Bevacizumab accelerates corneal wound healing by inhibiting TGF-β2 expression in alkali-burned mouse cornea

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This study investigated the effect of subconjunctival injections of bevacizumab, an anti-VEGF antibody, on processes involved in corneal wound healing after alkali burn injury. Mice were divided into three groups: Group 1 was the saline-treated control, group 2 received subconjunctival injection of bevacizumab 1 hr after injury and group 3 received bevacizumab 1 hr and 4 days after injury. Cornea neovascularization and opacity were observed using a slit lamp microscope. Corneal repair was assessed through histological analysis and immunostaining for CD31, α-SMA, collagen I, and TGF-β2 7 days post-injury. In group 3, injection of bevacizumab significantly lowered neovascularization and improved corneal transparency. Immunostaining analysis demonstrated a reduction in CD31, α-SMA and TGF-β2 levels in stroma compared to group 1. These results indicate that bevacizumab may be useful in reducing neovascularization and improving corneal transparency following corneal alkali burn injury by accelerating regeneration of the basement membrane. [BMB reports 2009; 42(12): 800-805]

INTRODUCTION

Neovascularization (NV) of the cornea involves abnormal formation of blood vessels in the cornea, leading to severe visual impairment. Corneal NV may be induced by inflammation, infection, degeneration and traumatic disorders of the cornea and is a high risk factor for graft rejection after allograft corneal transplantation (1-3).

The regulation mechanism of corneal NV is a complex process involving the equilibrium between pro- and anti-angiogenesis factors. Angiogenic stimulators such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)-α and β can cause capillary endothelial cells to proliferate, leading to NV (4). Angiogenic inhibitors, such as angiostatin, play an essential role in maintaining an avascular environment in the cornea (5, 6).

The simple cellular organization of the cornea provides a particularly useful model for the study of wound healing events. The corneal alkali burn injury model used in this study is well established for the study of anterior surface inflammation, NV and wound healing processes (7). Healing following alkali burn is characterized by the extended presence of inflammatory cells in injured corneas, recurrent epithelial defects and NV. These characteristics interfere with proper healing processes and result in the formation of scar tissue and recurrent corneal erosion (8, 9).

Bevacizumab (Avastin) is a recombinant humanized murine monoclonal antibody that binds to and inhibits the biological activities of all human VEGF-A isoforms (10). Bevacizumab has been reported to abolish ocular NV in patients with age-related macular degeneration (AMD) and proliferative diabetic retinopathy. Bevacizumab can improve visual acuity and optical coherence tomography in patients with macular edema resulting from central retina vein occlusion (11).

Corneal opacity can be induced by NV, irregular epithelium structure and improper synthesis of the extracellular matrix (ECM) (12, 13). Recent studies suggest that bevacizumab inhibits corneal NV following chemical injury in rats (14), but there are no reports on its effect in improving corneal opacity and fibrotic response. Therefore, the purpose of this study was to investigate the effect of subconjunctival injections of bevacizumab on corneal wound healing following alkali burn injury.

RESULTS

Decrease of alkali burn-induced corneal haze and neovascularization following bevacizumab treatment

After removal of the alkali-immersed application stick from the eye, the injured central corneal stroma appeared opaque with a distinct edematous margin (Fig 1B). The opaqueness continued to increase following the alkali burn, reaching an average of grade 2.5 by post-operative day 7 (Fig. 1E). On day 3, the onset of peripheral NV extended from the limbus toward the central cornea, continuing to grow new vessels until day 7 (average of grade 3.5) (Fig. 1B, F). Group 2 received a subconjunctival injection of bevacizumab at 1 hr and showed scarce corneal NV and moderate opacity at day 7 (Fig. 1C, E, F). Group 3 received bevacizumab 1 hr and 4 days after alkali injury. There was no NV (average of grade 0.3) and central corneal opacity was mild (average of grade 0.9) by day 7 (Fig. 1D-F).
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Fig. 1. Effect of bevacizumab on alkali-burn-induced mice corneal neovascularization (NV). Following alkali burn injury on the central cornea of mice, NV was monitored by slit lamp microscope. Representative photographs of alkali burn induced corneal neovascularization at days 7 are shown. (A) In the normal cornea, a clear cornea was shown. (B) Group 1 was not treated with bevacizumab. The treatment group was divided into groups 2 and group 3. (C) Group 2 received subconjunctival injection of bevacizumab at 1 hr and (D) group 3 received bevacizumab at 1 hr and 4 days after the alkali injury. Graphs displaying corneal haze and neovascularization scoring in eye after alkali-burn injury. Haze (E) and neovascularization (F) were graded in the increments of 0.5. *P < 0.01 compared to group 1 at 7 days after the alkali injury.

Immunological staining of corneas indicated significant levels of CD-31 in the peripheral and central areas of the stroma 7 days after alkali burn injury (Fig. 2b1, 2b2). However, bevacizumab treatment of group 2 produced lower levels of CD-31 immunostaining in the central and peripheral areas at day 7 (Fig. 2c1, 2c2). There were less neovessels observed in group 3 treated with bevacizumab 1 hr and 4 days following injury (Fig. 2d1, 2d2). Furthermore, fewer neovessels were detected in the peripheral regions of both bevacizumab-treated groups (group 2 and 3) at day 7 (Fig. 2c2, 2d2)

Reduction of the fibrotic response following bevacizumab treatment
Corneal sections were stained with hematoxylin-eosin in order to detect the effect of bevacizumab on corneal structure (Fig. 3a1-3d1). All corneal epithelia were completely destroyed after alkali burn injury. Group 1 showed an increase in stroma thickness at day 7 (Fig. 3b1). Cornea in group 3 demonstrated significant recoveries in epithelial tissue with morphological features very similar to those of normal cornea (Fig. 3d1).

Since the presence of myofibroblasts is dependent upon the release of TGF-β2 from the epithelium into the stroma, we examined the expression of TGF-β2. TGF-β2 expression was increased in the stroma of controls (Fig. 3a2-3d2), but decreased in bevacizumab-treated group 3, in which expression was localized only in the epithelium at day 7.

The characteristics of fibrotic repair were examined by immunostaining for α-SMA, a marker for fibrosis that allows identification of stromal remodeling (Fig. 3a3-3d3). Stromal cells beneath the corneal epithelium showed strong staining for α-SMA after disruption by alkali injury (Fig. 3b3). Treatment of stromal cells with bevacizumab 1 hr and 4 days after alkali

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Fig. 3. Markers of fibroblast activation are reduced in alkali-burned cornea after subconjunctival bevacizumab treatment. (A) Normal, (B) group 1, control injury, (C) group 2, bevacizumab treated at 1 hr, (D) group 3, bevacizumab-treated at 1 hr and 4 days. Hematoxylineoosin-stained crossections of alkali burn-induced cornea and bevacizumab-treated cornea were examined 7 days (a1-d1) after alkali burn injury. The 4 groups were immunostained for TGF-β2 (a2-d2) and α-SMA (a3-d3) at day 7 (Ep = epithelium, St = stroma, End = endothelium). Scale bar, 100 μm; original magnification ×100.

Fig. 4. PAS staining and immunostaining of basement membrane components and type IV collagen in alkali-burned cornea 7 days after bevacizumab treatment. (A) Normal, (B) group 1, control injury, (C) group 2, bevacizumab-treated at 1 hr, (D) group 3, bevacizumab-treated at 1 hr and 4 days. The 4 groups were PAS stained (a1-d1) and immunostained for collagen IV (green) (a2-d2) at day 7. Nuclei were localized by DAPI staining (blue) (a3-d3). Arrows, basement membrane region (Ep = epithelium, St = stroma). Scale bar, 100 μm; original magnification ×400.

The integrity of basement membrane and the effect of epithelial TGF-β2 on fibrotic activation in stroma
We next questioned whether a proper basement membrane is involved in the regulation of TGF-β2 release into the stroma, and whether bevacizumab treatment has any effect on basement membrane production. The basement membrane or Bowman’s layer, as revealed by periodic acid-Schiff (PAS) stain, was thinner and discontinuous in many regions of the alkali-burned cornea (Fig. 4b1). In addition, the surface of the epithelium of the alkali-burned cornea was rough and exhibited excessive sloughing (Fig. 4b1). Administration of bevacizumab 1 hr and 4 days after burn injury, however, did affect the integrity of the basement membrane as well as the re-epithelialization and stratification of the cornea (Fig. 4d1). PAS histology of the cornea revealed that the basement membrane was lacking in certain regions of the epithelium after alkali injury, but was reestablished by bevacizumab treatment (Fig. 4b1-4d1). This was further explored by examining the expression of type IV collagen, a basement component (Fig. 4b2). Immunostaining of alkali-burned corneas for type IV collagen showed degradation of the basement membrane and loss of epithelial integrity 7 days post-injury. In contrast, corneas in the bevacizumab-treated group showed clear linear patterns of subepithelial immunoreactivity, indicating regeneration of the basement membrane (Fig. 4d2). TGF-β2 immunoreactivity was initially confined to the epithelia of normal burn injury produced no staining for α-SMA (Fig. 3d3).
Corneal NV and subsequent opacification remain the most frequent causes of blindness following alkali burn injury. This process is characterized by an ingrowth of neovessels originating from the limbus and is often accompanied by an inflammatory response. VEGF is an important factor in this process because it stimulates corneal NV (15). Therefore, one possible treatment could involve the competitive binding and inhibition of VEGF with a specific, neutralizing anti-VEGF antibody (16).

This study shows that subconjunctival application of the anti-VEGF agent, bevacizumab, is useful for the inhibition of corneal NV and for accelerating the regeneration of epithelial basement membrane. As a result, the transfer of TGF-β2 into stroma becomes blocked, resulting in corneal clarity after alkali burn injury.

We previously reported that alkali burn causes severe cell death, edema, NV and opacification of corneal stroma by transdifferentiation of keratocytes into α-SMA-positive myofibroblasts (17). Neovascularization of corneal stroma potentially contributes to opacification. Blockage of VEGF action by the subconjunctival administration of bevacizumab 1 hr and 4 days after alkali burn injury caused reduced levels of stromal NV, resulting in improved corneal clarity.

The transient conversion of keratocytes into myofibroblasts, as characterized by α-SMA expression, is an important histological feature of corneal stromal wound healing after exposure to alkali and is associated with the upregulation of matrix components in stromal scarring (18-20). In vitro studies have shown that α-SMA expression in dermal fibroblasts is regulated by cytokines and the extracellular matrix. Our immunohistochemical studies indicate that fibroblastic cells in the injured stroma have characteristics of myofibroblasts, as they are strongly stained with antibodies against α-SMA. Stromal cells in neither the normal cornea nor the bevacizumab-treated cornea reacted with anti-α-SMA antibody, indicating that stromal fibroblast cells did not undergo any phenotypic changes in the stroma.

Several studies have suggested that an epithelial-stromal interaction is responsible for fibrotic repair in cornea. Several secreted factors produced by the epithelium are capable of controlling fibrosis, and among these factors, TGF-β2 has been identified as the major mediator of the fibrotic phenotype (21-24). In the early stages, wounds treated with TGF-β1 and TGF-β2 demonstrated increased fibronectin, collagen III and collagen I deposition compared to controls (25).

Our results demonstrate that the epithelium and stroma of alkali-injured cornea expressed TGF-β2 7 days post-injury. Bevacizumab-treated cornea showed that TGF-β2 expression was confined to the epithelium and was suppressed by bevacizumab treatment in the corneal stroma. Since TGF-β normally inhibits the growth of epithelial cells, its suppression by bevacizumab would increase epithelial cell proliferation, thereby enhancing reepithelialization as seen in our cornea model. Epithelial healing in the cornea depends on cell migration, proliferation and the integrity of the epithelial basement membrane. Stramer et al. developed a mouse model for penetrating keratectomy and demonstrated that TGF-β2 is released by corneal epithelia into the corneal stroma following a disturbance of the basement membrane (24). They further demonstrated that corneal epithelial cells release TGF-β2 into the stroma when cultured on an artificial stromal surface, which prevents the synthesis and deposition of basement membrane. Once in the stroma, TGF-β2 induces the transformation of keratocytes into myofibroblasts. When basement membrane synthesis was stimulated in this model, the fibrotic phenotype was absent and TGF-β2 was expressed only in the epithelium (24). In a study using conditional knockout mice of transcription factor AP-2α, the basement membrane exhibited intermittent breaks and stained positive for α-SMA, and TGF-β2 was detected in the stroma (26).

Our data showed that the stroma stained strongly for TGF-β2 upon loss of the basement membrane. However, TGF-β2 expression was decreased in the stroma upon reconstitution of the basement membrane after bevacizumab treatment. Additionally, only the epithelial cells stained positive, suggesting that regenerative repair occurred in stromal cells after alkali burn injury in the cornea. Our immunohistochemistry analysis showed that α-SMA expression in the corneal stroma after alkali burn was inversely correlated with reconstitution of the basement membrane by bevacizumab treatment, which itself correlated with the decline of TGF-β2 levels within the stroma. This observation suggests that bevacizumab regulates the integrity of the epithelial basement membrane and moreover determines whether the corneal repair process is fibrotic or regenerative by controlling the release of TGF-β2 into the stroma.

In summary, our findings suggest that subconjunctival administration of bevacizumab can reduce NV and haze in the cornea after alkali burn injury. Bevacizumab treatment rapidly recovered the integrity of the basement membrane after alkali burn injury and prevented interaction between the epithelium and stroma, which in turn promotes stromal cell activation. As
a result, the basement membrane inhibits the fibrotic repair process by controlling the release of TGF-β2 into the stroma. We therefore conclude that the subconjunctival injection of bevacizumab plays an important role in regenerating the basement membrane in the cornea after alkali burn injury by creating a physical barrier to TGF-β2. It also plays a critical role in maintaining corneal homeostasis and minimizing fibrotic repair.

MATERIALS AND METHODS

Experimental animals
Twenty 5- to 7-week-old male C57BL/6 mice (20 g) were purchased from Daehan Biolink (Seoul, South Korea). All animal experiments were conducted in accordance with the Animal Care and Use Committee criteria and the Association for Research and Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Corneal alkaline burn injury
Anesthesia was achieved by intraperitoneal injection of ketamine hydrochloride (30 mg/kg) and xylazine hydrochloride (5 mg/kg). Alkali burn injury was performed by pressing an application stick soaked in 0.5 N NaOH onto the central cornea for 10 seconds. The corneal surface was then carefully rinsed with 10 ml of physiological saline solution for 5 minutes.

Drug preparation and treatment protocol
Bevacizumab (2.5 mg/ml) (Avastin, Roche, Basel, Switzerland) was delivered into both eyes (5 μg) by subconjunctival injection. The animals were separated into three groups according to the different time frames of bevacizumab treatment. Group 1 (n = 10, 20 eyes), the control, received a sham injection of 2 μl of saline, group 2 (n = 10, 20 eyes) received bevacizumab 1 hr after alkali burn injury and group 3 (n = 5, 10 eyes) received bevacizumab 1 hr and 4 days after injury. This treatment regimen was based on our previous report that additional treatment of bevacizumab over several days after injury was effective in inhibiting NV (17).

Assessment of corneal haze and neovascularization
The degree of corneal haze produced in mice was examined on days 1, 3 and 7 after alkali burn injury using slit lamp microscopy. Corneal opacity was scored using a 0-to-4 scaling on days 1, 3 and 7 after alkali burn injury using slit lamp microscopy. Corneal opacity was scored on a scale of 0–4, where 0 = hardly visible, 1 = severely opaque, 2 = moderately opaque, 3 = slightly hazy, iris and lens visible; 4 = completely opaque, with no view of iris and lens. Corneal haze was graded in increments of 0.5.

REFERENCES
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