the CRT was >250 μm and if the change in CRT following the previous injection was <50 μm.

At the 24-month primary endpoint, significant proportions of patients randomized to 0.3 mg ranibizumab, 0.5 mg ranibizumab, and sham improved by at least +15 letters in RISE (45%, 39%, 18%) and RIDE (34%, 46%, 12%). Mean improvements in BCVA and CFT in RISE and RIDE were +13, +12, +3 letters and +12, +11, +0.5 letters, and −250 μm, −253 μm, and −133 μm, and −259 μm, −270 μm, and −125 μm respectively. Patients receiving ranibizumab required fewer lasers (means: 0.3–0.8) than those receiving sham injections (means: 1.8 and 1.6).

The median diabetic retinopathy severity scores (DRSS) improved from moderately severe NPDR to mild NPDR in patients receiving ranibizumab but remained at moderately severe NPDR throughout the study in patients randomized to sham/laser. Fewer patients receiving ranibizumab experienced a 2-step worsening in the DRSS (1.7–2.1% vs. 9.6%) and fewer ranibizumab eyes developed vitreous hemorrhage. A dose-dependent risk of stroke was noted in the ranibizumab arms. The US FDA approved (2012) the 0.3 mg ranibizumab dose for the treatment of center-involving DME and the label was subsequently (2015) expanded to include the treatment of diabetic retinopathy in eyes with DME.

Patients originally randomized to ranibizumab continued receiving monthly injections during year 3 and those randomized to sham became eligible to receive monthly PRN 0.5 mg ranibizumab [10]. Patients in the ranibizumab arms had stable BCVA during year 3, whereas those in the sham arms improved to +4 (RISE) and +5 (RIDE) letters above the baseline acuities.

Following the 36-month visit, 582 patients from RISE and RIDE were enrolled in the extension study [9]. All patients were eligible to receive 0.5 mg ranibizumab every 4 weeks if DME was identified by the investigator or BCVA worsened by at least 5 letters compared to month 36. Patients received a mean of 4.5 injections (annualized: 3.8) during a mean follow-up of 14.1 months but 25% of patients required no injections during the extension. Best corrected visual acuity in all groups remained stable throughout the extension and mean CFT increased slightly. Few patients developed PDR and those originally randomized to ranibizumab had a lower overall rate of progression to PDR than those originally randomized to sham.

Patients with macular non-perfusion at baseline had lower VA scores than those with good perfusion but by the trials' completion, those with non-perfusion experienced greater improvements in BCVA. Non-perfused areas did not increase in size when exposed to ranibizumab.

3.3.4 RESOLVE and RESTORE

The phase II RESOLVE trial and phase III RESTORE trials were performed in Europe, Asia, and Australia and served as the basis for regulatory approval in these areas. The 12 month RESOLVE trial randomized 152 patients to receive 3 monthly 0.3 mg ranibizumab, 0.5 mg ranibizumab, or sham injections [41]. After 1 month, the dose of ranibizumab was doubled for the following reasons: CRT > 300 μm; or CRT > 225 μm if the reduction was <50 μm since the previous injection. At 3 months, patients were eligible for rescue laser and additional monthly PRN injections or sham. At the 12-month primary temporal endpoint, mean BCVA improved by +10.3 letters in the pooled ranibizumab groups but declined by −1 letter in the sham group. Visual acuity gains of +2 lines and +3 lines were achieved by 60.8% and 33% of ranibizumab treated eyes but by only 18.4% and 5% of sham treated eyes. Improvements in mean CST were −194 μm for the pooled ranibizumab groups and −48 μm for the sham group. Doubling the dose of ranibizumab was required by 86% of all eyes and 68% of those receiving ranibizumab (70–78% of these occurred at the 1-month exam). The mean number of injections was 10.2 and only 4.9% of ranibizumab treated eyes (compared to 34.7% of sham eyes) required rescue laser.

The multi-center (75 sites), phase III RESTORE trial randomized 345 patients to ranibizumab + sham laser, ranibizumab + laser, or sham injections + laser. Ranibizumab injections were given monthly ×3 then PRN, and laser was performed at baseline then every 3 months PRN [44]. At the 12-month visit, patients in the ranibizumab monotherapy, ranibizumab + laser, and sham/laser groups had improvements in mean BCVA (+6.1, +5.9 and +0.8 letters), BCVA score > 73 letters (53%, 44.9%, and 23.6%) and mean CRT (−118.7 μm, −128.3 μm and −61.3 μm). A mean of 7 ranibizumab/sham injections were administered. Health related quality of life scores (measured by the NEI VFQ-25 questionnaire) improved more in the ranibizumab monotherapy and ranibizumab + laser groups compared to sham/laser ($P < 0.05$ for each). Subgroup analyses showed that patients with baseline BCVA of ≥73 ETDRS letters or CRT <400 μm had similar visual acuity improvements with laser photocoagulation as with ranibizumab injections. There were no cases of endophthalmitis and no additional cases of cardiovascular or cerebrovascular events with ranibizumab therapy.

Following the 12-month visit, 240 patients were enrolled in the 24-month extension trial. All patients were eligible to receive 0.5 mg ranibizumab injections according to BCVA, disease progression criteria, and the investigators' discretion. Additional laser photocoagulation was performed according to ETDRS guidelines. At the pre-planned 24-month interim analysis, patients who originally received ranibizumab monotherapy and ranibizumab + laser maintained gains in mean best corrected visual acuity (+7.9 letters, +6.7 letters from baseline), CRT (−140.6 μm, −133.0 μm) and NEI VFQ-25 composite scores (5.6, 5.8) [37]. Patients originally treated with sham/laser experienced significant improvements (+5.4 letters, −126 μm, 4.3), most of which occurred after becoming eligible for ranibizumab. Similar numbers of injections were performed in each group (3.9, 3.5, and 4.1). No cases of endophthalmitis occurred and the incidences of non-ocular SAEs were low.

Two hundred and eight (86.7%) patients completed the 24-month extension study [56]. Improvements in mean BCVA at the 36-month visit were +8.0 letters (ranibizumab monotherapy), +6.7 letters (ranibizumab + laser) and +6.0 letters (sham/laser + PRN ranibizumab after 12 months). Patients in the three treatment arms required 4.0 to 6.8 (mean for each group) injections over the final 2 years.

3.3.5 DRCR.net PROTOCOL I

The 5-year, double-masked, multi-center DRCR.net Protocol I trial produced the first level I evidence that supported the use of ranibizumab in eyes with DME. Eight hundred fifty-four eyes with center-involving DME were randomized to receive 0.5 mg ranibizumab with prompt macular laser photocoagulation, 0.5 mg ranibizumab with deferred laser (for at least 6 months), intravitreal triamcinolone with prompt laser, or sham injections with prompt laser [19]. During the first year of the trial, patients received ranibizumab injections according to the 4:2:7 rule – 4 monthly injections, followed by 2 injections if fluid persisted, followed by 7 monthly visits during which injections were performed at the investigator's discretion. Laser photocoagulation and intravitreal triamcinolone (4 mg) injections were repeated quarterly as needed. Patients randomized to the deferred laser group were not obligated to receive laser if the macula was dry.

At 1 year, the median improvements in BCVA in the ranibizumab + prompt laser, ranibizumab + deferred laser, triamcinolone + laser, and sham + laser groups were +9, +9, +4, and +3 letters respectively, with most gains seen by the 8-week visit. The BCVA in patients receiving triamcinolone improved rapidly during the first 3 months but then worsened through 12 months because of corticosteroid-induced cataracts. Eyes that were pseudophakic at baseline had similar 1-year improvements in VA with triamcinolone as with ranibizumab. Diabetic retinopathy was less likely to progress in eyes treated with ranibizumab. In subgroup analyses, none of the following factors affected final visual outcomes: prior treatment for DME, baseline BCVA, baseline CST, baseline severity of DR. Three patients receiving ranibizumab (0.8%) developed endophthalmitis, and patients receiving triamcinolone were most likely to develop cataracts and elevated intraocular pressure.

During year two of the trial, the interval between visits could be extended to 8 weeks if treatment had been withheld at 3 consecutive visits, and to 16 weeks if treatment was not performed at the 8-week visit. Patients in the triamcinolone + laser and laser/sham groups became eligible to receive ranibizumab for persistent edema without improved vision as early as week 74. The 2-year anatomic and functional outcomes were similar to those seen at 1 year. Fifty percent of ranibizumab treated eyes improved by at least +10 letters and 33% improved by at least +15 letters [20]. Compared to the group randomized to sham/laser, the changes in mean BCVA for patients receiving ranibizumab + prompt laser, ranibizumab + deferred laser, and triamcinolone + prompt laser were +3.7, +5.8, and −1.5 letters. Forty-three eyes in the sham/prompt laser group were switched to ranibizumab within the

first 2 years because of "failure of treatment", whereas none of the patients randomized to ranibizumab were regarded as treatment failures.

By the 3-year visit, the median numbers of injections given to patients in the ranibizumab + prompt laser and ranibizumab + deferred laser groups were 12 and 15 respectively [21], and the median numbers of lasers were 3 and 0 respectively. Improvements in mean BCVA were +2.9 letters better in patients treated with ranibizumab + deferred laser than in those receiving ranibizumab + prompt laser ($P = 0.02$). The percentages of eyes with CST <250 μm were 36% in both ranibizumab groups.

At the 5-year visit, the improvements in mean BCVA from baseline were +7.2 letters in the ranibizumab + prompt laser group and +9.8 letters in the ranibizumab + deferred laser group ($P = 0.09$) [25]. The proportions of eyes experiencing loss of ≥ −10 letters (9% and 8%), improvement of ≥ +10 letters (46% and 58%), and improvement of ≥ +15 letters (27% and 38%) were similar. From baseline through 5 years, 56% of patients in the deferred group did not require laser. The mean numbers of ranibizumab injections during the trial were 13 and 17; 54% and 45% of eyes did not receive ranibizumab during year four, and 62% and 52% did not receive injections during year five.

3.3.6 RETAIN

The RETAIN trial evaluated the efficacy of a treat-and-extend strategy (T&E), the most commonly used anti-VEGF regimen, in patients with DME [51]. Three hundred seventy-two patients were randomized to receive T&E 0.5 mg ranibizumab plus laser (G1), T&E 0.5 mg ranibizumab (G2), or monthly PRN 0.5 mg ranibizumab (G3). Monthly injections were given to all patients until the vision stabilized, at which time patients in G1 and G2 could be extended at 1-month intervals up to a maximum of 3 months. At 24 months, median BCVA improvements in the G1, G2, and G3 groups were +8, +7, and +8 letters. Compared to the PRN regimen, patients receiving T&E required 40% fewer clinic visits, and 70% were extended to a treatment interval of at least 2 months.

3.3.7 Ranibizumab for PDR

Ranibizumab not only restores the blood-retinal barrier but it also suppresses neovascularization (Fig. 13.4). The DRCR.net Protocol S trial enrolled 305 patients with PDR to compare panretinal photocoagulation with intravitreal 0.5 mg ranibizumab [64]. Complete PRP was performed at baseline and ranibizumab was given every 4 weeks PRN. Eyes in both treatment arms with co-existing DME were eligible to receive ranibizumab. At 2 years, improvements in BCVA for the ranibizumab and PRP groups were +2.2 and +0.2 letters respectively (95% CI, −0.5 to +5.0). The group receiving ranibizumab experienced less peripheral visual field sensitivity loss (−23 dB vs. −422 dB; $P < 0.001$), fewer vitrectomies (4% vs. 15%;

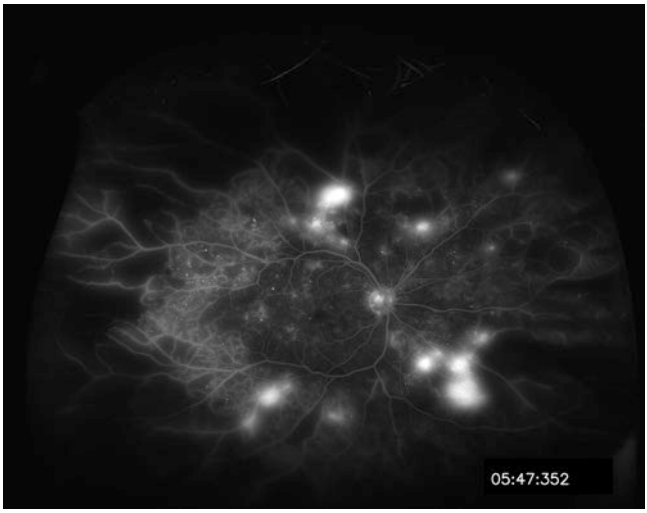

Fig. 13.4 This ultra-widefield fluorescein angiography frame shows broad areas of capillary nonperfusion of the peripheral retina. Several areas of pre-retinal neovascularization are present, as evidenced by significant fluorescein dye leakage

$P < 0.001$), and less DME (9% vs. 28%). A median of 7 ranibizumab injections were administered through year one and 10 injections through year two. Forty-five percent of eyes randomized to PRP required additional laser after baseline and 53% required ranibizumab for DME. Only one eye developed endophthalmitis after a ranibizumab injection. The authors concluded that ranibizumab may be a reasonable alternative to PRP over the course of 2 years.

3.4 Aflibercept

Aflibercept is a fusion protein comprized of the second binding domain from VEGFR1 and the third binding domain from VEGFR2 bound to the Fc fragment of an IgG molecule. Aflibercept has a high binding affinity for $VEGF_{165}$ ($K_D = 0.5$ pM) and also binds VEGF-B and placental growth factor [34]. Aflibercept passes unaltered from the vitreous into the systemic circulation where its half-life is approximately 6 days. Aflibercept decreases plasma VEGF concentrations below the lower detection limit of some assays (10 pg/ml) for at least 7 days [6].

3.4.1 Pilot Study

In a small pilot study, 5 patients with DME each received single intravitreal injections of 4 mg aflibercept. Excess macular thickness decreased from a median of 108–59 μm and the BCVA improved by a median of +9 letters at 4 weeks. By

6 weeks, the therapeutic effects had waned as excess macular thickness had increased to 74 μm and the improvement in BCVA had dropped to +3 letters [24].

3.4.2 DA VINCI

The prospective, multi-center, phase II DA VINCI trial randomized 221 patients with center-involving DME to 5 treatment arms: 0.5 mg every 4 weeks (0.5q4), 2 mg every 4 weeks (2q4), 2 mg every 8 weeks after 3 monthly loading injections (2q8), 2 mg PRN after 3 monthly loading doses (2PRN), and quarterly laser PRN/sham injections [23]. Patients receiving aflibercept were not eligible for rescue laser until 6 months. At 1-year, the mean BCVA improvements were +11.0, +13.1, +9.7, +12.0, and −1.3 letters for each arm, the proportions improving by at least +15 letters were 40.9%, 45.5%, 23.8%, 42.2%, and 11.4%, and the mean improvements in CST were −165.4 μm, −227.4 μm, −187.8 μm, −180.3 μm, and −58.4 μm. Patients in the 2PRN and 2q8 groups received an average of 7.4 and 7.2 injections respectively. Patients in the laser/sham group received more laser treatments than those in the aflibercept arms (2.5 vs. 0.5–0.8). Improvements in DRSS were experienced by 31%–64% of aflibercept treated patients but by only 12% of laser treated patients. Worsening in DRSS was experienced by only 0%–14% of aflibercept treated patients compared to 24% of laser treated patients.

3.4.3 VIVID and VISTA

The VIVID and VISTA trials [36] were similarly designed, double-blind, randomized, phase III trials that enrolled a total of 872 patients with center-involving DME. Eyes were randomized 1:1:1 to receive intravitreal 2 mg aflibercept injections (IAI) every 4 weeks (2q4), or every 8 weeks (2q8) after 5 monthly loading doses, or laser photocoagulation/sham injection. Patients were eligible for laser photocoagulation every 12 weeks if ETDRS defined edema was present. All study eyes were eligible for rescue treatment (IAI for patients randomized to laser and laser for patients randomized to IAI) beginning at 24 weeks if they lost ≥10 letters of BCVA on 2 consecutive visits or ≥15 letters at any visit from the previous best measurement and the BCVA was worse than baseline. Patients receiving IAI continued to receive injections through 148 weeks and patients randomized to laser/sham were eligible for IAI during year 3. VISTA enrolled a greater proportion of Black patients and VIVID enrolled a greater proportion of Asian patients. More eyes in VISTA, compared to VIVID, had previously received anti-VEGF injections (42.9% vs. 8.9%).

Mean BCVA changes from baseline to the 52-week primary temporal endpoint for the groups receiving IAI 2q4, IAI 2q8, and laser/sham were +12.5, +10.7, and +0.2 letters ($P < 0.0001$) in VISTA and +10.5, +10.7, and +1.2 letters ($P < 0.0001$) in

VIVID. When eyes receiving rescue therapy were included in the analysis, improvements in the IAI groups ranged from +10.7 to +12.4 letters whereas those in the laser groups improved by +4.2 and +3.5 letters. The corresponding proportions of eyes improving by ≥ +10 letters were 64.9%, 58.3%, and 19.5% ($P < 0.0001$) in VISTA and 54.4%, 53.3%, and 25.8% ($P < 0.0001$) in VIVID. The proportions improving by ≥ +15 letters were 41.6%, 31.1%, and 7.8% ($P < 0.0001$) in VISTA and 32.4%, 33.3%, and 9.2% ($P < 0.0001$) in VIVID. The proportions that lost ≥ −15 letters were 0.6%, 0.7%, and 9.1% ($P < 0.0001$) in VISTA and 0.7%, 0%, and 10.6% ($P < 0.0001$) in VIVID. Significantly more patients treated with IAI 2q4 and 2q8 experienced a 2-step improvement in DRSS in both VISTA (33.8% and 29.1% vs.14.3%) and VIVID (33.3% and 27.7% vs. 7.5%). Mean changes in CRT were −185.9 μm, −183.1 μm, and −73.3 μm in VISTA and −195.0 μm, −192.4 μm, and −66.2 μm in VIVID. The mean (+/− SD) in NEI VFQ-25 scores for the IAI 2q4 groups were significantly different from the laser groups only for the near activities subscale scores in VISTA (9.0 +/− 20.6 vs. 5.4 +/− 20.4; $P = 0.0168$). For patients treated with laser/sham, the mean numbers of lasers were 2.7 and 2.1 in VISTA and VIVID respectively. More patients in the laser group than the IAI groups received rescue therapy (VISTA: 31.2% vs. 0.7% and 2.6%; VIVID: 24.1% vs. 4.4% and 8.1%).

Incidences of ocular and non-ocular adverse events, and serious adverse events, including Anti-Platelets Trialists Collaborative defined vascular events and deaths, were similar among all groups. Serious non-ocular adverse events were uncommon (hypertension: 9.7%; cerebrovascular accidents: 1.1%; and myocardial infarction: 1.1%).

The improvements in mean BCVA from baseline to week 100 in the 2q4, 2q8, and laser arms in VISTA (+11.5, +11.1, and +0.9 letters) and VIVID (+11.4, +9.4, and +0.7 letters) resembled those at the 52-week primary endpoint [11]. The proportions of eyes that gained ≥ +15 letters were 38.3%, 33.1%, and 13.0% ($P < 0.001$) in VISTA and 38.2%, 31.1%, and 12.1% ($P < 0.001$) in VIVID. Significantly more eyes receiving aflibercept than laser achieved ≥2-step improvements in DRSS in both VISTA (37.0%, 37.1%, and 15.6%) and VIVID (29.3%, 32.6%, and 8.2%).

Eyes receiving aflibercept maintained previous improvements in VA and CRT through the 148-week visit. Eyes in the laser groups were eligible for monthly aflibercept after week 100, but experienced mean BCVA improvements of only +1 letter. Rescue or as-needed aflibercept was given to 82% of laser treated eyes in VIVID and 87% of eyes in VISTA. Eyes in the laser groups that required rescue therapy achieved better final visual acuities than those that did not require rescue therapy [Justus Ehlers, Macula Society, Miami Beach, FL, February 26, 2016]. Only 12% of aflibercept treated eyes required laser at some point during the trials. Eyes with limited responses at 12 weeks (<10% improvement in CRT) ultimately went on to mean visual acuity improvements of +7.8 letters [Rishi Singh, Macula Society, Miami Beach, FL, February 25, 2016].

Eyes in the laser groups averaged −84 μm of macular thinning at week 100, but after crossing over to aflibercept this increased to −110 μm by week 148. In contrast,

the IAI 2q4 and 2q8 groups achieved significantly better thinning of the macula (−200 and −190 μm).

Aflibercept received US FDA approval for the treatment of center-involving DME (2014) and then for the treatment of diabetic retinopathy in patients with DME (2015). It has been approved by the European Medicines Agency and the recent approval in Egypt brings the total to 31 countries.

The ability of intravitreal aflibercept to prevent DR progression in eyes without DME and PDR is being evaluated in the PANORAMA trial and in the DRCR.net Protocol W trial. Patients with moderately severe NPDR are randomized to receive sham injections or aflibercept every 8 or 16 weeks.

3.5 Comparison Trials

The only trial to directly compare aflibercept, bevacizumab, and ranibizumab treatment of DME was the prospective, randomized, multi-center DRCR.net Protocol T trial [22]. Six hundred sixty patients at 89 sites received 1.25 mg bevacizumab, 0.3 mg ranibizumab, or 2 mg aflibercept every 4 weeks unless the BCVA reached 20/20 or better with a CST below the eligibility threshold, or if the BCVA changed by fewer than 5-letters over the past 2 injections, or if the CST changed by less than 10%. Beginning at week 24, injections were withheld if the changes in BCVA and CRT were ˂5 letters and ˂10% over the previous 2 injections regardless of absolute BCVA. At 24 weeks, patients were eligible for laser photocoagulation if they had persistent edema.

By 52 weeks, the mean numbers of injections given to patients in each arm were 9 (aflibercept), 10 (bevacizumab) and 10 (ranibizumab) ($P = 0.045$) and laser photocoagulation was performed in 37%, 56%, and 46% of eyes ($P < 0.001$). Mean changes in BCVA were +13.3 letters (aflibercept), +9.7 letters (bevacizumab), and +11.2 letters (ranibizumab) ($P ˂ 0.001$, aflibercept versus bevacizumab; $P = 0.03$, aflibercept versus ranibizumab). A pre-planned subgroup analysis showed that for eyes with baseline BCVA of 20/32 to 20/40, mean BCVA changes were +8.0 (aflibercept), +7.5 (bevacizumab), and +8.3 letters (ranibizumab). For eyes with baseline BCVA of 20/50 or worse, however, the mean changes in BCVA measured +18.9 (aflibercept), +11.8 (bevacizumab), and +14.2 letters (ranibizumab). The mean changes in CST for all ranges of BCVA were −169 μm, −101 μm, and −147 μm. Only 2 eyes developed endophthalmitis and there were no significant differences in the rates of serious adverse events ($P = 0.40$), hospitalization ($P = 0.51$), death ($P = 0.72$), or major cardiovascular events.

Gains in best corrected visual acuity were sustained by all groups during year 2 but the differences in BCVA gains between the drugs narrowed. Even among eyes with baseline BCVA of 20/50 or worse, aflibercept produced significantly better visual acuity gains than bevacizumab, but not significantly better than ranibizumab [Wells J, Macula Society, Ft. Lauderdale, FL, Feb. 23, 2016].

4 Corticosteroids and Clinical Trials

Glucocorticoids were the first drug class shown to improve DME in randomized clinical trials. Corticosteroids counteract the effects of angiogenesis via several mechanisms. They bind to the promoter region of the VEGF gene and downregulate VEGF synthesis [30, 57]. The resultant free VEGF levels drop significantly, though remain 100-fold higher than after the intravitreal injection of anti-VEGF drugs. By promoting vasoconstriction, steroids reduce the hydrostatic pressure gradient and decrease exudation by favorably altering the Starling's Law equilibrium.

Corticosteroids reduce inflammation by repressing several key pro-inflammatory transcription markers such as nuclear factor-kappa B (NFκB) and activator protein 1 [61, 67], inhibiting phospholipase A2 [59], downregulating the release of prostaglandins and histamines [66] and inhibiting the synthesis of endothelial nitric oxide synthase (eNOS), a potent vasodilator [38]. Corticosteroids decrease the synthesis of several chemokines, intercellular adhesion molecules, and growth factors that inhibit the migration and margination of polymorphonuclear leukocytes.

Steroids maintain and restore the blood-retinal barrier by preventing phosphorylation of tight junction proteins [32, 63]. Corticosteroids increase fluid movement through the retina by stabilizing Müeller cells and improving aquaporin-4 (AQP-4) and potassium channels [53, 69].

4.1 Triamcinolone

Pilot studies showed that intravitreal injections of triamcinolone acetonide (IVTA) effectively reduce DME [7, 40]. In the prospective, randomized, DRCR.net Protocol B trial, 1 and 4 mg IVTA every 4 months were compared to laser photocoagulation. At 4 months, patients receiving 4 mg IVTA were more likely to have +10-letter improvements in BCVA compared to laser (27% vs. 17%) and had greater mean decreases in CRT (−98 μm vs. −39 μm). At the 2-year primary endpoint, however, the mean BCVA improvement in the laser group, 1 mg, and 4 mg IVTA groups were +1, −3, −2 letters. Elevated IOP was seen in 13%, 16%, and 33% of eyes. More patients receiving triamcinolone required glaucoma medications (4 mg: 13%; 1 mg: 6%; laser: 3%) and more developed cataracts (4 mg: 61%; 1 mg: 23%; laser: 13%) [18].

4.2 Dexamethasone

Single intravitreal injections of dexamethasone phosphate have been used to control inflammation associated with bacterial endophthalmitis but its short half-life (5.5 h) prevents it from being used to treat chronic conditions. The dexamethasone

posterior-segment drug delivery system (DEX, Ozurdex®, Allergan Inc., Irvine, CA, USA) is a biodegradable, sustained release reservoir that releases 0.7 mg of dexamethasone over 3 months. The cylinder is pre-loaded into a single use applicator and then injected across the pars plana into the anterior vitreous through a 22-gauge needle (Fig. 13.5).

The long-term safety and efficacy of the dexamethasone insert in eyes with DME was compared to sham injections in the randomized, double-blind, multicenter, phase III MEAD registration trials [8]. From baseline to 3 years, more patients receiving the 0.7 mg and 0.35 mg inserts compared to sham experienced ≥ +15-letter improvements in VA (22.2% vs. 18.4% vs. 12.0%; $P < 0.018$). The mean improvement in BCVA among patients receiving the 0.7 mg insert was +7 letters. Patients that were pseudophakic at baseline experienced rapid improvements in BCVA followed by stability throughout the balance of the trial, whereas those who were phakic at baseline experienced rapid improvements in VA, a decline beginning at week 24 due to the formation of cataracts, and finally an improvement in VA after week 52 as cataracts were removed.

More patients receiving the insert experienced IOP readings of at least 25 mmHg (29.7% vs. 4.3% sham). The increase in IOP was transient in most cases with a peak at 6 weeks followed by a rapid return to baseline. In most cases, pressure elevations were managed with topical medications or by observation, and only 3 patients (0.4%) required incisional glaucoma surgery. IOP elevations tended to occur early, as 75% of spikes were diagnosed after the first 2 insertions and 85% after the first 3.

Among eyes that were phakic at baseline, 66.0% of those treated with DEX experienced development or progression of cataracts (cortical, nuclear, or subcap-

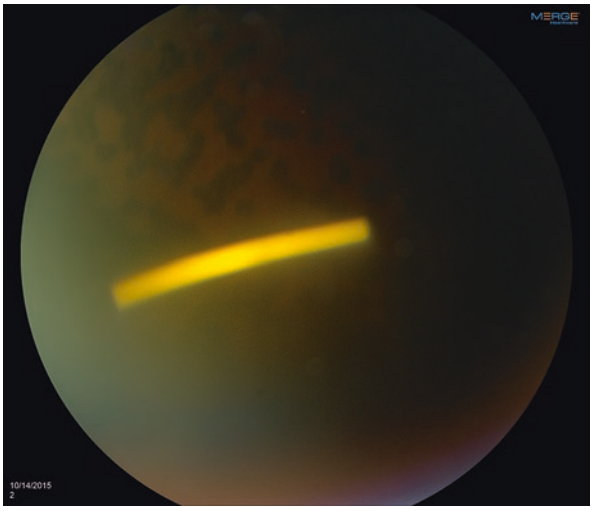

Fig. 13.5 The photograph, taken immediately after an injection, shows a dexamethasone insert suspended in the posterior vitreous just above the retina

sular), compared with 20.4% of sham-treated patients. Nearly 56% of DEX treated patients compared with 7.2% of sham treated patients underwent cataract surgery. The incidence of cataract-related adverse effects increased throughout the duration of the study with most cataract surgeries performed during the second and third years.

In addition to cataract progression and IOP elevation, the most frequent adverse events were conjunctival hemorrhage (23.5%), vitreous hemorrhage (10.0%), macular fibrosis (8.3%), conjunctival hyperemia (7.2%), eye pain (6.1%), vitreous detachment (5.8%), and dry eye (5.8%). Retinal tear, retinal detachment, vitreous loss, and endophthalmitis occurred in approximately 2% of patients.

4.3 Fluocinolone

The fluocinolone insert (FA, Iluvien®, Alimera Sciences, Alpharetta, GA) is a nonbiodegradable, tubular device that releases fluocinolone into the vitreous for 3 years. The insert was evaluated in two parallel, randomized, phase III registration trials (FAME) that randomized 956 patients to receive sham (185), a 0.2 mg (375), or a 0.5 mg insert (393) [12]. Six weeks after randomization, subjects were eligible for rescue laser, and 1 year after randomization, additional inserts or sham injections could be given if necessary.

The mean duration of DME at baseline had been 3.5–3.9 years. Significant visual acuity improvements were noted in both FA treatment groups at 3 weeks and at every time point thereafter. The proportions of patients improving by at least +10 letters were 28.7% (low dose), 28.6% (high dose), and 16.2% (sham; $P = 0.002$ for each). The mean improvements in BCVA at month 24 were +4.4, +5.4 and +1.7 letters ($P = 0.02$ and $P = 0.016$ compared to sham). A final VA of 20/40 or better was achieved in 33%, 31%, and 16% of eyes ($P = 0.0185$ and $P = 0.0064$ compared to sham) whereas a final acuity of ≤20/200 was achieved in 14% of insert eyes and 12% of sham eyes. For eyes with at least 3 years of DME prior to the study, 34% in the low dose group improved by at least 15 letters (versus 13.4% of sham; $P < 0.001$) [16]. Eyes receiving the insert had significantly greater improvements in foveal thickness at all time points. Final CST of ≤250 μm was achieved in 40%, 47%, and 51% of eyes. Because of recurrent or persistent edema, 23.5% (low dose) and 26.4% (high dose) of eyes required at least 2 insert injections. Fewer insert than sham patients required laser photocoagulation treatments (36.7%, 35.2%, and 58.9%). Significantly more phakic patients receiving the insert (74.9% and 84.5% vs. 23.1% sham) required cataract surgery and their final BCVA improvements were similar to eyes that were already pseudophakic at baseline. Incisional surgery to control glaucoma was required in 3.7%, 7.6%, and 0.5% of eyes. A secondary analysis showed that eyes receiving the 0.2 mg insert experienced less progression of PDR compared to controls (17% vs. 31%; $P < 0.0001$) [3].

5 Future Therapies

Most patients suffering from the ocular complications of angiogenesis respond well to initial anti-VEGF regimens but up to 40% have an incomplete response to therapy. Ongoing pharmaceutical development is attempting to provide new delivery methods (sustained release devices, nanoparticle systems, encapsulated cell technology, iontophoresis delivery, mucous penetrating platforms, adenovirus delivered gene therapy), new injectable anti-VEGF drugs (abicipar, conbercept, RTH256, ziv-aflibercept), new routes of administration (oral, topical), and drugs that target other contributory molecules (angiopoietin-2, integrins, interleukins, vascular adhesion proteins, phospholipase A2, RAF proto-oncogene serine/threonine-protein kinase, hypoxia-inducible gene, plasma kallikrein, mTOR, Tie 2, and insulin-like growth factor).

Vascular endothelial growth factor inhibition will continue to be first-line therapy for the complications of DR well into the future, and new medications will probably be used as second-line therapy or in combination with anti-VEGF drugs.

6 Conclusions

Currently available data shows that aflibercept, bevacizumab, and ranibizumab each improves visual acuity in most patients with DME. For patients with visual acuities of 20/40 or better, any of these drugs is a reasonable therapy. Most physicians in the United States choose bevacizumab, primarily because of its low cost. For eyes with BCVA of 20/50 or worse, physicians often select a higher binding-affinity drug (aflibercept or ranibizumab). Corticosteroids effectively resolve macular edema but their use is frequently accompanied by cataracts and glaucoma. Randomized head-to-head trials between corticosteroids and anti-VEGF drugs are needed to better understand the potential of corticosteroids as primary therapy. Consequently, corticosteroids are generally regarded as second-line or third-line therapies by most physicians except in specific circumstances.

Only ranibizumab has been shown to be equally effective as laser for the treatment of PDR, but physicians are likely to use off-label bevacziumab for this condition. Trials investigating the use of these drugs for new DR-related indications are ongoing and favorable results may convince some physicians to start anti-angiogenesis therapy for earlier stages of diabetic retinopathy.

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Part IV
Therapeutic Implications of Angiogenesis in Cardiovascular Disorders and Peripheral Vascular Disease

Chapter 14
Therapeutic Angiogenesis, Cell Therapy and Peripheral Vascular Disease

Brian H. Annex

Abstract Peripheral Arterial Disease (PAD) is one of the major complications of systemic atherosclerosis where occlusions along the major arterial pathway that supplies blood to the lower extremities is interrupted and blood flow to the distal limb becomes dependent on the presence, extent, and function of collateral blood vessels. Estimates are PAD is present in ~8.5 million Americans at or over the age of 40 and the two major clinical manifestations of PAD are intermittent claudication (IC) and critical limb ischemia (CLI) (Go et al., Circulation 129(3):e28–e292, 2014). Across the two major clinical manifestations of PAD the types of leg symptoms, amputation rates, and mortality differ greatly (Norgren et al., J Vasc Surg 45(Suppl S):S5–S67, 2007). Medical therapies for PAD subjects are designed to limit complications from systemic but no medical therapies are reliably able to improve blood flow to the ischemic limb. Here we will review how trials of therapeutic angiogenesis using gene or cell therapy have fared to treat PAD.

Keywords Peripheral arterial disease • Critical limb ischemia • Angiogenesis • Gene therapy • Cell therapy

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1 Frequency and Clinical Manifestations of PAD

Peripheral arterial disease (PAD) is caused by atherosclerosis that results in a narrowing of, or frequently complete occlusion in, one or more arteries that supply the lower extremities. A reduced ankle-brachial blood pressure index (ABI) defines the presence of PAD and this finding is present in ~8.5 million Americans at or over the age of 40 [1]. There are two major clinical manifestations of PAD: intermittent claudication (IC) and critical limb ischemia (CLI), though interestingly many patients with PAD are asymptomatic when asked standard medical questions. The time-honored Rose Criteria is used to define IC by the presence of leg pain/cramping with walking that relieves with rest. Patients with reduced ABI, with or without classic symptoms of IC, have similar endurance capacities on exercise testing. CLI is defined as pain present at rest, with or without, present or imminent tissue loss, of ischemic ulcers or gangrene, and CLI is classified as Rutherford Class 4–6 or Fontaine Class III and IV.

Importantly PAD should not be viewed as a disease this runs as a continuum from asymptomatic, to IC, to CLI. Patients with CLI are quite different than those with IC. The 1 year mortality rate in patients with CLI is approximately 25%, and the overall amputation rate over 1 year is approximately 30%, while patients with IC have amputation rates of 1–2%/limb/year [2]. Medical therapies for PAD are viewed as those that affect the limb or are designed to limit complications from atherosclerosis and reduce general cardiovascular mortality from myocardial infarction and stroke. There are no medications that have demonstrated the ability to reliably improve blood flow to the ischemic limb and thus treat the underlying problem in PAD.

The primary problem in PAD is reduced blood flow, or more accurately, perfusion to the lower limb with the clinical goal of treatment to augment blood flow to relieve ischemic (rest) leg pain, promote wound healing, reduce limb loss, and improve exercise capacity. Currently, endovascular and/or surgical revascularization remains the cornerstone of therapy in patients with CLI and those with IC and life-style limiting claudication. A large fraction of patients with PAD are not suitable candidates for revascularization based on their vessel anatomy, or the procedure is often unsuccessful due to graft failure and/or stent thrombosis or re-stenosis. With no specific medical treatment able to improve blood flow distal to a vascular occlusion, investigational approaches have emerged. Angiogenesis is defined as the growth and proliferation of blood vessels from an existing vascular structure while therapeutic angiogenesis seeks to use angiogenesis to enhance tissue perfusion distal to a vessel occlusion. This chapter summarizes key approaches designed to induce therapeutic angiogenesis using cell or gene therapy.

2 Angiogenesis

The arterial anatomy of the leg is such that the vast majority of the blood flow to the distal leg is carried along a single dominant vessel and in the setting of an occlusion along that pathway perfusion becomes dependent on the growth of new blood

vessels or neo-vascularization which is a physiological process that occurs as an adaptation to ischemia. The net final product from the extent of angiogenesis, arteriogenesis, and vasculogenesis is termed the degree of neovascularization [3–5]. Angiogenesis is the formation of new blood vessels/capillaries (8–12 μm in diameter) from pre-existing vessels and angiogenesis results from a combination of endothelial cell proliferation, differentiation and migration. Angiogenesis is actively under positive and negative regulation from local factors, the tissue microenvironment (i.e. hypoxia), and the extent to which genetic and epigenetic factors regulate modulate the balance of the conditions. Arteriogenesis involves the de-novo formation, and/or remodeling, of pre-existing vessels that are in the 20–50 μm diameter range while vasculogenesis is the formation of new vascular structures and involves the contribution of cells outside of the native vasculature."Therapeutic angiogenesis" whether achieved by drug, gene, or cell should be viewed as being agnostic to the exact processes but are able to improve the delivery of oxygene and the removal of toxins from the distal bed.

3 Therapeutic Angiogenesis and Peripheral Arterial Disease (PAD)

In patient with PAD arterial occlusion result in hypoxia in the distal muscle bed and in the adult should result in activation of the hypoxia inducible factor 1 − α, a transcription factor to induce hypoxia mediated angiogenesis. At least in principle, this should result in the generation of several pro-angiogenic cytokines and receptors, which would initiate endothelial cell sprouting, differentiation and proliferation, thereby initiating the process of angiogenesis to alter shear forces and drive arteriogenesis to enhance tissue perfusion. To some extent this process occurs in all patients and in some the response is likely to be so extensive, that patients are absolutely or relatively symptomatic. Indeed, it is highly likely that greater the functional performance of PAD patients the greater is the vascular remodeling that follows vessel occlusion. We showed that in human subjects with PAD (intermittent claudication, IC) the lower the capillary density in the gastrocnemius (ischemic) muscle the worse was the functional performance and this was independent of the ankle-brachial blood pressure index in those patients [6]. Moreover while supervised exercise training is widely regarded as the most effective treatment for IC, in patients with PAD who undergo supervised exercise training, angiogenesis in the ischemic calf muscles precedes changes in functional capacity and despite the improvement in functional capacity this without an appreciable increase in blood flow [7], indicating a role of micro-circulation in enhancing muscle performance independent of macro-vascular measurable blood flow. Thus, capillaries and angiogenesis are important in PAD and may be a site for therapeutic modulation.

4 Gene and Cell Approaches to Induce Therapeutic Angiogenesis

Therapeutic angiogenesis seeks to improve tissue perfusion by creating an environment in the ischemic tissue that allows for an increase in blood vessels through some combination of angiogenesis, arteriogenesis, and vasculogenesis. Experimental approaches have included the direct injection of growth factors as protein into vessels of tissue from patients' legs, extra-vascular or intra-muscular delivery of DNA within some vector to increase angiogenic growth factors in ischemic tissue and stem/progenitor cell therapy to promote angiogenesis in the ischemic tissue. Direct protein therapy rapidly fell out of favor due to difficulty in routes of delivery, limited uptake by muscle cells, and short half -life of proteins. Gene and cell therapy and are currently the focus of investigations in therapeutic angiogenesis.

In gene therapy a patients own cells must ultimately provide the machinery for protein production and the nucleic acids encoding the protein must reach (transduce) the cells in the organ and be transcribed into product. Gene therapy has an interesting history in medicine with its origin dating back to 1960s, when the first evidence was provided that nucleic acids could be taken up and expressed in mammalian cells was obtained [8]. Theoretically, gene therapy has numerous advantages over protein delivery which include more prolonged and controlled expression of the transgene products certainly when compared to exogenous protein delivery.

4.1 Nucleic Acid Delivery in Gene Transfer

Vectors for gene transfer revolve around viral and non-viral vectors and each has different properties which should not be equated to advantages/disadvantages. Viruses and discussion will be largely focused on adenoviral vectors have the potent capability to infect (transduce) host cells and have their limited genetic package utilize mammalian cell machinery for gene expression. These viruses can readily achieve high transfection efficiency and have robust expression of the target gene; albeit for limited time span. Adenoviral vectors for human use have defective replication and they retain an extra-chromosomal location and thus largely, or totally, avoiding incorporation into the host genome and the oncogenic risks to the recipient. Other important characteristics of the adenoviruses are that they are immunogenic which limits redelivery and existing antibodies have the (theoretical) potential to limit transduction efficiency. Lentivirus (retrovirus that can infect both dividing and non-dividing cells and can enter cells through intact cell membrane by membrane fusion) have the ability to provide high levels of gene expression. Adeno-associated viruses (AAV) are emerging as promising vectors for gene therapy with AAV serotype 9 being known to have selectivity for skeletal muscle and even greater selectivity to ischemic skeletal muscle [9]. Sendai virus and even other vectors may eventually come into use.

Non-viral gene delivery systems such as plasmid DNA have been extensively used in humans for research. When compared to viral vectors there is no inherent ability for the nucleic acids to obtain access to translational machinery with the exception of direct physical, or chemical, facilitation. There are examples of physical forces to facilitate transfection such as electroporation and particle bombardment but in reality most of the activity is simple hydrodynamic forces from the needle injection. Certain lipid/polymers serve aide in chemical transfer but need to consider within clinical trial design. Plasmid vectors have lower transfection efficiency but actually have the advantage of low immunogenicity and the potential for repeat dosing.

5 Gene Therapy Mediated Therapeutic Angiogenesis in Critical Limb Ischemia (CLI)

The primary problem in PAD is reduced blood flow/perfusion and the goal of therapeutic angiogenesis is to improve blood flow to relieve pain, improve wound healing, and limit the risk of amputation. Table 14.1 summarizes agents that have been tested to promote angiogenesis. Table 14.2 summarizes many of the key clinical trials PAD patients with CLI.

5.1 Vascular Endothelial Growth Factor Gene Therapy

Vascular Endothelial Growth Factor (VEGF) includes a family of genes that are the most extensively studied angiogenic growth factors. First identified in 1983 by Senger and Colleagues [24]. Today, the VEGF family of ligands consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and PLGF. The ligands have varying affinities both for binding and activating VEGF receptors 1, 2 and 3 with VEGFR2 being the dominant VEGF receptor in post-natal angiogenesis and the role of VEGFR1 being the least well understood. The lengths of the VEGF-A isoforms identified are 121, 145, 165,189 and 206 amino acids and the properties vary with

Table 14.1 Factors tested for therapeutic angiogenesis

Growth factors	VEGF A-E, PLGF, FGF-1, 2, 3, 5, Angiopoietin-1 and 2, HGF, PDGF, GM-CSF, Neurotrophin, IGF-1 and 2.
Chemokines	MCP-1, SDF-1
Trascription factors	HIF-α, EGR-1, Prox-1

VEGF Vascular endothelial growth factor, *PLGF* Placenta growth factor, *FGF* Fibroblast growth factor, *HGF* Hepatocyte Growth Factor, *PDGF* Placenta-derived growth factor, *GM-CSF* Granulocyte-macrophage colony stimulating factor, *IGF* Insulin-like growth factor. *MCP* Monocyte chemoattractant protein, *SDF* Stromal Derived Factor, *HIF* Hypoxia-inducible Factor, *EGR* Early growth response protein, *Prox* Prospero homeobox

Table 14.2 Clinical trials of growth factors in CLI

Reference	Year	Study type	Gene	Vector	Route of Delivery	Improvements in
Isner et al. [10]	1996	Phase I	VEGF	Plasmid	Intra-arterial	Collateral vessels and distal flow
Baumgartner et al. [11]	1998	Phase I	$VEGF_{165}$	Plasmid	Intramuscular	Distal flow, ulcer healing
Simovic et al. [12]	2001	Phase I	$VEGF_{165}$	Plasmid	Intramuscular	Symptom score, neurological exam, ABI, collaterals
Kim et al. [13]	2004	Phase I	$VEGF_{165}$	Plasmid	Intramuscular	Collaterals
Makinen et al. [14]	2002	Phase II	VEGF	Adenovirus	Intramuscular	Vascularity
Kusumanto et al. [15]	2006	Phase II	$VEGF_{165}$	Plasmid	Intramuscular	Ulcer healing Hemodynamics
Comerota et al. [16]	2001	Phase I	FGF-1	Plasmid	Intramuscular	Pain Ulcer healing ABPI Transcutaneous oxygen pressure
Nikol et al. [17]	2008	Phase II	FGF-1	Plasmid	Intramuscular	Risk of amputation
Belch et al. [18]	2011	Phase III	FGF-1	Plasmid	Intramuscular	No benefit
Morishita et al. [19]	2004	Phase I	HGF	Plasmid	Intramuscular	Pain, ABI, ulcer size
Powell et al. [20]	2008	Phase II	HGF	Plasmid	Intramuscular	Transcutaneous oxygen pressure
Pwel et al. [21]	2010	Phase II	HGF	Plasmid	Intramuscular	Rest pain
Shigematsu et al. [22]	2010	Phase III	HGF	Plasmid	Intramuscular	Rest pain, ulcer size, QOL
Rajagopalan et al. [23]	2007	Phase I	HIF-1α	Adenovirus	Intramuscular	Rest pain, ulcer size

VEGF Vascular Endothelail Growth Factor, *FGF* Fibroblast Growth Factor, *HGF* Hepatocyte Growth Factor, *HIF-1α* Hypoxia-inducible transcription factor-1α

VEGF 189 and 206 have extensive matrix and heparin binding ability while 121 and 165 no or limited heparin binding and thus are found in the circulation [25, 26]. Genetic deletion studies of all VEGF-Receptors are embryonically lethal [27–30] such a critical role in developmental vasculogenesis. Even the well-studied VEGF ligands are becoming more complex and reports have described that not all VEGF-A is angiogenic with anti-angiogenic forms having a potential role in PAD [31].

VEGF 121 and VEGF 165 are the isoforms that have been most extensively studied and many preclinical trials with VEGF gene therapy showed promising results in pre-clinical models of PAD [32–34], The late Dr. Jeffery M. Isner et al. conducted the first human clinical trial using VEGF more than 20 years ago [10]. Back then, a

patient was given a plasmid DNA encoding VEGF 165 to vessel wall of the distal popliteal artery using the hydrogel polymer coated balloon. The report used digital subtraction angiography and 4 weeks after gene therapy showed an increase in collateral vessels, intra-arterial ultrasound showed increased resting and maximum flows. Only a couple of years later, Baumgarther et al. administered the same plasmid (VGEF 165) by intramuscular (IM) injection into ischemic limbs of patients with CLI and showed improvements in ankle brachial index (ABI), collateral vessels by angiography in the VEGF treated patients [35]. Other studies followed. Simvoic et al. [12] used VEGF165 as a plasmid in patients with CLI and IM VEGF165 resulted in increased ABI, less symptoms, improved motor and nerve functions, indicating a role of VEGF165 in chronic ischemic neuropathy. Then, Kim et al. [13] used IM VEGF 165 gene into patients with CLI and reported reduced leg pain, improved rates of ulcer healing, greater ABI and increased collaterals even 6 months out.

Randomized trials are needed to establish efficacy. The first such report of VEGF 165 gene therapy was published by Makinen et al. [14], where is a 2002, randomized, double-blind, placebo-controlled trial patients received either an adenoviral of VEGF-plasmid vs. ringers lactate injections via an intra-arterial catheter during a lower extremity angioplasty with imaging (digital subtraction angiography, DSA) as the primary endpoint. Vascularity by DSA was significantly increased in either VEGF compared to controls. Then, Kusumanto and colleagues reported a phase II, double-blinded placebo controlled study of VEGF gene carrying plasmid (phVEGF 165) vs. saline in patients with CLI and superimposed diabetes [15]. In these 54 patients, the primary end-point studied was amputation rate at 100 days was no altered but the secondary endpoints were a 15% or more increase in ABI/toe brachial index, clinical improvement and safety were improved along with improvements in ulcer healing.

5.2 Fibroblast Growth Factor (FGF)

Though the VEGF family is complex, the fibroblast growth factors (FGF) family of >20 structurally related angiogenic growth factors and receptors is far more complex [36]. Beyond a role in developmental angiogenesis FGF ligand and receptor signaling also plays a significant role in post-natal angiogenesis with FGF-1 (acidic) and FGF-2 (basic) have been the focus of therapies for promoting angiogenesis [17, 18, 37, 38].

In 2002, the first phase I clinical trial using FGF gene therapy was reported by Comerota et al. [16]. Here, a “naked” or simple plasmid vector encoding FGF-1 (NV1FGF) was given IM to the ischemic limbs of patients with CLI to assess safety and tolerability of increasing and repeated (though only 2 doses) of NV1FGF. Of course, the trial also examined changes in hemodynamic and clinical parameters at 12 months follow-up. NV-1FGF was safe, well tolerated and there were significant improvements in ABI, reductions in pain, better aggregate ulcer size reductions were noted along with an increase in transcutaneous oxygen pressure compared when compared to pretreatment values. This studied was followed by a phase II

clinical trial with FGF in patients with CLI [17]. Here, in this double-blinded, randomized placebo-controlled trial, investigators injected IM NV1FGF or placebo in ischemic limbs of patients with CLI. Interestingly, though there was no significant improvement in ulcer healing, the use of NV1FGF did significantly reduced the secondary but important end-points of all amputations and major amputations compared to placebo. Unfortunately for patients with CLI what followed was a large phase III trial, that was published in 2011 [18]. Here, 525 CLI patients who were deemed unsuitable for revascularization were randomized to IM placebo or (NV1FGF1) DNA plasmid. The study showed no difference in the primary endpoint of time to major amputation or death and an increase in peripheral edema drove greater adverse effects in plasmid DNA vs. placebo and development was halted.

5.3 Hepatocyte Growth Factor (HGF)

Hepatocyte growth factor is (HGF, originally called "Scatter Factor") is a potent mitogen or migration factor in many cells, acting through the tyrosine kinase receptor encoded by the MET proto-oncogene [39, 40]. Morishita et al. reported the first Phase I human study using HGF in PAD in 2004 [19]. The group studied the safety and efficiency of IM HGF plasmid DNA in 6 patients with CLI. Follow-up was at 3 months and no significant complications or adverse effects were detected. Interestingly, no edema was observed as opposed to all other gene therapy trials. This open-label study had reductions in pain, increases in ankle pressures and reduced ulcer size. What followed was a phase II IM study of HGF in patients with CLI which showed that HGF plasmid was safe and as well tolerated as placebo [20, 21]. The study showed significant improvements in transcutaneous oxygen pressure [20], and decrease rest pain [21] with HGF compared to placebo. Investigation with HGF was investigated in a multicenter, randomized, double-blind placebo-controlled trial [22]. Placebo or HGF plasmid was injected to ischemic limbs of patients with CLI. After 12-weeks follow-up, there was improvement in rest pain and reduction of ulcer size in the HGF group, and HGF plasmid group also had improved quality of life. There were no major safety problems. Investigations using HGF have continued but ultimately a multinational phase III trial NCT02144610 of the hepatocyte growth factor (HGF) plasmid for critical limb ischemia (CLI) (http://www.anges-mg.com/en/pdf.php?pdf=100886.pdf) was halted for slow enrollment.

5.4 Hypoxia Induced Factor-1 Alpha (HIF-1α)

Hypoxia Induced Factor-1 α is a transcription factor that is a central mediator in the cellular adaptation/response to ischemia or (as its name-sake) hypoxia. HIF-1 is a complex heterodimer with a constitutively expressed β subunit (HIF-1β) and oxygen regulated α subunit HIF-1α [41, 42]. The control of this protein is also important for

its potential action because under normoxic conditions, the HIF-1α subunit undergoes rapid proteosomal degradation, while under hypoxic condition the HIF-1α is stabilized, accumulates and dimerizes with HIF-1β [43]. What follows is this heterodimer, translocates to the nucleus and binds to conserved hypoxia response elements (HREs), which then drives transcription of potentially hundreds of genes that encodes proteins involved in the processes of neovascularization, making HIF-1α an attractive therapeutic target to modulate angiogenesis. It is interesting to note that that HIF-1α over-expression designed to promote therapeutic angiogenesis only has the potential for success in situations where endogenous activation is submaximal.

Rajagopalan et al. reported the first, phase I, trial using a constitutively active form of HIF-1α [23]. This report of 34 no-option patients included both a randomized, double-blinded, placebo-controlled study and an open-label extension study. The study reported no serious agent attributed adverse events at 1 year of follow-up, the study of the "limb"included complete rest pain resolution in 14 of 32 patients and complete ulcer healing in 5 of 18 patients. Overall, the therapy was well tolerated, with the most common adverse events reported being peripheral edema. HIF-1α then switched to patients with intermittent claudication where the agent was unable to promote therapeutic angiogenesis but did induce a significant degree of edema [44].

5.5 Overview/Summary of Gene Therapy for Angiogenesis

Despite wide-spread positive results from pre-clinical studies and encouraging data from small phase I and phase II clinical trials, therapeutic angiogenesis for CLI must be viewed as a failure and while some research in this area is ongoing, successful programs will need to identify systems for improved gene delivery. Studies that will seek to combine growth factors and /or delivery of growth factors with cell therapy need to be considered as opposed to single gene therapy approach. Alternatives in trial design such as gene therapy as an adjunctive therapy to surgical/endovascular therapies, as opposed to stand alone studies to promote neovascularization need to be considered. Perhaps, out-of-box ideas such as the use of therapy to be used in conjunction with surgical and endovascular therapies to limit graft failure and to limit in-stent re-stenosis need to be considered.

6 Cell Therapy for Therapeutic Angiogenesis

Stem cells can be derived from multiple sources and this area has opened new avenues of investigation for disease treatment. Embryonic tissue provides a rich source for stem cells but for this any clinical use is complicated by ethical issues and the strong potential for these cells to differentiate into uncontrolled tumors and cause immune reaction such as a graft vs. host disease. Adult tissue provides a source of

stem cells with many with many if not all adult tissue containing some cells with pluripotentcy that can be modulated by the micro-environment. Adult tissue also contains committed stem cells or better referred to as progenitor cells, where local environmental stimuli can lead to differentiate into specific cell populations. Tissue regeneration using stem or progenitor cells has led to many studies in CLI.

Theoretically, cell therapy administration has numerous advantages over the use of a specific growth factor as the "cells' can contain or produce numerous cytokines not only a single cytokine and a fixed dose. Stem/progenitor cells can have multiple effects on angiogenesis by having the capacity to localize to tissue and then differentiate into endothelial cells at sites of high ischemia, and/or the cells can differentiate into supporting cells with paracrine effect on proliferation. Cell therapy therefore has the potential to be a more efficient and durable treatment for therapeutic angiogenesis but theory must be tested and as yet has not reached reality.

6.1 Different Modes of Delivery Cell Therapy

Currently, several different modalities for the delivery of cells for cell therapy are under investigation. There is direct intramuscular or intra-arterial injection for delivery of cells such as bone marrow-derived mononuclear cells (BMMNCs). The same IM approach can be used for cytokine-mobilized and apheresed/concentrated peripheral mononuclear cells (PB-MNC). Finally, attempts can be made to mobilize cells (self stem cells) at sites of ischemia.

6.2 Clinical Trials of Cell Therapy in Patients with CLI

Within any population of bone marrow-derived mononuclear cells or peripheral blood mononuclear cells are a fraction of endothelial progenitor cells (EPCs) that can incorporate and enlarge existing vascular networks and augment limb perfusion. While simple in theory, there is no definitive agreement to identify these cells. Different and varying cell surface markers are used to identify and sort putative EPCs by different laboratories. Despite the variability, the surface markers of CD-34, CD-133, and KDR (VEGF−/receptor 2) are used by many groups. BMMNCs also contain cells that are of the monocyte/macrophage lineage that contribute to angiogenesis by secreting angiogenic cytokines and matrix metalloproteinases. Bone marrow derived cells can augment neovascularization by promoting pericyte or other support cells to stabilize new vascular endothelial networks.

Table 14.3 contains many of the clinical trials that have established the safety and feasibility of IM injections into the ischemic limb of bone marrow derived stem cells in patients with CLI. *In-toto,* these studies have shown that IM of bone-marrow derived stem cells is clinically safe with no untoward signal of increases in major adverse events. Much like the gene therapy reports of improvements in pain,

Table 14.3 Clinical trials of cell therapy in CLI

RefReference	Disease	Source of cells/cell preparation	Route of administration/average no.of cells	Results
Tateishi-Yuyama et al. [45]	CLI	BMMNC: no further fractionation	Intramuscular/3.7 × 10^{10}	Improved ABI and TcO2, Increased pain-free walking time Increased collateral by angiography
Nizankowski et al. [46]	CLI	BMMNC: cell enriched for CD34 and AC 133 +ve cells	Intramuscular	Increased laser Doppler flux Increased ABI and TcO2 Improved ischemic ulcers Improved symptoms
Kajiguchi et al. [47]	CLI	BMMNC/PBMNC(1): no further fractionation	Intramuscular/4. × 10^{6}–7 × 10^{7}	No change in ABI Increased TcO2 Improved subjective symptoms
Saigawa et al. [48]	CLI	BMMNC: no further fractionation	Intramuscular/6 × 10^{7} /kg	Increased ABI and TcO2 Increased vessel formation by digital angiography
Huang et al. [49]	CLI	PBMNC; G-CSF stimulation and apheresis on day 5	Intramuscular/3 × 10^{9}	Increased laser Doppler flux, increased ABI, increased collaterals by angiography, improved ulcer healing.
Kawamura, A et al. [50]	CLI	PBMNC; G-CSF stimulation and apheresis	Intramuscular/1.9 × 10^{8}	Improved subjective symptoms, improved thermography, improvement in 3D–CT
Isida et al. [51]	CLI	PBMNC; G-CSF stimulation and apheresis	Intramuscular/3.9 × 10^{10}	Improved ulcer healing, Increased ABI, increased TCO2, enhanced acetylcholine mediated dilatation, no change in nitroprusside mediated dilatation

(continued)

Table 14.3 (continued)

RefReference	Disease	Source of cells/cell preparation	Route of administration/average no.of cells	Results
Lenk et al. [52]	CLI	PBMNC: circulating progenitor cells, G-CSF stimulation and MNC cultured.	Intra-arterial/3.9 × 10^7	Increased peak walking time, increased ABI, TcO2, increased adenosine dilatation, increased acetylcholine dilatation
Miyamoto et al. [53]	CLI	BMMNC and EPCs	Intramuscular/3.5 × 10^9	Improved ABI, pain free walking time
Powell et al. [54]	CLI	BMMNC; enriched for CD90 +ve and CD45 +ve cells	Intramuscular/136+/−41 × 10^6	Improved wound healing
Benoit et al. [55]	CLI	BMMNC, no further fractionation	Intramuscular	Reduced amputation rate
Lu et al. [56]	CLI	BMMSC cultured and expanded(based on CD29, 71, 90, 105, 34 and 45 +ve cells) and BMMNC	Intramuscular	Improved ulcer healing, increased pain free walking time, improved TCO2
Powell et al. [57]	CLI	BMMNC: cultured and expanded, enriched for CD90+ and CD 14+ cells	Intramuscular/35–295 × 10^6	Decreased major amputation, improved would healing
Idei et al. [58]	CLI	BMMNC; no further fractionation	Intramuscular/1.8 × 10^9	Decreased major amputation
Iafrati et al. [59]	CLI	BM-aspirate concentrate	Intramuscular	Decreased major amputation, Imprved rest pain
Walter et al. [60]	CLI	BMMNC: Enriched for CD34+, CD45+ and CD133+ cells	Intraarterial/	Decreased ulcer healing
Murphy et al. [61]	CLI	BMMNC: Enriched for CD34+, CD133+ and KDR +ve cells	Intramuscular 1.3–2 × 10^9	Increased perfusion index by PET-CT, improved rest pain, increased first toe pressure
Losordo et al. [62]	CLI	PBMNC; GCSF stimulation and mobilization of BM cells, apheresis and enriched for CD 34+ cells	Intramuscular/ 1 × 10^5 cells/kg (low dose) 1 × 10^6cells/kg (high dose)	Favorable trend towards reduced amputation

MMNC Bone marrow-derived mononuclear cells,*PBMNC* Peripheral blood-derived mononuclear cells, *BMMSC* Bone marrow-derived mesenchymal stem cells

improvements in quality of life measures, improve rates of limb salvage, increases in ABI and transcutaneous oxygen levels have been reported.

An alternative approach for cell therapy is the mobilization of stem cells out from the bone marrow and to the periphery using chemokine agents and then harvesting the mobilized mononuclear cells which can then by apheresed and concentrated; eventually cells could be modulated before administration. The collected cells can then be transplanted to ischemic tissue using IM or intra-arterial injections. Cell mobilization avoids bone marrow aspiration of course the approach provides lower number of cells and carries the risks from the mobilization agents. Table 14.3 contains several clinical trials of cell therapy where studies have suggestions for ABI a d wound healing mprovements with and increased formation of collaterals.

Cell therapy approaches using the mobilization of "self" stem cells to sites of leg ischemia have also been reported for example using injection of cytokines. Granulocyte colony stimulating factor when given IM appears relatively safe and this approach effectively mobilizes stem cells from bone marrow to peripheral blood, where cells can access sites in ischemic tissue in response to chemotactic factors present in ischemic tissue. Conceptually this method seems simpler but of course the relative lack of blood flow and vessels in ischemic limbs may affect delivery of adequate number of cells for neovascularization. A few small clinical trials have reported clinical outcomes [63, 64].

6.3 Overview of Cell Therapy Trials for Therapeutic Angiogenesis

In CLI, various studies of stem cell therapy have been reported and in general the approach has proven to be safe and feasible in smaller clinical trials. Still few randomized, controlled, clinical trials have been completed [45, 49, 54, 58–60, 62, 63, 65, 66]. Trials completed to date have been complicated to interpret due to the multiple variables of cell lineage, routes of delivery, doses and relative-dose comparisons, routes of delivery and duration of follow-up. These studies have been conducted on the most extremely of the CLI severity and studies must be moved into larger patient populations for sizable enough randomized clinical trials, with more standardized methods of stem cell selection and delivery to establish if cell therapy can be a therapy for vascular regeneration.

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Chapter 15
Cell-Based Therapy in Ischemic Heart Disease

Adnan Khan, Akshay Menon, and Jörn Tongers

Abstract Despite continuous advances in primary prevention and secondary management of arteriosclerotic disease, ischemic cardiovascular disease constitute an increasing socioeconomic burden. A solid body of evidence has previously indicated a regenerative capacity of stem and progenitor cell-based therapy in preclinical and early-phase clinical studies. Clinical application of stem and progenitor cells in ischemic heart disease have included patients with coronary artery disease after revascularized acute myocardial infarction, ischemic cardiomyopathy, or refractory angina. Larger scale clinical studies subsequently generated mixed data partly due to differences in study design and employed techniques. While the therapeutic application of different cell populations appears safe, therapeutic efficacy of stem and progenitor cells needs yet to be proven at a larger scale in properly designed randomized-controlled trials. Vast efforts have been undertaken to overcome practical limitations and conceptual challenges that were encountered in praxis over time. Multiple strategies such as supportive use of biomaterials, combination of different cell sources, genetic modification of cells prior to application, and addition of factors turned out to be promising overly in the preclinical evaluation To optimize and fully leverage the regenerative potential of cell-based therapies further aspects including identification of a potentially ideal cell linage as well as timing, repetition and dosing of cell delivery need to be addressed.

Keywords Ischemic heart disease • Cell therapy • Stem and progenitor cells • Limitations

Abbreviations

ADRC	Adipose derived regenerative cell
AMI	Acute myocardial infarction
BMC	Bone marrow-derived mononuclear cell

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CAD	Coronary artery disease
CSC	Cardiac stem cell
EPC	Endothelial progenitor cell
ESC	Embryonic stem cell
GM-CSF	Granulocyte-macrophage colony stimulating factor
HPSC	Hematopoietic stem cell
ICM	Ischemic cardiomyopathy
iPS	Inducible pluripotent stem cell
MSC	Mesenchymal stem cell
SM	Skeletal myoblast

1 Background

Ischemic cardiovascular diseases that subsume coronary artery disease (CAD), cerebrovascular, and peripheral artery disease constitute a leading cause for quality of life impairment, morbidity and mortality in developed industrial countries [1]. For the purpose of the present chapter, we will exclusively focus on ischemic heart disease that comprises of refractory angina, ST-elevation myocardial infarction (STEMI, equals acute myocardial infarction, AMI), and ischemic cardiomyopathy (ICM). Gold standard therapy of these conditions comprise of optimized medical therapy, (immediate) complete revascularization, and, where indicated, modern heart failure management to reduce symptoms, minimize myocardial ischemia as well as ischemia-related ventricular dysfunction, and adverse remodeling. Beyond, it is crucial to attenuate CAD progression by secondary prevention including medication and risk profile optimization. Despite modern treatment, a significant portion of patients remain symptomatic (i.e., angina) and/or prognostically threatened lacking revascularization options. In addition, recurrent hospitalizations due to acute coronary syndromes and acute heart failure cause socioeconomic burden [2].

Despite substantial progress on disease prevention and progression, interventional and surgical revascularization strategies are often required to improve perfusion of manifest ischemic tissue. In cases of later-stage ischemic cardiovascular disease, revascularization of ischemic tissue may not be a viable option any longer despite practical and technical experiences as well as development of modern devices and materials. In light of this unmet medical need, regenerative strategies have been developed in the last two decades. Previously, the therapeutic concept of cardiovascular gene therapy that aims at overexpression of certain target genes via encoding plasmids or viruses had emerged. In the context of ischemic cardiovascular disease, therapeutic overexpression of several proangiogenic factors has been advocated based on promising preclinical data and early phase clinical studies. Due to many conceptual and practical limitations, at least in part, angiogenic gene therapy has not made its clinical breakthrough.

More recently the field of cell-based therapies has emerged. Since the identification of stem cells by Asahara and co-workers in 1997, the therapeutic concept to repair or regenerate ischemic tissue by the application of stem or progenitor cells has been developed. Earlier the idea was that stem/progenitor cells would differentiate into organotypic cells and integrate structurally into the organ architecture and thereby replace dysfunctional areas. In the meanwhile, the community reached a consensus that paracrine effects via secretion of factors such as proangiogenic and/or anti-apoptotic proteins constitute the key mechanism of the underlying beneficial effects ascribed to cell-based therapies. At present, multiple stem/progenitor cell populations are being discussed to leverage a regenerative potential. Almost independent of a given stem/progenitor cell population, transplanted cells are challenged by the harmful environment of ischemic tissue. As a consequence, poor viability, impaired homing, low retention, and impaired functionality of cells have been recognized as limiting factors [3, 4]. Traditionally, bone-marrow mononuclear cells (BMC) have most extensively been studied in the clinical arena. More recently, resident stem cells and inducible pluripotent cells have gained momentum, although practical and ethical concerns are pertinent.

After years of smaller, uncontrolled clinical studies, the field of cell-based in ischemic cardiac disease stands at the verge to proof its efficacy in randomized-controlled studies (Table 15.1). Underpowered, smaller-size, methodologically

Table 15.1 Randomized-controlled trials in ischemic heart disease

RCT	Indication	N	Route of delivery	Cell source	Key results
REPAIR-AMI	AMI	204	IC	BMC	At 4 months: ↑ LV-EF At 1 year: ↓ cardiovasc. events At 2 years: ↓ cardiovasc. events ↑ regional contractility
BOOST	AMI	60	IC	BMC	At 6 months: ↑ LV-EF At 18 months: ↑ diastolic function At 5 years: no effect
Cao et al.	AMI	86	IC	BMC	At 6 months ↓ LV-EF At 1 year ↓ LV-EF At 4 years ↓ LV-EF

(continued)

Table 15.1 (continued)

RCT	Indication	N	Route of delivery	Cell source	Key results
Janssens et al.	AMI	67	IC	BMC	No effect
Lunde et al.	AMI	100	IC	BMC	No effect
TIME	AMI	120	IC	BMC	No effect
LATE-TIME	AMI	87	IC	BMC	No effect
END-HF	ICM	28	EM	BMC	No effect
FOCUS-CCTRN	ICM	92	TE	BMC	No effect
MSC-HF	ICM	60	IM	MSC	↑ LV-ESV ↑ SV ↑ myocardial mass
TAC-HFT	ICM	65	TE	MSC BMC	↓ NYHA ↑ 6-min walk ↓ infarct size ↑ reg. myocard. function
PRECISE	ICM	27	TE	ADRC	↑ MVO_2 ↑ LV mass ↑ LV wall motion
van Ramshorst et al.	RA	50	IM	BMC	↓ angina ↑ quality-of-life
PROTECT-CAD	RA	28	EM	BMC	↑ exercise time ↑ LV-EF ↓ NYHA
ACT34-CMI	RA	167	IM	$CD34^+$	↑ angina ↑ exercise tolerance
RENEW	RA	112	IM	$CD34^+$	*Early terminated* ↑ *total exercise time* ↓ *angina*
PROGENITOR	RA	28	TE	$CD133^+$	↓ angina episodes ↓ angina class ↑ SPECT summed core

Summary of randomized controlled cell-therapy trials in patients with ischemic heart disease. *AMI* acute myocardial infarction, *ICM* ischemic cardiomyopathy, *RA* refractory angina, *BMC* bone marrow-derived mononuclear cells, *MSC* mesenchymal stem cells, *ADRC* adipose tissue derived regenerative cells, *EM* endomyocardial, *IC* intracoronary infusion, *TE* transendocardial.

flawed studies will not be of value for the progress of the field. Thus, we here give a critical overview on the randomized-controlled studies of the cell-based therapies in ischemic heart disease. Furthermore, we will make an attempt to appraise limitations, remaining challenges and potential solutions to potentially leverage the regenerative efficacy that has been proposed (Fig. 15.1).

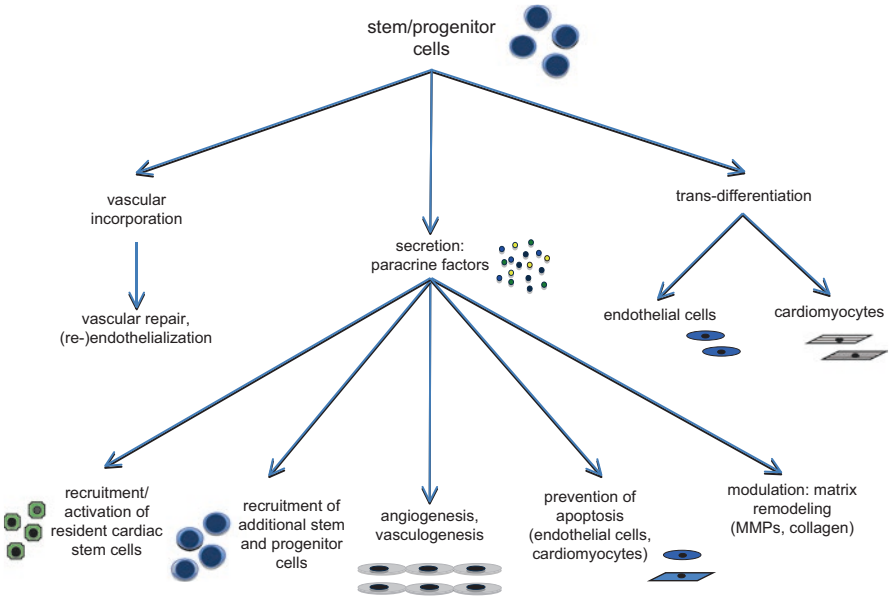

Fig. 15.1 Mechanisms of regenerative efficacy proposed for stem and progenitor cells-mediated repair of ischemic tissue

2 Methodological Aspects of Clinical Cell-Based Therapy

The heterogeneity of existing early-phase clinical data may be related in part to relevant differences between investigated patient populations, design and methodology of previous studies. Particularly difference making aspects to consider are population, delivery route, timing, dosing and repetition of cell transfer.

2.1 Cell Populations

Stem cells (stemness) are defined by the capacity of self-renewal and the ability to differentiate into further developed progenitor cells that itself can differentiate into lineage specific mature cells. Progenitor cells, in turn, entail a limited differentiation capacity, capable of dividing for a limited number of cell cycles, and also having the ability of self-renewal. Until today, various cell sources such as unselected bone marrow cells (BMC), resident cardiac stem cells (CSC), mesenchymal stem cells (MSC), and skeletal myoblasts have clinically been evaluated for cell-based repair of the ischemic myocardium. Each population shows its own set of advantages vs. disadvantages, and practical feasibility vs. limitations [5]. Some populations have been employed in clinical studies with success, others have yet merely been investigated

in the preclinic with great promise. Of the cell populations shortly characterized below, only BMC, HPSC, MSC, and skeletal myoblasts have clinically been used yet.

Bone Marrow Cells Unselected BMC have by far most been studied at bedside. BMC are easy to harvest and their preparation is straightforward not requiring prolonged ex-vivo manipulation. Thus, BMC contain a broader range of cell types including small fractions of stem and progenitor cells. Opposed to selected, non-expandable stem cell populations, BMC use is not restricted by the number of cells that can be obtained.

Selected Hematopoietic Stem and Progenitor Cells Stem and progenitor cells are defined by surface markers. CD133 and CD34 surface markers are commonly used to isolate and enrich for hematopoietic stem cells. Isolating $CD34^+$ cells, for example, from the pool of circulating mononuclear cells has been proposed to increase regenerative potency [6]. This observation has raised particular interest in selection strategies with a vague preference for selected stem vs. unselected BM cells. In physiological states, merely low levels of hematopoietic stem cells (HPSC) are circulating. To augment levels of circulating cells and thereby optimize the yield, pharmaceutical agents have been used to mobilize HPCS from the bone marrow into the circulation before consecutive enrichment via leukapheresis.

Mesenchymal Stem Cells Mesenchymal stem or stromal cells (MSC) are derived from stromal cells localized in adipose tissue and bone marrow stroma. These cells have generated interest because of their anti-apoptotic and anti-inflammatory paracrine properties [7]. This potential immunosuppressive paralleling effect has led to the notion that MSC may be particularly useful for allogeneic applications. As one of the features of an ideal cell therapy, allogenic application of MSC (off-the-shelf) would be of interest. On the downside, use of MSC requires ex-vivo expansion given limited cell numbers.

Emerging Cell Populations Beyond the "established" cell populations, other promising ones are also emerging, but neither of these have been assessed clinically yet.

The adult heart has been shown to undergo an age-dependent cardiomyocyte turnover of 0.5–1.0% annually [8]. This turnover arises from resident cardiac stem cells generating cardiomyocytes and other cardiac cell types including endothelial and smooth muscle cells [9]. Because of very low number of cardiac-derived resident stem cells (CSC), these need to be expanded ex-vivo after isolation from cardiac specimen for therapeutic utilization [10].

Resident stem cells in the skeletal musculature are resting under normal conditions and are capable of generating new myocytes following injury. These so called skeletal myoblasts (SM) are easily accessible via muscle biopsies for ex-vivo expansion. Preclinical evidence suggests that SM may be driven into cardiomyocytes in a targeted fashion. A regenerative potential has already been described in models of ischemic myocardium [11, 12].

The capability to differentiate into any cell lineage is the defining feature of embryonic stem cells (ESC) [13, 14]. Limited availability as well as ethical and

regulatory concerns have been discussed heavily. So far, their therapeutic use has been restricted by these and other aspects. One hampering concern, however, is the potential risk of developing tumors such as intra- and extra cardiac teratoma [15, 16]. It has been challenging to come up with effective methods to regulate and control the differentiation of ESC, although different concepts are explored to prevent tumor formation while enhancing cardiopoietic differentiation of ESC. Whether ESC functionally integrate electromechanically and structurally into the ischemic myocardium, remains to be answered.

The development to reprogram adult cells into stem cells in 2006 has revolutionized the field [17]. These cells termed inducible pluripotent stem cells (iPS), are able to form all three germ layers. This invention enables to produce autologous iPS from its patient won somatic cells using nuclear reprogramming with ectopic stemness factors [17, 18]. The ability of human iPS to differentiate into functional cardiomyocytes has already been demonstrated [19]. Although this concept is still distant from the translation to the bedside, hectic research activity is progressing this therapeutic concept forward.

2.2 Routes of Cell Delivery

Different modes of cell delivery have been established aiming at the treatment of ischemic myocardium: for cell transfer. The route mainly depends on the revascularization status of the myocardial region. The overall aim is to safely deliver the therapeutically efficacious number of functional cells to the therapeutic target zone with the least possible risk but via the conceptual meaningful route. Intracoronary infusion and direct or respectively indirect intramyocardial injection have been most accepted. The advantage of intracoronary infusion allows homogenous distribution, but on the other side requires target vessel revascularization. Similar to the methods of coronary interventions, an angioplasty balloon catheter is inflated to stop blood flow and minimize spillback. This stop-flow techniques enables contact of cells to the vessel wall and the efficiency of adhesion and transmigration [20]. It is particularly suitable for patients after acute revascularization of AMI. If the target vessel is not patent or chronically occluded, intramyocardial injection of cells is conceptually more suitable. Intramyocardial injection can be achieved from the endomyocardial side via needle-tipped delivery catheters or from the epimyocardial side with limited risk. Despite the choice of several injection sites, this route of delivery leads to a more heterogenous distribution of cells. Guidance of cell injection to ischemic but viable myocardium by means of electromechanical mapping (EEM, e.g. NOGA-mapping) can enhance efficacy.

The vast majority of adult stem and progenitor cells are confined to the bone marrow niche. By systemic application of certain mobilizing cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), the circulating fraction can be augmented. Adopting the scenario of bone marrow transplant before its ablation in hematology, it was previously conceptualized that higher circulating levels

per se may contribute to ischemic tissue repair. Earlier phase clinical studies failed to prove efficacy of this strategy in ischemic heart disease (summarized in: [21]). The safety profile, on the other hand, was at least questionable in CAD. Given the unfavorable risk-benefit profile of mobilization strategies, RCTs have not been reported yet and may not be advocated. Furthermore, there are also no convincing clinical data for other pharmaceutical mobilization agents aiming at endogenous stem/progenitor cell trafficking (e.g., dipeptidylpeptidase IV inhibition, CXCR4-antogonism AMD3100) available.

3 Randomized-Controlled Evidence on Cell-Based Therapy for Ischemic Heart Disease

Traditionally bone-marrow mononuclear cells (BMC) have most extensively been studied for the clinical application. More recently, resident cardiac stem cells and inducible pluripotent cells have gained momentum although practical and ethical concerns are limiting their clinical use. Cell-based therapy has been employed to treat the following pathologies: acute myocardial infarction (AMI), chronic, refractory angina pectoris, and ischemic cardiomyopathy (ICM).

3.1 Acute Myocardial Infarction

Randomized-controlled trials have shown mixed results in early-phase clinical studies. In addition to a proof-of-concept, these studies suggested safety and feasibility of intracoronary BMC-infusion after revascularized AMI [20, 22–24]. Two RCTs embarking on intracoronary infusions (Reinfusion of Enriched Progenitor Cells and Infarct Remodeling in Acute Myocardial Infarction, REPAIR-AMI; Bone Marrow to Enhance ST-Elevation Infarct Regeneration, BOOST) reported an increase in left-ventricular ejection fraction (LV-EF) 4–6 months after BMC transfer while left-ventricular end-diastolic volume (LV-EDV) remained unchanged [25, 26]. In REPAIR-AMI, the improvement of LV-EF persisted until 12 months follow-up. Although the study was not powered accordingly, the authors also described fewer adverse cardiovascular events over 2 years. However, in case of BOOST trial, the initial improvement in left ventricular function was not sustained. By contrast, two other RCTs did not show an improvement of LV function or dimensions at 4–6 months after BMC application [27, 28]. Notably, Janssens and co-workers found smaller infarct sizes 4 months after very early BMC infusion within 24 h after AMI [27]. Similarly, Lunde et al. did not find an effect of a single BMC intracoronary infusion two to three weeks after AMI as assessed by global LV-function in their ASTAMI (Autologous Stem cell Transplantation in Acute Myocardial Infarction) trial. The REGENT investigators did not find a significant difference in

LV function and dimensions 6 months after intracoronary cell delivery neither in the unselected nor in the $CD34^+/CXCR4^+$ cell treated group. Notably, they observed a trend in cell-treated patients with severe LV impairment and longer delay of the cell application after AMI. The randomized, double blind, placebo-controlled TIME (Timing in Myocardial infarction Evaluation) trial was designed to assess the aspect of timeliness of autologous intracoronary BMC therapy on global and regional LV function comparing cell application 3 vs. 7 days after AMI. There was no difference in LV-EF 6 months impendent of the time point of cell delivery. Furthermore, there was no significant effect of therapy on regional left ventricular function in both infarct and border zones [29]. Along the same line, Late-TIME was meant to address the relevance of BMC therapy 2–3 weeks after AMI. Regional as well as global LV function turned out to be unchanged after 6 months [30].

Long-term follow-up data on the REPAIR-AMI and BOOST collectives were subsequently reported. A sustained improvement of LV function was found after 12 months, [25] and was related with a significant reduction of major adverse cardiovascular events after AMI in REPAIR-AMI [31]. These observations were robust up to 2 years [32]. In the BOOST trial, there was no relevant difference in LV-EF after 18 months [33]. While there was a persistently improved diastolic function in an echocardiographic substudy [34]. Furthermore, there was not cardiac benefit neither in systolic and diastolic LV function 5-year after a single BMC infusion after AMI [35]. It needs to be acknowledged that subgroup analyses revealed a greater benefit from BMC infusion in patients with a more severe LV dysfunction at baseline. On the other hand, in a study conducted by Cao et al. intracoronary BMC infusion improved LV-EF beyond initial 6 months follow-up up to 4 years after inclusion while there was no effect in viability [36].

Differences in protocol and design including time lag between coronary revascularization and cell injection, type, cell number and isolation procedure, follow-up design differences may reasons for the heterogeneity of results. Exemplary, intracoronary BMC infusion has generally taken place within the first seven post-AMI days, while in the Janssen's trial cell transfer took place within 24 h [27]. Furthermore, there were differences in LV imaging. MRI was not performed before 2–3 weeks after cell transfer while only echocardiography was done at baseline in the ASTAMI trial. In this study cells by contrast were prepared differently according to the Lymphoprep technique [28].

3.2 Ischemic Cardiomyopathy

Several RCTs have evaluated the regenerative use of different cell populations in patients with severe LV dysfunction and chronic heart failure due to ischemic cardiomyopathy:

BMC In contrast to the borderline efficacy of BMC-infusion after AMI, the use of BMC in ischemic cardiomyopathy is not supported by evidence so far. In the

END-HF no significant changes in LV-EF and LV-ESV were detected 6 months after viability-guided endomyocardial injection of autologous BMC [37]. Similarly, in the FOCUS-CCTRN trial LV-ESV, myocardial perfusion and maximal oxygen consumption remained unchagned following transendocardial BMC injection in patients with chronic ischemic heart failure [38].

MSC The MSC-HF trial aimed at investigating the effects of intramyocardial injection of BM-derived, expanded MSCs in severe ischemic heart failure [39]. There were significant improvements of LV-EF and myocardial mass associated with a decrease in LV-ESV after 6 months. Similarly, in TAC-HFT (Transendocardial Autologous Mesenchymal Stem Cells and Mononuclear in Ischemic Heart Failure) trial, infarct size was decreased and regional myocardial function improved by transendocardial injection of MSC in patients with ischemic cardiomyopathy with LV dysfunction while there were no effects in the paralleling BMC group. These observations were associated with a clinical improvement in 6 min walk 1 year after transendocardial cell injection [40].

ADRC The regenerative utility of adipose tissue-derived cells (ADRC) has been studied in patients with severe ischemic cardiomyopathy by the PRECISE investigators. They describe an improvement in LV function, myocardial perfusion, and exercise capacity following transendocardial after application of autologous ADRC [41].

All in all, there is no evidence for the efficacy of BMC-therapy, while there are sign of bioactivity for MSC and ADRC for in patients with ICM.

3.3 Refractory Angina

A substantial number of patients suffer from refractory angina despite optimal medical and/or surgical therapy [42]. Early phase clinical trials revealed mixed results.

BMC In a randomized-controlled trial conducted by von Ramhorst et al., angina frequency and quality of life were improved 3 months after intramyocardial injection of BMC [43]. The PROTECT-CAD (Prospective Randomized Trial of direct Endomyocardial Implantation of Bone Marrow Cells for Treatment of Severe Coronary Artery Diseases) studied patients with intractable angina. In this collective patients refractory to conventional therapy angina symptoms, exercise time, and LV function were improved after BMC-injections [44].

HPSC In contrast to unselected BMC, the selection for hematopoietic stem cell markers is considered to increase efficacy. Along this line, the ACT34-CMI investigator surrounding Losordo locally injected $CD34^+$ cells in refractory patients. Six months after NOGA-guided intramyocardial application of autologous cells, exercise tolerance and angina frequency were improved [42]. The subsequent phase-3 RENEW (Efficacy and Safety of Targeted Intramyocardial Delivery of Auto $CD34^+$

Stem Cells for Improving Exercise Capacity in Subjects With Refractory Angina) trial was designed to similarly evaluate the efficacy of intramyocardial deliver of autologous $CD34^+$ cells in refractory angina and chronic myocardial ischemia. Unfortunately it was prematurely terminated by the sponsor due to strategic internal reasons despite favorable trends in total exercise time and angina frequency for intramyocardial $CD34^+$ application [45]. Selecting hematopoietic stem cells for the surface marker CD133 was also employed in the randomized controlled multicenter PROGENITOR (Endothelial Progenitor Cells and Refractory Angina) trial in patients with refractory angina. Transendocardial administration of $CD133^+$ stem cells was related to less angina episodes and functional class. Additionally myocardial perfusion was also enhanced based on the SPECT summed score [46].

4 Challenges and Limitations in Clinical Cell-Based Therapies

The field of cell-based therapies faces the dilemma that robust preclinical and early-phase clinical studies suggested therapeutic efficacy delivering stem or progenitor cells to potentially regenerate ischemia tissue. Subsequent larger-scale and randomized-controlled trials, however, have not uniformly proven efficacy. On the other end available clinical evidence suggest safety of using BMC, HPSC, MSC and SM in elder and fragile cardiac patients suffering from ischemic heart disease. As outlined above, part of the heterogeneity may be due to methodological differences amongst the studies. At this point the field will only progress if upcoming trials are thoughtfully designed, randomized-controlled, and properly powered. This is particularly true if trials are aimed to address clinical or prognostic endpoints.

4.1 Beside-to-Bench: Modeling of Ischemia

To systematically address limitations focused preclinical studies aligned to the remaining clinical challenges may be warranted. In order to understand behavior and demands of stem and progenitor cells challenged by the ischemic microenvironment after transfer, for example, true models of ischemic milieu are needed. Further understanding of schema and its impact on exposed cells may provide us with new avenues to optimize cell-based therapy. In-vivo models are known to show relevant variability of the ischemic phenotype. Available in-vitro models do not closely mimic the ischemic milieu. Current models only reflect of the multiple features such as hypoxia, acidosis, or hypoglycemia. More complex models that reflect the totality of ischemic features including an altered metabolic state, inflammation, cytokine storm and many others are not yet available. Thus, some efforts may be redirected to preclinical work I order to facilitate the field.

4.2 Ideal Cell Population

Many cell populations have been proposed. It is entirely uncertain, which population may be more suitable than others. Suitability may also be determined by the clinical indication. This may be particularly true in the setting of autologous cell therapy, where cells harvested from patients may be dysfunctional due risk profile and/or comorbidities and may impair the regenerative capacity [47–55]. Different unselected or selected cell types may show varying patters of bioactivity or biological behavior. In the ischemic target zone [56]. Over the last years the notion of the underlying mechanism by which cells exert their regenerative potential shifted to the paracrine concept [57]. It is unknown, however, whether this paracrine theory can be generalized.

4.3 Selection of Study Endpoints

The field of angiogenic gene therapy has been struggling to proof efficacy according to established endpoints, which is even more difficult in peripheral-artery disease. In terms of therapeutically recover or prevent adverse remodeling of the myocardium multimodal measures of LV function and dimensions are established. A significant and lasting improvement of global LV function may be the gold standard but presumably difficult to achieve. Additional parameters such as infarct size, regional contractility, diastolic function, and myocardial perfusion are of interest particularly for indications such ischemic cardiomyopathy and refractory angina. Based on if at all smaller impact of cell-therapy meeting clinical or prognostic endpoints may be even more difficult. Notably, there is evidence that infarct size is a strong predictor of outcome in ischemic heart disease. Beyond, none of the established variables assessing the myocardium does address the microcirculation, a function that is thought to be beneficially affected by cell-therapy in light of preclinical findings of pro-angiogenic effects.

4.4 Timing and Dosing of Cell Transfer

Efficacy of stem/progenitor cells appears to be dose-dependent [6, 58], and to follow a dose-effect relationship [58]. For each any every constellation dose titration may be needed before making a final call on the field of cell-based therapy. Cell population, route of delivery, indication and disease severity may also influence the optimum dose. Furthermore, it appears to be over asking to gain a global functional impact from a single, one-time injection. Repetitive cell applications may boost efficacy. Previous studies have not addressed this aspect. In addition, timing of application may also be relevant. In the REPAIR-AMI trial, patients showed a better response in

LV-EF when BMC were administered later than day 5 [25]. This may be explained by an even more detrimental ischemic environment early after acute ischemia. In contrast, homing of proangiogenic progenitors has been shown early (≤14 days) [59].

4.5 Local Retention of Viable Cells

The number of viable cells has been reported to vary from roughly 30% to nearly 1% with a rapid decrease within the first 7 days after application [60, 61]. Imaging of labeled stem and progenitor cells in the preclinic early on suggested that cells do not remain in the therapeutic target zone [62, 63]. Retention of viable cells (cell fate) is multi-factorial. Mode and route of application does also affect cell viability. Clinical observations showed a rapid washout of labeled BMC with a 1 h after application. The signal then further declines over the next 3–4 days. Along the same line, 4% of radiolabeled $CD34^+$ cells in the myocardium 1 h after intracoronary infusion in ischemic cardiomyopathy [64]. Uptake and retention of cells is probably also influenced by the state of ischemia. Presence and magnitude of inflammatory factors may beneficially affect homing and retention while the even more hostile microenvironment early after ischemia may alter injected cells.

5 Perspective

The combination of a lack of satisfying efficacy data, technical limitations and unmet conceptual challenges have triggered intensive research. These activities overly aim at the protection of cells within the hostile ischemic environment. This may be achieved at different levels of cell therapy cascade.

5.1 Cell Modification

An intriguing strategy to manipulate stem and progenitor cells prior to the ischemic exposure more viable and thereby resistant is to precondition or modify the cells upfront. This strategy is adopted from previous pre-clinical observations [52]. Any functional modification aiming at improved homing, adhesion, transmigration, survival, engraftment, differentiation, cell-cell interaction, and retention might help to increase the potency of cell-based therapy. Various techniques to precondition or modify cells have been evaluated with success, but none of theses has yet been tested in the clinical setting. These strategies include exposure to hypoxia, pretreatment with small molecules and drugs, epigenetic reprogramming, and transfection for overexpression of certain genes of potential benefit by means of plasmids or viruses such as hemeoxygenase-1 [65, 66].

5.2 Supportive Biomaterials

Given the complex nature of biology cell-cell and cell-matrix interactions are crucial for viability and functionality of cells. Cells loosing these interfaces are negatively affected. This is of particular relevance in the setting of stem and progenitor cells harvest, isolation and ex-vivo expansion. It is therefore critical to maintain the homeostasis of cells for the purpose of an efficient cell transfer [67]. In recent years the field of biomaterials and nanotechnology has rapidly evolved. Building up on biological and synthetic compounds this technology allows to design smart materials that combine mechanical, 3-dimensional support resembling the extracellular microarchitecture as well to integrate signals of bioactivity by incorporation of epitopes, functional groups, compounds, viruses, genes and biological factors. Rigidity, shape, structure, and dimensions of these materials are relevant to resemble local architecture as close as possible. In addition to mechanical support of cells novel materials that incorporate bioactive signals such as insulin growth factor (IGF), stromal derived factor-1 (SDF1), or the integrin binding domain of fibronectin (RGDS) have been shown to positively influence cellular functions [68]. Ideally such materials would be build from biological compounds, be injectable, biodegrade into harmless by products, and form a 3-dimensional matrix.

5.3 Combined Approaches

To ultimately accomplish the wholly grail of repairing cardiac tissue is challenging given shortcomings and limitations at present is challenging. In light of highly complex mechanisms in embryology and biology, it may be questionable to believe that a single application of genes or cells results in a regeneration of damaged tissue. Following this notion recent studies aim at combined biological repair, which may be more efficacious than the one-time, single-strategy approaches. Combination of various types of stem/progenitor cells plus stromal cells seems conceptual attractive. Unselected and selected cells, for example showed different spatial patterns of homing [56]. To add another level of complexity, combination with gene therapeutic approaches may be warranted [3].

Such combinatorial approaches of tissue regeneration, however, need to be precisely timed, may require various types of stem/progenitor and stromal cells, the addition of (paracrine) factors, and specifically designed biomaterials in order to really advance the this exciting field to the next level of “true ischemic tissue repair”.

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Chapter 16
Angiogenesis and Atherosclerosis

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Abstract Atherosclerotic heart disease is the leading cause of morbidity and mortality worldwide. Despite recent advances in our understanding of atherosclerosis the role of angiogenesis in atherosclerosis is still being debated. The use of therapeutic angiogenesis has been widely regarded as an attractive approach in treatment of ischemic heart disease. On the other hand, there is growing evidence that neovascularization contributes to the progression of atherosclerotic lesions, and that it may play key role in intraplaque hemorrhage, plaque destabilization and rupture. Most trials on therapeutic angiogenesis using growth factors like VEGF (vascular endothelial growth factor)/FGF (fibroblast growth factor) have used single agents and are inconclusive. Bench and bedside research continues to bring insight into new mechanisms of atherosclerosis and tumor growth. Further understanding of different facets of angiogenesis may help in the development of novel and specific therapies.

Keywords Plaque neovascularization atherosclerosis • Arteriogenesis • Therapeutic angiogenesis • Growth factors

1 Introduction: Angiogenesis in Health and Disease

Atherosclerotic heart disease is the leading cause of morbidity and mortality worldwide. Despite recent advances in our understanding of atherosclerosis the role of angiogenesis in atherosclerosis is still being debated. Angiogenesis is the process of

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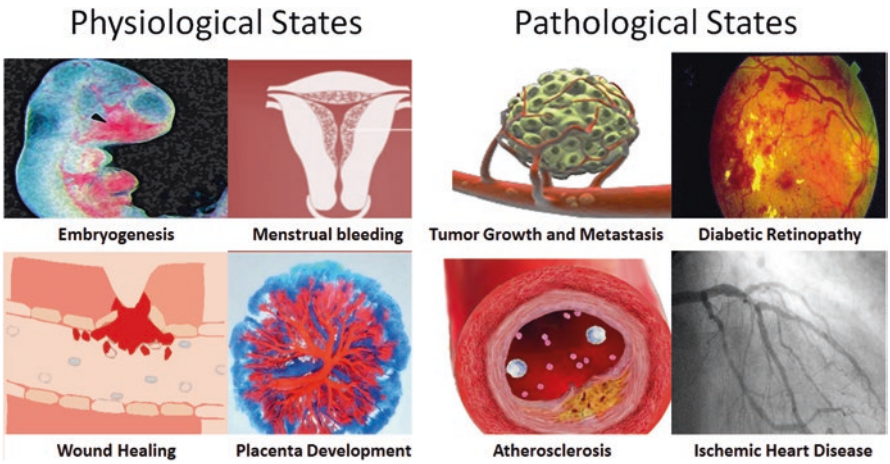

Fig. 16.1 Role of angiogenesis in health and diseases

formation of new blood vessels and it plays an important role in many physiological and pathological processes in human body. Angiogenesis plays an important role in many physiological processes in adults (Fig. 16.1). This includes wound healing, tissue repair, exercise-induced skeletal and cardiac muscle hypertrophy, normal menstrual cycle in females, embryogenesis and organogenesis in pregnancy.

Physiological angiogenesis is highly regulated with checks and balances at many steps. Dysregulation of this process, also known as pathological angiogenesis can lead to many disease states such as dysfunctional uterine bleeding, benign conditions such as vascular malformations, hemangiomas/hemartomas to malignancies such as colon and lung cancers. Exaggerated angiogenesis is the key in the pathogenesis of many other diseases like proliferative retinopathy, psoriasis, rheumatoid arthritis and other chronic inflammatory diseases. On the other hand, insufficient vessel growth results in disease processes like ischemic heart disease, cerebrovascular disease, peripheral arterial disease, delayed wound healing and scleroderma. Hence, understanding mechanisms and regulation of angiogenesis is necessary for understanding the pathophysiology of disease processes and their management.

2 Mechanism of New Vessel Formation

Angiogenesis is a process involving a complex interplay of various growth signals and their cellular receptors (Fig. 16.2). Formation of new blood vessels depends on three major factors: angiogenesis switch, expression of receptors, and second messenger systems in the target cells and the matrix environment. The process begins when the delicate balance between angiogenesis and anti-angiogenesis factors shift towards angiogenesis (Fig. 16.3). This is generally characterized by up- regulation of growth factors and/or their receptors in the endothelial cells.

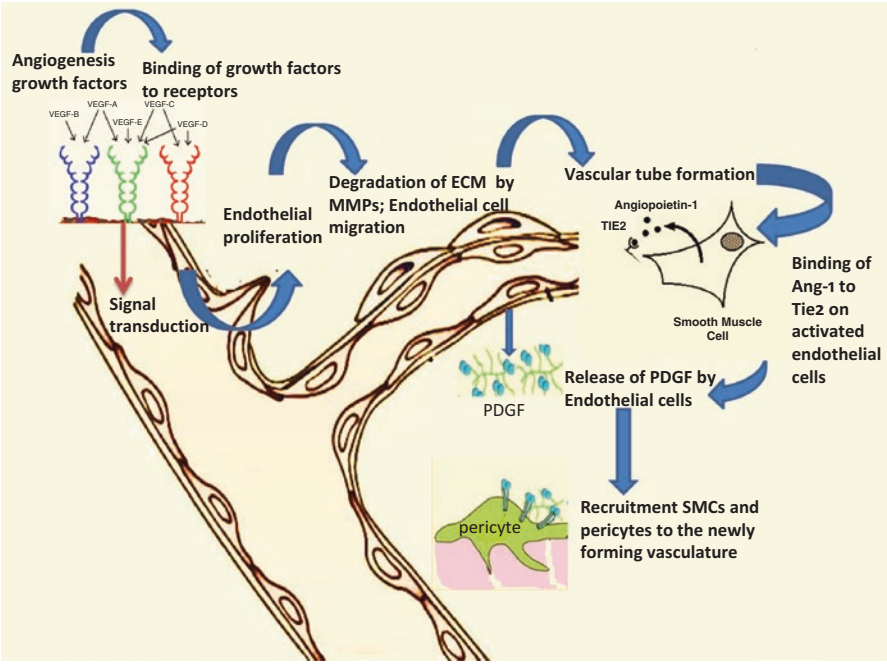

Fig. 16.2 Summary of mechanisms of angiogenesis. *ECM* Extracellular Matrix, *MMPs* Matrix Metalloproteinases, *VEGF* Vascular Endothelial Growth Factor, *PDGF* Platelet Derived Growth Factor, *Ang-1* Angiopoietin-1, *Tie-2* Tyrosine kinase with immunoglobulin-like and EGF-like domains 2

Our understanding of blood circulation in human body has been constantly evolving since first described by William Harvey in sixteenth century. The blood flows in conduits was first described by the studies of Malphigi when he described the capillary circulation in the lung of a frog and later von Reckingausen postulated that these vessels are not just conduits but also lined by cells [1]. The endothelial cell surface in an adult human is composed of approximately 1–6 × 10^{13} cells and covers a surface area of approximately 1–7 m^2 [2, 3]. Endothelial cells are one of the most quiescent and genetically stable cells of the body with a very prolonged turnover time of about 100 days. When these quiescent cells detect pro-angiogenic signal/s, their cell-cell junctional contacts develop gaps, proteases are activated, basement membrane is degraded, and the cells acquire motile behavior; this initiates new blood vessel sprouting. There are several pro-angiogenic factors such as vascular endothelial growth factor A (VEGF-A) which is secreted by tissues in response to inflammation, hypoxia or ischemia [4]. Platelet derived growth factor (PDGF), fibroblast growth factor (FGF), interleukins and other pro-angiogenic factors act synergistically with VEGF.

VEGF binds to three structurally related receptor tyrosine kinases (RTKs) named VEGFR-1, VEGFR-2 and VEGFR-3. VEGFRs have an extracellular ligand binding

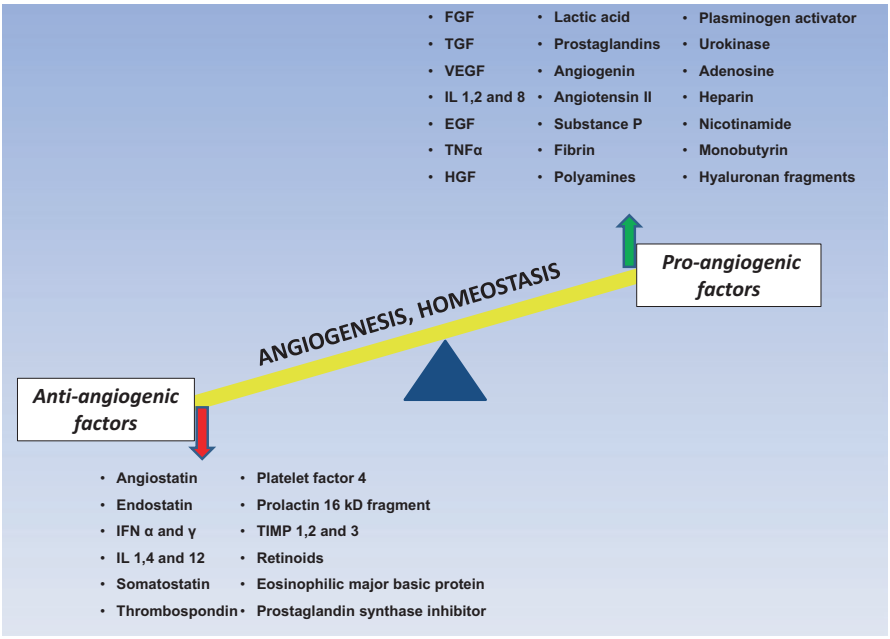

Fig. 16.3 Angiogenesis is regulated by balance in pro-angiogenic factors and ant-angiogenic factors. Increase in pro-angiogenic factors stimulates angiogenesis while increase in anti-angiogenic factors leads to arrest of angiogenesis

portion consisting of 7 immunoglobulin-like domains, a transmembrane region, a juxta-membrane domain, an intracellular split tyrosine kinase domain and a C-terminal tail [5]. VEGFR-3 is a regulator of lymphoendothelial function. VEGF-A, the principle regulator of angiogenesis, binds to VEGFR-1 and VEGFR-2. VEGFR-1 is expressed in both endothelial and non-endothelial cells. It plays important role during vasculogenesis in embryo and it also has a role in inflammation-induced angiogenesis by recruiting inflammatory cells followed by deposition of angiogenic growth factors. VEGFR-2 is expressed only on endothelial cells and regulates endothelial proliferation, migration and formation of the vascular tubes [5]. Integrins mediate cell matrix adhesion by binding to extracellular components. VEGF induces complex formation between integrin αVβ3 and VEGFR-2 which is required for angiogenesis [5].

Proteinases, like matrix metalloproteinases (MMPs), chymase, and heparanase, degrade extracellular matrix, liberate growth factors from matrix. Proliferating solid cords of endothelial cells reach at distant sites once the matrix has been disrupted. PDGF is a chemoattractant for smooth muscle cells. Once recruited angiopoietin 1 and transforming growth factor β1 (TGFβ1) stabilize the newly formed vessels by increasing interactions between endothelial cells and peri-endothelial vascular smooth muscle cells and pericytes [6]. These newly assembled endothelial cells

develop lumen, and anastomose with other buds or capillaries. They persist as long as they are required or differentiate into mature venules and arterioles as per metabolic requirements of local tissue. A complex interaction of proliferation, migration and interaction of endothelial cells, perivascular smooth muscle cells and pericytes is essential for angiogenesis [6]. Angiogenesis is regulated not only by activating signals but also by inhibitory signals, such as thrombospondin-1, interferon-α, platelet factor-4, and angiostatin (Fig. 16.3).

3 Angiogenesis in Atherosclerosis: A Historical Perspective

The association of neovascularization and atherosclerosis was first noted by Koester over a century ago [7]. Paterson postulated in 1930s that rich vascular channels namely vasa vasorum (VV) surrounding and penetrating atherosclerotic lesions were the source of the plaque hemorrhages [8, 9]. Later, Barger et al. [10] hypothesized that adventitial VV of coronary arteries played important role in atherosclerotic plaque pathophysiology and may play a role in supplying oxygen and nutrients to the atherosclerotic lesions. Moreno et al. [11] emphasized a correlation between neovascularization in arterial wall and atherosclerosis progression in coronary arteries in human autopsy studies. They found that microvessel density was higher in lipid-rich and ruptured plaques as well as in lesions with intraplaque hemorrhage and a thin fibrous cap. This was further substantiated by Virmani et al. [12] who found that the number of VV was increased 2-fold in vulnerable plaques and up to 4-fold in plaque ruptures compared with stable plaques with severe luminal narrowing. Other characteristic features found were arborization of VV around the necrotic core, the formation of immature vessels and loss of basement membrane around functional capillaries [12]. The deficiency of PDGF receptors may contribute to some of these findings [12]. Of note, Barger and Beeuwkes [13] suggested in 1990 that the atherosclerotic plaque with neovascularization is fragile, prone to rupture and therefore leads to plaque destabilization and clinical manifestation of the disease.

More convincing evidence for the concept that angiogenesis is not just a bystander; rather a key player in atherosclerosis progression came from mouse models of atherosclerosis. Moulton et al. [14, 15] found that angiogenesis inhibitors endostatin, TNP-470 and angiostatin reduced plaque area in $ApoE^{-/-}$ mice significantly. In 2001, Celletti et al. [16] reported that intraperitoneal administration of recombinant human VEGF protein enhanced atherosclerotic plaque progression in $ApoE^{-/-}ApoB100^{-/-}$ mice. Similarly, Khurana et al. [17] in two different animal models showed that pro-angiogenic molecules such as VEGF or a proline/arginine-rich peptide (PR39) can promote the growth of the intimal lesions. Additional evidence that VEGF has a pro-inflammatory and pro-angiogenic cytokine function in plaque development was provided by Zhao et al. [18].

4 Determinants of Atherosclerotic Plaque Angiogenesis: Role of Hypoxia, Oxidative Stress and Endothelial Dysfunction

The process of plaque angiogenesis in atherosclerosis is regulated by multiple signaling such as hypoxia, oxidative stress (release of reactive oxygen species or ROS) and local inflammation. Hypoxia within the thickened atherosclerotic plaque is a major driving force for growth of new blood vessels in and around the plaque [19]. NADPH oxidase-mediated ROS production plays an important role in angiogenesis which is an adaptive response to hypoxia. Hypoxia leads to an increase in hypoxia inducible factor (HIF)-1 and -2 which in turn upregulates the expression of VEGF-A, PDGF and other growth factors. ROS also upregulates the VEGF expression at both the mRNA and protein levels [20–22].

NADPH oxidases (Nox1, Nox2 and Nox4) are the major source of ROS in the cardiovascular system. Nox1 in smooth muscle cells and Nox4 in endothelial cells are upregulated by hypoxia. Hypoxia-induced upregulation of Nox1 increases HIF-1 and Nox4 stabilizes HIF-2α [20]. Additionally, Nox1 upregulates VEGF and increases the production of MMPs. Nox4 increases the expression of VEGF and PDGF in endothelial cells. PDGF in turn upregulates Nox1 in smooth muscle cells leading to recruitment of smooth muscle cells and pericytes around newly formed blood vessels. Nox2 in monocytes and endothelial cells also plays an important role in angiogenesis [20]. It has also been seen that Nox2 and Nox4 are activated or upregulated in endothelial cells during diabetes and dyslipidemia and their activation may be the basis of endothelial dysfunction in these conditions [20]. NADPH oxidase-induced angiogenesis may play a tissue protective role in chronic ischemia; however, time for angiogenic response in the setting of acute ischemia is limited, and acute burst of ROS during the early stages of reperfusion may be detrimental to the tissues. Indeed, in experimental studies Nox1 and Nox2 deficient mice had decreased tissue injury after ischemic stroke [23, 24].

Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), a receptor for oxidized low density lipoprotein (ox-LDL), is highly expressed in endothelial cells. Endothelial cells when exposed to ox-LDL show expression of LOX-1 expression, up-regulation of adhesion molecules, pro-inflammatory proteins, tissue factor and tissue remodeling proteins like metalloproteinases and collagens. Many of the MMPs are stimulators of angiogenesis [21, 22]. Small concentrations of ox-LDL (<5 μg/ml) promote capillary tube formation by transcription of LOX-1, which activates NADPH oxidase/mitogen-activated protein kinases/NF-κB pathway (Fig. 16.3) [25]. Ox-LDL has been reported to markedly increase the expression of VEGF and activate a peroxisome proliferator activated receptor (PPAR)-gamma; both are attenuated by anti-LOX-1 antibody [26].

Hu et al. demonstrated that capillary sprouting induced by angiotensin II is also related to the elevated expression of VEGF, and anti–LOX-1 antibody markedly inhibits angiotensin II-induced VEGF expression [27]. Moreover, angiotensin II–induced capillary sprouting from aortic rings from LOX-1 null mice is minimal compared to the sprouting from aortic rings of wild-type mice [27]. They also suggested that angiotensin II, which is over-expressed in atherosclerotic region, plays a major

role in angiogenesis in the rapidly growing plaque by LOX-1 up-regulation. These observations collectively suggest that LOX-1 may turn out to be an important regulator of angiogenesis in atherosclerosis. Hypoxia, a primary driver of angiogenesis in atherogenesis is also found to induce angiogenesis through up-regulation of LOX-1 and the LOX-1-mediated p47(phox) subunit of NADPH oxidases [28]. The factors that regulate angiogenesis are shown in a simple form in Fig. 16.3.

5 Plaque Neovascularization and Its Consequences

Recent studies suggest that plaque angiogenesis makes the plaque vulnerable by promoting plaque inflammation, destabilization, progression and rupture [12]. Histological characteristic of plaque angiogenesis reveal that these microvessels often lack pericytes and smooth muscle cells, and have poorly formed endothelial cell junctions [12]. Therefore, the neovascular network in coronary atherosclerotic plaques is more fragile and prone to rupture. Rupture of plaque capillaries can trigger intraplaque hemorrhage, leading to plaque destabilization and its consequences. Close observation of such sprouting vessels in the atherosclerotic plaque reveals their clustering in the shoulder regions, and to some extent at the plaque base (the intima–media border) and in the fibrous cap of the atherosclerotic plaque. These are areas that are more prone to fissuring and rupture. Such sprouting vessels supply oxygen and nutrients to the core of the atherosclerotic plaques; thereby increasing its size [10, 12]. Finally, these microvessels are generally surrounded by macrophages (foam cells), mast cells, T cells, red blood cells, albumin and/or lipoproteins. All this suggests that these vessels actually bring in more inflammatory cells to the atherosclerotic plaque, and thus may play a role in the perpetuation of atherosclerosis.

Similar concept of plaque neovascularization leading to plaque instability holds true for atherosclerotic disease elsewhere. For example, recent studies of carotid artery plaque emphasize the significance of intraplaque hemorrhage in causation of symptomatic cerebrovascular ischemia [29, 30]. Horie et al. [30] showed that in patients with moderate to severe carotid atherosclerosis the patients with early appearance of neovascularization exhibited larger infarctions and had more severe intra plaque hemorrhages. Relative to the origin of the hemorrhage, pathologic studies suggest that angiogenesis is significantly increased in carotid artery plaques from symptomatic patients when compared with matched individuals with asymptomatic plaques. It has been shown that plaque angiogenesis and angiogenetic factors, such as VEGF, are critical in the progression of atherosclerotic carotid plaque and intraplaque hemorrhage [31].

Another example of pathological angiogenesis in human body leading to leaky vessels and hemorrhages is diabetic retinopathy. In diabetic retinopathy, there is enhanced secretion of VEGF in the vitreous fluid of the eye as well as enhanced expression of VEGFR-1 and VEGFR-2 receptors [32, 33]. This leads to formation of new capillaries, which are leaky, have immature extracellular matrix and have greater tendency to rupture leading to retinal hemorrhage and subsequently, blindness. Evidence from a variety of angiogenesis-related diseases supports the fact that neovascularization leads to the formation of frail and leaky blood vessels.

6 The Angiogenesis Paradox: To Be or Not to Be?

Angiogenesis research has gained momentum from different perspectives. On one hand, data from histopathology of atherosclerotic plaques suggests that angiogenesis has an important role in the pathogenesis of atherosclerotic disease and its clinical sequlae. On the other hand, drugs that inhibit angiogenesis paradoxically increase the incidence of thromboembolic events. In normal tissue, there is a system of checks and balances between pro-angiogenic factors like VEGF, bFGF and IL-8, and anti-angiogenic molecules like thrombospondin-1 and -2, endostatin and angiostatin [6]. If this balance tips in favor of pro-angiogenic molecules, abnormal angiogenesis ensues contributing to plaque destabilization and rupture. Hence, at first glance, anti-angiogenic agents might be of benefit to prevent neovascularization and normalize the leaky vasculature that promotes plaque destabilization and hemorrhage. A variety of agents have been developed in the last few years that can inhibit the process of angiogenesis at various stages. Action of some of these agents vis-a-vis angiogenesis are shown in Fig. 16.3.

Our understanding of these agents and their effects on cardiovascular system mainly comes from their use in oncology. Some of these agents have been approved for management of various cancers for over a decade now, and bevacizumab is a prototype example of these agents. Bevacizumab binds to VEGF and blocks its activity. Pooled analysis of five randomized controlled trials with a total of 1745 patients with various metastatic carcinomas treated with either conventional chemotherapy vs. chemotherapy with bevacizumab demonstrated significantly more thromboembolic events in the latter group (hazard ratio = 2.0, p = .031). Most thromboembolic events were coronary or cerebrovascular. Further, upon multivariate analysis, a history of atherosclerosis was found to be an important risk factor for such adverse events. Similarly increased cardiovascular risk with bevacizumab has been replicated in other studies as well [34, 35]. Of note, thalidomide, and lenalidomide have also been associated with high (8–17%) incidence of thrombotic complication [36, 37].

Recently, it has been suggested that VEGF not only plays a significant role in human disease states as mentioned earlier, but it is of utmost importance in maintaining vascular homeostasis [38]. Endothelial-derived VEGF constitutes only a small proportion of total body VEGF and does not contribute significantly to the overall angiogenic response. Nonetheless, there is compelling evidence that such autocrine VEGF signaling is required for endothelial cell survival, and removal of endothelium-derived VEGF in vivo results in systemic endothelial apoptosis [39, 40]. In murine models [38], removal of endothelium-derived VEGF resulted in devastating systemic vascular pathologies in the form of multiple hemorrhagic and thrombotic events, and sudden deaths. Interestingly, in mice with endothelium-specific loss of VEGF, systemic vascular catastrophe could not be compensated for by VEGF secreted from adjacent types or circulating VEGF [38].

A role for VEGF in adaptive cardiac growth is also being recognized. VEGF is upregulated in myocardium in pathological conditions such as myocardial ischemia, and hemodynamic overload. Data from murine models of pressure overloaded

heart suggests that VEGF derived angiogenic response is essential for coronary vascular network growth to provide compensatory cardiac hypertrophy [40]. A reduction in available VEGF contributes to the rapid progression from compensatory cardiac hypertrophy to its failure in pressure overloaded hearts. Further, treatment with VEGF receptor decoy has been shown to attenuate cardiac hypertrophy induced by pressure overload. Hence, stress-induced heart growth may depend on the status of the vascular bed in a manner that is similar to tumor growth [40]. In summary, VEGF signaling seems essential for adaptive cardiac growth, and disruption of such signaling may impair cardiac function under stress.

7 Understanding Different Angiogenesis Pathways: Collateral Arteriogenesis vis-à-vis Plaque Angiogenesis

As opposed to plaque angiogenesis, collateral arteriogenesis is characterized by the enlargement of arteriolar anastomoses to collateral vessels through growth and proliferation results in formation of new arteries possessing fully developed tunica media. It is not a merely a passive dilation of collateral, but an active proliferation and remodeling of the arteriole resulting in formation of large vessels that can take over the role of an artery when occluded [41, 42]. While plaque neovascularization results in the formation of the immature, pericyte-lacking capillaries, collateral arteriogenesis involves endothelial tubulogenesis, in association with pericyte recruitment and smooth muscle cell proliferation and envelopment resulting in mature vessel formation. Hence, arteriogenesis is the preferred type of neovascularization for purposes of restoring myocardial perfusion. The pre-existing collateral arterioles can give rise to conductance vessels with up to 20-fold increase in their diameter. In some patients, occluded coronary arteries can be completely compensated for by collateral arteries [41, 42].

While the major stimulus for plaque angiogenesis is hypoxia, arteriogenesis involves interplay between shear stress and circulating monocytes. In response to ischemia, shear force and blood flow increases through these collaterals. This leads to the activation of normally quiescent endothelial cells in the collateral arterioles. The activated endothelium releases monocyte chemoattractant protein-1; simultaneously there is upregulation of receptors for docking the monocytes [41, 42]. The adherent monocytes, in turn, release a variety of growth factors and cytokines, such as monocyte chemoattractant protein-1, granulocyte-monocyte colony-stimulating factor, transforming growth factor-β1 that creates the environment for collateral artery growth. Among these factors, PDGF and bFGF are directly mitotic for endothelial- and smooth muscle cells [41, 42]. In plaque angiogenesis, VEGF is the primary growth factor while compensatory arteriogenesis is mediated by an array of growth factors, among which FGF and PDGF appear to be of key significance [43]. The major differences between these two processes have been summarized in Table 16.1.

Table 16.1 Key differences between two major processes operant in adult neovascularization: angiogenesis and collateral arteriogenesis

Characteristics	Arteriogenesis	Angiogenesis
Stimulus	Shear stress	Ischemia
Key growth factor	VEGF	FGF, PDGF, MCP-1
Substrate	Pre-existing arterioles	Pre-existing capillaries
Result	New arteries	Increased capillaries
Time frame	Days to weeks	Days
Max increment in blood flow	10–20 fold	1.5–1.7 fold
Ability to compensate for an occluded artery	Yes	No

8 Specificity and Heterogeneity in the Expression of Angiogenesis

Human vasculature comprises of a variety of endothelial cells forming a pattern well-suited to the functional requirement of the specific organ. Although endothelial cells have a number of characteristics in common, they exhibit great variation phenotypically, genetically and in functionality. For instance, liver sinusoids are lined by discontinuous endothelial cells that permit greater movement of materials between intercellular gaps, kidneys are lined by fenestrated endothelial cells that facilitate selective permeability required for filtration, absorption and secretion, central nervous system is lined by continuous thin endothelial cells that form an effective blood-brain barrier [44]. Endothelial cells also differ in their surface phenotype and protein expression [45], antigen expression [46] and response to growth factors [47].

It is a common clinical observation that when saphenous vein is grafted to coronary artery, it acquires artery-like properties and exhibits an increased tendency to develop atherosclerosis. Studies suggest that endothelial cells in regions of disturbed flow acquires a pro-inflammatory phenotype in response to systemic insult [48, 49]. Hence, endothelial cells are not only heterogenous, but also very dynamic. These differences have been attributed mainly to genetic control (nature) and environmental influences under which these cells thrive (nurture) [50, 51]. Understanding this heterogeneity in endothelial cell biology and the factors governing such heterogeneity is essential as microvascular involvement in various pathologies is strongly governed by the behavior of these cells.

Besides the differences in endothelial cell biology at different sites, there is also a significant heterogeneity in angiogenic response induced by endothelial growth factors in different sites. For example, a recent study by Pettersson et al. involving adenoviral vector delivery of VEGF to the heart, skin, fat and skeletal muscle showed that there was a common initial angiogenic response in the form of pericyte-poor leaky microvessels among all these tissues followed by tissue specific progression of angiogenesis [52]. In muscle (cardiac and skeletal), smaller caliber or disorganized tangles of daughter vessels were formed. Although similar response occurred in skin and fat with a greater intensity, some of the resultant

vessels acquired muscular coat and progressed to form conduits that closely resembled medium-sized arteries and veins that persisted indefinitely. Interestingly, these microvessels were similar to the collateral vessels formed by the process of arteriogenesis in ischemic hearts [52]. These findings led the authors to postulate that the clinical benefits in ischemic limb salvage via VEGF therapy in animal models might have resulted from spillage of injected cytokine or its encoding DNA into perimuscle fat tissue [52].

Finally, data from experimental animal models suggest that heterogeneity in angiogenic response and differential sensitivity to angiogenesis inhibitors is largely governed by genetic factors [53–55]. Rohan et al. showed in the corneal micropocket assay of various inbred mice that there exists nearly 10-fold range of response angiogenesis by growth factor among different strains [56]. Further, differential sensitivity to angiogenesis inhibitors was seen between strains. Although, at this time there is paucity of information on genetic control of angiogenic heterogeneity, it is possible that a similar heterogeneity as described in inbred mouse strains by Rohan et al. [56] is seen in human beings also. This might explain the individual differences in angiogenic potential and the different rate of progression of angiogenesis related diseases in different individuals.

9 Therapeutic Angiogenesis: Promises and Problems

Ischemic heart disease continues to exert a tremendous burden on healthcare system worldwide. It is the leading cause of morbidity and mortality. As per the latest Heart and Stroke statistics by American Heart Association the cardiovascular diseases still accounted for 31% of all-cause mortality in United States or approximately 1 of every 3 deaths in the United States [57]. While most of them undergo surgical or catheter-based revascularization, many of them are not optimal candidates for any form of revascularization procedures for various reasons. Similarly, cerebrovascular diseases resulting in transient ischemic attacks and strokes leads to a major socioeconomic burden on the society [57]. In this regard, therapeutic angiogenesis has attracted interest as an alternative treatment for patients with ischemic heart disease or cerebrovascular disease.

Therapeutic angiogenesis vis-a-vis ischemic heart disease refers to induction of new blood vessels that can effectively supply blood to the area of myocardium jeopardized by occluded native coronary arteries. Results of numerous preclinical studies have provided evidence that angiogenic growth factors can promote collateral artery development and hence reduce ischemia in animal models of peripheral and coronary circulation [58]. Yet, clinical trials on therapeutic angiogenesis have not been as impressive [59].

The first clinical trial (VIVA trial) by Henry et al. [60] used a combination of intracoronary and intravenous Recombinant human vascular endothelial growth factor protein (rhVEGF) infusions. rhVEGF seems to be safe and well tolerated. However, at 60 days, there were no significant differences in exercise tolerance or

angina class between the treated and control groups. In the same trial it was found that high-dose rhVEGF resulted in significant improvement in angina class ($p = 0.05$) and but nonsignificant trends in exercise treadmill test time ($p = 0.15$) and angina frequency [60].

Similarly, FIRST (The FGF-2 Initiating Revascularization Support Trial) trial was a multicenter double blinded placebo controlled trial that randomized patients to receive a single intracoronary infusion of recombinant FGF2 (rFGF2). Unfortunately, rFGF2 did not show any improvement in exercise tolerance or myocardial perfusion, although there was some symptomatic improvements on day 90 after rFGF2 administration, but the improvement was not observed on day 180 [61]. Similarly, several studies with granulocyte colony stimulating factor (G-CSF) have shown that though it is safe and feasible to administer but it does not improve cardiovascular outcomes [62, 63]. Although preclinical studies of VEGF and FGF gene delivery using plasmid and adenoviral vectors have shown impressive improvements in heart function and perfusion in different animal models of myocardial ischemia, results of clinical studies have not yielded impressive results [64, 65].

The key limitations of VEGF/FGF protein therapy are the relatively short half-life and method of delivery of these growth factors which may have prevented exposure of the target tissue to VEGF/FGF at concentrations and for times sufficient to promote a viable and sustained improvement in collateral blood flow. For example, only 3–5% of the dose is typically retained in the myocardium 150 min after intracoronary injection of basic FGF [66]. However, after pericardial administration 19% of FGF was present at 150 min. The optimal preparation and delivery strategy for therapeutic neovascularization is the subject of ongoing clinical investigation.

Another major factor which might explain the failure of these trials is the use of a single growth factor in all the studies. Major trials on therapeutic angiogenesis have hovered around a single growth factor. Unlike angiogenesis, the process of collateral arteriogenesis involves a cocktail of chemokines, growth factors and proteases. It is difficult to envision that the complex process of endothelial tubulogenesis, combined with pericyte recruitment and smooth muscle cell proliferation, can be achieved with a single agent given as a single dose. Combination therapy for collateral arteriogenesis has not been studied at this time but is an interesting avenue for future molecular cardiologists!

10 Summary

Angiogenesis is operative in human beings right from the embryonic stage and involves a complex interplay of various cells, growth factors, cytokines and the environment in which such process takes place. While this process plays a key role in many physiological processes and in the development of cardiovascular system, inadvertent angiogenesis is also a major pathologic factor in the development of many disease states including tumors and atherosclerosis.

Use of therapeutic angiogenesis has been widely regarded as an attractive approach in treatment of ischemic heart disease. On the other hand, there is growing evidence that neovascularization contributes to the progression of atherosclerotic lesions, and that it may play key role in intraplaque hemorrhage, plaque destabilization and rupture. Despite the failure of early clinical trials with angiogenic agents, bench and bedside research interest in using angiogenic stimuli has continued to bring about more insight into the mechanisms of this phenomenon. Understanding the basis of only limited success in early trials of angiogenic therapy may help in the development of novel and specific therapies. Again combination of various growth factors and gene therapy are the future avenues for research in this field.

Conflict of Interest None

Disclosures None

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Chapter 17
microRNAs, Angiogenesis and Atherosclerosis

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Abstract MicroRNAs are short non-coding regulatory RNA molecules that control post-transcriptional gene expression and are involved in several physiological and pathological processes in different species, as well-conserved characters. Their dysregulation has been described in various cardiovascular diseases, including atherosclerosis, coronary heart disease and acute myocardial infarction. A possible role as novel biomarkers has been proposed for some circulating microRNAs with potential prognostic implications. Though still in their infancy, microRNA-based therapies have been enthusiastically welcomed as innovative treatments. This chapter briefly outlines the role of microRNAs in the diagnosis and prognosis of atherosclerosis and coronary heart disease as well as their therapeutic use for patients afflicted with these diseases.

Keywords microRNA • Atherosclerosis • Coronary artery disease • Acute myocardial infarction • Biomarkers

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Abbreviations

ACS	Acute coronary syndrome
AMI	Acute myocardial infarction
apo	Apolipoprotein
CAD	Coronary artery disease
CV	Cardiovascular
DGCR8	Di George syndrome critical region 8
DNA	Deoxyribonucleic acid
EC	Endothelial cell
ECM	Extracellular matrix
EPC	Endothelial progenitor cell
HDL	High-density lipoprotein
hs-cTNT	High-sensitivity cardiac troponin T
HUVEC	Human umbilical vein endothelial cells
KO	Knockout
LDL	Low-density lipoprotein
LNA	Locked nucleic acid
MAP kinase	Mitogen-activated protein kinase
miRNA	microRNA
mRNA	messenger RNA
qRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
SOCS1	Suppressor of cytokine signaling 1
STAT3	Signal transducer and activator of transcription 3
TF	Tissue factor
TGF-β	Transforming growth factor-β
TRBP	TAR RNA-binding protein
VEGF	Vascular endothelial growth factor
VLDL	Very low-density lipoprotein
VSMC	Vascular smooth muscle cells
UTR	Untranslated region

1 Introduction

The proportion of non-coding to coding genome increases with developmental complexity. Therefore, in mammals the vast majority of the genome is never translated into proteins but is extensively transcribed, generating ribonucleic acid (RNA). In the past RNA was regarded as an intermediate product formed in the pathway from deoxyribonucleic acid (DNA) to proteins (messenger RNA, mRNA), or as a constitutive element (ribosomal RNA); however, the discovery of its regulatory tasks changed the way RNA would be regarded forever. Both short (<200 nucleotides, nt) and long

(>200 nts) non-coding RNAs are now recognized as playing important roles in gene regulation and function [1]. MicroRNAs (miRNAs), short single-stranded RNAs (18–24 nts) that act as post-transcriptional regulators of mRNA decay or inhibition, represent the vast majority of short non-coding RNAs. A single miRNA can have hundreds of mRNA targets that are often functionally related. Different miRNAs can also regulate a single mRNA, creating a dense regulatory network for approximately two thirds of all genes. [2]. There are specific software programs that predict which mRNAs are the targets of a specific miRNA (TargetScan, http://www.targetscan.org; miRanda, http://www.microRNA.org; TarBase (http://www.microrna.gr/tarbase).

The history of microRNAs began in 1993, when Lee et al. [3] described the first miRNA -lin 4- in the nematode *C. elegans* that acts by negatively regulating the level of lin-14 protein. The authors demonstrated that lin-4 does not encode for a protein, but it contains sequences complementary to a repeated sequence element in the 3'-untranslated region (UTR) of lin-14 mRNA, regulating it via an antisense RNA-RNA interaction. Seven years later, the second miRNA -let-7- involved in *C. elegans* larval development, was discovered [4]. Since then, thousands of miRNAs have been described in animals, plants, and viruses and have been catalogued in the online database miRBase [5]. Moreover, their role in physiological and pathological processes have been progressively elucidated.

2 microRNA Biogenesis

miRNAs are primarily transcribed in the nucleus by RNA polymerase II as primary miRNAs (pri-miRNAs) with hundreds or thousands of nucleotides folded in a canonical hairpin structure, including a 5' cap and a 3' poly-A tail [6]. The pri-miRNA is then cleaved by the ribonuclease III Drosha/DGCR8 (Di George Syndrome Critical Region Gene 8) microprocessor complex into a 70 nts hairpin precursor miRNA (pre-miRNA), which is then transported to the cytoplasm by Exportin-5. The pre-miRNA is then processed by another ribonuclease III, Dicer and its cofactor TRBP (TAR RNA-binding protein), producing a short, double-stranded microRNA duplex, formed by the miRNA guide and the passenger strands. The Argonaute protein family and in particular Ago 2, undergoes conformational changes to allow the binding of the miRNA-miRNA* duplex [7]. At this point, miRNA strand selection (the -3p or -5p strand) depends on several factors, including thermodynamic features, as the strand with the weakest binding at its 5'-end is more likely to be incorporated into the RNA-Induced Silencing Complex (RISC) to target mRNA expression. However, both strands are potentially functional [8].

Other non-canonical miRNA biogenesis pathways have also been described [9]. miRNAs can: (1) act intracellularly; (2) transfer through gap junctions in proximal cell-to-cell communication; or (3) be released into the bloodstream, packaged in microvesicles, exosomes, microparticles, apoptotic bodies or in association with RNA-binding proteins (as Ago 2) or lipoprotein (as High-Density Lipoprotein, HDL). Figure 17.1 summarizes miRNA biogenesis.

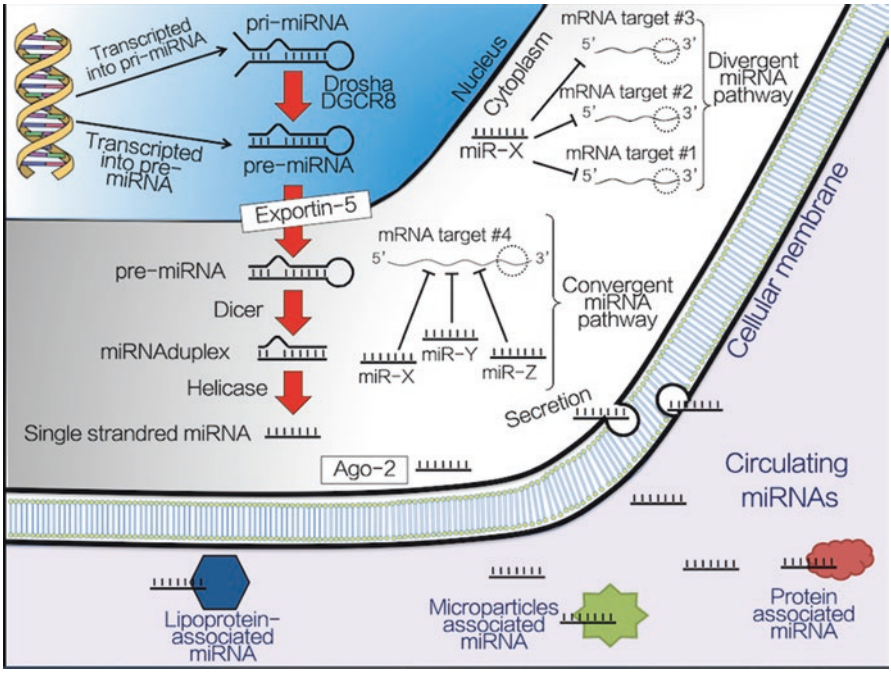

Fig. 17.1 microRNA biogenesis. In the canonical microRNA biogenesis pathway, the DNA is transcribed into pri-microRNA in the nucleus then the Drosha/DGCR8 complex cleaves it to produce pre-microRNA, which is transported in the cytoplasm by Exportin-5, where another RNase III, Dicer cleaves it into a microRNA duplex, to finally obtain a single stranded microRNA. The mature microRNA can act intracellularly or in a cell-to-cell interaction or can be secreted packed in microvesicles, exosome, microparticles or associated to lipoproteins. (Adapted from Cavarretta and Frati [10])

3 Atherosclerosis-Associated microRNA

Cardiovascular disease is the leading cause of death worldwide, and atherosclerosis is recognized as a major contributor. Several miRNAs are associated to the progression and clinical complication of atherosclerosis. Most studies have been performed in small animal models of atherosclerosis using miRNA transgenic mice, apolipoprotein E–deficient mice (Apo E−/−), and low-density lipoprotein receptor knockout mice (LDLR−/−). A smaller number of studies have also been conducted on human atherosclerotic plaques.

3.1 miR-126

The endothelial cells (EC)-enriched miR-126 is a pivotal regulator of vascular homeostasis and angiogenesis with vasculoprotective and atheroprotective features. Both mature strands, miR-126-3p and miR-126-5p, are abundant in EC. miR-126

contributes to vascular homeostasis by inhibiting angiogenesis and maintaining the quiescent EC phenotype associated with increased vascular integrity, inhibiting EC proliferation and motility. Platelets are also an important source of circulating miR-126, thus controlling vascular homeostasis and inflammation [11]. miR-126 inhibits vascular cell adhesion molecule 1 (VCAM-1), which has an important role in the development of atherosclerosis. In fact, decreasing endogenous miR-126 levels increases leukocyte adherence to ECs [12]. Moreover, in vascular injury and hypoxic ischemia, up-regulation of miR-126 demonstrates a proangiogenic activity as it stimulates recruitment and migration of endothelial progenitor cells (EPCs) to injured sites [13]. This activity is reduced in diabetic patients, where miR-126 expression has been found down-regulated in EPCs with enhanced Spred-1 expression, a negative regulator of mitogen-activated protein kinase (MAP kinase) signaling [14]. In particular, the link between miR-126 and atherosclerosis has been demonstrated in miR-126$^{-/-}$ mice, where miR-126-5p is the predominant atheroprotective regulator, targeting Delta-like 1 homolog (Dlk1), which controls endothelial cell proliferation and lesion formation [15]. In ECs, DLK1 acts as a negative regulator of angiogenesis by inhibiting NOTCH signaling [16]. The treatment with anti-miR-126-5p has been shown to cause an increase in the atherosclerotic area and impaired EC recovery and proliferation, though treatment with anti-miR-126-3p has not [15]. In diabetic patients, low miR-126 levels are associated with markedly increased tissue factor (TF) protein and TF-mediated thrombogenicity. Furthermore, the reduction of miR-126 expression is accompanied by increased vascular inflammation, as evident from the heightened levels of vascular adhesion molecule-1 and fibrinogen, as well as increased leukocyte counts [17]. miR-126 expression responds to shear stress and mechanical forces in a flow-dependent manner so that it has been called a "mechanosensitive athero-miR," similarly to the vascular-related miRs: miR-17–92, miR-21, miR-663, miR-92a, miR-143/145, miR-101, miR-126, miR-712, miR-205, and miR-155 [18]. Briefly, ECs at athero-protected regions show the capacity to proliferate and regenerate (proliferative reserve), which is amplified by laminar flow and high shear stress, due to an increased miR-126-5p expression [19]. In athero-sensitive regions, such as arterial bifurcations, ECs are damaged by turbulent flow. This causes an increased regenerative EC proliferation, but with a less significant proliferative reserve, not capable of compensating for the hyperlipidemia-induced suppression of the EC proliferation [20].

3.2 miR-143/145

Both miRs are distinct in sequence, yet, are transcribed together as one primary cluster, which is a master regulator of the differentiation, plasticity and contractile phenotype of vascular smooth muscle cells (VSMC). This promotes the VSMC phenotypic switch from a contractile/nonproliferative to a migrating/proliferative state [21]. The consequent increase in migratory ability generates the basis of neointimal formation and progression in atherosclerosis. In a miR 143/145 knockout (KO)

mouse model, loss of miR-143 and miR-145 expression results in reduced vascular tone and blood pressure control because of an incomplete differentiation of VSMCs [22]. Overexpression of miR-145 in apolipoprotein E knockout mice resulted in an increased fibrous cap area and plaque collagen content with a reduced necrotic core area, and an overall reduction of aortic plaque size, shifting the balance toward plaque stability [23]. Furthermore, in miR-143/145/LDL-R double KO mice, the atherosclerotic plaque size and macrophage infiltration was significantly reduced. This was associated with an improvement in total plasma cholesterol, specifically with the reduction of LDL and very-low-density lipoprotein (VLDL) cholesterol [24]. miR 143/145 has also been demonstrated to control ECs as it is exchanged intercellularly through fine nanotubules from VSMCs to ECs; transforming growth factor-β (TGF-β) and vessel stress trigger this intercellular traffic [25]. Interestingly, the transfer of miR-143/145 via exosomes has also been described to occur in the opposite direction, from ECs to VSMCs as an anti-atherosclerotic signal induced by laminar flow in ECs [26]. Further studies are needed to better clarify this intercellular communication between ECs and VSMCs [27].

3.3 miR-17-92 Cluster

The miR-17-92 cluster is a polycistronic miR gene that encodes for 7 mature miRs that can be grouped into 4 families: miR 17/20, miR-18, miR-19 and miR-92a. This cluster has been studied in oncogenesis as a promoter of angiogenesis [28], but it is also highly expressed in ECs. Vascular Endothelial Growth Factor (VEGF)-mediated up-regulation of the miR-17-92 cluster via ERK/ELK1 activation has recently been proved to be necessary for EC proliferation and angiogenic sprouting in vitro and physiological angiogenesis in vivo [29]. In particular, among the others, miR-92a controls the angiogenesis that targets the mRNAs corresponding to several proangiogenic proteins, including the integrin subunit alpha5 [30]. In mouse models of limb ischemia and myocardial infarction, systemic administration of a miR-92a antagomir led to enhanced blood vessel growth and functional recovery of damaged tissue [30]. The miR-17-92 cluster is also regulated by changes in shear stress, and members of this cluster are mechano-sensitive miRs. Specifically, turbulent flow in cooperation with oxidated LDL triggers the expression of miR-92a in a signal transducer and activator of transcription 3 (STAT3)-dependent manner and miR-92a is up-regulated in atherogenic sites in the aortic arch endothelium of swine [31]. Moreover, in LDL R(−/−) mice in vivo the blockade of miR-92a expression reduced endothelial inflammation and altered the development of atherosclerosis, decreasing plaque size and promoting a more stable lesion phenotype [31]. miR-17-92 cluster is also critical in physiological and ischemia-triggered arteriogenesis, as EC-specific deletion of miR-17-92 resulted in increased arterial vasculature density in ischemic limbs and consequently improved blood flow recovery [32]. In addition, overexpression of miR-19b plays a key role in the attenuation of TNF-α-induced endothelial cell apoptosis via the Apaf1/caspase-dependent pathway, and miR-19b levels in patients with coronary artery disease (CAD) are reduced [33].

3.4 miR-155

MiR-155, an inflammation-related microRNA, is highly expressed in macrophages, lymphocytes, human umbilical vein endothelial cells (HUVECs) and VSMCs. In macrophages, miR-155 expression promotes cardiac inflammation, hypertrophy, and failure in response to pressure overload. miR-155 also influences cardiomyocyte growth through paracrine signaling [34]. In a mouse model of acute viral myocarditis, miR-155 was up-regulated, and its systemic inhibition by a systemically delivered locked nucleic acid-modified antisense oligonucleotides (LNA)-anti-miR led to reduced cardiomyocyte/macrophage infiltration, decreased T lymphocyte activation and reduced myocardial damage [35]. In angiotensin II-stimulated HUVECs, Ets-1, a key endothelial transcription factor for inflammation and tube formation, and its downstream genes, including VCAM1, MCP1 (monocyte chemotactic protein 1) and FLT1, a member of the vascular endothelial growth factor receptor (VEGFR) family, were upregulated. This effect was partially reversed by overexpression of miR-155 [36]. In ischemia-reperfusion injury, miR-155 aggravates the inflammatory response, leukocyte infiltration and tissue damage via modulation of suppressor of cytokine signaling 1 (SOCS-1)-dependent generation of reactive oxygen species [37]. Expression of miR-155 is involved in atherogenic programming of proinflammatory macrophages to sustain and enhance vascular inflammation, because miR-155 is specifically expressed in atherosclerotic plaques, where it represses B cell lymphomaBLC6, a transcription factor that attenuates proinflammatory NF-κB signaling [38]. Systemic delivery of an antagomiR-155, in an Apo E−/− mice, significantly reduced atherosclerotic plaque and decreased lipid-loading in macrophages [39]. Since opposing effects of miR-155 have been reported, as pro- and anti-inflammatory, the question of what specific effects miR-155 has remains unanswered [40]. Last but not least, miR-155 and miR-29b were found significantly up-regulated in ECs of atherosclerotic abdominal aortic aneurysm tissue (AAA) and significantly reduced in plasma of patients affected by AAA [41].

3.5 miR-21

Expression of miR-21 has been extensively demonstrated in ECs and VSMCs where it targets phosphatase and tensin homolog (PTEN) and B cell lymphoma 2 (BCL2), thus promoting VSMC proliferation and inhibiting apoptosis [42]. In addition, miR-21 promotes differentiation of VSMC in response to transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP) [43, 44]. While its role in cardiac fibrosis has been extensively reported [45], its exact relationship with atherosclerosis is still to be defined. In VSMC, miR-21 regulates the contractile phenotype, especially in dedifferentiated VSMCs, with respect to mature differentiated VSMCs. In a humanized rat model of balloon-injured human internal mammary arteries, anti-miR-21 eluting stents were implanted and local miR-21 suppression was

effective in reducing neointimal lesion formation, while systemic administration of anti-miR-21 showed off-target effects, due to the miR-21 ubiquitary expression in the liver, kidney, heart and lung [46]. This promising antiproliferative effect of the anti-miR-21 local delivery needs to be further investigated.

3.6 miR-29 Family

The miR-29 family has been implicated in vascular remodeling as a key regulator of extracellular matrix (ECM) deposition. Up-regulation of miR-29 family members was associated with a profound down-regulation of matrix metalloproteinase and other ECM proteins in abdominal aortic aneurysm. In the Angiotensi-II-induced aneurysm model in ApoE$^{-/-}$ mice, the inhibition of miR-29 by locked nucleic acid-modified antisense oligonucleotides (LNA-29) prevented aortic dilation and increased the protein levels of elastin, a miR-29 target [47]. Moreover, this miR family regulates fibrosis after acute myocardial infarction by targeting mRNA coding for ECM proteins such as collagens, fibrillin and elastin [48], and in hypertrophic cardiomyopathy it is associated with both hypertrophy and fibrosis [49].

4 Circulating microRNAs

Given the fact that the majority of miRNAs are located intracellularly, the discovery of circulating miRNAs in blood in 2008 [50] has created a new perspective on miRNAs: their potential use as novel biomarkers. Since then, miRNAs have been extensively described in all body fluids [51], including serum, plasma, urine, breast milk [52], and saliva [53], and they gained momentum. Circulating microRNAs can be released into the bloodstream by different mechanisms, including cell necrosis, apoptosis and active secretion. Despite the extracellular RNase activity, miRNAa are stable molecules in the circulatory system, as they are packaged in exosomes (50–100 nm), microvesicles (100–1000 nm), microparticles or apoptotic bodies (1–5 μm), and due to their association with proteins and lipoproteins [54]. *De iure* circulating miRNAs are the ideal biomarkers: they have a simple chemical composition, less complexity in comparison with proteins, a small size, and a long half-life within the sample, which allows for a rapid and cost-effective laboratory detection by real-time polymerase chain reaction (qRT-PCR). Moreover, they are obtained non-invasively from body fluids and are resistant to extreme pH changes, prolonged storage at room temperature and repeated cycles of freeze-thaw [55, 56]. Up- or down-regulated levels of circulating miRNAs have been linked to several cardiovascular diseases, including acute myocardial infarction, atherosclerosis, heart failure and cardiomyopathies [57] as they are disease-specifically modulated and consistent among individuals of the same species. Pros and cons of miRNAs clinical use as biomarkers have been extensively reviewed elsewhere [10].

4.1 Circulating microRNAs as Prognostic Biomarkers of Coronary Artery Disease

Several miRNAs such as cardiomyocyte-enriched (miR-133, miR-208a), endothelial cell-enriched (miR-126, miR-17-92a cluster), vascular smooth cell (mir-143/145) and inflammatory cell-enriched (miR-155), platelet-enriched (miR-199a) miRNAs were associated to coronary artery disease (CAD) in stable patients [58], but in particular lipometabolism-related miR-122 and miR-370 increased as the severity of CAD quantified by the Gensini score increased [59]. In a population-based study, Zampetaki et al. identified miR-126 as a possible prognostic marker of incident myocardial infarction [60]. Jansen et al. confirmed this result [61], by demonstrating that microvesicles-associated miR-126 and miR-199a could predict the occurrence of CV events in patients with stable CAD. Using miR-1, miR-126 and miR-485-3p, D'Alessandra et al. [62] were able to identify patients with stable angina in comparison to control subjects, but failed to discriminate between stable and unstable angina, suggesting that these miRs probably reflect atherosclerosis. In addition, the expression of miR-146a was positively correlated with CAD when compared to controls, and it significantly decreased after a 12-month therapy with statin and angiotensin-converting enzyme inhibitor or angiotensin receptor blocker [63]. In a recent study, only transcoronary levels of miRs were associated with coronary atherosclerotic plaque phenotypes evaluated by optical coherence tomography in a cohort of 52 patients undergoing coronary angiography. Levels of miR-29b-3p were found to be inversely associated with plaque fibrosis. In addition, miR-126-3p and miR-126-5p concentrations in the coronary vessels were negatively associated with high plaque load or vulnerable plaques [64]. The lack of correlation with systemic miR concentration does not encourage the use of these miRs as biomarkers for vulnerable plaque. Table 17.1 resumes selected studies on coronary artery disease. Various confounding factors may have influenced the systemic measurement of circulating miRs, as anti-platelet drug or heparin use [67].

4.2 Circulating microRNAs as Prognostic Biomarkers of Acute Myocardial Infarction

The diagnosis of acute myocardial infarction (AMI) relies on the use of cardiac troponin, even if this biomarker is not able to discriminate between AMI caused by atherosclerotic plaque rupture or because of supply/demand mismatch or other conditions presenting with an unspecific troponin elevation, such as non-ischemic heart failure, renal failure or myocarditis. The possible role of miRNAs in the diagnosis of AMI has been extensively evaluated by different authors, who independently reported a possible role for the cardiomyocyte-enriched miRNAs: miR-1, miR-133a, miR-133b, miR-208 and miR-499. These were found to be up-regulated in the plasma of AMI patients [68–72]. Unfortunately, when these results were translated in a large multicenter study of 1155 unselected patients with acute chest pain, none of the proposed miRNAs provided an additional diagnostic value when combined

Table 17.1 Selected studies on circulating microRNAs in coronary artery disease

miR	Regulation	Specimen	Study population	Reference
miR-126, miR-17, miR-92a, and miR-155	Down-regulated in CAD patients	Plasma and serum	44 stable CAD patients + 25 healthy controls	[65]
miR-126	Positively associated with incident AMI	Plasma	820 participants (Bruneck study)	[60]
miR-208b, miR-499 and miR-320a	Up-regulated miR-208b in AMI	Plasma	1155 chest pain patients	[66]
miR-122, miR-370	Up-regulated in hyperlipidemia, associated with the severity of CAD	Plasma	255 hyperlipidemia patients with or without CAD + 100 controls with normal lipidemia	[59]
miR-29b, miR-126	Inversely associated with plaque fibrosis	Plasma and serum from coronary sinus and aorta	52 patients undergoing coronary angiography	[64]

with cardiac troponin T, though miR-208b provided the highest diagnostic accuracy [66]. In meta-analysis of 19 studies, miR-499 and miR-133a were identified as possible biomarkers of AMI, showing a sensitivity of 0.88 (95%CI:0.86–0.90; P = 0.0000) with a specificity of 0.87 (95%CI:0.84–0.90; P = 0.0000), and a sensitivity of 0.89 (95%CI:0.83–0.94; P = 0.0047) with a specificity of 0.87 (95%CI:0.79–0.92; P = 0.0262), respectively [73]. The prognostic role of miRNAs in AMI is even more interesting. The cardiomyocyte-enriched miRNAs [66] failed to predict long-term mortality at a 2-year follow-up after the AMI. Similarly, miR-133a and miR-208b levels were significantly associated with the risk of death in acute coronary syndrome (ACS) patients, but in an adjusted analysis their independent association with outcome was lost [74].

Very recently Karakas et al. [75] demonstrated that peripheral-blood miRNAs (miR-132, miR-140-3p, and miR-210) could predict CV mortality in a cohort of 1112 patients with CAD at a 4-year follow-up.

5 MicroRNA-Based Therapeutic Strategies

The observation that administration of oligonucleotides, that mimic or inhibit the activity of specific miRNAs in the animal models, can have therapeutic effects has led to considerable interest in the therapeutic target of miRNAs in the clinic. To prevent the instability of the delivered oligonucleotides, protective features such as the encapsulation in lyposomes or polymer-based nanoparticles and addition of chemical

modifications as conjugation of cholesterol groups occur [76]. Two main miRNA-based strategies have been taken: miRNA replacement approach and miRNA targeting approach. In the first approach, a small oligonucleotide that mimics the activity of a specific miRNA is delivered to restore the lost suppressor function of miRNA. Many pre-clinical studies are ongoing, but two miRNA-based therapeutics have reached clinical trials. The first liposome-formulated mimic of miR-34, MRX34, was tested in a phase I trial for treatment of metastatic cancer with liver involvement and unresectable primary liver cancer, but the study was terminated early because of the occurrence of five serious adverse events related to the immune system (NCT01829971). Greater success will probably be achieved by targeting the specific miRNA-mRNA interaction or a specific miRNA pathway for therapeutic fine-tuning, rather than studying a broad inhibition of all miRNA targets. In the inhibition strategy, the main approaches are antagomirs, locked nucleic acids and miRNA sponges. A LNA-based antisense miR-122 inhibitor (miravirsen), led to successful results with long-lasting suppression of hepatitis C virus viremia with negligible side effects in patients with chronic hepatitis C, even after a long-term follow-up. The study is currently in phase II [77, 78]. The recent Food and Drug Administration approval of the first antisense oligonucleotide drug, Mipomersen, that inhibits translation of apolipoprotein (apo) B-100 mRNA, thereby reducing hepatic synthesis of apo B-100 and lowering its concentration. This has been regarded as a huge step forward in the treatment of homozygous familial hypercholesterolemia [79, 80] because it significantly reduced LDL cholesterol, apo B, and lipoprotein(a) in a double-blind randomized study. This success could pave the way for other oligonucleotides-based drugs. The role of miRNAs in cholesterol homeostasis and their impact on atherosclerosis progression are two key points. Preclinical studies in non-human primates showed that inhibition of miR-33a and miR-33b by an anti-miRNA oligonucleotide increased hepatic expression of ABCA1, a key regulator of HDL biogenesis, and induced a sustained increase in plasma HDL levels over 12 weeks, with reduction of VLDL levels [81]. Indeed, miR-33a/b could be an attractive target to promote atherosclerosis regression. Furthermore, in a swine model of ischemia/reperfusion injury, administration of LNA-modified antisense miR-92a showed a cell-protective, proangiogenic, and anti-inflammatory effects with reduction of infarct size and improved recovery of cardiac function [82]. Unfortunately these promising results have not yet progressed to human trials.

6 Conclusion

Atherosclerosis-related processes have been shown to be linked to miRNA expression in several cell cultures, from animal to human studies, but the complexities of the miRNA signature in human atherosclerosis remain veiled. We elucidated how the most studied miRNAs expressed in atherosclerotic lesions may have a clinical role as diagnostic and prognostic biomarkers. Furthermore, though they are still in their infancy, miRNA-based therapies may progress to clinical trials. As this is a relatively novel field of research, the full clinical potential of miRNAs in atherosclerosis and coronary artery disease remains to be discovered in a time of evolving personalized medicine.

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Chapter 18
Trials of Angiogenesis Therapy in Patients with Ischemic Heart Disease

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Abstract Therapeutic angiogenesis is a novel method to create endogenous bypass conduits around the occluded coronary arteries. After the success in animal studies, therapeutic angiogenesis has been studied in humans with ischemic heart disease not responding to (or in addition to) conventional treatments. The most commonly studied angiogenic cytokines are vascular endothelial growth factor, fibroblast growth factor and granulocyte colony stimulating factor. Delivery as a protein, or vector with gene encoding for specific protein have been tested in clinical trials. These cytokines, using a multitude of delivery routes ranging from direct intramyocardial transfer either from epicardial or endocardial side, intracoronary infusion, systemic administration via subcutaneous route, have been introduced to myocardial tissues. Small sample size phase I studies have shown promising results. But large sample size, controlled studies have failed to demonstrate any significant improvement in various clinical, radiographic and angiographic outcomes in ischemic heart disease patients. Angiogenesis is influenced by a multitude of variables including duration of exposure, type of vector and need for co-factor. They also vary based on the individual patient characteristics. Further studies accounting for these variables are needed to fully determine the potential of therapeutic angiogenesis in ischemic heart disease.

Keywords Angiogenesis • Vascular endothelial growth factor • Fibroblast growth factor • Granulocyte colony stimulating factor • Ischemic heart disease

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1 Introduction

Ischemic heart disease (IHD) is the end result of insufficient blood supply to myocardium due to narrowing of coronary blood vessels. Both macrovascular and microvascular disease may contribute to this. Clinically significant macrovascular coronary artery disease is treated by revascularization using percutaneous or surgical approaches. Many patients, nearly one-fifth, are not a candidate for interventional procedures due to increased risk, poor target arteries or associated medical comorbidities [1]. Moreover, interventional procedures treat only a segment of coronary artery and the rest of the diseased vessel remains. The stent itself is at a higher risk for stenosis compared to the native artery [2]. Treatment options for microvascular coronary artery disease is limited to medical management. Hence, scientists for a long time evaluated therapeutic angiogenesis as a novel method to create endogenous bypass conduits around the occluded arteries. Collateral blood vessels exist in humans even in the absence of coronary artery disease (CAD). Well-developed collateral function was related to improved survival in patients with CAD, though it is a poor prognostic marker, likely from an increased prevalence in patients with severe CAD [3].

Data generated from in vitro and in vivo animal models for the past three decades supports the feasibility of therapeutic angiogenesis in IHD. Angiogenic cytokines such as basic fibroblast growth factor (b-FGF or FGF-2), vascular endothelial growth factor (VEGF) and granulocyte colony stimulating factor (G-CSF) were used in various experiments to promote neovascularization of myocardium. Variety of techniques are used to deliver these angiogenic cytokines to the target tissue. Intracoronary infusion, direct injection into the myocardium either from epicardial or endocardial end and gene therapy are some of them. Gene therapy involves transferring genes encoding the angiogenic cytokines through viral vectors or via non-viral methods using naked plasmid DNA. Gene therapy can produce sustained exposure to angiogenic factors, as growth factors are secreted in vivo for a given period of time. Genes can be transferred directly to specific biological site, limiting their side effects. However introduction of foreign genetic material and exposure to viral vectors may mediate an immune and inflammatory response. There is also a potential for long term, low level systemic exposure to secreted angiogenic factors. Duration and level of gene expression are unpredictable in gene therapy. Potential long term adverse effects can arise, due to excessive vascular growth in non-target tissues such as in retina in diabetic patients and in malignant tumors.

2 Human Trials of Cardiovascular Protein Therapy

Human trials of angiogenic therapy have been conducted since late 1990s. Here we review all the major trials of therapeutic angiogenesis reported in English literature. Biology of therapeutic angiogenesis including role of growth factors has been discussed in previous sections. The important trials involving the various cardiovascular protein therapies are shown in tables (18.1–18.5).

2.1 Fibroblast Growth Factor (FGF)

The first study evaluating the effects of FGF on humans was conducted on patients undergoing CABG. FGF-1 was injected into the myocardium close to the internal mammary artery (IMA) – left anterior descending (LAD) anastomosis (Table 18.1). Twenty patients received active FGF-1 and another 20 who were controls received denatured FGF-1. Twelve weeks later, IMA bypass grafts were selectively imaged by intra-arterial digital subtraction angiography. The imaging demonstrated formation of capillary network sprouting around the injection site in all the patients who received active FGF-1 and the capillary sprouting was not present among any of the controls. The study was limited, as it did not measure any clinical outcome and provided data for a relatively small period of time, but it was valuable in proving the safety of these procedures [4].

Incorporating FGF into heparin alginate slow-release beads provided a sustained and controlled release of the growth factor. Sellke et al. looked into 8 patients who underwent implantation of these devices into epicardial fat in patients undergoing CABG who had atleast one major arterial distribution not amenable to revascularization [5]. These devices were implanted into the regions of unrevascularizable territory. The results of this phase 1 trial showed that there was no mortality or evidence of renal, hematologic or hepatic toxicity related to sustained release of bFGF. But one patient suffered perioperative myocardial infarction in the area of bFGF administration. Three month stress myocardial perfusion imaging (MPI) done on 7 of the patients showed varied results in terms of neovascularization but their contractile function either improved or remained similar and all patients were angina free. Three of the patients had clear enhancement of perfusion to the unrevascularized myocardium, 1 had new fixed defect and minimal overall change in the other three. A randomized double blind placebo controlled trial was done using the same technique in 24 patients using different bFGF doses (10 μg of bFGF (n = 8) Vs. 100 μg of bFGF (n = 8) vs. placebo (n = 8)). It showed a significant improvement of defect size per stress MPI in the 100 μg-bFGF group (19.2–9.1%, p = 0.01), no significant change in the 10-μg group and a trend towards worsening defect size in placebo group (20.8–23.9%, p = 0.06). There was no treatment related mortality, or signs of systemic toxicity from bFGF administration. The study concluded that combination CABG/bFGF therapy did not have an excess rate of complication [6].

Multiple paraenteral routes were explored for delivery of b-FGF, so that it could be used for treatment of patients who does not require CABG, due to the inherent risk associated with thoracotomy. First trial on intracoronary delivery of FGF in humans was conducted to evaluate its safety, tolerability, pharmacokinetics and pharmacodynamics [7]. Twenty-five patients with CAD and stable angina were randomized in 2:1 ratio to single dose bFGF or placebo. bFGF ranging from 3–100 μg/kg were delivered to the left main coronary artery. Sustained hypotension and bradycardia were the common side effects with doses of 30–100 μg/kg. Doses <30 μg/kg were generally well tolerated. Systemic angiogenesis was not observed in any patients. Exercise time and electrocardiographic indexes of ischemia did not

Table 18.1 Trials of angiogenesis with FGF protein therapy

Author (year)	Agent	Delivery mode	N	Control Yes(Y)/ No(N)	Follow up	Outcome/endpoints	Adverse events	Comments
Schumacher 1998 [4]	FGF-1	Intramyocardial	20	Y	12 weeks	Capillary density	None	First study No clinical outcomes reported Long term data was not available
Sellke 1998 [5]	b-FGF	Epicardial heparin-alginate beads	8	N	6 months	Angina, SPECT-MPI	Perioperative MI	First study to report clinical end points
Laham 1999 [6]	b-FGF	Epicardial heparin-alginate beads	16	Y	16.0 ± 6.8 months	Angina, SPECT-MPI, MRI	Clinical (none)	Evidence for dose effect
Unger 2000 [7]	b-FGF	Intracoronary injection	17	Y	29 days	Exercise time and exercise induced ECG changes	Hypotension Bradycardia Atrial fibrillation	No systemic angiogenesis
Laham 2000 [8]	FGF-2	Intracoronary infusion	52	N	6 months	Clinical TMST Angina MRI	Hypotension	Dose dependent hypotension
Simons 2002 [9]	r FGF2	Intracoronary infusion	337	Y	180 days	Exercise tolerance Seattle angina question MRI	Hypotension	Largest trial to date for FGF induced myocardial angiogenesis No benefit was noted with FGF therapy

change at 29 days, between the two groups. This study was not primarily designed to assess therapeutic efficacy. Results suggests that prolonged exposure to the growth factors may be required for therapeutic angiogenesis of target tissue. Tolerable dosage of intracoronary bFGF was determined through the studies by Laham et al. [8]. Twenty minutes infusions of FGF were well tolerated upto a dose of 36 μg/kg in patients with IHD not amenable to CABG or PTCA. Again, systemic hypotension was the main side effect of bFGF infusions. This study also suggested an improvement in Seattle angina questionnaire, exercise tolerance by treadmill testing, and regional wall thickening by MR imaging at 180 days. In addition to adding on previous data on feasibility and safety, it provided evidence for dose effect of FGF therapy in myocardial angiogenesis.

Based on the results of above mentioned phase I studies, Simons et al. conducted a phase II FGF Initiating RevaScularization Trial (FIRST) to evaluate further the efficacy and safety of recombinant FGF2 (rFGF-2) [9]. This was a multicenter, randomized, double-blind, placebo-controlled trial of a single intracoronary infusion of rFGF-2 at 0, 0.3, 3, or 30 μg/kg (n = 337 patients). Efficacy was evaluated at 90 and 180 days by exercise tolerance test, myocardial nuclear perfusion imaging, Seattle Angina Questionnaire, and Short-Form 36 questionnaire. Exercise tolerance was increased at 90 days in all groups and was not significantly different between placebo and FGF-treated groups. FGF-2 reduced angina symptoms as measured by the angina frequency score of the Seattle Angina Questionnaire (overall $p = 0.035$) and the physical component summary scale of the Short-Form 36 (pairwise $p = 0.033$, all FGF groups versus placebo). These differences were more pronounced in highly symptomatic patients (baseline angina frequency score ≤ 40 or Canadian Cardiovascular Society score of III or IV). None of the differences were significant at 180 days because of continued improvement in the placebo group as well. Adverse events were similar across all groups, except for hypotension, which occurred with higher frequency in the 30 μg/kg rFGF2 group. The authors concluded that single intracoronary infusion of rFGF2 does not improve exercise tolerance or myocardial perfusion but does show trends toward symptomatic improvement at 90 (but not 180) days. Lack of improvement with FGF despite it being a potent angiogenic factor may represent variability in effect secondary to delivery route. Intracoronary delivery may result in transient exposure of myocardium to FGF and thus a non-sustained effect. But, unlike FIRST trial, previous studies with intramyocardial administration of FGF which had resulted in significant clinical improvement were performed on a small sample size of patients.

Further human studies using FGF alone, were limited due to lack of efficacy from clinical trials. Recent work by Jang et al. on improving the 'bioefficacy' of FGF-2 holds promise for the field of therapeutic angiogenesis using FGF. Proteo-liposomal preparation of FGF-2 with a proteoglycan syndecan-4, markedly enhanced neovascularization in animal model by improving biological activity [10]. Another interesting strategy is dual delivery of growth factors PDGF and FGF in self-assembling peptide fibers that provide microenvironment to help recruit endothelial cells [11]. Studies in rats showed myocardial protection, stable vessel formation and improvement in cardiac function. These newer strategies are yet to be tested on human IHD patients.

2.2 Vascular Endothelial Growth Factor (VEGF)

Recombinant human VEGF (rhVEGF) was studied for its effects of angiogenesis in IHD patients (Table 18.2). Initial studies demonstrated safety of intracoronary infusions of rhVEGF. In a small group of 14 patients, serial SPECT studies demonstrated improvement in myocardial perfusion with high doses of intracoronary rhVEGF at 60 days of therapy [12]. But quantitative analysis failed to demonstrate statistical significance.

Similar to the FGF, dose related hypotension was the most common side effect of rhVEGF therapy. In the phase 1 clinical study by Henry et al., designed to determine the safety and tolerability, intracoronary rhVEGF was well tolerated at infusion rates upto 0.005 μg/kg/min [13]. Seven out of 15 patients who received rhVEGF in this study, demonstrated improved perfusion on SPECT-MPI and increased collateral density score on follow up angiograms at 60 days.

The above mentioned clinical trials played pivotal role in helping design the subsequent large clinical trials. Vascular endothelial growth factor in Ischemia for Vascular Angiogenesis (VIVA) was a relatively large sample size (178 patients) double blind, placebo controlled trial in stable IHD patients who were unsuitable for standard revascularization [14]. Endpoints included exercise treadmill testing, quality of life assessments and nuclear perfusion imaging at 60 days. There was no improvement beyond placebo in all measurements by day 60. By day 120, high dose rhVEGF resulted in significant improvement in angina (p-0.05), but non-significant favorable trends in treadmill exercise time (p-0.15) and angina frequency (p-0.09). There were no difference in clinical event rates of death, myocardial infarction, angina requiring hospitalization between the placebo and rhVEGF groups. Similar to the previous phase I trials, this study also demonstrated excellent short-term safety of using intracoronary rhVEGF. The study also demonstrated benefit with regard to symptomatic improvement in angina at longer follow up duration in patients treated with high dose rhVEGF. However further studies needs to be done to assess the true therapeutic efficacy of this approach.

2.3 Granulocyte Colony Stimulating Factor (G-CSF)

Like VEGF and FGF, G-CSF has been shown to promote neovascularization in IHD. In contrast to VEGF and FGF which promote local angiogenesis, G-CSF injection promotes mobilization of progenitor cells from bone marrow into ischemic myocardium. These cells once localized to tissue of interest lead to secretion of angiogenic cytokines which promote local angiogenesis. An important difference between G-CSF therapy and treatment with FGF and VEGF is that in addition to neovascularization, G-CSF also leads to replacement of necrotic myocytes by stem cells. Thus G-CSF therapy may really represent a dual pronged strategy in management of IHD patients. In-stent restenosis and accelerated atherosclerosis were some of the concerns in using this treatment modality.

Table 18.2 Trials of angiogenesis with VEGF protein therapy

Author (year)	Agent	Delivery mode	N	Control Y/N	Follow up	Outcome/ end points	Adverse events	Comments
Hendel 2000 [12]	rhVEGF	Intra-coronary	14	N	60 days	Safety endpoints, SPECT-MPI	Not reported	Dose dependent effect, higher dose = more efficacy
Henry 2001 [13]	rhVEGF	Intra-coronary	20	N	60 days	Safety endpoints, SPECT-MPI, Coronary angiogram	Hypotension	
Henry 2003 [14]	rhVEGF	Intra-coronary + intravenous	178	Y	120 days	Exercise treadmill test, Angina class, Quality of life, SPECT-MPI	Transient hypotension	Higher dose demonstrated benefit at 120 days but not at 60 days

Table 18.3 Trials of angiogenesis with G-CSF or GM-CSF protein therapy

Author (year)	Agent	Delivery mode	N	Control Y/N	Follow up	Outcome/ end points	Adverse events	Comments
Seiler 2001 [16]	GM-CSF	Intracoronary + subcutaneous	21	Y	2 weeks	Collateral flow index	Low grade fever, skin rash	
Zbinden 2005 [17]	GM-CSF	Subcutaneous	14	Y	2 weeks	Collateral flow index	Acute coronary syndrome	
Kuethe 2005 [15]	G-CSF	Subcutaneous	14	Y	3 months	Wall motion and perfusion on SPECT-MPI	No severe side effects	Marked improvement in G-CSF arm
Valgimigli 2005 [18]	G-CSF	Subcutaneous	20	N	6 months	SPECT-MPI perfusion and LV function	Transient transaminitis	Non-significant improvement in EF and LVEDV
Zohlnhofer 2006 [19]	G-CSF	Subcutaneous	114	Y	6 months	SPECT-MPI, MRI	Bone pain and muscle discomfort	No improvement in clinical outcomes
Ellis 2006 [20]	G-CSF	Subcutaneous	18	Y	30 days	ECHO	No severe side effects	No significant difference in EF between treatment and placebo
Kang 2004, 2007 [21, 22]	G-CSF	Subcutaneous G-CSF followed by intracoronary stem cells	27	Y	6 months, 2 years		In-stent restenosis at culprit lesions	No significant improvement in 2 year follow up
Engelmann 2006, 2010 [23, 24]	G-CSF	Subcutaneous	44	Y	1 months, 3 months, 1 yr	MRI	No increased adverse events	No improvement in myocardial function or survival
Ince 2005 [25]	G-CSF	Subcutaneous	50	Y	4 months	ECHO	No severe effects	Improvement in EF

Achilli 2010, 2014 [26, 27]	G-CSF	Subcutaneous	49	Y	6 months, 3 years	ECHO, SPECT, MRI, angiography	No increased MACE.	No beneficial effects.
Kang 2006, 2012 [28, 29]	G-CSF	Subcutaneous	96, 169	Y	6 months, 5 years	MRI, angiography	No increased rate of in-stent restenosis	No long term improvement in LVEF between groups
Chih 2012 [30]	G-CSF	Subcutaneous	14	Y	42 weeks	MRI, EST	No increased side effect	No improvement in angina
Hibbert 2014 [31]	G-CSF	Subcutaneous	86	Y	6 week, 9 months	SPECT	No increased MACE in G-CSF group	Lower EF in G-CSF group
Brenner 2016 [32]	G-CSF + Sitagliptin	Subcutaneous	174	Y	12 months	MRI	No increased MACE in treatment group	No difference in LVEF in treatment and placebo group
Roman 2015 [33]	G-CSF	Subcutaneous + intracoronary stem cell inj	120	Y	12 months	MRI	No increased MACE	No benefit in LVEF or LVESV

The first human study evaluating safety and efficacy of G-CSF in IHD was performed by Kuethe et al. [15] in 2005 on patients with acute myocardial infarction. In this non randomized open label study, 14 patients in the treatment group was administered G-CSF subcutaneously for a mean duration of 7 ± 1 days, 48 h after successful PCI following acute myocardial infarction. Nine patients served as controls. No severe side effects were observed in the treatment group. Myocardial perfusion and regional wall motion measured using SPECT at discharge and 3 months showed significant improvement between treatment and control groups (Table 18.3). This initial trial provided proof for utility of G-CSF in promoting angiogenesis following recanalization of coronary arteries.

Similar to the prior study, Valgimigili et al. randomized 20 patients with STEMI to receive GCSF or placebo, subcutaneously for 4 consecutive days [18]. G-CSF therapy induced a significant increase of white blood count, CD34(+) cells, and CD34(+) cells co-expressing AC133 and VEGFR-2. The treatment and placebo groups showed similar pattern of perfusion defect recovery at 3 and 6 months. Trend towards improvement in ejection fraction ($p=0.068$) and lowering of left ventricular end-diastolic volume ($p=0.054$) was observed in the study. No clinical or angiographic adverse events were observed throughout the study.

Ellis et al. [20] performed randomized double blind trial on 18 patients for evaluating rupture free survival and recovery of Left ventricular function. The study included patients who were post MI and was reperfused after 4 h. They were given subcutaneous G-CSF within 48 h. One month follow up showed no difference in left ventricular systolic or diastolic function in treatment and placebo group.

A larger sample size phase two study that evaluated effects of autologous bone marrow stem cell mobilization induced by G-CSF was the G-CSF STEMI trial [23]. This randomized double blinded, placebo controlled study included 44 patients with late revascularized STEMI (STEMI >6 h and <7 days of onset of complaints and clinically stable). They were treated with subcutaneous administered G-CSF after PCI. At 1 and 3 months their myocardial function and infarct size were analysed. It was concluded that G-CSF was not superior to placebo in improving myocardial function at any of the follow up periods in patients with subacute myocardial infarction in whom delayed PCI was performed. Myocardial perfusion at 1 month appeared to be more in the treatment group but the difference was non-significant at 3 months follow up. One year follow up of the G-CSF STEMI trial [24] demonstrated no difference in event-free survival such as death, MI, CABG or target lesion revascularization between the two groups. Also ejection fraction, myocardial perfusion, infarct size, left ventricular end systolic and diastolic volumes remained similar between the two groups. The study again demonstrated the safety of G-CSF treatment over 1 year period. Hence G-CSF administration after subacute STEMI did not improve myocardial function or survival when used as a single agent. It should be noted that around half of the study subjects were lost at 1 year follow up.

Front Integrated Revascularization and STem Cell Liberation IN Evolving Acute Myocardial Infarction (FIRSTLINE-AMI) trial tested the impact of integrating G-CSF with PCI in the acute myocardial infarction [25]. Fifty patients were randomly assigned to either placebo or subcutaneous G-CSF treatment group in this

non-blinded study. Within 89 ± 35 min after successful PCI, G-CSF was administered for 6 days in addition to the standard care. Ejection fraction at 4 months in G-CSF group was significantly improved compared to a control group $p < 0.01$). This trial was done in a predominantly male population (92%) and included patients with only one vessel coronary disease.

REgenerate VItal myocardium by Vigorous Activation of bone marrow stem celLs (REVIVAL-2) trial was a larger phase 2, double-blind, randomized placebo-controlled trial to assess the efficacy of stem cell mobilization with G-CSF in patients with myocardial infarction [19]. One-hundred and fourteen patients diagnosed with ST-segment elevation acute myocardial infarction who had successful reperfusion by PCI within 12 h after onset of symptoms were randomized after 5 days to receive subcutaneously either a daily dose of 10 μg/kg of G-CSF (n = 56) or placebo (n = 58) for 5 days. Treatment with G-CSF produced a significant mobilization of stem cells. Between baseline and follow-up, left ventricular infarct size according to scintigraphy was reduced by a mean (SD) of 6.2% (9.1%) in the G-CSF group and 4.9% (8.9%) in the placebo group ($p = 0.56$) and left ventricular ejection fraction was improved by 0.5% (3.8%) in the G-CSF group and 2.0% (4.9%) in the placebo group (p-0.14). Angiographic restenosis occurred in 19 (35.2%) of 54 patients in the G-CSF group and in 17 (30.9%) of 55 patients in the placebo group ($p = 0.79$). The most common adverse event among patients assigned to G-CSF was mild to moderate bone pain and muscle discomfort. Thus stem cell mobilization by G-CSF therapy in patients with acute myocardial infarction and successful mechanical reperfusion had no significant influence on infarct size, left ventricular function, or coronary restenosis when compared to standard care. Seven year follow up of the REVIVAL-2 trial in the form of a long term outcome analysis showed that the combined incidence of death or myocardial infarction was similar in G-CSF and placebo groups ($p = 0.85$) [34]. Thus long term follow up data show that G-CSF does not improve clinical outcomes of patients with acute myocardial infarction.

In a slightly different version of the above trial, Kang et al. examined the efficacy of intracoronary infusion of peripheral blood stem cells collected after G-CSF therapy in the MAGIC trial. This trial involved 27 patients with myocardial infarction who underwent coronary stenting for the culprit lesion and were prospectively randomized into three groups; cell infusion (n = 10), G-CSF alone (n = 10), and control group (n = 7). At 6 month follow up exercise capacity as assessed by treadmill exercise time, myocardial perfusion and systolic function improved significantly in patients who received cell infusion. However, an unexpectedly high rate of in-stent restenosis at culprit lesion in patients who received G-CSF was noted and thus the trial was terminated prematurely. Two-year follow up data from this study supported a persistent improvement in group which received intracoronary infusion of stem cells, though it was not statistically significant when compared with control group [21].

In MAGIC trial the G-CSF was injected prior to revascularization. Hence there is a potential to destabilize the coronary plaques due to mobilization of progenitor cells and inflammation induced by G-CSF, which could explain the high in-stent restenosis rate in that study. MAGIC trial incorporated a heterogenous group of patients including those with chronic infarction. The study had incomplete follow up.

Therapeutic success with MAGIC trial strategy in comparison to strategy used with REVIVAL trial may be related to more direct availability of stem cells to injured myocardium. But waning of significant benefit at 2 years in MAGIC trial raises the question of performing repeated injection of stem cells/G-CSF therapy to promote myocardial angiogenesis and regeneration.

STEMI-AMI trial (STEM cell mobilization In Acute Myocardial Infarction trial – 2010) evaluated patients with anterior STEMI who underwent PCI [26]. The symptom to reperfusion time of the study group was 2–12 h and EF of the patients after PCI was <45%. They were randomized to placebo (n = 25) or G-CSF group (n = 24). G-CSF group received subcutaneous G-CSF (5 μg/kg b.i.d). At 6 month follow up there was no difference in improvement of ejection fraction or perfusion between the placebo and G-CSF groups. G-CSF was generally well tolerated by the patients and there were no significant difference in major adverse cardiac event (MACE) between the groups. Three year follow up of the patients again did not show any significant adverse or beneficial clinical outcome [27].

From the results of prior studies it was becoming clear that the benefit of G-CSF therapy appears to become less significant as the time of administration increased from the onset of myocardial infarction. MAGIC Cell-3-DES trial [28] evaluated the differential effect of intracoronary infusion of peripheral blood stem cells (PBSC) in acute MI vs Old MI. Ninety six patients who underwent coronary revascularization with DES were randomly allocated to 4 groups (25 Acute MI cell infusion and 25 Acute MI controls; 16 Old MI cell infusion and 16 Old MI controls). PBSC's were mobilized for 3 days using G-CSF and administered to infarcted myocardium by intracoronary infusion. The study showed that PBSC infusion in Acute MI had a significant improvement in LVEF compared to controls at 6 months. There was no improvement in LVEF or ventricular remodeling old MI cell infusion group. Thus Old MI patients had no short term benefit from PBSC therapy. Unlike the previous versions of MAGIC trial which saw an increase in in-stent restenosis rate, the MAGIC cell-3-DES trial did not have an increase in ischemia or thrombosis. It is believed that DES was effective in preventing neointimal growth aggravated by G-CSF and G-CSF in turn facilitated re-endothelialization of DES. A 5 year follow up of the study including additionally recruited patients (total sample size of 169 patients) suggested a decline in major adverse cardiac events i.e. non fatal MI, hospitalization for heart failure/ angina, cardiac death in cell infusion group (acute + old MI, n = 79) compared to the control group (n = 84) (22.8 Vs. 39.3, p=0.015) [29]. The improvement of LVEF in Acute MI group compared to control which was present in the short term follow up was not observed after 2 years. Hence there was no long term improvement in LVEF in cell infusion and control groups in both acute MI and old MI groups.

Chih et al. in 2012 evaluated efficacy of repeated low dose G-CSF in patients with severe chronic ischemic disease who were having Canadian Cardiovascular Society (CCS) Class III-IV angina [30]. In this 14 patient randomized, double blind cross over trial, G-CSF was administered for 5 days every fortnightly for three cycles. Groups were crossed over at 6 weeks. At 42 weeks after the study onset, G-CSF, when compared with placebo had no effect on myocardial ischemia by MRI or EST despite effective endothelial progenitor cell mobilization. There were no improvement in angina between the two groups.

The latest in the series of studies of G-CSF in acute myocardial infarction is the Cardiovascular Percutaneous Intervention TriAL group's G-CSF for STEM cell mobilization post Myocardial infarction trial (CAPITAL STEM MI) [31]. In this prospective, randomized placebo control trial, patients with anterior wall MI were randomized to G-CSF group (n = 43) or placebo group (n = 43) after PCI. G-CSF group received subcutaneous G-CSF (10 μg/kg daily) on day 3 or 4 of STEMI. Six week follow up demonstrated similar EF in G-CSF and placebo group. Six month follow up showed a lower ejection fraction in the G-CSF group compared placebo group. Major adverse cardiac events (MACE) were similar in both groups at 6 months.

Combining G-CSF with sitagliptin has showed to increase homing of bone marrow derived progenitor cells to injured myocardium in animal studies. Brenner et al. performed a randomized placebo controlled, double blind, phase III trial on the efficacy and safety of G-CSF + sitagliptin in acute MI patients (SITAGRAMI trial) [32]. After revascularization, 174 patients were randomized in 1:1 fashion and either received G-CSF + sitagliptin or placebo. No difference in right or left ventricular ejection fraction was observed between the two groups at 12 months. MACE also remained similar in both groups.

Roman et al. compared different methods of stem cell delivery on STEMI reperfusion (TECAM trial – Trial of Hematopoeitc stem Cells in acute myocardial infarction) [33]. In this open label, prospective study, 120 patients were randomized to G-CSF mobilization group, intracoronary injection of bone marrow autologous mononuclear cell (BMMC) group, combination of both and conventional treatment group. Again, no significant change in LVEF and LVESV was observed between the groups.

Thus majority of the large sample size human studies show no major beneficial effect of using G-CSF in patients with CAD. Most of the studies found that G-CSF therapy as a safe procedure, but few studies have shown concern regarding its safety due to its pro-atherogenic effect. Currently undergoing STEMI-AMI OUTCOME trial tries establish whether G-CSF improves hard clinical long term outcomes [35]. This is a multicenter randomized trial which includes a large sample size of 1530 patients. Completion of this rigorous controlled phase III trial will conclusively assess efficacy of G-CSF treatment in STEMI with adequate statistical power.

3 Granulocyte Monocyte Colony Stimulating Factor (GM-CSF)

Granulocyte Monocyte-Colony Stimulating Factor (GM-CSF) was also evaluated for safety and efficacy in improving collateral flow in CAD patients (Table 18.3). In 2001, Seiler et al. [16] reported, in 21 patients with extensive coronary artery disease not eligible for CABG, effect of granulocyte-macrophage colony-stimulating factor (GM-CSF, Molgramostim) on quantitatively assessed collateral flow index (CFI) in a randomized, double-blind, placebo-controlled fashion. The study

protocol involved both intracoronary and intravenous infusion of GM-CSF. The treatment-induced difference in CFI was +0.11 ± 0.12 in the GM-CSF group and −0.07 ± 0.12 in the placebo group (P = 0.01) which was suggestive of improvement. This was the first clinical study investigating the potential of GM-CSF to promote angiogenesis in IHD patients.

Same group reported, in 2005, on the safety and efficacy of short-term subcutaneous GM-CSF therapy for promoting coronary collateral growth [17]. This was a randomized, double-blind, placebo-controlled trial of a 2-week period with subcutaneous GM-CSF (10 μg/kg; n = 7) or placebo (n = 7) in 14 men with chronic stable IHD. Efficacy measurements were made in a stenotic as well as a normal coronary artery before and after GM-CSF administration. There was significant improvement in CFI in the treatment group. Among 11 determined cytokines, chemokines and their monocytic receptor concentrations, the treatment-induced change in CFI were predicted by the respective change in tumor necrosis factor-alpha concentration. In this trial, 2 of the 7 patients in the GM-CSF group and none in the placebo group suffered an acute coronary syndrome during the treatment period raising questions about its safety. It was speculated that the precipitation of coronary syndromes with GM-CSF might have been related to its pro-atherogenic effects.

4 Human Trials Using Other Agents that Promote Angiogenesis

1. **Dipyridamole**
   Belardinelli et al. [36] evaluated the effect of oral dipyridamole in inducing coronary collateral growth in IHD. They randomized 30 male patients with coronary artery disease and left ventricular systolic dysfunction (ejection fraction >40%) into three matched groups to receive dipyridamole alone (n = 10) orally for 8 weeks or exercise training at 60% of peak $VO_{(2)}$ three times a week for 8 weeks along with dipyridamole (n = 10), or neither exercise testing nor dipyridamole (n = 10). Thallium uptake of the collateral-dependent myocardium, coronary collateral score and wall thickening score increased significantly only in groups receiving dipyridamole. Further studies are needed to better define the role of oral dipyridamole as sole therapy or in combination with growth factors in treatment of ischemic heart disease.
2. **Erythropoeitin**
   Low dose erythropoietin was successful in improving neo-angiogeneis and cardiac regeneration in experimental models. In a human placebo controlled, randomized, double blind pilot trial involving 28 patients, the efficacy and safety of low dose epoetin-β was evaluated [37]. The study included patients who had symptomatic heart failure due to IHD involving proximal segment of LAD, RCA or circumflex coronary arteries. The patients received weekly 35 IU/kg of epoetin-β, 3 weeks after successful PCI, for 6 months. At 6 months no adverse events due to treatment were reported. There was a significant increase in ejection

fraction in epoetin group compared to placebo when measured using ECHO ($p=0.019$) and cardiac MRI ($p=0.042$). Futher larger studies are essential to determine the generalizability of study results.

3. **Physical exercise**
   Association between coronary collaterals and exercise training is unclear. Impact of exercise training on coronary collateral circulation in patient with stable coronary artery disease trial (EXCITE trial) grouped 60 patients with significant coronary artery disease (ffr < 0.75) to high intensity exercise group, moderate intensity exercise group and control group (current recommendations of 2–3 sessions of 20 min each per week) [2]. This prospective, open label randomized study showed that collateral flow index (CFI) significantly improved in exercise group (10 h per week) when compared to control group in 4 weeks. In addition the exercise threshold, peak oxygen uptake and ischemic threshold increased significantly in exercise group. There was no difference in these parameters between high intensity and moderate intensity groups. Angiography failed to show increase in epicardial collateral vessels in response to prolonged exercise. This may be because, increase in CFI may be mediated by higher recruitment of preexisting vessels or by an improvement in endothelial function.

5 Human Trials of Gene Therapy

5.1 VEGF Gene Trials

VEGF gene transfer in humans was first evaluated by Losordo et al. in 1998 [38]. In 5 patients with angina who failed conventional therapy, mini-thoracotomy was performed and naked plasmid DNA encoding VEGF (phVEGF165) was injected into the myocardium (Table 18.4). All patients had significant improvement in angina frequency and severity at 60 days, which correlated with increased myocardial perfusion on SPECT-MPI imaging. Coronary angiography also showed improved Rentrop score. This study provided the first evidence for favorable clinical effects of direct myocardial injection of naked DNA encoding VEGF.

Following this landmark study, there were three more phase 1 clinical studies where phVEGF165 was delivered directly into ischemic myocardium via mini left anterior thoracotomy in patients with medically intractable angina. These studies demonstrated trend towards reduced clinical symptoms, reduced evidence of ischemia on SPECT imaging and improved collateral filling of at least one occluded vessel on angiography. More importantly, intramyocardial injection of phVEGF165 did not appear to produce any increased adverse events, atleast in short term [39–41].

In a study done by Rosengart et al. [42] the viral vector expressing the 121-amino-acid form of human VEGF was administered to individuals with clinically significant coronary artery disease. It was administered directly to an ischemic area of the myocardium as an adjunct to conventional CABG surgery in a region that could not

Table 18.4 Trials of angiogenesis with VEGF gene therapy

Author (year)	Agent	Delivery route	N	Control Y/N	Follow up	Outcomes/ end points	Adverse effects	Comments
Losordo 1998 [38]	phVEGF	Intramyocardial	5	N	60 days	SPECT myocardial perfusion, coronary angiography	None	First human study with viral vector
Symes 1999 [39]	Ph-VEGF 165	Intramyocardial	20	N	60 days	SPECT myocardial perfusion, coronary angiography	None	
Vale 2000 [40]	Ph-VEGF 165	Intramyocardial	13	N	60 days	NOGA electromechanical mapping	None	
Sarkar 2001 [41]	Ph-VEGF A165	Intramyocardial	7	N	12 months	SPECT-MPI, tissue velocity imaging	None	
Rosengart 1999 [42, 43]	AdVEGF121	Intramyocardial	21	N	30 days, 11 years	Coronary angiography, SPECT-MPI and treadmill exercise	Safe in the long term	Continued improvement in angina and exercise capacity at 6 months
Laitinen 2000 [44]	VEGF	Intracoronary	15	Y	6 months	Coronary angiography	No adverse events	Established safety and efficacy of intracoronary route for viral vector delivery
Hedman 2003, 2009 [45, 46]	VEGF	Intracoronary	103	Y	6 months, 8 years	Myocardial perfusion	No serious adverse events long term	
Vale 2001 [47]	phVEGF-2	Intramyocardial	6	Y	365 days	NOGA electromechanical mapping, SPECT-MPI	None	

Losordo 2002 [48]	phVEGF-2	Intramyocardial	19	Y	12 weeks	CCS angina class, Seattle angina questionnaire	None	
Kastrup 2005 [49]	phVEGF-A165	Intramyocardial	80	Y	3 months	Myocardial perfusion, wall motion abnormality, CCS angina class	5 adverse events in treatment group	In a sub-study improvement in perfusion with VEGF therapy in NOGA defined ischemic areas
Ripa 2006 [50]	VEGF165 + G-CSF	Intramyocardial + subcutaneous G-CSF	32	Y	3 months	SPECT-Mpi	No major side effects	
Stewart 2009 [51]	VEGF165	Intramyocardial	93	Y	6 months	SPECT-MPI, exercise treadmill time, angina symptoms	No difference in major events	First multicenter trial of VEGF gene therapy via Intramyocardial route
Kastrup 2011 [52]	VEGF121	Intramyocardial	17	Y	52 weeks	Exercise capacity, myocardial perfusion	None	
Favaloro 2013 [53]	pVEGF165	Intramyocardial	10	N	2 years	SPECT, stress ECHO	None	High doses of VEGF found to be safe
Ruel 2008 [54]	VEGF + L arginine	Intramyocardial VEGF + oral L-arginine	19	Y	3 months	PET- perfusion and wall motion	None	

be bypassed, in 15 patients (group A) or through a mini-thoracotomy as sole therapy in 6 patients (group B). There was no evidence of systemic or cardiac adverse events related to vector administration. In both groups, coronary angiography, and stress sestamibi scan assessment of wall motion 30 days after therapy suggested improvement in the area of vector administration but no improvement in relative blood flow. All patients reported improvement in angina class after therapy, but in group A. This could not be solely attributed to vector administration (had concomitant CABG). In group B, in which gene transfer was the only therapy, treadmill exercise assessment suggested improvement in most individuals assessed 30 days after therapy and at 6 month follow up. Trends toward improvement in angina class and exercise treadmill testing at 6-month follow-up in the sole therapy group suggest that the effects of this therapy are persistent for or ≥6 months. Long term follow up these patients (median follow up 11.8 years) suggested that the incidence of malignancy and retinopathy were similar to that of age matched population [43]. The study lacked a control group and was of a small sample size, hence it is difficult to reliably assess the efficacy of VEGF gene therapy.

Stewart et al. performed the phase 2 trial, Randomized evaluation of VEGF for Angiogenesis (REVASC), that enrolled 67 patients with severe angina pectoris with no option for revascularization. They used adenovirus containing vascular endothelial growth factor (AdVEGF121) for intramyocardial injection. Of the 67 patients enrolled, 35 continued maximum medical treatment and 32 received AdVEGF121. Exercise time to 1 mm ST-segment depression, the predefined endpoint, was increased in the AdVEGF121 group compared to control at 26 weeks ($p=0.026$), but not at 12 weeks. Total exercise duration and time to level 2 angina were also significantly improved in the AdVEGF121 group compared to control at weeks 12 ($p=0.008$ and $p=0.006$) and 26 ($p=0.015$ and $p=0.003$). Significant improvements in the Canadian Cardiovascular Society class score was evident in the AdVEGF121 group as compared to control as early as 6 weeks and continued to improve at 12 and 26 weeks ($p=0.001$ at all timepoints). Despite the inability to blind for treatment assignment due to performance of mini-thoracotomy, this study provided the first large scale objective data for the efficacy of therapeutic angiogenesis in patients with severe symptoms who are not candidates for traditional revascularization procedures.

Through studies mentioned above, efficacy and safety of gene transfer in ischemic myocardium was established. But the use of operative thoracotomy to deliver DNA precluded the use of randomization against placebo effect. Though minithoractomies were well tolerated even in individuals with advanced heart disease, the procedure is associated with risk of general anesthesia and surgical manipulation. Animal models had demonstrated equivalence of gene expression and protein secretion whether AdvVEGF121 is injected from endocardial or epicardial side of heart [48]. Thus catheter based techniques of left ventricular injections and intracoronary infusion of viral vectors encoding angiogenic growth factors were tried and thus initiated trials of non-operative gene transfer in ischemic myocardium.

Laitinen et al. studied the safety and feasibility of catheter-mediated VEGF plasmid/liposome (P/L) gene transfer in human coronary arteries after PCI in a randomized, double-blinded, placebo-controlled study [44]. Ten patients received

VEGF P/L, three patients received beta-galactosidase P/L, and two patients received Ringer lactate. Catheter-mediated intracoronary gene transfer performed after angioplasty was found to be safe and well tolerated. There were no VEGF plasmid or recombinant VEGF protein present in systemic circulation after gene transfer. However, there were no differences in the degree of coronary stenosis between treatment and control groups on angiography after 6 months.

Left ventricular injections of using a steerable deflectable catheter with the guidance of NOGA left ventricular electromechanical mapping was performed in patients with chronic myocardial ischemia. In this single blind, placebo controlled pilot study of catheter based myocardial gene transfer, patients were randomized to receive 200 μg of naked plasmid DNA encoding phVEGF-2 or placebo [47]. phVEGF-2 transfected patients had reduced angina and improved myocardial perfusion. But due to FDA regulations, the needle was not deployed into the myocardium in the placebo group though all the other steps were performed. Hence it is not clear whether the injection itself is contributing to improvement in recruitment of progenitor cells leading to improved myocardial perfusion.

Another double blind placebo control trial involving injection of naked plasmid DNA encoding phVEGF-2 into left ventricular myocardium via catheter in escalating doses of 200 μg (n = 9), 800 μg (n = 9) or 2000 μg (n = 1) was reported by Losordo et al. [48]. Placebo group received injections of saline into the left ventricular myocardium. At 12 week follow up, angina class was decreased ($p = 0.04$). Mean duration of exercise, functional improvement by ≥2 Canadian Cardiovascular Society classes and Seattle Angina questionnaire data showed strong trend favouring phVEGF.

The first randomized double blind, placebo controlled trial evaluating the use of intracoronary infusion of VEGF gene for promoting myocardial angiogenesis was the Kuopio Angiogenesis trial (KAT) [45]. This phase 2 study involved 103 patients who had CCS II-III angina. PTCA was performed in these patients and 90% of them received stents. Following PCI, 37 patients received VEGF-adenovirus, 28 patients received VEGF plasmid liposome and 38 patients received ringer's lactate. There were no differences in clinical restenosis rate or minimal lumen diameter as measured by quantitative coronary angiography between the groups at 6 months follow up. Significant improvement was detected in myocardial perfusion in the VEGF-Adv-treated patients. But, no statistically significant differences were observed between the study groups in CCS classification, working ability or need for oral nitrates. Eight year safety follow up of the study demonstrated no significant between group mortality, MACE, cancer or diabetes [46]. Hence intracoronary VEGF gene transfer can be considered a relatively safe procedure.

The first study to use placebo plasmid as a control was the EUROINJECT-ONE trial. In this phase 2 trial, 80 patients with severe stable IHD with no other treatment option were randomized to direct intramyocardial plasmid phVEGF-A165 or placebo plasmid [49]. Intramyocardial delivery was performed with the help of catheter based delivery system. The study failed to show significant improvement in stress-induced myocardial perfusion abnormalities compared with placebo (38 ± 3% and 44 ± 2%, respectively). However, improved regional wall motion, as assessed

both by NOGA (p-0.04) and by ventriculography (p = 0.03) was evident in VEGF group compared to placebo. There were no adverse events attributable to phVEGF-A165 therapy. Further sub-study of this randomized trial was done to analyze changes in myocardial perfusion in NOGA-defined regions with intramyocardial injections of plasmid encoding human phVEGF-A165 [55]. The projection of NOGA-guided injection area onto the SPECT maps permitted quantitative evaluation of myocardial perfusion in regions treated with angiogenic substances. No differences were found between VEGF and placebo groups at baseline with regards to the perfusion defect severity. At follow-up, a trend toward improvement in perfusion defect severity at stress was observed in VEGF group as compared with placebo (68.5 ± 11.9% versus 62.5 ± 13.5%, p = 0.072).

Ripa et al. [50] performed a pilot study of combined VEGF165 gene therapy and stem cell mobilization in patients with IHD who were symptomatic but were not candidates for revascularization. Sixteen patients received intramyocardial injections of VEGF165 plasmid followed by administration of G-CSF 1 week later to mobilize progenitor cells from the bone marrow. The historical control groups consisted of 16 VEGF plasmid–treated patients and 16 control plasmid–treated patients from the Euroinject One trial. The number of circulating progenitor cells (identified via CD34+ cells) increased significantly after G-CSF treatment, but there was no improvement in the primary end-point of change in myocardial stress perfusion. The authors speculated that the homing of mobilized stem cells to the ischemic area may have been inadequate and suggested that co-transfer of a plasmid encoding stromal cell– derived factor 1, a progenitor cell–homing factor or higher doses of VEGF plasmid may be required to get full therapeutic benefit. Other explanations for a lack of benefit must also be considered. SPECT scanning has not been validated for documenting changes in perfusion that may occur following local therapy, which could potentially result in sub-segmental, non-transmural alterations in flow. In addition, the timing of G-CSF administration may not have coincided with the peak of VEGF gene expression, thereby diminishing the possibility of synergy.

The NORTHERN trial (NOGA angiogenesis Revascularization Therapy assessment by RadioNucleotide imaging) was a double blind, placebo-controlled trial in which VEGF165 DNA was delivered to left ventricular myocardium. Seventy-two no-option and 21 patients with single vessel coronary occlusion or diffuse in-stent restenosis patients were randomized to receive gene therapy (n = 48) or saline placebo (n = 45). Primary end point, which was the change in myocardial perfusion did not differ between the VEGF-treated and the placebo group at 3 or 6 months, assessed by SPECT imaging. A significant reduction in the ischemic area and improvement in perfusion scores was seen in both groups over time. Also, there was no difference between placebo and treatment arms exercise treadmill time and angina symptoms in both groups at 3 and 6 months [51].

NOVA trial, another randomized double-blind placebo-controlled multicenter gene therapy trial, was conducted to study the efficacy of adenovirus carrying VEGF121 (BIOBYPASS) in patients with refractory advanced coronary artery disease [52]. Seventeen patients with severe CAD were randomized to receive BIOBYPASS (n = 12) or placebo (n = 5) as 12 intra-myocardial injections into

the ischemic area using the NOGA mapping. Direct intramyocardial injection of BIOBYPASS was safe but did not improve exercise capacity, time to ischemia threshold or myocardial perfusion compared to sham injection in patients with refractory myocardial ischemia.

Previous controlled trials showed little benefit for VEGF gene transfer in CAD. Favaloro et al. postulated that this may be due insufficient doses of VEGF. Based on success in animal studies, higher doses of VEGF genes were tried in the GENESIS-1 trial [53]. This phase 1, open label trial was performed to assess safety of high doses of VEGF in no-option severe CAD patients. The trial had a 2 year follow up. High dose intramyocardial pVEGF165 was found to be safe at 2 year follow up. Further controlled trials with high dose VEGF gene are yet to be performed.

5.2 FGF Gene Trials

These trials involved transfer of genes for human fibroblast growth factor (FGF) (Table 18.5). First trial in this series was the Angiogenic GENe Therapy (AGENT) trial which evaluated the safety and anti-ischemic effects of 5 ascending doses of adenovirus (Ad) containing a human FGF gene in patients with chronic stable angina [56]. Seventy-nine patients underwent the study and they were randomized in a 3:1 ratio (Ad5-FGF n = 60; placebo n = 19). Single intracoronary infusion of Ad5-FGF5 was found to be safe and well tolerated. Serious adverse events during follow-up (mean, 311 days) were not different between placebo and treatment group. Patients who received Ad5-FGF4 tended to have greater improvements in exercise time at 4 weeks (1.3 versus 0.7 min, NS). A protocol-specified, subgroup analysis showed the greatest improvement in patients with baseline ETT <10 min (1.6 versus 0.6 min, $p = 0.01$).

AGENT 2 was a randomized, double blind trial which was performed to assess the effect of adenoviral gene for FGF in improving myocardial perfusion [57]. Of the 52 patient in study with stable angina and reversible ischemia, 35 patients were given intracoronary injection of Ad5FGF4. At 8 weeks, Ad5FGF4 resulted in significant reduction in ischemic defect on SPECT. Additionally, there was significant change in reversible perfusion defect size compared to placebo after removing an outlier.

AGENT 3 and 4 trials were parallel randomized placebo controlled double blind trials designed to evaluate the efficacy of low and high dose of Ad5FGF4 for therapeutic angiogenesis in myocardial ischemia [58]. Patients who remained symptomatic despite anti-anginal medications and who did not require immediate PCI or CABG were enrolled in AGENT-3, whereas those who were unsuitable for PCI or CABG were enrolled in AGENT 4 study. The primary end point was change from baseline in total exercise time at 12 weeks, and at secondary time points of 4 weeks and 6 months. These randomized, double blind, placebo controlled trials included 532 patients. Both the studies were halted when an interim analysis could not find any significant difference between the active groups and the placebo for the primary end-point in both trials. There were no differences between the dose group

Table 18.5 Trials of angiogenesis with FGF gene therapy

Trial	Agent	Delivery route	N	Control Y/N	Follow up	Outcomes/end points	Adverse events	Comments
Grines 2002 [56]	FGF	Intracoronary infusion	79	Y	311 days (mean)	Exercise treadmill time	No difference in adverse event rate	
Grines 2003 [57]	FGF	Intracoronary infusion	52	Y	8 weeks	Myocardial perfusion		
Henry 2007 [58]	FGF	Intracoronary infusion	532	Y	12 weeks	Exercise treadmill time	No significant difference	No significant benefit with treatment. Upon subgroup analysis, women had benefit with treatment
Kukula 2011[59]	FGF/ VEGF	Intramyocardial	52	Y	12 months	SPECT-MPI, exercise capacity, angina control		

and placebo for any of the secondary end points except for Canadian Cardiovascular Society angina class in high dose group. There was significant improvement of angina class in all patients over placebo at 12 week, 6 month, and 12 month in high dose group ($p < 0.05$). This was be driven by the female population as the male only subgroup, had no significant change in Canadian Cardiovascular Society class. This may be explained by the significantly lower placebo effect in the female subgroup. Other secondary end points such as time to 1 mm ST segment depression and time to angina also showed gender specific significance or nearly so effects in females at 12 weeks and 6 months in both dose groups. Though these trials were stopped prematurely, subgroup analysis on pooled data revealed gender differences in angiogenic response which may suggest effect of differential hormonal milieu on the biology of angiogenesis.

5.3 Other Gene Therapy Trials

VIF-CAD is a randomized, placebo-controlled, double-blind trial that was designed to study therapeutic angiogenesis by percutaneous intramyocardial transfer of bicistronic (vascular endothelial growth factor/fibroblast growth factor [VEGF/FGF]) plasmid (Pvif) in patients with refractory heart ischemia [59]. Fifty-two patients with refractory angina were randomized to receive VEGF/FGF plasmid ($n = 33$) or placebo plasmid ($n = 19$) into myocardial region showing stress-induced perfusion defect. Stress and rest perfusion defect at 5 months did not differ in groups. Canadian Cardiovascular Society functional class improved after 5 months ($p = 0.02$) in the treatment group, but results were non-significant at 12 months ($p = 0.06$).

Similarly, Endothelial Modulation in Angiogenic Therapy (EMAT) phase 1 double blind, placebo controlled trial tested safety and efficacy intramyocardial VEGF165 combined with L-arginine, in patients undergoing CABG [54]. It was thought that L-arginine being a nitric oxide donor may potentiate angiogenesis. Nineteen patients with surgical 3-vessel coronary disease and a severely diffusely diseased left anterior descending artery were randomized to receive placebo injection + placebo oral supplementation, placebo injection + oral L-arginine supplementation, intramuscular VEGf165 + placebo oral supplementation, or intramuscular VEGf165 + oral L-arginine supplement. Patients who received the combination of VEGF and L-arginine had improved anterior wall perfusion on positron emission tomography ($p = 0.02$), a trend toward smaller rest and stress perfusion defects ($P = 0.10$), and better anterior wall contractility ($P = 0.02$, Kruskal-Wallis) at 3 months versus baseline. Thus concomitant endothelial modulation with L-arginine not only has the potential to make angiogenesis effective but also may have implications for cell therapy trials.

6 Conclusion

The success of therapeutic angiogenesis in *in vitro* and animal models could not be extrapolated into human studies. Most of the large sample size, placebo controlled trials did not show any significant beneficial effect in clinical or imaging outcomes in IHD patients. This reflects the complex biology of IHD in humans and helps us realize that simplistic preclinical models of therapeutic approach may not always be successful in real life situations. Future research in co-delivery of growth factors, prolonging time of exposure of ischemic myocardium to a given growth factor without causing systemic effects, discovering newer selective and efficacious angiogenic factors are some of the few approaches that can help define this novel field in coming years.

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Part V
Therapeutic Implications of Angiogenesis in Miscellaneous Disease States

Chapter 19
Perspectives in New Advances in Retinal Neovascularization Pathogenesis and Therapeutic Approaches

Temitope Sasore and Jian-Xing Ma

Abstract Ocular neovascularization (NV) is the primary cause of catastrophic loss of vision in vast majority of ocular diseases including age-related macular degeneration, proliferative diabetic retinopathy and retinopathy of prematurity. The development of abnormal blood vessels in these patients is driven by a complex signaling process involving pro-angiogenic mediators such as vascular endothelial growth factor (VEGF) and anti-angiogenic factors, such as pigment epithelium-derived factor. Current anti-VEGF drugs such as ranibizumab, aflibercept and "off-label" bevacizumab are effective in only 30–40% of patients and are typically associated with undesirable route of administration, increased risk of infection and high clinical costs. This therefore increases the urgency to discover and develop additional therapeutics that are safer and more efficacious. In the last few years, several studies have contributed to understanding the underlying pathogenesis of ocular NV and the roles of different signaling cascades. Thus, this article aims to review molecular mechanisms regulating ocular NV and emerging therapeutic strategies to treat this group of diseases.

Keywords Angiogenesis • Inflammation • Vascular endothelial growth factor (VEGF) • Retina • Choroid • Ocular neovascularization (NV) • Age-related macular degeneration (AMD) • Diabetic retinopathy (DR) • Choroidal neovascularization (CNV)

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1 Introduction

Ocular neovascularization (NV), the final common pathway seen in ocular disorders such as age-related macular degeneration (AMD), proliferative diabetic retinopathy (PDR), retinopathy of prematurity (ROP), occlusive retinal vasculopathies and other ocular inflammatory diseases is characterized by abnormal or excessive in-growth of blood vessels, a process known as angiogenesis. Together, these debilitating eye complications represent the leading causes of human blindness and remain a huge socioeconomic burden for health care systems and patients worldwide. For example, recent research by the National Eye Institute estimates that the number of expected cases of AMD will increase from ~2 million to over 5 million by the year 2050 (https://nei.nih.gov/eyedata/amd). The burden placed by vision loss, not only on the healthcare system, will continue to grow unless greater steps are taken to understand and treat eye conditions that cause vision impairment. The "common denominator" shared by the aforementioned diseases is the excessive growth of unwanted vessels either at early or later stages of life, which often lead to vision impairment and blindness. Over the years, researchers have successfully mimicked some of these ocular pathologies in animal models, for example the oxygen-induced retinopathy (OIR) model to study ROP, and the laser-induced choroidal neovascularization (CNV) to study AMD [1, 2]. The understanding of these neovascular-related complications led to the identification of the well-recognized anti-vascular endothelial growth factor (VEGF) molecules, ranibizumab and aflibercept as treatment strategies [3]. However, the limitations of these drugs include high costs, limited population of responders (over two-thirds of patients fail to respond), an invasive route of administration and adverse drug reactions certainly calls for identification of improved therapies [4]. Thus, this review will discuss the current and emerging therapies for the treatment of ocular NV.

2 Ocular Neovascularization and Related Eye Diseases

Under normal physiological milieu, angiogenesis is tightly regulated by a stringent balance between pro-angiogenic factors, such as VEGF and anti-angiogenic molecules such as pigment epithelium-derived factor (PEDF). In contrast, excessive blood vessel growth is usually preceded by an imbalance between both pro- and anti-angiogenic molecules [5], consequently giving rise to a wide range of vascular diseases such as AMD, PDR, RVO, and ROP. Together, these ocular complications make up the leading causes of irreversible blindness and visual impairment worldwide.

2.1 Age-Related Macular Degeneration

AMD, the most prominent form of vision loss affecting elderly individuals aged over 50, is an ocular condition caused by defective function of the retinal pigment epithelium (RPE) which in turn leads to the development of degenerative lesions in central region of the retina, known as the macula. Located in the macula, are specialized photoreceptors cells responsible for detailed and sharp focus. As such, the breakdown of these light-sensitive cells in patients with AMD results in a gradual and steady loss of central vision. Clinically, AMD can be diagnosed as either "dry" AMD or "wet" AMD. Dry AMD, also known as non-exudative or atrophic AMD, is the most common form and occurs in 80–90% of AMD patients [6]. This is usually characterized by the accumulation of ophthalmoscopically visible yellow deposits known as drusen between the RPE and the Bruch's membrane [7]. It is understood that the presence of localized drusen ("soft" or "hard") is the result of undigested material from dysfunctional phagocytic cells that increases with aging and accumulates in the RPE [8, 9]. Typically, dry AMD begins with its early stages featuring a few drusen deposits causing slight blurred vision [9]. However, this can then progress slowly to a more advanced dry AMD (without turning into the wet form) where drusen deposits grow in size. This classic feature of late stage dry AMD causes breakdown or damage of light-sensitive retinal cells (atrophy) and as a result leads to loss of central vision.

Dry AMD can sometimes progress to wet AMD, also known as exudative or neovascular AMD, which is more severe but less prominent as it occurs in 10–20% of all AMD patients [10, 11]. In wet AMD, there is an abnormal growth of blood vessels from the choroid layer through the Bruch's membrane and into the macula, a process known as choroidal neovascularization (CNV) [12]. As these vessels are fragile, they often leak blood content and fluid into the retina, therefore leading to damage of light-sensitive cells and scarring of the macula. These pathological features usually result in the classic hallmark; presence of blind spots and loss of central vision. Wet AMD is responsible for ~90% of severe visual loss in AMD [12]. Interestingly, it is possible to experience both forms of AMD at the same time, in one or both eyes. In addition, the onset and progression of either type does not follow any particular pattern.

2.2 Proliferative Diabetic Retinopathy and Diabetic Macula Edema

DR reflects disruptions of the retinal vasculature resulting from elevated blood glucose. In addition to chronic hyperglycemia, there is evidence suggesting hyperlipidemia and hypertension also contribute to the development of DR [13]. Characteristic pathologies of this disease include pericyte loss, basement membrane changes, microaneurysms (vessel wall swelling), capillary occlusion, vascular leakage or blood retinal barrier breakdown and retinal NV [14]. DR is commonly classified

into non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR) [15]. The former, characterized by presence of hard exudates, vascular leakage and aneurysm, is subdivided into mild, moderate and severe stages. PDR on the other hand, is a more advanced stage of the disease and is characterized by the development of abnormal retinal microvessels (retinal NV).

These proliferative changes, in most cases, trigger the onset of macular edema (DME) whereby the fragile and thin vessels can leak fluid into the macula, causing it to swell [15]. DME can develop at any stages of DR but mostly occurs as the severity of DR increases. PDR and DME, the two sight-threatening features of diabetes, make up the most prevalent causes of blindness and visual impairment amongst working-age individuals (20–65) of most developed countries [16].

2.3 Retinopathy of Prematurity

Retinopathy of Prematurity (ROP) is a common vasoproliferative disorder of the retina and a major cause of blindness in ~50,000 premature infants of developed countries [17]. In the normal human fetus, the development of retinal vasculature occurs in-utero and commences from approximately 16th week of gestation till the 40th week of gestation, where the eyes become fully vascularized. However, preterm delivery of infants results in incomplete vascularization of the retina and as a consequence predisposes the immature retina to debilitating complications. ROP can be classically segregated into two distinct phases, consisting of phase I whereby initial retinal vessel growth is ceased and secretion of pro-angiogenic factors, such as insulin-like growth factor-1 and VEGF, is down-regulated at the time of preterm birth [18]. As development continues, the avascular retina becomes increasingly hypoxic therefore triggering increased metabolic activity. In phase II of ROP, the hypoxic condition from the prior stage stimulates the secretion of pro-angiogenic factors and as such triggers retinal NV as in other proliferative retinopathies [19]. With increasing severity, this second stage progresses as an uncontrolled fibrovascular proliferation into the vitreous and ultimately leads to tractional retinal detachment and the associated blindness [20].

3 Angiogenesis

In the developing mammalian embryo, vascular development occurs via two distinct, yet interrelated processes termed, vasculogenesis and angiogenesis [21]. The former involves differentiation of mesodermal cells into hemangioblasts. The peripheral hemangioblasts then differentiate into endothelial precursor cells, angioblasts, leading to the formation of tube-like endothelial structures [22]. The latter on the other hand, is characterized by the subsequent sprouting and remodeling from pre-existing vessels into a mature vascular network [23]. Altogether, vascular

development is typically an essential requirement for biological processes such as wound healing, organ development and the female reproductive cycle.

Similar to other organs, development of the mammalian eye is influenced by the intense process of angiogenesis which is necessary for the oxygenation of ocular tissues [24, 25]. The molecular basis of angiogenesis is characterized by an orderly cascade of complex events regulated by angiogenic molecules and degrading enzymes [26]. The release of pro-angiogenic factors such as VEGF, fibroblast growth factor and angiopoietin 2 are largely in response to hypoxia or other endogenous stimuli. Upon their release, these proangiogenic factors bind to surface receptors of neighboring vessels, thereby promoting endothelial cell activation [27]. Subsequently, enzymes such as matrix metalloproteinases are secreted by activated endothelial cells. These extracellular proteases are responsible for the degradation of the basement membrane. Therefore, this allows the proliferation and migration of endothelial cells towards angiogenic stimuli such as VEGF. The proliferating cells connect with nearby endothelial cells, and specific adhesion molecules, such as intergrins (αvβ3, αvβ5) are released to accommodate cell migration and neovessel sprouting [28]. As the sprouts elongate, proliferating endothelial cells are reorganized to form tube-like structures with a central lumen. Each individual blood vessel tube buds with adjacent tubes, thereby producing a functional vascular network capable of circulating blood. Additionally, pericytes and smooth muscle cells are then recruited to stabilize the newly formed vessels [27].

4 Mammalian Ocular Angiogenesis and the Role of VEGF in Mammalian Retina

The mammalian retina receives its nutrition from two discrete circulations, the retinal and choroidal circulations [29, 30]. The choroidal and hyaloid vessel which conducts ~80% of retinal circulation nourishes the outer retina, to ensure the oxygenation of the retina during the initial development of the eye as the inner retinal vasculature is absent [29]. In contrast, the remaining ~20% circulation is carried by the central retinal artery emerging from the optic nerve head, to nourish the inner retinal layers during the late eye development [24]. During development, the mammalian ocular vascular network undergoes key physiological changes.

The matured retinal vasculature is made up of two laminar layers: the primary superficial layer and the deep vascular layer involved with the development of astrocytes and Müller cells, respectively. By virtue of its induction by hypoxia-inducible factor 1, a transcription factor which binds to the hypoxia responsive element in the promoter region of the VEGF gene, VEGF is the principal mediator needed for stimulating retinal vascular development [31]. Studies by Miller et al. and Alon et al. report a correlation between the spatial and temporal changes in VEGF mRNA levels in a rat model of retinal ischemia [32, 33]. Furthermore, Aiello et al. also assess the anti-angiogenic effect of VEGF-neutralizing proteins in a mouse OIR

model. Here, authors report the human Flt or murine Flk chimeric protein resulted in complete inhibition of retinal NV in treated mice [34]. Ozakia and colleagues demonstrated that PTK787, a VEGF inhibitor, blocked the phosphorylation of VEGF, completely inhibited retinal NV in a murine OIR model and partially inhibited retinal vascularization during development, therefore suggesting that VEGF plays a vital role in retina NV [35].

5 Current Therapies for Ocular Neovascularization

The importance of ocular NV is crucial to the pathology of the aforementioned ocular complications, with growth factors such as VEGF implicated in the disease process. As such, therapeutic targeting of VEGF in the posterior eye has been a central focus for the treatment of these diseases.

5.1 Vascular Endothelial Growth Factor Inhibitors (Anti-VEGFs)

Pegaptanib (Macugen®; Eyetech, Palm Beach Gardens, FL) a ribonucleic acid aptamer directed against the VEGF165 isoform, was the first anti-angiogenic therapy approved for neovascular AMD in 2004 [36]. Bevacizumab (Avastin®; Genentech/Roche, San Francisco) is a full-length, humanized monoclonal antibody that binds to all VEGF-A isoforms. In 2004, Avastin was approved exclusively for the treatment of metastatic colon cancer and often used as off-label to treat ocular NV following its tolerability and efficacy evaluation [3]. In 2006, in an effort to improve retinal penetration and systemic half-life, Ranibizumab (Lucentis®, Genentech, San Francisco), a corresponding Fab fragment of full-length Bevacizumab was specifically designed and approved by FDA for treatment of CNV due to AMD [37]. It is a humanized, recombinant, monoclonal antibody Fab fragment which binds and neutralizes all identified VEGF-A isoforms. Aflibercept (Eylea® (VEGF Trap-Eye), Regeneron), which was approved by FDA in 2011 for treatment of exudative AMD, is a humanized, recombinant VEGF-receptor fusion protein that binds to all forms of VEGF-A, VEGF-B and the associated placental growth factor with high affinity, thereby preventing activation of cognate VEGF receptors [38].

Table 19.1 summarizes the features of current anti-VEGF drugs.

Table 19.1 Molecular characterization of select FDA-approved anti-VEGF drugs

Name	Molecular weight (KD)	Half-life (days)	Binding specificity	Fc fragment	Structure components
Pegaptanib	50	10	VEGF-164/165	No	Pegylated oligonucleotide aptamer
Bevacizumab	149	5.6	VEGF	Humanized IgG	Full length humanized anti-VEGF monoclonal antibody
Ranibizumab	48	3.2	VEGF-A	No	Humanized monoclonal antibody with only Fab
Aflibercept	115	4.8	VEGF-A, VEGF-B, PIGF	Human IgG	Chimeric receptor comprised of the second Ig domain of VEGFR-1, the third Ig of domain VEGFR-2 in the Fab, and a human IgG fc

5.2 Photodynamic Therapy (PDT) and Laser Photocoagulation

Besides the broadly used anti-VEGF's, visudyne photodynamic therapy (PDT) and laser photocoagulation represent other therapeutic strategies for the clinical management of ocular NV [39]. PDT is a two-step procedure whereby a pharmacological photosensitizer (e.g. verteporfin (Visudyne®)) is first administered intravenously, followed by its subsequent activation using a laser light. This visible light induces a photo-oxidative damage of vascular endothelium, thereby selectively destroying unwanted retinal vessels. However, PDT appears to stimulate the release of VEGF and other inflammatory mediators [40], an initial problem in ocular NV. Thus, combination of PDT therapy with an intravitreal steroid (e.g. triamcinolone acetonide) or anti-VEGF (e.g. ranibizumab) adjunct is increasingly being studied and applied to inhibit the expression of VEGF and other inflammatory mediators [41]. Laser photocoagulation, on the other hand, uses laser burns to directly reduce retina vessel leakage or, in some case, destroys tissue in the peripheral retina, therefore reducing oxygen demand and alleviating ischemia in central retina. These procedures are often used to halt disease progressing to a more serious condition such as PDR and DME.

5.3 Limitations

Typically, these anti-VEGF biologicals are administered through intravitreal injections into the vitreous of the patients' eye. Despite their therapeutic benefits in some AMD patients, long-term visual improvements of anti-VEGF therapies are impeded in 60–70% of patients due to sub-optimal dosing, genetic variations, rapid drug

clearance, tachyphylaxis and poor access to clinics [4, 38, 42]. As such, patients usually require monthly in-clinic injections in order to obtain significant therapeutic efficacy. Owing to their invasive route of administration, this classic treatment modality is associated with potentially severe complications including increased risk of infectious endophthalmitis (up to 1.6% occurrence following intravitreal anti-VEGF injection) [43], ocular hemorrhage (up to 10% occurrence) [44]; intraocular inflammation (1.4–2.9% occurrence) [45], retinal detachments (less than 1%) [46] and not to exclude the enormous yearly cost of ranibizumab and aflibercept.

Although the application of verteporfin PDT and laser photocoagulation are less common, the major drawback of these treatment strategies is that they can entail small retinal scars which can cause blind spots in patients' field of view and may induce vision loss [41]. A complementary approach to circumvent these drawbacks is to engineer the development of VEGF-independent molecules that are more efficacious and could be delivered topically or as sustained release implants, therefore reduce the frequency of intravitreal injections.

6 Emerging Therapies to Treat Ocular Neovascularization

Aside from the anti-VEGF drugs, there are several potential treatment strategies emerging through the pipeline and hold promise for improving treatment of ocular NV. These include endostatin, PDGF inhibitors, PEDF, integrin receptor blocker, complement cascade inhibitors, gene therapies and anti-immune/inflammatory molecules.

6.1 Endostatin

Endostatin, another endogenous inhibitor of angiogenesis, has been demonstrated to have significant inhibitory effect on retinal NV [47]. Here, authors showed that endostatin prevented endothelial cell migratory and tubular network formation processes, as well as the secretion of VEGF in endothelial cells. Moreover, intraocular injection of endostatin convincingly reduced neovascular areas in mouse OIR model. However, as endostatin is unstable in properties and is unable to penetrate through the BRB, efforts are being made to improve the permeability of endostatin. Recently, Li et al. used a genetic engineering method to fuse Tat PTD, a protein transduction domain of the Tat protein of HIV-1, with endostatin. The successful generation of Tat PTD-endostatin (Tat PTD-Es) not only resulted in increased ocular barrier penetrance following topical administration, but also maintained inhibitory effects on CNV [48]. Tat PTD-Es has been modified by the introduction of a tripeptide of arginine-glycine-aspartic (RGD) to its structure, which improves its binding specificity to $\alpha_v\beta_3$ integrin that is highly expressed on endothelial cells in

pathologic conditions [49]. Tat PTD-Es-RGD similarly demonstrates high BRB permeability and inhibits abnormal retinal angiogenesis and therefore could offer an innovative therapeutic option for the prevention of retinal NV through eye drop formulations.

6.2 Pigment Epithelium-Derived Factor

Pigment epithelium-derived factor (PEDF), a glycoprotein secreted by most cells, is well understood to have neurotropic and anti-angiogenic activity in mammalian retina [50]. As a potent endogenous inhibitor of ocular angiogenesis, PEDF halts the development of neo-vessels by inducing apoptosis of endothelial cells activated for new vessel formation [51]. Studies reporting decreased PEDF levels in the vitreous, aqueous humors and retinas of PDR-affected eyes highlight the importance of PEDF to human blindness [52, 53]. As several ocular NV pathologies are characterized by neuronal loss, PEDF presents as an attractive therapeutic protein as a result of its multifunctional activity.

Emerging as a therapeutic strategy, Mori et al. demonstrated that adenovirus-mediated gene transfer of human PEDF by intraocular injection destabilized CNV in mouse eyes [54]. Amaral and Bacerra also reported that PEDF34-mer (Asp(44)-Asn(77)), a functional PEDF N-terminal peptide, exerted significant PEDF-like anti-angiogenic effect in a rat model of laser-induced CNV [55]. According to the study, subconjunctival administration of 0.1 and 1 pmol/d of the synthetic peptide dose-dependently reduced CNV lesion volumes compared to vehicle [55]. Furthermore, supporting evidence has also shown that PEDF over-expression delays photoreceptor and neural retinal cell death – a contributing factor in retinal diseases [56]. Conversely, it has been postulated that increased circulatory PEDF in Type 1 and Type 2 diabetes patients may exacerbate systemic symptoms of diabetes such as impaired wound healing due to impaired peripheral angiogenesis [57, 58]. However, the local delivery of PEDF into the affected eye of PDR or AMD patients to bolster the declining levels of PEDF in ocular tissues may result in the inhibition of unwanted vessel growth and potentially overcome unwanted side effects.

In theory, this approach may hold up for ocular neovascular diseases including PDR and AMD, and also serve as an adequate means to combat the activity of pro-angiogenic stimuli, such as VEGF.

6.3 Platelet-Derived Growth Factor Inhibitors

Platelet-derived growth factor (PDGF) is a potent mitogen known to be active on several cell types, in particular fibroblasts and vascular smooth muscle cells [59]. This growth factor is involved in enhancing vascular growth by promoting migratory and proliferative responses of endothelial cells as well as recruitment of

pericytes [60]. Four PDGF ligands namely A, B, C and D make up the PDGF family [61]. The aforementioned polypeptide chains which function as homodimers (PDGF-AA, BB, CC and DD) and heterodimers (PDGF-AB) recognize and bind to tyrosine kinase receptors PDGFRα and PDGFRβ [61]. Several studies have documented the role of PDGF in retinal NV. According to Seo et al., PDGF expression specific to photoreceptor results in severe retinal NV and retinal detachment [62]. Supportive evidence by Freyberger and colleagues also reveal increased PDGF levels in vitreous fluid of PDR patients [63]. Thus, the inhibition of PDGF remains an attractive option to treat ocular NV.

From a therapeutic standpoint, the antagonism of PDGF by a designed Ankyrin repeat protein (DARPin) which selectively binds to PDGF-BB has been shown to suppress retinal angiogenesis [64]. In this study, intraperitoneal injection (10 mg/kg) or intraocular injection (1.85 μg) of the anti-PDGF-BB DARPin significantly reduced subretinal and retinal NV in mouse laser-induced CNV and OIR, respectively. Furthermore, E10030 (Fovista – Ophthotech, New York, USA), an anti-PDGF pegylated aptamer, is in advanced stages of clinical trial for treatment of wet-AMD. Following the successful completion of a phase I safety and tolerability study which recorded no dose-related toxicities of E10030 in combination with ranibizumab in NVAMP subjects, data from the phase II study reported a similar favorable safety and efficacy profile in wet AMD participants [65]. In this study, E10030 in combination with anti-VEGF demonstrated statistically and clinically significant superiority in visual acuity gain compared to ranibizumab alone. Taking together these promising findings, a phase III study which will assess the safety and efficacy of E10031 in combination with anti-VEGF drugs compared to anti-VEGF alone has been initiated (Phase 3).

6.4 Integrin Receptor Blocker

Integrins, which are a group of heterodimeric transmembrane proteins expressed by endothelial cells, are composed of α and β subunits and orchestrate the attachment between a cell and its surrounding extracellular matrix components including fibronectin, laminin, collagen, thrombospondin and fibrinogen [66]. The role of specific integrins $\alpha_v\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ has been reported in AMD [28]. In particular, integrin $\alpha_v\beta_3$ is known to be highly expressed within the endothelial cells of developing retinal blood vessels of DR patients and conversely, choroidal and retinal NV can be suppressed by $\alpha_v\beta_3$ antagonists [28], thus indicating that integrin α_v is a promising therapeutic target to treat ocular NV. Additional evidence has emerged from the use of a potent α_v integrin antagonist, JNJ-26076713, in rodent models of ocular vasculopathy [67]. Oral administration of this peptide which targets both $\alpha_v\beta_3$ and $\alpha_v\beta_5$, significantly attenuates retinal NV and reduces retinal vascular permeability in mouse OIR and diabetic rats, respectively [67].

Currently in clinical trial are two $\alpha_v\beta_1$ integrin antagonists, JSM6427 and Volociximab, for the treatment of AMD. Reports from Phase 1 clinical trial reveal JSM6427 to increase mean best corrected visual acuity in patients with exudative

AMD (https://clinicaltrials.gov/ct2/show/record/NCT00536016). Interestingly, α_v integrin mediates its effect in association with other pro-angiogenic factors, in particular VEGF. In line with this, it may appear prudent to develop combination therapy including α_v integrin antagonists and VEGF inhibitors. For example, a Phase 1 clinical trial assessing the safety and efficacy of intravitreal Volociximab in combination with ranibizumab for treatment of neovascular AMD showed to improve visual acuity in human subjects (https://clinicaltrials.gov/ct2/show/NCT00782093). Taken together, the potential of integrin antagonists may be positive indication for therapeutic intervention for ocular NV.

6.5 Thrombospondin-1 (TSP)

Thrombospondin-1 (TSP1), a large extracellular glycoprotein, is a member of the TSP gene family typically secreted by RPE and vascular endothelial cells [68, 69]. TSP1 is widely known to orchestrate a wide array of cellular processes including cell migration, regulation of TGF-β during inflammation, wound healing and angiogenesis. TSP1 has been shown to be a major mediator of ocular homeostasis and congruently, retinal vascular development and NV are mitigated by increased levels of TSP1 [70]. The observations of low levels of TSP1 in choriocapillaries of AMD patients, and vitreous of DR patients also highlight the significant role that TSP1 plays in overall retinal vascular homeostasis [71, 72]. Recently, Wang and colleagues investigated the impact of TSP1 deficiency in a mouse model of CNV and the antiangiogenic influence of TSP1 peptide agonist. Here, it was evident that TSP1-deficient mice developed significantly larger areas of CNV compared to WT animals. Furthermore, this effect was shown to be reversed in TSP1-deficient mice following treatment with TSP1 mimetic peptide but to a greater extent in WT mice [73]. The archetypal phenomenon of AMD and DR is the display of both angiogenesis and inflammation in an exacerbated manner. Thus, TSP1 has also been reported to exert anti-inflammatory activity in the eye via the upregulation of TGFβ in RPE cells [74]. Altogether, these findings suggest that TSP1 plays a key role in the progression of CNV in AMD and that its modulation through TSP1 mimetic peptides could be perhaps complement current gold standard therapies for ocular NV diseases.

6.6 Peroxisome Proliferator-Activated Receptor Alpha (PPARα) Agonist

Peroxisome proliferator-activated receptor-alpha (PPARα), a member of the nuclear receptor superfamily, is a ligand-activated transcription factor expressed in several tissues including the liver, intestine, kidney and skeletal muscle [75]. In association with its role in modulating lipid and glucose metabolism, this transcription factor

has also been reported to have anti-inflammatory and anti-angiogenic activities [76, 77]. The activation of PPARα is initiated via the binding of endogenous or synthetic ligands such as fatty acids or fibrate. Compelling evidence for therapeutic effects of PPARα agonist in retinal vascular leakage and NV is obtained from two large clinical trials; the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) and the Action to Control Cardiovascular Risk in Diabetes (ACCORD) which revealed preventive effects of Fenofibrate (PPARα agonist) in diabetes-related microvascular complications including DR in type 2 diabetes [78, 79].

In light of this, our group first demonstrated that fenofibrate significantly inhibits hallmarks of PDR and DME in rodent DR models [77]. Here, oral administration of fenofibrate suppressed retinal vascular leakage, leukostasis and levels of pro-inflammatory factors in STZ-diabetic rats and Akita mice. Furthermore, in STZ-diabetic rats and a separate OIR model of ischemic retinopathy, intravitreal injection of fenofibrate also attenuated retinal inflammation/hyperpermeability and retinal NV, respectively. Therefore, this indicates that the protective effect of fenofibrate on retinal inflammation and angiogenesis are independent of its systemic effect, and instead may be attributed to a direct ocular effect. Moreover, compared with current anti-VEGF drugs which are administered via invasive intraocular injections, the robust ocular effects of fenofibrate on DR and DME achieved by oral delivery along with its distinct pharmacokinetic behavior make PPARα agonists highly advantageous for the treatment of ocular NV diseases.

6.7 Wnt Pathway Blocker

Wnts are a family of secreted cysteine-rich glycoproteins which regulate gene expression via both canonical and non-canonical Wnt signaling pathways. Of the two distinct cascades of Wnt signaling, the former has been reported to play significant roles in vascular development and angiogenesis [80]. Typically within the canonical pathway, the binding of Wnt ligands to the co-receptor complex of frizzled (Fz) receptors and low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6), induces the subsequent phosphorylation and activation of downstream kinase nodes, leading to transcription of Wnt target genes such as VEGF, PDGF and TNF-α [81]. As previously reported by our group, retinal levels of total β-catenin, a key signaling factor of the canonical Wnt pathway, is significantly more abundant in patients with NPDR [82]. Additional confirmation of Wnt pathway activation in pathologic retina stemmed from data which also showed increased retinal levels of β-catenin and LRP5/6 co-receptors in several rodent models of retinal NV [82].

With this, it would appear prudent to inhibit the Wnt signaling pathway as a therapeutic strategy to treat ocular NV. In light of this, our group has convincingly shown the anti-inflammatory and anti-angiogenic activities of DKK1, a specific inhibitor of the Wnt pathway. Intraocular injection of this peptide decreased retinal levels of inflammatory marker, ICAM-1, and retinal vascular leakage in STZ-diabetic rats. In the same study, local injection of DKK1 into the vitreous of OIR

rats also appeared to reduce neovascular areas and tufts, as well as VEGF levels in rat retina, thereby demonstrating anti-angiogenic efficacy *in vivo* [82]. In a separate study, we also demonstrated the inhibitory effect of a monoclonal antibody (Mab) specific for the E1E2 domain of LRP6, Mab2F1, on canonical Wnt signaling and its therapeutic potential for DR [83]. In summary, Mab2F1 blocks the accumulation of β-catenin and overexpression of angiogenic/inflammatory factors in retinal cells. *In vivo* studies also reveal its anti-angiogenic and anti-permeability effects in OIR rats and late stages of STZ-induced diabetic rats [83]. Altogether, these studies showcase the therapeutic and beneficial effects of canonical Wnt signaling pathway inhibitors in ocular NV.

6.8 Corticosteroid Implants

As noted, inflammation is a common pathological feature in PDR and DME. In light of this, corticosteroids have been noted to exert anti-inflammatory activity by blocking macrophage release of angiogenic factors and suppressing ICAM-1 expression, thereby stabilizing the BRB through increased tight junction proteins [84]. Three sustained-release corticosteroid implants currently in the development for treatment of DME include Ozurdex (Allergan), Iluvien (Alimera Science) and Retisert [85]. Ozurdex is a tiny biodegradable implant that slowly releases 0.7 mg dexamethasone into the vitreous and has been approved by the FDA for the treatment of DME secondary to BRVO or CRVO [86]. Both Iluvien (a nonbiodegradable polymer) and Retisert (a nonbiodegradable implant) release 0.19 mg and 0.59 mg fluocinolone acetonide into the vitreous, respectively [85]. The latter, which is typically inserted intravitreally, releases active steroid and has been approved in some European countries to treat chronic DME but not in the United States. Moreover, clinical efficacy studies in the United States have reported that Iluvien significantly reduced foveal thickness for up to 36 months [87].

6.9 Complement Cascade Inhibitors

Typically, the complement system contributes to innate immunity and mediates the inflammatory responses seen in physiological and pathological conditions. There are several elegant studies implicating the link between ocular NV and the complement system as shown by increased levels of plasma C3adesArg in NVAMD subjects; and deposits of complement C5b-9 complexes in choriocapillaries of DN subjects [88, 89]. Complement targeted drug molecules are recognized as a promising therapeutic strategy for ocular NV diseases. A number of these compounds are currently in early stages of clinical trials. For example, POT-4/Compstatin (Potentia Pharmaceuticals/Alcon) is a “gel-like” synthetic peptide which binds and inhibits the cleavage of complement component 3 (C3) to its active form C3a and C3b [90].

Intravitreal injection of compstatin has been shown to suppress drusen formation in cynomolgus monkeys, primate model with early-onset macular degeneration [91]. Successful phase 1 safety and efficacy studies demonstrate therapeutic efficacy in AMD patients with subfoveal CNV (NCT00473928. http://www.clinicaltrials.gov/ct2/show/NCT00473928) and phase 2 clinical studies is in the pipeline to test efficacy of intravitreal POT-4 in neovascular AMD.

ARC1905 (Optotech Corporation) is a pegylated aptamer designed to target and prevent the cleavage of C5 into its active C5a and C5b forms [92]. A phase 1 clinical trial assessed the safety and tolerability of this anti-C5 aptamer in combination with anti-VEGF, Lucentis, in patients with wet AMD (NCT00709527. https://clinicaltrials.gov/ct2/show/NCT00709527)

6.10 Other Small Molecule Inhibitors

Activation of cysteinyl leukotriene (CysLT) receptors, which are expressed in several tissues, mediates increased vascular permeability and ischemic retinopathy [93]. Of recent, Kennedy et al. identified an inhibitor of CysLT1 and CysLT2 receptor (CysLT1/2R), quininib, which demonstrated significant anti-angiogenic activity *in vitro* in EC tubular network assay, *ex vivo* in a mouse aortic ring assay and in zebrafish developmental angiogenesis assays. This CysLT1/2R antagonist was also reported to be safe and effective on preventing retinal NV in mouse OIR model when injected intravitreally [94]. Furthermore, to enhance its ocular release for up to 4 weeks, quininib was formulated into hyaluronan (HA) microneedles which showed to maintain its ocular anti-angiogenic and safety profile. In addition, intravitreal quininib-HA also attenuated CysLT-induced retinal vascular permeability in rats [95].

The phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) signaling pathway has also been reported to be an alternative or adjunct target to treat ocular NV [96, 97]. Individual or combinations of PI3K/Akt/mTOR inhibitors including LY294002, NVP-BEZ235, PI-103 and rapamycin (Sirolimus) display significant anti-angiogenic effect in developmental and pathological angiogenesis in zebrafish and mouse retina [96, 98–100]. In particular, the development of sirolmus has transitioned up to Phase II clinical testing for treatment of AMD and DME (NCT01445548, http://www.clinicaltrials.gov/ct2/show/NCT01445548).

7 Conclusion and Perspective

Significant advances have been made towards understanding the molecular mechanisms regulating physiological and pathological angiogenesis. This progress has led to a comprehensive understanding of ocular NV which has yielded the discovery of current drug molecules to treat ocular NV-related diseases. The most common of these therapeutics are FDA approved anti-VEGF's such as ranibizumab and

aflibercept, and the off-label bevacizumab. It must be ceded that the future development of anti-VEGF therapies to treat ocular NV is undergoing a paradigm shift as a result of their limited therapeutic efficacies, high clinical costs, invasive intravitreal injections and unwanted side effects. Furthermore, ongoing research in the field of ocular anti-angiogenic therapy has offered what may prove to be alternative ways of treating NV-related blindness. A vast majority of the emerging drug therapies include, but not limited to those highlighted in this review. Among these, the PPARα agonist, fenofibrate, seem to be the most promising drug molecule for the prevention of ocular NV. Fenofibrate boasts high anti-angiogenic efficacy in animal models which recapitulate many of the clinical manifestations of CNV and retinal NV in humans. Moreover, the biopharmaceutical characteristic of the fenofibrate enables it to be delivered orally or as microparticles facilitating its sustained release over extended periods. In conclusion, indeed great strides have been made in the identification of novel therapies for the treatment of retinal NV, and the discovery of these new drug targets brings us one step closer toward the goal of delivering innovative therapies that are safe and more efficacious for patients affected by ocular NV.

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Chapter 20
The Role of Sex Steroids in Angiogenesis

Yuen Ting Lam, Laura Lecce, Christina A. Bursill, and Martin K.C. Ng

Abstract Sex steroids such as estrogen and testosterone are key mediators of angiogenesis. They are implicated in both physiological and pathological angiogenesis such as during the menstrual cycle, wound healing and cancer growth and progression. Sex steroids regulate many aspects of angiogenesis through both classic genomic transcription modulation and rapid non-genomic signaling pathways. In this capacity, sex steroids modulate endothelial and progenitor cell functions such as proliferation, migration and attachment, which are all essential components involved in neovascularization. Since sex steroids are known to augment angiogenesis which is vital to tumor progression and growth, common treatment of hormone responsive tumors is through sex steroid receptor antagonists or hormone deprivation. Due to the involvement of sex steroids in necessary physiological functions as well as the potential to promote pathological angiogenesis, it is fundamental that the mechanisms behind sex steroid-mediated neovascularization are understood.

Keywords Angiogenesis • Androgens • Estrogen • Testosterone • Endothelial cells • Growth factors

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Abbreviations

AR	Androgen receptor
ARE	Androgen response element
bFGFR	Basic fibroblast growth factor receptor
BSA	Bovine serum albumen
CDK	Cyclin-dependent kinases
CFU	Colony-forming unit
cGMP	Cyclic guanosine monophosphate
DHT	Dihydrotestosterone
ECM	Extracellular matrix
EDC	Estrogen-dendrimer conjugates
EGF	Epidermal growth factor
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cells
ER	Estrogen receptor
ERE	Estrogen response element
ERK	Extracellular signal regulated kinases
FAk	Focal adhesion kinase
FGF-2	Fibroblast growth factor-2
Flt1	VEGF receptor-1
Grb2	Growth factor receptor-bound protein 2
HGF	Heptocyte growth factor
HIF-1	Hypoxia-inducible factor-1
HRE	Hormone-responsive elements
HUVEC	Human umbilical vein endothelial cells
IL-1	Interleukin-1
IL-1Ra	IL-1 receptor antagonist
KDR/Flk-1	VEGF receptor-2
MAPK	Mitogen-activated protein kinases
mTOR	Mammalian target of rapamycin
NF-κB	Nuclear factor-κB
PAF	Platelet-activating factor
PAI	Plasminogen activator inhibitor
PDGF	Platelet-derived growth factor
PI3K	Phosphatidylinositol 3-OH kinase
RhoA	Ras homolog gene family member A
ROCK	Rho-associated protein kinase
SDF-1	Stromal cell-derived factor-1
SHC-1	SHC-transforming protein 1
Sos	Son of sevenless
TAM	Tumor-associated macrophages
TERT	Telomerase reverse transcriptase
TNF-α	Tumor necrosis factor-α
VEGF	Vascular endothelial growth factor

1 Introduction

There is accumulating evidence that sex steroids regulate key events in angiogenesis (the formation of new blood vessels from pre-existing ones) and vasculogenesis (involvement of bone marrow-derived progenitor cells to new blood vessel formation). The direct modulation of pathological angiogenesis such as tumor neovascularization by estrogens and androgens are extensively studied. While estrogens are also well known for its direct effects on the recurrent hormone-regulated neovascularization of the female reproductive tract, emerging data also demonstrates a role for androgens on the regulation of angiogenesis. This chapter will discuss the role of sex steroids in angiogenesis and the mechanisms by which they function.

2 Sex Steroids and Angiogenesis: Basic Mechanisms

2.1 Overview of the Actions of Sex Steroid

Classic genomic regulation involves the binding of sex steroids to specific intracellular hormone receptors which regulate gene expression and protein synthesis as illustrated in Fig. 20.1. Ligand-bound receptors become active through

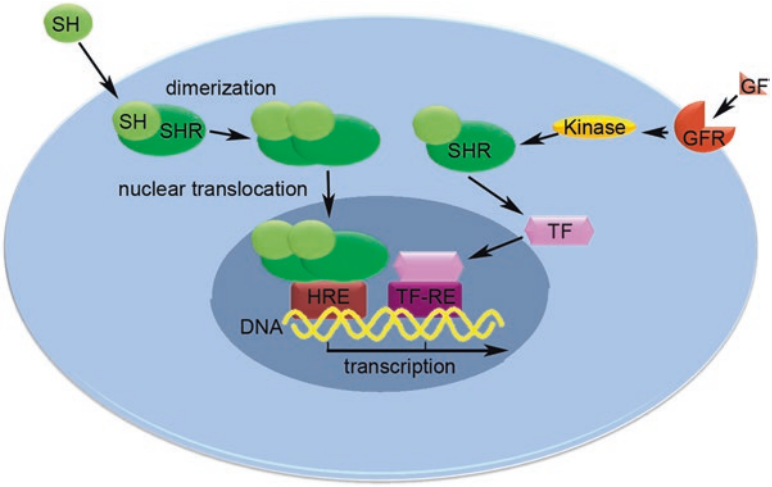

Fig. 20.1 Genomic actions of sex hormones. Sex hormones (*SH*) bind to sex hormone receptors (*SHR*) which translocate to the nucleus following dimer formation. The SHR binds to a hormone response element (*HRE*) within the promoter region of specific genes to initiate transcription. Transcription can also be initiated through growth factor (*GF*) binding to growth factor receptors (*GFR*) which activate specific kinases. These active kinases then lead to SHR activation of other transcription factors (*TF*) which bind to transcription factor response elements (*TF-RE*) causing gene transcription

dimerization before translocation to the nucleus. Once in the nucleus, the hormone receptors interact with their specific hormone responsive elements (HRE) present on DNA. This interaction causes the induction or repression of target genes through gene transcription which ultimately regulate many cellular functions. Sex steroids also act via rapid non-genomic signaling pathways which involve sex steroid binding to specific membrane-associated receptors. This modulates other membrane-associated or cytoplasmic proteins, and subsequently activates signaling cascades.

2.2 Estrogen-Mediated Neovascularization

Estrogen directly modulates the growth and survival of blood vessels *in vivo*. In oophorectomized rabbits, the withdrawal of endogenous estrogen causes vascular degeneration in the bladder, which is restored by exogenous estrogen administration [1]. Estrogen administration in ovariectomized rats improves reendothalialization following endothelial injury [2]. Estrogen administration also enhances the recovery of blood perfusion after ischemic injury by capillary regeneration within the tissue, which is associated with an increase in hypoxia inducible factor-1 (HIF-1) and vascular endothelial growth factor (VEGF) expression, that are key inducers of angiogenesis [3]. Furthermore, estrogen-mediated neovascularization is accomplished by complex molecular pathways that regulate specific endothelial and progenitor cell functions which collectively enable new blood vessel regrowth.

2.3 Estrogen and Genomic Pathways of Regulation

Estrogen receptors (ERs) are ligand-activated nuclear transcription factors that are expressed in human vascular endothelial cells [4, 5]. ERs mediate the effects of estrogen on gene regulation which modulate many facets of the angiogenic features of endothelial cells [4–6]. Of the two known ERs, ERα is most often implicated in angiogenesis and plays a larger role in endothelial progenitor stimulation than ERβ [7]. In the absence of both receptors, estrogen-promoted vascular protective mechanisms are ineffective [8]. Estrogen induces temporally distinct cascades of transcriptional events in the whole vasculature. ERs mediate the earliest modulation of gene expression by estrogen. These ER-mediated gene targets are transcriptional factors that are enriched with direct ER binding sites in their promoters. Subsequently, these early-induced transcription factors recruit others transcription factors that are involved in lipid metabolism and cellular growth and proliferation, leading to a propagation of transcriptional cascades that eventually modulate the long-term effects of estrogens on vascular functions [9].

2.4 Estrogen and Non-genomic Rapid Signaling

In addition to the classic genomic pathway, increasing evidence suggests estrogen stimulates endothelial cell functions, such as vasodilation, cell growth, migration and survival via non-genomic signaling [10–12]. Using membrane-impermeable estrogen conjugates, estrogen- bovine serum albumen (BSA) or estrogen-dendrimer conjugates (EDC), estrogen has been shown to bind to membrane-associated ERα and activate phosphatidylinositol 3-OH kinase (PI3K) signaling events and Akt phosphorylation. This is achieved through the binding of ERα with Gαi, c-Src (cellular-src) tyrosine kinase, and p85 (the regulatory subunit of PI3K). Subsequently, endothelial nitric oxide synthase (eNOS) is activated, leading to a rapid release of nitric oxide (NO) from endothelial cells as shown in Fig. 20.2 [13, 14]. Furthermore, estrogen stimulates sphingosine kinase 1 signaling via ERα and increases intracellular production of sphingosine-1-phosphate which in turn is an upstream mediator of PI3K/Akt/eNOS signaling activation [15]. Via the ER membrane-binding mechanism, mitogen-activated protein kinases (MAPK) pathways are also rapidly induced by estrogen, which corresponds to an increase in cyclic guanosine monophosphate (cGMP), the second messenger of NO [16]. On the other hand, the presence of eNOS itself, rather than its enzymatic activity for NO production, is critical for estrogen-induced rapid signaling of extracellular

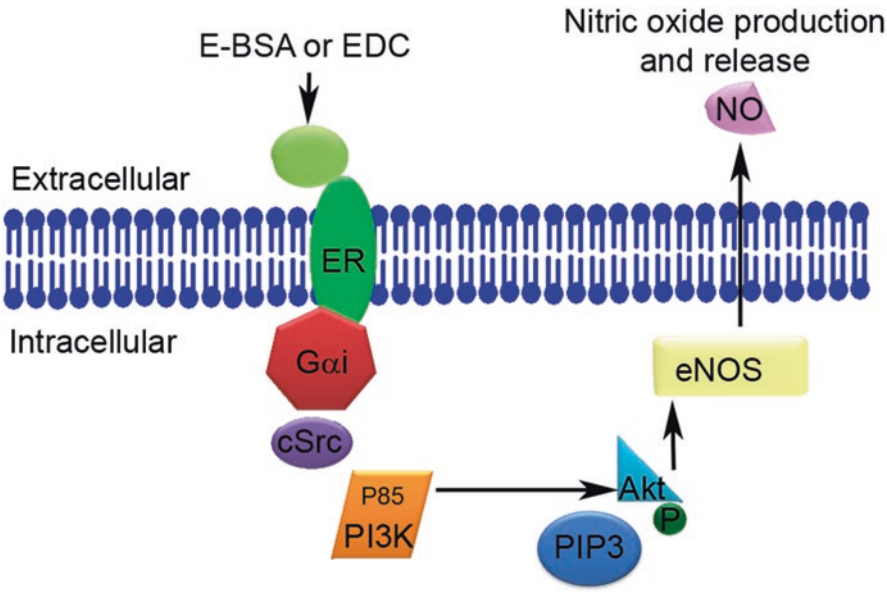

Fig. 20.2 Non-genomic estrogen signaling. Estrogen binding to membrane associated estrogen receptors (*ER*) activates PI3K signaling though Gαi, Src tyrosine kinase and p85 subunit binding. This leads to the phosphorylation of Akt and activation of PIP3 which increases nitric oxide (*NO*) production through endothelial nitric oxide synthase (*eNOS*)

signal-regulated kinases 1 and 2 (ERK1/2) phosphorylation [17]. Altogether, multiple signaling pathways are rapidly activated by estrogen via ERα, which in turn stimulate eNOS activation and result in the production and release of NO. NO is known to be vascular protective by controlling vascular tone and vasodilation, but is also critical for the stimulation of angiogenesis via the induction of cell growth, proliferation and migration of endothelial cells.

Recently, a "KRR" triple-point region in the ERα has been identified to cause ERα-mediated rapid signaling. Mutation in the "KRR" causes a defect in estrogen-induced rapid signaling, but ERα-mediated transcriptional regulation is maintained through the classic genomic pathway. Endothelial cells expressing the KRR mutant version of ERα have impaired estrogen-induced proliferation and migration [18]. The vascular protective effects of estrogen are lost in transgenic mice expressing the KRR mutant ERα [19]. Interestingly, the numbers of genes modulated by estrogen are markedly reduced in human endothelial cells expressing the KRR mutant ERα, when compared to wild type ERα. A distinct set of transcription factors are upregulated by estrogen-induced rapid signaling via the membrane bound ERα and act as downstream effectors to induce gene transcription. Therefore, the transcriptional regulation of estrogen-responsive genes is also dependent on ERα-mediated rapid signaling.

Membrane-impermeable estrogen signaling that provides vascular protection, but does not stimulate cancer growth, making selective activation of membrane-associated ERs a potentially attractive therapeutic target. For example, membrane-impermeable EDC does not stimulate the growth of Ishikawa cells (a uterine endometrial carcinoma cell line) or MCF-7 breast cancer cells, which are normally stimulated by membrane permeable estrogen [20]. A natural estrogen produced by the human fetal liver during pregnancy, estetrol, differentially regulates estrogen effects by uncoupling nuclear and membrane ERα activation [21]. Estetrol antagonizes estradiol-induced mammary tumor formation in rat [22] and prevents cell invasion of human T47D breast carcinoma cells stimulated by estradiol [23].

2.5 Estrogens and Endothelial Cells

Estrogen regulates many facets of endothelial cell function. Through receptor binding, estrogen initiates the rapid signaling pathway (Table 20.1) and/or modulates gene transcription, which is associated with proangiogenic growth factors and accompanying receptors. These growth factors collectively enhance the angiogenic capacity of endothelial cells by promoting cell proliferation, migration, tubule formation and attachment to extracellular matrix [24].

Table 20.1 The role of estrogen in endothelial function

Estrogen	Proliferation/cell survival	Migration/Adhesion
	Increases ↑ Cyclins CDK MAPK/ERK1/2 PI3K/Akt HIF1α VEGF/VEGFRs eNOS bFGF	*Increases* ↑ RhoA/ROCK/RhoGEF1 Notch1/Jagged1 c-Fos/c-Jun PAI-1 VEGF/VEGFRs eNOS FAK LIM kinase Cofilin Focal adhesions
	Decreases ↓ p27	

2.5.1 Estrogen and Endothelial Cell Attachment, Migration and Tubule Formation

Estrogen promotes endothelial cell migration and capillary formation by stimulating pathways that regulate changes in adhesion molecules and actin cytoskeleton. Estrogen upregulates the mRNA and protein expression of integrins β1, α5 and α6 which allow endothelial cells to attach to extracellular matrix (ECM) such as laminin and fibronectin [25]. Estrogen enhances integrin-mediated signaling via the phosphorylation of focal adhesion kinase (FAK). Estrogen-mediated activation of ERα signaling recruits Gα/Gβ proteins and triggers the formation of ERα complex with c-Src, PI3K and FAK, where FAK is phosphorylated. Estrogen-induced FAK phosphorylation also leads to rapid remodeling of actin cytoskeleton which mediates the interaction between cell movement and ECM. As FAK is activated by phosphorylation, it is translocated toward plasma membrane and forms focal adhesion complexes that facilitate actin remodeling and promote endothelial cell migration [26].

Estrogen also upregulates the expression and activity of Ras homolog gene family member A (RhoA), and RhoA-regulatory proteins such as RhoGEF1 in an ER-dependent manner [27]. RhoA is a small GTPase protein that regulates actin cytoskeleton by activating Rho-associated protein kinase (ROCK), Lim kinase and cofilin. Estrogen induced G-protein/PI3K and ROCK-II signaling upregulate c-Fos and c-Jun (the AP-1 early response transcription factor). This leads to an increase in plasminogen activator inhibitor (PAI-1) expression, which plays a role in the horizontal migration of endothelial cells [28]. Additionally, estrogen modulates cytoskeleton remodeling by post-translational modification of cofilin, a family of actin-binding proteins that disassembles actin filaments. Estrogen directly nitrosylates (covalent addition of a nitric oxide moiety) cofilin at the cysteine residue Cys80 with NO derived from eNOS, and activates cofilin by dephosphorylation of the serine residue Ser3 [29]. Altogether, these proteins allow endothelial cells to

form stress fibers which function in cell spreading during migration and attachment. Regulation of Notch1 and Jagged1 pathways, which ensure effective communication between adjacent cells, are also involved in estrogen-induced endothelial tubule formation and capillary stabilization [30].

2.5.2 Estrogen and Endothelial Cell Proliferation

Estrogen promotes cell proliferation in culture by downregulating genes associated with cell cycle inhibition, such as p27 which specifically inhibits cyclin-dependent kinases (CDK) [27]. Estrogen-stimulated cell proliferation is further enhanced by increasing gene expression that promotes the cell cycle such as Cyclin-D1, Cyclin-A2, Cyclin-B1, CDK1, CDK2 and CDK4. VEGF production is also increased by estrogen through transcriptional upregulation as ERs bind to the estrogen response element (ERE) in the VEGF gene promoter [6]. VEGF stimulates endothelial cell proliferation and migration mainly via its binding to VEGF receptor 2 (VEGFR2/KDR), which activates the MAPK/ERK pathway and FAK signaling. Additionally, estrogen activation of MAPK and ERK1/2 signaling pathways and phosphorylation of basic fibroblast growth factor receptor (bFGFR) promotes cell proliferation [31]. Cell proliferation requires a cross-talk between ERα-mediated genomic transcriptional regulation and non-genomic rapid signaling induced by estrogen. Post-translation modification of ERα upon estrogen binding, such as monoubiquitination (attachment of a single ubiquitin) within the ERα ligand binding domain, synchronizes ERα-mediated transcriptional activity and PI3K/Akt signaling cascades. For example, activation of PI3K/Akt phosphorylates CREB1 transcription factor, which in turn drives estrogen-induced Cyclin-D1 transcription activation via non-ERE [32, 33]. Together, estrogen modulates transcriptional activation via both ERE and non-ERE pathways and promotes cell survival and proliferation.

2.6 Estrogen and Endothelial Progenitor Cells (EPCs)

Endothelial progenitor cells (EPCs) are directly involved in vascular repair by facilitating reendothelialization and angiogenesis [34–36]. In women, there is a correlation between a higher plasma estrogen level and increased circulating EPC levels [37]. Male EPCs only contain ERα, whereas female EPCs express both receptors, even so, both male and female EPCs are responsive to estrogen [38].

Similar to its effects in differentiated endothelial cells, estrogen stimulates the key angiogenic activities of EPCs including migration, proliferation and tubulogenesis as outlined in Fig. 20.3 [39, 40]. The effects are mediated by both ERα and ERβ with a stronger contribution by ERα, at least in part, due to its higher expression in EPCs. Increasing evidence indicates that the non-genomic action of membrane ERα plays a significant role in mediating the effects of estrogen on EPCs. Membrane impermeable estradiol-conjugated BSA promotes EPC proliferation via ERα by increasing

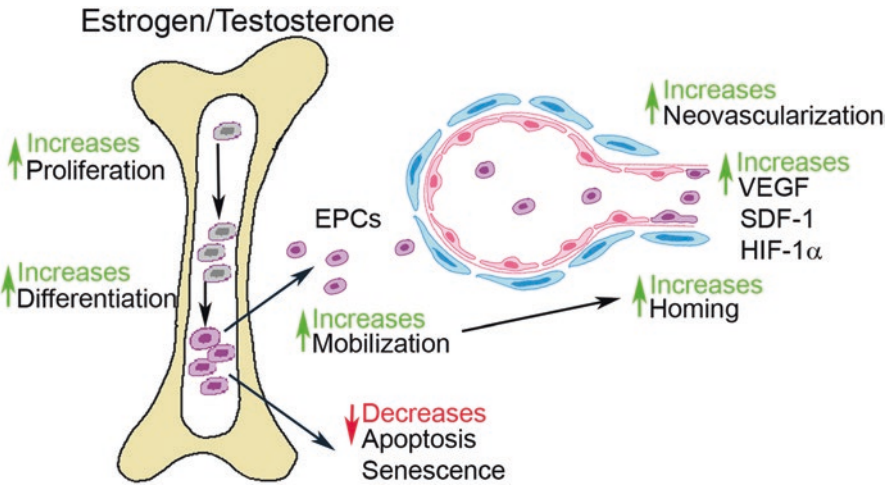

Fig. 20.3 The role of sex steroids in endothelial progenitor cell functions. Endothelial progenitor cells (*EPC*) are stimulated by sex steroids which lead to an increase in EPC proliferation, differentiation, mobilization, and homing. Sex steroids also upregulate hypoxia inducible factor-1 α (*HIF-1α*), and its downstream proangiogenic factor, such as vascular endothelial growth factor (*VEGF*) and stromal cell-derived factor-1 (*SDF-1*). The upregulation of proangiogenic factors enhances EPC mobilization and homing, resulting in increased neovascularization. Sex steroids also decrease apoptosis and senescence of EPCs

caveolin expression and activating PI3K and ERK1/2 signaling pathways [41]. Estrogen also stimulates capillary formation by human EPCs via an ERα-genomic independent mechanism. Actinomycin, an antibiotic that inhibits gene transcription, does not suppress estrogen's action on EPCs. In fact, estrogen activates receptor tyrosine kinases (RTKs), including receptors for VEGF, hepatocyte growth factor (HGF) and stromal cell-derived factor-1 (SDF-1) via ERα. The activation of RTKs increases the expression of heme oxygenase 1, which is an angiogenesis-stimulating enzyme, as well as phosphorylation of Akt and Erk1/2 signaling pathways [40].

Estrogen also prolongs the lifespan of EPCs in culture through a number of mechanisms. Estrogen inhibits EPC apoptosis by decreasing caspase-8 activity. EPC senescence is also delayed by an estrogen-mediated increase in telomerase activity and telomere length [42]. Estrogen increases the catalytic subunit of telomerase, known as telomerase reverse transcriptase (TERT) via the activation of PI3K/Akt signaling pathway [42]. ER-mediated activation of the PI3K/Akt pathway is also involved in estrogen-enhanced EPC proliferation and migration [43], that are necessary processes in facilitating EPC-mediated angiogenesis.

Estrogen stimulates EPC mobilization from the bone marrow to the circulation and promotes cell homing to sites of vascular injury or in response to ischemia. Estrogen replacement enhances EPC-mediated reendothelialization in ovariectomized mice subjected to carotid artery denudation [37, 44] and enhances recovery after myocardial infarction (MI) by augmenting EPC incorporation into the ischemic sites [45]. The enhanced EPC recruitment is due to an increase in myocardial

expression of SDF-1α induced by estrogen following rat MI [46], in which SDF-1α is a cytokine that regulates EPC mobilization and homing. Additionally, *ex vivo* preconditioning of EPCs with estrogen also enhances cell recruitment to infarcted myocardium when administrated to mice, improving cardiac function following MI. The augmented EPC migratory activity by estrogen is associated with an upregulation of the SDF-1α receptor, CXCR4. Treatment of EPCs with CXCR4 inhibitor AMD3100 abrogates the beneficial effects of EPCs from estrogen preconditioning [47]. The estrogen contribution to EPC mobilization and homing is mediated by both ERα and ERβ, with a greater role indicated for ERα [39]. In support of this, ERα expression in both endothelial cells and bone marrow-derived progenitor cells is required to mediate estrogen-induced augmentation in reendothelialization following carotid injury. Absence of ERα expression in either the bone marrow or endothelial cells attenuates estrogen-enhanced reendothelialization [48]. Furthermore, estrogen-mediated augmentation of EPC mobilization and homing appears to be eNOS-dependent, in which the effects of estrogen are absent in eNOS knockout mice after arterial injury or in response to ischemia [44].

2.7 Androgens and Angiogenesis

Increasing evidence indicates that androgens play a significant role in regulating angiogenesis. Depletion of endogenous androgens caused by castration decrease a range of angiogenic cytokines, such as VEGF, placenta growth factor, fibroblast growth factor-2 (FGF-2) and FGF-8 in mice [49]. The alteration in cytokine levels is associated with vascular regression [50]. Castrated male mice exhibit reduced neovascularization following hindlimb ischemia, which is associated with decreased mRNA expression of angiogenic mediators such as HIF-1α, SDF-1α and KDR in the ischemic tissues of castrated mice. Androgen replacement rescues castration-induced impairment in neovascularization [51, 52]. Topical application of testosterone on rats fitted with a human skin graft also improves angiogenesis with increased vascular density in the dermis of the skin graft compared with placebo treated rats [53].

Despite the regulation of angiogenesis by androgens *in vivo*, the underlying mechanisms for the effects of androgens on endothelial cells remains controversial. Similar to ERs, the androgen receptor (AR) is a ligand bound transcription factor. Dihydrotestosterone (DHT), a potent natural androgen that is non-aromatizable to estrogen, has a higher affinity for AR than testosterone. Upon the binding of androgens to AR, AR is then translocated to the nucleus and modulates gene transcription via an androgen response element (ARE) [53]. Androgen enhancement of ischemia-induced HIF-1α upregulation and angiogenesis is dependent on the transcriptional activation of AR. Following ischemia, male knockout mice with a transcriptionally inactive AR, that is incompetent to exert androgen-induced genomic modulation, display attenuated angiogenic responses with reduced HIF-1α levels when given

DHT treatment [54]. DHT enhances VEGF secretion in fibroblasts and subsequently stimulates angiogenic functions of endothelial cells via a paracrine-mediated mechanism. Androgen responsiveness is attenuated in fibroblasts from older men with reduced VEGF secretion. Age-related androgen insensitivity is associated with impairment in AR nuclear translocation and AR transcriptional activation [55]. Through AR, androgens also enhance endothelial cell proliferation by increasing VEGF mRNA and protein secretion that upregulates Cyclins A and D1 which in turn activate CDK to promote cell cycle [56]. In addition to its genomic action, AR also mediates androgenic effects via a non-genomic action which is involved in cell growth and survival. AR forms a triple complex with Src and p85α, the regulatory subunit of PI3K, to mediate androgen-induced activation of PI3K/Akt and MAPK signaling [57]. Furthermore, the AR/Src/p85α complex also interacts with KDR to induce eNOS phosphorylation upon VEGF stimulation [58]. AR itself also plays a role in vascular biology. Knockout mice with a total deletion of AR exhibit an impairment in angiogenesis following hindlimb ischemia [58]. Male AR knockout mice also display increased neointimal hyperplasia caused by carotid ligation, which is associated with increased outgrowth of vascular smooth muscle cells [59]. Mice with selective deletion of AR from vascular smooth muscle cells exhibit an impairment in angiogenic vascular remodeling and limits blood perfusion following ischemia [60].

2.8 Androgens and Progenitor Cells

Progenitor cell homeostasis is closely related to endogenous androgens. Circulating EPC levels are reduced in the peripheral blood of hypogonadal men [61]. In castrated rat, circulating CD34+ progenitor cell levels are decreased following MI. Testosterone replacement increases the mobilization and homing of CD34+ progenitor cells in the castrated rats with increased HIF-1α, VEGF and SDF-1 in the ischemic myocardium [62]. DHT augments the production of Sca1+/CXCR4+ progenitor cells in the bone marrow and enhances progenitor cell mobilization into the circulation of male mice following hindlimb ischemia [54]. *Ex vivo*, DHT increases the formation of progenitor cell colonies isolated from the bone marrow in mice after ischemia [51]. *In vitro,* DHT increases the proliferation and adhesion of cultured EPCs from the peripheral blood of healthy men, in a dose- and time-dependent manner via AR-mediated PI3K/Akt activation [63, 64]. Androgens induce AR nuclear translocation in EPCs [63] that differentially modulates the expression of over 300 genes. These genes are related to early growth response, angiogenesis, cell cycle and signaling cascades [65]. In male knockout mice with an AR incompetent of inducing genomic action, DHT-induced augmentation of Sca1+/CXCR4+ progenitor cell production and mobilization is abolished following hindlimb ischemia [54]. The effects of androgens on progenitor cell dynamics in response to ischemia is therefore dependent on AR transcriptional activation.

2.9 Sex Specificity of Steroid Action

There are striking sex differences regarding the incidence of cardiovascular disease, atherogenesis, angiogenesis and cardiovascular adaptation and repair in response to ischemia and infarcts [66–69]. Similarly, sex differences in ischemia-induced neovascularization are observed in murine models. Female mice have lower levels VEGF and eNOS in the ischemic muscle tissues and exhibit impaired blood flow recovery compared to males post-ischemia [70]. These differences have led researchers to explore the impact of sex-steroids on the regulation and function of the cardiovascular system. In both male and female mice gonadectomy severely impairs neovascularization *in vivo* [51]. Exogenous DHT or estrogen improve angiogenesis in gonadectomized male and female mice, respectively, following hindlimb ischemic injury. Interestingly, ovariectomized female mice do not benefit from exogenous DHT treatment following ischemia [51]. In contrast to this, male mice receiving estrogen treatment recover faster from hindlimb ischemic injury than placebo treated mice through progenitor cell recruitment [71].

Gender specific differences in sex hormone-mediated angiogenesis are due, at least in part, to differences in sex steroid receptor expression in vascular tissues. Both male and female mice exhibit high binding affinity to estrogen in aortic tissue, and surprisingly male mice contain twice as many binding sites for estrogen than females, which is independent of the fact that females have higher circulating estrogen levels [72]. In male mice, ERα mRNA is expressed at low levels in vascular endothelial and smooth muscle cells, whereas ERβ mRNA is greatly increased on endothelial cells following aortic denudation injury [73]. These studies demonstrate that the male vasculature can respond to an estrogen stimulus. In contrast to this, endothelial cells from women express much lower AR levels. Male endothelial cells have a 2-5-fold higher AR expression level than female cells [51, 74]. Monocyte-derived macrophages from men also express significantly higher levels of AR than those from women [75]. In macrophages these differences in AR expression translate to marked sex differences in transcriptional responses to androgen exposure, with male-donor macrophages exhibiting an upregulation of genes that are involved in angiogenesis while genomic responses in female-donor cells to androgens are much less striking [76]. Despite the evidence that AR-mediated androgen responses seem to be sex-specific, the AR itself regulates angiogenesis independent of gender. Loss of total AR impairs angiogenesis in both male and female mice following hindlimb ischemia in the absence of exogeneous androgen treatment. The sex-independent functions of AR are associated with its ability to interact with KDR through the recruitment of p85 and Src [58].

3 Sex Steroids and Angiogenesis: Role in Health and Disease

3.1 Estrogen and Menstruation and Angiogenesis

Blood vessel growth and regression is a major part of the ovarian and menstrual cycles which are regulated by the female hormones estrogen and progesterone [77–79]. An increase in estrogen production also accompanies the development of the placenta during pregnancy, establishing an extensive vascular network [80]. These processes are mediated by complex signaling and the production of numerous growth factors and angiogenesis-promoting proteins as demonstrated in Fig. 20.4.

Estrogen regulates angiogenesis in the endometrium by influencing the proliferation and stimulation of many endometrial cells including uterine endothelial and epithelial cells, as well as stromal cells, and smooth muscle cells associated with the vascular wall [81]. Estrogen stimulates cell proliferation by increasing VEGF expressions in isolated endometrial vascular cells via both ERα and ERβ [82]. ER expression fluctuates throughout the menstrual cycle, and thereby allowing vascular growth and regression [83]. The production and secretion of VEGF in the endometrium is attributed to the glandular epithelial cells, which produce large amounts of VEGF with increased estrogen stimulation [84]. VEGF then exerts its proangiogenic effects by binding VEGFRs on endometrial endothelial cells, causing an increase in proliferation, tubulogenesis and blood vessel growth in the endometrium. Human endometrial endothelial cells are more sensitive to VEGF stimulation than dermal, coronary, and umbilical endothelial cells, which results in higher levels of cell proliferation and angiogenic capacity [85]. This may be attributed to that fact that uterine endometrial cells having higher mRNA expression levels of the VEGFRs, KDR and Flt-1 [86]. Furthermore, estrogen-induced VEGF production is augmented in luminal epithelial cells via ERα-mediated activation of HIF-1α and the PI3K/Akt pathway [87, 88]. Along with VEGF, FGF-2, epidermal growth factor (EGF) and platelet derived growth factor (PDGF) are also produced in the normal human endometrium and influence endometrial angiogenesis [89]. Their receptors VEGFRs, FGFR2, EGFR and PDGFR are all expressed within and close to endometrial blood vessels with the strongest expression coinciding with the start of the secretory phase as sub-epithelial capillary density increases.

Furthermore, bone marrow-derived circulating EPC levels peak at the periovulatory (the most intensive angiogenesis phase) and middle luteal phases of the menstrual cycle. The circulating EPC levels are correlated with serum levels of estrogen, VEGF and angiogenesis-related factor granulocyte colony stimulating factor (G-CSF) [90]. Estrogen stimulates the proliferation of peripheral blood-derived EPCs from women during the menstrual phase, but not the luteal phase. This is associated with upregulation of ERα expression in the EPCs from women during their menstrual phase, which is subsequently downregulated in the luteal phase [91].

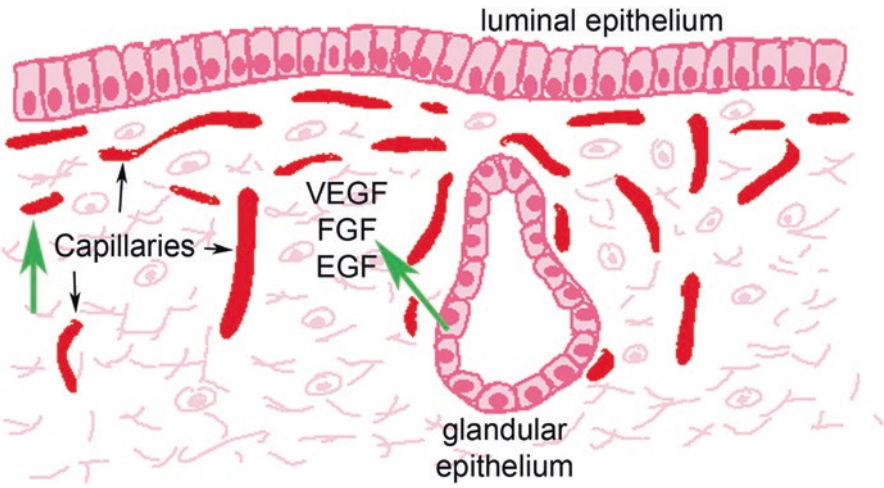

Fig. 20.4 Estrogen in endometrial angiogenesis. During specific phases of the menstrual cycle, estrogen stimulates the growth of uterine blood vessels and capillaries through glandular epithelial cell secretion of growth factors such as vascular endothelial growth factor (*VEGF*), fibroblast growth factor (*FGF*) and epidermal growth factor (*EGF*)

3.2 Sex Steroids and Tumor Angiogenesis

Sex steroids promote angiogenesis through cell proliferation, migration and capillary formation, which are all key factors in tumor growth, progression and metastasis. It is therefore a major concern regarding the relationship between sex steroids and cancer development and progress. The roles of estrogen and testosterone in tumorigenesis have been mostly studied in the context of breast and prostate cancer respectively. However, they may also be implicated in the progression of tumors in other endocrine organs such as in the uterus, ovaries [92], pituitary, adrenal glands, thyroid and parathyroid [93]. Figure 20.5 details the association of sex steroids in tumor growth and angiogenesis, and includes common treatments that target hormone-mediated angiogenesis to restrict tumor growth and metastasis.

3.2.1 Estrogen and Tumor Angiogenesis

The balance of local estrogen levels in hormone-dependent tumors and in non-pathological tissues related to the vascular system is critical in regulating estrogen-sensitive cancer development. Steroid sulfatase (STS) is an enzyme that hydrolyses biologically inactive estrogen sulfates to active estrogens. STS activity is found to be higher in breast cancer tissues than in normal breast tissues. STS reactivity is also positively associated with tumor size. On the other hand, estrogen sulfotransferase (EST) converts active estrogens to estrogen sulfates and is associated with decreased risk of recurrence and improved prognosis [94].

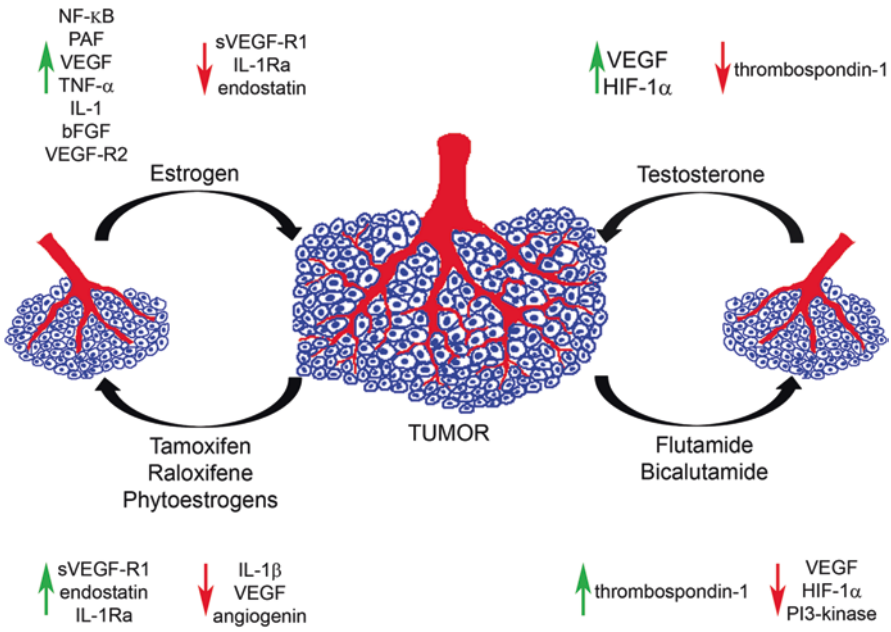

Fig. 20.5 The involvement of sex steroids in tumor growth and angiogenesis. Estrogen and testosterone both stimulate the growth of hormone specific tumors such as breast and prostate respectively by increasing tumor angiogenesis. Tumor angiogenesis is augmented through hormone-induced upregulation of proangiogenic mediators and growth factors, in addition to the downregulation of anti-angiogenic factors. Treatment of hormone responsive tumors with anti-estrogens or anti-androgens suppress tumor growth by reducing tumor angiogenesis

Estrogen exposure induces neovascularization, which corresponds with the growth and enlargement of tumor blood vessels in ovariectomized Fisher 344 rats that develop spontaneous pituitary tumors [95]. This vessel growth is stimulated by an elevated production of VEGF from both endothelial and non-endothelial tumor cells. The VEGF production is accompanied by increased VEGFR2 levels on tumor endothelial cells which respond by increased tumor angiogenesis. Similarly, in MCF-7 breast cancer cells, treatment with estrogen also increases VEGF secretion, which leads to an increased expression of VEGFR2 on HUVECs cultured with breast cancer conditioned medium [96]. Additionally, estrogen reduces negative regulators of VEGF-mediated angiogenesis such as soluble VEGFR1 production [96, 97]. In matrigel plugs containing breast cancer cells given to mice with an estrogen or placebo implant, decreased VEGFR1 expression in estrogen treated mice was accompanied by a significant increase in angiogenesis [97]. Estrogen-induced downregulation of VEGFR1 is blocked by pretreatment of ER-positive breast cancer cells with an ER antagonist. While ERα mediates estrogen-induced tumor cell proliferation and growth, ERβ has an opposite effect. Overexpression of ERβ is associated with a reduction in tumor volume in estrogen-treated mice which exhibit reduced microvessel density and decreased expression of angiogenic factors such as VEGF and PDGF-β. [98].

Estrogen also enhances expression of many other proangiogenic factors including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and bFGF [99]. The expression of these proangiogenic factors is mediated through the activation of nuclear factor-kappaB (NF-κB), via platelet activating factor (PAF). Importantly, long term estrogen deprivation, such as in post-menopausal women, causes breast tumors to develop a heightened sensitivity to estrogen [100]. These cells adapt through an upregulation of ERα at the membrane, which activates growth factor pathways via SHC transforming protein-1 (SHC1), growth factor receptor-bound protein-2 (Grb-2) and son of sevenless homolog-1 (Sos-1). Estrogen deprivation also upregulates growth factors related to cell proliferation as well as other proangiogenic pathways, including MAPK, PI3K and mammalian target of rapamycin (mTOR) pathways.

In addition to the effects of estrogen on ER-mediated tumor cell growth and proliferation, estrogen also impacts the tumor microenvironment. This occurs without directly influencing the ERα-negative tumor cells [101, 102]. For example, estrogen promotes the outgrowth of xenograft tumors from patient-derived ER negative breast cancer cells in mice by enhancing angiogenesis through the mobilization and recruitment of proangiogenic, bone marrow-derived hematopoietic myeloid cells. In mice transplanted with ERα-knockout bone marrow, estrogen treatment has no effect in promoting tumor angiogenesis and growth. Therefore, ERα expression in bone marrow-derived cells alone is sufficient to mediate estrogen-induced mobilization of hematopoietic myeloid cells to the tumor cells, whereas ERα expression in the host cells or non-bone marrow-derived cells is not necessary [102]. Estrogen also increases the proliferation and angiogenesis of ERα-negative high grade serous ovarian cancer (HGSOC) xenografts in mice by promoting infiltration of tumor-associated macrophages (TAM). Consistent with this, immunohistochemical analysis of ERα-negative tissue samples from premenopausal HGSOC patients display a greater TAM infiltration than those from postmenopausal women [101].

The prevention of angiogenesis is a known therapeutic target to block tumor growth [103]. Tamoxifen and raloxifene are selective estrogen receptor modulators (SERMs), that are the most commonly used ER-antagonists to treat hormone-responsive breast cancers. Patients treated with tamoxifen have the best prognosis when tumors are ER-positive. Tamoxifen directly reduces endothelial cell proliferation in culture and decreases endothelial cell proliferation and migration *in vivo* when given orally to rats with a matrigel plug containing VEGF [104]. Tamoxifen reduces the effects of estrogen on tumor angiogenesis by reducing the secretion of VEGF and proangiogenic factor, angiogenin, from the tumor cells. This leads to a decrease in tumor vessel area and the overall tumor vasculature [105, 106]. It also inhibits estrogen-mediated reduction of soluble VEGFR1, which functions as a negative regulator of VEGF stimulated angiogenesis [96]. Along with phytoestrogens (dietary estrogens) such as flaxseed and enterolactone, tamoxifen decreases tumor microvessel density by inhibiting endothelial cell proliferation which is associated with a reduction in proangiogenic factor IL-1β levels and increase in IL-1 receptor antagonist (IL-1Ra) levels [107]. Phytoestrogens are also able to counteract estrogen-induced tumor growth and angiogenesis in ovariectomized mice injected with human breast cancer cells by decreasing VEGF secretion from cancer cells [108].

Despite being a cornerstone in breast cancer therapy, tamoxifen treatment only reduces the risk of recurrence by 30–50% [109] and is associated with side effects such as thromboembolic events, endometrical cancer and joint pain [110]. Therefore, there is a need to identify novel estrogen-dependent targets to improve cancer therapy. *In vivo* sampling of human chemokines by microdialysis reveals high levels of extracellular CCL2 and CCL5 in ER-positive breast cancer tissues compared to normal breast tissues. Estrogen promotes the activation and infiltration of TAM by increasing the release of CCL2 and CCL5 from cancer cells. These effects are inhibited by anti-CCL2 or anti-CCL5 therapy, resulting in an inhibition of tumor growth [111].

In recent years, estrogen-related receptor alpha (ERRα), an orphan nuclear receptor that regulates gene expression in association with coactivators and corepressors, has shown to be involved in cancer initiation and progression of a wide variety of endocrine-related cancers [112]. Knockdown of ERRα with siRNA suppresses angiogenesis and tumor growth via reduction of VEGF expression and induction of cell cycle arrest and caspase-3-mediated apoptosis, which inhibits cell proliferation [113]. Given that endogenous ligands for ERRα are absent naturally, it is considered a suitable direct target for pharmacological intervention.

3.2.2 Testosterone and Tumor Angiogenesis

The pathogenesis of prostate cancer is highly dependent on the presence of androgens. This is highlighted by the fact that men who undergo castration before puberty, or men with 5α-reductase deficiency have never been reported to develop prostate cancer [114]. Androgen deprivation therapy (ADT) is the standard systemic treatment for patients with prostate cancer, in which ADT suppresses androgen production and AR activity. Androgens directly stimulate proliferation and inhibit apoptosis of prostate cancer cells, but also largely regulate prostate tumor growth by promoting angiogenesis. Castration induces a regression of prostatic vasculature. In castrated rats, prostatic endothelial cell proliferation, weight of total blood vessels and endothelial cell numbers decrease in addition to a decrease in epithelial cells and total organ weight [115]. Castration in mice implanted with androgen-sensitive Shionogi tumors showed that androgen withdrawal leads to vascular regression from the tumor periphery within 24 h, followed by tumor regression after 1–2 days [116]. Vascular regression and endothelial cell apoptosis precedes epithelial apoptosis. The initial vascular regression is attributable to a sharp decrease in VEGF expression induced by androgen ablation, followed by a decrease in microvessel density and a subsequent decrease in overall tumor size in mice with androgen-sensitive tumors [117–119]. Androgens upregulate VEGF mRNA and protein secretions in normal prostate cells as well as androgen-responsive LNCaP prostate cancer cells via PI3K-mediated HIF-1α activation [117, 120–122]. Non-steroidal antiandrogens such as flutamide and bicalutamide inhibit DHT-induced upregulation of HIF-1α, as does inhibiting the PI3K signaling pathway. In hormone-independent PC3 cells, androgen-induced HIF-1α-mediated upregulation of VEGF is not observed, thereby VEGF reduction by androgen withdrawal is ineffective [121].

Alternatively, ligand-bound AR forms a nuclear complex with transcription factor Sp1 and upregulates VEGF gene expression, in which three AR binding sites are located within the promoter region of VEGF [123]. In addition to an elevation in proangiogenic cytokine levels, prostate cancer tissues also have increased levels of CD34+/CD31+ EPCs compared with normal tissues. ADT is able to decrease these EPC levels transiently [124].

Following the initial vascular regression induced by ADT, tissues become hypoxic and this is eventually accompanied by an angiogenic burst. Mice implanted with Shionogi tumor exhibit a re-expression of VEGF levels and tumor regrowth after 2 weeks of castration [116], mirroring the development of a more malignant form of androgen-independent prostate cancer termed castration-resistant prostate cancer (CRPC) [125]. The recurrent prostate cancers are often found to have a significantly higher expression of AR. This indicates that androgen-independent tumors continue to require a functional androgen signaling pathway to regulate tumor growth and angiogenesis following androgen withdrawal. Androgen-independent cancer cell line PC3 secretes a wide range of CXC chemokine ligands that are involved in metastatic migratory functions, a crucial feature during the transition of androgen-dependent prostate cancer into the more aggressive androgen-independent state [126]. Although ADT decreases CD34+/CD31+ EPCs levels in the prostate cancer tissues, the decreased EPC levels gradually recover over time and increase as the cancer progress into CRPC [124]. Within the prostate microenvironment, endothelial cells increase interleukin-6 (IL-6) secretion, leading to activation of transforming growth factor-β (TGF-β)/metalloproteinase-9 (MMP-9) signaling that promotes the invasion of prostate cancer cells [124]. ADT also modulates the prostate tumor microenvironment by augmenting the infiltration of inflammatory mast cells. The recruited mast cells increase stem/progenitor cell populations and MMP9 expression by suppressing AR signaling [127]. Furthermore, ADT-induced hypoxia upregulates gene expression involved in epithelial-to-mesenchymal transition (EMT), exerting selection pressure for cancer clones with a more proangiogenic, stress-resistance genotype that are advantageous in tumor cell invasion and metastatic spread [128]. In mice transplanted with human prostate xenografts, castration upregulates transcription factors that are involved in reprogramming, self-renewal and pluripotency in differentiated somatic cells, namely Oct4, Sox2, Klf4 and NANOG [129]. Androgen deprivation enhances the “stem-ness” features in prostate cells that contributes to the recurrence of aggressive cancer cells.

Other angiogenic mediators are required for the continuation of vascular repression following ADT. Thrombospondin-1 (TSP-1) functions as an angiogenesis inhibitor and is strongly expressed in normal prostate tissues. In human prostate cancer tissues, TSP-1 expression is downregulated while proangiogenic VEGF and FGF-2 levels are increased [130]. Patients that undergo ADT show an increase in TSP-1 expression and a decrease in tumor microvessel density [131]. Similarly, in rats, androgen withdrawal via castration increases TSP-1 synthesis and decreases vascularization of the normal prostate, which is reversed with androgen replacement [131]. TSP-1 inhibits angiogenesis via activation of TGF-β, which in turn suppresses epithelial and stromal cell proliferation, contributing to the continued

vascular regression [132]. For patients with androgen-dependent prostate cancer, combining ADT with TSP-1 therapy that targets the TGF- β pathway, or anti-angiogenic therapy such as a VEGF-inhibitors or VEGFR tyrosine kinase inhibitors [133], could delay the recurrence time of androgen-independent cancer.

Polymorphisms in the AR gene, especially the CAG repeat length within the first exon that encodes the polyglutamine tract in the N-terminal domain of the AR protein, are related to the variable outcomes of ADT and relapse of CRPC. The length of the CAG repeat in the AR gene is also associated with prostate cancer-specific mortality and serves as a molecular marker to determine ADT efficacy [134]. Despite low serum testosterone levels, patients with a shorter CAG repeat length in the AR gene have higher AR density and higher microvessel density within the tumor with increased metastatic potential [135]. The CAG repeat length alters AR transactivation and transrepression functions by modifying transcriptional coactivators and corepressors, respectively, enhancing AR response to low androgen levels. A shorter CAG repeat length is associated with greater transactivation function of the AR [136–139]. On the other hand, a long CAG repeat length is more effective in recruiting a corepressor, one of which is known as the silencing mediator for retinoic acid and thyroid hormone receptor (SMRT), that dampens AR transcriptional activation upon androgen binding [140].

Interestingly, genes associated with ER signaling are also used to predict the recurrence of prostate-specific antigen and 5-year cancer-specific survival [141]. Estrogen administration has been shown effective for advanced, androgen-insensitive prostate cancer [142, 143]. While androgen modulation of tumor microenvironment stimulates endothelial cell growth via a paracrine-mediated mechanism, co-administration of estrogen attenuates androgen-induced endothelial cell growth *in vitro* as well as angiogenesis in xenograft prostate tumors [144]. Estrogen has a biphasic effect on prostate tumor growth. A lower dose of estrogen increases tumor growth in mouse xenograft model using human PC3 cancer cells, while a higher dose of estrogen inhibits tumor growth. High estrogen levels suppress prostate tumor growth by modulating krüppel-like zinc finger transcription factor 5 (KLF5)-dependent transcription through ERβ, subsequently lowering the levels of proangiogenic PDGFA [145].

4 Conclusion

It is evident that sex steroids are fundamentally involved in the regulation of key angiogenic processes. This chapter has detailed the known mechanisms by which sex steroids mediate basic endothelial and progenitor cell functions that are necessary in both physiological and pathological neovascularization. Given the complex nature of sex steroid-mediated angiogenesis, further research is necessary in understanding the pathways which contribute to angiogenesis in both health and disease. This will lead to improved treatment of hormone-responsive cancers, as well as the potential to utilize sex steroids in promoting angiogenesis following injury such as during wound healing.

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Chapter 21
Brain Angiogenesis After Stroke

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Abstract Stroke remains a major health problem worldwide, and is the leading cause of serious long-term disability. Although many advances have been made in terms of the basic molecular mechanisms underlying neuronal death, clinically effective neuroprotective drugs in stroke have not yet been discovered. Recent findings now suggest that strategies to enhance angiogenesis after focal cerebral ischemia may provide unique opportunities to improve clinical outcomes during stroke recovery. This chapter aims at summarizing current knowledge on mechanisms and potential targets for angiogenic therapies in brain after stroke. Crosstalk between cerebral endothelial cells and their neighboring cells may provide substrates for plasticity and remodeling in the recovering brain. A better understanding of the molecular interplay between all these complex pathways may lead to novel therapeutic approaches for this devastating disease.

Keywords Stroke • Angiogenesis • Cerebral • Endothelial • Cell • Neurovascular unit • Neurovascular niche • Oligovascular niche • Biphasic response • Brain remodeling

1 Introduction

Stroke is the second leading cause of death and a leading cause of adult disability worldwide. Under stroke conditions, brain function is perturbed due to cerebral ischemia caused by thrombosis or hemorrhage. In the central areas of ischemic regions, blood flow deficits are severe and brain cells die rapidly. In the peripheral penumbral areas, blood flow deficits are relatively mild, so that therapeutic salvage is theoretically possible (Fig. 21.1). However, therapeutic options for clinical

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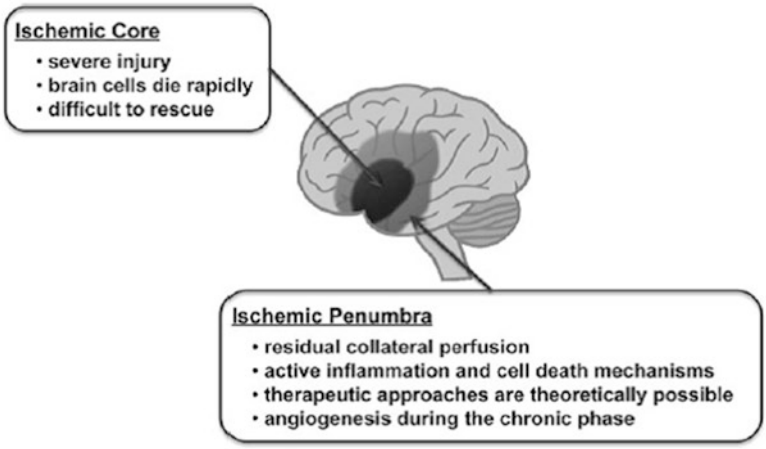

Fig. 21.1 Schematic of stroke brain. Under stroke conditions, brain function is perturbed due to cerebral ischemia (lack of blood supply to the brain). In central areas of ischemic regions (ischemic core), blood flow deficits are severe and brain cells die rapidly. In peripheral areas (ischemic penumbra), blood flow deficits are relatively mild and cell death progresses are slower. Hence, therapeutic salvage is theoretically possible, and angiogenesis may occur during the chronic phase

management in stroke remain quite limited. In the acute phase, thrombolytic reperfusion with recombinant tissue plasminogen activator (t-PA) is still only used in less than 3% of all ischemic stroke patients worldwide [1]. During the chronic phase after stroke, standard treatments involving rehabilitation provide some support for recovering patients. However, many high-profile failures in a wide spectrum of pharmacologic neuroprotection trials have led to some pessimism in the field [2]. In recent years, accumulating data suggest that damaged brain can be surprisingly plastic, and intriguing mechanisms of neurogenesis and angiogenesis might provide novel substrates for brain repair [3, 4]. In this chapter, we will focus on key findings that emphasize interactions between growth factors, progenitor cells, and neurovascular/oligovascular signaling as potential mechanisms that may be augmented to stimulate cerebral angiogenesis and enhance stroke recovery.

2 Brain Angiogenesis After Stroke

Angiogenesis is a key restorative mechanism in response to ischemia in several non-CNS tissues. For example, myocardial infarction and limb ischemia can trigger endogenous angiogenesis in each organ, and therapeutics that enhanced angiogenesis can sometimes reduce injury in these disorders. Also in the brain, angiogenic responses may play important roles on brain remodeling after ischemic injury. Increasing evidence in both human stroke patients and animal stroke models suggests that the post-stroke penumbra is extremely resilient and is a site of intense

remodeling and active angiogenesis. Autopsy studies show that brain ischemia stimulates angiogenesis in part via stereo-typed hypoxia-inducible factor (HIF-1) [5]. Proliferation of endothelial cells starts at several days after ischemic events [6]. Studies using mice with middle cerebral artery occlusion demonstrated that endothelial cell proliferation might begin as early as 12–24 h after ischemia and persist for up to several weeks thereafter [7, 8]. Studies using human brain samples also suggested that active angiogenesis takes place at 3–4 days after stroke, and the number of vessels appeared to be correlated longer survival times in ischemic stroke patients, suggesting that active angiogenesis may be beneficial [5, 9, 10]. In contrast, older patients who tend to do worse after stroke seem to have reduced new vessel formation after stroke [11, 12]. Furthermore, patients who develop dementia after stroke may suffer from reduced blood flow in adjacent cortical regions [13]. This raises the possibility that angiogenesis may improve cerebral perfusion and function as part of a network repair. However, the purpose of this angiogenic response remains speculative. Lyden and colleagues have proposed a "clean-up hypothesis," whereby newborn vessels serve to facilitate macrophage infiltration and clear up and remove cellular debris from pan-necrotic tissue [14, 15]. They demonstrated that microvessel density was always associated with increased numbers of macrophages. Ischemic brain areas without macrophages displayed no vascular changes compared with normal animals. This alternate hypothesis would suggest that post-stroke brain angiogenesis is only transient and not permanently involved in neuronal recovery. Nevertheless, the data in aggregate support a beneficial role for brain angiogenesis during recovery phase after ischemic stroke.

2.1 Growth Factors for Brain Angiogenesis

After focal cerebral ischemia, brain cells manufacture and secrete angiogenic peptides to stimulate angiogenesis. The precise regulatory mechanisms that underlie angiogenesis after ischemia still remain elucidated. But, several growth factors have been found to be upregulated after stroke to promote angiogenesis for brain remodeling. So far, at least 20 growth factors are known to induce angiogenesis, and here we will review the well-characterized growth factors.

2.1.1 Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) is a major mediator for angiogenic responses in the brain. VEGF can accelerate angiogenesis and neurogenesis in the delayed stroke phase [16, 17]. VEGF can trigger remodeling responses in both endothelial cells and neurons. VEGF was increased within hours after ischemia and had a strong effect on the new vessel growing. Beginning minutes after stroke in rodents, VEGF signals appear in neurons for days after ischemic onset, and can be found in astrocytes for up to a few weeks [18, 19]. In human, mRNA and proteins

of VEGF165, VEGF189, and the receptor flk-1 were found to be upregulated in brain tissue or serum of patients following acute ischemic insult [20]. Elevated serum levels of VEGF were correlated to infarct volumes and clinical disabilities. An increase in angiogenesis by VEGF in rats was also associated with reduced neurological deficits after focal cerebral ischemia [19]. Boosting VEGF also seems to promote recovery. An intra-cerebroventricular injection of VEGF via osmotic pump, starting 24 h after onset of focal cerebral ischemia, stimulated angiogenesis and decreased infarct volume in rodent models of focal cerebral ischemia [21]. In addition to these biochemical and pharmacologic findings, genetic data have also been obtained. In transgenic mice overexpressing human VEGF165, brain microvessel density was significantly elevated compared with wild-type mice before ischemia, and the increase in microvessel density 3 days after stroke onset was improved [22]. These data show that VEGF promotes revascularization after stroke. Recently, encapsulated cell grafts overexpressing VEGF were implanted into rat striatum before induction of focal cerebral ischemia resulting in brain edema [23]. Angiogenesis was significantly increased around the area of the encapsulated graft after 24 h concomitant with a reduction in infarct size, but interestingly, there was no increase in cerebral blood flow at 1, 7, and 14 days compared with control untreated animals. These data suggest that the link between increased vascularization, increased blood flow, and recovery may not be interrelated or concomitant.

2.1.2 Fibroblast Growth Factor-2 (FGF-2/bFGF)

FGF-2 is a potent stimulator of endothelial cell migration, proliferation, sprouting, and tube formation. FGF-2 signaling also promotes mitogenesis and differentiation in neural progenitor cells in vivo [24]. In rat MCAO models, FGF-2 was elevated in neuron adjacent to infarct after 1 day following cerebral ischemia. Besides, endothelial cell, reactive astrocyte, and macrophage also expressed FGF-2 in the first 2 weeks after ischemia [6, 25]. The clinical findings were consistent with these experimental models. In human, mRNA and protein of FGF-2 were reported to be upregulated in the brains, serum, and cerebrospinal fluid (CSF) of patients who died of acute ischemic stroke [26]. The cellular localization of FGF-2 was found to be in endothelial cells in peri-infarct region of surviving patients after stroke, confirming its important role in angiogenesis in human as well as animal models [26]. Many studies examined the effect of FGF-2 administration on brain damage and recovery in animal stroke models. In rodent, FGF-2 administration 2 h after focal cerebral ischemia markedly reduced infarct volume [27]. Injection of FGF-2 enhanced recovery of sensorimotor function in rat stroke models [28, 29]. Later administration increased neuronal sprouting and enhanced neural recovery, though it could not reduce infarct volume [28, 30]. This effectiveness after later administration considered FGF-2 as a suitable therapy for human patients where treatment is often delayed; then human clinical trials have been carried out [31]. However, human clinical trials conducted in the USA were curtailed because of high dose toxicity.

2.1.3 Platelet-Derived Growth Factor-Beta

Platelet-derived growth factor-beta (PDGF-beta) and its receptor PDGFR-beta are essential for the recruitment of pericytes to cerebral endothelium. This step contributes to maintain the homeostasis of microvessel and the mechanical stability of capillary [32]. Thus, following cerebral ischemia, PDGF-beta is thought to support angiogenesis and vascular remodeling by mediating interactions of endothelium with pericytes. Analysis of the expression pattern following experimental cerebral ischemia showed that PDGFR-beta was specifically upregulated in vascular structures in the infarcted area mainly associates with pericytes 48 h after MCAO [33]. In human, PDGF-beta and its receptor can be detected on microvessel endothelial cells around cystic infarction for weeks after stroke following stroke [34].

2.1.4 Transforming Growth Factor-Beta

Transforming growth factor-beta (TGF-beta) controls proliferation, cellular differentiation, and other functions in most cells. TGF-beta contributes to angiogenesis by stabilizing newly formed capillary sprouts. Many studies of TGF-beta in mice and humans have demonstrated its pivotal role in modulation of angiogenesis. In rodent, TGF-beta was upregulated after hypoxia/ischemia, resulting in reduced infarct size and neuroprotection [35]. TGF-beta was detected to occur in astrocytes, activated microglial cells, and microvessels after cerebral ischemia. In the human brain, TGF-beta mRNA was upregulated in microvascular cells of the penumbra region of patients following ischemic stroke [36, 37]. Further human studies demonstrated that TGF-beta level was increased in CSF but not in serum [38]. Upregulation of TGF-beta seems to be associated with NOS1 in the serum and tissue of patients after stroke, suggesting that this interaction mediates pro-angiogenic function of TGF-beta [39, 40]. When TGF-beta was injected into animals prior to ischemia, it decreased infarct size, showing its neuroprotective role [41]. However, it had no beneficial effect if injected after the ischemic injury [42].

2.2 Biphasic Responses of Angiogenic Factors After Stroke

The biphasic nature of many mediators in neurobiology is now well known. For example, trophic factors such as NGF and BDNF promote cellular survival via their primary receptors TrkA and TrkB, respectively. But in contrast to these neuroprotective effects, both factors can also be neurotoxic via overactivation of the p75NTR receptor. As noted, the responses and regulatory mechanisms that underlie brain remodeling are highly complex. Growth factors seen above (and also other angiogenic factors) promote brain angiogenesis after stroke. Angiogenesis is an essential step for restoring brain function in injured brain, but those angiogenic factors may not be always supportive for the brain. As introduced, one major mediator in

vascular responses after stroke is VEGF. VEGF is the prototypical biphasic mediator. VEGF can trigger remodeling responses in both endothelial cells and neurons [16, 17], and accelerate angiogenesis and neurogenesis responses in the delayed stroke phase. By contrast, in the acute phase in stroke, VEGF increases blood–brain barrier (BBB) permeability, which causes cerebral hemorrhage and brain edema [43, 44]. In fact, VEGF administration worsens BBB leakage by ischemic insults [19]. Within the context of the vascular remodeling, similar biphasic properties of many factors and mediators may also emerge. Therapies that can boost these endogenous signals and substrates of vascular remodeling might be a new direction for stroke treatments [45]. However, it remains to be fully elucidated how these approaches can be utilized in clinic. It is worth noting that most molecular targets for stroke therapy have biphasic roles in stroke pathophysiology [46, 47]. Here we will overview three major examples of the so-called biphasic responses in vascular remodeling after stroke.

2.2.1 Matrix Metalloproteinase

In recent years, dysregulation of neurovascular proteases has been implicated as central in neurovascular injury and remodeling after stroke. Hence, neurovascular proteases such as matrix metalloproteinases (MMPs) may have the biphasic properties after brain injury. The MMP family of extracellular proteases has been well studied in our field. MMPs comprise a family of zinc endopeptidases with major roles in the physiology and pathology of the mammalian CNS. To date, MMP-2 (gelatinase A), MMP-3 (stromelysin 1), MMP-7 (matrilysin), MMP-9 (gelatinase B), and MMP-13 (collagenase-3) are known to contribute to infarct extent and⁄or BBB disruption in the acute phase after stroke [48–52]. On the contrary, however, these same proteases may have a beneficial role during neurovascular repair. In a mouse stroke model, peri-infarct cortical areas demonstrate a secondary elevation in MMP-9 in endothelial and glial cells within networks of regrowing microvessels [53], and inhibition of MMPs during this delayed phase actually made outcomes worse with the development of hemorrhagic and malformed blood vessels and enlarged volumes of infarction and cavitation. Beyond the peri-infarct zone, other brain areas were also involved. Secondary MMP-9 signals co-localized with streams of migrating neuroblasts from the subventricular zone, and inhibition of these MMPs also blocked the movement of these neuroblasts, originally headed toward damaged brain [54].

2.2.2 High-Mobility Group Box 1

Besides VEGF and MMPs, attention is currently focused on the roles of high-mobility group box 1 (HMGB1) as well on brain remodeling after stroke. HMGB1, a highly conserved non-histone nuclear DNA-binding protein, is widely expressed in most eukaryotic cells including neural cells in several animal species including humans [55]. Traditionally, HMGB1 acts as a nuclear and cellular danger signal

[56]. HMGB1 can exert different functions depending on its cellular localization. It can be passively released from damaged cells or actively secreted from stimulated cells. Release of HMGB1 is observed after traumatic brain injury and ischemic stroke. In rodent middle cerebral artery occlusion models, levels of HMGB1 in the ischemic core are immediately decreased, and in turn, serum HMGB1 is rapidly increased [57–59]. In clinical stroke patients, HMGB1 is upregulated in serum of up to day 7 after stroke onset [60]. HMGB1 is also increased in CSF of subarachnoid hemorrhage patients on day 3, 7, and 14 after onset [60]. In addition, plasma HMGB1 in patients is acutely elevated 30 min after severe trauma in comparison to healthy subjects [61]. However, in contrast to the negative effects, HMGB1 may also possess beneficial actions. HMGB1 signaling can promote endothelial activation [62] and sprouting [63]. And it has also been reported that HMGB1 may increase neurite outgrowth and cell survival in neurons [63–66].

2.2.3 c-Jun N-Terminal Kinase (JNK)

The concept of biphasic angiogenic responses may apply more broadly to a large spectrum of other mediators such as intracellular signals. The stress-activated protein kinase JNK pathway is known to trigger many cell death pathways including caspases, and many studies have shown that JNK inhibitors are neuroprotective in rodent stroke models [67]. However, more recent data clearly support a beneficial role for JNK in CNS disease and repair [68]. JNK signaling is involved in neuronal precursor cell migration, microtubule assembly, and axonal guidance during brain development. After injury, this signal can contribute to dendritic sprouting and axonal regrowth. More recently, JNK has also been shown to mediate angiogenesis [69]. JNK mediates the regulation of both VEGF and MMPs, and blockade of JNK cascades with inhibitors can suppress angiogenesis [70, 71]. Whether similar pathways are activated in cerebral neurovascular repair and remodeling remains to be determined, but very recent paper reported that delayed JNK inhibition worsened vascular remodeling in rat stroke model PMID: 22,699,892.

2.3 Endothelial Progenitor Cell in Brain Angiogenesis

Interactions between angiogenesis and functional remodeling after stroke can be also manifested in terms of circulating endothelial progenitor cells (EPCs). EPCs are immature endothelial cells circulating in peripheral blood and are under maturation process to become endothelial cells [72]. Hence, EPCs possess functional and structural characteristics of both stem cells and mature endothelial cells. As discussed above, angiogenesis in the penumbra area is an important natural response to stroke. Although circulating EPCs represent only ~0.01% of cells in the blood under steady-state conditions, EPC numbers are highly affected by stroke onset. Emerging studies are beginning to elucidate the relationship between stroke outcome and the

number of circulating EPCs. In rodent models of focal cerebral ischemia, there was a strong correlation between the volume and severity of infarcts and the absolute number of circulating EPCs [73]. In clinical stroke patients, an increase in circulating EPCs after acute ischemic stroke was associated with good functional outcome and reduced infarct growth and maturation [74, 75]. Importantly, EPC levels were significantly lower in patients with severe neurological impairment compared with patients with less severe impairments at 48 h after ischemic stroke [75]. In mouse cerebral ischemia models, bone marrow-derived EPCs homed to the ischemic core and participated in cerebral neovascularization [39]. Recent experiments suggest that HMGB1 and interleukin-1beta can promote EPC homing and proliferation, respectively [76, 77]. Moreover, very recent study indicates that HMGB1 from reactive astrocytes recovers neurological function through EPC accumulation in the injured area after stroke [78]. These observations raise the possibility that EPCs can be used as a therapeutic approach for promoting repair [79]. However, the precise mechanisms of the EPC contribution to postnatal angiogenesis remain to be elucidated. It has been reported that bone marrow-derived EPCs did not incorporate into the adult growing vasculature [80, 81]. These reports suggest that EPCs support angiogenesis indirectly through growth factor release.

3 Neurovascular and Oligovascular Signaling for Brain Angiogenesis

Thus far, we have discussed the mechanisms of brain angiogenesis after stroke. But again, regulating mechanisms for brain angiogenesis after stroke are quite complex, and it may not be sufficient to focus on only endothelial cells (and their progenitor cells) to understand how new blood cells appear in the remodeling brains. In recent years, the concept of the "neurovascular unit" has emerged as a new paradigm for understanding the pathology in the CNS diseases including stroke [82–86]. This modular concept is defined at an intercellular level that comprises dynamic interactions between cerebral endothelial cells, glia, neurons, and other brain cell types (Fig. 21.2). Dysfunctional crosstalk between neurons, glia, and vascular compartments contributes to multiple aspects of acute pathophysiology in CNS disease. Impaired glutamate release–reuptake mechanisms in neurons and astrocytes can amplify excitotoxicity [87]. Perturbed signaling between cerebral endothelium, astrocytes, and pericytes can disrupt BBB integrity [85]. Dysfunctional coupling between neuronal activation and vascular responses can promote deleterious spreading depression [88]. Moreover, disordered signaling between all neurovascular and gliovascular elements can underlie the evolution of neuroinflammation and cell death [89]. In addition to the acute phase, the concept of the neurovascular unit has now been applied to discuss the mechanisms of the chronic phase after stroke (Fig. 21.3). The evolution of brain injury and neurodegeneration comprises a dynamic balance and imbalance between initial triggers of injury and evolutionarily

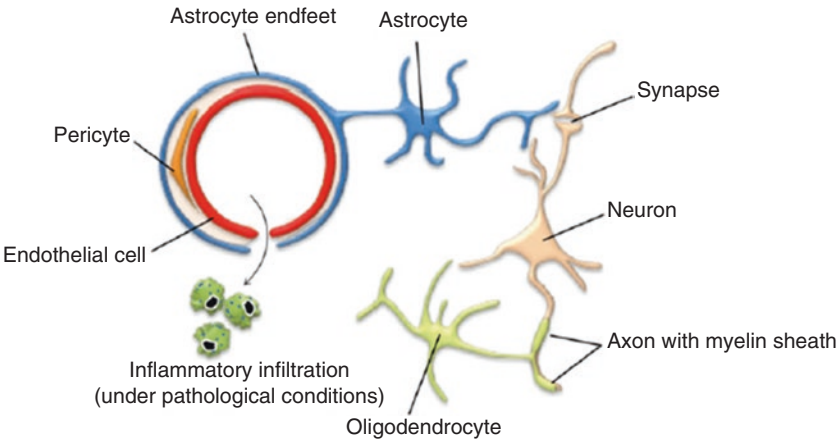

Fig. 21.2 Schematic of the neurovascular unit. This schematic depicts a cerebral blood vessel and surrounding brain cells such as astrocyte, neuron, pericyte, and oligodendrocyte

conserved responses of brain plasticity, remodeling, and compensation [90]. And just as cell–cell signaling in the entire neurovascular unit mediates acute injury, delayed recovery should also recruit analogous non-cell-autonomous mechanisms in the brain. Hence, dissecting these various signals and substrates within the neurovascular unit may reveal opportunities for developing novel therapeutic targets for stroke. In this section, we briefly outline the principles of the neurovascular unit and discuss recent data that may help us find common mechanisms of injury and repair after stroke, focusing on brain angiogenesis.

3.1 Neurovascular Damage in the Acute Phase

The fundamental mechanisms of brain cell death in the acute stroke phase are multifactorial. Accumulated data over the past two decades have implicated excitotoxicity, oxidative stress, and, in some circumstances, apoptotic-like pathways [82, 91]. When brain fails to generate sufficient ATP by reduction of blood flow supply, energy failure occurs and ionic gradients are lost. Glutamate reuptake processes are impaired, and accumulated glutamate promotes excessive calcium entry and release. Calcium-dependent synthases and proteases contribute to neuronal death by degrading key cytoskeletal and enzymatic proteins. Abnormality of calcium homeostasis also generates nitric oxide and peroxynitrite, which directly strike neighboring cells. Moreover, mitochondrial functions such as oxidative phosphorylation fail and reactive oxygen radicals are released that further compromise cells by attacking proteins, lipids, and nucleic acids. In parallel with these ionic and free radical pathways, deleterious molecules such caspases may also promote cell death by suicidal endogenous mechanisms. However, most of the cell death pathways outlined here

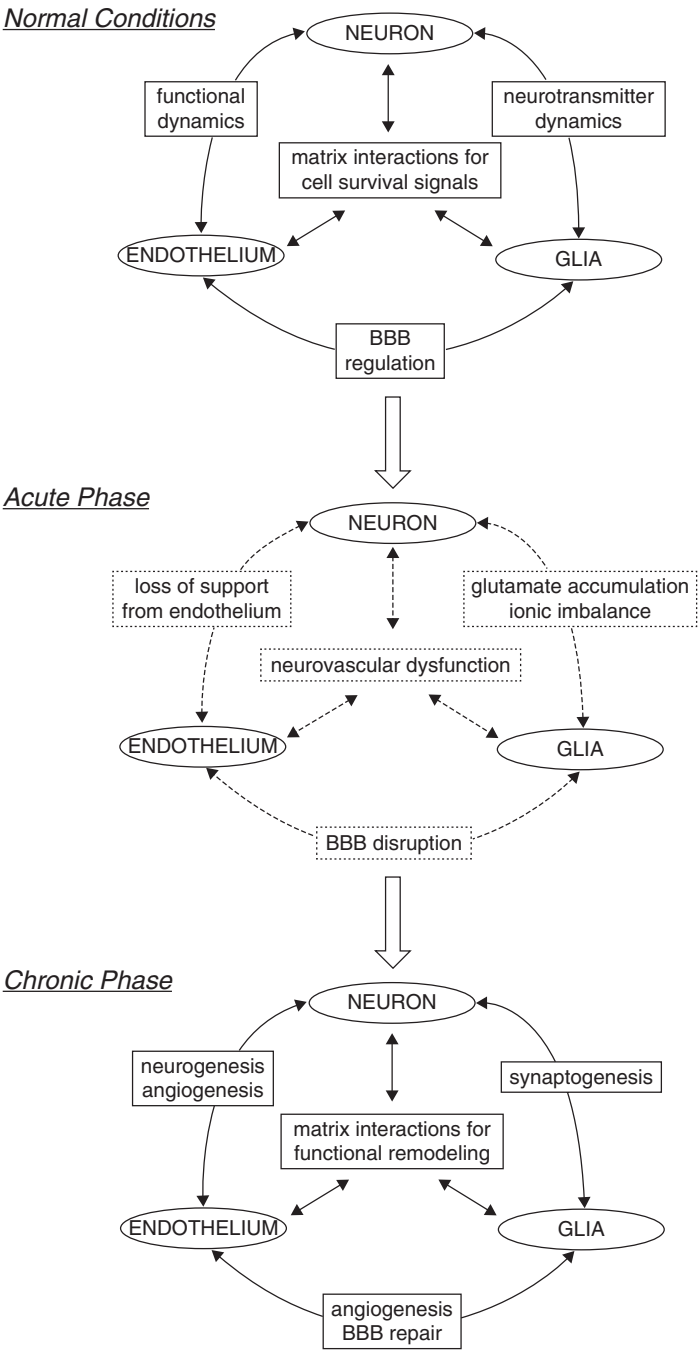

Fig. 21.3 Schematic of the neurovascular unit under normal conditions, acute and chronic phase after stroke. Homeostatic signaling in the neurovascular unit sustains normal brain function. Dysfunctional neurovascular signaling mediates injury after stroke. During the recovery phase after stroke, neurovascular signaling may also be critically important with repair mechanisms that may involve neurogenesis, synaptogenesis, and angiogenesis

are well documented for neurons. Whether similar mechanisms should be targeted for glial and vascular compartments remains to be carefully assessed. Besides basic cell death mechanisms, one of the most important facets of early neurovascular damage is manifested as perturbations in BBB function. The BBB homeostasis is remarkably dependent on endothelial–astrocyte–matrix interactions [85, 92]. Perturbation of the neurovascular matrix (type IV collagen, heparan sulfate proteoglycan, laminin, fibronectin, etc.) disrupts the cell–matrix and cell–cell signaling that maintain neurovascular functions. Many proteinases might contribute to extracellular matrix proteolysis, and the extracellular protease systems become dysregulated under diseased conditions. As discussed, roles of the MMP family have been focused in the neurovascular damage after stroke [93]. MMP levels are increased in both experimental models of stroke [94–96] and stroke patients [49, 97]. Those excessive MMP activities might be deleterious. MMPs can degrade the extracellular matrix that comprises the basal lamina, thus damaging the BBB directly. In experimental stroke models, MMP inhibition reduces infraction and edema [98, 99]. In addition to BBB disruption, MMP-induced proteolysis of the neurovascular matrix might also promote programmed cell death by detachment of cells from the extracellular matrix (so-called anoikis) [100, 101]. These findings suggest that MMPs (and other extracellular proteases) mediate neurovascular damage during the acute stages of stroke. Ultimately, these neurovascular perturbations can also be interpreted as dysfunctional crosstalk between components of the neurovascular unit. However, none of above cell death pathways or neurovascular mechanisms have been successfully exploited for treating acute stroke patients. Although many translational barriers are involved, the heterogeneity of patients and tight timelines during acute pathology makes it difficult to block these early targets efficiently. Therefore, a recent emphasis in the field is beginning to assess opportunities for promoting neurovascular recovery (especially for angiogenesis) after stroke.

3.2 Neurovascular Repair in the Chronic Phase

Most stroke patients show some degree of recovery over time. For example, functional MRI studies demonstrate that peri-infarct areas are highly plastic [3, 102]. Representational areas shift as latent networks are unmasked, and parallel circuits are recruited adjacent to damaged regions [103]. One of the best early examples of cell–cell signaling in the neurovascular unit may be found in the original observations of the so-called neurovascular niche for neurogenesis and angiogenesis. From an evolutionary perspective, the underlying molecular mediators of neurogenesis and angiogenesis overlap and are highly conserved [104]. Molecular mechanisms of angiogenesis and neurogenesis have been evolutionarily conserved so that similar mediators and pathways are involved in both phenomena [105]. It is now accepted that cell–cell signaling between cerebral endothelium and neuronal precursor cells helps mediate and sustain pockets of ongoing angiogenesis and neurogenesis in adult brain [3, 84, 85, 106, 107]. Crosstalk between the vascular and neuronal

compartments in the neurovascular niche is mediated by an exchange of soluble signals. This phenomenon is partly mediated by the ability of cerebral endothelium to secrete a rich repertoire of trophic factors [108–110]. In the normal brain, the neurovascular niche defines these complex mechanisms of cell–cell signaling between cerebral endothelium and neural precursors in the subventricular and subgranular zones of ongoing neurogenesis. In the context of post-stroke recovery, these close relationships between neurogenesis and angiogenesis are maintained. Neuroblasts migrate along perivascular routes [111]. Promotion of neurogenesis enhances vascular regrowth, and conversely, angiogenic stimulation enhances neurogenesis [112, 113]. Angiogenesis in peri-infarct regions has been detected in rodent models of cerebral ischemia [114] as well as in human stroke [36]. Hence, brain recovery after stroke comprises interdependent neurovascular plasticity and remodeling processes that recruit multiple common mediators and signals [105].

3.3 Cell–Cell Trophic Coupling in White Matter

For the most part, the concept of the neurovascular unit is used to guide investigation in gray matter. However, cell–cell trophic interactions are likely to be important in white matter as well. White matter is vulnerable to ischemic stress, and white matter damage is a clinically important part of stroke [82, 115]. Therefore, without considering white matter mechanisms, we may not be able to protect/recover the brain function against ischemic insults. Compared to the cellular mechanisms of neurovascular damage/repair in gray matter, white matter pathophysiology remains relatively understudied and poorly understood. However, the idea of the neurovascular unit is now applied to the white matter stroke research. The main components of white matter are the neuronal axon, oligodendrocyte (myelin), astrocyte, and endothelium. As in the neurovascular unit in gray matter, astrocytes and cerebral endothelial cells work together to maintain BBB in white matter [115]. In addition, astrocytes are in close apposition to OLGs within white matter [116], and couple with OLGs through gap junctions to maintain their functions [117]. Furthermore, astrocyte-derived soluble factors were also reported to nourish oligodendrocyte lineage cells [118, 119]. And, of course, myelin–axon interactions are essential for white matter homeostasis. OLGs not only myelinate axons but also maintain their functional integrity and survival through OLG-specific proteins and/or trophic factor release [120, 121]. Similar to gray matter, during the acute phase of stroke, several deleterious factors/cascades are activated. For example, MMPs are upregulated, and direct attack of MMPs on myelin components affects OLG survival and function [122]. Even if outright cell death does not occur, metabolic dysfunctions in OLGs might still affect the normal replenishment of myelin and synthesis of myelin-associated proteins, which eventually impair myelin–axon interactions. In the chronic phase, some endogenous responses might work for repairing white matter damage. However, it remains to be fully elucidated how angiogenesis and oligodendrogenesis occur during the chronic phase after stroke. As in the neurovascular

niche, an oligovascular niche in the white matter may play an important role in supporting trophic interactions between brain endothelium and oligodendrocytes [123]. Brain endothelium-derived VEGF promotes OPC migration through focal adhesion kinase and reactive oxygen species-dependent mechanisms [124, 125]. On the contrary, after white matter injury, oligodendrocytes produced MMP-9 which may promote vascular remodeling [126]. Future studies should carefully examine the precise mechanisms of the cell–cell trophic coupling in white matter for better understanding the brain angiogenesis after stroke.

4 Therapeutic Implication

Therapeutic options for clinical management in stroke still remain quite limited. The treatment of only one FDA-approved drug t-PA is not easy due to its narrow therapeutic time window and related risks of brain hemorrhage [127]. Because recent preclinical studies have revealed that brain injury activates cellular signaling for angiogenesis and neurogenesis, strategies to promote angiogenesis are part of larger neurorestorative approaches in order to increase the diversity in therapeutic options for a variety of patients. Although there are no agents and manipulations in clinical use that can boost angiogenesis after stroke yet, we should discuss some key promising seeds here.

4.1 Combination Therapy with VEGF

As introduced, VEGF is the prototypical mediator for brain angiogenesis after stroke. Therefore, in theory, VEGF therapies could promote neurorestoration either directly as a neuroprotective agent or indirectly by inducing angiogenesis [21, 128, 129]. However, the function of VEGF as a vascular permeability factor also means that an untitrated response may lead to blood–brain barrier leakage, brain edema, vasodilation, and aberrant systemic hemodynamics [130–133]. In addition, VEGF-induced angiogenic vessels are hemorrhagic, aggravating inflammatory responses in the recovering penumbra [134, 135]. Nevertheless, recent findings now suggest that combinatorial therapy with other agents would be beneficial. The untoward side effects of VEGF were partially obviated by treatment with a combination of angiopoietins [136]. An alternate strategy might be to apply HIF prolyl hydroxylase inhibitors. These reagents may raise HIF-1 levels and increase expression of several hypoxia-response proteins that could avoid vascular leakage [137]. Moreover, several downstream effectors of VEGF have been tested as selective modulators for VEGF signaling [138]. The Roundabout (Robo) protein 4/Slit2 axis has been shown to selectively inhibit VEGF-165-induced migration, tube formation, and permeability in vitro and VEGF-165-stimulated vascular leak in vivo by blocking Src family kinase activation [139]. Thus, targeting the Robo4-Slit2 signaling or in recovering vessels may open newer therapeutic options along with VEGF to minimize tissue injury and maximize its beneficial effects [140].

4.2 Cell Junction Molecule

In addition to VEGF signaling, cell junction molecules would be interesting target for promoting brain angiogenesis. Cell junction molecules exist at the interface of multiple cellular decisions and play important roles in vascular permeability, quiescence, invasion, and differentiation [141–143]. Anti-integrin therapy for tumor angiogenesis has gained ground in the recent times [144]. GPIIb/IIIa and αvβ3 integrins mediate endothelial–platelet interaction, and several antagonists targeted against these molecules have been found to have anti-angiogenic effects in vivo [145, 146]. Those findings suggest that modulation of cell adhesion molecules and their signaling might be a useful strategy in stroke therapy because of their ability to alter responsiveness to growth factors—either potentiate growth factor signaling or attenuate its effects where necessary [147–149]. Hence, the use of soluble adhesion molecules may deliver survival signals, alter growth factor responsiveness, and facilitate pertinent cell–cell communication within the remodeling penumbra. For example, soluble N-cadherin fragments were found to stimulate migration of endothelial cells through the FGF receptor [150, 151]. During vasculogenesis, N-cadherin mediates adhesion, recognition, and signaling between pericytes and endothelial cells and is required for normal vascular morphogenesis. The significant diversity in expression of cell junction molecules, the expression of tissue-specific isoforms, and their spatiotemporal functions in the CNS can be exploited for better vascular morphogenesis and neurogenerative outcomes.

4.3 Cell-Based Therapy

As noted in the section of EPCs, beyond the cell signaling targets, cell-based therapies would be promising approaches for the treatment of brain injury [152, 153]. Cell therapies using neural stem/progenitor cells (NSPCs) may replace lost brain cells, promote endogenous neurogenesis, and improve functional recovery [152]. NSPCs stabilize vasculature during ischemia, suggesting therapeutic application of NSPCs to promote revascularization and repair after brain injury [154]. Of course, there is little evidence to assess the applicability of NSPCs to stroke patients, and therefore, well-designed clinical trials are necessary to evaluate safety, toxicity, and efficacy as well as optimal cell type, route, and time of delivery for NSPCs [155, 156]. Another candidate for cell-based therapy would be mesenchymal stem cells (MSCs) isolated from bone marrow, adipose tissue, umbilical cord blood, placenta, and pancreas. MSCs exert powerful immunomodulatory effects, which include inhibition of proliferation and function of T cells, B cells, and natural killer cells. Those effects reduce immune reactions and increase tolerance of MSC recipients [157]. Moreover, MSCs secrete various growth factors including BDNF, VEGF, and FGF, which promote angiogenesis after stroke [158–162]. Hence, genetically engineered MSCs (and NSPCs) with overexpression of growth factors may be an improved source for cell

therapy for stroke [163]. Finally, recent landmark experiments have shown that transient overexpression of a small number of transcription factors can reprogram differentiated cells into induced pluripotent stem (iPS) cells that resemble embryonic stem cells [164]. These iPS cells avoid the ethical issue inherent in embryonic tissues or oocytes and have the potential to generate patient-specific cell types for cell replacement therapy. iPS cells may offer promising opportunities for the treatment of brain injury. But again, pharmacological and cell-based therapies to induce rapid angiogenesis run the danger of leading to dysfunctional tissue architecture and exacerbating neuronal damage. How these promising experimental approaches can be tested long term in stroke patients remains to be carefully assessed.

5 Conclusion

The adult mammalian brain can be surprisingly plastic, especially after stroke and brain injury. Under normal conditions, newborn neurons in the subventricular and subgranular zones migrate to olfactory regions and the hippocampus. After brain injury, the birth rate of new cells seems to increase, and neuroblasts are rerouted toward damaged tissue. Along with neurogenesis, the recovering brain also exhibits complex patterns of vascular remodeling. This chapter provided an abbreviated summary and survey of major pathophysiological concepts in stroke, focusing on mechanisms of brain angiogenesis. Thus far, drugs that can be cyto-protective against stroke are not yet developed. Therefore, an emerging emphasis on promoting recovery after brain diseases is beginning to take shape in our field. Although there are many difficulties in translating findings in basic research into clinical applications, therapies that can boost endogenous angiogenic properties would be promising approaches for stroke patients in the future.

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Chapter 22
Stimulated Microgravity and Induction of Angiogenesis; A New Perspective in Wound Healing

Selvaraj Vimalraj, Kasiviswanathan Dharanibalan, and Suvro Chatterjee

Abstract The current research interest in the therapeutic management of wound healing is to attain a complete and rapid healing of chronic wounds with minimal scar. There is an urge to apply a novel approach to prompt the wound healing process because of the huge economic burden worldwide. Hence, the current article initially focuses on the management and care of wounds from classic to currently available techniques and vulnerability of wound. Several propositions for better wound healing has been proposed, one of them is simulated microgravity which heals the wounds by promoting microgravity. Stimulated microgravity induces changes in cytoskeleton; thereby it regulates the behavior of endothelial cells in terms of cell proliferation, adhesion, migration, production of extracellular matrix and translocation of bioactive molecules inside the cells. Additionally, we have listed around 40 genes which are potentially involved in angiogenesis and are differentially expressed in endothelial cells under microgravity conditions. The coordinated cellular and molecular events in endothelial cells in microgravity promote angiogenesis which in turn facilitates wound healing process.

Keywords Microgravity • Angiogenesis • Wound healing • Therapeutics • Endothelium • Actin remodeling

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1 Introduction

Wound healing is a process by which the integrity and homeostasis of the tissue is restored by activating several coordinated intra and extra cellular signaling pathways [23]. In addition to immune cells, neutrophils, monocytes, lymphocytes dendritic cells, keratinocytes, fibroblasts and endothelial cells also endure phonotypical changes which results in cellular proliferation, apoptosis, differentiation and migration in wound milieu [23]. If these processes are not coordinated well in response to injury it causes impairment in wound healing process. It could also lead to the malignant transformation in the tumorogenic wound environment [45]. A hyperactivated healing process promotes scar formation due to disorganized extracellular matrix and patch formation in the wound [61, 50].

Gravity has its influence in the evolution of organism by shaping up genetic makeup and physiology. Hence, alterations in the gravity could have the potency to manipulate the functions of the system [31]. The long term exposure to microgravity leads to mechanical unloading of tissues organization which results in alterations of physiological function. The spaceflight and space mission lead to bone loss, muscle loss, cardiac dysfunction, impairment in wound repair and immune system dysfunction [4]. Alteration of gravity in a controlled manner can be used in therapeutic applications. For instance, there are reports which indicate that alteration in the gravity could facilitate bone fracture repair [54]. The mechanical stretch involved in the asymmetric migration of keratinocytes by increasing EGF secretion thereby it facilities wound healing process [33]. We have reported that the short term microgravity, 2 h exposure promotes endothelial monolayer of wound [48, 49]. Microgravity exposure is involved in the regulation of vasoactive, inflammatory and adhesion signaling molecules by remodeling the cytoskeleton and the distribution of caveolae. These molecular events regulate angiogenesis, cell survival, apoptosis, proliferation, differentiation and migration [34, 48, 49, 11]. Since these molecular and cellular events are key components of wound healing steps we anticipate that a controlled use of stimulated microgravity may facilitate a faster and efficient wound healing.

2 Wound Healing

Wound healing is the process by which injured tissue repairs itself after trauma. Normal skin consists of two layers, epidermis (outer layer) and dermis (inner layer) which acts as a protective barrier against the external environment. A series of biochemical cascades are activated at the time of injury. This process consists of four consecutive steps that are hemostasis, inflammation, tissue growth and remodeling phase [19]. Homeostasis is an immediate post-injury phase which promotes attachment of platelets to the injury site. This event facilitates blood clotting by forming fibrin-mesh to avoid excessive bleeding. In inflammation phase, damaged and dead cells are removed by phagocytosis process. Next, platelet derived growth factors are

secreted at the site of injury to initiate migration and division of cells in tissue growth phase. Simultaneously, formation of granulated tissue, epithelization, collagen deposition and wound contraction occur. Also, a new extracellular matrix formed to provide a new tissue at the site of injury followed by migration of cells at the top of wound area. Where cells are no longer alive in proliferation or tissue growth phase, those are removed by apoptosis process.

3 Wound Inflammation, Chronic Inflammation Switch Over to Tumor

It is suggested that to avoid infection at the site of wound the process of its healing should be speedy one [3]. However, impairment in wound healing process leads to chronic skin disorders like ulcers (e.g., diabetic ulcer) and hypertrophic scarring. Hypertrophic scars are raised, red, hard and have abnormal sensations including pain and tenderness [13, 1]. Hypertrophic scars are associated with dermal wounds and it not only causes disfigurement of skin but also cause loss of functionality if occurring over a joint [27]. Hypertrophic scarring is common when epithelialization is delayed in the healing process or when wound occurs in areas of high tension like deltoid or sterna regions [27, 38, 39]. Scarring may also occur if wound is deep seeded as fibroblasts of scar site which resembles fibroblasts derived from deep dermis [58].

However, a significant number of studies have been done to understand the relationship between chronic wounds with cancer. In many cases, the formation of malignant occurs at the site of chronic injury. The site of chronic wound is prone to develop malignant tumors and the chronic inflammation is considered to be an important risk factor for the pathogenesis of malignant disease [45, 46]. Disturbance in vascular integrity releases the plasma proteins and deposition of fibrin at the site of injury. But, these changes are non-permanent process and are not involved in tumor growth. In inflammation phase, secretion of cytokines induces the growth factors, especially M1 like macrophage phenotype. But, in cancerous tissue cytokines induce differentiation of M1 to M2 like macrophage phenotype. Understanding differentiation of macrophages of M1 to M2 phenotype at the site of injury will give better perception of wound nature [26].

4 Wound Care and Management: A Chronology

The primary aim for any open wound care is to attain a quick closure of ruptured skin, decrease the potential risk for infection at the exposed area and minimized scar formation. Various methods have emerged to cater the above condition ranging from topical use of antiseptics to skin grafts. Wounds can be classified based on their etiology into acute and chronic wounds. Rapid wound healing is very important for

severe trauma or burns wound to obtain scar less healing. Chronic wounds take a long time to heal rather than acute wounds. But, in both pathological conditions, formation of biofilm, accumulation of microorganisms in the site of injury is an important factor which delays the complete wound healing.

Traditionally, burns were allowed to heal on its own without the application of any topical agents. Hydro-therapy was used for sub eschar suppuration and debridement [51, 24]. Topical aids such as sulfamylon cream, mafenide acetate (11.1%), para-aminomethylbenzene sulphonamide penetrate deep into full-thickness eschars that give a major advantage compared to other antimicrobials specifically when treating full-thickness of burn wounds. Silver acts as nanoparticles and can penetrate through the cell wall of bacteria and thus bring about anti-bacterial effect. Moyer and Monafo introduced 0.5% silver nitrate (AgNO3) in 1964 as a topical agent for anti-infection prophylaxis. Further, silver sulfadiazine (1% cream) was developed [16] by amalgamating silver nitrate and sulfadiazine. These approaches are effective if begun immediately post burn injury. Moreover silver ion precipitates on contact with chloride ion to form $AgCl_2$. It does not penetrate the eschar and thus has no antibacterial action within burned tissue. Another drawback of these drugs is depletion of silver ions at the site of wound due to "leeching" across the open wound. Surgical methods have been applied for wound care management. The tangential excision of deep partial thickness burns, excision to fascia of extensive full thickness burns and implanting of autologous skin graft has shown improvement in survival of patients, especially where excision was done within the first 72 h after injury. In patients with absence of sufficient donor site for autologous grafts, biologic dressing like cadaver allograft is also used to close wounds. Such dressings help in re-establishment of skin barrier function and prevent contamination of underlying tissue with exogenous bacteria [5]. However, if the allograft is placed over non-viable tissues the sub-graft suppuration may occur which result in grafts rejection. Stem cells based therapies are potential approach for wound healing through release of secretary molecules, growth factors and cytokines that triggers new vessel formation and controls inflammation. However, there are barriers in the clinical translation of stem cell based therapies for wound healing. Immunogenicity and tumorigenicity are the possible risk of stem cells based therapies. Additionally, identification of suitable stem cells population and choosing or optimizing the standard delivery vehicles are considerable challenges for wound healing management [12]. Addressing these problems and speeding up the wound healing process still warrant new therapeutic strategies.

5 Proposing Microgravity as a Technique for Wound Healing by Promoting Angiogenesis

Alteration of haemostatic phase by regulating growth factors, activating platelets involved in proliferation and function of inflammatory cells by microgravity leads to a decrease in wound healing process [14].

The sustained microgravity stimulation impaired the wound healing process by decreasing growth factors and collagen content in the rat [10]. Microgravity promotes nitric oxide (NO) supported angiogenesis via the iNOS–cGMP–PKG pathway in macrovascular endothelial cells [34, 48, 49]. Maier et al., [34] have reviewed how microgravity and hypergravity is involved in endothelial cells functions and discussed the controversial statement of microgravity on endothelial cells. Microgravity alters the expression of signaling molecules involved in vasolation, inflammation and cell adhesion, which result from alteration in cytoskeleton remodeling. These molecular events regulate cell proliferation, apoptosis, migration and angiogenesis. The methods employed to stimulate the microgravity, experimental condition and different cells used for the study are the main reason for controversial outcome of microgravity involved systems' function.

6 Microgravity Perturbs Vascularization in Wound Milieu?

Angiogenesis is a process of formation of new blood vessels from preexisting blood vessels. This process has been initiated by endothelial activation and release of nitric oxide synthase (NOS). NOS is a well known mediator for pro angiogenic characteristics. Activation of vascular endothelial growth factor (VEGF), fibroblast growth factors (FGF), increased cell proliferation and migration along with increased flow in micro circulation is mainly regulated by NOS derived NO [15]. Recent reports have indicated that the ECs on the interior surfaces of vessels are highly sensitive to microgravity and undergo series of morphological and functional changes [8]. Several studies have been directed to understand the implications of microgravity in endothelial functions. It has been observed that simulated microgravity increase the rate of proliferation of endothelial cells [6]. Also, expression profile of many angiogenic molecules, such as NOS, VEGF and endothelin-1 are modified under microgravity conditions. These studies indicate that microgravity conditions intrigue angiogenesis.

7 Experimental Approach

7.1 Cell Viability Under Simulated Microgravity

The cell viability was measured after 2 h of microgravity treatment followed by Fluorescein diacetate (FDA) staining of EAhy926 (Fig. 22.1a). The procedure for FDA staining was performed as described elsewhere [44].

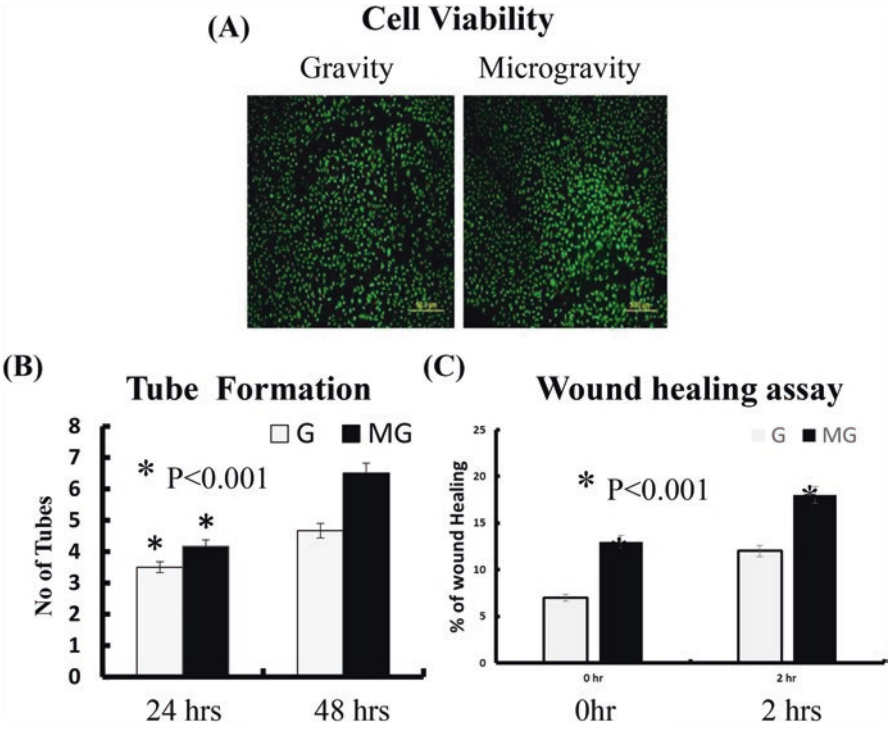
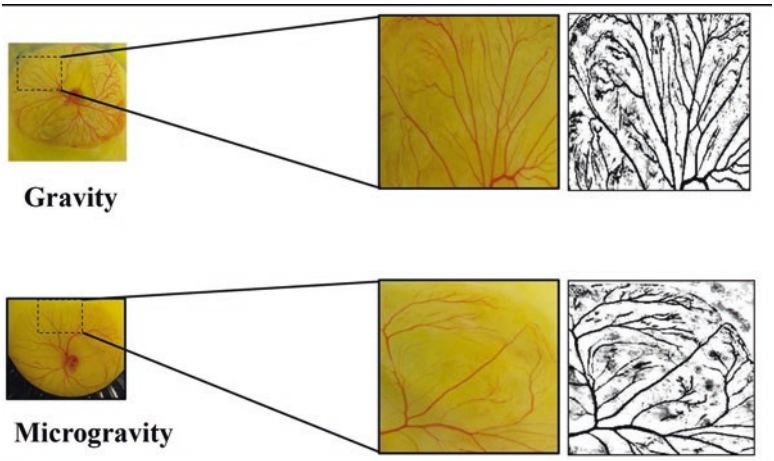


Fig. 22.1 Microgravity elevates the distinctive features of angiogenesis. (**a**) Cell viability. After 2 h of microgravity stimulation the cells were washed with 1× PBS (pH 7.4) and then cells were treated with Fluorescein Diacetate (FDA -30 ug/ml) solution to visualize the viable cells. A significant increase in migration (Boyden chamber assay, wound healing assay) (**b**), tube formation (**c**) was observed under microgravity exposed EAhy926 cell suspension. *P < 0.001 vs gravity control and P < 0.001 vs microgravity control

7.2 Migration of Endothelial Cells Under Simulated Microgravity

In order to examine the dynamics of migratory response of ECs under microgravity conditions, we have used Boyden's chamber assay. Results showed that 60% of cells were migrated from upper chamber to lower under microgravity compared with 1G (gravity) treated cells. Wound healing assay for studying migration of ECs monolayers revealed that microgravity sensitized cells promotes the migration rather than 1G group (Fig. 22.1b). Tube formation characteristics of ECs analyzed by subjecting ECs suspension to microgravity for 2 h then seeded in matrigel coated coverslips. Then the number of tube formation was evaluated after 24 and 48 h (Fig. 22.1c). Further, to document the refined angiogenesis pattern under microgravity condition day 1 fertilized chicken eggs were treated for 2 h under microgravity conditions and then incubated for 3 days at 37 °C in CO_2 incubator prior to



Treatment Groups	Total Length of tubule complexes	Total size of tubule complexes	Total no of Junctions
Gravity	2812.593	10753.67	52.6666
Microgravity	3769.497	14016.67	86.6666

Fig. 22.2 Microgravity promotes the angiogenesis. Fertilized chicken eggs (1 day) were treated for 2 h microgravity conditions then incubated for 3 days at 37 °C to investigate the CAM angiogenesis. Vasculature pattern of chick eggs from gravity and microgravity quantified by using angioquant software also total number of junctions, length of tubule complexes and size of tubule complexes are tabulated (*$P < 0.05$)

experiment. Quantification of vasculature pattern was validated by Angioquant software (Fig. 22.2). These results clearly indicate that the microgravity is involved in the promotion of angiogenesis.

7.3 Prediction of Microgravity Stimulated Genes Involved in Angiogenesis

A series of studies have been performed in recent years to understand the effects of microgravity on endothelial functions [21]. Differential gene expression has been well documented in ECs under simulated microgravity [6, 21, 25, 35]. Available reports indicate that eNOS can induce angiogenesis process through PI3K-Akt dependent pathway in low gravity [47]. Additionally, members of GTPase family genes are shown to be modulated by MAPK intracellular signaling pathways under simulated microgravity [32]. Present section explored that a set microgravity stimulated genes are involved in the activation of endothelium. We anticipate that results of this study would offer mechanistic insight of microgravity mediated endothelial activation.

Microgravity involved in differential expression of genes in a system thereby it influence several physiological role. Based on the literatures we have listed around 40 genes, potentially involved in angiogenesis and are differentially expressed in ECs by microgravity influence [9, 20, 22, 30, 36, 47, 52, 55]. The differentially expressed genes are tabulated in Table 22.1. These differentially expressed genes were further subjected into ToppCluster (http://toppcluster.cchmc.org/) [28] and Cytoscape (http://www.cytoscape.org/), bioinformatics tools to identify their functional annotations (Fig. 22.3). The differentially expressed genes by microgravity in ECs were used to generate regulatory network clusters by hierarchical clustering in ToppCluster. The resulted files in the format of xgmml (XGMML is an XML application based on GML which is used for graph depiction) were then subjected to Cytoscape for the preparation of visualizable networks [37, 56, 57]. In the detailed network result, round boxes were represented in different size based on their relative importance which means the number of edges joined to particular gene with their interacting genes (Fig. 22.3a). Additionally, the same data were represented in bar diagram for better understanding, molecular function (Fig. 22.3b), molecular process (Fig. 22.3c), Pathways interaction (Fig. 22.3d). The molecular functions of genes implicated under microgravity conditions are listed in Table 22.1. In Molecular function (Fig. 22.3b), More than 60% of the genes are involved in molecular functions related to angiogenesis. Such as cell migration, cell proliferation, actin polymerization, cell polarity, histone deacetylation process. The total list of the genes involved includes enzyme, sequence-specific DNA, cytokine activity, regulatory region DNA binding regulatory region nucleic acid binding, collagen binding, type 2 fibroblast growth factor receptor binding, type 1 fibroblast growth factor receptor binding, DNA insertion or deletion binding, MutLalpha complex binding, chaperone binding, CCR5 chemokine receptor binding, cAMP-dependent protein kinase activity transmembrane receptor protein tyrosine kinase activator activity, vascular endothelial growth factor receptor 2 binding, satellite DNA binding. In molecular process (Fig. 22.3c), *in silico* approach revealed the functionally active and distinct role of genes which implicated in angiogenesis under simulated microgravity conditions. Most of the genes associated with a response to hormone, single organism cell adhesion, regulation of cellular component movement, regulation of locomotion, regulation of phosphorylation, cell migration, response to steroid hormone, regulation of cell migration, and positive regulation of cell proliferation. Additionally, a list of genes involved to initiate actin cytoskeleton reorganization, actin filament organization, actin cytoskeleton organization, actin filament-based process, actin filament bundle assembly, actin filament bundle organization, actin filament reorganization, actin filament severing to enhance the mobility and polarity rearrangement of the cells. Systematic analysis shows that a list of 237 potential molecular processes are positively regulated the angiogenesis process, such as positive regulation of cellular protein metabolic process, positive regulation of protein metabolic process, positive regulation of cell proliferation, positive regulation of catalytic activity, positive regulation of molecular function, positive regulation of cellular component organization, positive regulation of multicellular organism process, positive regulation of protein phosphorylation, positive regulation of phosphorylation, positive

Table 22.1 List of genes stimulated by microgravity

Gene name	Abbreviation	Function	References
RhoA	Small GTPase protien RhoA family	Regulation of cytoskeletal dynamics, transcription, cell cycle progression and cell transformation.	[30]
p21	Cyclin-dependent kinase inhibitor 1 or CDK-interacting protein	Cell cycle arrest protein.	[52]
Hsp70	Heat shock proteins	Protein folding processes.	[9]
Cav	Caveolin	Signal transduction.	[20]
eNOS	Endothelial nitric oxide synthase	Nitric oxide signaling.	[47]
VEGF	Vascular endothelial growth factor	Vaculogenesis and angiogenesis.	[30, 55]
EDN1	Endothelin 1	Receptor binding and hormone activity.	[22]
ABL2	ABL proto-oncogene 2	Rassignallin pathway and ERK signaling.	
AMPK 1	5′ AMP-activated protein kinase	Cellular energy homeostasis.	
Integrins	Integrins	Cell signaling.	
TNFRSF12A	TNF receptor superfamily member 12A	Promotes angiogenesis and the proliferation of endothelial cells.	
FN	Fibronectin	Cell adhesion molecule.	
LM	Laminin	Cell adhesion molecule.	[36]
α-actin	Alpha actin	Cell motility, structure and integrity.	
β-actin	Beta actin	Cellular processes.	
α-Vbeta 3 intergrin	AlphaVbeta 3 intergrin	Endothelial cell migration.	
IL-1α, IL-1β, IL-1 RA, IL-6, IL-8	Cytokines family	Involved in immune function.	
IP-10	Interferon gamma-induced protein 10	Promotion of T cell adhesion to endothelial cells.	
Eotaxin	Eosinophil chemotactic protein	Chemotaxis.	
Rantes VCAM-1	Vascular adhesion molecule 1	Leukocyte-endothelial cell signal transduction.	
ICAM-1	Intercellular adhesion molecule 1	Cell signaling	
IFN-α	Interferon alpha	Activates NK cells	

(continued)

Table 22.1 (continued)

Gene name	Abbreviation	Function	References
TNF-α	Tumor necrosis factor alpha	Inflammation	[55]
TXNIP	Thioredoxin interacting protein	Regulator of cellular redox signaling	
TP53INP1	Tumor protein p53-inducible nuclear protein 1	Regulates transcription of cell death genes and p53 pathway	
ID1	DNA-binding protein inhibitor ID-1	Cell growth, senescence, and differentiation	
SLITRK4	SLIT and NTRK like family member 4	Neurite-modulating activity	
DPM1	Dolichyl-phosphate mannosyltransferase subunit 1, catalytic	Transferase activity	
CD58	Lymphocyte function-associated antigen 3	T-cell activation	
CASP8	Cysteine-aspartic acid protease	Involved in the programmed cell death induced by Fas and various apoptotic stimuli	
BNIP3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3	Cellular anti-apoptosis proteins.	
CCNF	G2/mitotic-specific cyclin-F	Phosphorylation-dependent ubiquitination.	
NDUFS4	NADH:ubiquinone oxidoreductase subunit S4	Cellular ATP production.	
NDUFA2	NADH:ubiquinone oxidoreductase core subunit S2	ROS generation.	

regulation of developmental process, positive regulation of phosphate metabolic process, positive regulation of phosphorus metabolic process, positive regulation of cell migration, positive regulation of immune system process, positive regulation of cell motility and positive regulation of cellular component movement. Also, its displays a list of genes associated with immune response are negatively regulated, negative regulation of interleukin-8 production, negative regulation of lymphocyte activation, negative regulation of cell development, negative regulation of CD4-positive, alpha-beta T cell differentiation, negative regulation of protein catabolic process, negative regulation of cell morphogenesis involved in differentiation, negative regulation of T-helper cell differentiation, negative regulation of receptor binding negative regulation of type 2 immune response, negative regulation of activated T cell proliferation, negative regulation by host of viral transcription, negative regulation of collateral sprouting, negative regulation of neuron projection regeneration, negative regulation of sprouting angiogenesis and negative regulation of necroptotic process. In pathway Interactions (Fig. 22.3d), the involvement of genes implicated

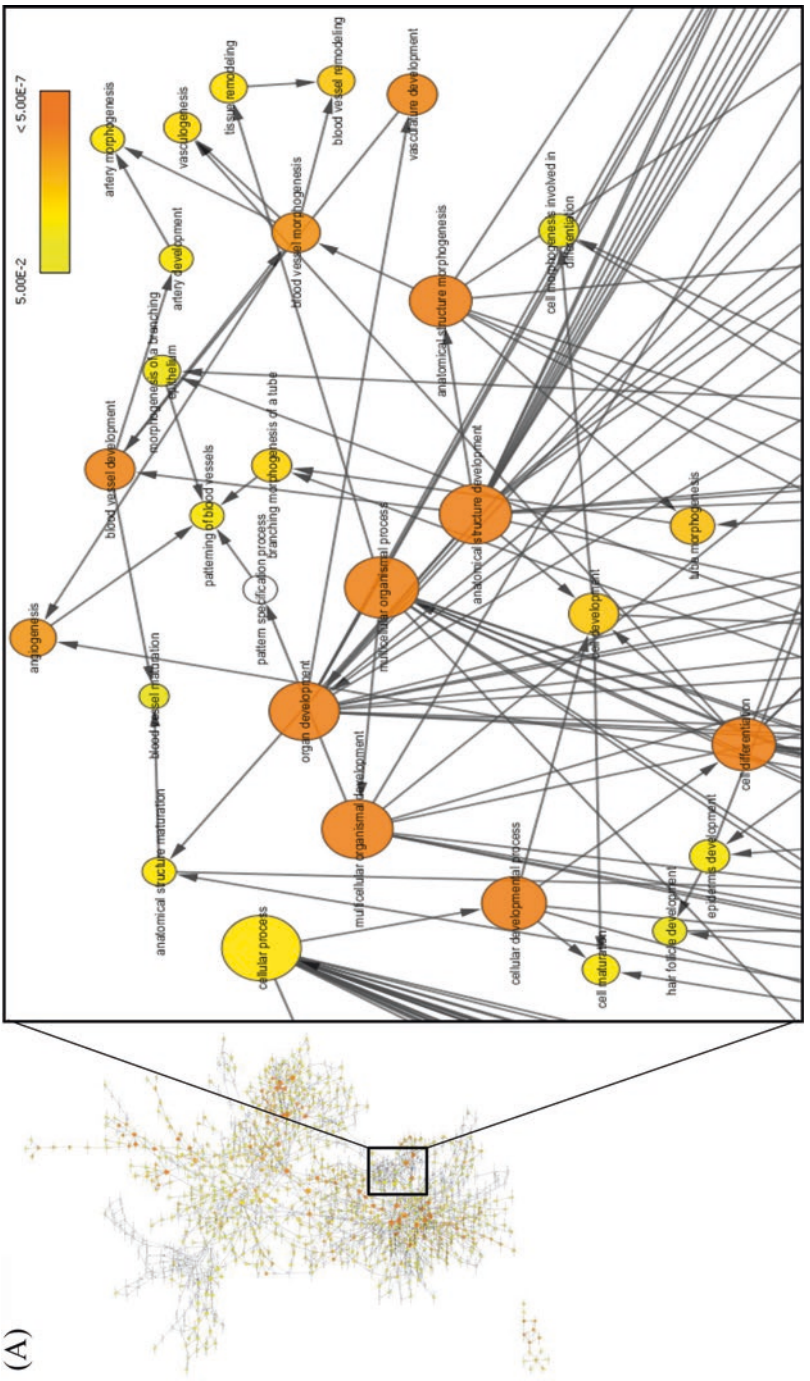


Fig. 22.3 The Cytoscape graph network was generated from ToppCluster network analyzer, online tool. The microgravity influenced differential expression of genes in ECs, involved in angiogenesis process and their interacting molecules is represented in the complete network (**a**). The microgravity influenced differential expression of genes in ECs, involved in angiogenesis process and their (**b**) molecular function (**c**) biological process (**d**) pathways

Fig. 22.3 (continued)

under microgravity mediated angiogenesis in other cellular pathways shown in Fig. 22.3d. The cellular processes which involve ≥80% of alcohol related angiogenesis genes among the total list of the genes involved includes Leukocyte trans endothelial migrating signaling via TRKA from the plasma membrane, signaling events mediated by VEGFR1 and VEGFR2, oncostatin M signaling pathway, phosphorylation of proteins involved in G1/S transition by active Cyclin E:Cdk2 complexes, G2/M DNA damage checkpoint, Cellular responses to stress, TSH signaling pathway, cell cycle, mitotic, EGF receptor (ErbB1) signaling pathway, retinoic acid receptors-mediated signaling. Also <60% of genes are involved in Rap1 signaling pathway, platelet activation, signaling and aggregation, plasma membrane estrogen receptor signaling, neuropeptides VIP and PACAP inhibit the apoptosis of activated T cells, G2/M DNA replication checkpoint, Chk1/Chk2 (Cds1) mediated inactivation of Cyclin B: Cdk1 complex, E2F transcription factor network, cell cycle: G1/S check point, recruitment of mitotic centrosome protein complexes and centrosome maturation as signaling pathway. This analysis framed a list of molecular process which interacts with wide range of molecular process such as blood vessel formation, blood vessel maturation, patterning of blood vessels, anatomical structural morphogenesis, vasculature development, cell differentiation, cell maturation, epidermis development, hair follicle formations and tissue remodeling. We performed REACTOME functional analysis based on the genes which are differentially expressed under microgravity (Fig. 22.4). This analysis gives the information that the functions activated by microgravity and the functions involved in the activation of endothelium. The functions includes signal transduction, immune response, DNA replication, cell cycle, diseases, homeostasis, cellular response to stress, cell death, metabolism of proteins, extracellular matrix organization, gene expression, developmental biology and cell-cell communication.

This pathway diagram exhibits the entire network interaction of pathways and incorporates the additional data to generate the experimental hypothesis. Hence, these current knowledge based studies will help to discover the unexpected functional relationships of genes implicated in microgravity conditions and its interplay with other pathways. Therefore, we concluded that genes up regulated in microgravity condition are mainly involved in angiogenesis process. And also, overlapping functions of genes implicated in microgravity condition are documented in this study. These results give a clue that microgravity may play a role in the regulation of angiogenesis thereby it regulates wound healing.

7.4 Microgravity Activates Endothelium

In physiological milieu, endothelial cells play an important role in maintaining the durability and functionality of vascular wall. It controls wide range of biological functions such as vaso-relaxation and contraction, smooth muscle cell growth, supplies nutrition for tissue growth and homeostasis [41, 63]. The imbalance between vaso-relaxation and contraction modulated by endothelium is known as endothelial activation. Also, it is considered that the main factor for many vascular diseases

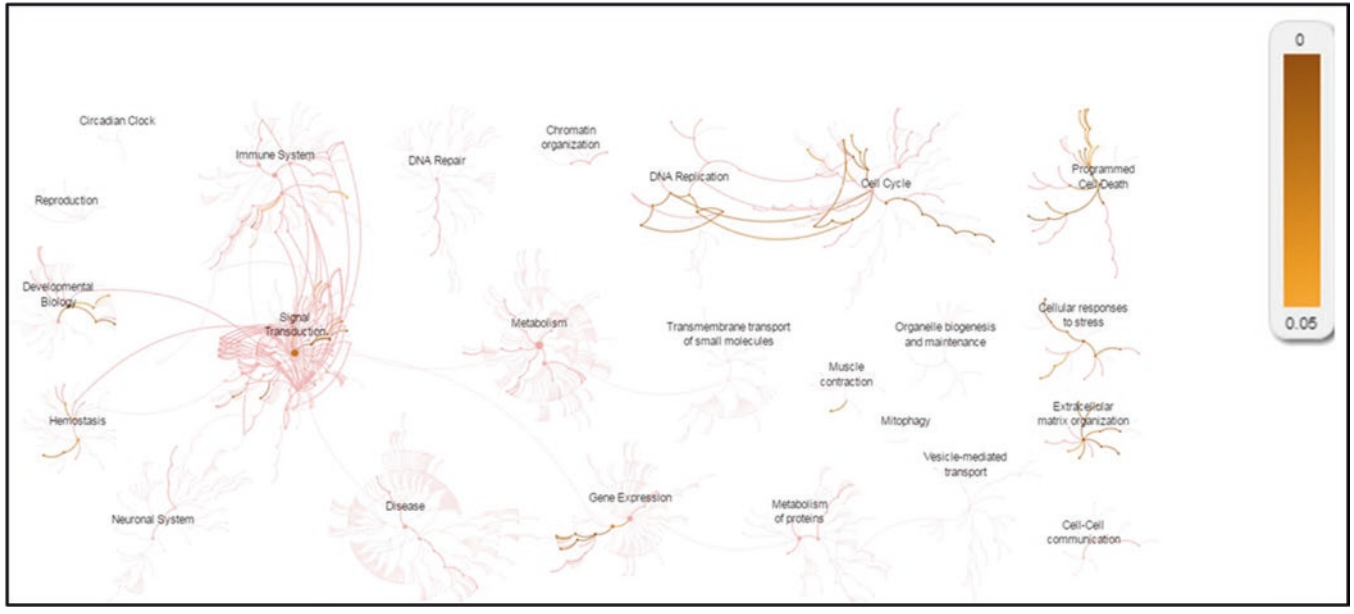


Fig. 22.4 Graphical representation of list of genes upregulated under simulated microgravity from the literature mining and its role in angiogenesis associated pathways using cytoscape 3.0

such as atherosclerosis, hypertension, diabetes, coronary artery diseases and thrombosis [17, 41]. Activation of endothelium by external stimuli is carried out in two ways. (1) Endothelium activation type I, it is an immediate response to the external agents which occurs rapidly without activation of genes. (2) Endothelium activation type II, it is activating genes and protein synthesis and occurs leisurely [42]. Endothelium activation synchronous happens in many ways rather than external pathological agents. Changes in endothelium integrity, local permeability, oxidative stress leads to chronic endothelial activation. Discharge of fluids from intravascular space exposed through sub endothelium happens in loss of vascular integrity [17, 41, 42, 53]. This adverse effect activates the leucocyte adhesion molecules, ICAM1, VCAM1 and E-selectin and it moves into tissues and then prothrombotic effects of endothelium enhances the loss of surface anticoagulant, activation of plasminogen activator inhibitor type 1, activation of platelet activating factor, NO and cytokines [53]. Among these, NO plays an important role in endothelial activation [43]. Reduced bioavailability of NO is considered as a marker for endothelial dysfunction. NO a gaseous molecule widely known as endothelium derived relaxation factor (EDRF) which interacts most of the biological functions such as vasodilation, activation of leukocytes to bind with endothelium, platelet aggregation and immune response. Arginine derived NO synthesized by three NOS isozymes; iNOS (inducible Nitric Oxide Synthase), eNOS (Endothelial Nitric Oxide Synthase) and nNOS (Neuronal Nitric Oxide Synthase) [18, 42, 53]. In this, iNOS produce NO through phagocytes (monocytes, macrophages and neutrophils) as part of immune response [18, 29, 53]. Intracellular NO free radicals have less half life time, acting as toxic molecule to pathogens in divergent pathways. On the other hand, NO plays a unique role in angiogenesis process. In our report, it is emphasized that the simulated microgravity promotes NO supported angiogenesis via the iNOS–cGMP–PKG pathway in macrovascular endothelial cells [48, 49]. The NO production analysis by DAR-4AM fluorescence probe and wound healing assays was performed in EAhy926 cells under microgravity with and without NO donor and inhibitor (Fig. 22.5a, b). The result indicated that the increased NO production and migration under microgravity in EAhy926 cells.

In last decade, new mechanism has been identified which describes the role of mechanical force in endothelial activation [7].EC activation in cultured bovine aortic under cycle strain and shear stress has been proved [2]. Regulation of differential gene expression and proliferation in mechanical stress were proven in human dermal microvascular endothelial cells (HDMEC). These mechanical force perceived by the cell membrane converted into biochemical force which regulates the wide range of intracellular molecular process, known as mechanotransduction [59]. Mechanotransduction studies have been conducted *in vitro* models by using different methods such as clinostat, drop tower, microfluidics and rotatory wall cell culture. Among these, clinostat a well-defined established model to promote the mechanotransduction in *in vitro* models. Output of mechanotransduction process well equated with microgravity conditions, in other ways mentioned as free fall state. Free fall or microgravity or low gravity state has been observed in space station and well documented for its extreme physiological changes in astronauts. Astronauts experience

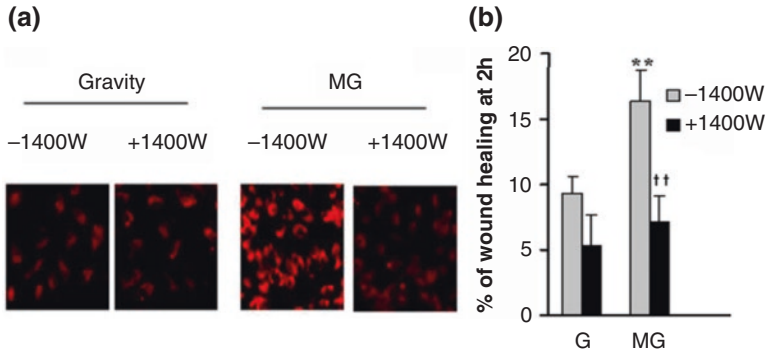


Fig. 22.5 NO production and wound healing in macro-vascular EC, Eahy926 under microgravity. (**a**) The cells were kept under microgravity with and without NO donor (1400 W) and measured NO production by DAR-4AM fluorescence probe. (**b**) Wound healing assay with and without 1400 W was assayed in microgravity treated Eahy926. **P < 0.001 vs gravity; P < 0.001 vs microgravity (Adapted from Siamwala et al. [48]. License Number: 3932580979460)

the free fall condition in space travel along with its adverse effects. Longer stays in these harsh environment mainly creates muscle loss, neurovestibular system, cardiac function, bone mass loss problems, however, mechanisms behind these defects are not understood well [60].

On the other hand, weightless or free fall or microgravity condition has its own potential to enhance the endothelial cell proliferation and migration. Also, it has been shown that ECs are more sensitive in low gravity conditions. These observations are analyzed and well documented using different in vitro models [47]. Freefall and parabolic flight can be used to simulate microgravity but this way of microgravity stimulation is typically too short to alter cell functions. Now, there are several alternative methods under practice to generate microgravity in Earth. Clinostats are convincingly successful ground based apparatus to stimulate microgravity.

7.5 Effect of Microgravity on Endothelial Cells in Relation to Wound Healing

Various reports by the use of a variety of *in vitro* models have thrown light on the fact that ECs are highly sensitive to microgravity and undergo morphological, functional and biochemical changes under microgravity condition [34]. Promotion in wound healing can be characterized by migratory, proliferators, cytoskeleton and extracellular matrix properties of cells. Shi et al. [47] has demonstrated that, after 24 h of exposure to simulated microgravity in a clinostat, human umbilical vein endothelial cells (HUVEC) migration is significantly promoted through the eNOS pathway up regulation by means of PI3K-Akt signaling. Simulated microgravity increased cellular migration of sensitized ECs by 25% compared to non-sensitized ECs by modulating actin and releasing nitric oxide [48, 49]. Microgravity enhances

the proliferation of ECs without inducing apoptosis and compromising cell viability [48, 49]. The cytoskeleton plays a key role in the adaptation to mechanical stress, including alterations of gravity. This property of cytoskeleton to undergo changes can be a key to explain the effects of weightlessness on cells [40]. Reports suggested that [6] actin microfilaments in HUVEC exposed to microgravity showed elongated and extended podia. The disorganization of actin microfilaments clustered in the perinuclear area and decrease in stress fibers compared to normal HUVECs. Further studies [48, 49] has also shown the key migratory structures of cells, filopodia and lamellipodia, formed by EC to be more in simulated microgravity compared to gravity. In detail, Eahy926 cells were kept under microgravity for 2 h followed by time measured NO production image using DAR-4F Mfluorescent probe. The result showed that NO production by Eahy926 is increased under microgravity. To further investigate the possibility of the simulated microgravity increase NO production by actin remodeling, the Eahy926 cells were analyzed NO production under microgravity with and without cytochalasin D (CD) and followed by performed dual staining of actin and nucleus with phalloidin and DAPI, respectively. The result indicates that the number of central microfilaments was observed more in cells treated with microgravity compared to gravity treated cells. Additionally, directionless, shorter central microfilaments were observed in microgravity treated cells (Fig. 22.6a). Notably, the CD simulated stress fiber formation was significantly attenuated in microgravity treated cells compared to gravity treated cells (Fig. 22.6b). This evidence concludes that the microgravity involved in endothelial cells' actin remodeling and thereby it stimulates NOS to induce NO motivated migration.

8 Consolidation of the Outcome of Cell Biology and Omics Results and Future Direction

The relationship between the endothelial cells and altered gravity suggests the options for bridging microgravity and wound healing via endothelial activation and thereby angiogenesis. The microgravity induced changes in the cytoskeleton can strongly affect the behavior of endothelial cells in terms of adhesion, migration, production of extracellular matrix and can interfere with other processes such as translocation of molecules inside the cells, trans endothelial migration and even inflammation and angiogenesis, in turn effecting wound healing process as wound healing is the amalgamation of the above processes. Further, it has been suggested that the unloading, weightlessness sensitization of endothelial cells' conditioned media have the pro-healing properties on bone cells [54] and they can be used in clinical management of wound healing. But the precautions should be taken for no gangrene, tumor/cancer formation and least scar. It is reasonable that the stimulated microgravity will be the way forward in future developments for advanced management of wound healing. However, being and emerging area of microgravity on wound healing is not conclusive yet. Several studies are underway in various

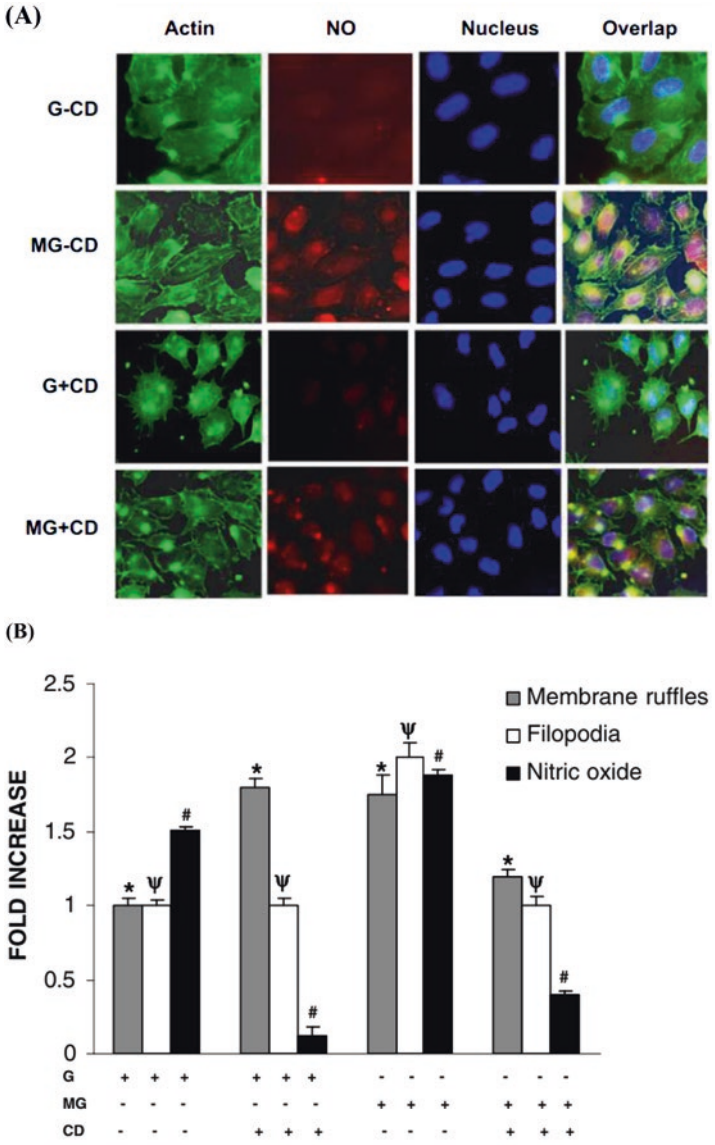


Fig. 22.6 Microgravity stimulates NO production by actin remodeling. (**a**) The cells were exposed to microgravity with and without CD and stained with phalloidin and DAPI. The images of actin pattern, NO, nucleus, and a merge of all three. (**b**) Data presented as fold increase of membrane ruffles, filopodia, and NO production in Eahy926 cells treated with microgravity with and without CD.* Membrane ruffles vs gravity, ψ filopodia vs gravity, # NO production vs gravity (Adapted from Siamwala et al. [49]. License Number: 3932501093561)

laboratories. Some of them are cell-based assays and limited number of animal studies. Elaborated evidences using higher model is required for further confirmation. We understand that there are several challenges still remain in the management of wound healing, and it is evident that a single strategy for the management of wound healing is not always solution for the problems encountered in wound healing process. Therefore, a combination of therapeutic strategies is required and this stimulated microgravity for wound healing management could be one of them in near future.

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Chapter 23
Role of Skeletal Muscle Angiogenesis in Peripheral Artery Disease

Naranjan S. Dhalla, Rebeca O. Camargo, Vijayan Elimban, Ravideep S. Dhadial, and Yan-Jun Xu

Abstract Peripheral artery disease is a major circulatory disorder, which is characterized by obstruction of arteries mainly due to atherosclerosis and thrombosis, leading to reduced blood supply and ischemia in the hind limb. On the basis of their actions on blood constituents and blood vessels, several interventions such as antiatherosclerotic, antithrombolytic, antihypertensive, antidiabetic, and vasodilating agents are commonly used for the treatment of this disease but none of these drugs are satisfactory. Since angiogenesis is an adaptive process, which is concerned with promoting blood flow in different organs, it is plausible that the development of angiogenesis in the skeletal muscle may serve as a novel target for the treatment of peripheral artery disease. Because the formation of several factors such as vascular endothelial growth factor and nitric oxide as well as reduction of oxidative stress and inflammatory cytokines is known to promote angiogenesis, manipulation of these mechanisms by newer interventions including stem cell therapy can be seen to produce beneficial effects. In this context, both exercise training and CO_2-bath therapy have been shown to induce angiogenesis and increase blood flow in the ischemic limb. Thus the development of angiogenesis-based therapies is suggested for improving blood flow in the treatment of peripheral arterial disease.

Keywords Skeletal muscle angiogenesis • Peripheral arterial disease • Exercise training • CO_2-bath therapy • Vascular endothelial growth factor • Oxidative stress • Inflammatory cytokines

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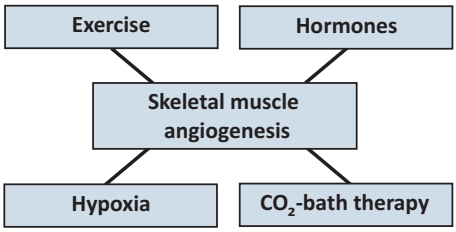

Fig. 23.1 Different interventions which are known to induce angiogenesis in skeletal muscle as an adaptive mechanism in peripheral arterial disease

1 Introduction

Angiogenesis, the formation of new blood vessels from the existing vasculature, has been shown to occur in several organs including skeletal muscle under different physiological and pathological conditions [1–6]. This phenomenon was first described during placental growth [7] and was observed in ear upon implanting a transparent chamber [8]. It is considered to play an adaptive role in wound healing following myocardial infarction, stroke, ulcers and neurodegeneration in addition to its involvement in the growth of female reproductive tract [9–12]. On the other hand, angiogenesis is also associated with cancer, inflammatory disorders, pulmonary hypertension and eye diseases and can be seen to play a pathogenic role [13–16]. Nonetheless, in view of the dynamic role of blood vessels in supplying oxygen and nutrients for the maintenance of skeletal muscle homeostasis and function [17], the development of angiogenesis, particularly arteriogenesis, plays a critical role in both health and disease [1, 2, 18–20]. There are several physiological, pathological and therapeutic conditions including exercise training, different hormones, hypoxia/ischemia and CO_2 water-bath therapy (Fig. 23.1), which have been experimentally shown to induce angiogenesis in the skeletal muscle. This article is therefore focussed to describe the general characteristics of the skeletal muscle angiogenesis. In addition, the epidemiology, pathophysiology and therapy of peripheral arterial disease are discussed to highlight the importance of angiogenesis as a target for drug development. Furthermore, the possible mechanisms for the occurrence of angiogenesis are outlined in peripheral arterial disease upon treatments with different interventions.

2 General Characteristics of Angiogenesis

It is now well known that collateral circulation due to the formation of new blood vessels in the skeletal muscle occurs in response to the development of severe ischemia as a consequence of decrease in blood supply due to atherosclerosis and/or thrombosis in peripheral arteries [21, 22]. It should be noted that the formation of new blood vessels (vasculogenesis) is considered to play a role in the maintenance of skeletal muscle homeostasis. The process of vasculogenesis is predominant during embryogenesis but is also known to occur in adulthood due to the presence

of progenitor cells [23]. Furthermore, blood vessels are known to have the capacity to grow (arteriogenesis) as well as form new blood vessels from the pre-existing capillary bed (angiogenesis). There are two forms of angiogenesis, namely the sprouting and non-sprouting angiogenesis. The non-sprouting form of angiogenesis is more common in the skeletal muscle but also occurs in the heart and brain. The non-sprouting angiogenesis happens either due to cytoplasm invagination or invasion of pericytes and myofibroblast; however, these processes are closely related to the formation of pillars in capillaries followed by their enlargement in size [20, 24]. Since there occurs less cell migration and proliferation in non-sprouting angiogenesis, there is a less expenditure of energy and thus this process is more efficient [25].

The sprouting angiogenesis occurs in a sequential process originating from endothelium and requires two specific cells, tip cells and stalk cells, for the formation of new blood vessels [26]. The endothelial cells normally stay in a quiescent state and are positioned in a layer of pharynx cells, followed by smooth muscle cells, pericytes and the basement membrane [27]; as these structures assure the maintenance of the flow inside the blood vessel. But when a stimulus, such as hypoxia interacts with quiescent cells, these become activated [28–30]. The formation of tip cells depends on the exposure of endothelial cells to vascular endothelial growth factor (VEGF) and Notch ligands such as delta-like ligand 4 (Dll4) and JAGGED [31–33]. Some endothelial cells are more sensitive to VEGF, thus leading to the selection of tip cells which become more polarized than stalk cells and are characterized for being on the tip of sprouts [32, 34]. The main function of tip cells is migratory and thus these are responsible for the guidance of sprouting in a process called filopodia. In addition to the expression of VEGF, the tip cells also express Dll4, platelet derived growth factor b (PDGFb), unc-5 homolog b (UNC5b), vascular endothelial growth factor receptor 2 (VEGFR-2) and VEGFR-3/Flt-4, neuropeptide Y, and a small amount of molecules of the Notch signaling pathway. In contrast, the function of stalk cells relies on proliferation, following the tip cells activity; these are also responsible for the formation of branches and the vascular lumen [30, 35].

After the activation of tip cells, electron density of the basement membrane of the deriver vessel is diminished [34]. Enzymes such as metalloproteinases (MMP) are released from endothelial cells, which then degrade the basement membrane [36, 37]. This step permits a loosening between cell junctions and migration of tip cells, and is assisted by the relation between MMP and integrins (transmembrane molecules present on the cell surface) [38]. When VEGF is released, it binds to VEGFR-2 and increases filopodia of tip cells in addition to enhancing Dll4, which binds to the Notch signaling pathway and decreases VEGFR-2. This mechanism regulates the amount of tip cells that are released as well as increases the formation of stalk cells [38]. With this well balanced process, the stalk cells start to operate in response to Notch, Notch-regulates Ankyrin repeat protein (NRARP), WNTs, placental growth factor (PIGF), and fibroblast growth factor (FGF) [29, 39]. It promotes the formation of a solid cord of endothelial cells by stalk cells and due to their intense proliferation, this cord is elongated [34]. The next step is the formation of vascular lumen, and for this purpose, stalk cells release vacuoles which develop a lumen of the new vessel [40, 41]. Lastly, the cells of the new endothelium regress to the quiescent state and the junction adhesion becomes tight again for diminishing the

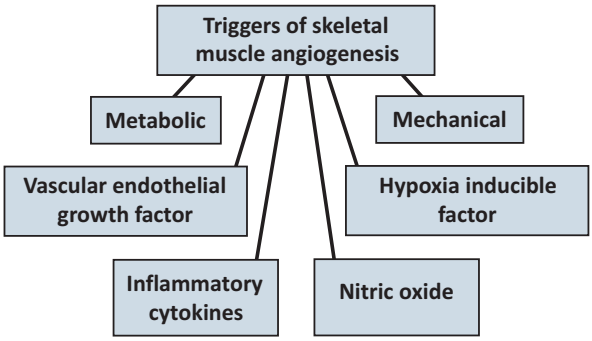

Fig. 23.2 Various factors which are considered to trigger angiogenesis in skeletal muscle during the development of peripheral vascular disease

cellular permeability. The levels of VEGF return to the normal state and pericytes become involved in the new vessels, concluding the angiogenesis process [30].

From the discussion outlined in this section, it is evident that angiogenesis is a complex but a highly regulated process. It involves the stimulation of quiescent endothelial cells by interventions such as hypoxia, exercise, some hormones and CO_2, which trigger the activation of tip cells and formation of filopodia in the presence of VEGF and different inflammatory cytokines. This leads to the activation as well as proliferation of stalk cells and the formation of a solid cord. The release of MMP results in the degradation of basement membrane. The process of maturation of new blood vessels is associated with the formation of lumen, quiescence of endothelial cells and normalization of VEGF levels. It is pointed out that although angiogenesis has been shown to develop in the ischemic skeletal muscle, the exact factors associated with angiogenesis in peripheral artery disease are not clear at present. During the development of peripheral arterial disease, the occurrence of angiogenesis may be triggered by several factors (Fig. 23.2) including VEGF, hypoxia inducible factor, inflammatory cytokines and nitric oxide. In addition, mechanical factors (changes in shear and stress as well as muscular activity) and metabolic factors (formation of AMP and adenosine) have been demonstrated to affect the occurrence of angiogenesis in the skeletal muscle. Thus in view of the complexities involved in the regulation of angiogenesis, and to understand the role of angiogenesis in the development of peripheral arterial disease, it is considered appropriate to describe the epidemiology, pathophysiology and therapeutics of peripheral vascular disease.

3 Pathophysiology and Therapy of Peripheral Arterial Disease

Peripheral arterial disease is the third most prevalent cardiovascular disease as it affects more than 200 million people worldwide [42]. It is mainly age-related because most of the incidence of the disease occur in individuals who are within the age-range of 60–70 years whereas the chances of its occurrence are very low in

people who are less than 50 years old [43]. Gender and ethnicity are other risk factors for the peripheral artery disease because males are more susceptible than females [44] and the incidence of this disease is about 2 fold in the African-Americans in comparison to the non-Hispanic White people [45]. The major symptoms of this disease include discomfort in legs and feet which are associated with cramps, achiness, burning and fatigue.

The spectrum of peripheral arterial disease varies from asymptomatic to critical ischemia as the most severe scenario, leading to a possibly gangrene and amputation of the limb. Related to presentation of this disease, the most relevant risk factors are hypertension, obesity, dyslipidemia, diabetes, smoking, cold temperature, emotional stress and physical inactivity [46–48]. It should be mentioned that atherosclerosis obliterans and arterial insufficiency are two forms of peripheral arteries disease whereas deep vein thrombosis and intermittent claudication are commonly associated with peripheral veins disease. All these clinical conditions and risk factors lead to the development of skeletal muscle ischemia and thus limiting locomotion and mobility [49, 50].

The arteries obstruction happens mainly because of the formation of an atherosclerotic plaque or thrombus. It is noted that atherosclerosis is a multifactorial chronic disease, which is progressive and inflammatory. It begins early in life and depending of genetic factors and environment risks, the disease may progress and cause reduction in blood flow [51, 52]. The most important cells involved with atherosclerosis are endothelial cells, smooth muscle cells and macrophages [53, 54]. The process is initialed by dysfunction of the endothelial cells, which decreases the production of nitric oxide (NO), an important vasodilator [55, 56]. Also, it increases the production of vasoconstrictors agents leading to platelet aggregation and formation of thrombosis. When the endothelium is damaged, free radicals are released, which make the endothelial cells more permeable [57], and low-density lipoproteins (LDL) start to accumulate on the subendothelial space. The interaction between LDL and free radicals results in the oxidation of LDL [55, 58], which transforms the complex of leucocytes, macrophages and phagocyte into foam cells. The accumulation of these cells forms the core of an atherosclerotic plaque, which along with apoptotic and fibrotic smooth muscle cells (vascular remodeling) narrows the vessel lumen [59]. The atherosclerotic plaque upon rupture can become hemorrhagic through vasa vasorum and thus increases the risk of thrombosis formation [60].

While atherosclerosis and vascular remodeling are associated with narrowing the lumen of peripheral blood vessels, the formation of thrombosis results in the obstruction of blood flow. Furthermore, endothelial dysfunction and subsequent insufficiency of NO formation is invariably associated with the inability of blood vessels to dilate and can be seen to reduce blood flow under stressful conditions. All these mechanisms (Fig. 23.3) are considered to play an important role in the pathogenesis of peripheral arterial disease by reducing blood flow and thereby leading to the development of ischemia in the skeletal muscle. This disease process is associated with a wide variety of complications such as blood clots in small arteries, leg pain, wounds and dead tissues in limbs, heart attack, stroke and even death (Fig. 23.4). It is therefore of paramount importance that some appropriate strategies

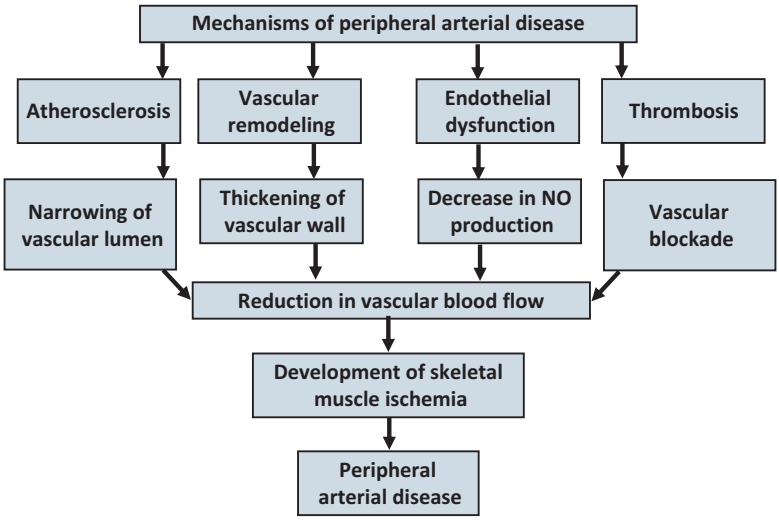

Fig. 23.3 Different mechanisms which are known to result in the development of peripheral arterial disease through the reduction of vascular blood flow and subsequent skeletal muscle ischemia

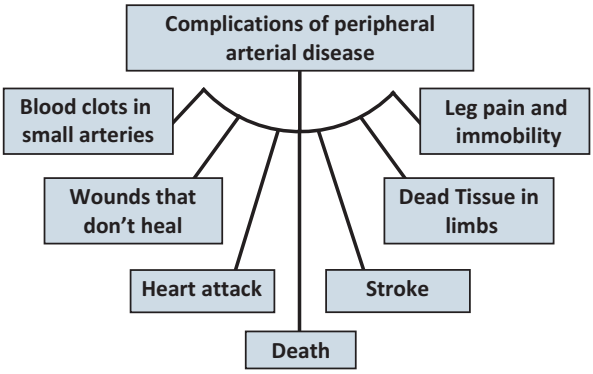

Fig. 23.4 Several complications which are known to occur during the development of peripheral arterial disease

be developed for improving the quality of life in this devastating disease. Some of the preventive, surgical and medical treatments which are currently being used in this regard are outlined in Fig. 23.5.

The therapeutics of this disease starts generally with some conservative measures. Reducing cardiovascular risks [44, 46] upon changing the smoking habits and increasing the physical activity; in addition, angioplasty and thrombolytic therapy are recommended for the management of peripheral artery disease. In some patients with severe disease, bypass surgery, and amputation are required [61]. Furthermore, several medical interventions, which are known to promote blood flow by their actions on the vascular smooth muscles or by affecting the vessel lumen, are used for the treatment of peripheral arterial disease (Fig. 23.6). Since none of the existing

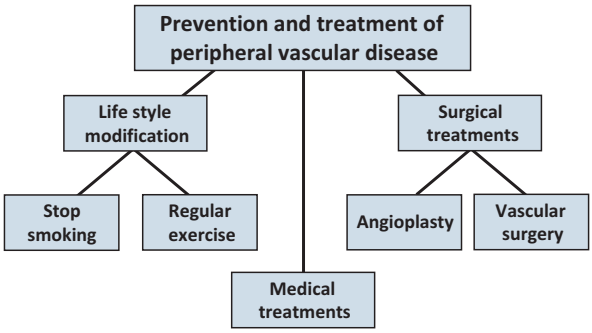

Fig. 23.5 Various preventative and surgical treatments for the improvement of blood flow in peripheral arterial disease

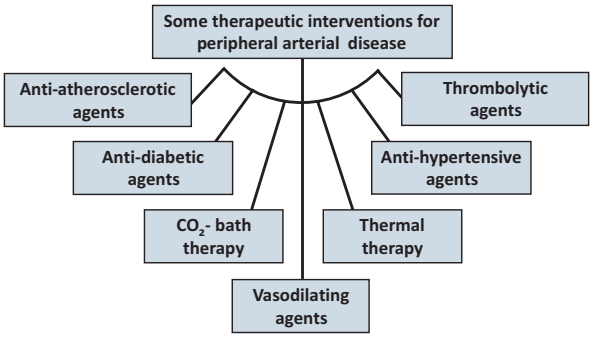

Fig. 23.6 Some of the pharmaceutical agents and medical device which are either being currently used or expected to be used for the treatment of peripheral arterial disease

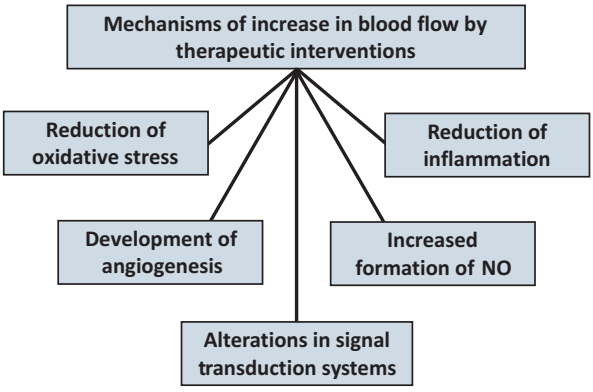

Fig. 23.7 Different mechanisms which are considered to serve as targets for the development of various therapeutic interventions to improve skeletal muscle blood flow in peripheral vascular disease

treatments are satisfactory, several new interventions including stem cell therapy are being developed to promote the formation of collateral blood vessels [62–64]. In particular, several mechanisms including angiogenesis, changes in signal transduction and increase in NO production, as well as reduction in both oxidative stress and inflammation have been proposed for the improvement of blood circulation in the ischemic limb (Fig. 23.7).

4 Mechanisms of Skeletal Muscle Angiogenesis

It has now become evident that angiogenesis in the skeletal muscle plays an adaptive role in promoting blood flow in the peripheral arterial disease. Several molecular mechanisms have been suggested for the development of angiogenesis [1, 6, 19, 20]. Different angiogenic growth factors, particularly VEGF, have been demonstrated to play a critical role in this process [28, 30, 31, 39, 61, 62]. Inflammatory cytokines such as TNF-α, reduction in the production of oxidative stress and the formation of NO have also been shown to participate in promoting the occurrence of angiogenesis [3–6, 29, 58]. Various studies using stem cells and other types of cells have been carried out for promoting angiogenesis in the skeletal muscle [23, 33, 48, 65]. Since exercise training, hypoxia, different hormones and CO_2-bath therapy have been reported to induce angiogenesis in the peripheral arterial disease, this section is devoted to discussion of mechanisms of angiogenesis in the skeletal muscle under these physiological and clinical conditions.

Exercise-Induced Angiogenesis In exercise training, certain events occur to stimulate angiogenesis; these events involving the skeletal muscle and the endothelium include metabolic factors, the release of angiogenic compounds and mechanical factors such as shear stress and muscular stretch [66]. The levels of VEGF are markedly increased due to the formation of AMP and adenosine due to exercise [67]. Furthermore, the occurrence of shear stress due to high pressure on capillaries [20] leads to the production of NO and VEGF [68]. The proteases, MMP-2 and MMP-9 are also increased during the muscle stretch in exercise [69, 70] and these then stimulate the production of VEGF [71], which is stored in the skeletal muscle cells [72]. VEGF thus produced due to the formation of adenosine, production of NO and protease activation can be seen to induce angiogenesis during exercise training. It should also be mentioned that VEGF receptors, VEGFR-1 and VEGFR-2 are also activated due to the formation of NO as well as other metabolites in the skeletal muscle [66, 72]. The exact sequence of events leading to exercise-induced angiogenesis involving VEGF and other angiogenic factors have been described recently [90].

Hormone-Induced Angiogenesis Several vasoactive hormones including angiotensin II have been shown to exert angiogenic effect. Likewise, sex hormones which exert vasoactive actions have been reported to induce angiogenesis [73]. For years, androgens have been thought as factors for increasing cardiovascular risk; however, testosterone is now considered as an important vasoactive substance, which imparts protection to atherosclerosis [74, 75]. Dihydrotestosterone (DHT), an analogue of androgen, has been reported to stimulate angiogenesis via increasing VEGF expression in a dose and sex-dependent manner. It means that the application of DHT in females does not increase VEGF or angiogenesis. Although DHT acts by binding to androgen receptors in endothelial cells in males, it is not clear whether it increases VEGF through the production of NO [76]. In contrast to androgens, the amount of research related to the role of estrogens in vasculature is extensive. Estrogens act cyclically in the female endometrium, pathologically in breast cancer and induce angiogenesis in response to ischemic stimulus [77]. There are three forms of estro-

gen, but the most important for angiogenesis is estrogen-2 as it promotes endothelial cell migration and proliferation. This form can act by both genomic and non-genomic pathways. In the genomic pathway, estrogen-2 stimulates transcription directly in the nucleus. On the other side, it binds to estrogen receptors in endothelial cells (ERα and ERβ). ERα is most active in the endothelium as it stimulates the production of VEGF by phosphatidylinositol 3-kinase and mitogen activated protein kinase pathways for promoting the angiogenesis process [36].

Hypoxia-Induced Angiogenesis Hypoxia has been reported to be an important factor for promoting angiogenesis [77–79]. There are three main molecules which are involved in this mechanism, namely NO, hypoxia-inducible factor (HIF) and VEGF [80–82]. HIF is a heterodimer transcriptional factor and is a part of the Per/ARNT/Sim (PAS) subfamily. There are three different types of HIF including HIF-1, HIF-2, HIF-3. Each type is formed by two subunits: HIF-α and HIF-β bind to each other, the α subunit being sensitive to oxygen; the most important of these for the formation of new blood vessels are HIF-1α and HIF-2α [83, 84]. When the levels of oxygen are normal, HIF-1α remains unstable and it is broken by propyl-4-hydroxylase-2 (PHD2) [85, 86]. However, in hypoxic situations, this enzyme production decreases and HIF-1α becomes more stable [87]. After this, HIF-1α is transported to the nucleus, forming a dimer with HIF-1β and binding to the hypoxia response element present in the DNA region where there are genes for VEGF production; it then stimulates the production of VEGF and several other angiogenic factors [88]. Although the complete mechanism of HIF-2α activation is not entirely understood, it has been suggested to play a role in increasing the formation of NO [82].

The most recognized angiogenic factor in the ischemic/hypoxic tissue is VEGF and its role is essential in angiogenesis [89–91]. VEGF family is compounded as dimeric glycoproteins by seven members, namely VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PIGF presented in mammals; VEGF-E in parapoxvirus and VEGF-F in snake venom. It is noted that VEGF-A is the most important molecule which is related to angiogenesis, causing cell migration, sprouting, proliferation, and lumen formation. It is normally called VEGF and is known to have at least six isoforms: VEGF-121, VEGF-145, VEGF-165, VEGF-183, VEGF-189 and VEGF-206; the most vasoactive in skeletal muscle is VEGF-165 [92, 93]. The role of VEGF-B is not completely understood yet, but it does not seem to be essential in angiogenesis [94]. On the other hand, VEGF-C and VEGF-D have been shown to have some role in both angiogenesis and lymphogenesis [95, 96]. PIGF has four different isoforms: PIGF-1, PIGF-2, PIGF-3, and PIGF-4 and is considered to increase the arteriogenesis process.

When activated, VEGF binds to different tyrosine kinase receptors: VEGFR-1, VEGFR-2, and VEGFR-3 as well as neuropilin-1(Nrp-1) and neuropilin-2 (Nrp-2). All receptors are present in the vascular endothelium but VEGFR-3 is more specific for lymphatic endothelium, as well as Nrp-2. In addition, Nrp-1 is more characteristic for arterial endothelium whereas Nrp-2 is for venous endothelium [92, 97]. Different VEGF receptors have affinity for different angiogenic factors. For example, VEGFR-1 binds to VEGF-A, VEGF-B and PIGF whereas VEGFR-2 has affinity for

VEGF-A, VEGF-C, and VEGF-D. On the other hand, VEGFR-3 binds to VEGF-C, and VEGF-D, Nrp-1 and Nrp-2 bind to VEGF-A and PIGF (especially VEGF-165 and PIGF-2). VEGF-B binds only to Nrp-1 and VEGF-C binds only to Nrp-2 [98, 99]. After binding to their respective receptors, VEGF increases the formation of NO and promotes sprouting angiogenesis [100]. It is pointed out that hypoxia is an important stimulus that evokes an adaptive response to ischemia through the HIF-dependent production of inflammatory cytokines such as TNF-α and growth factors, which induce angiogenesis in the ischemic skeletal muscle [101].

CO_2-Induced Angiogenesis CO_2 is essential in the human body as it maintains pH homeostasis during respiration and has been shown to increase blood flow and angiogenesis [102]. Since the middle ages, CO_2 baths have been used for the treatment of different disorders. In 1859, the beneficial health effects of CO_2 were described and in 1880, CO_2 balneotherapy was recommended for cardiovascular disorders in patients by immersion in the well "Carbonated Springs of Bad Nauheim" in Germany [103, 104]. Balneotherapy consists of body immersion in the natural thermal mineral water and it has been used in Europe for ages [105, 106].

When applied locally by baths, CO_2 increases vasodilation and blood flow; this process is complex and may have several mechanisms [107, 108]. Due to local acidosis, CO_2 increases the vessel diameter and decreases the hematocrit. Other explanations are related to the stimulation of the parasympathetic system and inhibition of the sympathetic system as well as the increase in NO production [109, 110]. Other effects of CO_2 include bradycardia by reducing the sympathetic activity, lowering of core temperature and elevation of the thermal sensation caused by heat exchange due to increase of cutaneous blood flow and inhibition of cold nerve receptors [111]. It has been demonstrated that CO_2 stimulates local VEGF production and VEGF mRNA expression for promoting angiogenesis. Although the exact mechanism for this action of CO_2 is not well elucidated, it is thought to be related to the production of NO as well as the progenitor endothelial cells activity [112, 113]. Clinically CO_2 has been shown to improve physical tolerance in patients with peripheral arterial disease as well as blood flow in the skin and the hind limb muscle [105, 107, 109, 111]. Recent experimental studies [114–116] have provided the evidence that the increase in blood flow in the ischemic skeletal muscle by CO_2-bath treatment was associated with the development of angiogenesis. Thus it appears that the CO_2-bath therapy may increase the blood flow in the ischemic limb by inducing angiogenesis due to the production of both NO and VEGF.

5 Conclusions

From the foregoing discussion, it is evident that reduction in blood supply mainly due to atherosclerosis and thrombosis in the peripheral arteries leads to ischemia in the limb muscles, which results in the occurrence of peripheral artery disease. These changes are invariably associated with the development of angiogenesis as an

adaptive mechanism for maintaining the skeletal muscle homeostasis and muscle function in the ischemic limb. In view of the insufficiency of this adaptive response, several surgical and medical treatments, which are known to reduce the blockade to blood flow, increase the lumen of vessels or affect remodeling of the vessel wall, are used for the treatment of peripheral arterial disease. However, none of these interventions are satisfactory and it is thus important that some newer strategies be developed to deal with this devastating disease. Accordingly, several therapies such as exercise training, stem cell therapy, and CO_2-water bath therapy, which are known to promote the production of VEGF and other growth factors as well as NO and induce angiogenesis, are being recommended for the treatment. Thus angiogenesis seems to be a novel target for the development of newer drugs, interventions and therapies for improving blood flow to the ischemic limb in peripheral arterial disease.

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