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Current and Upcoming Therapies for Corneal Neovascularization

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Abstract

The cornea is unique because of its complete avascularity. Corneal neovascularization (CNV) can result from a variety of etiologies including contact lens wear; corneal infections; and ocular surface diseases due to inflammation, chemical injury, and limbal stem cell deficiency. Management is focused primarily on the etiology and pathophysiology causing the CNV and involves medical and surgical options. Because inflammation is a key factor in the pathophysiology of CNV, corticosteroids and other anti-inflammatory medications remain the mainstay of treatment. Anti-VEGF therapies are gaining popularity to prevent CNV in a number of etiologies. Surgical options including vessel occlusion and ocular surface reconstruction are other options depending on etiology and response to medical therapy. Future therapies should provide more effective treatment options for the management of CNV.

Keywords

Eye; Cornea; Vascularization; Neovascularization; Angiogenesis; Limbal stem cell deficiency; Inflammation

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INTRODUCTION

Corneal neovascularization (CNV) can result from a variety of etiologies such as contact lens wear; corneal infections; and ocular surface inflammation and injury including limbal stem cell deficiency (LSCD).[1–3] CNV may result in decreased visual acuity from the sequelae of blood vessels invading the cornea and causing opacification of the stroma and irregularity of the corneal surface. Surface irregularity results in higher order aberrations; this can be accompanied by extravasation of fluid and lipids, which leads to corneal edema and lipid keratopathy as well as alteration of the stromal architecture.[4] These changes minimize corneal clarity and thus impede vision. In a comprehensive ophthalmology clinic, CNV was found in 4.14% of patients, of which 12% showed a decrease in visual acuity.[5] While CNV can be helpful in certain circumstances (e.g. uncontrolled corneal infections, conditions involving stromal necrosis), it is more often a pathologic consequence of various ocular surface and corneal disorders. Notably, CNV is an important risk factor for corneal graft rejection and subsequent failure.[6]

DEFINITION AND TERMINOLOGY

The cornea is unique because it is completely avascular and alymphatic, which is essential for its clarity and optimal vision. When blood and lymphatic vessels from the pericorneal vascular plexus grow into the cornea, the result is a pathologic condition termed corneal hemangiogenesis and corneal lymphangiogenesis, respectively.

It has been proposed that the term corneal vascularization is appropriate in contrast to neovascularization, as the latter refers to a condition in which new blood vessels arise from pre-existing ones. To avoid confusion with choroidal neovascularization and due to the absence of pre-existing blood vessels in the cornea, the term “corneal vascularization” should be used for corneal vessel formation.[7] However, in reviewing the literature, majority of studies term this pathology “corneal neovascularization”. To follow prior studies in this field, the term “corneal neovascularization (CNV)” is used throughout this review.

In many pathologic conditions, lymphatic vessels grow into the cornea parallel to blood vessels. Lymphangiogenesis plays a critical role in many processes such as immunity, infection, and metastasis.[8]

VASCULOGENESIS VERSUS ANGIOGENESIS

Vasculogenesis comprises the *de novo* formation of vessels from vascular endothelial precursor cells (i.e. hemangioblasts and angioblasts) which are derived from mesodermal precursors (via mesodermal induction).[9] In contrast, angiogenesis is a process in which endothelial cells of pre-existing vessels proliferate and form new vessels.[9] In CNV, the endothelial cells of newly formed corneal vessels originate from pre-existing limbal vessels (i.e. angiogenesis). However, pericytes, another crucial cell type in blood vessel formation, originate from bone-marrow derived precursors (i.e. vasculogenesis).[10] Ozerdem and colleagues believe that both angiogenesis and vasculogenesis are involved in CNV and that targeting both mechanisms would be most effective in managing this condition.[10] Similar to blood vessels, lymphatic vessels may arise *de novo* from bone-marrow derived cells (i.e.

CD11b-positive macrophages) or they may extend from pre-existing limbal lymphatic vessels.[8, 11]

CORNEAL VASCULAR PRIVILEGE

Previous studies have identified a number of mechanism(s) by which the limbal vascular plexus does not invade the cornea under normal physiologic conditions. It is believed that an imbalance between angiogenic and anti-angiogenic mechanisms in the cornea results in CNV.[12]

The first proposed mechanism for CNV was proposed by Cogan, who claimed corneal swelling and subsequent disintegration of the corneal lamellae were the sole factors responsible for CNV.[13] However, further investigation revealed that corneal swelling is necessary but not sufficient for the development of CNV.[14, 15]

While there is no anatomical boundary between the limbal vascular plexus and the clear cornea, the angiostatic function of the limbus has been proposed as a mechanism for corneal avascularity, especially since LSCD is often associated with CNV.[16–18] It is unclear whether the limbus exerts its barrier function via a physical or functional mechanism, or both. The physical barrier effect of the limbus has been proposed by Friedenwald as a “growth pressure theory,” in which continuous selfrenewal of the limbal stem cells prevents invasion of the conjunctival epithelium and subsequent vascularization of the cornea.[19] However, using a murine hemilimbal corneal injury model, Tobaigy showed factors other than the limbal barrier are involved to maintain corneal avascularity.[20]

Although earlier reports supported the angiogenic properties of corneal epithelium,[21, 22] the predominantly anti-angiogenic role of the corneal epithelium has been widely accepted in more recent studies.[23] Clinically, the association of a persistent corneal epithelial defect (PED) with CNV and its resolution after epithelial transplantation further supports the role of corneal epithelium in preventing CNV.[24]

Interestingly, the corneal epithelium releases pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), which are then sequestrated by the basement membrane (BM) under normal conditions.[22, 25] For example, Ambati and colleagues found that the cornea contains a high quantity of VEGF-A, a potent pro-angiogenic molecule. However, it is almost completely bound to the soluble VEGF-receptor 1 (also known as soluble fms-like tyrosine kinase-1 sflt-1), thus preventing its angiogenic effects.[26] They concluded that sflt-1 is a crucial factor in corneal avascularity.[26] Ambati and colleagues have also reported that expression of sflt-1 is significantly lower in vascularized corneas (secondary to alkali burn, ocular cicatricial pemphigoid, interstitial keratitis, and aniridia) when compared to normal human corneas.[27] Inhibitory PAS (Per/Arnt/Sim) domain protein is another corneal epithelial derived factor with antiangiogenic properties, specifically against hypoxia inducible factor (HIF)/Hypoxia induced CNV.[28] In addition, VEGF receptor 3, which is constitutively expressed by the corneal epithelium, is an inhibitor of corneal angiogenesis.[29]

The corneal epithelial BM also contains anti-angiogenic factors such as tissue inhibitor of metalloproteinase 3 (TIMP-3) and collagen XVIII/endostatin.[30, 31] Angiostatin, restin, arrestin, endostatin, canstatin, tumstatin, thrombospondins, interleukin-1 receptor antagonist, pigment epithelial derived factor (PEDF), vasoactive intestinal peptide (VIP) and α-melanocyte stimulating hormone (α-MSH) are also anti-angiogenic molecules, which have been found in the cornea and/or the aqueous humor.[4, 32–34] Given that the cornea contains both angiogenic and antiangiogenic factors, damage to the basement membrane (BM) due to LSCD or persistent epithelial defects may result in the release of pro-angiogenic factors and loss of anti-angiogenic factors, and thus lead to CNV.[35]

Several molecules with anti-lymphangiogenic properties have been identified in the cornea and aqueous humor. These include alternatively spliced VEGF receptor-2 (soluble VEGFR-2), tumor necrosis factor superfamily member 10 (Tnfsf10/Trail), tissue plasminogen activator (tPA), and thrombospondin 1 in the cornea as well as VIP and α-MSH in the aqueous humor.[33, 36–38]

HEMANGIOGENESIS VERSUS LYMPHANGIOGENESIS

The lymphatic system is a network of vessels throughout the body that allows lymphatic fluid to return to the systemic blood circulation. In addition to lacking blood vessels, the cornea is also devoid of lymphatic vessels. The paucity of blood vessels prevents immune cells from accessing corneal antigens, and the lack of lymphatic vessels prevents cellular and cytokine traffic to the regional lymph nodes.[39] While several anti- (hem) angiogenic factors are known to be present in the normal cornea (as mentioned above), the anti- (lymph) angiogenic factors are still yet unknown. However, in vascularized human corneas, the degree of lymphangiogenesis is significantly correlated with the degree of hemangiogenesis.[40, 41] In contrast, corneal lymphangiogenesis may also occur in an avascular cornea. Transient physiologic lymphangiogenesis has been reported to reduce corneal edema and increase corneal transparency in cases with corneal edema.[42]

Lymphatic vessels can provide a drainage pathway for both antigenic material (cells, cellular debris) and more importantly antigen presenting cells.[43, 44] Besides enabling the transport itself, lymphatic vessels enhance the speed and amount of antigenic material or antigen presenting cells that reach the regional lymph nodes.[45] This is particularly important after corneal transplantation.[46] Corneal lymphangiogenesis provides a route of exit from the graft to regional lymph nodes, which has been shown to be essential in promoting alloimmunization and subsequent graft rejection.[47, 48] Corneal lymphatics in vascularized human host beds adjacent to grafted tissue could enhance the traffic of graft-derived antigens to regional lymph nodes, thereby promoting rejection.[39]

MECHANISMS OF CORNEAL NEOVASCULARIZATION

Cogan originally described histologic evidence of a mechanism following corneal inciting injury, which led to local corneal edema accompanied by aneurysmal engorgement of venules and capillaries. After a few days, these were replaced by tiny spicule-like masses of hemorrhages in a radiating pattern which could later either form capillary channels or

regress /disappear. The channels typically formed an extension in front of a pre-existing loop. With repetition of this cycle, there was progressive movement of the vessels toward the central cornea.[13] Conversely, Shi and colleagues described three pivotal steps to CNV in five models (suture-mediated, alkali injury, fungal infection, or implantation of immunogen or tumor cells).[49] These pivotal steps included a sprout period, a vigorous stage, and a regressive stage. Although in all of the models the initial findings (corneal edema and vascular dilation) were similar to Cogan's investigations, Shi and colleagues identified more details of the events leading to CNV. First, the BM of the perilimbal capillary network is degraded by proteases released by endothelial cells. Thereafter, endothelial cells (ECs) migrate and invade the extracellular matrix (ECM) and begin to proliferate. Finally, the lumen of the new vessels forms, and the BM is remodeled.[49]

The mounting evidence in literature clearly show CNV and corneal lymphangiogenesis are the result of sprouting from pre-existing limbal blood and lymphatic vascular arcade.[50, 51] Furthermore, there are also reports that corneal edema is neither necessary nor sufficient for CNV.[52, 53]

INFLAMMATION

Inflammation is the core mechanism of CNV induced by any etiology including chemical injury, infection (Figure 1A), immune disorders (Figure 1C), LSCD (Figure 2), and hypoxia.[12, 54] There is an interplay between inflammatory cells (especially macrophages and neutrophils) and angiogenic growth factors (i.e. VEGF family) in inflammation induced CNV. VEGF-A mediated recruitment of macrophages may initiate a process called "immune amplification cascade" that promotes corneal hemangiogenesis and lymphangiogenesis.[50] Inflammatory cells that are recruited during corneal injury, especially macrophages, produce pro-angiogenic factors and proteolytic enzymes which promote limbal vascular endothelial cell proliferation and migration.[50, 55–57] Pro-inflammatory cytokines and chemokines are strong mediators of angiogenesis in humans and are over-expressed in corneal inflammation.[58–66]

IL-1 is a key cytokine in inflammatory angiogenesis.[64] It is released mainly from injured corneal epithelium and directly stimulates proliferation as well as migration of endothelial cells.[58, 66] IL-1 also enhances production of strong pro-angiogenic molecules VEGF and bFGF,[67] increases the expression of adhesion molecules and inflammatory mediators by human corneal epithelial cells, and recruits leukocytes via production of chemokines.[68] IL-6 is another proinflammatory cytokine which can promote CNV by increasing VEGF production in corneal and inflammatory cells in herpes simplex virus (HSV) keratitis.[61] Recombinant human IL-8 (rhIL-8) under physiologic concentrations proved to be a potent stimulator of CNV *in vivo*.[65,69] IL-17A has also been implicated in the pathogenesis of HSV infection associated with CNV.[69]

Matrix metalloproteinases (MMPs) have a dual role in corneal angiogenesis. Release of MMPs (especially MMP-2/gelatinase A) from injured corneal epithelium and leukocytes degrades the basement membrane,[70] resulting in the release of sequestrated pro-angiogenic factors as previously discussed. MMPs also degrade the ECM components, creating a physical space and facilitating endothelial cell migration during angiogenesis.[71]

Simultaneously, some MMPs show anti-angiogenic activity by catalyzing the production of angiostatic mediators such as endostatin and angiostatin from their precursors.[72–74]

LIMBAL STEM CELL DEFICIENCY (LSCD)

LSCD, whether congenital or acquired, is commonly associated with CNV.[75] According to Friedenwald's theory of growth pressure, loss of the physical barrier effect of the limbus is the main cause of CNV following LSCD.[19] However, Tobaigy and Azar demonstrated that when half of the limbus and more than half of the corneal epithelium were removed, vessels grew from the opposite side of the defect.[20] This observation led to the conclusion that factors other than the physical barrier are responsible for the limbal angiostatic effect.[20]

Previous studies have shown that damage to limbal stem cells (LSCs) induces long-standing inflammation and recruits macrophages,[76] which are important sources of VEGF.[77] Additionally, LSCD is followed by amplification of tissue growth factor beta (TGF-β) signaling which exacerbates inflammation and VEGF production.[63, 78] The vicious cycle of increasing inflammation causes destruction of the remaining LSCs and progression towards total LSCD. The amplification of VEGF and TGF- β, key angiogenic factors, contributes to CNV. Finally, the establishment of CNV in the absence of the limbal barrier leads to the invasion of the conjunctival epithelium toward the corneal surface, resulting in corneal conjunctivalization.

HYPOXIA

Hypoxia is a classic stimulator of angiogenesis. In the hypoxic cornea, VEGF is expressed by corneal epithelial and endothelial cells, as well as the endothelium of limbal vessels in an attempt to enhance the supply of oxygen to the cornea.[77, 79] However, in an animal model of closed-eye contact lens-induced CNV, increased expression of VEGF did not correlate with inflammation.[80] Since VEGF is present in the tear film of the normal non-vascularized cornea and is almost completely inactivated by sflt-1, other factors may also mediate CNV in the hypoxic state.[80] Hypoxia inducible factor-1 alpha (HIF-1α) plays an essential role in the response to hypoxia and contributes to angiogenesis.[81, 82] Similar to VEGF, there is an inhibitory mechanism for HIF-1α in hypoxic conditions which prevents HIF-1α induced angiogenesis in the cornea.[28] Although bFGF, a second angiogenic growth factor, was not found to play a significant role in hypoxia-induced corneal angiogenesis,[83, 84] other angiogenic cytokines and chemokines that are upregulated by hypoxia may contribute to hypoxic CNV.[85] While nitric oxide (NO) is a potent vasodilator which is upregulated by hypoxia and has both pro- and anti-angiogenic activities in a dose dependent manner,[86, 87] the effects of hypoxia on corneal NO production and angiogenesis have not yet been fully elucidated.

Inflammation induced by hypoxia might possibly be the most relevant factor for corneal angiogenesis. Hypoxia induces the production of potent inflammatory metabolites of arachidonic acid by the corneal epithelium via the cytochrome p450 (CYP) pathway.[88] The most important metabolites are 12-hydroxy-5,8,11,14-eicosatetraenoic acid (12-HETE) which promotes corneal edema and 12-hydroxy-5,8,14-eicosatrienoic acid (12-HETrE) which incites an inflammatory cascade resulting in neutrophil chemotaxis and CNV.[89]

IMPAIRED CORNEAL INNERVATION

Ferrari and colleagues elucidated the relationship between corneal innervation and angiogenesis in mice models of bFGF-induced corneal vascularization. They concluded that when vessel growth is stimulated, nerves disappear and, conversely, denervation induces angiogenesis. They suggested that this phenomenon is mediated, at least in part, by the reduction of angiostatic molecules (including epithelial-derived PEDF and epithelial VEGFR3) constitutively expressed under normal physiologic conditions by the cornea.[90]

MOLECULAR PATHWAYS

VEGF

The VEGF family comprises five members that regulate vasculogenesis, angiogenesis, and lymphangiogenesis.[91] VEGF-A (also known as VEGF) is a crucial factor for vessel formation and maturation in embryotic and adult tissues. It is overexpressed in various vasculogenic corneal pathologies including LSCD[92], inflammation[93], chemical injuries[94], hypoxia[95], edema[96] and infections.[97] The main sources of VEGF in the human cornea are epithelial, stromal, and endothelial cells as well as macrophages/inflammatory cells, vascular ECs, and pericytes.[77]

VEGF exerts its angiogenic effects via two major receptor tyrosine kinase (RTK); the VEGFR-1 (flt-1), and VEGFR-2 (kinase insert domain-containing receptor; flk-1/KDR) which are mainly expressed by the vascular ECs. Although VEGFR-1 has higher affinity for the VEGF, its kinase activity is much weaker than VEGFR-2.[98, 99] On the other hands, VEGFR-1 is negatively regulated in the cornea by the sflt-1[100] and a low affinity VEGF isoform called VEGF165b[101] which competes with VEGF-A. Thus, angiogenic activity of the VEGF is mostly related to the VEGFR-2 pathway, while VEGFR-1 has a regulatory rather than angiogenic effect.

Once VEGF binds to its receptor, VEGFR-2, it induces dimerization and autophosphorylation of the receptor at the 1175-phosphotyrosine site which activates phospholipase C, gamma 1 (PLCg1) and finally the PKC-Ca^{++}-c-Raf-MEK-MAPK pathway.[102] It leads to activation of the angiogenic cascades including proteolytic activities, proliferation, migration and tube formation of ECs, and maturation of new blood vessels.

VEGFR-1 has no kinase activity in vascular ECs and does not stimulate EC proliferation directly.[103] In turn, its activation in macrophages activates the receptor for activated C kinase 1 (RACK1) dependent PI3-AKT pathway[104], which stimulates macrophage migration and promotes tissue remodeling which is essential for tube formation and maturation of the vessels.

There are also non-tyrosine kinase-type receptors such as neuropilins (NRP)[105] heparin sulfate proteoglycans (HSPGs)[106] that regulate VEGF activity via receptors.

PDGF

The Platelet-derived growth factor (PDGF) family comprises 5 dimeric ligands including PDGF-AA, -AB, -BB, -CC and –DD. Although platelet granules are the primary source of

PDGF, it can also be released by other cells such as monocytes, vascular endothelium, and vascular smooth muscle cells.[107] It can also be expressed by corneal fibroblasts, epithelial, and endothelial cells.[108] PDGF receptor (PDGFR) is a tyrosine kinase receptor, composed of alpha and beta chains, that is dimerized upon stimulation by the related ligand. Once PDGF attaches to its receptor, the kinase activity leads to receptor autophosphorylation, which in turn activates multiple signaling molecules including steroid receptor coactivator (SRC), Phosphoinositide 3-kinase (PI3K), and phospholipase Cγ 1 (PLCγ).[109] PDGF-BB and PDGFR-β, which are the predominant subtypes in corneal cells, have the greatest role in the angiogenic process.[110]

The most important physiologic role of PDGF in angiogenesis is recruitment, proliferation, and viability of pericytes.[111, 112] Furthermore, it stimulates expression of VEGF by pericytes which can enhance endothelial cell survival.[113] Inhibition of the PDGF signaling pathway by PDGFR-β or PI3K inhibitors results in loss of pericytes and decrease of corneal vessels which is correlated with reduced expression of PDGF, VEGF, and other angiogenic molecules.[114] In addition, PDGF inhibitors might increase susceptibility of the corneal vessels to anti-VEGF therapy through pericyte detachment and decreased endothelial pericyte coverage.[115]

BASIC FGF

Fibroblast growth factor (FGF) is another angiogenic growth factor with a complex effect on vascular development. Following corneal injury, FGFs, especially FGF-2 (bFGF), are expressed by the corneal epithelial and stromal cells, as well as inflammatory cells,[116] and they promote angiogenesis via their interaction with FGF receptors (mainly FGFR-1) of the vascular ECs. The subsequent dimerization of the FGFRs activates multiple signaling pathways including mitogen-activated protein kinase (MAPK), which in turn induces migration, proliferation, and tubule formation of the ECs; there is also increased protease activity and expression of the cell adhesion molecules (CAMs).[117] Additionally, bFGF promotes angiogenesis by induction of VEGF expression.[118]

NITRIC OXIDE

Nitric oxide (NO) is a free radical produced from L-arginine by three enzymes including neuronal NO synthase (nNOS; NOS-I), inducible NO synthase (iNOS; NOS-II), and endothelial NO synthase (eNOS, NOS-III).[119] NO, also referred to as endothelium-derived relaxing factor (EDRF), has been implicated in new vessel formation because of its vasodilatory effects, a phenomenon which usually precedes angiogenesis. However, the role of NO in angiogenesis is controversial probably due to the different isoforms of NOSs involved in the applied models.[120, 121]

Hypoxia and inflammation, which are important factors in promoting CNV, are potent stimulators of iNOS activity.[122, 123] In hypoxia induced angiogenesis, NO synthesized by iNOS is downstream to the HIF-1 and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) but upstream to the VEGF and bFGF pathways.[122] Studies have shown that iNOS is a crucial factor in angiogenesis related to tumors since its inhibition effectively blocks tumor angiogenesis.[124] Similar results were reported in a cauterization-induced CNV

model in mice.[125] On the other hand, it has been shown that iNOS is overexpressed in alkali-burn corneas and plays an inhibitory role in CNV.[126] The other isoenzyme eNOS is the predominant type of NOS in endothelial cells. It has been shown to be an important modulator of angiogenesis in ischemic conditions by regulating proliferation and migration of endothelial cells.[127] Upregulation of eNOS activity in ischemic conditions can be mediated by VEGF, bFGF, and substance P.[86, 128, 129] The effects of NO on the vascular endothelial and smooth muscle cells is mainly driven by the cGMP pathway which results in vascular dilation, permeability, and finally angiogenesis.[130]

RHO/ROCK PATHWAY

The main effect of Rho-associated protein kinase (ROCK) pathway is regulation of cell polarity and cell migration via stimulation of cellular protrusions and contractions as well as focal adhesions. This translates to a great impact on the angiogenic processes by inducing migration of endothelial cells and tube formation. Tumor angiogenesis has been enhanced by activation of ROCK.[131] One of the suggested underlying mechanisms of ROCK-induced angiogenesis is regulating actin-binding proteins including Moesin, Radixin, and Ezrin.[132, 133] Sonic Hedgehog (Shh) protein and VEGF are up-stream inducers of the ROCK pathway.[134–136]

The expression of Shh is up-regulated in tissues under ischemia, leading to enhanced vascularization.[134, 135] It has been shown that Shh directly induces angiogenesis by up-regulating the expression of pro-angiogenic genes e.g. MMP-9 and osteopontin (OPN) via Rho/ROCK signaling pathway.[137] Moreover, the Shh induces the expression of VEGF and Angiopoietin 1 (Ang1).[134, 135]

The induction of Rho/ROCK pathway with VEGF results in phosphorylation of focal adhesion kinases followed by migration of endothelial cells.[138] Treatment of endothelial cells with ROCK inhibitor (Y27632) has led to inhibiting migration and tube formation induced by VEGF.[136] *In vivo* studies have also indicated the role of ROCK in angiogenesis. Y27632 reduces hypoxia-induced angiogenesis in the lung.[139] Additionally, the VEGF-induced angiogenesis has been suppressed by fasudil (another ROCK inhibitor) both *in vitro* and *in vivo*.[140, 141]

WNT PATHWAY

The canonical Wnt signaling pathway involves various physiological activities such as cell proliferation, differentiation, migration, and apoptosis via regulating the expression of several genes.[142] The role of Wnt signaling in angiogenesis has been well established.[143] The signaling pathway of canonical Wnt starts by binding of Wnt ligands to a cell surface receptor complex. It results in cytoplasmic β-catenin stabilization via reducing its phosphorylation. Non-phosphorylated-β-catenin will then translocate into the nucleus and with association with T cell factor lead to activation of Wnt target gene transcription such as VEGF and other angiogenic factors.[144, 145]

The Wnt/β-catenin signaling pathway also plays a pivotal role in the processes involved in corneal wound healing including stem cell proliferation and angiogenesis.[146] In an animal study, alkali corneal burns resulted in increased Wnt signaling activity, which was associated

with elevated VEGF level in burned corneal buttons. In this study, inhibition of the Wnt signaling pathway was associated with a significant decrease in Wnt signaling activity, VEGF levels, and CNV.[147]

NOD1 PATHWAY

Nucleotide-binding oligomerization domain 1 (Nod1) receptor is a member of Nodlike receptors (NLRs) that can be expressed by human corneal epithelial cells and may play an important role in inflammatory responses as well as angiogenesis.[148] Upon stimulation, Nod1 activates nuclear factor-kappa B (NF-κB) via a kinase called RICK.[149] Activation of NF-κB leads to secretion of inflammatory cytokines/chemokines, which can mediate angiogenesis (as discussed above). Stimulation of Nod1 signaling in alkali-induced CNV models resulted in increased vascularization, while Nod1 blocking reversed the condition.[150] Hence, the Nod1 pathway might have an important role in CNV and could be considered as a therapeutic target in the future.

EVALUATION

CLINICAL EVALUATION

Inatomi and colleagues introduced a clinical classification for corneal vascularization which allows one to determine the severity of CNV. Grade 1 is the least severe category and indicates peripheral vascularization. Grade 2 indicates peripheral and mid-peripheral vascularization; grade 3 shows modest vascularization involving the entire cornea; finally, grade 4 is the most severe category and indicates massive vascularization of the entire cornea.[151]

Faraj and colleagues assessed parameters including location, depth, length, branching pattern, color, lipid leakage, nature of blood flow, and presence of hemorrhage to classify corneal vascularization. According to their study, CNV can be classified as active young, active old, mature, partially regressed, or regressed vessels. Their scoring system also considered the number of involved quadrants, depth, and location of the vessels in the setting of CNV secondary to infectious keratitis. With this grading system, herpes simplex keratitis (HSK) and acanthamoeba keratitis had the most and least severe CNV, respectively.[152] Furthermore, some CNV characteristics may be suggestive of specific etiology. For example, lipid keratopathy is more likely to be observed in CNV related to viral keratitis, contact lens use (Figure 1D, 2D), and deep anterior lamellar keratoplasty (DALK) (Figure 1B).[152]

Different parameters have been used for quantifying CNV, including the number of major thick-walled vessels originating from the limbus and reaching the cornea, vessel caliber, and involved CNV area.[153]

ANTERIOR SEGMENT IMAGING

Ziche and colleagues created a scoring system for CNV quantification which functions by multiplying the vessel density (number of corneal vessels) by the distance from the limbus (in millimeters).[154] Furthermore, image processing software such as ImageJ (available online at https://imagej.nih.gov/ij/) can be used to determine the extent of CNV most

accurately.[155] With this software, boundaries of vessels may be determined manually with cursors or by a semi-automated approach. The semi-automated method eliminates interobserver and intraobserver variability.

Tatham and colleagues developed a semi-automated software and compared its reproducibility with 3 different clinicians. Results indicated the semi-automated software to be roughly 95% reproducible during assessment of CNV while clinicians were only approximately 10% reproducible. As a result, Tatham and colleagues concluded that computer-aided analysis of corneal photographs provided a more reproducible method for quantifying CNV compared to clinicians.[156] In other studies, morphometric image analysis of standardized slit-lamp pictures has been used based on gray filter sampling for semi-automatic, semi-quantitative measurement of the extent and progression of CNV in several phase II and III clinical trials.[157, 158] Newer imaging technologies have allowed for anatomical and functional evaluation of non-visible and small corneal vessels that cannot be detected with high quality images. Anijeet and colleagues used anterior segment angiography with fluorescein and indocyanine green (ICGA), which showed three to four-fold more visibility of corneal vessels compared to color images. Results also indicated that fluorescein is more effective than ICGA to show leakage from vessel apices.[159] Kirwan et al also assessed fluorescein and ICGA findings in patients with keratitis. To do so, four parameters were used: vessel area, diameter, tortuosity, and dye leakage. Fluorescein was useful for detecting vessel leakage, while ICGA was excellent in vessel delineation even in the presence of a stromal scar. Both methods were significantly more sensitive in detecting corneal vessels compared with color images.[160]

More recently, optical coherence tomography angiography (OCTA) has been shown to provide a feasible, less invasive, more rapid method for evaluation of ocular vasculature including corneal vessels. Using a combination of OCT imaging and angiography has the advantage of determining the depth of vessels and acquisition of en-face views.[161, 162]

In vivo confocal microscopy (IVCM) has been shown to be useful in assessment of depth and activity of both blood and lymphatic vessels in the cornea. Based on these images, intravascular red blood cell traffic was present in active but not in inactive (ghost) vessels. Moreover, active vessels demonstrated barely visible walls and contained nucleated cells.[163]

Corneal lymphatic vessels are more difficult to visualize by non-invasive imaging. However, there are reports of detection of corneal lymphatics using IVCM[164] and more recently microscopic OCT (mOCT).[165] In both techniques, lymphatics were characterized as vessel-like structures with sparse, slowly moving immune cells.

HISTOLOGIC EVALUATION

Histologic features of CNV vary according to the pathologic condition and the chronologic stage of vessel formation. Disruption of the lamellar corneal architecture is a consistent finding in almost all cases of CNV. Cellular infiltration is another common feature of vascularized corneas, which may occur due to the underlying inflammatory disease or increased vascular permeability. A rabbit CNV model revealed that in early stages the vessel wall is composed of a thin layer of cells without muscle and nerve supply, indicating that the

vessels are capillaries rather than arterioles or venules. However, in later stages, venules, veins, and finally arterioles are seen.[166] Grading or scoring of CNV in a histologic section can be performed by counting the number of vessels per square millimeter[167] or by measuring the total vascularized area and the percentage of the cornea covered by vessels.[115]

Corneal vessels can be identified by hematoxylin-eosin staining, and cell surface markers can be used to determine corneal vessels in corneal buttons. The most commonly used markers for determining blood and lymphatic vessels in corneal buttons include CD31, LYVE-1 and podoplanin. It has been shown that CD31+/LYVE1+++/Podoplanin+ vessels are lymphatics, whereas CD31+++/LYVE-1-/Podplanin- vessels are blood vessels.[41, 168] Cursiefen and colleagues found histologic evidence of CNV in roughly 20% of corneal buttons obtained from patients who underwent keratoplasty.[169]

MANAGEMENT STRATEGIES

Management is focused primarily on the underlying etiology and pathophysiology causing the CNV; for example, if inflammation is the primary mechanism, the best treatment is to control the inflammation. It is important to discontinue contact lens use if contact lens–related hypoxia is suspected. Generally, management consists of medical and surgical strategies.

MEDICAL OPTIONS

Table 1 summarizes some of the medications for potential use as anti-angiogenic therapy although most of them have only been used in experimental studies.

ANTI-INFLAMMATORY AND IMMUNOSUPPRESSIVE AGENTS—Inflammation is a key factor in the process of corneal angiogenesis in most pathologic conditions.[170] Inflammatory cells that infiltrate the injured cornea are the major source of potent angiogenic mediators such as IL-1, TNF-α, and VEGF.[834] Also, products of inflammatory reactions, such as prostaglandins, have angiogenic properties.[171]

Corticosteroids are potent inhibitors of inflammation and have been used for their anti-inflammatory and anti-angiogenic properties in CNV.[172] Despite their side effects, corticosteroids are one of the most potent medications clinically available to control inflammation and consequently CNV. Thus, they can be considered first-line treatment for CNV, especially in the setting of inflammation. Corticosteroids vary in their potency and routes of administration; commonly available corticosteroids include difluprednate, fluorometholone, rimexolone, loteprednol, prednisolone, dexamethasone, triamcinolone, and fluocinolone. While these conventional corticosteroids often have undesirable ocular side effects, newer agents such as anecortave acetate have been reported to be equally effective yet safer.[173] We recommend aggressively treating inflammatory conditions associated with CNV such as HSV immune stromal keratitis and keratoplasty rejection with frequent potent corticosteroids (i.e. difluprednate every 1 hour). The topical corticosteroids are then tapered very slowly based on clinical response (over the course of months in the case of HSV immune stromal keratitis). It is essential to closely monitor the patients on long-term corticosteroids for early diagnosis and management of potential adverse effects.[174]

NSAIDs are also anti-inflammatory agents with known anti-angiogenesis properties. Both cyclooxygenase 1 (COX-1) and COX-2 are found in the cornea and have a role in angiogenesis. Indomethacin and ketoprofen are non-selective COX-1 and COX-2 inhibitors that have been found to significantly suppress bFGF and VEGF-induced angiogenesis in the mouse cornea.[175] Similar to corticosteroids, long-term use of topical NSIADs is limited by potential corneal side effects which necessitates close monitoring.[176] We do not use NSAIDs for CNV and to the best of our knowledge, there is no clinical study evaluating the efficacy of NSAID to prevent or management of CNV.

Cyclosporine is an immunomodulatory agent which is used for ocular surface immune disorders. Systemic cyclosporine A (CsA) can inhibit migration of primary endothelial cells and angiogenesis induced by VEGF.[177] In a rabbit model, the efficacy of topical CsA 0.05% was better than bevacizumab but less than dexamethasone for the treatment of immune-mediated CNV.[178] However, in a second study, a high dose subconjunctival CsA implant did not have a significant effect on CNV in human corneal transplants.[179]

Tacrolimus is another potent immunosuppressant which effectively blocks production of cytokines by T-cells and immunoglobulins by B-cells. Systemic tacrolimus has also been recommended.[180, 181] Turgut and colleagues reported that both systemic and topical administration of tacrolimus are effective in prevention of CNV in an experimental model.[182] Besides inhibiting VEGF production, tacrolimus also decreases the production of a number of angiogenic factors, such as FGF, epidermal growth factor (EGF), histamine, PDGF, prostaglandin E2 (PGE2), TNF-α, MMP-9 and MMP-13, IL-1, IL-6, and HIF.[182]

Sirolimus (rapamycin) and its derivative everolimus inhibit T-cell activity by preventing cell cycle progression from G1 to S phase, thereby blocking proliferation. In addition, rapamycin can promote T-cell anergy independently of the inhibition of proliferation even in the presence of T-cell receptor activation and co-stimulation by CD28 and IL-2.[183, 184] Systemic and topical administration of sirolimus has been associated with a significant decrease of corneal opacity and vascularization as well as decreased corneal IL-6 and TGF-β 1 levels after alkali ocular injury in mice.[185] Moreover, topical everolimus significantly decreased CNV induced with silver nitrate in rats, by decreasing the expression of VEGFR-2 and ERK 1/2.[186]

Tocilizumab, an IL-6 receptor antagonist, was effective in reducing CNV in animal models by decreasing corneal inflammation and VEGF expression.[187] Subconjunctival tocilizumab (2.5 mg) was proven as effective as the same dose of subconjunctival bevacizumab in reducing CNV area and VEGF levels.[188]

TNF-α inhibitors have been used in experimental studies for the treatment of CNV because of their simultaneous anti-inflammatory and antiangiogenic activities.[189] Infliximab is an anti-TNF-α monoclonal antibody which can bind both the monomeric and active trimeric form of TNF-α, thus blocking its activity. Topical infliximab was effective in reducing CNV and was correlated with TNF-α and VEGF activity.[190] It can also prevent corneal lymphangiogenesis and conjunctivalization after alkali-induced injury.[34, 191] In another study, a single intraperitoneal injection of infliximab 15 minutes after a corneal alkali burn

was associated with markedly reduced CNV.[192] Finally, etanercept is a recombinant TNF receptor which neutralizes both TNF-α and TNF-β. Subconjunctival etanercept had both anti-inflammatory and antiangiogenic effects in an animal model, which were enhanced when administered in combination with subconjunctival bevacizumab.[193]

ANTI-VEGF AGENTS—Several anti-VEGF agents such as bevacizumab have been primarily approved for use in multiple cancers for their antiangiogenic properties and are being used off-label for the treatment of ocular angiogenesis, including CNV. However, ranibizumab was primarily approved the FDA in 2006 for wet age-related macular degeneration. These agents are most effective for the treatment of actively growing vessels and have limited efficacy in well-established CNV.[194, 195]

Topical bevacizumab has been successfully used for the reduction of CNV in cases unresponsive to conventional anti-inflammatory medications in animal studies and clinical trials.[196] Topical bevacizumab (commonly 5 mg/ml-five times/ day) has demonstrated a beneficial effect by decreasing the affected area of vascularization.[197] Although topical bevacizumab has poor penetration into the cornea when the epithelium is intact, it penetrates a vascularized cornea well.[198] This same study showed that subconjunctival bevacizumab penetrates the stroma even with an intact epithelium[198] and has been used to treat CNV in a human case series as well as its associated lipid keratopathy.[199] However, following chemical injury in rats, topical bevacizumab demonstrated longer standing anti-angiogenic effects than subconjunctival bevacizumab.[200] A separate study has shown that lower doses of bevacizumab are required for subconjunctival injections compared to topical administration for equal efficacy.[201] In addition, results of a meta-analysis on seven clinical and 18 experimental studies revealed a significant reduction of CNV after treatment with either topical or subconjunctival bevacizumab.[202] However, CNV may recur following successful management with subconjunctival bevacizumab necessitating repeated injections, especially in cases with lipid deposition.[203]

Ranibizumab, another monoclonal antibody against VEGF-A, has also been used to reduce CNV. In an animal model, early subconjunctival administration of ranibizumab inhibited alkali-induced corneal vascularization. It not only significantly reduced VEGF levels in the cornea and bulbar conjunctiva, but also in the aqueous humor and the iris.[204] In another experimental model, both topical and subconjunctival administration of ranibizumab were associated with significant improvement of CNV.[205] In one clinical study, topical ranibizumab effectively reduced vessel caliber but not invasion area.[206] There is also a report of successful management of CNV due to HSV keratitis with subconjunctival and intrastromal injection of ranibizumab in a case refractory to bevacizumab.[207] However, in a different clinical study, subconjunctival ranibizumab proved to be less effective than bevacizumab in reducing CNV.[208–210]

FD006 is a novel anti-VEGF-A monoclonal antibody with strong antiangiogenic activity. It is a full-length IgG antibody (similar to bevacizumab), obtained by using antibody phage display technology.[211] Subconjunctival injection of FD006 significantly decreased the expression of VEGF, VEGFR-1, VEGFR-2, ICAM-1, and MMP-9 in alkali burned rat corneas. FD006 was found to be slightly superior to subconjunctival bevacizumab in

reducing CNV in animal models.[211] This might be due to higher affinity and a slower dissociation rate of FD006 compared to bevacizumab.

Pegaptanib has been approved by the food and drug administration (FDA) for treatment of choroidal neovascularization associated with AMD, but there is little evidence for its use in CNV. Akar and colleagues compared the efficacy of subconjunctival injection of bevacizumab, ranibizumab, and pegaptanib in the treatment of CNV in an animal model and found significant improvement of CNV in all treated groups. The outcome was best in the bevacizumab group followed by pegaptanib and ranibizumab.[210]

Aflibercept (VEGF $Trap_{R1R2}$) has strong anti-VEGF activity and is a soluble fusion protein with binding domains for both VEGFR-1 and 2. It was approved by the FDA in 2011 for intravitreal injection in treatment of diabetic macular edema. Systemic administration of VEGF $Trap_{R1R2}$ inhibited bFGF induced CNV.[212] More recently, topical use of the 0.1% and 0.01% concentrations of aflibercept was associated with a significant decrease in CNV and VEGF expression in suture induced CNV in rabbits.[213] In addition, in another experimental study, subconjunctival injection of Aflibercept at the time of corneal grafting was associated with significant reduction of donor vascularization and improvement of graft survival in a high-risk graft murine model.[214]

So far, the only topical antiangiogenic agent tested in phase II and III trials is aganirsen. It is an antisense oligonucleotide that inhibits CNV via preventing insulin receptor substrate-1 (IRS-1) expression as well as downregulating expression of VEGF and IL-1 β.[215] Administration of topical aganirsen eye drops (86 μg/day/eye) in patients with keratitis-related progressive CNV led to significant amelioration of CNV and reduced need for corneal transplantation.[158]

There are some concerns about the safety of VEGF neutralization at the ocular surface. Delayed epithelial wound healing and increased expression of MMPs have been reported with topical bevacizumab in a rat model of epithelial injury.[216] Furthermore, topical administration of bevacizumab was associated with increased risk of persistent epithelial defects (PEDs) that was dependent on the dose and duration of treatment.[217, 218] Most clinical studies have reported no serious side effects with subconjunctival bevacizumab. However, a few complications have been reported such as subconjunctival hemorrhage and PEDs.[219]

Overall, anti-VEGF agents have provided a new therapeutic approach for the clinical management of CNV. As expected based on the biology of angiogenesis, these agents are most effect in the setting of active CNV with “immature” vessels. As clinical experience has shown, their efficacy is limited in longstanding CNV where the vessels have matured and are less dependent on VEGF signaling.[209]

MMP INHIBITORS—Tetracyclines, such as doxycycline, are potent inhibitors of collagenase activity and MMP-induced extracellular matrix degradation. Doxycycline is also known for its anti-neovascular effects in the cornea.[220] Topical preparations of doxycycline including a 2% neutralized solution, temperature-sensitive hydrogel, and, more recently, eye

drops have been used successfully for inhibiting CNV in animal and human studies.[221, 222] Doxycycline inhibits angiogenesis by modulation of the PI3K/Akt-eNOS pathway in an MMP-independent mechanism.[223] Furthermore, it was shown to enhance the anti-VEGF effects of bevacizumab by decreasing the expression of VEGF and its receptors.[224]

Minocycline is a semisynthetic tetracycline with anti-collagenase and anti-angiogenic properties. In an animal model of alkali ocular injury, systemic minocycline was effective in promoting epithelial healing and decreasing CNV. In this study, corneal VEGF, VEGFR-1, VEGFR-2, bFGF, IL-1β, IL-6, MMP-2, MMP-9, and MMP-13 levels were significantly lower in the treated group.[225]

Tigecycline, another member of the tetracycline family, has also been used as a topical and subconjunctival injection for inhibiting CNV in a rat chemical burn model.[167] Results were promising for both routes, although subconjunctival injection was more effective than topical administration.[167]

MULTIKINASE INHIBITORS—Sunitinib is a multi-targeted receptor tyrosine kinase (RTK) inhibitor (TKI) which selectively and potently inhibits four RTKs involved in angiogenesis including VEGFR-2, PDGFR-β, FGFR-1, and EGFR.[226] It has been approved by the FDA for treatment of metastatic tumors because of its remarkable antiangiogenic activity.[227] Oral administration of sunitinib in inflammation-induced CNV in mice was effective in reducing angiogenesis, most likely by blocking the VEGF-A/VEGFR-2 pathway.[228] Topical application of 0.5 mg/ml sunitinib was also associated with a significant decrease of VEGFR-2 levels and CNV in rats.[186] Topical sunitimib is reported to be 3-fold more effective than bevacizumab in the treatment of CNV due to its inhibitory effect on both VEGF and PDGF pathways.[229] Furthermore, greater results were obtained by topical administration as compared with subconjunctival injection.[230] However, the safe dose of the topical sunitinib remains to be determined due to the possibility of epithelial cytotoxicity that was seen at concentrations greater than 3.3 μg/ml in an *in vitro* study.[231]

Pazopanib is a small TKI active against VEGF and PDGF that is approved for use in renal cell and soft tissue carcinoma. The safety and efficacy of a topical preparation of pazopanib in treatment of CNV due to different underlying etiologies were studied in phase I and II clinical trials and showed promising results.[232]

Sorafenib is another multikinase inhibitor which blocks both VEGFR2 and PDGFR.[233] Its short-term oral administration was effective in reducing experimental CNV most likely via inhibition of ERK and VEGFR-2 phosphorylation.[234] However, it was more toxic to corneal epithelial cells *in vitro* compared to sunitinib.[231] Similarly, regorafenib inhibits multiple RTKs including VEGFR-1, −2 and −3; PDGFR-β; and FGFR.[235] Topical regorafenib (1 mg/ml) decreased epithelial and endothelial VEGF levels as well as the percentage of CNV area in an alkali-burn CNV model.[236]

Lapatinib blocks 2 RTKs, including human epidermal growth factor receptor 2 (HER2) and epidermal growth factor receptor (EGFR), and it is a part of the treatment for early stages of HER2-positive breast cancer.[237] Oral administration of lapatinib effectively reduced CNV

by decreasing corneal epithelial and stromal VEGF expression in an animal model. It was more effective than trastuzumab, which only blocks HER2.[238]

ROCK INHIBITORS—Fasudil hydrochloride is a potent inhibitor of the Rho/ROCK pathway and has demonstrated antiangiogenic properties. *In vitro* studies showed that fasudil attenuated the Rho/ROCK pathway and VEGF-dependent phosphorylation of extracellular signal regulated kinase 1/2 (ERK1/2) and Akt.[140] In alkali burn-induced CNV mice models, administration of topical fasudil (100 μM) was associated with decreased expressions of VEGF, TNF-α, MMP-8, and MMP-9 as well as decreased incidence of CNV in treated mice. It also caused decreased inflammatory cell infiltration (especially PMNs) and reactive oxygen species production.[239]

AMA0526 is a newer, more specific, locally acting ROCK inhibitor with minimal systemic side effects.[240] *In vitro* studies demonstrated significant inhibition of vascular endothelial proliferation and migration. In animal models, its topical application was comparable to bevacizumab and dexamethasone in regards to preventing corneal vascularization and scarring, respectively.[241]

SURGICAL OPTIONS

CORNEAL VESSEL OCCLUSION

Several methods have been described for corneal vessel occlusion including conjunctival resection, cryotherapy, laser thermal cauterization, fine needle diathermy (FND), electrolysis-needle cauterization, and photodynamic therapy (PDT). Although these methods are most effective in the treatment of established CNV, they can also be applied to active vascularization in adjunction with anti-VEGF agents.[242]

Laser thermal cauterization using Argon, yellow dye, and Nd:YAG lasers have been used to ablate CNV in experimental[243–245] and clinical[246–248] studies. Histopathologic studies revealed disruption and degeneration of endothelial cell and clot formation in occluded vessels as well as intracorneal hemorrhage followed by increased cellularity and distortion of corneal lamella by red blood cells in treated corneas.[243, 244] In a recent study, frequency-doubled Nd:YAG laser (532 nm) was used to treat 40 eyes with quiescent CNV.[248] After 3 months, complete occlusion was observed in 53% of vessels, while 37% of vessels were re-canalized.[248] Complications include corneal hemorrhage, corneal thinning, vascularization exacerbation and vessel lumen reopening.[248–250]

FND was successful in treating lipid keratopathy associated with CNV in more than 80% of cases in a study.[251] Additionally, more than 80% of grafts survived longer than one year in vascularized corneas that were pre-treated with FND. The authors concluded FND can be considered an effective, low-cost, easy, and safe method for treatment of established CNV.[251] The long-term efficacy and visual acuity improvement of FND in treatment of HSK-associated CNV persisted for a mean 18.9 months after initial treatment.[252] Since FND upregulates VEGFs, it has been suggested that FND should be combined with topical anti-VEGFs to reduce recurrence rates.[253]

PDT with verteporfin is another approach for the selective occlusion of abnormal, leaky CNV vessels. This procedure creates a highly specific tissue damage that seals off the vessel. A case series showed evidence of vascular thrombosis and decreased CNV in about two thirds of eyes that underwent PDT.[254] Resolution of bilateral CNV and associated lipid keratopathy after PDT has also been reported.[255] PDT has limited clinical use due to high costs and potential complications related to laser irradiation and generation of reactive oxygen species.

OCULAR SURFACE RECONSTRUCTION

Amniotic membrane transplantation (AMT) has been shown to be effective in preventing inflammation and angiogenesis following ocular injuries. Temporary or permanent transplantation of human amniotic membrane (HAM) in acute chemical injury promotes re-epithelialization and reduces both inflammation and vascularization.[256] Chondrocyte-derived extracellular matrix (CDECM) has been introduced as an alternative to amniotic membrane in ocular surface reconstruction because of its potent anti-fibrotic and antiangiogenic effects.[257] Transplantation of CDECM in the acute phase of alkali injury of the cornea resulted in significant reduction of CNV and opacity in a rabbit model.[257]

Autologous or allogenic ocular surface stem cell transplantation (OSST) is the current standard method of managing unilateral or bilateral total LSCD. However, less invasive procedures might be used for CNV and/or conjunctivalization associated with partial LSCD. Sequential sector conjunctival epitheliectomy (SSCE) with or without AMT has been used successfully for management of CNV in eyes with partial LSCD.[258,259]

To treat total LSCD, limbal stem cells can be harvested from autologous or nonautologous sources. A conjunctival limbal autograft (CLAU) taken from the healthy fellow eye is considered the most effective surgical procedure in patients with total unilateral LSCD. It produces excellent results, often with complete regression of corneal neovascularization.[260–262] Cultivated limbal epithelial transplantation (CLET) is a suitable alternative in cases of total unilateral LSCD or in cases of bilateral LSCD when the damage is more severe in one eye.[263–265] Living-related conjunctival limbal allograft (lr-CLAL), keratolimbal allograft (KLAL), or combined procedures (i.e. Cincinnati procedure) are surgical alternatives in patients with bilateral LSCD.[266–268] A method that provides fresh tissue from a patient's first-degree blood relative, lr-CLAL utilizes tissue from one eye (or occasionally both eyes) of the best available HLA-matched donor. Lr-CLAL also has the advantage of providing viable conjunctival tissue, which may be used in patients with severe conjunctival deficiency. In comparison, KLAL utilizes cadaveric tissue, is more accessible, and offers more stem cells because two corneoscleral rims are used.[269] Simple limbal epithelial transplantation (SLET), cultivated oral mucosal epithelial transplantation (COMET), or allogeneic CLET are other surgical options for total LSCD.[270–272]

Typically, superficial corneal vessels regress after successful OSST, indicating successful grafting. However, re-growth of these vessels may be a marker of graft failure.[273] Moreover, deeper vessels may not disappear with OSST only, so concomitant treatment modalities including keratoplasty may be required.[274]

STEM CELL-BASED THERAPY

In the last decade, there has been intense focus on stem cells for regenerative therapies. Many sources of stem cells including bone marrow, fat, umbilical cord, dental pulp, hair follicle, and induced pluripotent stem cells have shown promising results in experimental models of ocular surface injury.[275, 276] Among these, mesenchymal stem cells (MSCs) hold the most promise for clinical application. MSCs are found in most adult tissues, including the limbus, and play animportant role in tissue repair and maintenance. A number of clinical trials aimedat evaluating the safety and efficacy of MSCs in the promotion of cardiac tissue regeneration, modulation of systemic immune diseases, and healing of soft tissue injuries are currently underway.[277, 278] In animal models of chemical injury, MSCs have been shown to accelerate corneal wound healing, attenuate inflammation, and modulate corneal neovascularization.[279–284] These effects have been shown to be mediated in part through secreted factors such as TSG-6.[285] Furthermore, in a recent study we showed cornea-derived MSCs (cMSCs) are directly antiangiogenic mainly through sflt-1 and PEDF.[286] Further study revealed cMSCs can change the immunophenotype and angiogenic function of the macrophages mainly through cMSC-derived PEDF.[287] The so-called cMSC-educated macrophages expressed significantly higher levels of anti-inflammatory and anti-angiogenic factors compared with control macrophages, both in vitro and in vivo.[287] Overall, stem cell-based therapies are likely to replace tissue transplantation in the future for certain ocular surface diseases that lead to CNV.

FUTURE DIRECTION

Although several novel agents have been used successfully to treat CNV in animal models and clinical studies, the need for an FDA-approved potent topical antiangiogenic agent is still unmet.[288–290] The only topical antiagiogenic agent that has made it to the phase III trial is aganirsen, an example of small interfering RNA (siRNA).[158, 215] Agents/interventions which are under investigation for possible application in treatment of CNV have been listed in table 1 for experimental and table 2 for clinical studies.

Gene-based antiangiogenic therapy is an emerging strategy that encompasses a variety of target genes and gene-delivery methods.[291] Adeno-associated viral (AAV) vectors have been utilized to transfect corneal epithelial cells with antiangiogenic genes such as endostatin and angiostatin, which successfully reduced CNV in animal models.[292, 293] AAV vectors have been reported to be an effective and safe mean for gene delivery to the cornea.[294] Nanoparticles are effective, non-viral, non-toxic, and a sustainable form of gene delivery. Nanoparticle-based vectors containing an expression plasmid of sflt-1[295] or small hairpin RNA (shRNA) against VEGF-A have been reported to regress CNV in experimental studies.[296] Downregulating proangiogenic genes using gene-silencing techniques is an evolving alternative for inhibition of ocular angiogenesis. Besides the example of aganirsen (mentioned above), siRNA targeting VEGF has demonstrated inhibition of CNV in other animal models.[156, 211, 297, 298] CRISPR-Cas9-mediated genome editing is a novel approach for manipulating the genome of living cells.[299] This rapidly evolving technique has great potential with unlimited applicability.

Using combined strategies targeting different mechanisms might be more effective in treatment of CNV. Combination therapy with a vessel occlusion method (PDT), an anti-VEGF agent (subconjunctival bevacizumab), and a corticosteroid (triamcinolone acetonide) or immunosuppressive agent (cyclosporine A) have been shown to be effective in two cases with extensive superficial and deep CNV.[300–303] Combination of PDT and subconjunctival bevacizumab was associated with significant improvement of CNV refractory to conventional treatments in two case series.[304, 305] Likewise, combination of FND and bevasizumab significantly reduced CNV prior to keratoplasty.[253] In addition, combination of two or more anti-angiogenic agents with different mechanisms of action (e.g. anti-VEGFs, TKIs, immunomodulators, MMP inhibitors, etc.) might have additional effects compared to monotherapy.[186, 193, 224, 306, 307] Recently, corneal crosslinking using ultraviolet A light (UVA) and topical riboflavin has shown promising results to regress both preexisting blood and lymphatic vessels.[308] However, randomized clinical trials are needed to investigate the shortterm and long-term safety and efficacy of these methods.

SUMMARY

CNV is a relatively common adverse consequence of severe ocular surface and corneal pathologies that can result in significant visual disability if not addressed promptly. Moreover, since it is an important risk factor for corneal graft rejection, its treatment before any keratoplasty procedure is recommended. Recent advances in elucidating the molecular pathways, clinical staging, anterior segment imaging, and therapeutic strategies have led to improved treatment strategies.

Studies show that the formation and invasion of vessels is the result of a complex mechanism which includes inflammation, hypoxia, corneal edema, innervation abnormalities, and LSCD. Although VEGF is the core molecule responsible for endothelial proliferation, other important mediators such as PDGF, bFGF, nitric oxide, MMPs, and proinflammatory cytokines have a critical role in the maturation, establishment, and invasion of vessels.

Semi-automated and automated analysis software which have been optimized for interpreting corneal photographs are useful tools for grading and classifying CNV, as well as evaluating interventions. Application of IVCM, AS-OCT, fluorescein, and ICG have led to increased understanding of the origin, nature, and course of vessels on the cornea, as well as improved targeted treatment and assessment of outcomes.

Currently, anti-inflammatory and anti-VEGF medications are the mainstay therapy of active CNV, while FND is used for ablation of established vessels. The advent of newer, more potent anti-angiogenic agents such as multi-kinase inhibitors, ROCK inhibitors, MMP inhibitors, and immunosuppressive medications may lead to improved treatment outcomes. However, targeting multiple angiogenic pathways may have a greater synergistic effect and should be considered the goal of future drug designs. The concomitant use of angiography guided thermo-ablation and photodynamic therapy may also increase the success rate of medical therapies which target CNV. In case of severe total LSCD with deep stromal CNV,

the best strategy is an OSST followed by a keratoplasty. MSC-based cellular therapy and gene-based antiangiogenic therapy are novel promising alternatives.

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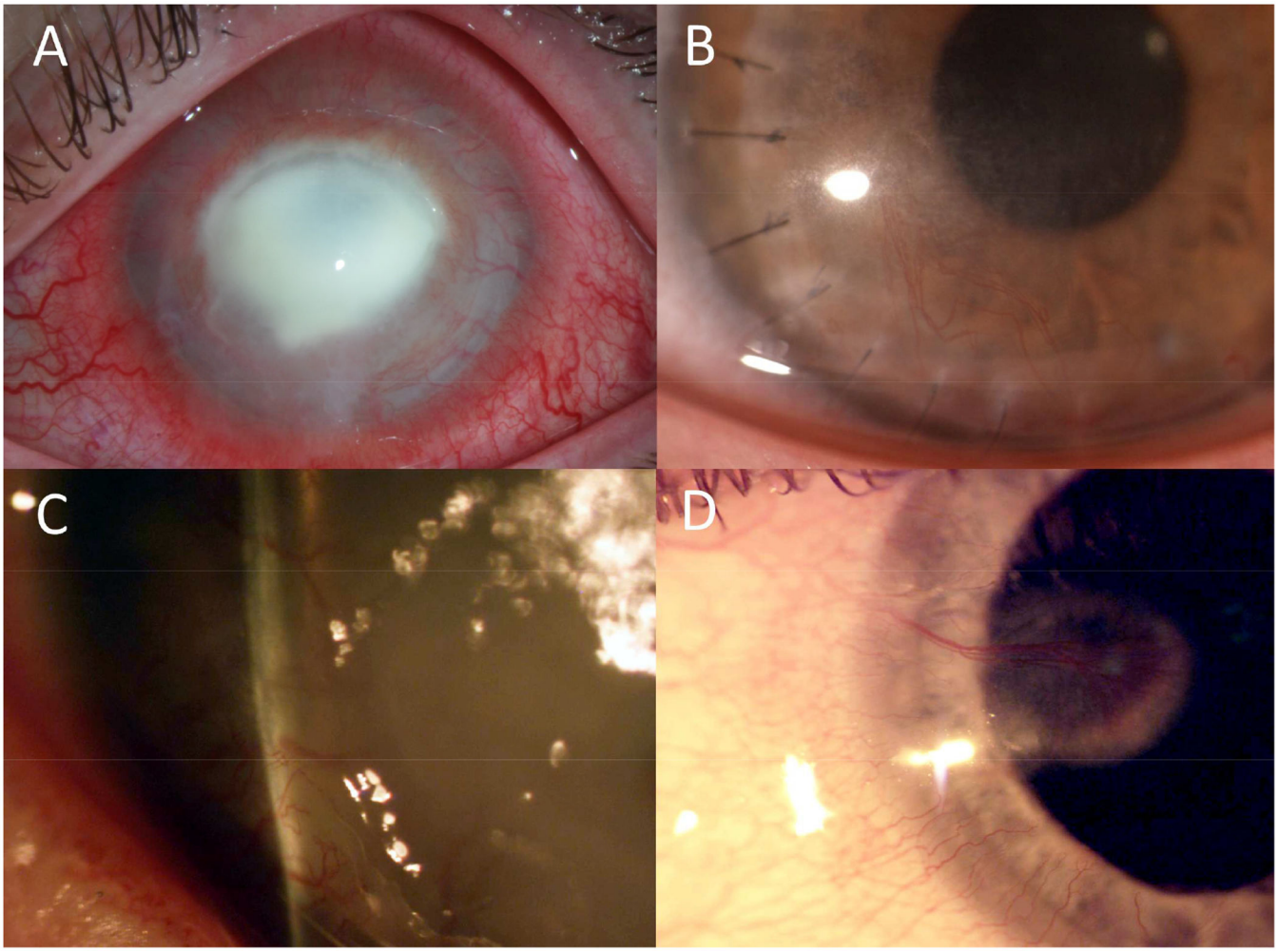

Figure 1.

Slit lamp photographs demonstrating CNV. Necrotic corneal ulcer and persistent epithelial defect with surrounding ring infiltrate and CNV secondary to acanthamoeba (1A, photograph courtesy of Marius Miron). Stromal CNV from acute stromal rejection of a deep anterior lamellar keratoplasty (1B). Regressing deep CNV with mild surrounding haze and lipid secondary to HSV immune stromal keratitis (1C). Deep frond of CNV with accompanying lipid deposition from long-term rigid gas permeable contact lens abuse (1D).

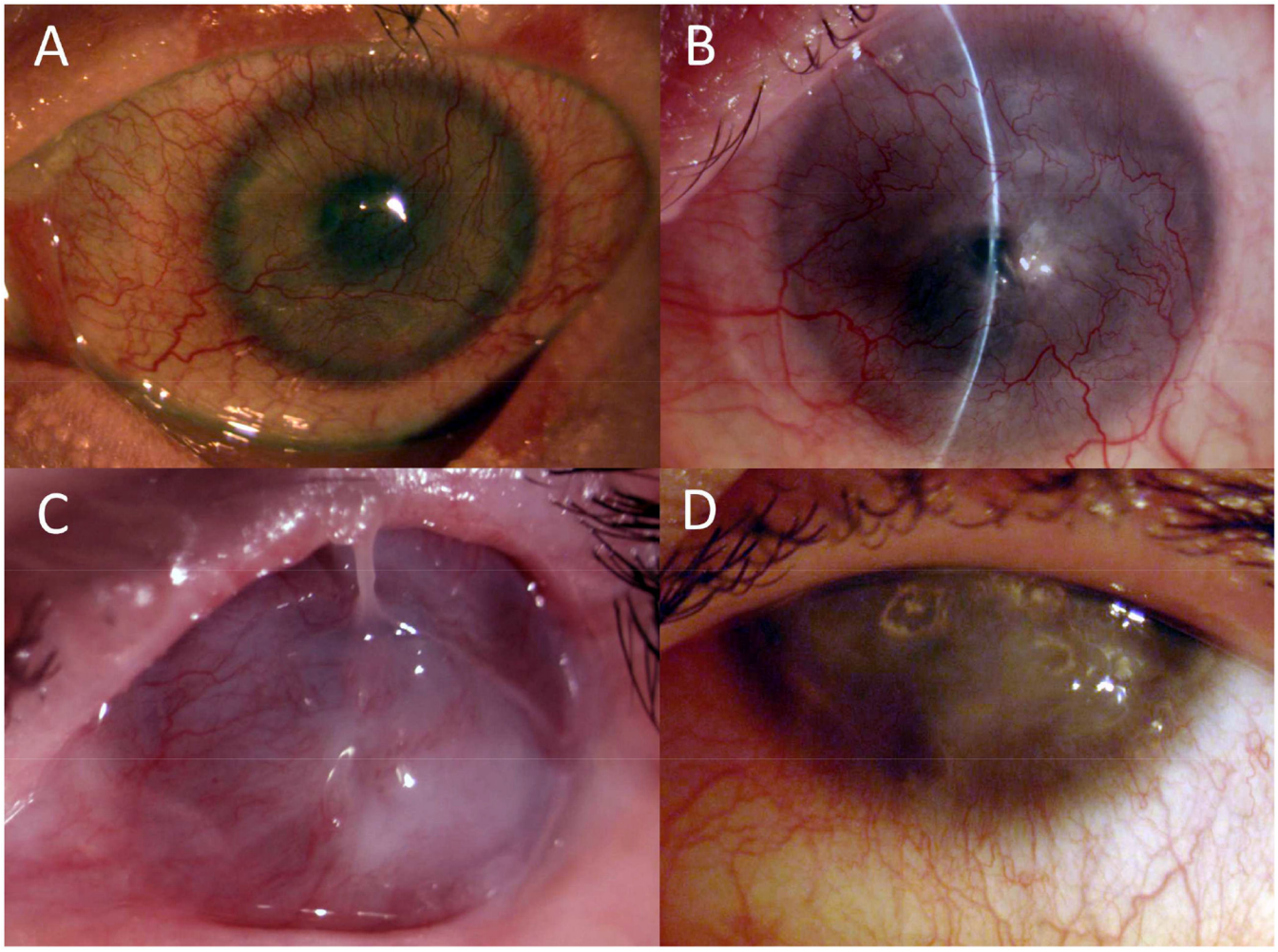

Figure 2.

Slit lamp photographs of CNV secondary to limbal stem cell deficiency from various etiologies are depicted: Stevens Johnson Syndrome (2A), alkaline chemical injury (2B), mucous membrane pemphigoid (2C), and long-term soft contact lens abuse (2D).

Table 1

Novel agents, with potential antiangiogenic effects, used for treatment of corneal neovascularization in animal models.

Agent[ref]	Model	Route	Proposed Mechanism(s)
Diospyros kaki Extract (EEDK)[309]	Alkali burn	Oral	Suppression of VEGF, FGF, IL-6, and MMP-2.
Recombinant PEDF[310]	Chemical injury	S.C.	Inhibits VEGF, bFGF and IL-8.
PEDF[311]	Chemical injury	Topical	Downregulates VEGF expression.
Gold nanoparticles[312]	Chemical injury	Topical	Decreases VEGFR-2 levels, inhibits ERK phosphorylation.
interferon-induced protein of 10 kDa (IP-10)[313]	Chemical injury	Topical	Decreases VEGF and bFGF expression, EC proliferation and tube formation.
Netrin 1[314]	Chemical injury	Topical	Laminin-related protein; Decrease inflammation, decrease VEGF, increase PEDF.
Netrin-4[315]	Chemical injury	Topical	Laminin-related protein Decreases leukocyte infiltration and VEGF and NF-κB signaling and EC migration, invasion and tube formation, increases PEDF.
LCB54–0009[316]	Suture induced and chemical injury	S.C.	Imidazole-based alkaloid Derivative; Antioxidant; decreases VEGF-A and HIF-1α level, inhibits VEGFR-2 and NF-κB signaling, decrease MMP-1, −2, −3 and −9 activity.
Epigallocatechin gallate[317]	Suture induced	Topical	Flavonoid; Decreases expression of VEGF and COX-2.
Largazole[318]	Chemical injury	Topical	Histone deacetylase inhibitor; Decreases expression of VEGF, b-FGF, TGFβ1 and EGF.
Serine Proteinase Inhibitor A3K (SERPINA3K)[319]	Suture induced	Topical	Inhibits Wnt signaling pathway and VEGF
TC14012[320]	Chemical injury	S.C.	CXCR4 antagonist and CXCR7 agonist; Reduce CXCR4, CXCR7, VEGF and MMP-2 and −9 mRNA levels *
Itraconazole[321]	Chemical injury	Topical, S.C., I.P.	Inhibits cholesterol biosynthesis, endothelial cell proliferation and capillary tube formation.
Lanepitant[322]	Chemical injury and Suture induced	Topical	NK1 receptor antagonist; Reduces corneal substance P level and leukocyte infiltration.
Low-molecular-weight heparin-taurocholate 7 (LHT7)[323]	Chemical injury	S.C.	Blocks VEGF-VEGFR.
Recombinant C-terminal fragment BIGH3 protein[324]	Micropocket assay	Local	Blocks phosphorylation of PI3K/Akt and ERK, inhibits tube formation, increase EC apoptosis.
Celastrol[325]	Suture induced	Topical	Reduce the expression of VEGF, MMP-9, and MCP-1.
ADP-ribosylation factor[326]	Chemical injury	Topical	Ras-related small GTPase; Downregulates corneal VEGF expression, increases EC apoptosis.
SB-328437[327]	Chemical injury	Topical	CCR3 antagonist; Reduces intracorneal MCP-1 and MCP-3 mRNA expression.
Ascorbic acid[328]	Suture induced	Topical	Anti-VEGF and anti-MMP.
Cetuximab[329]	Chemical injury	S.C.	Anti-EGFR mab; Inhibits EGFR, modulate VEGF and IL-8.
Peroxiredoxin-6[330]	Ultraviolet radiation	Topical	Anti-oxidant; Inhibits NF-κB, decrease VEGF expression, increase PEDF expression.
CL9189AP[331]	Chemical injury		Anti-SDF-α1 mab; Inhibits SDF-α1/CXCR4 pathway, down regulates VEGF and C-Kit expression.

Agent[ref]	Model	Route	Proposed Mechanism(s)
Methotrexate[332]	Suture induced	Topical, S.C.	Cytotoxic agent; Decreases VEGF and IL-6 expression.
N-acetyl-L-cysteine[333]	Chemical injury	I.P.	Anti-oxidant; Down-regulates NF-κB pathway.
Suramab[334]	Chemical injury	I.V.	Combined Bevacizumab and Suramin; Inhibits VEGF, bFGF, PDGF, IGF, TGF-β.
Canstatin[335]	Chemical injury	I.P.	NC1 domain of the α2 chain of type IV collagen; Inhibits VEGF, HIF-α and TNF-α, prevents EC migration and tube formation.
AMD3100[336]	Chemical injury	S.C.	Antagonist of CXCR4; Decreases inflammation, VEGFR-2 expression and EC proliferation.
Dihydroartemisinin[337]	Suture induced	Topical	Novel anti-malarial drug; Reduces expression of and phosphorylation of VEGF and VEGFR-2, ERK1/2 and p38.
Parstatin[338]	Chemical injury	S.C.	Proteinase-activated receptor 1; Inhibits FGF-2, VEGF, ERK1/2 and MAPK.
PTK/ZK, ZK991[339]	Suture induced	Oral	VEGFR-tyrosine kinase inhibitors; Block VEGF receptors.
IMD0354[340]	Suture induced	Systemic	Selective blocker of the IKK complex IκB kinase β (IKK2). Decreases inflammatory cell invasion, suppressed CCL2, CXCL5, Cxcr2, and TNF-a and VEGF-A expression.
IL-1R antagonist[341]	Suture induced	Topical	Inhibits IL-1 induced angiogenesis.
H-KI20[342]	Suture induced and Micropocket assay	Topical	A 20-amino acid peptide from HGF with anti-inflammatory and antiangiogenic preoperties.
Angiopoietin-like protein 2 (ANGPTL2)[343]	Chemical injury	Topical	Short heparin RNA inhibiting ANGPTL2 induced inflammation and angiogenesis.
Tissue inhibitors of matrix metalloproteinases[344]	Micropocket assay	Topical	Inhibits proliferation and migration of human ECs.
suberoylanilide hydroxamic acid (SAHA)[345]	Chemical injury	Topical	Downregulates the expression of VEGF, bFGF, TGFβ1 and EGF and inhibits migration, proliferation, and tube formation by ECs.
Suramin[346]	Suture induced	S.C.	Heparin analog; Decreases expression of VEGF, PDGF and bFGF.
FND[347]	Suture induced	FND	FND destroys not only blood but also lymphatic vessels, thereby promotes corneal high-risk graft survival.
PDT and verteporfin[348]	Suture induced	PDT after I.V. injection of verteporfin	Corneal PDT after i.v. verteporfin injection time-dependently regresses mature corneal BV and LV and promotes allograft survival after subsequent high-risk corneal transplantation.
UVA crosslinking and riboflavin[308]	Suture induced	Topical riboflavin and then UVA light exposure	Corneal crosslinking with UVA light and riboflavin regressed both preexisting blood and lymphatic vessels significantly via induction of apoptosis in vascular endothelial cells. In addition, macrophages and CD45+ cell counts were significantly reduced.

- ref = reference number, S.C. =subconjunctival, EC = endothelial cell, I.P. = intraperitoneal, I.V. = intravenous, BIGH3 = TGF-β1-inducible gene-h3, SDF = stromal-cell derived factor, LHT7 = Low-molecular-weight heparin (LMWH)–taurocholate derivative, HGF= hepatocyte growth factor, FND= fine-needle diathermy, PDT= photodynamic therapy, UVA= ultraviolet light A.

* Increases CNV in early stages and decreases in later stages.

Table 2

Interventions for treatment of CNV investigated or under investigation in clinical studies.

Intervention	Study design	Result	Ref./ID[†]
Bevacizumab (T)	Single group	Reduced corneal NV within the first month. Increased risk of epithelial defect in the second month.	Kim, 2008[349]
	Single group	Reduced CNV in nearly two thirds of the eyes treated. Ocular side effects including eyelid swelling/chalazion and superficial punctate keratitis.	Waisbourd, 2013[350]
	Single group	Significant reduction in mean neovascular area (47%) and vessel caliber (54%)	Dastjerdi, 2009[153]
	Single group	Significant reduction in mean neovascular area (47%) and vessel caliber (36%)	Cheng, 2012[196]
Bevacizumab (S.C.)	RCT	Regression of recent-onset CNV in all treated eyes.	Petsoglou, 2013[219]
	Single group	Regression of CNV in all eyes at 1 week continued to decrease for 1 month.	Benayoun, 2012[351]
	RCT	Ongoing	NCT00555594
Bevacizumab (I.S.)	Single group	Complete regression of CNV in 16/25 eyes and reduced opacity and improved visual acuity in 5/25 eyes.	Sarah, 2016[352]
Bevacizumab, (S.C. vs. T)	RCT	Reduced recurrence of CNV and increased graft survival in cases of high-risk corneal transplants. Less effective when used topically.	Bhatti, 2013[353]
Bevacizumab (S.C. and I.S.)	Single group	Significant regression of CNV in all eyes.	Yeung, 201 1[354]
Bevacizumab (S.C. followed by T)	RCT	Ongoing	NCT01072357
Ranibizumab (T)	Single group	Significant decrease in neovascular area lasted through 16 weeks.	Ferrari, 2013[206]
Bevacizumab vs. Ranibizumab (S.C. and I.S.)	Comparative interventiona case series	CNV regressed in both group. Bevacizumab had more effective and stable regression of CNV.	Kim, 2013[208]
Doxycycline (T)	Single group	Vessels disappeared or attenuated, shortened and less dense in five of six patients.	Jovanovic, 2014[222]
Topical heparin and steroid [*]	Single group	All eyes showed complete regression of CNV within 5 months.	Michels, 2012[355]
Topical Pazopanib	Single group	Neovascular area, invasion area and vessel caliber significantly decreased.	Amparo, 2013[232]
Aganirsen	RCT	Significantly reduced the relative corneal neovascular area. Decreased the need for corneal transplantation.	Cursiefen, 2014[158]
Topical IL-1R antagonist	RCT, crossover assignment	Ongoing	NCT00915590
FND	Single group	Complete regression of vessels in all patients. Reduced graft rejection in high-risk graft.	Pillai, 2000[356]
FND	Single group	Reduced lipid deposition, prevented rejection episodes, reduced intraoperative bleeding	Faraj, 2014[251]

Intervention	Study design	Result	Ref./ID†
Angiography guided FND	Single group	Decreased neovascular area. Corneal angiography enables selective treatment to the afferent vessels.	Spiteri, 2015[357]
FND and I.S. Bevacizumab	Single group	8/9 eyes had complete resolution of CNV and lipid deposition.	Elbaz, 2015[242]
Fluorescein-potentiated argon laser	Single group	Decreased corneal edema, CNV, and lipid keratopathy.	Gordon, 2002[358]
dihematoporphyrin ether (DHE) PDT	Single group	Immediate reduction in CNV in all patients. Three patients suffered significant systemic short-term phototoxicity reactions.	Sheppard, 2006[359]
PDT with verteporfin	Single group	Significant decrease of corneal vessel length and CNV in 90% of cases.	Verdiguel-Sotelo, 2010[360]
	Single group	Complete vascular occlusion in 42.4% and partial occlusion in 24.2% of eyes.	Al-Torbak, 2012[254]
	Single group	32% total occlusion, 60% partial occlusion and 8 % worsening. Significant decrease of mean neovascular area.	Diaz-Davalos, 2016[361]
PDT and S.C. Bevacizumab	Single group	Complete regression in 62.5% and partial regression in 37.5% of eyes.	Yoon, 2017[305]
	Single group	All eyes showed a significant decrease in CNV. After 1-year, complete and partial occlusion was achieved in 66.7% and 25.0% of eyes, respectively.	You, 2011[304]
PDT with topical dihematoporphyrin derivative	RCT	Ongoing	NCT00004430
Topical bevacizumab (Avastin™)	Single group	The mean reduction in vascularized area during treatment was 61%. The mean reduction in vessel diameter under topical Avastin™ therapy was 24%.	Koenig, 2009[197]
FND and bevacizumab	Single group	The vascularized area was reduced significantly ($P < 0.05$). Combined subconjunctival and eye drop antivascular endothelial growth factor treatment was significantly more effective in reducing the vascularized area compared with antivascular endothelial growth factor eye drop therapy alone ($P < 0.05$).	Koenig, 2012[253]

† CinicalTrials.gov identifier

* Remixolone or dexamethasone.

RCT= randomized clinical trial, S.C.= subconjunctival, T= topical, I.S.= intrastromal, FND= fine-needle diathermy, PDT= photodynamic therapy.