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Corneal transparency: genesis, maintenance and dysfunction

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Abstract

Optimal vision is contingent upon transparency of the cornea. Corneal neovascularization, trauma and, surgical procedures such as photorefractive keratectomy and graft rejection after penetrating keratoplasty can lead to corneal opacification. In this article we identify the underlying basis of corneal transparency and factors that compromise the integrity of the cornea. With evidence from work on animal models and clinical studies, we explore the molecular mechanisms of both corneal avascularity and its dysfunction. We also seek to review therapeutic regimens that can safely salvage and restore corneal transparency

Introduction

Sight has been a primary factor of advantage in natural selection and evolution thereby conferring great importance to the maintenance of corneal transparency [63]. The cornea's primary physiologic functions are transmission of incident light, refraction and provision of protection to intraocular structures from trauma and pathogens [20]. More than 60% of the total refractive power of the eye is attributed to the cornea making it vital to focusing light onto the retina for visual processing [115].

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For optimal vision, the cornea must efficiently transmit incident light by maintaining its transparency. The cornea is thus a highly complex sensory organ made largely of extracellular matrix components, developed and differentiated in a manner conferring optical transparency. In our review we assimilate pertinent structural features and molecular mechanisms by which the cornea is kept transparent and correlate them with pathological and surgical conditions under which corneal transparency is compromised. In light of past and current research endeavors, we have reviewed effective therapies and highlighted avenues for intriguing and promising research in the field of corneal angiogenesis.

Development of the cornea and relevant animal models in angiogenesis research

The development of the human eye is an orderly process of complex events most of which occur simultaneously. Some of these tissues induce the development of other ocular tissues and affect tissue formation through interactions among each other. If these critical processes are disturbed at any point from fertilization of the ovum until early postnatal period, severe congenital anomalies may result [58].

The eye is derived from three embryonic layers:

- Surface ectoderm and its derivative, the neural crest
- Neural ectoderm
- Mesoderm

Development of the eye can be divided into three main stages: embryogenesis, organogenesis and differentiation.

Embryogenesis is the first period during which the three primary germinal layers are established, the ectoderm, mesoderm and endoderm. The outer layer of cells forms the ectoderm; a longitudinal ectodermal thickening over the dorsal surface of the embryo known as the primitive streak forms over the embryo. The ectoderm anterior to the primitive streak, also known as neuroectoderm, forms a large portion of the eye and brain. It is the cells of the neuroectoderm that divide to form the neural tube. As folding of the neural tube occurs, a ridge of cells known as the neural crest cells form at the edges of the converging ends of the tube [17,94,140]. These cells migrate dorsolaterally to form portions of the eye and orbit.

The corneal epithelium and endothelium are first apparent at fifth gestational week, at the 12mm stage. At week 7 the anterior segment is formed by the invasion of the neural crest cells under the surface ectoderm, forming the corneal epithelium and occurs in three stages responsible for the formation of the corneal endothelium, corneal stroma and stroma of the iris respectively. The corneal endothelium forms as a two-cell cuboidal layer. These cells begin to secrete a basement membrane by week 8, which later becomes Descemet's membrane [134,193].

It is by the 8th gestational week that differentiation of tissues begins to form a fully functional eye. The stroma under the corneal epithelium thickens slowly and condenses to form Bowman's membrane at 4 months of gestation, at the 100mm stage [159]. The Bowman's layer is seen as an extension of filaments from the basal lamina of the epithelium.

The primitive cornea is formed once the surface ectoderm detaches from the lens vesicle and differentiates into a two-layered epithelium which rests on a basal lamina [167,171,181]. These cells develop junctional complexes by gestational week 6. At week 7, neural crest cells begin to move towards the lens vesicle in three stages as aforementioned. By the third

month of gestation, fibroblasts and collagen fibrils appear; fibroblasts begin to secrete glycosaminoglycans which form the ground substance of the cornea. Keratan sulfate production is also seen at this time. At month 4, tight junctions begin to form between the apices of the corneal endothelial cells. As development proceeds, the cornea enlarges with production of aqueous humor and concomitant dehydration of the stroma leading to the formation of a transparent ocular tissue- the avascular cornea [196].

Through exhaustive mouse studies of genes, transcription factors and growth factors, today we have a greater understanding of the molecular mediators of corneal development. Using the technique of identifying genes of interest by correlating ocular phenotype with chromosomal rearrangement anomalies, a number of genes including but not limited to *PAX6*, *PITX2*, *FOXC1*, *MAF*, *TMEM114*, *SOX2*, *OTX2* and *BMP4* have been implicated in ocular development [126]. In the developing embryo, a transcription factor of interest in the developing cornea is Kruppel-like transcription factor (*Klf4*) which is highly expressed in the cornea [141]. *Klf4* is responsible for maintaining corneal epithelial integrity and proper stromal hydration. In *Klf4* conditional null (*Klf4*CN) mice, corneas lose transparency and develop corneal edema and epithelial fragility. These findings are explained by a concomitant decrease in the levels of keratin-12 and aquaporin-5 in *Klf4*CN mice [179].

Genes that promote and maintain avascularity of the cornea and thus fundamental to transparency are of particular interest to investigators of corneal neovascularization. Two genes of particular interest are *PAX6* and *Destrin*.

Pax6 belongs to the *Pax* family of developmental genes which have a conserved paired-box motif. The paired-box motif was originally identified in *Drosophila melanogaster* and is known to play a key regulatory role in the development of the eye. Mutations in *Pax6* are inevitably associated with ocular malformations [78]. Heterozygotes develop aniridia whereas homozygotes have anophthalmia, loss of nares and abnormalities in the brain [164]. A clinical syndrome known as Peters anomaly which has anterior segment dysgenesis is associated with mutations in *Pax6* [78,138]. Peters anomaly can be identified by the presence of an avascular corneal opacity which is usually associated with but not limited to microcornea, microphthalmos, aniridia, cataractous lens, iris coloboma and myopia.

Aniridia and spontaneous corneal neovascularization in humans is associated with mutations in *Pax6* similar to mouse models of *Pax6* heterozygotes (*Pax6*^{+/-}) which have aniridia, small eyes, atrophic corneal epithelium, goblet cells in the cornea and in some cases spontaneous vascularization of the cornea can also be found [151,152]. These mice provide an excellent platform for the study of ocular surface disorders, aniridia-related keratopathy and corneal neovascularization.

Destrin, also known as actin-depolymerizing factor, is critical in the normal development of the cornea. Autosomal recessive mutations in *destrin* lead to corneal epithelial hyperplasia suggesting an interrelation between actin cytoskeleton remodeling and corneal epithelial cell growth [86]. *Corneal disease-1* (*corn1*) and *corn1* (*2J*), spontaneous mutation mouse models of *destrin*, have an irregular, thickened and vascularized cornea. The corneal thickening seen in *corn1/corn1* mice has been linked to aberrant levels of lumican expression in corneal epithelial cells [187]. Soon after birth *corn1* mice exhibit signs of developmental anomalies; corneal epithelial hyperplasia and corneal neovascularization are seen by post-natal day 20 [174]. These mice also spontaneously grow lymphatic endothelium-specific hyaluronan receptor (LYVE-1) LYVE-1⁺⁺⁺/CD31⁺ lymphatic vessels in the cornea [48]. Hence, the *corn1* mouse model is of remarkable value in the study of ocular surface diseases, corneal lymph- and hemangiogenesis.

Corneal architecture and its role in corneal transparency

The transmission and refraction of light through the cornea depends primarily on a highly specialized ultrastructure. The human cornea is approximately 540 μm thick [150] and is comprised mainly of the stroma, which lies between Bowman's layer anteriorly and Descemet's membrane posteriorly. The corneal epithelium forms the most superficial layer of the cornea while the endothelium lies deepest and is in contact with the aqueous humor of the anterior chamber. The epithelium and endothelium play important roles in maintaining corneal transparency by serving as a mechanical barrier to fluid diffusion and by creating a gradient that allows osmotic transport of water out of the stroma [59].

The stroma is comprised of keratocytes lying between lamellae of collagen bundles. Collagen type I and type V are the predominant forms in mammalian corneas [150], although collagen type IV is the main constituent of Descemet's membrane [19,96,137,180]. These collagen fibrils have a diameter of approximately 10-20 nm, while the wavelength of light is 500 nm [85]. Each collagen fibril lies at a fixed distance from the other (20 nm) and fibril density within the lamellae increases in the centre of the stroma relative to the periphery [26,131].

The extracellular matrix which lies between the collagen bundles and keratocytes is composed predominantly of water, proteoglycans, glycoproteins and inorganic salts. Proteoglycans consist of a core protein with a variable number of glycosaminoglycan side chains with sulphated groups. By interacting with collagen fibrils, proteoglycans determine both the architecture of the corneal matrix and its water-retaining properties [27]. As proof of this principle, *Lum*^{-/-} mice that lack the main keratan sulfate proteoglycan, lumican, develop corneal haze and abnormally thick collagen fibers in the posterior corneal stroma [33]. It is proposed that in the *Lum*^{-/-} mouse, corneal clouding occurs due to abnormal fibril assembly, lateral fusion of fibrils due to an absent lumican protein core, and altered interfibrillar spacing because of the lack of lumican-bound keratan sulfate [87].

The refractive index changes from 1.38 to 1.373 across the stromal anterior-posterior dimension and yet the total amount of light scattered is estimated at less than 1% [89]. Two complementary theories have been preferred to explain this phenomenon. According to the "lattice theory", the regular arrangement of collagen fibrils (which have a smaller diameter than the wavelength of light) causes destructive interference of any scattered waves except for those in the direction of the incident beam [124]. Goldman and Bendek modified this theory, proposing that the relatively miniscule distance between collagen fibrils relative to the wavelength of light minimized the possibility of scatter regardless of fibril arrangement [73]. Evidence from the *Lum*^{-/-} mouse experiments suggests that rather than the specific arrangement of collagen fibrils, it is the interfibrillar spacing and proteoglycan content that plays an important role in ensuring corneal transparency.

A further structural adaptation to minimize light scatter is thought to be the expression of corneal crystallins in keratocytes [91]. Keratocytes lie between stromal lamellae at a density of 23,000 cells/mm³ [125]. In vivo confocal microscopy reveals that while light scattering is (as expected) maximal at the anterior superficial epithelial and posterior endothelial surfaces, light scatter within the stroma is restricted to keratocyte nuclei [90]. Initially, keratocytes from rabbits were found to express water-soluble proteins that are identical to lens crystallins, for example, aldehyde dehydrogenase 1A1, which is homologous to neu-crystallin [92]. Other corneal crystallins, like aldehyde dehydrogenase 3A1, have been identified in most mammalian species and are thought to both absorb UV light directly and protect from UV-induced enzyme damage [62]. The role of corneal crystallins in reducing light scatter has been demonstrated by the use of reflectance confocal microscopy [91]. In

this study, corneal keratocytes were plated on collagen coated polyacrylamide gels (to reduce light scatter) and grown in serum-free conditions to maintain keratocyte differentiation. Upon the addition of TGF-beta1 the keratocytes differentiated into myofibroblasts. This was associated with a 50% ($p<0.05$) increase in light scatter and 45% decrease in ALDH1A1 expression compared to controls. Reduced expression of corneal crystallins also occurs following transcorneal freeze injury in rabbits [92] and reduced ALDH3A1 expression (with accompanying keratocyte differentiation into fibroblasts) was also found in patients with corneal haze following failed corneal transplant [145]. These data suggest a major role for corneal crystallin expression in the maintenance of stromal transparency.

The corneal stroma is carefully structured to minimize light scatter with important contributions from the extracellular matrix with its proteoglycans, carefully spaced collagen fibrils and crystallin-expressing keratocytes. Dysfunction in any of these components can cause a loss of transparency and crucial loss of function.

Corneal Haze in Response to Injury

Corneal wound healing is a complex process, involving cellular changes and signaling molecules from cells of every layer of the cornea with contributions by the lacrimal gland and infiltrating inflammatory cells. Except for minor epithelial abrasions, the injured normal adult cornea rarely returns to its original transparency and tensile strength.

As with injuries in other parts of the human body, the process of wound healing begins immediately after the injury. The corneal epithelium heals by a sequence of three processes: migration, mitosis, and differentiation [108]. If the injury breaches the acellular Bowman's layer, the corneal stroma becomes involved; this process of healing begins with apoptosis of the keratocytes immediately deep to the wound, followed by the activation, transformation, and migration of keratocytes that are further away from the wound [191]. The population of activated keratocytes becomes fibroblast-like and secretes extracellular matrix components. Later, some of these fibroblasts differentiate into myofibroblasts, which have contractile properties [93].

If the endothelium is not damaged by the injury, it mainly responds by secretion of signaling molecules (as with the other layers of the cornea). However, if the endothelial structure is compromised, the defect is repaired by migration and enlargement of the surrounding healthy endothelial cells. In humans, mitotic regeneration of endothelial cells is very limited [166]. In addition to the involvement of each layer of the cornea in response to injury, there is also participation of outside inflammatory cells. For example, circulating monocytes migrating into the wound can also transform into fibroblasts [29].

All layers of the cornea must heal to maintain corneal transparency, but the layer that most crucial to corneal haze in response to injury is the corneal stroma. The following sections will explore components of the corneal stromal keratocyte response to injury and its relation to corneal haze.

The development of corneal haze in response to injury is dynamic and can change over the course of years after the initial event [39,61,67,130]. In the period immediately after the injury, some of the corneal haze is due to corneal edema. Traditionally, it has been thought that the corneal haze that develops after this initial phase was from irregularly oriented collagen fibers and accumulation of macromolecules such as proteins, glycosaminoglycans, and lipids [128]. However, studies in the last decade have suggested that the intrinsic transparency of the corneal fibroblast also plays a role. One theory implicates the involvement of intracellular keratocyte crystallin proteins, which are analogous to the water-

soluble crystallin proteins of the lens. The keratocyte crystallin proteins are found in abundant amounts in quiescent keratocytes. In several mouse, rabbit and human studies, when the quiescent keratocyte transforms into an activated fibroblast, the expression of these proteins is markedly reduced, and this is associated with increased reflectivity of the keratocyte [92,99,145,176], and thus increased corneal opacity. In the human, corneal crystallin proteins that appear to be relevant to corneal haze include aldehyde dehydrogenase (ALDH) and transketolase (TKT).

Several authors who have been proponents of the keratocyte crystallin theory have also proposed other theories for increased keratocyte reflectivity. A proteomic study published in 2004 found that activated corneal fibroblasts had increased expression of proteins protecting against oxidative stress and protein misfolding. The authors concluded that protein misfolding from oxidative stress could be a contributor to keratocyte reflectivity [99]. Another protein expression study published in 2005 proposed that the increased expression of intracytoplasmic actin increased keratocyte light-scattering. Lastly, a confocal microscopy study in 2004 suggested that changes in the cellular organelles could increase keratocyte reflectivity [128]. The protein expression profile eventually regresses back to a phenotype similar to the original quiescent keratocytes from which they were derived, yet they do not exhibit the exact same phenotype. This partly explains the changes that result in the nearly normal clarity of the corneal injury site.

The transformation of the corneal fibroblast into the myofibroblasts occurs during the later stages of wound healing, and is characterized by the development of intracytoplasmic α -smooth muscle actin. Contraction of the myofibroblasts results in the irregular contour of the healing scar [93], which may contribute to corneal haze. A recent study showed that extracellular matrix metalloproteinase inducer/CD147 (EMMPRIN) specifically promoted α -smooth muscle actin expression by corneal fibroblasts and contraction of myofibroblasts [84].

As mentioned above, it has long been established that a major component in corneal haze in wound healing is a change in the composition and configuration of the extracellular matrix. In the normal uninjured cornea, the extracellular matrix of the stroma is primarily composed of proteoglycans and Type I collagen. The two main types of proteoglycans found in the normal uninjured stroma are keratan sulfate proteoglycan (KSPG), which predominates in the adult cornea, and dermatan sulfate proteoglycan (DSPG), which predominates in the fetus. It is thought that the proteoglycans regulate the diameter and spacing of the collagen fibrils [108]. When the corneal stroma is injured, KSPG and DSPG from areas adjacent to the wound enter to fill in the wound [29]. Subsequently, there is production of new proteoglycans; however, KSPG synthesis is significantly downregulated while DSPG synthesis is greatly upregulated. Specifically, there is upregulation in production biglycan, a form of DSPG that is seen to accumulate in corneas with chronic pathological conditions [13,68].

After the corneal wound site is cleared of debris, the production of collagen by the keratocyte begins. The predominant form is collagen type I, but collagens type III, IV, V, and VI are synthesized as well [76,108]. Electron microscopy of injured rabbit corneas shows that the collagen matrix is initially a mix of parallel and randomly oriented arrays that eventually remodels into a lamellar pattern. However, the final lamellar pattern that was observed two years after injury consisted of shorter and narrower lamellae [43]. The new proteoglycans and collagen laid down in the corneal scar are not of the same transparency and strength as the original stroma.

Matrix metalloproteinases (MMPs) foster scar remodeling from a hazy, disorganized array into clearer lamellae. MMPs are proteins which cleave extracellular proteins. In corneal wound healing, five MMPs play central roles in corneal wound healing: fibroblast collagenase (MMP-1), gelatinase A (MMP-2), stromelysin (MMP-3), neutrophil collagenase (MMP-8), and gelatinase B (MMP-9). All of these are expressed early in the corneal wound healing process, implying that they contribute to the initial tissue destruction after an injury. However, MMPs-1, 2 and 3 are specifically expressed by the corneal fibroblasts and have sustained expression many months after the initial injury. These three degrade collagen, and one of them degrades proteoglycans (MMP-3), so they likely play a role in the selective catabolism of the scar matrix. Then, new collagen and proteoglycans can be synthesized in an arrangement that is closer to the original lamellar pattern prior to the corneal injury [65,108].

More complex than the actual corneal wound healing processes described above are the molecular signals that modulate the actions of the stromal keratocyte. There are a myriad of signals which act in autocrine, paracrine and exocrine pathways. Among the many proteins, the key signal molecule in the process of corneal wound healing is transforming growth factor beta (TGF- β). Among the three isoforms of TGF- β , the TGF- β_1 isoform is the main one involved in corneal wound healing. One study showed that TGF- β_1 postponed re-epithelialization, stimulated migration keratocytes to the vicinity of the wound of the stroma, promoted keratocyte proliferation and stimulated myofibroblast transformation. On the other hand, TGF- β_3 and IL-10 seemed to oppose the action of TGF- β_1 [32]. TGF- β has been found to interact with a number of other signaling molecules. Fibroblast growth factor-2 (FGF-2) inhibits expression of TGF- β_1 while connective tissue growth factor (CTGF) and epidermal growth factor (EGF) work synergistically with TGF- β_1 [71,80], but the exact roles are unclear.

The spatial orientation of these factors frames the understanding of these signaling pathways. A number of studies have shown that corneal epithelial-stromal interactions greatly influence the transparency of a healed corneal wound. This is extremely important from the perspective of refractive surgery, in which different methods vary by the layer of the cornea manipulated. There are two possible theories which are not mutually exclusive. The first theory is that the epithelial cells secrete specific growth factors or cytokines depending on the degree of damage. The second theory is that the epithelial substratum, including the basement membrane and acellular Bowman's layer, may have a barrier function for some of the molecular signals. Therefore, only if the Bowman's layer is breached will a significant stromal response occur [112,203]. Many future studies will need to be done to elucidate a complete picture of the sequence and spatial orientation of signaling pathways involved in corneal wound healing.

Corneal Haze in the Setting of Photorefractive Keratectomy (PRK)

Despite the increase in successful postoperative outcomes in PRK since its introduction in the late 1980s, corneal haze remains a major complication of the procedure when compared to other methods of corneal refractive surgery. Like the processes leading to corneal haze in response to penetrating injuries, the pathophysiology of PRK-associated corneal haze is not fully understood. Risk factors for post-PRK corneal haze are many; they include deeper ablation, small ablation diameters with abrupt transition edges, pregnancy, oral contraceptives, exposure to UV radiation, viral keratitis, systemic viral illness, trauma, ocular surface disorders, increased corneal temperature during ablation, a rough stromal bed, and dark-colored irides [108]. All of these factors can theoretically alter the pro-inflammatory/wound repair milieu of the cornea. The increased keratocyte response, in turn, leads to distortion of the photoablated stromal surface and increased reflectivity from the

activated/transformed keratocytes and augmented fibrosis [129]. This is the reason that steroids, cooled BSS, antioxidants, chemotherapeutic agents such as mitomycin C (MMC), and TGF- β inhibitor have been shown to decrease or prevent the development of PRK haze [105,108,129,175,185]. In rabbits, post-PRK corneal haze is linked with increased myofibroblasts generation due to heightened TGF- β signaling to the stroma[139].

However, it is likely that the basal epithelial-stromal interaction is most crucial factor in the pathophysiology of post-PRK corneal haze; this relationship explains the almost nonexistent risk of corneal haze after laser in-situ keratomileusis (LASIK). Methods to facilitate re-epithelialization or placement of a basement membrane over the corneal stroma have been proven to decrease corneal haze. Using alcohol rather than mechanical epithelial removal, using urokinase-type plasminogen activator (to presumably enhance epithelial migration), and creating a smooth post-ablation surface result in reduced corneal haze [44,108,168,169,170]. The use of amniotic membrane and amniotic membrane-derived ointments also show positive results in reducing corneal haze. It is hypothesized that the therapeutic effect of the amniotic membrane lies in its thick basement membrane as well as its anti-inflammatory factors [40,42,104,144]. Finding reliable methods to eliminate post-PRK corneal haze may help PRK regain popularity as a corneal refractive procedure as well as aid the understanding of corneal wound healing.

Corneal Avascularity and Angiogenesis

Corneal avascularity is an essential element of corneal transparency [5,35]. The disruption of this state of avascularity has a significant impact; four percent of the U.S. population have decreased vision from neovascularization [35]. Corneal neovascularization (KNV) is under the control of local pro- and anti-angiogenic factors [10,34,97,120]. The natural balance of these factors maintains corneal avascularity. KNV is induced by the upregulation of angiogenic cytokines due to inflammation or hypoxic conditions [10,34,96,120]. The remaining portion of this section will discuss the process of angiogenesis as well as specific pro- and antiangiogenic factors.

The overall process of angiogenesis involves the degradation of the extracellular matrix and the vascular basement membrane by matrix metalloproteinases (MMP) allowing endothelial cells to invade and form vessels [100,162,177]. Under inflammatory conditions, the invasion of endothelial cells into the cornea is largely stimulated by the actions of macrophages which enhance inflammation through the recruitment of additional macrophages while also producing pro-angiogenic factors [4,161,183]. Chemokines recruit macrophages to areas of inflammation by binding to specific receptors; one of these receptors is CCR5 [4]. Among these pro-angiogenic factors released by the macrophages are macrophage migratory inhibitory factor and basic fibroblast growth factor (bFGF). Macrophage migratory inhibitory factor promotes interactions between endothelial cells increasing the angiogenic effects of leukocytes, while bFGF stimulates the proliferation and migration of endothelial cells [183].

The most significant role of macrophages in KNV is their secretion of vascular endothelial growth factor (VEGF) [4]. It is a potent pro-angiogenic factor that is required for KNV [3,4,6]. VEGF binds to its receptors flt (fms-like tyrosine kinase, also known as VEGFR-1) and KDR (VEGFR-2) which are expressed on the vascular endothelium. Flt is a transmembrane receptor consisting of seven immunoglobulin domains with an intracellular tyrosine kinase [184]. Upon VEGF binding, receptor transduction promotes endothelial cell proliferation and migration [4]. The major source of VEGF in the cornea originates from invading macrophages [4,96]. VEGF further amplifies inflammatory CNV by recruiting more macrophages [135].

While VEGF and bFGF promote endothelial cell proliferation and migration, the VEGF-A isoform is needed to initiate vascular formation [69]. The formation of vasculature is then stabilized and remodeled by the binding of angiopoietin-1 to Tie-2 receptors on endothelial cells [69,178]. In subtle contrast to angiopoietin-1, angiopoietin-2 promotes destabilization of vasculature which can bud into new vessels in the presence of VEGF [120,189].

In addition to inflammation-induced angiogenesis, KNV is also induced under hypoxic conditions in attempt to supply oxygen to the cornea. Under hypoxic conditions, pro-angiogenic factors are upregulated as anti-angiogenic factors are down regulated. For example, pigment epithelium-derived factor effectively inhibits KNV under normoxic conditions by hindering endothelial cell migration toward angiogenic inducers [54]. Under hypoxic conditions, pigment epithelium-derived factor is down-regulated, creating a permissive environment for KNV [54]. In addition to crippling the eye's defense against KNV, hypoxic conditions upregulate VEGF, thus stimulating angiogenesis [16,47,88,116]. This process stimulates KNV in about half a million Americans who wear soft contact lenses [116].

The eye has developed multiple methods to protect the cornea from vascularization. The Fas ligand (FasL) provides a barrier against both inflammatory cells and new blood vessels [177]. FasL is highly expressed on the cornea and it induces apoptosis to invading inflammatory cells and endothelial cells that are Fas positive [75,177]. Additionally, thrombospondin-1 (TSP-1) reduces angiogenesis in response to corneal injury [135]. TSP-1 activates CD36, a transmembrane glycoprotein found on macrophages and endothelial cells [53,135]. Activated CD36 on a macrophage reduces VEGF secretion, thus inhibiting endothelial cell proliferation, migration, and the recruitment of additional macrophages [135]. Furthermore, activated CD36 on endothelial cells targets them for apoptosis [135].

The most crucial element discovered to date in maintaining corneal avascularity is soluble VEGF receptor-1 (sFlt-1). If one of the redundant defense mechanisms against CNV mentioned before is impaired, avascularity is still maintained. However, sFlt-1 is required to prevent neovascularization [3,5,35,64]. sFlt-1 is formed by the splicing of Flt mRNA into a shorter fragment [83]. It inhibits the angiogenic effects of VEGF by acting as a decoy receptor while also inactivating the membrane bound VEGF receptor, Flt [6]. The presence of IFN- γ , a cytokine, further enhances sFlt-1 expression while also sequestering VEGF IFN- γ [107].

Lymphangiogenesis

A clear human cornea lacks both blood and lymphatic vasculature. Corneal lymphangiogenesis is induced with corneal neovascularization and it correlates with the degree of corneal neovascularization, though it has been found that the lymphatics of the cornea regress sooner and more fully than blood vasculature [49,50].

Lymphangiogenesis is the creation of new lymphatics by lymphangioblasts or the sprouting of lymphatics from the preexisting limbal lymphatics [106,142]. In the cornea, lymphatics have been observed to arise within the cornea which then connect to the limbal lymphatic vessels and also by growing from the limbal lymphatics [123]. Proof of lymphangiogenesis in the cornea has been hindered due to the invisibility of lymphatics [47]. This has recently changed with the discovery of specific lymphatic markers such as vascular endothelial growth factor receptor 3 (VEGFR3), LYVE-1, podoplanin, prox1, and D2-40 (an antibody against an endothelial marker) [47,50,155].

The formation of lymphatics begins with the homeobox gene prox1 [69]. Endothelial cells expressing this gene bud from veins and differentiate as lymphocyte endothelial cells

(LECs) which leads to the formation of the lymphatic system [69,190]. Without these budding endothelial cells expressing the homeobox gene *prox1*, the lymphatic system fails to develop [190]. *Prox1* also upregulates the gene encoding the tyrosine kinase receptor VEGFR-3 which regulates the growth and maintenance of lymphatic vessels [47,98,121].

VEGFR-3 is expressed on LECs and is activated by its ligands VEGF-C and VEGF-D [47,50,98,121,188]. This activation leads to the proliferation, migration, and survival of LECs [121]. VEGF-C is essential for sprouting the first lymphatic vessels from embryonic veins [46,98]. Pro-inflammatory cytokines upregulate the release of VEGF-C and are not affected by hypoxia as is VEGF-A [160].

Pro-inflammatory conditions recruit macrophages which further enhances lymphangiogenesis in two ways. First, macrophages release VEGF-C and VEGF-D which induces local sprouting of preexisting LECs [47,102,123]. Second, CD11b positive macrophages transdifferentiate into LECs which initially form aggregates that are later integrated into sprouting lymphatic vessels [102,123]. Without macrophages, lymphangiogenesis does not occur [123].

Fibroblast growth factor-2 (FGF-2) stimulates both lymphangiogenesis and angiogenesis, though it has been shown that lymphatic vessels are considerably more sensitive to FGF-2 than blood vessels [37,110]. FGF-2 has proved to stimulate lymphangiogenesis in the mouse cornea via two routes: first, by upregulating VEGF-C expression and second, by binding to its receptors promoting migration and proliferation of LECs [37,110,173].

It has also been found that VEGF-A induces a lymphangiogenic response and that neuropilin-2 is important in lymphatic development [136,202]. Despite the many pro-lymphangiogenic factors there are relatively few known anti-lymphangiogenic factors. It was recently discovered that neostatin-7, the C-terminal 28 kDa endostatin-spanning proteolytic fragment of collagen XVIII, has anti-lymphangiogenic properties [28,36,106]. Neostatin-7 reduced bFGF-induced corneal lymphangiogenesis, binds to VEGFR-3 in vitro, and corneal lymphangiogenesis and VEGF-C expression is enhanced in collagen XVIII knockout mice [106]. Over expression of endostatin, another proteolytic fragment of collagen XVII, has also shown ability to diminish lymphangiogenesis in carcinogen induced skin tumors in mice [28]. It has been proposed by Takashi Kojima et al. that endostatin-containing fragments may be among the factors that prevent new lymphatic vessel formation after wound healing [106].

Anti-angiogenic therapies

An increased understanding of the anti-angiogenic regulators and the pro-angiogenic factors that maintain the avascularity of the homeostatic cornea has lead to a number of anti-angiogenic pharmaceutical strategies. These strategies have targeted both the general inflammatory cascade as well as the inhibition of selective pro-angiogenic factors.

Steroids

Steroids, have remained the mainstay therapy of corneal neovascularization in the clinic. Specifically, topical and subconjunctival administration of triamcinolone has been shown to inhibit corneal neovascularization [133,158]. However steroids sometimes do not successfully inhibit neovascularization, and carry with them serious side effects including inherent risk of infection, glaucoma, and cataract formation [9]. Corticosteroids are therefore inadequate, and alternative therapeutic options are required.

VEGF inhibitors

Vascular Endothelial Growth Factor (VEGF) plays a key role in angiogenesis in the human cornea. Comprised of 5 isoforms, VEGF promotes several steps within normal vascular growth including the induction of angiogenesis, endothelial cell proliferation, enhanced inflammatory response, proteolytic activities and increased vascular permeability[21,23,35].

Several cellular components within the human cornea have been found to excrete VEGF when under duress or inflammation including corneal endothelial and epithelial cells, fibroblasts, macrophages, and limbal vessel vascular endothelial cells[3,70,146]. VEGF antagonists disrupt these pathways, thus preventing and regressing corneal neovascularization.

Bevacizumab (Avastin) is a full-length, recombinant monoclonal antibody that binds all VEGF isoforms. It effectively inhibits the VEGF–receptor interaction, hence inhibiting vascular permeability and capillary formation[165]. A related compound, Ranibizumab (Lucentis), is a high affinity recombinant monoclonal antibody derived from the same parent murine antibody as bevacizumab. It is much smaller than the parent molecule and neutralizes all isoforms of VEGF-A[5].

Bevacizumab and ranibizumab have been widely used in the management of diabetic retinopathy and age-related maculopathy (AMD)[7,165]. Initial reports in treating corneal neovascularization have shown bevacizumab to be safe and effective in both the animal model[22,197,198], as well as multiple human clinical reports[42]. Of specific clinical importance is the role VEGF inhibitors play in the prophylactic treatment of penetrating keratoplasty which will be discussed in a later section.

Studies have investigated various drug delivery approaches of bevacizumab in neovascularization treatment. Two series consisting of patients with corneal neovascularization that effectively responded bevacizumab eye drops have been described[22,56]. However, difficulty that older patients may experience with the use of eye drops may make single injection a superior drug delivery method[31]. Subconjunctival administration of bevacizumab has been shown to inhibit corneal neovascularization in experimental rabbit models[143]. In human patients, short term results of subconjunctival injection of bevacizumab has been shown to be well tolerated, and shows regression of corneal neovascularization[12,200].

A recent study suggests that bevacizumab not only inhibits hemangiogenesis but also lymphangiogenesis[22] by the inhibition of the VEGF-A isotype as discussed previously. This isotype plays a particularly important role in corneal lymphangiogenesis. This gives rise to the hope that bevacizumab may be of particular usefulness in preventing immune rejections after penetrating keratoplasty as lymphangiogenesis is a component of graft rejection (as will subsequently be discussed), and in preventing tumor metastasis via lymphatic vessels[47,111].

Furthermore, bevacizumab may play a complementary role in preventing corneal neovascularization in herpetic stromal keratitis[31] as endothelium and stroma infected with herpes contain elevated levels of VEGF. In recurrent pterygium, the efficacy of bevacizumab is debatable. Short-term results of an initial study demonstrated that subconjunctival bevacizumab did not significantly induce regression of corneal vessels in recurrent pterygium[12]. However, latest evidence indicates successful use of bevacizumab in the management of recurrent pterygium[192].

Matrix metalloproteinase-2

Matrix metalloproteinase-2 (MMP-2) has also recently been implicated as a regulator of angiogenesis in inflammation-related corneal neovascularization, and are the target of new treatment modalities[113,162]. Propolis extracts containing artemisin C and caffeic acid phenyl ester (CAPE)[182], has been shown to inhibit cell proliferation, migration and capillary tube formation in the human cornea. Propolis reduced the number of newly formed vessels, and inhibiting expression of MMPs as well as VEGF production from various cells[103]. Doxycycline, an inhibitor of MMP-2, has also been found to effectively suppress corneal neovascularization while in combination with Triamcinolone acetonide[5].

Agents under investigation

In addition to anti-VEGF therapy and MMP investigations, studies are ongoing for other promising treatment modalities. A number of various drugs and chemical compounds have been proposed including heparin[117], cyclosporine A[52], nonsteroidal anti-inflammatory drugs[57], methotrexate[95], FK506[18], and thalidomide[2,101]. Pigment epithelium-derived factor (PEDF) has recently been identified as an angiogenic inhibitor that typically aids in maintaining the quiescent vasculature in disease free cornea, and thus may be of therapeutic value in pathological neovascularization[54].

Combination treatments

Argon laser coagulation provides a potential treatment in closing pathologic blood vessels in the cornea[74]. Combination of bevacizumab injections and argon laser coagulation prior to penetrating keratoplasty may be a promising tool to enhance transplant survival[72]. A recent study demonstrated the inhibition of corneal neovascularization with topically administered combinations of triamcinolone acetonide with low molecular weight heparin[9]. Triamcinolone acetonide and doxycycline combinations also demonstrated efficient suppression of corneal neovascularization; yet these drugs were ineffective at similar concentrations used alone[9].

Photodynamic therapy (PDT) is a widely practiced and efficacious mode of treatment for vascular disorders ranging from tumors to vascular diseases of the eye[109]. PDT involves administration of a photosensitizer which is activated by localized application of light of a suitable wavelength, targeting the tissue of interest. Upon excitation, the photosensitizer releases highly reactive oxygen species that are destructive to blood vessels and neoplastic cells; these changes are irreversible. The changes induced in the microvasculature involve damage to the endothelial cells and basement membrane. This breach of architectural integrity generates a focal thrombogenic response, sealing off the lumen[109]. PDT has been safely and successfully used to regress corneal neovascularization in mice, rats, rabbits and humans, but multiple sessions may be required[30,60,66,81,149,199].

While VEGF and MMPs as targets comprise the mainstay of current drug therapy and research, an increasing understanding of other factors involved in the complex angiogenic pathway may provide the basis for future drug strategies. FHL2 gene expression in the plays an important role in developing vasculature following corneal injury[41] as does nitric oxide synthase. Cyclooxygenase-2 (COX-2) plays an important role in the progression of angiogenesis and is the known rate-limiting enzyme in angiogenic prostaglandin synthesis, including PGE₂, which induces VEGF[118,122]. Significant positive correlation between VEGF and COX-2 has been seen in both head and neck squamous cell carcinoma as well as non-small cell lung carcinoma[122].

Protection of Corneal Transplants from Rejection

When transparency of a native cornea cannot be maintained at a functional level for the patient, corneal transplantation is often the next intervention. Once transplanted, the major cause of corneal graft failure is allograft rejection. Despite this fact, corneal transplantation has a very high success rate. Over 90% of low-risk corneal transplants retain clarity years after transplantation using only local immunosuppression [108]. The success of penetrating keratoplasty (PKP) is determined by factors which confer the same immune privilege found in the pristine cornea. These factors include limiting access to the graft by the host immune system, suppressing antigen expression and migration of antigen-presenting cells, and inhibiting or modulating the host immune response. While most research to date has revealed that the protection against allograft rejection is a combination of avoiding higher-risk conditions and using agents to suppress host response, there is growing evidence that there are active agents in the host and the allograft that prevent allograft rejection.

Blocking access to the donor cornea from the host immune system is the first line of defense against corneal allograft rejection, of which corneal avascularity is an essential component. Increased blood vessel vascularity of the host bed as well as neovascularization of the donor graft increases the risk for corneal rejection, presumably by increasing delivery of host antigen-presenting cells and immune cells once the host immune system is activated. In normal low-risk grafts, it is common practice to avoid exposing suture knots and ends which may stimulate neovascularization, to remove sutures associated with stromal neovascularization, and to treat neovascularization aggressively using topical steroids. Furthermore, larger grafts and decentered grafts increase the risk for rejection as such procedures place the donor graft closer to host limbal vessels [108].

Because vascular endothelial growth factor (VEGF) plays a significant role in corneal avascularity, many have experimented with VEGF inhibition to prevent or treat corneal transplant rejection. The results are mixed. A few studies have shown favorable results from the use of anti-VEGF therapy post-transplant for animals and humans with high-risk vascularity in the recipient corneal bed [11,72]. A recent case report showed successful regression of graft neovascularization with the use of subconjunctival bevacizumab (an anti-VEGF monoclonal antibody) along with aggressive subconjunctival and topical steroid therapy [79]. On the other hand, a recent case series showed limited success of subconjunctival bevacizumab monotherapy for vascularized rejecting corneal grafts, in which all 3 grafts eventually failed [8].

Despite the longstanding notion that hemangiogenesis is intimately linked with corneal allograft rejection, lymphangiogenesis also plays an important role in PKP graft rejection. This could be a major reason for host immune activation against a corneal allograft despite the lack of hemangiogenesis into the graft, as the new lymphatics would allow antigen presenting cells and antigenic material to access the host immune system. Several studies showed that drainage of antigen-presenting cells into specific lymph nodes induced corneal allograft rejection [148,195]. Later studies have shown histological evidence of lymphatic vessels within a majority of corneal allografts in mice and humans [47,155].

Lymphangiogenesis, like hemangiogenesis, has also been linked with VEGF signaling. Specifically, lymphangiogenesis has been associated with VEGF isoforms A and C (VEGF-A, VEGF-C) as well as VEGF receptor-3 (VEGF-R3) [127]. Inhibition of VEGF resulted in decreased hemangiogenesis and lymphangiogenesis and increased corneal graft survival in several studies [24,45,82]. Inhibition of cell-surface integrin receptor $\alpha_1\beta_1$ via antibody blockade and knock-out mice also inhibits hemangio- and lymphangiogenesis and improves

graft survival, presumably by suppressing the ability of endothelial cells to migrate through the extracellular matrix for angiogenesis [38].

While increased hemangiogenesis and lymphangiogenesis lead to host immune activation against a corneal allograft, the converse seems to be true as well. Steroids traditionally have been used to bring about the regression of allograft neovascularization. More recently, immunosuppressants such as rapamycin (Sirolimus) have been shown to prevent rejection and neovascularization of allografts [172].

The fact that the development of blood and lymph vessels does not occur in all corneal transplants indicates that there may be anti-angiogenic factors native to the transplanted cornea. Several molecules show promise of playing this role. FasL is a molecule that modulates cell apoptosis through binding to its cell surface receptor, Fas. Interestingly, there are two forms of FasL, membrane-bound FasL (mFasL) and soluble FasL (sFasL), which is cleaved from mFasL by matrix metalloproteinases. Each form appears to antagonize the other; mFasL promotes apoptosis while sFasL opposes mFasL action [163]. Several studies have looked at FasL in the cornea. One study in 1997 concluded that the presence of FasL decreases corneal rejection. Another study showed that Fas-FasL interaction is involved in corneal neovascularization, although the authors could not find a single unifying theory to explain their results [177]. Yet another study found that higher sFasL levels were correlated with corneal allograft rejection [157]. The authors noted that sFasL is produced by the endothelial cells. The conflicting results among these studies and even within one of these studies may be due to the opposing actions of mFasL and sFasL. Future studies are needed to distinguish mFasL from sFasL action as well whether their actions are directly linked to corneal transplant rejection through neovascularization.

The soluble VEGF receptors, sVEGFR-1 (sFlt-1) and sVEGFR-2 (sFlk-1), inhibit neovascularization [5,114,201]. The interactions among VEGF, Flt-1, Flk-1, sFlt-1, and sFlk-1 and their production within cells of the cornea have been demonstrated on normal and vascularized corneas; future studies on transplanted corneas are needed.

Donor and host antigen-presenting cells play an important part in activating the host immune system. It has recently been confirmed that the central cornea does in fact contain antigen presenting cells (APCs). Interestingly, they do not express major histocompatibility (MHC) antigens until they are stimulated as in corneal transplantation [77,119]. Previously, there was no demonstrated benefit of HLA matching in corneal allograft rejection[1]. However, recent case series and mono-center trials indicate growing evidence for the protective effect of HLA-I, HLA-A and HLA-DR typing on corneal allograft survival[14,15,25,132,156]. Several studies have also noted increased graft survival with pre-transplant treatment of donor corneas with UV light or hyperbaric oxygen [153,154,186]. The suppression of host APC action is also crucial in preventing host immune cell activation. Inhibition of various cytokines, such as interleukin (IL-1) and tumor necrosis factor α (TNF- α) inhibits host APC migration into the donor graft, increasing graft survival [51,55,194].

The effector arm of the host immune reaction in allograft rejection involves T-lymphocytes. Immunosuppressants like Cyclosporine A and FK-506 traditionally used to prevent and control corneal allograft rejection target T-cell activation and proliferation. Some of the newer experimental treatments for corneal allograft rejection have been focused on monoclonal antibodies to block the cell-to-cell interactions needed to activate T-cells. In addition, several experiments have examined modulating the T-cell phenotype away from the Th1 pathway, which is thought to be more responsible for corneal graft rejection than the Th2 pathway [108].

As noted above, several studies have shown that exogenous modulation of cytokine levels can protect the corneal allograft. Fewer studies have examined cytokine profiles in the host that promote corneal graft survival. Lower levels of TNF- α and TNF- β , and higher levels of TGF- β_2 occur in human and animal eyes with graft acceptance [147]. A deeper understanding of cytokine profiles that inhibit graft rejection will likely usher in new treatments for corneal graft rejection.

Conclusion

The cornea uniquely melds the roles of refractive power and structural integrity into an optically transparent tissue. This review has sought to outline the key processes and molecules involved in the genesis, maintenance, and disruption of corneal transparency. The cornea's development is guided by a complex concert of gene activation at key times in embryogenesis (especially *Pax6* and *destrin*), and its transparency is maintained during life by precise collagen lamellar architecture, homeostatic fluid regulation by the epithelium & endothelium, and an ensemble of anti-hemangiogenic and anti-lymphangiogenic factors led by soluble VEGFR-1. These elements are closely linked to corneal immune privilege which enables the best long-term survival rates of any solid tissue transplant. Immune cell inflammation after corneal injury or surgery can induce haze, scarring, and transplant rejection. Much future research will be needed in the areas of minimizing haze formation during wound healing, the mechanisms inhibiting lymphangiogenesis, and preventing & reversing corneal transplant rejection.

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Table 1

Molecules involved in corneal transparency and avascularity.

Corneal crystallins
-aldehyde dehydrogenase (ALDH)
-transketolase (TKT)
Promotor of transformation of keratocyte to corneal myofibroblast
-extracellular matrix metalloproteinase inducer (EMMPRIN)/CD147
Matrix metalloproteinases (MMPs) involved in corneal healing
-Specific expression by corneal fibroblasts; sustained expression
-MMP-1/fibroblast collagenase
-MMP-2/gelatinase A
-MMP-3/stromelysin
-Short-term expression
-MMP-8/neutrophil collagenase
-MMP-9/gelatinase B
Signal proteins modulating keratocyte action
-Transforming growth factor beta-1 (TGF- β_1)—appears to promote healing/scar formation
-Transforming growth factor beta-3 (TGF- β_3)—appears to inhibit healing/scar formation
-Interleukin-10 (IL-10)—appears to inhibit healing/scar formation
-Fibroblast growth factor-2 (FGF-2)—inhibits TGF- β_1 expression
-Connective tissue growth factor (CTGF)—synergizes with TGF- β_1
-Epidermal growth factor (EGF)—synergizes with TGF- β_1
Molecules involved in penetrating keratoplasty graft rejection
-Pro-hemangiogenic/lymphangiogenic
-Vascular endothelial growth factor (VEGF) isoforms and its associated receptors
-Vascular endothelial growth factor-A (VEGF-A)—specifically also promotes lymphangiogenesis
-Vascular endothelial growth factor-C (VEGF-C)—specifically also promotes lymphangiogenesis
-Vascular endothelial growth factor receptor-3 (VEGF-R3)—specifically also promotes lymphangiogenesis
-Cell surface integrin $\alpha_1\beta_1$ —presumably involved in endothelial migration
-Anti-angiogenic
-Fas ligand (FasL)—two forms, mFasL (membrane-bound) and sFasL (soluble); unclear roles of each
-Thrombospondin 1 (TSP-1)
-Pigment Epithelial Derived Factor (PEDF)
-Interferon gamma
-Soluble VEGF receptors
-sVEGFR-1 (sFlt-1)
-sVEGFR-2 (sFlk-1)
-Other signal molecules that promote rejection
-Tumor necrosis factor alpha (TNF- α)

- Tumor necrosis factor beta (TNF- β)
- Interleukin-1 (IL-1)
- Other signal molecules that inhibit rejection
- Transforming growth factor beta-2 (TGF- β_2)

Table 2

Anti-angiogenic treatments and compounds.

ANTI-ANGIOGENIC FACTOR	MECHANISM OF ACTION
Ranibizumab (Lucentis)	<i>Monoclonal antibody fragment targeting vascular endothelial growth factor-A (VEGF-A)</i>
Bevacizumab (Avastin)	<i>Monoclonal antibody targeting vascular endothelial growth factor-A (VEGF-A)</i>
Triamcinolone acetonide	<i>A corticosteroid that inhibits the inflammatory response</i>
Artepillin C	<i>Matrix metalloproteinase 2 (MMP-2) and vascular endothelial growth factor (VEGF) inhibition</i>
Caffeic acid phenyl ester (CAPE)	<i>Matrix metalloproteinase 2 (MMP-2) and vascular endothelial growth factor (VEGF) inhibition</i>
Doxycycline/Triamcinolone acetonide combination	<i>Matrix metalloproteinase 2 (MMP-2) inhibition</i>
Heparin	<i>Immunoreactivity with basic fibroblast growth factor (bFGF), inhibiting neovascularization</i>
Cyclosporine A	<i>Induces significant reduction in interleukin 2-induced corneal neovascularization</i>
Methotrexate	<i>Possible inhibition of both macrophage invasion during early angiogenesis and endothelial cell proliferation</i>
Tacrolimus (FK506)	<i>Induces immunosuppression by inhibiting both T-lymphocyte signal transduction and IL-2 transcription</i>
Thalidomide	<i>Inhibits basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) induced corneal neovascularization</i>
Pigment epithelium-derived factor (PEDF)	<i>A naturally occurring angiogenic inhibitor shown to have neurotrophic activity</i>
Argon laser coagulation	<i>Ablates neovascular vessels</i>
Rapamycin	<i>May inhibit proinflammatory cytokines</i>