·Review·

Molecular underpinnings of corneal angiogenesis: advances over the past decade

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Abstract

• **The cornea is maintained in an avascular state by maintaining an environment whereby anti-angiogenic factors take the upper hand over factors promoting angiogenesis. Many of the common pathologies affecting the cornea involve the disruption of such equilibrium and the shift towards new vessel formation, leading to corneal opacity and eventually-vision loss. Therefore it is of paramount importance that the molecular underpinnings of corneal neovascularization (CNV) be clearly understood, in order to develop better targeted treatments. This article is a review of the literature on the recent discoveries regarding pro-angiogenic factors of the cornea (such as vascular endothelial growth factors, fibroblast growth factor and matrix metalloproteinases) and anti-angiogenic factors of the cornea (such as endostatins and neostatins). Further, we review the molecular underpinnings of lymphangiogenesis, a process now known to be almost separate from (yet related to) hemangiogenesis.**

• **KEYWORDS:** cornea; neovascularization; angiogenesis; lymphangiogenesis; vascular endothelial growth factor
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INTRODUCTION

Corneal avascularity comprises its main functionality index as a lens. This article reviews the literature on corneal neovascularization (CNV) promoted by proteins such as vascular endothelial growth factors, fibroblast growth factor, and matrix metalloproteinases, and subsequently inhibited by endostatins, angiostatins, and related anti-angiogenic factors. It also reviews the normal versus pathogenic changes in corneal immunity leading to new vascular formation.

CNV is induced by various stimuli mainly associated with inflammation, trauma, transplantation, and infection of the ocular surface [1-2]. Both corneal hemangiogenesis and lymphangiogenesis are promoted or inhibited by a balance of mediators, including the dynamics between pro-angiogenic and anti-angiogenic substances. In corneas diseased by inflammation, infection, degeneration, transplantation, or trauma, the normal balance is shifted towards the pro-angiogenic status, leading to corneal hemangiogenesis and/or lymphangiogenesis.

MAJOR ANGIOGENIC PROTEINS OF THE CORNEA

Vascular Endothelial Growth Factor (VEGF) VEGF-A is known to be linked to blood vessel formation in a wide range of events; including embryonic and physiologic growth, vascular pathologies and malignant tumor neovascularization. It acts directly on blood vessels by stimulating endothelial cell mitosis, migration, dissolution of original vessel membrane, and formation of new capillary tubes [3].

Following the discovery of VEGF, a series of subtypes were identified and named in alphabetical order: VEGF-A, VEGF-B, VEGF-C, and VEGF-D. Nevertheless, the chief VEGF was and still is VEGF-A. A wide range of heterogeneous cells were proven to secrete VEGF-A, including macrophages, pericytes, T-cells, astrocytes, fibroblasts, and retinal pigment epithelial cells [3].

VEGF has multiple isoforms, with its encoding gene comprised of eight exons. With differential pre-mRNA splicing, a single VEGF gene gives these different isoforms.

Examples of these isoforms include VEGF121, VEGF165 and VEGF189. These numbers refer to number of amino acids composing the protein. These isoforms have different properties based on the presence of absence of the C-terminal protein domains encoded by exons 6 and 7. Also, these isoforms constitute a reservoir of growth factors acting without gene transcription. Moreover, interaction of VEGF165 isoform with heparan sulfate proteoglycan-Glypican-1 had been reported to play a role in extending the half-lives of the isoform in the process of hypoxia induced angiogenesis[4].

Alternative splicing of VEGF gene yields five isoforms of VEGF-A, including (VEGF115, VEGF 121, VEGF 165, VEGF 189, and VEGF 206) [5]. The shorter isoforms have more distinctive functions like the mitogenic activity of VEGF121 and VEGF165, and the more powerful angiogenic activity of VEGF121 than other longer isoforms [6]. VEGF proved its importance based on the inhibition of neovascularization in rat model following stromal implantation of an anti-VEGF-A blocking antibody[7].

VEGF binds to different surface receptor proteins (VEGFR). VEGFR-1 is a transmembrane receptor tyrosine kinase while VEGFR-2 is a major signaling receptor for VEGF. Additionally, heparan sulfate proteoglycan (HSPG) is low-affinity class of VEGF receptors that modulate the activities of wide range of heparin-binding growth factors, morphogens and chemokines [4].

Basic Fibroblast Growth Factor Among the 23 heparin binding peptides fibroblast growth factor (FGF) family, basic fibroblast growth factor (bFGF) is a member that is hugely expressed in developing and adult tissues during cellular differentiation, angiogenesis, mitogenesis and wound repair. Moreover, it is upregulated after tissue injury and in stromal fibroblast/vascular endothelial cell co-cultures. FGFs mediate their action through interaction with peptide receptors (fibroblast growth factor receptors, FGFR), namely FGFR-1, -2, -3, and -4. Potential FGF- mediated intracellular signaling events are possibly present, also different FGFR isoforms show unique biological functions[8].

Besides, the diversity of FGFR biological response is manifested by its tissue-specific expression, which is regulated by differences in ligand function and specificity. The growth factor receptors being regulated are of great significance in the management of complex physiological processes[9-11].

FGFs share in a diverse set of actions modulated FGF-Receptor isoforms. FGF-1 is found in normal corneal epithelium, and differs from FGF-2 in that it is upregulated in injured cornea and in co-cultures of keratocytes and vascular endothelia. On the one hand, bFGF binds to Bowman's membrane and Descement's membrane in healthy corneas, yet on the other hand, it binds to vascular basement membranes of neovascularized corneas[12].

In fact, it is believed that bone marrow acts as a store of bFGF and VEGF, sequestering them in order to balance anti-angiogenesis [13]. Besides, the maturation of new blood vessels and the level of FGF binding are related.

Newly formed corneal vessels show similar binding capacities in comparison to normal limbal vessels. This has been linked to heparin sulfate proteoglycans and thus stresses on the role of extracellular matrix (ECM) components in the regulation of corneal angiogenesis[14].

The levels of FGF-1, 2, 3, 7, and 22 are found to be increased in alkali wounding model at 7 and 14d post-wounding[2]. It is thought that bFGF function in corneal angiogenesis is mediated through its effect on VEGF-A, -C and -D production. bFGF promotes angiogenesis greatly through this action.

Angiogenic Interaction Between Vascular Endothelial Growth Factor and Basic Fibroblast Growth Factor From the increased researches in the past decade on characterization of interaction between multiple membrane-bound receptors, a new hypothesis emerged. It stated that, membrane-anchored receptors associate and coordinate with each other to cooperatively induce an array of intracellular signaling cascades, instead of transmitting signals across the membrane individually. Instead of working individually it was observed that there is an interplay between FGF and VEGF signaling for the maintenance of endothelial junctions and vascular integrity during angiogenesis [15-16].

Also, a recent study about mustard intoxicated subjects who developed CNV found a significantly increased levels of growth factors, specifically VEGF-A165, bFGF and platelet derived growth factor-BB (PDGF-BB) [17].

FGF-VEGF signaling balance is assumed to lie at the center of the regulation of permeability and angiogenesis. They are both important angiogenic growth factors. As soon as VEGF activates VEGFR-2 (a.k.a. FLK-1/KDR), the receptor undergoes auto-phosphorylation on specific tyrosine residues, followed by the addition of Tyr (P) residues on signaling proteins and adapter that contain the Src homology domain 2 (SH2)[18].

As a result the receptor complexes and adapter activate multiple intracellular pathways through some effectors like focal adhesion kinase (FAK) and mitogen-activated protein kinases (MAPK)[19-20].

Flk-1/KDR can also trigger other cascades including PI3K-dependent AKT/PKB and phospholipase C-g (PLC-g)[21-22]. Flk-1/KDR-mediated intracellular signaling seems to be similar to bFGF signaling pathway; however, various lines of evidence suggest that bFGF-induced angiogenesis is independent of Src kinase activity unlike VEGF signaling [23].

In spite of the extensive research on bFGF- and VEGF induced angiogenesis, the complete intracellular signaling pathways that respond to each of them to induce angiogenesis

are not fully understood. Specifically, how these pathways interact with molecular regulators is not well documented. It is suggested recently that membrane-type 1 metalloproteinase (MT1-MMP) may be one of many factors involved in connecting the two pathways of VEGF and FGF signaling [2]. It has been showed that MT1-MMP increased bFGF- induced VEGF upregulation and CNV in mice synergistically [24].

Besides, MT1-MMP raises bFGF-induced VEGF upregulation in enzymatically inactive MT1-MMP corneal stromal fibroblasts; which suggest that linking the VEGF and FGF signaling pathways may be in part due to MT1-MMP enzymatic activity.

MINOR ANGIOGENIC PROTEINS OF THE CORNEA

Decorins Decorins are members of the small leucine-rich proteoglycan (SLRP) family, which in turns belongs to a family of differently functioning molecules that are involved in the regulation of collagen fibrillogenesis, direct modulation of cell behavior and binding and inactivation of cytokines. Decorins consists of a protein core containing leucine repeats with a glycoseaminoglycan (GAG) chain of either dermatan sulfate or chondoroitin sulfate [25-27].

It has been demonstrated that decorins may regulate corneal angiogenesis [2,28]. Their effects on corneal angiogenesis in mice have been heavily studied, as well as the effects of biglycan and fibromodulin [29]. Using chemical cauterization, it was verified that in decorin-deficient mice (unlike biglycan and fibromodulin-deficient corneas), the growth of corneal vessels is significantly diminished compared to wild type (WT).

Recently, it was observed that bFGF induce MT1-MMP expression but diminish decorins expression [30]. Furthermore, it was demonstrated that MT1-MMP cleaves decorins *in vitro*, and that cell lysates from MT1-MMP-deficient keratocytes do not show decorins processing activity.

Ephrins and Eph Receptors One of the largest known families of receptor tyrosine kinase (RTK) is the Eph/ephrin complex. It consists of 14 receptors and 8 ligands, and its family members are subcategorized to class A and class B depending on their structure and ligand binding receptor characteristics [31-32].

In several vascular endothelial cells, EphB1-B4 and ephrinB1 and B2 were found to be expressed [33-34]. In adult mice it was demonstrated that EphB1 and ephrinB2 induce corneal angiogenesis [35], and that ephrinB1 induces vascular endothelial cell migration, assembly, and adhesion [36].

Recently, immunohistochemical studies were used to demonstrate that ephrinB1 and EphB1 are expressed in bFGF-induced vascular corneas [2], which proves that Eph and ephrin receptors play a role in corneal angiogenesis [37].

EphrinB1 is expressed in corneal-resident keratocytes and neutrophils. In order to test Eph and ephrin receptors' role in angiogenesis, recombinant ephrinB1-Fc (which induces EphB receptor activation) was used. It was found to promote bFGF-induced tube formation in an *in vitro* aortic ring assay; as well as corneal angiogenesis *in-vivo* in a corneal pocket assay. These results suggest that ephrinB1 plays a synergistic role in CNV [2]. Ellenberg *et al* [2] also compared ephrinA/EphA expression to ephrinB/EphB expression in vascularized corneas. bFGF pellets were implanted to induce CNV. The eyes of WT, ephrinB2$^{tlacZ/+}$, and EphB4$^{tlacZ/+}$ heterozygous mice were harvested and sectioned 7d after pellet implantation. Confocal immunohistochemistry was performed to compare the expression of the Eph/ephrinA family and Eph/ephrinB family. EphA1, EphA3, ephrinA1, ephrinA2, EphB1, EphB4, ephrinB1, and ephrinB2 were detected in WT mouse corneal epithelial cells and keratocytes.

Using immunohistochemistry it was found that EphA2 was only located in the epithelial cells, while EphA3, ephrinA1, EphB1, EphB4, and ephrinB1 were localized in corneal epithelium and stroma. However, in neovascularized corneas; ephrinB1 was mainly localized to keratocytes around the vessels, and ephrinB2, EphB1, and EphB4 were mainly located simultaneously with CD31 in the vascular endothelial cells. These studies strengthen the suggestion that Eph/ephrin family of receptor tyrosine kinases and their ligands may play a role in the regulation of corneal angiogenesis [2].

Activin Receptor –like Kinase Activin receptor-like kinase-1 (ALK-1) is one of the seven type I receptors recognizing transforming growth factor beta (TGF-β) family proteins [38]. It has been suggested that ALK-1 plays a role in the maturation phase of angiogenesis [39]. The transfection of a constitutively active form of ALK-1 inhibit not only, endothelial cell proliferation at the G1 phase of the cell cycle, but also endothelial cell migration through a modification of the dynamics of endothelial cell cytoskeleton [40].

Supporting these results is a zebrafish ALK-1 mutant, *vgb*, whose vessel dilation phenotype is reminiscent of ALK-1$^{-/-}$ mice. Its affected vessels showed an increased number of endothelial cells, supporting a role for ALK-1 in the inhibition of endothelial cell proliferation.

It have been demonstrated using the pellet induced CNV model that over expression of ALK-1 (using naked DNA injection) in mouse cornea does not induce CNV [2]. Besides, it can prevent growth of new bFGF-induced stromal vessels. All of that strengthen the possibility that ALK-1 plays an important role in angiogenesis.

Recently, Ellenberg *et al* [2] have described a proteomic approach to investigate the differential protein expression patterns and identify the physiologically relevant angiogenic and anti-angiogenic factors involved in the hyaloid vascular system regression. Differentially expressed proteins were identified using two-dimensional gel electrophoresis from the

lens and vitreous of P1 and P16 mice followed by nanoflow chromatography coupled with tandem mass spectrometry [41]. Using this approach, the following factors expressed at P16 may be involved in angiogenesis: tumor necrosis factor-α (TNF-α), hepatoma-derived growth factor (HDGF), FGF-22, and kininogen.

Integrins A major family of type I transmembrane cell surface receptors are the integrins. Currently, 18 individual α subunits and 8 β subunits have been identified. Integrins are heterodimers composed of one α and one β subunit [42-43].

It has been noticed that a significant upregulation of αvβ3 and α5β1 takes place on activated vascular endothelium during angiogenesis. It is suggested that α5 integrins play a key role during the development of the vascular system [44-45]. Testing that hypothesis with genetic ablation of integrin α5 leads to severe vascular abnormalities. α5β1 integrin as well as its extracellular ligand fibronectin which is able to provide proliferative signals to vascular cells both are upregulated in tumor new blood vessels and plays a role in tumor angiogenesis and growth. Besides, angiogenesis have been inhibited *in-vivo* and *in-vitro* using integrin αvβ3 and αvβ5 antagonists.

On the other hand, treating animals systemically with an α5β1-inhibiting small molecule showed significant inhibition of CNV. Combining them to integrin αv and α5 does not promote the anti-lymphangiogenic effect *in vivo* [46-47].

Matrix Metalloproteinases Corneal extracellular matrix (ECM) remodeling by matrix metalloproteinases (MMPs) has also been implicated in corneal angiogenesis and in the maintenance of corneal avascularity. MMPs are described as a group of proteolytic enzymes that are zinc-binders, and participants in ECM remodeling, neovascularization, and lymphangiogenesis.

MMPs is a large family that involve 25 enzymes described so far, not less than 15 of which have been identified in the cornea; (MMP-1, -8 and -13) represent the collagenases, MMP-2 and -9 represent the gelatinases A and B, MMP-3, -10 and -11 represent the stromelysins, MMP-7 is the matrilysin and MMP-12 is the macrophage metalloelastase while MMP-14, -15, -17, -24 and -25 all represent the membrane type of MMPs[48-52].

After several studies it became clear that MMP-mediated proteolysis induce several important biological functions including: 1) changing structural matrix proteins into signaling molecules (*e.g.* collagen XVIII that is present in the cornea and having an NC1 domain which is anti-angiogenic); 2) changing the structure of matrix proteins like cleaving perlecan and decorin-corneal ECM proteoglycans; 3) changing the architecture of the tissue (*e.g.* cleaving E-cadherin); 4) changes in chemotaxis; 5) inducing proliferation like its action through epidermal growth factor receptor ligand processing; 6) ensuring the cell survival (*e.g.* neuronal survival factor); 7) activating some latent signaling molecules (*e.g.* TNF-a shedding and IGF binding protein cleavage); 8) changing the range of signaling molecule action (*e.g.* changing the range of VEGF diffusion); 9) causing tissue differentiation (*e.g.* adipose tissue maturation)[52-55].

Increased expression of MMPs in corneas during angiogenesis has already been demonstrated [56-57]. However it is still vague what is their definitive role in regulation of angiogenesis because they can act as pro- and anti-angiogenic factors at the same time, which might be explained by their ability to degrade the ECM, allowing tissue invasion by endothelial cells bearing MMP, and to generate anti-angiogenic fragments from their precursors [52,58-59].

In the following sections, additional information on the roles of MMP-2, MMP-7, and MT1-MMP in corneal angiogenesis are highlighted.

Matrix Metalloproteinase-2-Gelatinase A Gelatinase-A (MMP-2) has always been linked to angiogenesis. It was demonstrated that it is pro-angiogenic through facilitating vascular invasion by direct matrix degradation or through releasing matrix bound cytokines or growth factors [52,60-61].

MMP-2 expression by epithelial cells and stromal keratocytes has been confirmed by in situ hybridization [49]. Besides, its physiologic role in angiogenesis has been defined: when MMP-2 deficient mice were used to determine the role of MMP-2 in vascular endothelial cell migration and tube formation *in vitro* using aortic rings, it was demonstrated that bFGF mediated angiogenic response was diminished in mice lacking the functional MMP-2 gene compared to WT animals [50].

On the other hand, endothelial cells from MMP-2 lacking mice failed to display normal outgrowth after adding 5 ng/mL bFGF, which lead to the suggestion that the difference in bFGF-induced angiogenesis between MMP-2 lacking mice and WT mice may be due to the difference of vascular endothelial cells; as it could be inconvenient for endothelial cells lacking functional MMP-2 to traverse the basement membrane [2].

The MMP-2-null mice developed almost normally, and bFGF induced corneal angiogenesis even in the MMP-2-mutant mice, clearly indicating that the angiogenic process is not totally dependent on MMP-2. In another experiment, MT1-MMP null mice showed complete absence of corneal angiogenesis which lead to the suggestion that MT1-MMP by itself has an essential role in the process of angiogenesis [51]. Based on these data, further research is needed to explain the discrepancy between MMP-2 and MT1-MMP effect on angiogenesis.

Studies show that through intramolecular processing, MMPs can modulate the bioavailability of VEGF; a group of MMPs can cleave the matrix-bound isoforms of VEGF releasing

soluble fragments to promote capillary dilatation of existent vessels [59,62-63].

In recent researches, MMP-2 could cleave connective tissue growth factor (CTGF) and heparin affin regulatory peptide (HARP) and inactivate them upon proteolysis. As these two are angiogenic and mitogenic cytokine inhibitors in complex with VEGF, cleaving those releases the VEGF. As a result, MMP-2 possesses potential pro-angiogenic activity by releasing intact VEGF from HARP or CTGF cytokine inhibitory complexes [62].

Matrix Metalloproteinase–7 (Matrilysin) Matrilysin also called MMP-7 is expressed in basal epithelial cells in the migration and proliferation phases of corneal wound healing after excimer keratectomy [48-49]. Matrilysin has catalytic action against a wide range of ECM substrates *e.g.* gelatins (I, III, IV, and V), fibronectin, elastin, collagen IV, laminin and entactin-nidogen [64]. At the same time, it can cleave factors which modulate angiogenesis like CTGF, sVEGFR-1, plasminogen and collagen XVIII. It has been positively stained in basal epithelium of pterygium specimen suggesting its involvement in pathogenesis and angiogenesis in pterygium [65].

The anti-angiogenic role for MMP-7 in cornea is based on the fact that MMP-7 cleavage of corneal collagen XVIII yields a 28-kDa fragment which contains the endostatin domain of collagen XVIII that shows potent anti-angiogenic function [58]. Also in MMP-7 knock-out (KO) mice, in the keratectomy wounding model, a decrease in the levels of anti-angiogenic factors tilts the balance towards corneal angiogenesis [2].

Recent researches show that the induction of new vessel formation in diseased corneas involves not only upregulation and activation of angiogenic factors such as VEGF and bFGF but also suppression of anti-angiogenic factors.

Based on the observation that MMP-7 cleaves plasminogen and collagen XVIII *in vitro* to generate anti-angiogenic factors *e.g.* angiostatin and endostatin respectively suggest that the reduction of MMP-7 derived endostatin and/or angiostatin in the cornea may contribute to CNV after excimer keratectomy in MMP-7 KO animals [2].

Membrane –type 1 Metalloproteinase MT1-MMP is the most important MMP in angiogenesis. MT1-MMP expression in the cornea has been detected in the epithelium and stromal keratocytes during wound healing [49]. Its importance becomes evident in that it is the only MMP that its absence is lethal demonstrated by genetic KO of MT1-MMP in mice lead to death within three to four weeks, unlike other MMPs who's genetic KO might affect angiogenesis but is never lethal to the animal.

MT1-MMP proved to be important for angiogenesis and its absence causes delayed vascular development and impaired CNV by bFGF [51]. Further studies have been carried out to understand its role using genetic KO mice, antibodies against MT1-MMP, animal models and other methods [2]. It was noticed that there is an enhanced MT1-MMP expression in alkali wounded CNV. In addition using reverse transcription-polymerase chain reaction (RT-PCR) to evaluate the expression of growth factor receptors in WT, MT1-MMP KO, and MT1-MMP knockin (KI) mouse cornea stromal fibroblasts, it was found that no significant difference existed in the expression patterns of PDGFa, PDGFb, and VEGFR-1 in either type of cells.

On the other hand, endothelial growth factor receptor (EGFR) expression was decreased in MT1-MMP KO cells when compared to the WT and MT1-MMP KI cells, suggesting that MT1-MMP play a role in EGFR expression regulation. Since EGFR is also fibroblast proliferation and migration regulator, it may be responsible for some of MT1-MMP pro-angiogenic effects [66].

Angiogenic Interaction Between Membrane –type 1 Metalloproteinase and Vascular Endothelial Growth Factor/ Fibroblast Growth Factor There have been new studies that elaborated the signal pathways involved in the interaction between these molecules and their role in CNV[67]. It was suggested that MT1-MMP may link the two signaling pathways of VEGF and FGF[24], but its specific role in linking them remains vague. The pro-angiogenic role of MT1-MMP has been reported to be in part mediated through the upregulation of both VEGF transcription and translation[68].

Immuno-histochemistry and RT-PCR analysis of human glioma tissue samples proved a functional link in tumor angiogenesis between MT1-MMP and VEGF by giving evidence to the link between their expressions[69].

Further evidence is the correlation between VEGF stimulation and hypoxia-induced upregulation of MT1-MMP in the murine bone marrow -derived stromal cells. Putting all these data together gives a strong evidence of linkage between signaling pathways of MT1-MMP and VEGF, which may play a role in regulating corneal angiogenesis [59]. The FGF and MT1-MMP interaction has been well documented: FGF-1 induction of MT1-MMP transcription in LNCaP prostate carcinoma cells has been reported. Besides, FGFR-1 and STAT3 involvement in FGF-1 mediated MT1-MMP expression has also been reported [70]. bFGF induced CNV increased when bFGF pellets have been used in combination with naked MT1-MMP DNA plasmid injection [2]. The interplay between MT1-MMP, VEGF, and bFGF has been demonstrated by experiments in which VEGF and MT1-MMP expression increased after implantation of bFGF-pellets in murine cornea.

ANGIOGENESIS INHIBITORS

Corneal angiogenesis privilege (CAP) is shown to be secondary to the interaction of multiple anti-angiogenic factors. Most notable of which are angiostatin, angiostatin-like

fragments[71], and endostatin[72]. Other potent anti-angiogenic factors that modulate CAP include restin, arresten, canstatin, tumstatin, and pigment epithelial-derived factor (PEDF)[73].

They can be generally classified into, endostatin/endostatin analogues, and plasminogen/serine protease inhibitors.

Endostatin/Endostatin Analogues

Endostatin Endostatin has been shown to inhibit *in-vitro* VEGF mediated endothelial migration and proliferation[74], as well as decrease tumor progression in *in-vivo* murine models. Moreover, endostatin has been successfully administered in corneal assays, with significant reduction in bFGF mediated angiogenesis[75].

The mechanism of action of endostatin is relatively complex. It exerts its actions through primarily associating with tropomyosins, integrins, VEGF receptors, MMPs, and glypicans. Its action on VEGF is in the form of; blockage of VEGF cell surface receptor KDR/FLK1, downstream inactivation of ERK, MAPK, and P125FAK, ultimately, arrest of cell cycles in G1, with inhibition of mitogenic responses in vascular endothelial cells[76].

In addition to VEGF antagonism, endostatin enhances vascular endothelial apoptosis *via* increasing the activity caspase 3 [77].

Alongside angiogenesis inhibition, endostatin has been shown to affect lymphogenesis as well. Recombinant endostatin was shown to inhibit the proliferation and migration of lymphatic endothelial cells, *in vitro* [78]. One possible mechanism for this activity is the ability of endostatin to inhibit distribution of VEGF-C-producing tumor-associated inflammatory cells and to induce the apoptosis of VEGFR-3 expressing cells [79].

Neostatin There are two major types of neostatins, both of which have been proven as potent angiogenesis inhibitors. Neostatin-7 (formed *via* MMP-7's cleavage of collagen XVIII), and neostatin-14 (formed *via* MT1-MMP-mediated cleavage of collagen XVIII) [79].

Both MMP-7 and MT1-MMP are expressed by corneal epithelial cells [48], where collagen XVIII is actively secreted as well [59]. Moreover, it was demonstrated that recombinant neostatin-7 blocks bFGF-induced corneal angiogenesis and lymphangiogenesis [80]. This strongly shows the important role of corneal epithelium in maintaining CAP.

Other Miscellaneous Molecules Arresten, canstatin, and tumstatin are three type IV collagen-derived proteins that were shown to have potent anti-angiogenic activity[81-84].

Arresten actions are mediated *via* $\alpha 1\beta 1$ integrin receptors. It successfully inhibits bFGF-induced proliferation, migration, and tube formation of cultured endothelial cells[82,85-86].

Another molecule, canstatin, acting *via* $\alpha 3\beta 1$, $\alpha\gamma\beta 3$, and $\alpha\gamma\beta 5$ integrin receptors, has diverse functions. It causes suppression of tumor growth, inhibition of endothelial cell proliferation and migration, and induction of endothelial cell apoptosis [87-89].

Also, tumstatin which is a 28 kDa protein derived from type IV collagen $\alpha 3$ chain, exerts its action *via* $\alpha\gamma\beta 3$ and $\alpha 6\beta 1$ integrins receptors. It shares in suppression of tumor growth, inhibition of endothelial cell proliferation and migration, induction of endothelial cell apoptosis, and inhibition of protein synthesis [84,90-91].

Tumstatin can inhibit protein synthesis through the inhibition of phosphorylation of FAK, induced in endothelial cells *via* attachment to vitronectin, and by inhibiting the activation of PI3-kinase through $\alpha\gamma\beta 3$ binding [84,92].

Plasminogen-derived and Serine Protease Inhibitors of Angiogenesis

Angiostatin Angiostatin is a complex molecule that can inhibit primary and secondary tumor growth. One of the enzymes responsible for the generation of angiostatin in Lewis lung carcinoma has been identified as a macrophage derived metalloelastase (MMP-12) [93]. However, human matrilysin (MMP-7) and neutrophil gelatinase B (MMP-9) can also convert plasminogen to angiostatin fragments [94].

The suppressive action of recombinant angiostatin on *in-vivo* tumor growth and metastasis in animal models has also been demonstrated [95]. Moreover, angiostatin was proven to be a non-toxic inhibitor of neovascularization when injected to tumor-bearing mice[96].

One mechanism that explains these actions is down-regulation of endothelial cells migration and proliferation, through binding to ATP synthase and decreasing endothelial cell ATP production [97]. Another way is induction of endothelial cell apoptosis and arrest at the G2 to M transition phase [98].

Another possible mechanism for angiostatin is its binding to integrin $\alpha v\beta 3$; thereby inhibiting its actions. Typically plasmin binds to $\alpha v\beta 3$ through its kringle domains, promoting endothelial cell migration. This process can be disrupted through anti-integrin $\alpha v\beta 3$ agents (*i.e.* angiostatin) and a serine protease inhibitor [99].

Angiostatin and angiostatin-like molecules play an important role in maintaining corneal avascualrity after injury, and it was shown that their expression in corneal epithelium increases remarkably at these events [100]. Moreover, corneal segmental neovascularization was demonstrated after excimer laser keratectomy followed by treatment with anti-angiostatin (anti-LBS or anti- K1e3) antibody injection, further affirming the role of angiostatin [2].

Pigment Epithelium Derived Factor A member of the serine protease family, PEDF is a potent anti-angiogenic factor, expressed within endothelial and corneal epithelial cells [101]. It typically works through binding to surface receptors, such as glycosaminoglycans and collagen I[102].

Table 1 Differences between hemangiogenesis and lymphangiogenesis

Parameters	Hemangiogenesis	Lymphangiogenesis
Corneal privilege mechanism	Upregulation of anti-angiogenic factors and downregulation of angiogenic factors Soluble VEGFR-1 and ectopic VEGFR-3 act as "VEGF traps" to prevent hemangiogenesis[117]	Soluble VEGFR-2 and VEGFR-3 act as "VEGF trap" and selectively suppresses physiologic growth of lymphatics[117,121]
Formation	Sprouting or budding from post-capillary veinules	LEC's arise from either: Bone marrow-derived cells, such as transdifferentiated macrophages[106] Primitive veinules and local lymphangioblasts (described in detail in the text)
Role of macrophages	Providing a temporary scaffold for the new vessels[122] Provide paracrine support for vascular networks[123] Interact physically with the blood vessels[124]	Transdifferentiate into endothelial cells, thus participate - structurally- in lymphatic vessels[122] Secrete paracrine factors, most importantly VEGF-A[128] Act as "guide cells" that guide tip cells into finding and anastomosing with tip cells from other sprouting lymphatics[125]
Expression of VEGFR-3	Expressed during early stages of endothelial development. Fully developed blood vessels seldom contain VEGFR-3, with the exception of fenestrated blood vessel of endocrine glands[116]	Highly expressed by LEC's
Role of VEGF-C and VEGF-D	Hemangiogenic properties through binding to VEGFR-2[126]	Both copies of the VEGF-C gene are needed (haploinsufficiency)[130] Bind to VEGFR-3 and promote venous endothelial differentiation into lymphangioblasts[110,130] VEGFR-3 blockade results in a hemangiogenesis-dominant cornea[131] VEGF-C plays a key role in development of the lymphatic vascular tree, but is not needed to maintain lymphatics after that have already been developed[132] Deletion of VEGF-D, in contrast, does not affect lymphatic vascular development[130]
Role VEGF-A[124-125]	Binds to VEGFR-1 and -2 to promote hemangiogenesis	Binds to VEGFR-1 and -2 to promote lymphangiogenesis Also acts indirectly through recruitment of macrophages
Response to VEGF-A[118].	Early appearance of vessels in response to low VEGF-A levels	Delayed appearance of vessels and higher VEGF-A concentrations required[1]
Role of FGF-2	Stimulates angiogenesis only at high doses (80-100 ng)[133]	Low dose (12.5 ng) selectively stimulates lymphangiogenesis[134]
Regression after pathologic invasion of cornea[131]	Late	Early
Corneal transplant rejection[115]	Less important role	Important role

[1]The delayed appearance of lymphatics is attributed to the fact that sprouting blood vessels upregulate VEGFR-2, which traps VEGF-C and prevents it from forming new lymphatics at the early phases. This is hypothesized to allow more time for the immune cells to reach the inflammatory site before being cleared by lymphatics.

Furthermore, the recombinant PEDF inhibited CNV as well[103]. These findings point towards an essential function of PEDF in maintaining avascular environment of the cornea. Other works established similar roles for PEDF in the vitreous, aqueous humor, and retina[2].

Given its effectiveness against multiple inducers of angiogenesis, as VEGF and interleukin-8 (IL-8), and its multiple sites of action, PEDF derivatives can prove highly effective in reversing pathological ocular angiogenesis processes.

MOLECULAR UNDERPINNINGS OF LYMPHANGIOGENESIS

The process of lymphangiogenesis was thought to have the same molecular underpinnings as hemangiogenesis for a long time. It is only relatively recently that lymphangiogenesis started to have a separate entity, especially after the discovery of the key lyphatic endothelial cells (LEC) marker, lymphatic vessel endothelial hyaluronan receptor (LYVE-1) [101]. The formation of lymphatics can be summarized in the following steps [104]:

1) Endothelial cells differentiate from angioblasts into venous endothelial cells and arterial endothelial cells.

2) Venous endothelial cells highly express VEGFR-3 and a subset of them begins to express LYVE-1 as well. This subset represents the precursors of LEC's [105]. Bone marrow-derived cells, including macrophages, may also transdifferentiate into endothelial cells [106].

3) LEC precursors begin to express the transcription factor SOX18, which is present upstream of another transcription factor, Prox-1 [107]. SOX 18 induces the expression of Prox-α and triggers a set of incompletely-understood events that eventually determine the differentiated fate of LEC's [108]. In fact, Prox-1 is known to be expressed in a polarized manner in differentiating LEC's and is believed to be a "master switch" in LEC differentiation.

4) LEC precursors express Neuropilin-2 (NP-2) [109]. NP-2, while not triggering downstream signaling itself, sensitizes the LEC to VEGF-C stimulation and acts synergistically with VEGFR-3[109].

5) At this stage, the proliferating lymphatics begin to form

lateral extensions from the veinules, known as "lymphatic sacs" [107].

6) LEC's start expressing the transmembrane protein, podoplanin [110-112]. In turn, podoplanin binds to CLEC-2 receptors on platelets and activates SLP76 and Syk, leading to platelet aggregation [112].

7) The aggregated platelets block the connection between the developing lymphatic and the veinule from which it budded, eventually leading to physical separation of the two entities.

8) FoxC2 and NFACT-1 (nuclear factor of activated T cells), both of which are transcription factors downstream of VEGFR-3, cooperate in controlling genes that are important in further differentiation of the lymphatic tree, including lymphatic valves [113].

TGF-β and TNF-α both act to inhibit lymphangiogenesis [114-115]. On the other hand, VEGF-A, VEGF-C, FGF-2, IGF-1, IL-1B, HGF and PDGF have all been shown to simulate both hemangiogenesis and lymphangiogenesis (as has been discussed in detail earlier). It is noteworthy that the growth factors and cytokines that stimulate hemangiogenesis and lymphangiogenesis greatly overlap. Nonetheless, VEGF-C/VEGFR-3 signalling seems to be more specific to lymphangiogenesis and VEGF-3 ceases being expressed in adult blood vessels, with the exception of fenestrated vessels of endocrine glands [116-117]. Another factor that differentiates lymphangiogenesis from hemangiogenesis is the role of α5β1 integrins in lymphangiogenesis. An α5β1 antagonist results in a hemangiogenesis-dominant response to CNV induction [118]. Table 1 highlights some of the differences between hemangiogenesis and lymphangiogenesis [106,116-134].

CONCLUSION

The cornea is maintained in an avascular state through a balance between naturally present pro-angiogenic and anti-angiogenic chemical mediators. CNV continues to be an incompletely understood process that requires further research and funding to reveal its molecular pathways.

While hemangiogenesis and lymphangiogenesis were seldom distinguished in the past decades, recent attention has been geared towards understanding the differences between these two different, yet interdependent processes.

We think that more attention is needed in the upcoming decade to address and enhance our understanding of the molecular and genetic processes hidden under the mere manifest blood vessel.

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