

CORNEAL ANGIOGENIC PRIVILEGE: ANGIOGENIC AND ANTIANGIOGENIC FACTORS IN CORNEAL AVASCULARITY, VASCULOGENESIS, AND WOUND HEALING (AN AMERICAN OPHTHALMOLOGICAL SOCIETY THESIS)

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ABSTRACT

Purpose: To determine the molecular basis of corneal avascularity during wound healing and determine the role of angiogenic and antiangiogenic factors in corneal vasculogenesis.

Methods: The expression of proangiogenic factors (vascular endothelial growth factor [VEGF]; basic fibroblast growth factor [bFGF]; matrix metalloproteinase-2 [MMP-2]; and membrane-type 1-MMP [MT1-MMP]) and antiangiogenic factors (pigment epithelium-derived factor [PEDF]; angiostatin; restin; and endostatin) was analyzed in avascular corneas and in models of corneal neovascularization (bFGF pellet implantation, intrastromal injection of MT1-MMP cDNA, and surgically induced partial limbal deficiency).

Results: Immunohistochemistry demonstrated the presence of antiangiogenic factors (PEDF, angiostatin, restin, and endostatin) and proangiogenic molecules (VEGF, bFGF, MMP-2, and MT1-MMP) in the cornea after wounding. Proangiogenic MMPs were upregulated in stromal fibroblasts in the vicinity of invading vessels following bFGF pellet implantation. Corneal neovascularization (NV) was also induced by intrastromal injection of MT1-MMP naked cDNA in conjunction with de-epithelialization. Partial limbal deficiency (HLD-) resulted in corneal NV in MMP-7 and MMP-3 knockout mice but not in wild type controls.

Conclusions: Corneal angiogenic privilege is an active process involving the production of antiangiogenic factors to counterbalance the proangiogenic factors (which are upregulated after wound healing even in the absence of new vessels). Our finding that the potent antiangiogenic factors, angiostatin and endostatin, are colocalized with several MMPs during wound healing suggests that MMPs may be involved in the elaboration of these antiangiogenic molecules by proteolytic processing of substrates within the cornea.

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INTRODUCTION

Corneal clarity and avascularity are important for the proper optical performance of the cornea.¹ Several studies have examined the process of new blood vessel formation in the cornea since Arnold's classic work in 1872 showing that vascular processes utilize the striae of the intercellular cement substance for corneal neovascularization (NV).¹⁻⁹ Recent investigations have focused on understanding the mechanisms that are operative in maintaining corneal avascularity under homeostatic conditions and in avascular wound healing.⁹⁻¹³ These studies suggest that corneal angiogenic privilege involves several active cascades and is not a passive process.

Corneal NV is a sight-threatening condition usually associated with inflammatory or infectious disorders of the ocular surface. NV is the formation of new vascular structures in areas that were previously avascular. Three overlapping mechanisms may be involved in NV regulation: vasculogenesis, the formation of new blood vessels from bone marrow-derived angioblasts (mainly during embryogenesis); recruitment of progenitor vascular endothelial cells; and angiogenesis, the formation of new vessels from preexisting vascular structures.¹⁴⁻¹⁸ Angiogenesis is common in tumor growth and in corneal and retinal disorders.^{7,19} As has been demonstrated in cancer angiogenesis research, a balance exists between angiogenic factors, such as fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF), and antiangiogenic molecules, such as angiostatin, endostatin, or pigment epithelium-derived factor (PEDF), in the cornea.^{19,20}

Following corneal injury, wound healing often proceeds without corneal NV. However, corneal NV may be induced during wound healing in several inflammatory, infectious, degenerative, and traumatic corneal disorders.¹ Diseases associated with corneal NV include inflammatory disorders, corneal graft rejection, infectious keratitis, contact lens-related hypoxia, alkali burns, stromal ulceration, aniridia, and limbal stem cell deficiency (Table 1). In these conditions, the balance between angiogenic and antiangiogenic factors may be tilted in favor of NV due to the upregulation of angiogenic factors and/or the downregulation of antiangiogenic factors.^{6,11,15}

CORNEAL NEOVASCULARIZATION AND AVASCULARITY: EPIDEMIOLOGY AND RISK FACTORS

The corneal blood supply arises from the ciliary arteries, branches of the ophthalmic artery that subsequently divide and end in the pericorneal plexus in the limbal area. Corneal NV involves the sprouting of new vessels essentially from capillaries and venules of the pericorneal plexus. Three clinical entities of corneal NV can be discerned: (1) deep NV overlying Descemet's membrane seen in herpetic and luetic interstitial keratitis, (2) stromal NV mainly associated with most forms of stromal keratitis, and (3) vascular pannus composed of connective tissue proliferating in the superficial corneal periphery and mainly associated with ocular surface disorders.^{1,3,4,7}

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TABLE 1. POTENTIAL MECHANISMS OF CORNEAL NEOVASCULARIZATION

CAUSE	CNV GRADE	MECHANISM OF CORNEAL NEOVASCULARIZATION*					REFERENCES
		EPITHELIAL DEFECT	ACTIVATION OF KERATOCYTE	INFLAMMATION	STEM CELLS DEFICIENT	HYPOXIA	
Contact lens wearing	+	-	-	-	-	+	218
Trauma	++	+	+	+	+	-	219
Infections	+++	+	+	++	-	-	26
Local immune diseases	++	-	+	+++	+	-	220
Systemic immune diseases	+++	-	+	++	+	-	221
Degenerative disorders	+	-	-	++	-	-	222
Interstitial keratitis	++	-	+	++	-	-	223
Stromal ulceration	++	+	+++	+++	-	-	224
Diseases associated with limbal cells deficiency	+++	++	-	-	+++	-	225
Chemical burns	+++	+++	++	++	+++	-	226
Aniridia	+++	-	-	-	++	-	227
Congenital	++	-	-	-	+	-	228

CNV = corneal neovascularization.

*Mechanisms of corneal neovascularization: - = not applicable; + = evidence of limited involvement; ++ = moderate involvement; +++ = strong involvement.

Neovascular and infectious diseases of the cornea and other parts of the eye represent a major public health burden. Although the exact incidence and prevalence rates of corneal NV in the United States are unknown, the incidence rate was estimated at 1.4 million patients per year based on an extrapolation of the 4.14% prevalence rate at the Massachusetts Eye and Ear Infirmary in 1996.²¹ Twenty percent of corneal specimens obtained during corneal transplantation show histopathologic evidence of NV.^{1,4,7,21} Corneal NV may not only reduce visual acuity but also worsen the prognosis of subsequent penetrating keratoplasty (PK).^{1,4,7,22}

The optical quality of the cornea with corneal NV may be reduced by five possible mechanisms: (1) opacity caused by the circulating blood cells in the vascular channels, (2) irregular architecture of the vascular walls inducing higher-order aberrations, (3) alterations in the spacing of stromal collagen between blood vessels (Figure 1), (4) fluid leakage, edema, and lipid deposition in the tissue surrounding permeable blood vessels, and (5) in the case of superficial pannus, corneal surface irregularity. Improved visual acuity with a hard contact lens is helpful in establishing the latter etiology.

Leakage of lipids into the stroma following herpetic corneal NV results in corneal opacification.²³ Lipid keratopathy overlying the

pupillary zone causes a drastic reduction in visual acuity. Peripheral corneal NV associated with stromal scarring may, in rare circumstances, reduce vision by indirectly inducing astigmatism in the central cornea, but isolated peripheral NV does alter visual acuity.

Risk factors for corneal NV after PK have been assessed in patients without active inflammation, previous corneal NV, or persistent epithelial defects.^{7,24,25} The risk of corneal NV was increased when suture knots were buried in the host stroma, when active blepharitis was present, or when a large recipient bed was used.⁷

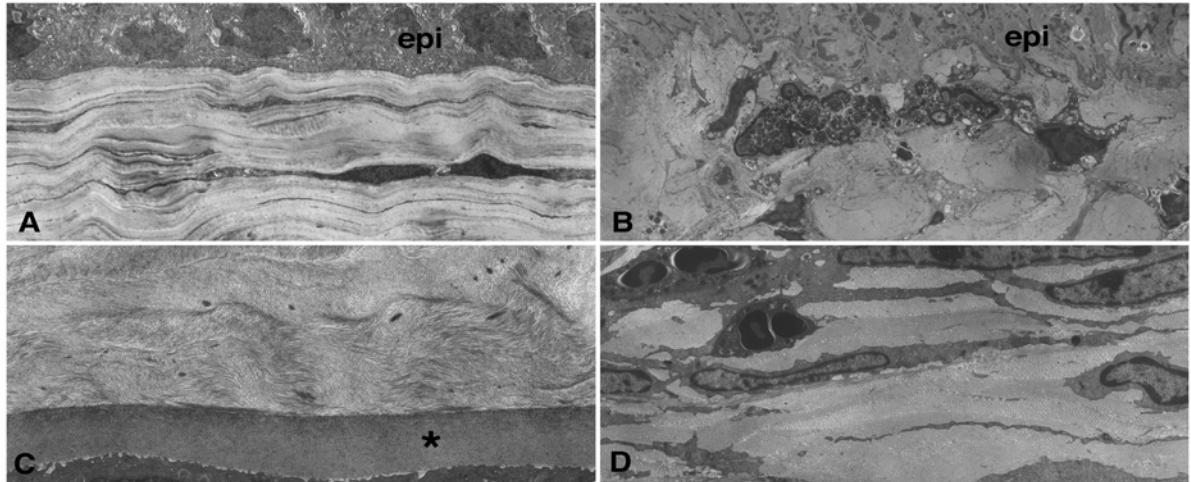


FIGURE 1

Electron micrography of normal and vascularized mouse corneas. Normal and vascularized corneas were fixed in half-strength Karnovsky fixative (A, C). The normal cornea shows uniform arrangement of collagen fibers (B, D). The vascularized cornea shows irregular collagen distribution. (epi, epithelium; asterisk (*), Descemet's membrane)

STROMAL WOUND HEALING AND CORNEAL AVASCULARITY

With appropriate medical treatment, bacterial corneal infections often heal without corneal NV. Similarly, *Acanthamoeba* keratitis is rarely associated with corneal NV even in relatively severe and long-standing cases.²⁶ Additionally, the process of wound healing after surgical corneal trauma (such as after keratorefractive surgery) is usually avascular.²⁷ This process involves epithelial proliferation, migration, and stratification as well as stromal wound healing, which occurs in four phases.

In the first phase of stromal wound healing, the keratocytes adjacent to the area of epithelial debridement undergo apoptosis, leaving a zone devoid of cells.²⁸ In the second phase, adjacent keratocytes proliferate to repopulate the wound within 24 to 48 hours after wounding. The keratocytes transform into fibroblasts and migrate into the wound area. Transformation of keratocytes to fibroblasts can be recognized at the molecular level as reorganization of the actin cytoskeleton (with development of stress fibers and focal adhesion structures). There is also activation of new genes for encoding extracellular matrix (ECM) components. Quiescent keratocytes also differ from wound fibroblasts in their inability to synthesize collagenase in response to treatment with agents that stimulate remodeling of the actin cytoskeleton.²⁹ This inability is due to the failure to activate an autocrine interleukin (IL)-1 α feedback loop.³⁰ The transformation of keratocytes to fibroblasts and their migration into the wound area may take up to a week and are not accompanied by corneal NV.

In the third phase of stromal wound healing, fibroblasts may be transformed into myofibroblasts (evidenced by α -smooth muscle actin staining). Myofibroblasts appear as stellate cells; they are highly reflective but are limited to the wound area. Laser wounds that remove Bowman's membrane and incisional wounds result in myofibroblast generation (which may take up to a month to become apparent). Corneal NV is absent in this phase of stromal wound healing.

The final phase of stromal healing involves stromal remodeling and is greatly dependent on the original wound. Wounds that have completely healed contain few, if any, myofibroblasts, presumably because they revert to the fibroblast phenotype or undergo apoptosis during wound healing.³¹

In contrast to incisional, keratectomy, and laser wounds (which are avascular), other wounding models have been associated with corneal NV.²⁷ These models include intrastromal implantation of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) pellets, hemilimbal injury, and mouse corneal transplantation in severe combined immunodeficiency (SCID) mice (Table 2). In these models, stromal wound healing is accompanied by NV: the latent phase of corneal NV coincides with the first stage of healing; the onset of corneal NV occurs during the second stage of stromal wound healing and continues through the third stage.

Transforming growth factor (TGF)- β and bFGF influence the proliferation and migration of vascular endothelial cells but have

opposing effects on the stromal cell phenotype during wound healing. TGF- β 1 (and activin A) stimulates myofibroblast transformation.³²⁻³⁴ In contrast, bFGF inhibits this transformation^{35,36} and induces the conversion of myofibroblasts to the fibroblast phenotype.³⁷⁻⁴¹ These two growth factors participate in different signaling pathways of the regulation of Smad proteins downstream of TGF- β receptor-I and -II.³⁷⁻³⁹

TABLE 2. MODELS OF CORNEAL ANGIOGENESIS

MODEL	BFGF PELLETT	VEGF PELLETT	HLD	SCID-PK
Epithelial defect	None	None	100%	None
Limbal injury	None	None	50%	None
Stromal wounding	Limited	Limited	None	Incision wound
Expected keratocyte morphology	Keratocyte fibroblast	None	Fibroblast/myofibroblast (?)	Fibroblast/myofibroblast (?)
Stromal scarring	–	–	+	+
Associated inflammation	SCID, WT, +	SCID, WT, +	+	+
Stimulus of neovascularization	bFGF (+fibroblast)	VEGF	Limbal injury/epithelial defect	Suture inflammation
Onset of neovascularization	3-5 days	3-5 days	4-7 days	7-10 days
Clinical relevance	Minimal	Hypoxia	Limbal deficiency PED	Graft rejection, non-immune graft failure

bFGF = basic fibroblast growth factor; HLD = hemilimbal deficiency; WT = wild-type; SCID = severe combined immunodeficiency; PK = penetrating keratoplasty; PED = persistent epithelial defect; VEGF = vascular endothelial growth factor; - = absent; + = present.

POSSIBLE FACTORS EXPLAINING CORNEAL ANGIOGENIC PRIVILEGE

The avascularity of the cornea is important for allowing light transmission without substantial aberrations. Several mechanisms may contribute to corneal avascularity and the pathogenesis of corneal NV: (1) mechanical considerations of corneal anatomy (including constant dehydration resulting in tightly packed collagen lamellae and the presence of compact keratocyte networks among the lamellae), (2) the angiostatic nature of corneal epithelial cells, (3) the immune privilege of the cornea and its dependence on the induction of anterior chamber-associated immune deviation (ACAID), in which antigen-specific, delayed-type hypersensitivity (DTH) is suppressed, (4) lower corneal temperature, extensive innervation, and movement of the aqueous humor across the cornea, (5) low levels (or even absence) of angiogenic factors under homeostatic conditions and during avascular corneal wound healing, (6) low levels of proangiogenic MMPs, (7) the barrier function of the limbal cells, and (8) active production of potent antiangiogenic factors that counterbalance the proangiogenic stimuli during homeostasis and avascular wound healing.

CORNEAL HYDRATION AND CORNEAL AVASCULARITY: INITIAL HYPOTHESES AND SUBSEQUENT EXPERIMENTS REFUTING THE ROLE OF CORNEAL HYDRATION IN THE PATHOGENESIS OF CORNEAL NV

The potential relationship between corneal edema and corneal NV can be appreciated by understanding the microanatomy of the cornea and the histopathology of the invading vessels during corneal NV. The corneal stroma is about 500 μ m thick and consists of regularly arranged lamellae of collagen bundles (200 to 300 centrally and 500 in the periphery).⁴² These bundles are surrounded by a proteoglycan ground substance.^{43,44} The lamellae are arranged in layers parallel to each other as well as to the central corneal surface. They are generally thought to run from the limbus, although this arrangement is less precise in the anterior third of the stroma, where there is greater interweaving of the lamellae, which pass forward obliquely to be inserted into Bowman's layer.

Many unique structural features of the cornea have evolved over time, providing clarity, smooth surface transparency, impermeability to large molecules, tectonic strength, and protection. The combination of tissue transparency and tensile strength is

achieved by having collagen fibrils of uniform diameter and maintaining them at close periodicity, which is highly dependent on the state of stromal hydration. Stromal hydration is normally about 3.5 g H₂O/g dry weight and increases linearly with increasing corneal thickness. The corneal stroma has an inherent tendency to imbibe water and swell. This property reflects the water-binding capacity of the proteoglycans in the ECM. Swelling pressure is inversely related to corneal thickness. For example, a cornea of 150% normal thickness has a swelling pressure of only 15 mm Hg as compared to 55 mm Hg in the nonedematous cornea. Conversely, compression of the cornea, by any mechanism, is associated with an increase in stromal swelling pressure.^{3,45-49}

In human corneal NV, the blood vessel lumina vary widely in diameter, ranging between 2 and 313 μ m (averaging 27 μ m), and in basement membrane thickness, ranging between 0.02 and 1.98 μ m.⁵⁰ Three months after the onset of corneal NV, pericytes are seen in approximately 80% of the corneal vessels. The pericytes coverage index (PCI) approaches 100% in more established corneal vessels.⁵⁰

The relationship between corneal NV and corneal edema was reported by Cogan in 1949.⁴⁹ He observed that in thermal injury, corneal edema always preceded corneal NV and postulated that the pathogenesis of corneal NV is the swelling of the corneal stroma after injury. Cogan also observed that corneal NV occurred after removal of the corneal endothelium and repeated water injection within the stroma. New vessel formation occurred earlier and more intensely at the site of greatest swelling of the cornea. Engorgement of the limbal vessels was followed by the appearance of saccular aneurysms, which then burst. New vessels then proliferated into the resulting hemorrhages between the corneal lamellae. Cogan hypothesized that distention and bursting of the vessels, which preceded formation of the capillary sprouts, were caused by the decrease of external pressure and subsequent decrease of blood vessel wall support.⁴⁹ He also noted that less compact tissues, such as the conjunctiva, showed early evidence of tissue swelling, engorgement of the vessels, and diffuse hemorrhages within the first 24 hours after a burn.

These observations supported Clark and Clark's⁵¹ work showing that once new vessels developed in a glass chamber, an increase in the volume of the chamber, comparable to a decrease in tissue pressure, resulted in engorgement of vessels and massive capillary proliferation. Cogan's experiments led him to conclude that factors other than corneal swelling (such as inflammation, necrosis, changes in hydrogen ion concentration, and hypoxia) were not significant causes of corneal NV.^{3,49}

Ashton⁵² observed the types of swelling associated with vascularization and determined that in the early stages of corneal swelling, there is accumulation of fluid between intact corneal lamellae, which maintains their normal thickness. In severe swelling, particularly in chemical injuries, the individual lamellae seem to disintegrate into fibrillar strands, becoming widely separated by mucoid material. Corneal NV can occur in the early stages of corneal swelling, but in more severe swelling, there is little to oppose vascular growth, which may be extremely dense. Ashton argued that Cogan's theory that swelling is the sole factor responsible for corneal NV is too simplistic. Ashton supported his concept with experiments performed by Langham⁵³ showing that intracameral injections of alloxan caused corneal blood vessels to cease their growth in the central part of the cornea, while the thickness of the cornea was still above normal. Ashton concluded that cessation or reversal of vascularization depended on the removal of certain vasoformative stimuli and/or the recovery of normal tissue compactness.^{54,55}

Although Langham's work using the intracameral alloxan technique⁵³ supported the pathogenetic role of corneal swelling in corneal NV and despite its confirmation by Heydenreich,⁵⁶ Bessiere and Teulieres⁵⁷ were unable to induce vascularization after the intracorneal injection of Vaseline or oil. Ashton⁵² explained this apparent contraindication by suggesting that such lipid substances may act as physical barriers to vessel growth.

Levene and coworkers⁵⁸ created corneal NV in rabbits using an encircling equatorial rubber band. Stromal swelling and cellular infiltration were associated with new vessel growth. The lactic acid concentration of the peripheral ring increased to that of the normal central disc, and the level of both corneal portions remained constant throughout the three stages of corneal NV (stage I: pregrowth; stage II: active vessel growth; stage III: cessation of vessel growth). The degree of thickening paralleled the vascular growth and persisted throughout the entire course of events. The thickening was caused by stromal hydration, and the affected areas showed a decrease in intensity of eosin staining. The authors concluded that corneal NV is only one component of a broader response to injury. Thus, swelling can occur without vascularization, but new vessel growth without edema was not conclusively demonstrated.

After the concept of corneal edema as a necessary factor for corneal NV had been established, Maurice and associates⁵⁹ tested the stimulus required to provoke vascularization in rabbit cornea. Various experiments were designed to control the swelling of the corneal stroma independently of any trauma. These experiments showed that edema was a necessary, but not sufficient, condition for the growth of vessels into the cornea. Further consideration suggested that the liberation of a certain chemical substance (vessel stimulating factor [VSF]) from the wounded tissue is necessary to provoke vessel growth.

Many clinical observations were published to elucidate the relationship between corneal edema and corneal NV. Rowson and associates⁶⁰ reported four cases of hydrops in keratoconic corneas; all developed corneal NV after the development of the "inferior" hydrops near the limbus. It took 4 weeks for the corneal NV to develop, and it persisted after the disappearance of the hydrops, consistent with the relationship between corneal edema and corneal NV. Baum and Martola⁶¹ studied patients with chronic, long-standing corneal edema reaching the limbal area without having evidence of corneal NV. Peripheral corneal thickness adjacent to the limbus was measured and compared to edematous peripheral corneas with clinically evident corneal NV. Both groups had comparable peripheral corneal thickness.

A similar situation is seen in patients with Brown-McLean syndrome,⁶² in which there is long-standing peripheral corneal edema sparing the superior and central cornea. Most patients are women who have had intracapsular cataract surgery. Corneal NV seldom occurs despite the long-standing presence of stromal edema in the vicinity of limbal vessels.⁶³⁻⁶⁵ These observations suggest that corneal edema alone might not be sufficient to produce corneal NV.

CORNEAL EPITHELIAL CELL BIOLOGY AND AVASCULARITY: EXPERIMENTAL STUDIES SUGGESTING AN ANTIANGIOGENIC ROLE OF THE CORNEAL EPITHELIUM

The corneal epithelium normally does not induce corneal NV, and ophthalmologists have long recognized that blood vessels can grow into corneas devoid of epithelium.⁶⁶ Early research suggests that the corneal epithelium may be a source of angiogenic factors⁶⁷⁻⁷⁰: (1) corneal epithelial injury and vascularization frequently coexist; (2) the vascular endothelium in thermally injured skin has a higher labeling index (³H-thymidine) beneath foci of incomplete re-epithelialization compared to nonepithelialized injured sites^{71,72}; and (3) homogenates of freshly excised or cultured normal corneal epithelial cells induce NV when infused into the peripheral cornea.⁶⁶

Eliason and Elliott⁶⁸ performed experiments using rabbit vascular endothelial cells in culture. Rabbit corneal epithelial homogenate, medium exposed to corneal epithelial cultures (epithelial-conditioned medium), and corneal keratocyte homogenate were tested for their mitogenic potential. Both corneal epithelial homogenate and epithelial-conditioned medium significantly increased proliferation of vascular endothelial cells. Keratocyte homogenate, when prepared in the same manner, was not mitogenic.

Additional experiments by the same group suggested that the corneal epithelium is the source of a heat-stable, growth-stimulating factor that can act directly upon vascular endothelial cells. More recent experiments using cultured vascular endothelial cells and tissue explants question Eliason's conclusion (Table 3). Kaminski and Kaminska⁷³ and Ma and associates⁷⁴ have reported that corneal epithelium has an angiostatic rather than an angiogenic effect (Table 4).

In the three-cell-type coculture system consisting of human umbilical vein endothelial cells, fibroblasts, and epithelial cells, limbal epithelial cells (but not conjunctival epithelial cells) exhibited a strong inhibitory effect on fibroblast-induced vascular endothelial cell tube formation.⁷⁵ A similar corneal epithelial antiangiogenic effect was noted by Nakayasu and associates.⁷⁶

TABLE 3. COMPARISON OF VARIOUS POTENTIAL SOURCES OF VASCULAR ENDOTHELIAL CELLS WITH RESPECT TO THE FEASIBILITY OF EXPERIMENTAL INVESTIGATIONS

	CPAEC	MPAEC	HUVEC	AORTIC RING
Ease of culture	+	+	+	-
Tube formation assay	+	+	+	+
Proliferation assay	+	+	+	-
Migration assay	+	+	+	+/-
Co-culture assay	+	+	+	+
Use of KOs	-	-	-	+
Reproductivity	+/-	+/-	+/-	+
Quantification	+/-	+/-	+/-	+

CPAEC, calf pulmonary artery endothelial cells; HUVEC, human umbilical vein endothelial cells; KO, knockout; MPAEC, mouse pulmonary artery endothelial cells; - = difficult to perform; ± = intermediate ease; + = no difficulty.

TABLE 4. EFFECT OF EPITHELIAL AND STROMAL CELLS ON ENDOTHELIAL PROLIFERATION, MIGRATION, AND TUBE FORMATION

CELL TYPE	EFFECT	REFERENCES
Epithelial cells	Inhibitory	73, 74
Epithelial cells	Stimulatory	67, 68, 70
Limbal cells	Inhibitory	74
Stromal keratocytes	Inhibitory	67, 68
Stromal keratocytes	Stimulatory	74, 76, 240
Conjunctival epithelium	Inhibitory	74
Skin keratinocytes	Stimulatory	241, 242

POST-TRAUMATIC CORNEAL INFLAMMATION AND INNATE IMMUNITY

In an experimental study of corneal NV in hamster cheek pouches, Klintworth⁷⁷ found that the vascular invasion of a variety of corneal explants was preceded by leukocytic infiltration into the corneal stroma. Subsequently, a series of comparative studies was conducted by Fromer and Klintworth.⁷⁸⁻⁸⁰ They designed experimental models of corneal NV via exposure of the cornea to noxious agents, including alloxan, sodium hydroxide, and silver nitrate. These investigators confirmed that a leukocytic infiltration preceded

the onset of vascular invasion.⁷⁸ Rats with leukocyte elimination (either by total body X-irradiation or by subconjunctival injection of methylprednisolone acetate) did not develop corneal NV after silver nitrate burning, further implying that leukocytes serve a crucial function in corneal vascularization.⁷⁹ In further experiments in which different leukocytes were injected intracorneally to total body X-irradiated rats, it was found that polymorphonuclear leukocytes had the potential of initiating corneal NV by releasing chemical mediators.⁸⁰

However, Eliason⁶⁷ and Sholley and associates⁷⁵ reported conflicting results in a similar series of experiments and questioned the role of the polymorphonuclear leukocyte in the NV process. Eliason suggested that the epithelium was the source of a vasostimulating substance⁶⁷ and concluded that the epithelial homogenate was able to provoke corneal vascularization in the absence of leukocytes.⁸¹ Schanzlin and associates⁸² developed a quantitative model examining the inflammatory cell response to thermal corneal injury. They reported that neovasculogenic peripheral burns elicited a polymorphonuclear cell response that peaked within 24 hours at the site of injury as well as in the perilimbal conjunctiva. At 24 hours after thermal burning, a wave of polymorphonuclear cells began to migrate across the stroma of the cornea, reaching the cautery site within 72 hours. This wave of polymorphonuclear cells preceded the appearance of NV at the limbus. It appeared that the migration of the new corneal blood vessels followed the polymorphonuclear cell infiltration through the corneal stroma. In contrast, the nonvasculogenic central corneal burns elicited a mononuclear cell response in the first 24 hours. This cellular reaction was not accompanied by corneal NV. These findings supported the concept of Fromer and Klintworth that polymorphonuclear neutrophil releases a vasculogenic substance.

Ryu and Albert⁸³ performed sequential histopathological observations after implantation of viable and nonviable tumor cells in the rabbit corneal stroma. In the majority of the eyes containing either viable or nonviable cells, vascularization was preceded by an inflammatory response. In all cases, the extent of the corneal NV is correlated with the degree of inflammation. In rabbits made immune-deficient by radiation, there was negligible inflammation and vascularization when the tumor was implanted.

Although conventional therapy for corneal NV relies mainly on anti-inflammatory corticosteroids, steroids do not inhibit angiogenesis directly.⁸⁴ Where inflammation is not the cause of angiogenesis, hydrocortisone has little or no effect on capillary growth. Examples of noninflammatory angiogenesis are tumor-induced neovascularization and developing vessels in the chick embryo chorioallantoic membrane. Hydrocortisone can be converted to a potent angiogenesis inhibitor by coadministration with heparin or with a sulfated cyclodextrin.⁸⁴⁻⁸⁷ Hydrocortisone-cyclodextrin drug pairs suppressed virtually all inflammatory cell infiltration (induced by endotoxin), whereas tetrahydrocortisol-cyclodextrin pairs only partially reduced inflammation. These results demonstrate that corneal NV and corneal inflammation are related, but distinct, processes.⁸⁸

These studies demonstrate a clear association between corneal inflammation and NV. However, corneal inflammation may occur without NV and corneal NV may occur in the absence of inflammation.

ROLE OF CORNEAL TEMPERATURE, UV AND VISIBLE IRRADIATION, INNERVATION, AND AQUEOUS HUMOR ON CORNEAL AVASCULARITY

It is not clear from the literature whether corneal temperature, UV and visible irradiation, innervation, and aqueous humor contribute to increased or decreased corneal NV. Except for the aqueous humor theory that may pertain to corneal hydration, these factors may be difficult to put into context without additional investigation regarding potential contributions to the maintenance of corneal avascularity.

ANGIOGENIC FACTORS AND CORNEAL NV: THE ROLE OF VEGF AND FGF IN CORNEAL NV

Several angiogenic molecules expressed during corneal wound healing have been identified, including VEGF and bFGF. VEGF is upregulated in inflamed and vascularized corneas in both human and animal models.⁸⁹⁻⁹¹ VEGF was initially identified as a stimulator of vascular permeability (called VPF, for vascular permeability factor) and was subsequently demonstrated to be an endothelial cell-specific mitogen and angiogenic factor. VEGF expression has been correlated with embryonic, physiologic, and pathologic blood vessel growth in vivo.⁹²⁻⁹⁴ The spatial and temporal expression patterns of VEGF and its tyrosine kinase receptors, *flt-1* and *flk-1/KDR*, in several systems suggest that VEGF is a key mediator of vasculogenic and angiogenic events associated with a wide range of biological events.⁹⁵⁻⁹⁷ Local and systemic signals (responsible for orchestrating the growth and regression of new blood vessels) regulate VEGF gene expression, including cAMP, steroid hormones, protein kinase C agonists, polypeptide growth factors, oxygen, free radicals, glucose, cobalt, and iron. These agents (protein kinase C agonist polypeptide growth factor, oxygen, free radical) modulate bFGF gene expression via transcriptional regulation through transcription activator protein-1 (AP-1), AP-2, p53, and NFκB.⁹⁸⁻¹⁰⁰

The secreted VEGF growth factor peptides are generated by alternative splicing into five isoforms: VEGF115, VEGF121, VEGF165, VEGF189, and VEGF206.¹⁰¹ Additional VEGF members include VEGF-B, VEGF-C, and VEGF-D, which bind differentially to VEGF receptors and regulate angiogenesis and lymphangiogenesis.¹⁰²⁻¹⁰⁶

VEGF-B is an inefficient vascular endothelial cell mitogen. It binds to the receptor VEGFR-1, but not to VEGFR-2 or -3. VEGF-C and -D are mitogenic for vascular endothelial cells. They activate VEGFR-3 and are involved in the regulation of the growth and/or differentiation of lymphatic and blood vessel endothelium. VEGF is produced by pericytes, fibroblasts, macrophages, T cells, retinal pigment epithelial cells, astrocytes, and smooth muscle cells. VEGF121 and VEGF165 are efficiently exported from the cell; VEGF189 and VEGF206 are predominantly cell-associated and very poorly secreted. Vascular permeability activity was detected for all forms of VEGF, and vascular endothelial cell mitogenic activity was apparent only with VEGF121 and VEGF165 isoforms. In addition, VEGF121 is more angiogenic than VEGF165 and VEGF189. Thus, alternative splicing of VEGF RNA can produce

polypeptides with strikingly different secretion patterns, which suggests multiple physiologic roles for this family of proteins.^{107,108}

The expression of VEGF is highly regulated. Enhanced VEGF production has been shown in hypoxia and inflammatory response. An overproduction of VEGF has been identified in tumor cell proliferation. In addition, an induction of VEGF expression was found in malignant transformation of cultured cells. Similarly, several reports demonstrate an upregulation of VEGF in vascularized corneas. VEGF expression was localized to corneal epithelial cells, corneal endothelial cells, vascular endothelial cells of limbal vessels, and keratocytes. In addition, VEGF expression has markedly increased in epithelial cells of inflamed corneas, vascular endothelial cells, macrophage infiltrates, and fibroblasts in corneal scar tissue. VEGF concentrations were significantly higher in vascularized corneas than in normal control corneas.¹⁰⁸

Additionally, VEGF promotes several steps of angiogenesis, including proteolytic activities (dissolution of the membrane of the original vessel), vascular endothelial cell proliferation, migration, and capillary tube formation. The importance of VEGF in corneal angiogenesis was demonstrated by the inhibition of NV after stromal implantation of an anti-VEGF blocking antibody in a rat model. This result has been reproduced using VEGF blocking peptides in a rabbit corneal model.^{109,110}

bFGF, discussed above in the context of stromal fibroblast transformation and noninflammatory models of corneal NV, is another potent angiogenic factor. bFGF has been extensively used in models of corneal angiogenesis. It is a member of the FGF family, which encompasses 23 structurally related heparin-binding peptides widely expressed in developing and adult tissues during cellular differentiation, angiogenesis, mitogenesis, and wound repair. bFGF is upregulated after tissue injury and in stromal fibroblast/vascular endothelial cell cocultures.¹¹¹ The potential repertoire of FGF-mediated intracellular signaling events is significantly increasing, and different FGF receptor isoforms display different biological functions.¹¹² In addition, tissue-specific FGFR expression exhibited the diversity of its biological response, which is regulated through differences in ligand specificity and function. The regulation of growth factor receptor activity plays an important role in the regulation of complex physiological processes.¹¹³

FGF function is mediated by binding its cognate receptors (FGFR-1, -2, -3, and -4). FGF-1 is expressed in the normal corneal epithelium, and bFGF is upregulated after injury and during keratocyte-vascular endothelial cell coculture. Interestingly, bFGF binds to Bowman's and Descemet's membranes in normal corneas and vascular basement membranes in neovascularized corneas.¹¹⁴ The level of FGF binding is related to the stage of maturation of new vessels, as differential FGF binding has been demonstrated. The difference in binding seen between normal limbal vessels and newly-formed corneal vessels is probably due to a different expression of heparan sulfate proteoglycans, emphasizing the role of the ECM components in the regulation of corneal angiogenesis.¹¹⁵

PROANGIOGENIC MMPs AND CORNEAL NV

MMPs have also been implicated in the support of angiogenic processes (Table 5). Although their upregulation during corneal angiogenesis has been demonstrated, their role in the regulation of angiogenesis may appear ambiguous, because the same molecule could alternately act as a proangiogenic or antiangiogenic factor. Accordingly, activation of MMP-2 may release antiangiogenic fragments, either allowing the production of a potent angiostatic factor or facilitating angiogenesis. The dual function of MMPs during angiogenesis may be explained by their ability to degrade ECM, allowing tissue invasion by MMP-bearing endothelial cells and by their ability to generate or release antiangiogenic fragments from their precursors, which otherwise lack angiogenic properties.^{13,116-120} Several reports have suggested a role of MMP-2 and -9 and MT1-MMP in vascular invasion by direct matrix degradation or through the release of matrix-bound cytokines or growth factors. MMP-9 and MT1-MMP knockout (KO) mice have delayed blood vessel formation during development. MMP-9 enzymatic activity has been implicated in the mediation of corneal NV.^{90,121,122} Inhibition of MMP-9 activity in the cornea is associated with a reduction in the angiogenic response.¹²³ MMPs that have a transmembrane domain are known as membrane-type MMPs (MT-MMPs). MT-MMPs¹²⁴ may be responsible for processing many biologically important proteins on the cell surface. Perhaps the most commonly involved MMP in angiogenesis is the membrane-type MMP, MT1-MMP. Impaired corneal NV by bFGF was observed in MT1-MMP-KO mice.¹¹¹ Localization of several MMPs in the cornea during wound healing has been reported. Members of the MMP family include MT-1-, -2-, -3-, -4-, -5-, and -6-MMP (MMP-14, -15, -16, -17, -24, and -25).¹²⁵⁻¹²⁹ Various reports cite the regulation of MT1-MMP by cytokines such as tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), epidermal growth factor (EGF), and bFGF.

Concanavalin A mediates the upregulation of MT1-MMP in a c-Ras-dependent manner. Upregulation of MT1-MMP in fibroblasts grown in relaxed collagen lattices suggests that MT1-MMP synthesis may also be regulated by the cytoskeleton and mediated by the lack of stress fibers in those cells.¹³⁰ MT1-MMPs play a major role in the activation of proMMP-2. For example, MT1-MMP is important in ECM remodeling through activation of proMMP-2 and direct cleavage of some ECM macromolecules such as gelatin, type I collagen, and fibronectin (Table 6). Tissue inhibitor metalloproteinase-2 (TIMP-2) plays an important role in proMMP-2 activation by binding to MT1-MMP.

In situ hybridization has been used to determine the expression of MT1-MMP in the cornea,¹³¹ and it has been shown to be expressed by stromal keratocytes during wound healing. Studies using plasma membrane extract from HT1080 cells containing MT1-MMP or a transmembrane-deleted form of MT1-MMP have shown that, at low concentrations, TIMP-2 stimulates proMMP-2 activation, whereas at high concentrations it inhibits activation. In addition, cross-linking experiments using plasma membrane preparations demonstrated binding of TIMP-2 to MT1-MMP and to the hemopexin-like domain of proMMP-2. Williamson and associates^{132,133} experimented with mutant forms of TIMP-2, specifically altering or deleting the amino acid sequence in the AB loop in the molecule and performing kinetic analysis on these molecules to determine the rate of association and the degree to which these

TABLE 5. LOCALIZATION AND PROPERTIES OF MATRIX METALLOPROTEINASES IN THE CORNEA

MMP NO.	ENZYME NAME	CORNEAL LOCATION	ANGIOGENIC PROPERTY	REFERENCES
MMP-1	Interstitial collagenase-1	Epithelium, ant stroma, fibroblasts		229-232
MMP-2	Gelatinase A	Epithelium and stroma (normal), basal epithelium superficial stroma (wounded)	Pro-angiogenic	11,131,194,231
MMP-3	Stromelysin-1	Epithelium, basement membrane, stroma (diabetics)		233, 234
MMP-7	Matrilysin	Epithelium	Anti-angiogenic	12, 198
MMP-8	Neutrophil Collagenase-2	Epithelium		235
MMP-9	Gelatinase B	Basement membrane and superficial stroma	Pro-angiogenic	131
MMP-10	Stromelysin-2	Epithelium and stroma (diabetics)		
MMP-11	Stromelysin-3			
MMP-12	Macrophage metalloelastase	Corneal fibroblasts (in vitro)	Pro-angiogenic	236
MMP-13	Collagenase-3	Deep stroma		198
MMP-14	MT1-MMP	Basal epithelial cells and stromal keratocytes	Pro-angiogenic	111, 131, 237
MMP-15	MT2-MMP	Epithelium		238
MMP-16	MT3-MMP			
MMP-17	MT4-MMP	Not present in normal, Substantia propia (infected cornea)		239
MMP-18	Xenopus collagenase 4			
MMP-19	RASI-1			
MMP-20	Enamelysin			
MMP-23	CA-MMP			
MMP-24	MT5-MMP	Epithelium (normal), substantia propia (infected cornea)		239
MMP-25	MT6-MMP Leukolysin	Infiltrating leukocytes		239
MMP-26	Matrilysin-2			
MMP-28	Epilysin			

mutants bind to MMP-2 and MT1-MMP. The most dramatic results were produced when tyrosine-36 in the AB loop was substituted with other amino acids, resulting in decreased affinity and binding rates of MT1-MMP with mutant TIMP-2 when compared to wild-type TIMP-2. Based on these observations, a model for the activation of proMMP-2 has been proposed in which the catalytic domain of MT1-MMP binds to the N-terminal portion of TIMP-2, leaving the negatively charged C-terminal region of TIMP-2 available for binding the hemopexin-like domain of proMMP-2. It has been suggested that this ternary complex clusters proMMP-2 at the cell surface near a TIMP-2-free active MT1-MMP molecule, which is thought to initiate activation of the bound proMMP-2. ProMMP-2 activation would occur only at low TIMP-2 concentrations relative to MT1-MMP, which would permit availability of active MT1-MMP to activate the proMMP-2 bound in the ternary complex (Table 7).

TABLE 6. COMPARISON OF THE ACTIVITY OF MT1-MMP AND MMP-2 ON EXTRA CELLULAR MATRIX DEGRADATION

ECM COMPONENT	MT1-MMP ACTION	MMP-2 ACTION
Collagen I	+	+
Collagen III	+	+
Collagen V	-	+
Collagen VI	-	+
Fibronectin	+	+
Laminin	+	-

ECM, extracellular matrix; MMP, matrix metalloproteinase; MT, membrane-type ;
- = no degradation has been reported; + = degradation has been reported.

TABLE 7. LINES OF EVIDENCE SUGGESTING MMP-2 MEDIATED PATHWAYS OF MT1 MMP AND MMP-2 INDEPENDENT ENZYMATIC ACTIVITY

	MMP-2 MEDIATED PATHWAY	MMP-2 INDEPENDENT PATHWAY
Mechanism	MT1-MMP activates proMMP-2	Direct MMP-13 VEGF
TIMP-2	Concentration dependent	NA
Synthetic inhibitor (BB94)	++	+
MTI-MMP KO data	↓NV Tumor growth	↓NV
Bone effect	-	++
Vascular EC migration	++	±
ECM degradation	++	+

ECM = extracellular matrix; EC = epithelial cells; KO = knockout; MMP, matrix metalloproteinase; MT = membrane-type; NA = not available; NV = neovascularization; VEGF = vascular endothelial growth factor; - = no effect; ± = intermediate effect; + = limited effect; ++ = strong effect.

Certain synthetic MMP inhibitors work in unison with TIMP-2 to promote proMMP-2 activation using MT1-MMP due to the inhibitors' affinity for MT1-MMP.¹³⁴ Procollagenase-3 is activated in much the same way as proMMP-2. MT1-MMP is also capable of hydrolyzing a variety of ECM components, such as interstitial collagens, fibulin-1 and -2, fibronectin, and others. TIMP-2 and TIMP-3 are effective inhibitors of MT1-MMP, shown by the decrease in proMMP-2 and proMMP-13 activation in the presence of either inhibitor in a concentration-dependent manner.

Several angiogenic factors are localized to the cornea. In the in vivo models, implantation or injection of angiogenic factors, such as bFGF and VEGF, caused corneal NV. In injured or diseased corneas, a correlation between elevated angiogenic factors and corneal NV has been documented. This suggests that angiogenic factors play a role in corneal NV. Corneal clarity under normal conditions suggests that the angiogenic and antiangiogenic factors may be in equilibrium until injury tilts the balance more toward production of angiogenic vs antiangiogenic factors, resulting in commensurate neovascular sequelae.

LIMBAL BARRIER FUNCTION AND CORNEAL AVASCULARITY

By definition, the limbus is the transition zone forming the border between the opaque sclera and the transparent cornea. However, there are no definite, reliable boundaries of the limbus. Various anatomic definitions of the limbus have been offered by anatomists, pathologists, histologists, and surgeons.^{135,136} The broadest definition of the limbus is the zone between a line drawn between the termini of Bowman's layer and Descemet's membrane, which forms the anterior border, and a parallel line approximately 1 mm posterior to the anterior line, passing through the posterior end of Schlemm's canal.¹³⁷

The precise anatomy and function of the limbus have raised controversy since 1859, when Manz first described limbal epithelial structures in swine, which he believed to be glands. Duke-Elder¹³⁸ considered the limbal structures in humans to be functionless vestigials of the glands of Manz. In 1971, Davanger and Evensen¹³⁹ first offered the concept that epithelial cells in the limbal region are involved in the renewal of corneal epithelium.

In 1977, Thoft and Friend¹⁴⁰ coined the term "ocular surface" to describe the continuous epithelial sheet that covers the eye. This term implies that these morphologically distinct regions of epithelium are closely linked in their responses to pathologic insults.¹⁴¹

There are three distinct types of ocular surface: conjunctival, limbal, and corneal epithelium.^{142,143} The limbal epithelium behaves differently from the corneal and conjunctival epithelia in ocular surface wound healing. Like the regenerated epithelium of corneal origin, the regenerated epithelium of limbal origin increased in thickness with time and showed no goblet cell appearance after wounding. However, analysis of protein expression and glycogen content demonstrated that regenerated epithelium of limbal origin had characteristics intermediate between those of corneal and bulbar conjunctival origin.¹⁴²

The limbal epithelium shares many features with the corneal epithelium. It is a stratified, squamous, nonkeratinizing epithelium whose cell junctions have apical and basal specializations similar to those in the cornea.¹⁴⁴ The basal layer of the limbus appears unique and is believed to be the location of corneal epithelial stem cells.^{139,145}

The concept of the limbal location of corneal epithelial stem cells has received support from a large body of experimental and clinical data.¹⁴⁶⁻¹⁴⁸ According to this model, the limbal basal epithelium serves as the continuous source of corneal epithelial proliferation and differentiation by providing an unlimited supply of transient amplifying cells. Extrapolating on this concept, it has been assumed that the limbus may prevent corneal NV by acting as a barrier to conjunctival overgrowth of the cornea. In other words, the ability of the stem cells to replenish the rapid self-renewal of the corneal epithelium allows the perilimbal zone to preclude the invasion of conjunctival epithelium onto the corneal surface, thereby preventing corneal NV under normal circumstances. This concept of growth pressure was first mentioned in 1951 by Friedenwald.¹⁴⁹

The limbal barrier hypothesis has been used to explain corneal NV in the setting of experimental limbal damage^{150,151} and clinical limbal stem cell dysfunction.^{147,152,153} It is one of the explanations given as a basis of corneal pannus formation in aniridia.¹ It is also a theoretical basis for the efficacy of limbal stem cell transplantation as a definitive surgical treatment of ocular surface disorders.¹⁵⁴ However, recent findings suggest that the concept of the limbus as merely a physical barrier may not be an adequate explanation of corneal avascularity.¹⁵⁵

CORNEAL ANTIANGIOGENIC MOLECULES AS THE BASIS OF CORNEAL ANGIOGENIC PRIVILEGE: THE ROLE OF ANGIOSTATIN, RESTIN, ENDOSTATIN, ARRESTIN, CANSTATIN, TUMSTATIN, AND PEDF

Several antiangiogenic molecules have been either detected or tested in the cornea. They may be derived from larger precursors by proteolytic cleavage or directly produced in their active forms.¹⁵⁶ Angiostatin, a 38 kDa proteolytic fragment of plasminogen, is a potent antiangiogenic factor.¹⁵⁷ Implantation of angiostatin and angiostatin-like fragments in the eye inhibits corneal NV induced by bFGF or angiogenin.^{158,159} Endostatin, another antiangiogenic factor, is a 20 kDa proteolytic fragment of collagen XVIII.¹⁶⁰ Endostatin has been isolated from the conditioned medium of a murine hemangioendothelioma cell line; it inhibits bFGF and VEGF-induced vascular endothelial cell migration and proliferation in vitro and reduces tumor progression in mice.¹⁶⁰ Addition of endostatin or other antiangiogenic molecules into the pellets significantly suppressed bFGF-induced angiogenesis in corneal pocket assay.^{9,161,162}

Angiostatin

Angiostatin represents a molecule generated by a primary tumor to inhibit both primary and secondary tumor growth. Recombinant angiostatin has been used successfully to suppress tumor growth and metastasis in animal model systems.^{159,163-167} Initially, plasminogen is converted to A chain (N-terminal kringle domains) and B chain (serine protease plasmin) by cleavage of a single Arg⁵⁶¹-Val⁵⁶² peptide bond. The A chain of plasminogen is then further processed by several MMPs and cathepsin to generate kringle-containing fragments. One of the enzymes responsible for the generation of angiostatin in Lewis lung carcinoma has been identified as macrophage-derived metalloelastase (MMP-12). The cleavage sites for these enzymes have been determined. Human matrilysin (MMP-7) and human neutrophil MMP-9 also convert human plasminogen to angiostatin fragments. The cleavage sites in plasminogen by MMP-7 and MMP-9 are located between the fourth and fifth kringle domains. In addition, an angiostatin-like fragment (containing kringles 1-4) can be generated from plasminogen with stromelysin-1 (MMP-3). MMP-3 hydrolyzes the Glu⁵⁹-Asn⁶⁰, Pro⁴⁴⁷-Val⁴⁴⁸, and Pro⁵⁴⁴-Ser⁵⁴⁵ peptide bonds in plasminogen, yielding a 55 kDa NH₂-terminal fragment comprising kringles 1 through 4.¹⁶⁸⁻¹⁷⁰

Gabison and associates⁹ have demonstrated the involvement of angiostatin in corneal avascularity after wounding. They confirmed that angiostatin-like molecules were expressed in the corneal epithelium and in cultured corneal epithelial cells. Western blotting after incubation of scraped corneal epithelial cell lysate with purified plasminogen showed reduction of the plasminogen bands at 6, 12, and 24, hours, respectively. Corneal neovascularization was observed after excimer laser keratectomy when antiangiostatin antibodies were injected into the cornea, which was significantly higher than when plasmin B chain antibodies were injected. These studies suggest that angiostatin may contribute to the maintenance of corneal avascularity after excimer laser keratectomy.

Several angiostatin-associated proteins have been identified and implicated for their functions in the regulation of angiogenesis. Angiostatin binds to ATP synthase and downregulates vascular endothelial cell proliferation and migration. Angiostatin also binds to integrin $\alpha_v\beta_3$ and affects angiogenesis as well as developmental NV. Disruption of integrin $\alpha_v\beta_3$ ligation with neutralizing antibody LM609 or peptide antagonists of integrin $\alpha_v\beta_3$ affects blood vessel formation. In addition, plasmin specifically binds to integrin $\alpha_v\beta_3$ through its kringle domains like angiostatin and induces vascular endothelial cell migration. The induced vascular endothelial cell migration can be blocked by anti-integrin $\alpha_v\beta_3$ agents and a serine protease inhibitor.^{171,172}

Angiostatin induces vascular endothelial cell apoptosis, and cells have been shown to be arrested at the G₂/M transition interface in the presence of angiostatin.¹⁷³ Administration of angiostatin to tumor-bearing mice has not resulted in detectable systemic cytotoxicity; only angiogenic proliferation appears to be inhibited. Angiostatin, therefore, appears to be an effective and nontoxic inhibitor of NV.¹⁷⁴

Restin and Endostatin

Collagen XV and XVIII are identified as chondroitin sulfate and heparan sulfate proteoglycans, respectively. They belong to a family of collagen-like proteins, referred to as the multiplexins, and are localized mainly in a perivascular position. Collagen XV is expressed in hearts, skeletal muscles, placentas, and kidneys. Collagen XVIII is expressed in developing and postnatal eyes in basement membrane (BM). Collagen XV-deficient mice displayed a high propensity for exercise-induced muscle injury and progressive degeneration of skeletal muscle with collapsed capillaries. Mice lacking collagen XVIII develop normally and without evidence of abnormal vascular morphogenesis.¹⁷⁵ However, these collagen XVIII-deficient mice developed ocular abnormalities similar to Knobloch syndrome.¹⁷⁶⁻¹⁷⁹

The NC1 domains of collagen XV and XVIII have been isolated from circulating serum. Endostatin, a 20 kDa cleavage fragment of carboxyl-terminal 183 amino acids in the NC1 domain of collagen XVIII, was first identified in the conditioned medium of hemangioendothelioma cells. This fragment has been characterized with antiangiogenic properties. Restin, the carboxyl terminal of collagen XV, also possesses antiangiogenic properties.

Endostatin, endostatin-like fragments, restin, and restin-containing fragments have been isolated from tissue extracts and circulating blood, suggesting that these fragments are physiologic cleavage products. The hinge domain of NC1 fragments contains cleavage sites for MMPs and cathepsin L. MMP-3, -7, -9, -12, -13, and -20 have been characterized as cleaving at the hinge region of the NC1 domain. It has been proposed that MMPs cleave the hinge region of the NC1 domain and that the resulting endostatin-containing fragments are further processed by cathepsin L to generate mature endostatin.^{13,162,180-182}

Endostatin has been shown to associate with tropomyosins, integrins, VEGF receptor, MMPs, and glypicans in the antimigratory and proliferative effects on vascular endothelial cells. Endostatin causes endothelial cell cycle arrest in G₁. Binding of endostatin to tropomyosins may play important roles in a variety of cellular functions, including contraction, cytokinesis, intracellular transport, secretion, motility, morphogenesis, and cell transformation. Endostatin blocks VEGF-induced tyrosine phosphorylation of KDR/Flk-1 and activation of ERK, p38 MAPK, and p125^{FAK}, which are downstream events of KDR/Flk-1 signaling and are involved in the mitogenic and motogenic activities of VEGF in vascular endothelial cells. Endostatin inhibits the binding of VEGF to vascular endothelial cells and to its cell surface receptor, KDR/Flk-1. Binding of endostatin to KDR/Flk-1 but not VEGF suggests a direct interaction of endostatin with KDR/Flk-1, which blocks the binding of VEGF to vascular endothelial cells.¹⁸³ In addition, endostatin treatment increased the activity of the intracellular protease caspase 3, enhancing vascular endothelial cell apoptosis.¹⁸⁴

Arresten, Canstatin, and Tumstatin¹⁸⁵⁻¹⁸⁸

The BM assembly is a thin, sheet-like, highly specialized structure of ECM that separates the epithelial cells from the stroma. Type IV collagen, the major BM component, demonstrates a stage- and position-specific distribution of its isoforms during development. These BM proteins in the basement membrane act as regulators of specific biological functions such as cellular growth, differentiation, repair, and migration, as well as modulators of pathological events such as tumor cell differentiation, invasion, and metastasis.¹¹⁶⁻¹¹⁹ Type IV collagen is composed of six distinct polypeptide chains.

In human BM, there are at least three molecular forms of type IV collagen that are tissue-specific in their distribution: $[\alpha 1(\text{IV})]_2/\alpha 2(\text{IV})$, which is ubiquitous in all BM; $\alpha 3(\text{IV})/\alpha 4(\text{IV})/\alpha 5(\text{IV})$, which is abundant in lung alveoli and glomerular BM; and $\alpha 5(\text{IV})/\alpha 6(\text{IV})$, which is localized in the BM of mammary ducts and lobules, epidermis, prostate glands, and smooth muscle cells. Type IV collagen promotes cell adhesion, migration, differentiation, and growth and through these functions may play a crucial role in angiogenesis. Molecular defects in type IV collagen have been linked to Goodpasture's syndrome, an autoimmune disease characterized by glomerulonephritis and pulmonary hemorrhages, Alport's syndrome, a genetic disease with progressive glomerulonephritis, and diffuse esophageal leiomyomatosis, characterized by benign proliferation of smooth muscle.¹⁸⁹

Loss of BM components is a hallmark of invasive lesions, and a disrupted collagen IV labeling of BM has been shown to precede tumor invasion in lung cancers. This disruption of BM components that occurs during tumor progression may be due to their degradation by proteolytic enzymes. Enzymatic degradation of type IV collagen $\alpha 1(\text{IV})$ in BMs triggers cell motility and enhances local tumor progression. In lung cancers, stromal cells are the principal source of synthesis of $\alpha 1(\text{IV})$ chains, and interaction between the tumor cells and the ECM could modulate their invasive capacity.¹⁹⁰

The C-terminal globular noncollagenous (NC1) domain of type IV collagen could disrupt tumor angiogenesis and thereby inhibit tumor growth. Canstatin, the NC1 domain of $\alpha 2(\text{IV})$, inhibits endothelial cell tube formation, migration, and proliferation, induces apoptosis *in vitro*, and suppresses tumor growth *in vivo*. Similarly, synthetic peptides (amino acids 185-203) derived from the NC1 domain of $\alpha 3$ chain of type IV collagen [$\alpha 3(\text{IV})\text{NC1}$] have been shown to inhibit the proliferation of melanoma *in vitro*.¹⁹¹

The regulation of cellular signaling is mediated by the interaction of extracellular matrix proteins with integrin. Binding of the extracellular matrix proteins to integrin is divided into two groups; one is mediated by the binding of the peptide residues RGD (arginine-glycine-aspartic acid) to integrin, and the other does not depend on these RGD residues. Binding of extracellular proteins to cellular receptor integrin activates kinase activity of focal adhesion kinase (FAK). Activated FAK subsequently phosphorylates several cytoskeleton molecules and regulates cytoskeleton reorganization and cellular function.

Tumstatin, the C-terminal fragment of $\alpha 3(\text{IV})\text{NC1}$, engages in a novel antiangiogenic activity. Tumstatin binds to $\alpha_v\beta_3$ integrin in a RGD-independent manner, and this binding is essential for its antiangiogenic activity. The angiogenic activity of tumstatin (amino acids 54-132) was localized by using deletion mutagenesis of tumstatin. Tumstatin peptides inhibited protein synthesis by inhibiting the phosphorylation of FAK induced in endothelial cells by attachment to vitronectin and inhibiting the activation of PI3-kinase through $\alpha_v\beta_3$ binding.¹¹⁶⁻¹¹⁹

Pigment Epithelium-Derived Factor

In the human cornea, PEDF has been immunolocalized to the epithelium and endothelium. PEDF is also widely expressed in a broad range of human fetal and adult tissues, including almost all regions of the brain. PEDF belongs to the serine protease inhibitor family, and PEDF-blocking antibodies implanted in the cornea facilitated corneal NV.¹⁹² In addition, preclearing of human cornea stromal extracts with anti-PEDF antibodies abrogated the inhibition of vascular endothelial cell migration customarily induced by these extracts. Furthermore, recombinant PEDF inhibits bFGF-induced corneal NV. PEDF is also a major NV inhibitor and is responsible for excluding vessels from invading the cornea, vitreous, and retina.

The molecular mechanisms of PEDF in neurotrophic and antiangiogenic activities have been identified. PEDF interacts with receptors on the cell surface and activates the necessary signal transduction events for neurotrophic activities. Several PEDF-binding molecules have been characterized, including glycosaminoglycans and collagen I.¹⁹²

In the eye, there are several large compartments from which blood vessels are completely excluded: the vitreous, the aqueous humor that fills the anterior chamber, and the cornea. PEDF is an essential contributor to the maintenance of avascularity of these ocular tissues. Currently, PEDF is a good candidate for drug development to provide pharmacologic inhibition of ocular angiogenesis, given its effectiveness against multiple inducers of angiogenesis, including VEGF and interleukin-8 (IL-8).

SUMMARY AND HYPOTHESIS

Several of the potential mechanisms described above can be eliminated based on our review of the literature, because they have been disproved on the basis of experimental evidence. We hypothesize that proangiogenic stimuli are present in the cornea in the absence of corneal NV, that they are increased during wound healing, that the stromal keratocytes and fibroblasts are the primary cells expressing proangiogenic factors, and that the cornea has several active mechanisms of generating potent antiangiogenic factors that are produced primarily by the epithelial cells. In addition, we question the concept of the limbal barrier to corneal NV as the basis of corneal angiogenic privilege.

METHODS

CHARACTERIZATION OF PROANGIOGENIC FACTORS (VEGF AND bFGF) IN THE CORNEA

Wild-type mice (C57BL/6) were anesthetized with an intramuscular injection of a 1:1 mixture of ketamine (40 mg/kg body weight) and xylazine and also topical 0.5% proparacaine eye drops. Mouse eyes were treated with excimer laser keratectomy (193 nm argon fluoride, 160 mJ/cm², and 130 to 150 pulses). Immediately after the surgery, 0.5% erythromycin ophthalmic ointment was applied to each treated eye. Mice were sacrificed at 6 hours and 1, 3, 7, 10, and 20 days after wounding.

Electron Microscopy

For electron microscopy, normal and vascularized corneas were fixed in half-strength Karnovsky fixative (2% paraformaldehyde and 2.5% glutaraldehyde) in 0.2 M sodium cacodylate buffer (pH 7.4) overnight and postfixed in 1% osmium tetroxide in 0.2 M sodium cacodylate for 1.5 hours. Following dehydration in graded alcohol, the eyes were embedded in Epon. Ultrathin sections (80 to 90 Å) stained with 2% uranyl acetate and Reynold's lead nitrate were studied by electron microscopy.

Western Blot Analysis with Anti-bFGF and Anti-VEGF Antibodies

Three wild-type normal mouse corneas were dissected from eyes under a surgical microscope, and corneal epithelial cells were collected by scraping. Each sample was homogenized and lysed with extraction buffer containing 50 mM Tris-HCl (pH 8.5), 150 mM NaCl, 1% NP-40, and protease inhibitor combination (Sigma). Samples were electrophoresed on 4% to 20% SDS-polyacrylamide gels and then transferred onto membranes (Immobilon P; Millipore). The membranes were blocked for 30 minutes in blocking buffer (3% BSA in TBST buffer) and probed with primary antibodies, anti-bFGF (Oncogene, Cambridge, Massachusetts) and anti-VEGF (Calbiochem, San Diego, California) for 1 hour. After washing, the membranes were incubated with secondary antibodies (horseradish peroxidase [HRP]-conjugated) and developed with enhanced chemiluminescence (ECL) reagent. X-ray films were scanned using Adobe Photoshop, and densitometry (Quantity One; BioRad) was performed on the band representing the peptides of interest.

Confocal Immunohistochemical Staining With Anti-VEGF and Anti-CD31 Antibodies

Mechanically wounded wild-type mice were used for confocal immunohistochemical staining. Cryosections (8 µm) of mouse corneas were mounted on glass slides (Superfrost/Plus; Fisher Scientific) and kept at room temperature for 30 minutes. A blocking solution (1% BSA in PBS) was applied for 30 minutes at room temperature and followed by incubation with primary antibodies, rat anti-mouse CD31 monoclonal antibody (PharMingen, San Diego, California), and goat anti-mouse VEGF monoclonal antibody (Calbiochem) of 1:100 dilution for 60 minutes. Samples were then washed with PBS, and the secondary antibody, rhodamine-conjugated donkey anti-goat of 1:400 dilution or fluorescein isothiocyanate (FITC)-conjugated, affinity-purified donkey anti-rat IgG of 1:100 dilution (Jackson ImmunoResearch Laboratories), was applied for 30 minutes. After the PBS wash, the specimens were mounted with antifading medium (Vectashield; Vector Laboratories, Burlingame, California). The sections were examined with a confocal microscope (TCS4D; Leica, Heidelberg, Germany). Negative control samples (without primary antibody) were similarly processed using the same procedure. Wild-type mice were sacrificed at 1, 4, 7, 10, and 14 days after FGF-2 and VEGF pellet implantation, and the corneas were sectioned and immunostained.

Micropocket Pellet Production

Pellets were made with slow-release polymer Hydron. Briefly, a 15 × 15-mm² piece of nylon mesh (pores approximately 0.4 × 0.4 mm) was embedded in sterile saline solution containing the appropriate (1 to 3 µg/injection) concentration of FGF-2 or VEGF. The mesh and its contents were added to 10 µL of 12% Hydron in ethanol. The mesh generated ~30 to 40 uniformly sized 0.4 × 0.4 × 0.2-mm pellets.

Corneal Micropocket Assay

A mouse corneal micropocket assay was carried out.¹⁹³ Briefly, the normal wild-type mice were anesthetized by a combined ketamine and xylazine injection. Lidocaine eye drops (Allergan, Irvine, California) were used for local anesthesia. Corneal micropockets were created with a modified Graefe's knife in normal wild-type mice (*n* = 20). Pellet implantation was performed by making a linear stromal incision (0.5 mm) in the middle of the cornea. A lamellar micropocket was dissected toward the temporal limbus to introduce the pellet. Hydron pellets (0.4 × 0.4 mm²) containing 80 ng of FGF-2 and 80 ng of VEGF (R&D Systems) were implanted separately into these corneal pockets. Ofloxacin eye drops (Allergan) were instilled after surgery. The eyes were examined and photographed on postoperative days 1, 3, 4, 7, 10, and 14 by slit-lamp microscopy (Nikon, Melville, New York).

CHARACTERIZATION OF PROANGIOGENIC MMPS (MMP-2 AND MT1-MMP) IN THE CORNEA

Confocal Immunohistochemical Staining With Anti-MMP-2 and Anti-MT1-MMP Antibodies in Bovine Corneal Keratocyte/Fibroblast and Wounded/Unwounded Mouse Corneas

Bovine corneal keratocyte and fibroblast cell lines were generated. Mouse wounded corneas were generated by undergoing excimer laser keratectomy as described previously. Bovine corneal keratocyte and fibroblast cell lines and wounded and unwounded mouse corneas were immunostained with anti-MMP2 and anti-MT1-MMP antibodies as previously described.

Confocal Immunohistochemical Staining With Anti-MT1-MMP, Anti-CD31, Anti-type IV Collagen, and Anti-MMP-2 Antibodies in Vascularized Mouse Corneas

Vascularized mouse corneal vessels were immunostained with anti-MT1-MMP antibodies, anti-CD31 antibodies, anti-type IV collagen, and anti-MMP-2 antibodies and double-stained with anti-MT1-MMP antibodies and anti-CD31 antibodies and with anti-type IV collagen and anti-MMP-2 -bodies.

Confocal Immunohistochemical Staining With Anti-MT1-MMP, Anti-VEGF, and Anti-CD31 Antibodies in bFGF Pellet-Implanted Mouse Corneas

Wild-type mouse corneas were obtained on days 4 and 14 after FGF-2 pellet implantation. Corneas were sectioned, and the central and peripheral corneas were immunostained with anti-MT1-MMP, anti-VEGF, and anti-CD31 antibodies.

Injection of MT1-MMP Naked DNA Into VEGF-LacZ Mouse Corneas

VEGF promoter regulated LacZ mice (VEGF-LacZ mice) were used in this experiment. Under direct microscopic observation, a nick in the epithelium and anterior stroma of a VEGF-LacZ mouse cornea was made in the midperiphery with a 30-gauge needle. A 33-gauge needle was introduced into the corneal stroma and advanced 1.5 mm to the corneal center. Two microliters of MT1-MMP naked DNA plasmid solutions were forcibly injected into the stroma to separate corneal lamellae and disperse the plasmid. The mice were sacrificed on days 3 and 8 after the MT1-MMP naked DNA injection.

Limbal Injury and Injection of MT1-MMP Naked DNA Into VEGF-LacZ Mouse Corneas

Either MT1-MMP naked DNA injection, hemilimbal injury, or a combination of MT1-MMP naked DNA injection and hemilimbal injury was performed on VEGF-LacZ mice as described previously. The mice were sacrificed on days 4 and 14 after MT1-MMP naked DNA injection, hemilimbal injury, or combination of MT1-MMP naked DNA injection and hemilimbal injury.

INVESTIGATIONS OF LIMBAL BARRIER FUNCTION USING HEMILIMBAL DEFICIENCY MODELS OF CORNEAL ANGIOGENESIS

MMP Knockout Animals

The generation of MMP-2 KO mice has been described in detail.¹⁹⁴ Heterozygous littermates were mated to obtain homozygous animals. Genotyping of animals was performed by polymerase chain reaction (PCR) of DNA obtained from tail biopsies. Primers for WT alleles were located in the 5' region (5'-TCC ACC CGG TGC TGC CAG CAC TCT TCC AGC CCA GC-3') and exon 1 (5'-GCC GGG GAA CTT GAT GAT GG-3'), and primers for mutated alleles were located in PGK-neo cassette [(5'-CTT GGG TGG AGA GGC TAT TC-3') and (5'-AGG TGA GAT GAC AGG AGA TC-3')].

The MMP-7 KO mice were generated as described by Wilson and colleagues.¹⁹⁵ A 6.5-kb BamHI genomic fragment cloned from a 129/Sv library was used to generate the targeting construct. A 550-bp EcoRV-StuI fragment spanning exons 3 and 4 was replaced with a 1.6-kb phosphoglycerate kinase-neomycin (PGK-neo) cassette. The construct was electroporated into R1 embryonic stem cells, and targeted clones were obtained and injected into C57BL/6 blastocysts after karyotype analysis. To generate WT littermates for MMP-7 KO mice, MMP-7 KO mice were crossed with WT mice (C57BL/6) to generate F1 heterozygotes (MMP-7 +/-). MMP-7 heterozygotes were used to generate matrilysin-deficient mice and their WT littermates. Primers for WT alleles were located in exon 3 (5'-TCAGACTTACCTCGGATCGT-3') and exon 4 (5'-GTCCTCACCATCAGTCCAG-3'), and primers for mutated alleles were

located in PGK-neocassette [(5'-TTGAGCCTGGCGAACAGT-3') and (5'-TGGATTGCACGCAGGTC-3')].

Hemilimbal Deficiency Models

Several hemilimbal deficiency (HLD) models have been developed to assay corneal NV: HLD-, HLD+, and HLD+ with steroid treatment. In HLD-models, hemilimbal injury was combined with removal of half of the epithelium. In HLD+ models, hemilimbal injury was combined with removal of the entire epithelium. After administering the previously described method of anesthesia, the nasal limbi of wild-type mice were surgically removed using a No. 15 Beaver blade with and without epithelial removal. The mice were sacrificed on day 7 after wounding.

Confocal Immunohistochemical Staining With Anti-type IV Collagen and Anti-CD31 Antibodies

The HLD- surgery was performed on WT, MMP-2 KO, MMP-3 KO, MMP-7 KO, and MMP-12 KO mice. Mouse corneas were visualized and documented daily with slit-lamp biomicroscopy. The mice were sacrificed on day 7 after wounding, and the eyes were sectioned and immunostained with anti-type IV collagen and anti-CD31 antibodies as described previously. Corneal vessels were further characterized by electron microscopy as described previously.

ROLE OF ANTIANGIOGENIC FACTORS (PEDF, ANGIOSTATIN, RESTIN, AND ENDOSTATIN) IN THE MAINTENANCE OF CORNEAL AVASCULARITY

Synthesis of PEDF Peptide and Anti-rabbit PEDF Antibody Population

Dawson and colleagues¹⁹⁶ have shown that injection of anti-PEDF antibody into mouse stroma induces corneal NV. A 20-amino acid peptide corresponding to the C-terminal region of PEDF has been synthesized and used for immunization of rabbits for antibody production. Female New Zealand white rabbits were immunized using synthetic peptides. Rabbit anti-mouse polyclonal antibodies to PEDF were generated. They were affinity-purified and used for immunohistochemical and Western blotting experiments.

Confocal Immunohistochemical Staining With Anti-PEDF Antibody in WT Mice

Wild-type mouse corneas were sectioned and immunostained with anti-PEDF antibody as described previously.

Cleavage of Antiangiogenic Precursors by MMPs

Collagen XVIII immunocomplexes were prepared by incubating cell lysates with antihinge antibodies. Recombinant PEDF and GST-XV-NCI were isolated from eukaryotic and bacterial cultures. Collagen XVIII immunocomplexes, PEDF, and GST-XV-NCI were incubated with an active MMP-1, -2, -3, -7, or -9 enzyme in a substrate buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 50 mM ZnSO₄) for 1 hour at 37°C. MMP-1 and -3 enzymes were obtained from Sigma (St Louis, Missouri) and MMP-2, -7, and -9 from Calbiochem (San Diego, California). Aminophenylmercuric acetate (APMA; 1 mM at 37°C for 2 hours; Sigma) was used to activate inactive enzymes. Combinations of MMPs (MMP-1 and -7, MMP-2 and -7, MMP-3 and -7, MMP-9 and -7, MMP-1 and -3, or MMP-3 and -9) were also used. Various MMP-7 concentrations (0, 2, 4, and 6 µg/mL) and incubation times (0, 1, 5, and 12 hours) were used. The reaction was stopped by adding 2× SDS gel loading buffer and boiling for 2 minutes.

Generation of Rabbit Anti-mouse Plasminogen Antibodies

Female New Zealand white rabbits were immunized using synthetic peptides containing residues of the murine 38-kDa NC1 domain (Research Genetics, Huntsville, Alabama). The first peptide, DDILANPPRLPDRQYPGVPHH, contained 22 residues in the hinge domain adjacent to the endostatin domain. The second peptide, RRADRGSVPIVNLKDEVLSPSWD, contained 23 residues in the N-terminal of the endostatin domain of NC1. Rabbit anti-mouse polyclonal antibodies to the hinge and endostatin domains were generated. They were affinity purified and used for immunohistochemical and Western blotting experiments. Similarly, antiplasminogen antibodies were generated. Female New Zealand white rabbits were immunized using synthetic peptides containing residues of murine plasminogen⁹. These rabbit anti-mouse polyclonal antibodies were used for immunohistochemistry and Western blot experiments. Three additional antiplasminogen antibodies were used: anti-LBS, anti-K1-3, and anti-B chain.

Collagen XVIII immunocomplexes were prepared by incubating mouse liver lysate with anticollagen antibodies (antiendostatin or antihinge), and Western blot analysis was performed.

Confocal Immunohistochemical Staining With Antiplasminogen Antibody in WT Mice

Using a combination of generated antiplasminogen antibodies and commercially available antibodies, expressions of plasminogen and angiostatin-like molecules in rabbit ocular tissues were evaluated as previously described.

Purification of Angiostatin-like Molecules and Western Blot Analysis

Papilloma E6, E7-immortalized mouse corneal epithelial cells and keratocytes were cultured as previously described, with modification. Immortalized cells were grown to confluence, scraped, and lysed with lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, and a protease inhibitor combination (Sigma). Mouse corneal cell lysates were precleared using CL-4B and protein A Sepharose beads to remove any antibodies from the extract. Angiostatin-like molecules were isolated using lysine Sepharose. Precleared lysate was incubated with lysine Sepharose beads for 12 hours at 4°C. After centrifugation (14,000 rpm for 10 sec), elution from the lysine Sepharose beads was performed using 50 mM aminocaproic acid. Western blot analysis was done under reducing conditions. Samples were electrophoresed on 4-20% precast SDS polyacrylamide gels (Invitrogen, Carlsbad, California), then electrotransferred onto a 0.20-µm polyvinylidene fluoride membrane (Immobilon P; Millipore, Billerica, Massachusetts). The membrane was blocked for 30 minutes in 3% bovine serum albumin (BSA) in TBST and probed with antiplasminogen antibodies.

After washing, the membrane was incubated for 60 minutes with HRP-conjugated secondary antibodies and revealed using a chemiluminescent technique (NEN Life Science Products, Boston, Massachusetts).

Isolation of Plasminogen

Peptide-generated antibodies were also characterized using liver lysate as a substrate for purification and Western blot analysis. Anti-K1, -K4, and -K5 antibodies recognized plasminogen. Plasminogen (97 kDa) was identified by anti-K1 antibody. Protein was eluted from beads with 100 mM or 500 mM aminocaproic acid (ACA).

RESULTS

CHARACTERIZATION OF PROANGIOGENIC FACTORS (VEGF AND bFGF) IN THE CORNEA

To investigate the role of angiogenic factors that may be involved in corneal NV, VEGF and bFGF levels were determined by Western blot analysis and immunohistochemical studies. VEGF and bFGF are expressed in WT normal corneal epithelial lysate as demonstrated by a 13-kDa reactive band to bFGF (Figure 2A, lane 1) and a 25-kDa reactive band (Figure 2A, lane 2) visualized by anti-bFGF and VEGF, respectively. In addition, WT mice were mechanically wounded, and the vascularized mouse corneas were sectioned and immunostained with anti-VEGF and anti-CD31. VEGF was localized in the corneal stroma and colocalized with mouse stromal vessels as visualized by anti-CD31 (Figure 2B). The kinetics of corneal NV induced by bFGF were investigated by implantation of similar pellets of 50 ng of bFGF into WT mouse corneas. No vessels were seen in the normal cornea; corneal vessels were visualized by day 4 and progressed during days 7, 10, and 14 (Figure 3, A through F).

No vessels were found in corneas implanted with a pellet of 80 ng of VEGF (Figure 4A), whereas the same concentration of bFGF induced vessel formation after pellet implantation (Figure 4B). Immunostaining of vascular corneas showed a positive staining of CD31 in the corneal vascularized zone with the leading fronds of NV and in the zone of bFGF pellets (Figure 4C, D).

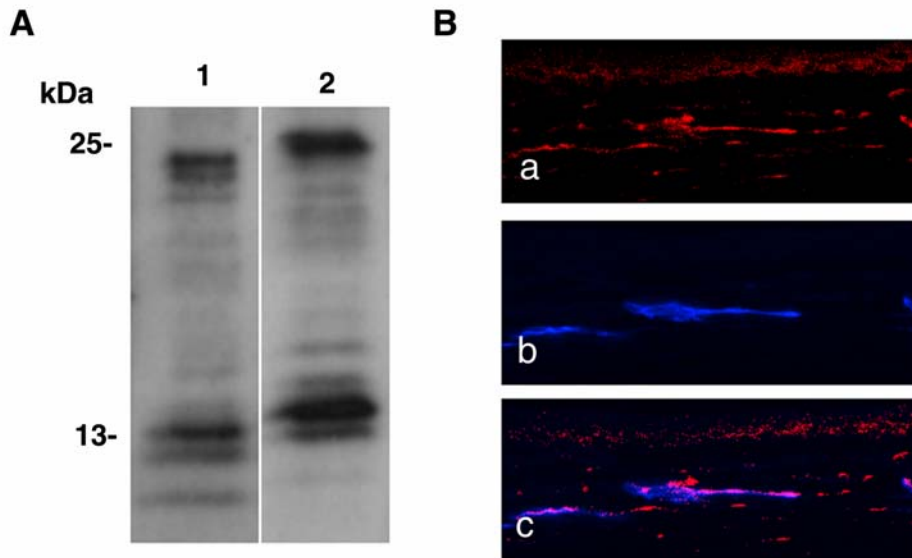


FIGURE 2

Characterization of bFGF and VEGF in mouse bFGF-induced corneas and neovascularization. Corneal epithelial cells were scraped and lysed in SDS sample buffer. A, Western blot analysis shows a reactive band at 13 kDa for bFGF (lane 1) and 25 kDa for VEGF (lane 2) when compared to commercially available bFGF and VEGF. B, Immunohistochemistry shows VEGF (keratocyte, a) and vascular endothelial cell marker (CD31, b) expression in corneal stroma and shows co-localization in the double staining (c).

CHARACTERIZATION OF PROANGIOGENIC FACTORS (MMP-2 AND MT1-MMP) IN THE CORNEA

Previous studies demonstrated that MMP-2 and MT1-MMP expression is induced during corneal wound healing. To further characterize the expression of MMP-2 and MT1-MMP, immunohistochemical studies on bovine corneal keratocyte (Figure 5A, B) and fibroblast cell lines (Figure 5C, D) demonstrated MT1-MMP (Figure 5A, C) and MMP-2 (Figure 5B, D) expression in both cytoplasm and the cell membrane. Similarly, MT1-MMP is expressed in the mouse unwounded stroma with an enhanced expression in the wounded areas (Figure 5E), and MMP-2 is expressed in the wounded area (Figure 5F).

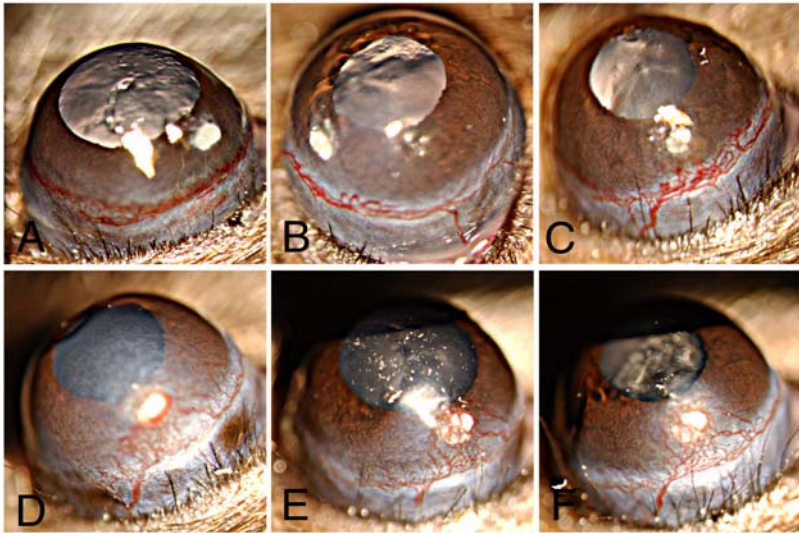
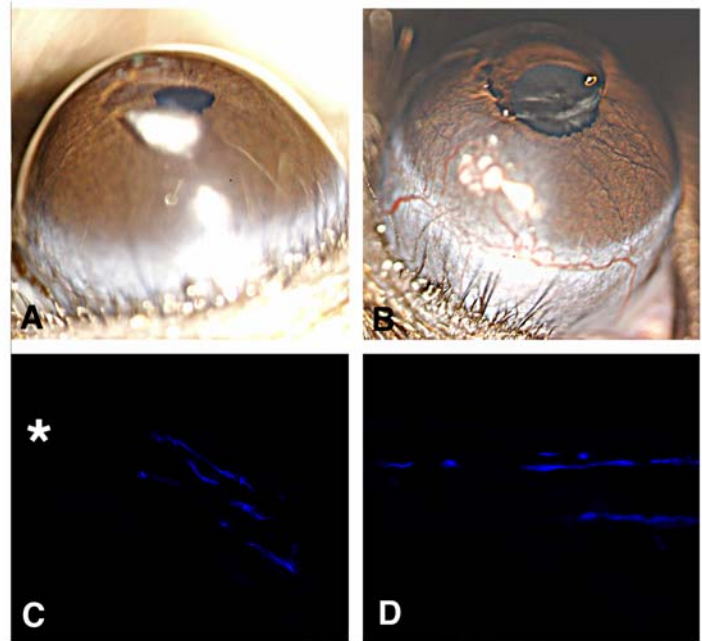


FIGURE 3

Kinetics of bFGF-induced corneal neovascularization. Pellets containing bFGF (50 ng/pellet) were implanted into mouse corneal stromata. Slit-lamp photographs were taken of corneal vessels (A, day 1; B, day 3; C, day 4; D, day 7; E, day 10; F, day 14).

FIGURE 4

Comparison of potency of VEGF and bFGF for induction of corneal neovascularization. A wild-type mouse corneal stroma was implanted with 80 ng/pellet VEGF (A) and the same concentration of bFGF (B). On day 14, immunohistochemistry revealed vascular endothelial cells near the bFGF pellet (C) and in the limbal zone (D). The presence of VEGF in the cornea was not sufficient to induce corneal NV after pellet implantation. (* indicates pellet location)



To examine the expression of MMPs and vascular endothelial cell markers, vascularized corneas were immunohistochemically stained with MMP-2, MT1-MMP, CD31, and type IV collagen. MT1-MMP colocalized with CD31 marker (Figure 6C) and MMP-2 colocalized with type IV collagen (Figure 6F).

To examine the correlation of MT1-MMP and VEGF in vascularized corneas, those corneas with bFGF pellet-induced vascularization were immunostained with anti-MT1-MMP, VEGF, and CD31 antibodies. MT1-MMP and VEGF were colocalized to the corneal stroma at days 4 (Figure 7A, C) and 14 (Figure 8A, C). A stronger MT1-MMP immunostaining pattern was revealed in the peripheral cornea at day 14 after bFGF implantation (Figure 8B). CD31 was expressed only in the peripheral cornea (Figure 7F, Figure 8F), not in the central cornea (Figure 7E, Figure 8E), on days 4 and 14 after bFGF implantation.

VEGF promoter-regulated LacZ mice were used to determine whether MT1-MMP stromal fibroblast mediated corneal NV is controlled via the transcriptional upregulation of VEGF intrastromal corneal injections (Figure 9). The intrastromal injection of naked DNA (MT1-MMP) combined with hemilimbal injury enhanced β -galactosidase activity in VEGF promoter-regulated LacZ mice corneas (Figure 10C, F). No corneal NV was observed in controls of hemilimbal injury (Figure 10A, D) or MT1-MMP DNA injection alone (Figure 10B, E).

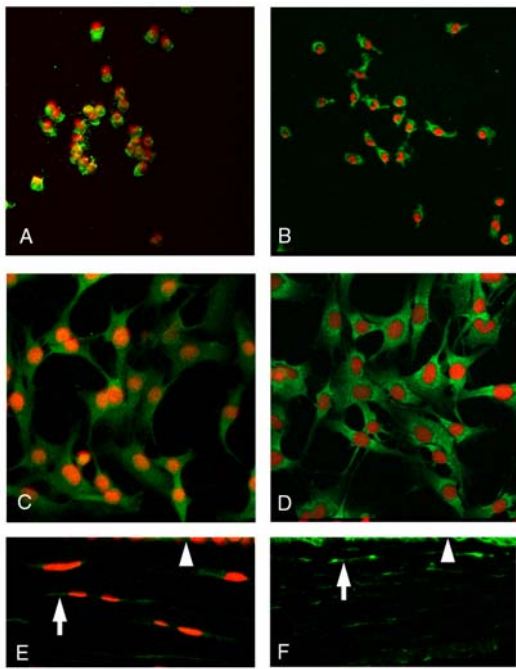


FIGURE 5

Characterization of MMP-2 and MT1-MMP in the cornea and cultured keratocytes and fibroblasts. Bovine keratocytes (A, B) and fibroblasts (C, D) were isolated and immunostained with anti-MT1-MMP (A, C) and anti-MMP-2 antibodies (B, D). MT1-MMP is localized in an unwounded cornea (E; arrow) and MMP-2 is in the stroma of a wounded cornea (F; arrow). Although these MMPs are proangiogenic, they are present in the cornea in the absence of corneal NV. (Arrowheads show the margin of the epithelium; PI was used for nucleus staining.)

INVESTIGATIONS OF LIMBAL BARRIER FUNCTION USING HEMILIMBAL DEFICIENCY MODELS OF CORNEAL ANGIOGENESIS

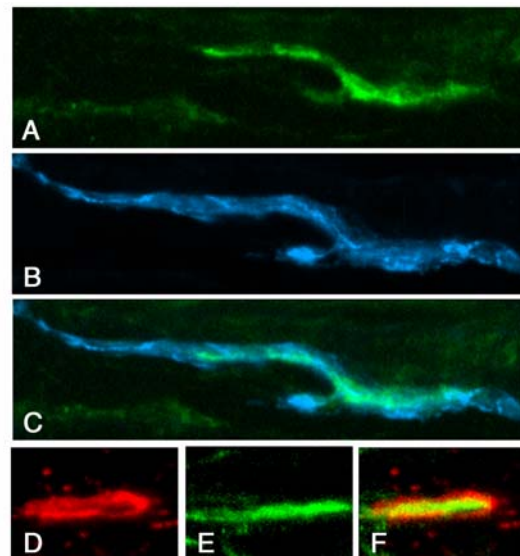
Several corneal wounding models have been developed to assay corneal NV, including hemilimbal removal and hemilimbal deficiency plus epithelial removal. Nasal limbi of WT mouse eyes were surgically removed (Figure 11A). Few or no vessels developed by day 7 after injury (Figure 11B, C). In contrast, when the nasal limbus and epithelium were removed (Figure 11D), vessels developed by day 7 after wounding (Figure 11E, F). Mouse corneal sections were immunolocalized by double-labeling with vascular marker collagen IV (Figure 11G) and CD31 (Figure 11H) 7 days after surgery to localize corneal vessels. Type IV collagen was expressed in the stroma and partially colocalized with CD31 (Figure 11I).

HLD models have been developed to study corneal NV (Figure 12). Total epithelial debridement alone does not induce NV (Figure 12A). Similarly, debridement of half the limbus and half the corneal epithelium (HLD-) fails to stimulate vessel growth. However, removal of half the limbus and total epithelial debridement (HLD+) induce corneal NV in WT mice as early as 4 days after injury (Figure 12C). Diminished NV was seen after similar wounding in WT mice treated with topical steroids (Figure 12D).

To further assess the role of MMPs in corneal NV, corneal wounding was applied to MMP-deficient mice. The HLD- surgery was performed on MMP-2 knockout (Figure 13, A through C), MMP-12 knockout (Figure 13, D through F), and WT (Figure 13, G through I) mice. No vessels were observed in corneas 7 days after surgery. Immunolocalization with double-labeling of collagen IV and CD31 confirmed the lack of vessels in these knockout and WT mice.

FIGURE 6

Colocalization of MMP-2 and MMP-14 in vascular endothelial cells. Vascularized corneal vessels were immunostained with anti-MT1-MMP(A) and CD31 antibodies (B) and double stained with both (C). They were also immunostained with anti-type IV collagen (D) and anti-MMP-2 antibodies (E) and double stained with both (F).



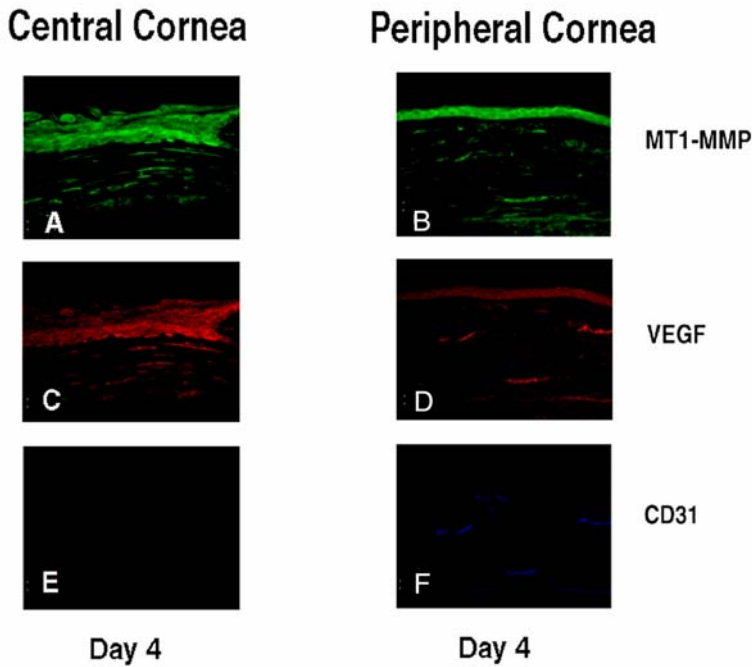
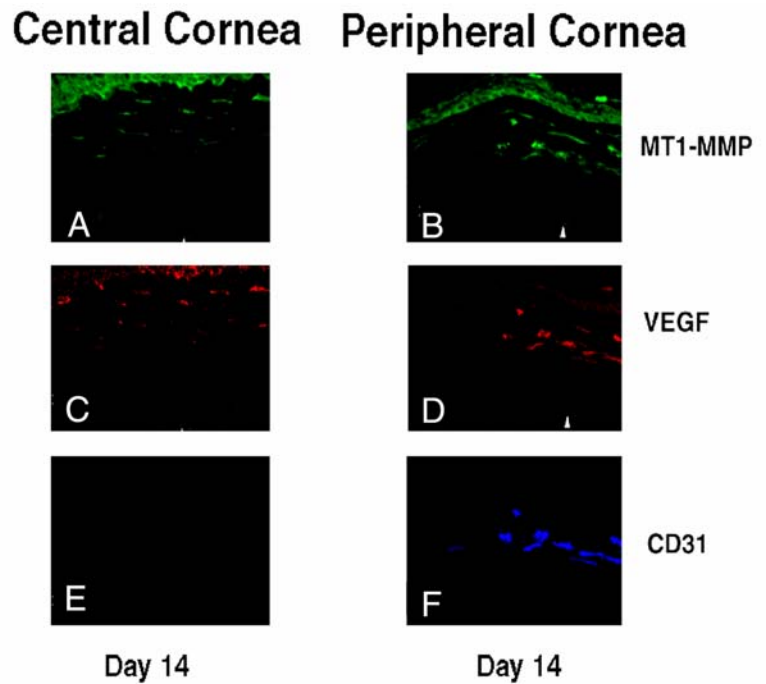


FIGURE 7
 Distribution of MT1-MMP, VEGF, and CD31 in mouse corneas 4 days following bFGF pellet implantation. FGF-2 pellets (50 ng) were implanted into mouse corneas. The corneas were harvested at 4 days postoperatively. The central avascular zone adjacent to the pellets and the peripheral neovascularization zone were examined. Individual sections were immunostained with MT1-MMP (A, B), VEGF (C, D), and CD31 (E, F), a vascular endothelial cell marker.

FIGURE 8
 Distribution of MT1-MMP, VEGF, and CD31 in mouse corneas 14 days after bFGF pellet implantation. FGF-2 pellets (50 ng) were implanted into mouse corneas. The corneas were harvested at 14 days postoperatively. The central avascular zone adjacent to the pellets and the peripheral neovascularization zone were examined. Individual sections were immunostained with MT1-MMP (A, B), VEGF (C, D), and CD31 (E, F), a vascular endothelial cell marker.



In contrast, when HLD- was applied to MMP-7 and MMP-3 knockout mice, corneal vessels were visualized at day 7 after surgery in MMP-3 knockout (Figure 14, A through C) and MMP-7 knockout mice (Figure 14, D through F).

Comparing to HLD- in WT mice (Figure 15C), corneal vessels were visualized in MMP-7 knockout mice (Figure 15A). These findings were confirmed by the absence of staining of type IV collagen in the WT mice (Figure 15D) when compared to MMP-7 knockout mouse cornea (Figure 15B). Vessels in MMP-7 knockout were further demonstrated by electron microscopy (Figure 15, F through H). The corneal thickness in wounded MMP-7 knockout mice was similar to the WT mouse cornea as measured by ultrasound biomicroscopy (UBM) (Figure 15J).

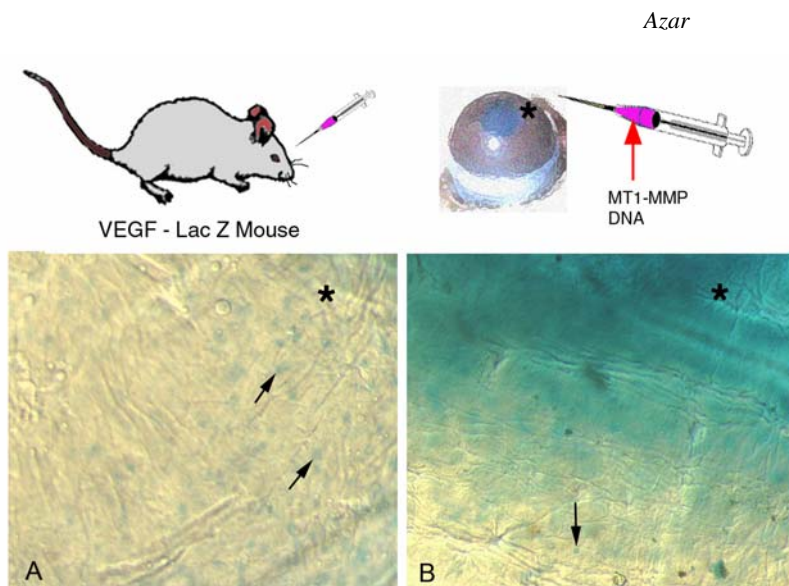


FIGURE 9

Injection of MT1-MMP naked DNA into mouse corneas induced corneal NV and VEGF expression. LacZ mice were injected with MT1-MMP DNA at 3 and 8 days (A, B, respectively). Arrows denote VEGF expression (β -galactosidase activity). Asterisks denote the area close to the injection sites.

ROLE OF ANTIANGIOGENIC FACTORS (PEDF, ANGIOSTATIN, RESTIN, AND ENDOSTATIN) IN THE MAINTENANCE OF CORNEAL AVASCULARITY

Several antiangiogenic factors have been proposed to play an important role in maintaining corneal avascularity. Several antibodies have been generated for immunohistochemistry studies, Western blot analysis, and functional blocking assays. Dawson and colleagues¹⁹⁶ have shown that the injection of anti-PEDF antibody into the mouse stroma induces corneal NV.

A 20-amino acid peptide corresponding to the C-terminal region of PEDF has been synthesized and used for immunization in rabbits (Figure 16A). Anti-PEDF antibodies were affinity purified by peptides. Corneal immunohistochemistry studies using anti-PEDF antibodies have shown that PEDF is mainly localized to the corneal epithelium (Figure 16B). Recombinant PEDF has been genetically engineered and isolated from cultured cells. Incubation of recombinant PEDF with MMP-2, MMP-7, MMP-9, or MMP-12 has shown that MMP-7 and -12 cleave PEDF but MMP-2 and -9 do not (Figure 16H).

Using our antiplasminogen antibodies (Figure 17A), in combination with commercially available antibodies, Western blot analysis showed the presence of an angiostatin-like fragment in the aqueous humor, cornea, lens, vitreous, retina, pigment epithelium, choroid, and sclera (Figure 17B). Plasminogen (97 kDa) was identified by an anti-K1 antibody. Note that the breakdown products seen in both liver and cell lysates are angiostatin-like molecules (Figure 18A). Protein was eluted from beads with 100 mM or 500 mM ACA. Lysine Sepharose purified the plasminogen and angiostatin-like molecules but not Butesin agarose (Figure 18B).

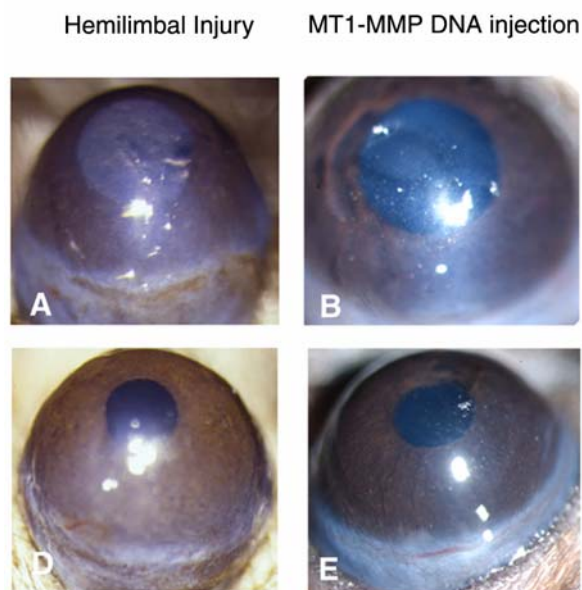


FIGURE 10

MT1-MMP DNA injection tips the balance towards corneal neovascularization during wound healing. Effects of limbal injury (A, D) and MT1-MMP DNA injection (B, E) and combination of limbal injury and MT1-MMP DNA injection (C, F). Combined hemilimbal injury and MT1-MMP DNA transfection resulted in corneal neovascularization.

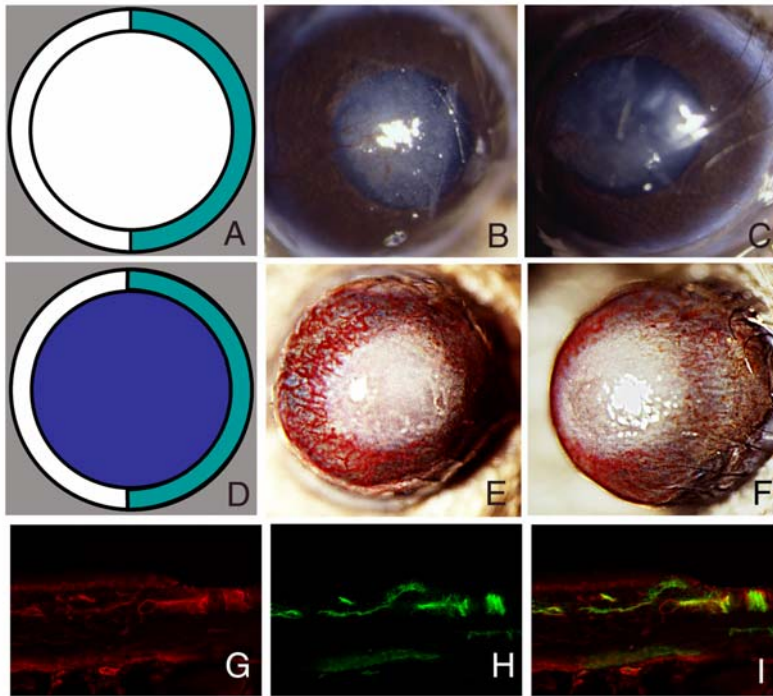
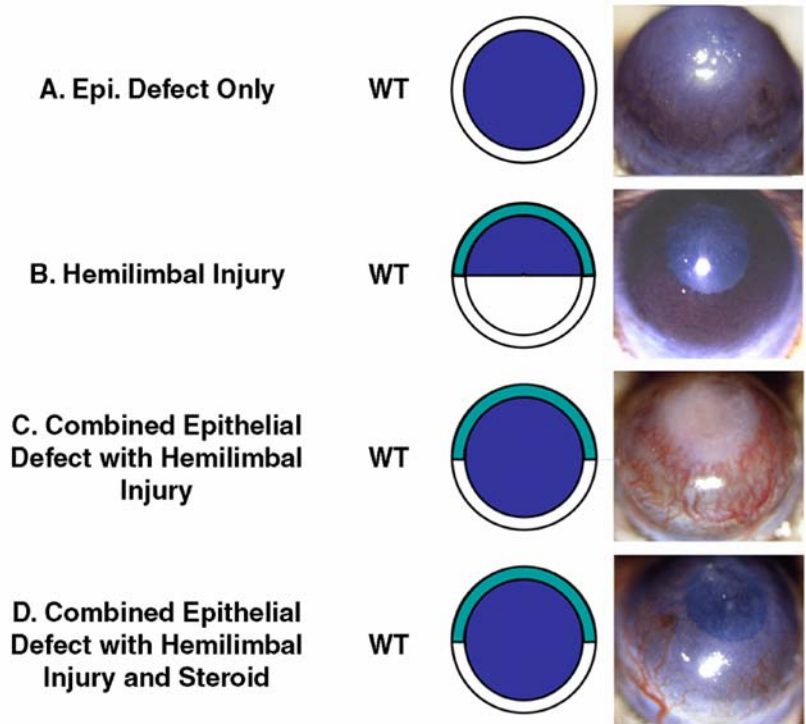


FIGURE 11

Hemilimbal deficiency: a model for injury-induced corneal NV. Diagrams depict limbal injury (A; green) and limbal plus epithelial removal (D; purple). The nasal limbi of wild-type mouse corneas were removed and photographed at day 7 after surgery (B, C). The nasal limbus and the epithelium of WT mouse corneas were removed and the corneas photographed at day 7 after surgery (E, F). Vascularized vessels were immunostained with anti-type IV collagen (G), anti-CD31 antibodies (H), and double staining (I).

FIGURE 12

Development of the hemilimbal deficiency (HLD) models. No vessels were induced when the epithelium was removed (A). In HDL models, hemilimbal injury is combined with removal of half of the epithelium (B). In HDL+ models, hemilimbal injury is combined with entire epithelium removal (C). HLD+ models with steroid treatment (D). WT = wild-type.



Corneal wounding may induce an interaction between keratocyte and corneal epithelial cells. Lysine Sepharose isolated proteins from coculture of corneal epithelial cells, and keratocytes were immunoblotted with anti-K1 or B-chain antibodies. The greatest level of protein identified by the anti-K1 antibody was observed in the epithelial/keratocyte coculture control (Figure 19A). The antibody against the B-chain of plasminogen did not identify the higher-molecular-weight proteins. Therefore, the anti-K1 antibody identified angiostatin-like molecules. Supernatant and cell lysates of epithelial/keratocyte coculture grown in either 0.5% or 10% fetal calf serum (FCS) were purified with lysine Sepharose. The pellets were eluted with ACA. The eluted fractions and pellets were blotted with anti-K1 antibody. The actively growing coculture (10% FCS) appears to increase the level of protein recognized by the anti-K1 antibody (Figure 19B).

Several types of collagen, including types XV and XVIII, play a role in regulating tumor formation and angiogenesis. The

structures of collagen XV and XVIII are very similar, containing a C-terminal globular domain that possesses antiangiogenic properties. A peptide corresponding to the C-terminal region of collagen XV has been synthesized and subjected to rabbit antibody production (Figure 20A). Anticollagen XV antibody is affinity purified by a peptide column. Collagen XV is localized to the mouse corneal epithelium (Figure 20B) when compared to a negative control (Figure 20C). Recombinant GST-collagen XV NC1 fragments have been generated and isolated from bacteria and incubated with various MMPs (MMP-1, -2, -3, -7, and -9). MMP-7 cleaved the collagen XV NC1 domain, but MMP-1, -2, -3, and -9 did not (Figure 20D).

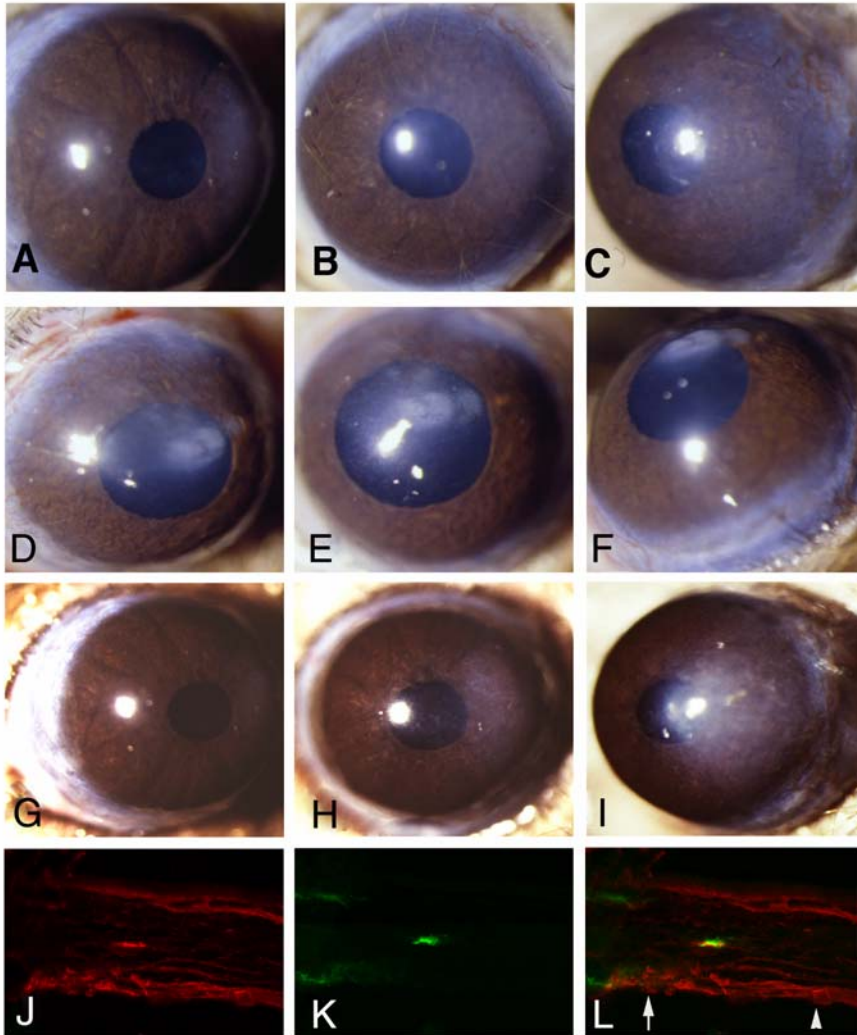


FIGURE 13

Hemilimbal injury (HLD-) of corneas in wild-type, MMP-2^{-/-}, and MMP12^{-/-} mice. Hemilimbal injury was applied to mouse cornea in MMP-2^{-/-} (A, B, and C), MMP12^{-/-} (D, E, F), and wild-type mice (G, H, I) at day 7 after wounding. Corneal vessels were visualized with type IV collagen (J) and CD31 (K) and then the images were merged (L).

Two anticollagen XVIII antibodies were generated by immunized rabbits using synthetic peptides containing residues of the murine 38-kDa NC1 domain (Figure 21A). Rabbit anti-mouse polyclonal antibodies to the hinge and endostatin domains were generated. Immunolocalization of collagen XVIII in human (Figure 21, B through E) and mouse (Figure 21, F through I) corneas using anti-NC1 antibody (Figure 21B, F; in collaboration with Dr Olsen, Harvard School of Dental Medicine, Boston, Massachusetts), antiendostatin antibody (Figure 21C, G), and antihinge antibody (Figure 21D, H) was performed. Negative control for collagen XVIII immunostaining was performed without the primary antibody in human (Figure 21E) and mouse (Figure 21I) corneas. cDNAs of collagen XVIII and G3PDH were amplified by PCR, and corresponding fragments were visualized by agarose gel electrophoresis (Figure 21J). In addition, competitive reverse transcriptase-PCR was used to quantify the level of collagen XVIII expression (Figure 21K).

Corneal collagen XVIII was immunoprecipitated with anticollagen antibodies (using Olsen's anti-NC11 antibody and antihinge antibody). A 28-kDa band was seen with MMP-7 cleavage. A heparin-isolated, 293-overexpressed NC1 fragment was blotted with antiendostatin (lane 5, Lin and associates¹³) and antiendostatin antibodies (lane 6, Cytimmune, MD), respectively (Figure 22A).

GST-endostatin and GST-hinge XVIII have been generated and isolated. These recombinant fragments have been confirmed by antiendostatin Western blot analysis (Figure 22B).

FIGURE 14

Hemilimbal deficiency model (HLD-) in MMP-3^{-/-} and MMP-7^{-/-} mice. Hemilimbal injury was applied to mouse corneas of MMP-2^{-/-} (A, B, C) and MMP-7^{-/-} (D, E, F) at day 7 after injury. Corneal vessels were visualized by slit-lamp photography.

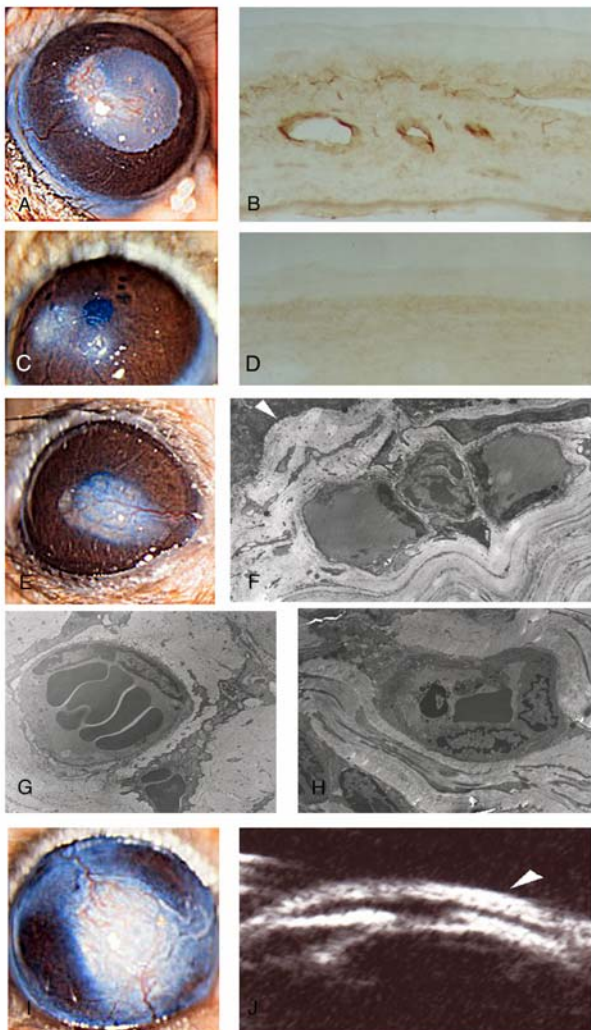
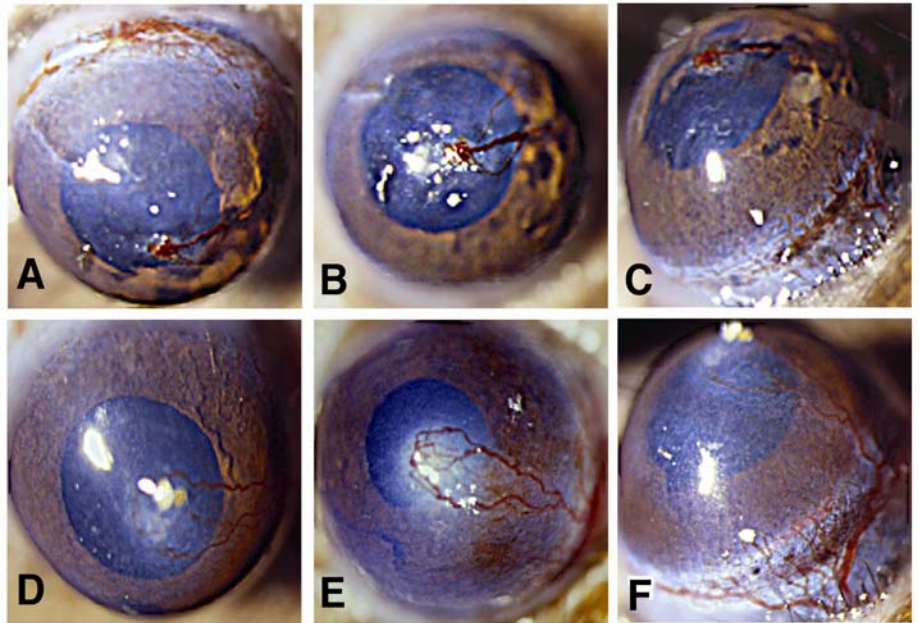


FIGURE 15

Characterization of corneal NV in hemilimbal deficiency model (HLD+) in MMP-7^{-/-} mice. Mouse corneas were injured and corneal sections were immunostained with type IV collagen (A, B). No vessels were found in the control (C, D). Vascularized MMP-7^{-/-} mouse cornea (D, I). Corneal vessels were further characterized using an electron microscope (F, G, H). Corneal thickness was determined by ultrasound biomicrography (J).

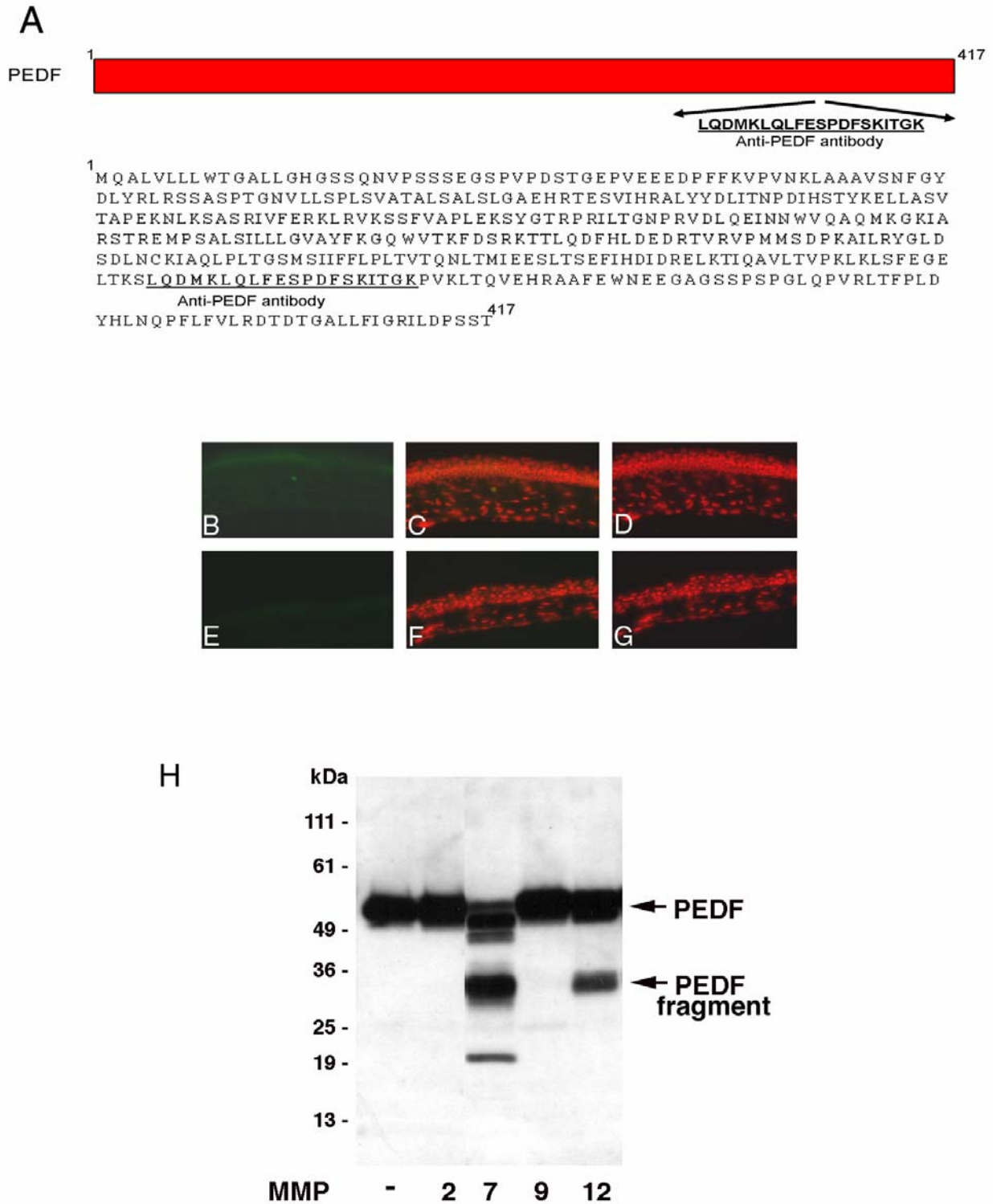


FIGURE 16

Characterization of PEDF in corneas. Diagram of PEDF and its primary structure (amino acids) (A). A peptide based on the C-terminal region of PEDF was synthesized and used for antibody production. Immunostaining of PEDF revealed that PEDF is localized to the mouse corneal epithelium (B). Wild-type negative control (anti-PEDF antibody omitted) (E). Propidium iodine was used for nuclear staining control (C, F). Double staining (D, merging of B and C; G, merging of E and F). Recombinant PEDF was incubated with various MMPs and assayed for cleavage (H).

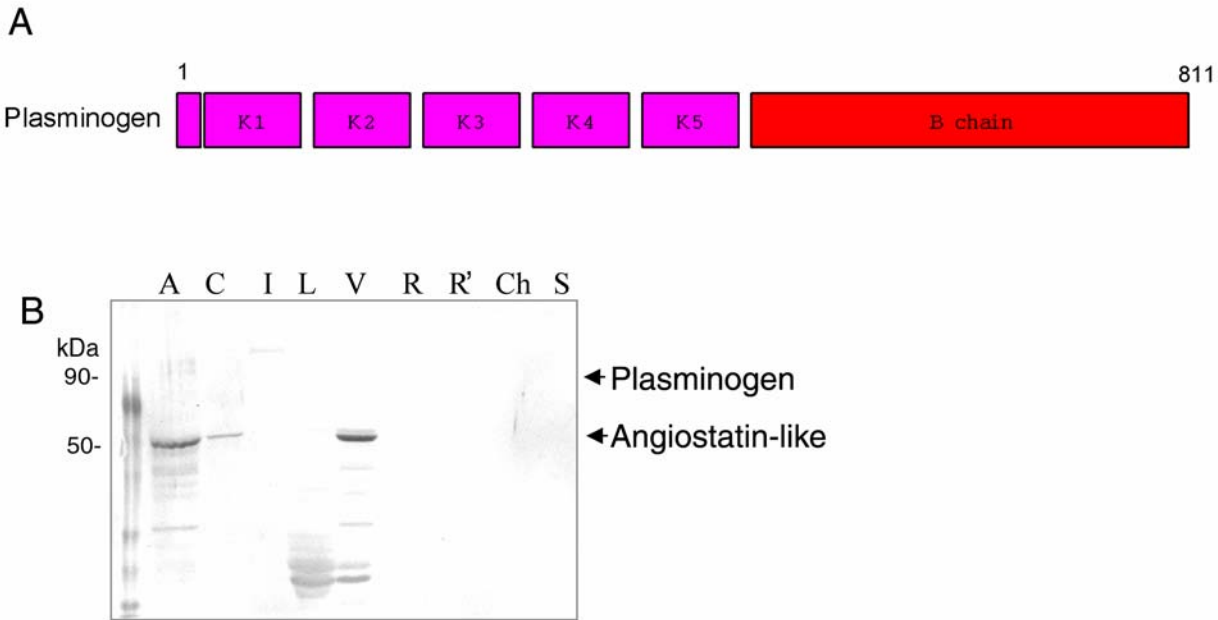


FIGURE 17

Characterization of corneal plasminogen, angiostatin-like molecules, and their function. The diagram depicts the structure and domain organization of plasminogen, including kringle domains and B chain (A). Six antibodies against various domains of plasminogen were generated. Using a combination of these antibodies and commercially available antibodies, expressions of plasminogen and angiostatin-like molecules in rabbit ocular tissues were detected in the aqueous humor (A), cornea (C), iris (I), lens (L), vitreous (V), retina (R), retina pigment epithelium (R'), choroid (Ch), and sclera (S).

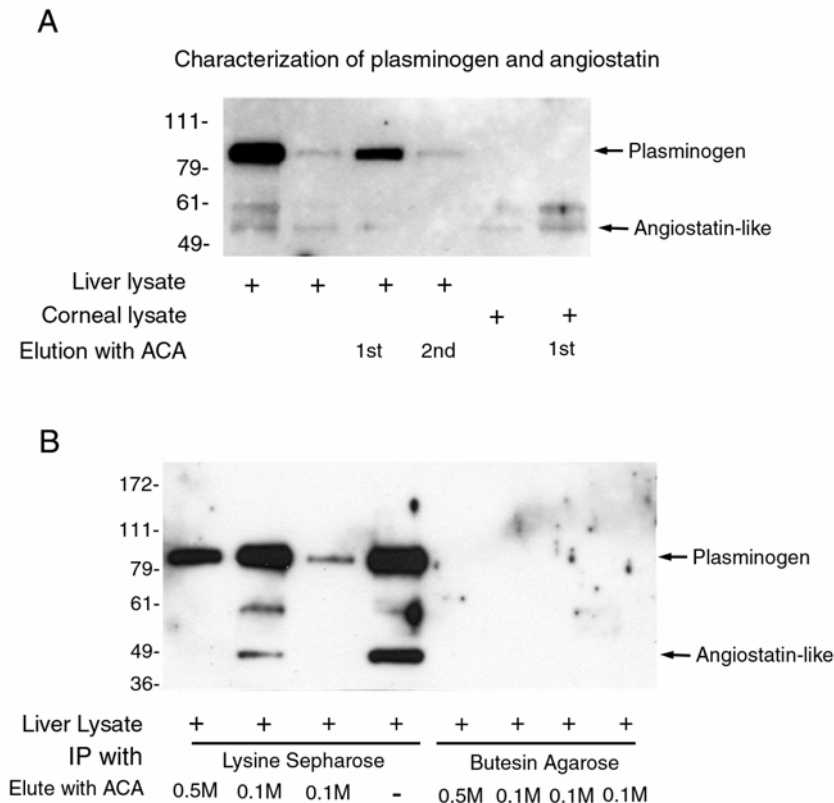
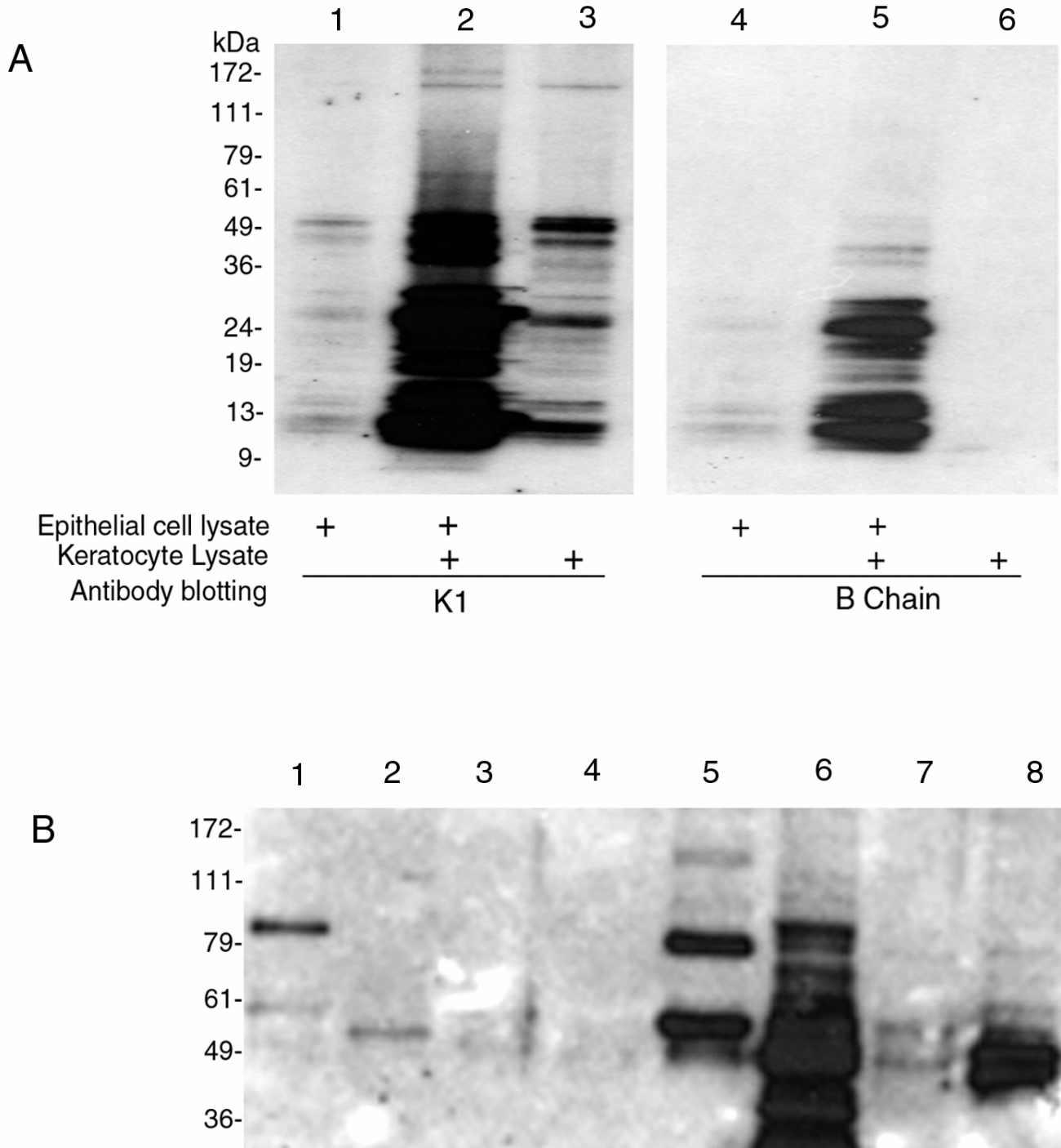


FIGURE 18

Characterization of antiangiostatin and B chain antibodies. Plasminogen was isolated by lysine Sepharose and eluted with aminocaproic acid (A). A 90-kDa plasminogen from liver lysates was determined by Western blot analysis by anti-K1 antibody, and 50 kDa angiostatin-like fragments were visualized in liver lysate and corneal lysate. Plasminogen and angiostatin-like molecules were isolated by lysine Sepharose and eluted with various concentrations of aminocaproic acid (0.5, 0.1 M) (B). Plasminogen from liver lysate did not bind to Butesin agarose.

**FIGURE 19**

Induction of angiostatin-like molecules by co-culture of corneal keratocyte and epithelial cells. Lysine Sepharose isolated proteins from co-culture of corneal epithelial cells, and keratocytes were immunoblotted with anti-K1 or B-chain antibodies. The greatest level of protein identified by the anti-K1 antibody was observed in the epithelial/keratocyte co-culture control (A; lane 2). The antibody against the B chain of plasminogen did not identify the higher-molecular-weight proteins (A; lane 5). Supernatants and cell lysates of epithelial/ keratocyte co-culture grown in either 0.5% or 10% FCS were purified with lysine Sepharose. The pellets were eluted with aminocaproic acid. The eluted fractions (B; lanes 1-4) and pellets (B; lanes 5-8) were blotted with anti-K1 antibody. The actively growing co-culture (10% FCS) appears to increase the level of protein that is recognized by the anti-K1 antibody (B; lanes 5, 6).

Corneal Angiogenic Privilege

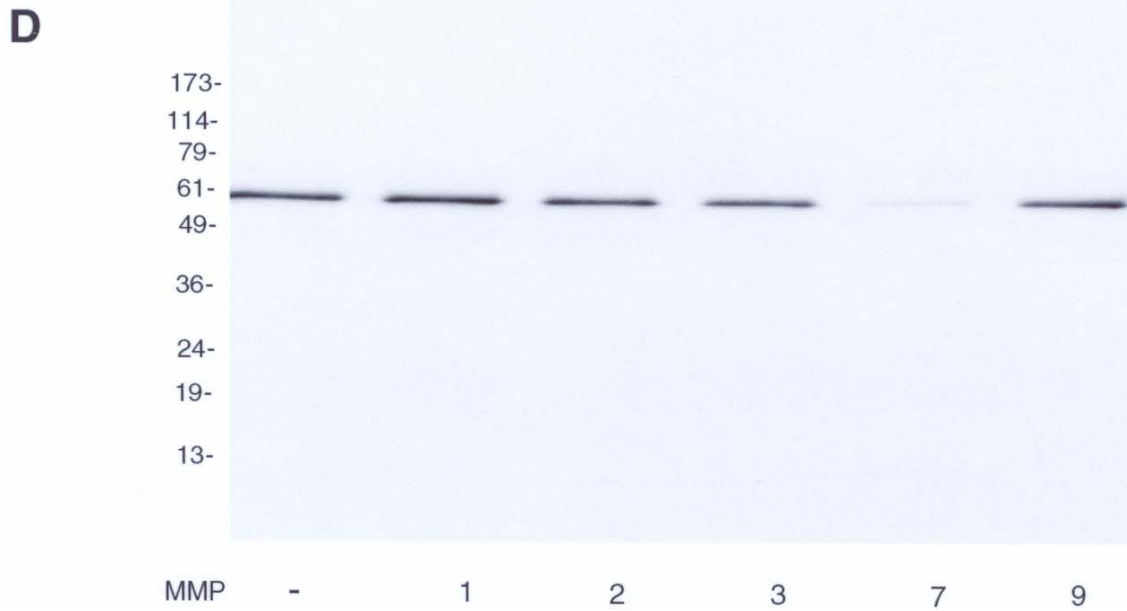
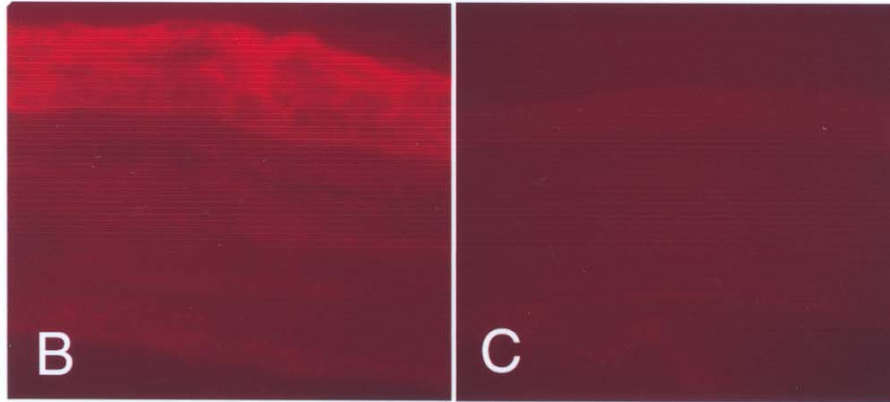
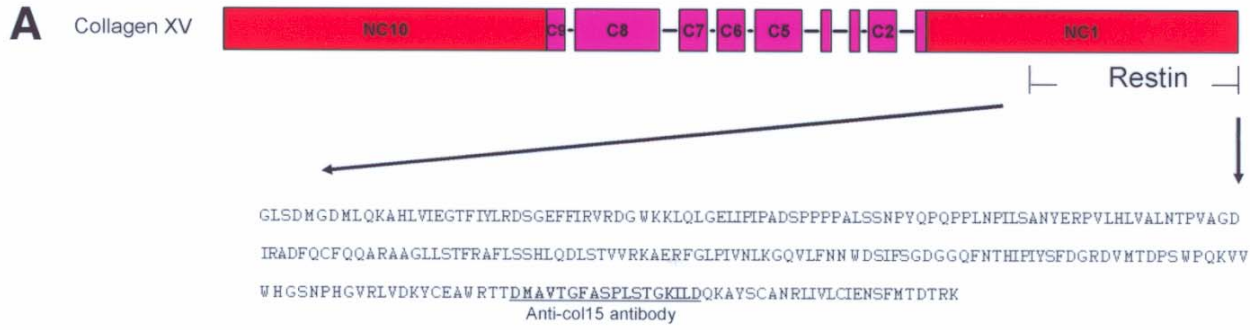


FIGURE 20

Characterization of mouse corneal collagen XV. The diagram depicts collagen XV and its C-terminal NC1 domain. The peptide based on the C-terminal region of collagen XV NC1 domain was synthesized and used for anti-collagen XV antibody production. Affinity-purified antibodies were used to localize collagen XV in the mouse corneal epithelium (B). Control with omitted primary antibody (C). Recombinant GST-XV NC1 was purified and incubated with various MMPs (D; MMP-1, -2, -3, -7, -9).

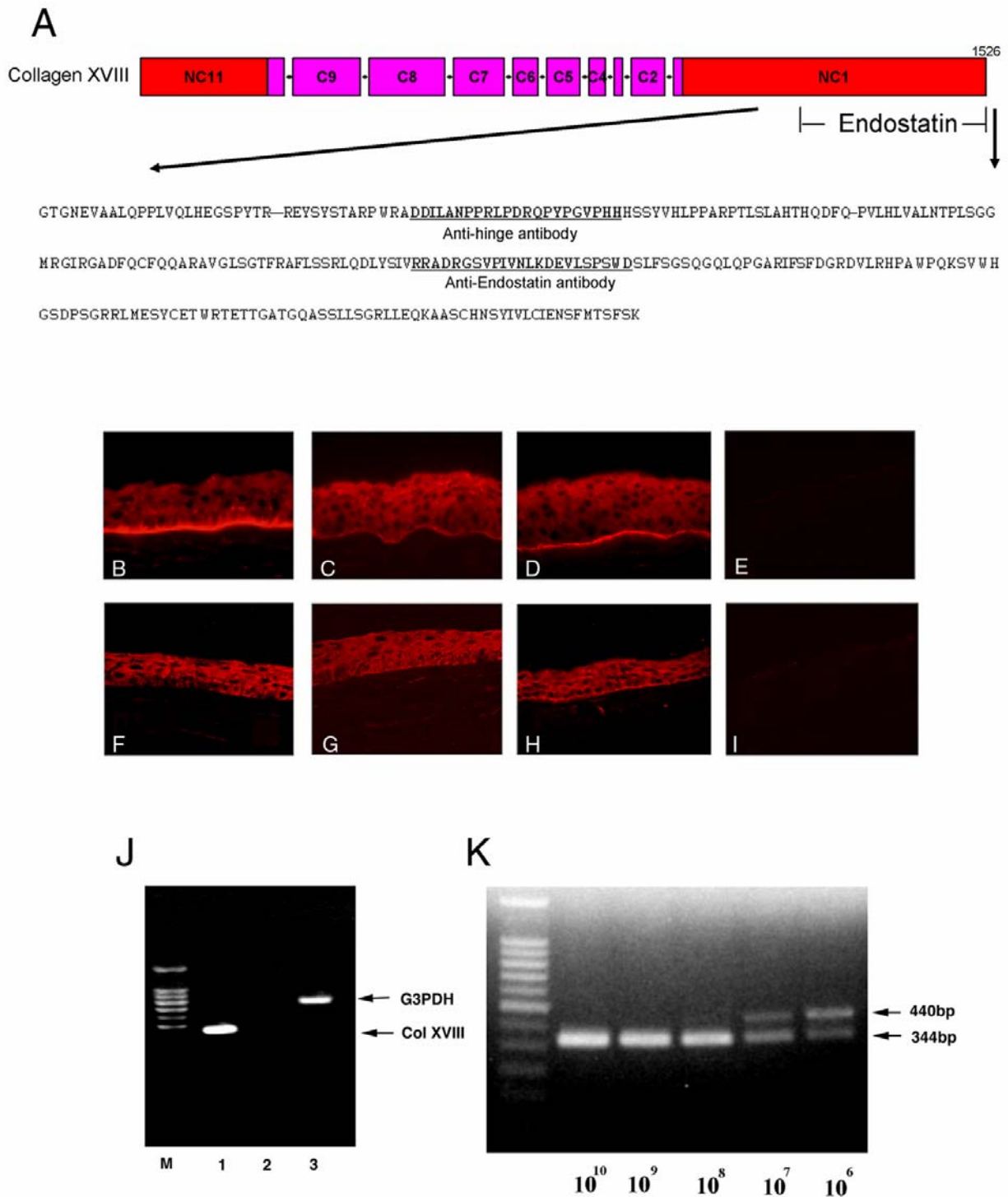


FIGURE 21

Characterization of corneal collagen XVIII. Diagram depicts collagen XVIII and its C-terminal NC1 domain. Two peptides corresponding to the C-terminal NC1 fragment were generated and used for antibody production. Immunolocalization of collagen XVIII in human corneas (B-E) and mouse corneas (F-I) using anti-NC1 antibody (B, F), antiendostatin antibody (C, G), and antihinge antibody (D, H) was performed. Negative control for collagen XVIII immunostaining was performed without primary antibody. Human cornea (E), mouse cornea (I). cDNAs of collagen XVIII and G3PDH were amplified by polymerase chain reaction, and the corresponding fragments were visualized by agarose gel electrophoresis (J). Competitive reverse transcriptase polymerase chain reaction was used to quantify the level of collagen XVIII expression (K).

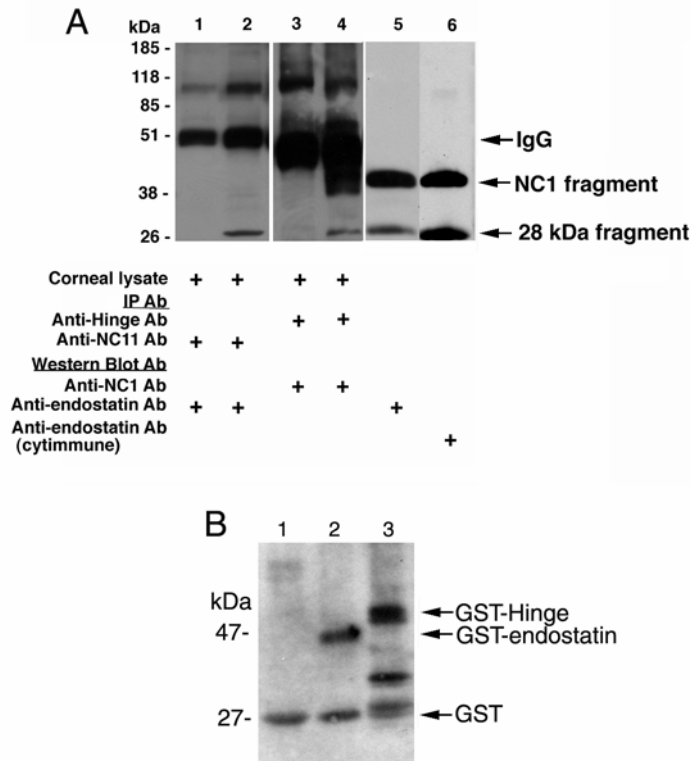


FIGURE 22

Characterization of anticollagen XVIII antibodies. (A) Corneal collagen XVIII was immunoprecipitated and cleaved with active MMP-7 and analyzed by Western blot analysis. Corneal cellular lysate was immunoprecipitated with anti-NC1 antibody and cleaved without (lane 1) and with (lane 2) active MMP-7 and blotted with antiendostatin antibody. Similarly, corneal cellular lysate was immunoprecipitated with antihinge antibody, and cleaved without (lane 3) and with (lane 4) active MMP-7 and blotted with anti-NC1 antibody. A 28-kDa band was seen with MMP-7 cleavage. Heparin-isolated, 293-overexpressed NC1 fragment was blotted with antiendostatin (lane 5) and antiendostatin antibodies (lane 6), respectively. (B) Isolation of glutathione S-transferase (GST), GST-endostatin, and GST-hinge domain of collagen XVIII. GST (lane 1), recombinant GST-endostatin (lane 2) and GST-hinge of collagen XVIII NC1 fragments (lane 3) were isolated and subjected to Western blot analysis with antiendostatin antibodies.

DISCUSSION

PROANGIOGENIC FACTORS AND MMPs IN CORNEAL ANGIOGENESIS

A well-accepted paradigm for angiogenesis in adult tissue involves differential recruitment of vascular endothelial cells and associated supporting cells, such as smooth muscle cells and pericytes, to different segments of the preexisting vasculature. This requires vascular endothelial cell migration, adhesion, and ECM degradation, which are mediated by several MMPs, including MT1-MMP, known to be expressed at the vascular endothelial cell surface.¹⁹⁷ Previous studies have reported that MMPs are expressed during the corneal wound healing process. Confocal microscopy of MMP-2, -7, -9, and -12 after keratectomy wounds demonstrated that MMP-2 is immunolocalized to the unwounded stroma and is upregulated in the superficial stroma and basal epithelium 1 to 14 days after wounding.¹³¹ MMP-7 is immunolocalized to the unwounded epithelial layer and upregulated after laser keratectomy.¹⁹⁸ MMP-9 is immunolocalized to the BM and superficial stroma 18 to 72 hours after wounding. MMP-12 was detected in the stroma 1 day after injury. Previous investigations have also noted that MMPs affect the extent of corneal NV after excimer laser keratectomy. Despite the presence of antiangiogenic factors in the cornea, upregulation of proangiogenic factors following severe burns, infections, and chronic ocular surface disorders tilts the balance toward angiogenesis. We observed that proangiogenic MMPs and VEGF are upregulated during corneal wound healing associated with NV and that transfection of stromal fibroblasts with MT1-MMP naked cDNA resulted in corneal NV. These findings support the hypothesis that keratocyte-specific, membrane-associated MT1-MMP may promote corneal NV.

LIMBAL BARRIER FUNCTION IN CORNEAL ANGIOGENESIS

Each stromal lamella is composed of a band of collagen fibrils arranged in parallel. Corneal avascularity is essential for optical clarity and also demands that oxygen be derived predominantly from oxygen in the tear film rather than from traversing red blood cells and that most nutritional requirements be fulfilled by the aqueous humor.

Why corneal NV reduces acuity can be addressed by understanding the basis of corneal transparency and scleral opacity. The combination of tissue transparency and tensile strength is achieved by collagen fibrils of uniformly small diameter maintained at close periodicity and is highly dependent on a constant state of relative corneal dehydration.

Significant scattering of light occurs when changes in the cornea structure cause refractive index fluctuations distributed over distances greater than half the wavelength of light. This occurs after blood vessel invasion into the stroma. The opaque nature of the

sclera is not a consequence of an absent lattice arrangement but of large variations in the diameters of adjacent collagen fibers so that neighboring regions have different refractive indices. In contrast, the collagen fibrils in the avascular corneal stroma are of uniformly small diameter and are closely spaced.

In cases of corneal edema and pannus formation, there is a loss of transparency. Stromal edema expands the ground substance, which greatly increases the spacing between collagen fibrils, leading to large fluctuations in refractive index and, consequently, scattering of light. Furthermore, the uptake of stromal water associated with corneal edema may be accompanied by a loss of stromal glycosaminoglycans.

ROLE OF ANTIANGIOGENIC FACTORS (PEDF, ANGIOSTATIN, RESTIN, NEOSTATINE, AND ENDOSTATIN) IN THE MAINTENANCE OF CORNEAL AVASCULARITY

In addition to the involvement of angiogenic and antiangiogenic cytokines in angiogenesis, they are involved in cell migration and cell differentiation in wound healing and tumor progression (even in the absence of angiogenesis). The signaling mechanisms in these processes are, in part, distinct from those in angiogenesis, although there is some overlap. Without studying signal transduction pathways, including NF κ B, it is impossible to determine corneal antiangiogenic vs antigrowth mechanisms, as occurs in avascular wounds and tumors. There are also likely other complex mechanisms that are related to corneal avascularity, including effects from tears and aqueous and the effect of TGF- β . Additionally, corneal neovascularization is a process of vasculogenesis (growth from preexisting limbal vessels) rather than true angiogenesis.¹⁹⁹

Table 8 summarizes various VEGF inhibitors, many of which may have potential therapeutic applications in the treatment of corneal NV. Endostatin, which inhibits cell migration and cell proliferation, enhances vascular endothelial cell apoptosis through endostatin-associated molecules that interact with integrin $\alpha\beta$ 3, VEGF receptor (KDR/flk-1), tropomyosin, glypican, and laminin.²⁰⁰ Evidence suggests that endostatin binds to cell surface receptors and ECM molecules to regulate/inhibit vascular endothelial migration, proliferation, and apoptosis. Kim and associates¹⁸³ demonstrated that endostatin binds directly to VEGF receptors but not to VEGF, and that binding of endostatin to VEGF receptor blocks VEGF-induced tyrosyl phosphorylation of VEGF receptors (KDR/flk-1), MAP kinases, and FAK in human umbilical vein endothelial cells. Rehn and associates²⁰¹ demonstrated that soluble endostatin binds to integrin α 5 and α v to inhibit human vascular endothelial cell migration. Javaherian and associates²⁰² demonstrated that oligomeric endostatin binds to laminin in the BM and to heparan sulfates on the cell surface to regulate migration and morphogenesis of vascular endothelial cells. MacDonald and associates²⁰³ showed that endostatin binds to tropomyosin and inhibits cell mobility. Lee and colleagues²⁰⁴ demonstrated that endostatin binds to the catalytic domain of MMP-2 but not to proMMP-2, hinge, or hemopexin-like domains. Karumanchi and associates²⁰⁵ demonstrated that endostatin binds to glypicans.

The proposed mechanisms for endostatin functioning are characterized as follows: (i) endostatin inhibits vascular endothelial tube formation by inhibiting nitric oxide synthase²⁰⁶; (ii) endostatin attenuates vascular endothelial cell migration by downregulating c-myc mRNA expression, which was abrogated with the introduction of the c-myc gene into vascular endothelial cells²⁰⁷; (iii) endostatin induces endothelial cell apoptosis by activating caspase-3 enzymatic activity, reducing antiapoptotic protein Bcl-2, and reducing MAP kinases activities¹⁸⁴; (iv) endostatin regulates the Wnt signaling pathway by promoting β catenin degradation in the *Xenopus* system²⁰⁸; (v) endostatin causes G1 arrest of endothelial cells by decreasing the hyperphosphorylation of retinoblastoma gene product and decreasing the mRNA and protein of cyclin D1.²⁰⁹

Collagens are structural proteins containing a triple helical domain. They participate in the assembly of various ECM polymers and function primarily in the formation of fibril networks and BMs. Type XVIII collagen is one of two nonfibril collagens (multiplexins) that have been cloned and sequenced. It belongs to the heparan sulfate proteoglycan family.^{210,211} It is composed of 10 collagen domains alternating with 11 noncollagen (NC) domains. The N-terminus (NC11) and C-terminus (NC1) NC domains have nontriple helical structures.²¹² The NC1 region contains three functionally distinct regions: an association domain (necessary for collagen XVIII oligomerization), a hinge domain (sensitive to protease degradation), and an endostatin domain (a 20 kDa fragment with potent antiangiogenic properties).²¹³ Collagen XVIII is localized mainly to the vascular and epithelial BMs.^{214,215} Several isoforms of collagen XVIII cDNA have been isolated. In mice, three isoforms have shown differential expression among tissues.^{214,216} In humans, the shorter of two isoforms is expressed in the heart, kidneys, placenta, ovaries, skeletal muscles, and small intestine; the longer variant is liver-specific. In the eye, collagen XVIII localization has been reported in the retina (inner limiting membrane and pigment epithelium), lens capsule,²¹⁷ and cornea.^{10,13} Recently, Fukai and associates¹⁷⁵ demonstrated a delayed regression of blood vessels in the vitreous along the surface of the retina after birth and a lack of or abnormal outgrowth of retinal vessels.

Cleavage of collagen XVIII by proteases at specific sites generates a fragment, endostatin, with antiangiogenic properties.^{160,176,191} Several proteases, including cathepsin L¹⁸¹, elastase, MMP7, and MMP14,^{180,215} have demonstrated involvement in the degradation of the C-terminal noncollagen domain (NC1) of collagen XVIII to produce endostatin. MMPs and other proteases may also be involved in the degradation of collagen XVIII.¹⁸¹ The corneal epithelium may be the source of these antiangiogenic molecules. It has long been demonstrated that the corneal epithelium inhibits angiogenesis.⁷³ Ferreras and associates¹⁸² reported the generation of endostatin-like fragments by cleaving collagen XVIII with MMP-2, -3, -9, -12, -13, or -14. Lin and colleagues¹³ demonstrated that MMP-7 cleaves immunoprecipitated corneal collagen XVIII and recombinant collagen XVIII NC1 fragments to generate a 28 kDa endostatin-spanning fragment.

Also under investigation is restoration of the ocular surface with conjunctival, limbal, or amniotic membrane transplantation to reduce the angiogenic stimuli. In support of this idea, evaluations of autograft and allograft conjunctival transplantation in the treatment of unilateral and bilateral ocular surface disorders have demonstrated decreased corneal NV. In addition, amniotic

membrane transplantation has been shown to have antiangiogenic properties as amniotic membrane contains antiangiogenic molecules and precursors (including thrombospondin I and collagen XVIII).

TABLE 8. VEGF INHIBITORS

VEGF INHIBITOR	MECHANISM	ADVANTAGES	SUBJECTS	REFERENCES
Macugen (pegaptanib, Eyetech)	VEGF aptamer (RNA based oligonucleotide which binds VEGF)	Direct inhibition of VEGF; intravitreal injection	Humans	243
rhu Fab (Genentech)	Anti-VEGF monoclonal antibody fragment	Small fragment; good penetration; intravitreal injection	Monkeys	244
Semaxanib (SU 5416, Sugen Inc)	Competitive inhibitor of Flk-1/KDR > Flt1-1	Specific receptor synthetic inhibitor	Humans	245
SU 6668 (Sugen Inc)	Flk-1/KDR, PDGFR β and FGFR-1 receptor inhibitor	Oral administration	Humans	245
Angiozyme (Ribozyme Pharmaceutical)	Ribozyme-cleave mRNA for Flt-1 and Flk-1/KDR	—	Humans	245
CGP41251	Protein kinase C and VEGF receptor kinase inhibition	—	Humans	246
siRNA targeting VEGF	Inhibits VEGF or VEGF receptors	Subconjunctival injection, intravenous injection	Mice	247
Soluble VEGF receptor	sFlt-1 binds VEGF	Intravitreal injection	Rats	248
VEGF peptide	Inhibits VEGF from binding the receptor	Intravitreal injection	Mice	249
Squalamine(Evizon)	Inhibit VEGF mediated endothelial	Systematic	Human	250
VEGF Trap	VEGF Trap binds VEGF	IV systematic	Human	251
Bevacizumab(Avastin)	Anti-VEGF monoclonal antibody(whole antibody)	Intravitreal injection	Human	252

The treatment of severe ocular surface disorders with corneal ulceration, NV, and conjunctival metaplasia may require limbal autograft transplantation. This technique has been used successfully in vascularized corneas; it is thought to act by a double mechanism: by treating the stem cell deficiency and therefore treating the angiogenic stimulus provided by chronic corneal ulceration and by directly inhibiting vascular endothelial cells. However, although these molecules and surgical therapies have shown efficiency in treating corneal NV, they do not prevent the treatment of the underlying corneal NV etiology: antibiotic or antiviral therapy for an infectious keratitis, suture or contact lens removal for iatrogenic corneal NV, lubrication and prolonged eyelid hygiene for meibomian gland dysfunction, avoidance of allergens in the context of atopic dermatitis, and systemic immunosuppressive therapy in case of severe ocular cicatricial pemphigoid.

In summary, the following conclusions can be derived from the study:

- Several angiogenic factors and proangiogenic matrix metalloproteinases (MMPs) are present in the unwounded avascular cornea.
- MT1-MMP and VEGF proteins colocalize to stromal fibroblasts during wound healing in vivo.
- Intrastromal injection of MT1-MMP naked DNA during corneal wound healing results in corneal NV.

- Intrastromal injection of MT1-MMP naked DNA in VEGF promoter-regulated LacZ mice demonstrates VEGF expression by stromal fibroblasts during corneal wound healing.
- The hemilimbal deficiency model questions the role of the limbus in maintaining corneal avascularity.
- Proteolytic processing of plasminogen generates 51 kDa angiostatin molecules in the cornea. Intrastromal injection of blocking antibodies against angiostatin enhances corneal NV after laser keratectomy.
- Purified recombinant endostatin and corneal-derived 28 kDa endostatin-like fragments reduce calf pulmonary artery endothelial cell proliferation.

These findings are consistent with our hypothesis that stromal fibroblasts promote corneal NV through their proangiogenic MMP activity by three possible mechanisms (breakdown of the ECM, degradation of corneal antiangiogenic factors, and transcriptional upregulation of VEGF) and that the corneal epithelium generates corneal antiangiogenic factors and antiangiogenic MMPs that counterbalance the NV stimuli and help maintain corneal angiogenic privilege.

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