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Accelerating repaired basement membrane after bevacizumab treatment on alkali-burned mouse cornea

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To understand the corneal regeneration induced by bevacizumab, we investigated the structure changes of stroma and basement membrane regeneration. A Stick soaked in 0.5 N NaOH onto the mouse cornea and 2.5 mg/ml of bevacizumab was delivered into an alkali-burned cornea (2 μl) by subconjunctival injections at 1 hour and 4 days after injury. At 7 days after injury, basement membrane regeneration was observed by transmission electron microscope. Uneven and thin epithelial basement membrane, light density of hemidesmosomes, and edematous collagen fibril bundles are shown in the alkali-burned cornea. Injured epithelial basement membrane and hemidesmosomes and edematous collagen fibril bundles resulting from alkali-burned mouse cornea was repaired by bevacizumab treatment. This study demonstrates that bevacizumab can play an important role in wound healing in the cornea by accelerating the reestablishment of basement membrane integrity that leads to barriers for scar formation. [BMB Reports 2013; 46(4): 195-200]

INTRODUCTION

The human cornea, a highly specialized and unique organ, is continually subjected to abrasive forces and mechanical trauma due to its anatomical location. Damage to the cornea may result in scarring or opacification that causes visual defects of transparency problems, even leading to severe visual impairment. However, many of those wounds and their problems in healing are highly related to the breakdown of corneal epithelium (1).

Corneal epithelial defects must be rapidly resurfaced to avoiding microbial infection and further damage to the underlying stroma. The epithelial healing is achieved both by migration of the epithelial sheet on (or over) the denuded surface and by epithelial stratification formed with enhanced cell proliferation quickly after resurfacing (2). Epithelial wound healing is also affected by complex epithelial-stromal interactions mediated by growth factors and extracellular matrix (ECM) components (3, 4).

Cell-cell and cell-matrix interactions play important roles in maintaining the stratified structure of the corneal epithelium (5). Cell adhesion and cell migration depend on the synthesis and assembly of the extracellular matrix, including the basement membrane at the epithelium-stroma junction (ESJ). During wound healing, the regeneration of a functional corneal epithelium depends on epithelial migration and the reconstitution of the ESJ, which anchors the epithelium to the stroma.

After an alkali burn, polymorphonuclear leukocytes infiltrate the injured corneas, and the proteolytic enzymes, oxidative derivatives, or both, released by the inflammatory cells can cause severe loss of the extracellular matrix (6). The stromal cells that survive after the alkali burn may proliferate and synthesize components of the extracellular matrix in the repairing process of injured corneas. Stromal ulceration takes place when the rate of degradation of extracellular matrix components (e.g. collagen, proteoglycans) exceeds the rate of synthesis (7). Many investigators have examined the metabolism of fibrillar collagens during the healing of the lacerated corneas in which increases in the synthesis of collagen I, III and V were reported (8).

Normal and abnormal processes of cellular invasion are initiated by degradation of basement membranes. The alteration of basement membrane (BM) components, collagen, lamin, and fibronectin, is an important marker of the healing process in corneas burned with alkali (9). The matrix metalloproteinases (MMPs) are involved in cleaving collagen types IV, V, VII, and X, fibronectin, laminin, and gelatins. A member of the MMP family of enzymes in both cellular invasion processes and degradation of epithelial BM, they are involved in the progression from alkali burns to ulceration (10). The MMPs and the tissue inhibitor of metalloproteinases (TIMPs) regulate the extracellular matrix, and both are important in the process of connective tissue remodeling (11). The myofibroblast is deeply

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involved in degrading some of the key matrix proteins (such as type I collagen), and may play an important role in tissue remodeling in corneal wounds through production of MMPs and TIMP (11).

Cornea alkali burns are one of the serious clinical problems leading to permanent visual impairment resulting from ulceration, scarring, and neovascularization (NV) during healing. Vascularization is important for wound healing and reproduction. Angiogenic agents include various growth factors and cytokines, such as TGF-β, b-FGF, VEGF, IL-8, and selectin E (12). TGF-β2 is released from corneal epithelia into the corneal stroma following a disturbance of the BM (13). Our previous study showed that subconjunctival application of the anti-VEGF agent, bevacizumab (avastin), is useful for the inhibition of corneal NV and lower TGF-β2 reactivity in the stroma and positive staining in the epithelium similar to a normal cornea (14). We assume that bevacizumab accelerates basement membrane regeneration and repaired basement membranes act as a barrier to TGF-β2 produced in the epithelium, so that the transfer of TGF-β2 into stroma may be blocked, which can have an effect on corneal clarity. In this study, we investigated the effects of bevacizumab on the structure and regeneration of the basement membrane using electron microscopy study.

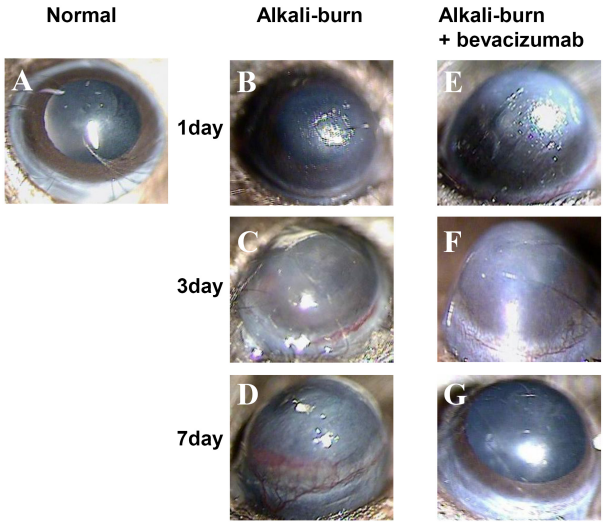

Fig. 1. Effect of bevacizumab on alkali burn-induced mice corneal NV. Following alkali burn injury on the central cornea of mice, NV was monitored by slit lamp microscope. Representative photographs of alkali burn induced corneal NV at day 1 (B and E), day 3 (C and F) day 7 (D and G) are shown. (A) The normal cornea, and a clear cornea was shown. (B-D) Alkali-burned cornea, haze and NV were shown. (E-G) Bevacizumab treated at 1 hour and 4 days after the alkali injury. Corneal haze and NV were reduced.

RESULTS AND DISCUSSION

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). On day 3, the onset of peripheral NV extended from the limbus to the central cornea, which were continuing to grow new vessels until day 7 (Fig. 1C, D). The Experimental group received bevacizumab at 1 hour and 4 days after alkali injury, in which there was no NV and central corneal opacity was mild by day 7 (Fig. 1G).

A necessary transmission of light is controlled by transparency of the cornea, a vascular tissue of eye. An organized extracellular matrix structure is important to the maintenance of the transparency. An alkali-burned cornea is an acute problem that may cause severe and permanent visual impairment by scarring (15-17). After alkali injury in the cornea, the influx of inflammatory cells, ie, macrophages, activation of corneal fibroblasts (keratocytes), and subsequent tissue scarring in association with myofibroblast generation and neovascularization (NV are all involved (18, 19). Bevacizumab has been reported to abolish ocular NV in patients with age-related macular degeneration and proliferative diabetic retinopathy (20). Bevacizumab can improve visual acuity and optical coherence tomography in patients with macular edema resulting from central retina vein occlusion (20).

Our previous study indicated that bevacizumab may be useful in reducing NV, and that improving corneal transparency following corneal alkali burn injury can be achieved by accelerating regeneration of the basement membrane (14).

The basement membrane or Bowman's layer, as revealed by periodic acid-Schiff (PAS) stains, was detatched from stromal collagen fibers in many regions of the alkali-burned cornea (Fig. 2B). In the bevacizumab treated group after burn injury, however, it was revealed in the cornea that the basement membrane was reestablished (Fig. 2C). This was further explored by examining the expression of type IV collagen.

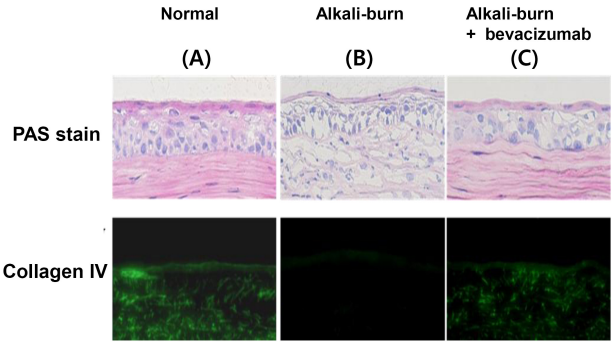

Fig. 2. PAS staining and type IV collagen immunostaining in alkali-burned cornea 7 days after bevacizumab treatment. (A) Normal, (B) group 1, control injury, (C) bevacizumab treated at 1 hour and 4 days.

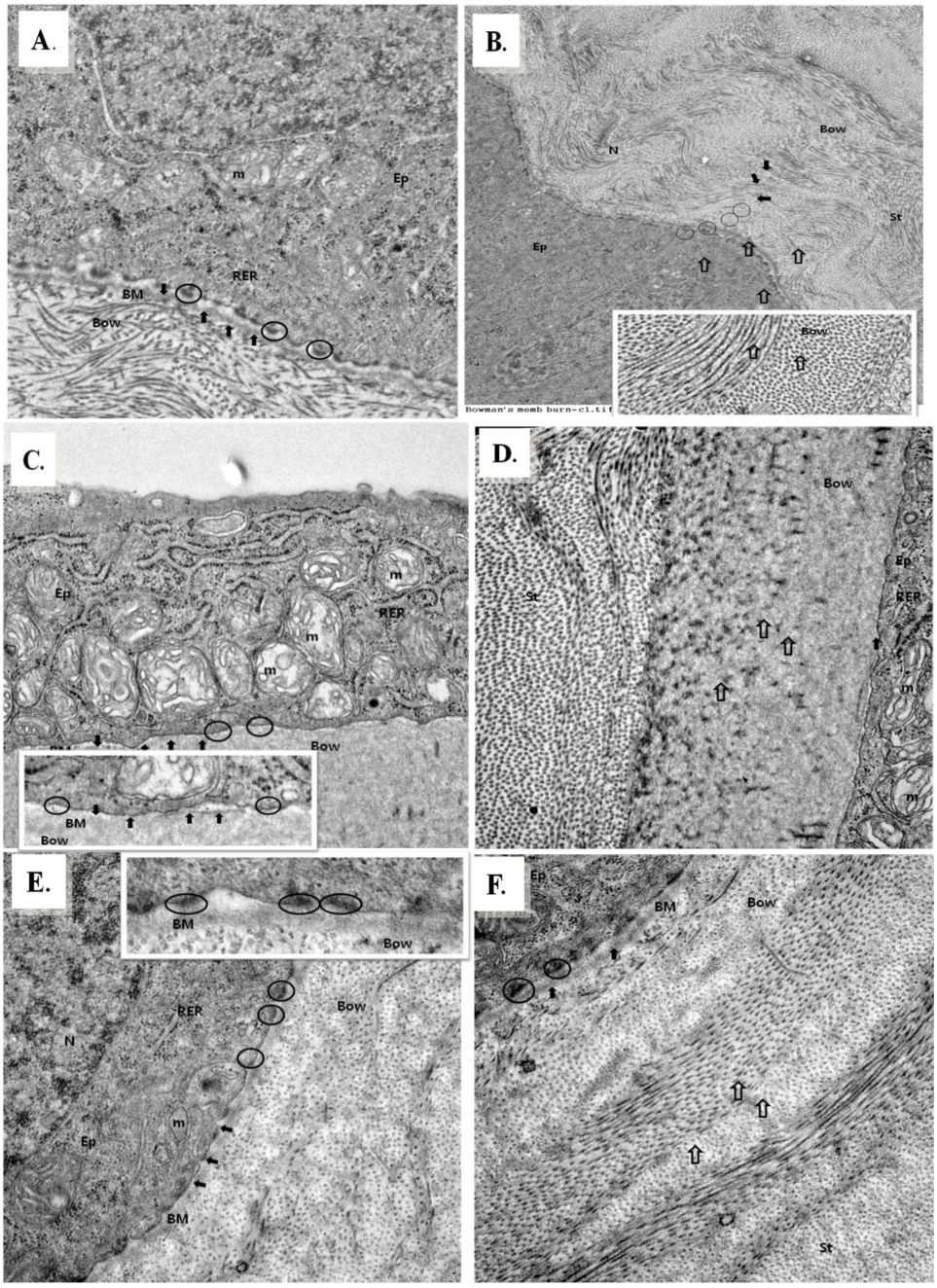

Fig. 3. An ultra-micrograph cross-section of the mouse cornea. Corneal epithelium (Ep) and stroma (St), Bowman's membrane (Bow), Rough endoplasmic reticulums (RER) and mitochondria (m). (A) control. The epithelial basement membrane (BM) has normal thickness (arrows) and normal density and uniform distribution of hemidesmodomes (circles). Rough endoplasmic reticulums (RER) and ribosomes in the cytoplasm. (B) control. regular collagen fibril bundles (open arrows) (original magnification, ×1,500). Regular collagen fibril bundles (open arrows) are shown in high magnification in the right inset (original magnification, ×10,000). (C) Alkali-burned. Uneven and thin epithelial basement membrane (arrows) and light density of hemidesmosomes (circles) shown in high magnification in the left inset (original magnification, ×10,000). (D) Alkali-burned. Edematous collagen fibril bundles (open arrows) are shown. (E) Bevacizumab-treated. The epithelial basement membrane has normal thickness (arrows) and normal density and uniform distribution of hemidesmodomes (circles) shown in high magnification in the right inset (original magnification, ×10,000). (F) Bevacizumab-treated. Basement membrane has normal thickness (arrows) and normal density and uniform distribution of hemidesmodomes (circles) and regular collagen fibril bundles (open arrows).

Immunostaining of type IV collagen was not observed in alkali burned cornea (Fig. 2B), however in the bevacizumab treated group expression of type IV collagen was observed. (Fig. 2C).

The epithelial basement membrane (BM) has normal thickness (arrows) and density and uniform distribution of hemidesmodomes (circles). Rough endoplasmic reticulums (RER) and ribosomes were found in the cytoplasm (Fig. 3A). Regular collagen fibril bundles (open arrows) are shown in high magnification in the right inset (Fig. 3B). Uneven and thin epithelial basement membrane (arrows) and light density of hemidesmosomes (circles) are shown in the alkali-burned cornea (Fig. 3C). The observation of the burned cornea shows edematous collagen fibril bundles (open arrows) (Fig. 3D). The epithelial basement membrane after being treated with bevacizumab has normal thickness (arrows) and density and uniform distribution of hemidesmodomes (circles) (Fig. 3E). In bevacizumab-treated cornea, the basement membrane has normal thickness (arrows) and normal density, uniform distribution of hemidesmodomes (circles) and regular collagen fibril bundles (open arrows) (Fig. 3F).

In this study, we further investigated the effects of bevacizumab on the structure and regeneration of the basement membrane using electron microscopy study and the relationship between maintaining process of uniform structure of cornea stroma. We previously suggested that subconjunctival administration of bevacizumab can reduce NV and haze in the cornea after alkali burn injury (14). Integrity of the basement membrane after alkali burn injury was rapidly recovered with bevacizumab treatment. The treatment also prevented interaction between the epithelium and stroma, which in turn promotes activation of stromal cells. The process of fibrotic repair was inhibited by the basement membrane with controlled release of TGF-β2 into the stroma. Therefore, it can be concluded that the subconjunctival injection of bevacizumab plays an important role in regenerating the basement membrane in the cornea after alkali burn injury. A physical barrier to TGF-β2 also plays a critical role in maintaining corneal homeostasis and minimizing fibrotic repair. In this study, we investigated the relationship between maintaining the uniform structure of cornea stroma and bevacizumab effects on basement membrane structure and regeneration processes.

Many of wounds and their problems in healing may be highly related to the breakdown of corneal epithelium due to a defect in adhesion to the basement membrane. The first corneal response to injury has very complex processes of inflammation. The integrity and transparency of the tissue is known as the consequence of this response. Inflammation induced by the injury provides inflammatory/fibrogenic growth factors/cytokines, which often lead to a fibrotic lesion causing failure of tissue remodeling and dysfunction of tissues caused by excess accumulation and contraction of extracelluar matrix (ECM) by myofibroblasts (21, 22). The fibrogenic process is stimulated by inflammatory cells derived cytokines, especially the fibrogenic cytokine, with TGF-β (23). Blocking TGF-β signals transduction molecules, e.g., Smad, by gene transfer or other technologies which could be an important strategy to prevent or treat the undesirable outcomes of the injured tissues and regeneration of functional tissues of fibrosis.

In the process of tissue repair of the corneal stroma, various growth factors/cytokines are involved. Among them, TGF-β family members play an important role in the development of scars on the cornea as well as regulation of epithelial migration. Although TGF-β family members are expressed in the corneal epithelium and stromal cells, they are in the inactive form in the physiological condition. In an injured cornea, it is rapidly activated and also expressed by infiltrating inflammatory cells, i.e., macrophages. Bavacizumab showed a reduction in inflammatory cell infilteration and cytokines in chemically burned corneas (24).

Healthy corneal epithelium is essential for maintaining transparency, avoiding infection and maintaining corneal integrity. To preserve the structure, the epithelium anchors to the stroma ECM through the BM. Cytoplasmic keratins, which is on intracellular intermediate filaments, participate in the formation of hemidesmosomes. It also plays an important role in the focal adhesion of epithelial cells to the basement membranes. Our data suggests that disassembly of hemidesmosomes must occur for barrier of TGF-β2 translocation to stroma in the process of wound healing.

It was shown that epithelium of posttraumatic recurrent corneal erosion splits between the basement membrane and Bowman's layer. The poor epithelial to stroma adhesion results from poor anchoring of collagen VII fibrils to the Bowman's layer (25). Persistent epithelial defects and recurrent erosions by diverse agents cause reduced adhesion of the epithelial layer to the Bowman's membrane, and could lead to ulceration and, in extreme cases, perforation. The platelet-activating factor inhibits epithelial wound healing by decreasing the attachment of the epithelial cells to various ECM components of the basement membrane, and by increasing keratocyte apoptosis (26).

The results suggest bavacizumab can plays an important role in corneal wound healing by affecting adhesion of epithelial cells. Our data suggest that bavacizumab critically acts on the basement membrane integrity through assembly of hemidesmosomes by acting as a barrier for inflammatory cytokine, such as TGF-β2. Helping to avoid loss of corneal transparency and visual acuity, bavacizumab could be of therapeutic importance for the case of prolonged ocular erosion. In conclusion, bevacizumab may play an important role in wound healing in the cornea by accelerating the reestablishment of basement membrane integrity which leads to a barrier for scar formation.

MATERIALS AND METHODS

Experimental animal

A total of fifteen mice, 5- to 7- week-old male C57BL/6 mice (20 g), purchased from DaeHan Biolink were used in this study. 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 ketamin hydrochloride (30 mg/kg) and xylazine hydrochloride (5 mg/kg). Alkali burn injury was performed by pressing application stick soaked in 0.5 N NaOH onto the central cornea for 10 seconds. The cornea surface was then carefully rinsed with 10 ml of physiological saline solution for 5 minutes.

Drug preparation and treatment protocol

2.5 mg/ml of bevacizumab (Avastin) was delivered into the eye (2 μl) by subconjunctival injections. The animals were separated into two groups. Group 1 (n = 5, 10 eyes), the control, received a sham injection of 2 μl of saline. Group 2 (n = 5, 10 eyes) received 2 μl per eye of bevacizumab (5 mg/ml) 1hour and 4 days after injury.

Assessment of corneal haze

The corneal haze in mice was examined and photographed on days 1, 3 and 7 days after alkali burn injury using a slit lamp microscope.

Tissue preparation

Mice were anesthetized and the experimental eyes were enucleated at 7 days after alkali burn injury and embedded in liquid OCT compound (Sakura FineTek, Torrance, CA, USA) within a mold. Cornea specimens were centered within the mold block to be sectioned transversely from the center of the cornea. The mold and tissue were rapidly frozen and stored at -80^{o}C until sectioning was performed. The corneal sections were cut into 4 μm thickness with a cryostat (Shandon cryotome FE, Thermo, USA) and placed on microscope slides and stored at -80^{o}C until staining. The sections were stained with hematoxylin and eosin stain and periodic acid-Schiff (PAS) stain.

Immunostaining

An air dried section was fixed with ice cold methanol (-20^{o}C) for 1 hour and rinsed in PBS and single and double immunofluorescence staining was performed. For single immunoflorescence labeling, the sections were stained with rabbit anti-collagen IV (1 : 80, chemicon, USA) overnight at 4^{o}C and stained again with secondary antibody Alexa 488 donkey anti-rabbit IgG (1 : 400, Invitrogen, USA) at room temperature for 1 hour (27). The sections were mounted by aqueous mounting medium (DAKO, USA).

Transmittance Electron Microscopy (TEM)

All corneal buttons were fixed in 2% neutral phosphate buffered glutaraldehyde. Strips of corneal tissue were dissected perpendicular to the corneal surface. The strips were taken from the center of the corneas to avoid peripheral artifacts. After incubation in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 1 hour, the strips were dehydrated by gradual ethanol and were embedded in epoxy resin. All of the specimens were processed using the same technique and by the same technician for uniformity in preparation without masking. Ultrathin sections (approximately 60-70 nm thick) were prepared by ultramicrotome and were stained with uranyl acetate in 30% ethanol for 10 minutes and 0.03% lead citrate in 0.01 N natrium hydroxide for 10 minutes. The sections were washed in tap water and were allowed to dry at room temperate (28). They were evaluated by transmission electron microscope (H-7600 operated at 80 kW, Hitachi Co., Japan) and photographed. The size of the spaced fibril bundles found in the Descemet's membrane was measured against a preset measure bar according to the microscope magnification.

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