Transmission Electron Microscopy Analysis of Epithelial Basement Membrane Repair in Rabbit Corneas With Haze

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Submitted: March 26, 2013
Accepted: May 15, 2013

Citation: Torricelli AAM, Singh V, Agrawal V, Santhiago MR, Wilson SE. Transmission electron microscopy analysis of epithelial basement membrane repair in rabbit corneas with haze. *Invest Ophthalmol Vis Sci*. 2013;54:4026–4033. DOI:10.1167/iov.13-12106

PURPOSE. To assess the ultrastructure of the epithelial basement membrane using transmission electron microscopy (TEM) in rabbit corneas with and without subepithelial stroma opacity (haze).

METHODS. Two groups of eight rabbits each were included in this study. Photorefractive keratectomy (PRK) was performed using an excimer laser. The first group had -4.5 -diopter (-4.5 D) PRK and the second group had -9.0 D PRK. Contralateral eyes were unwounded controls. Rabbits were sacrificed at 4 weeks after surgery. Immunohistochemical analysis was performed to detect the myofibroblast marker α -smooth muscle actin (SMA). TEM was performed to analyze the ultrastructure of the epithelial basement membrane and stroma.

RESULTS. At 4 weeks after PRK, α -SMA+ myofibroblasts were present at high density in the subepithelial stroma of rabbit eyes that had -9.0 D PRK, along with prominent disorganized extracellular matrix, whereas few myofibroblasts and little disorganized extracellular matrix were noted in eyes that had -4.5 D PRK. The epithelial basement membrane was irregular and discontinuous and lacking typical morphology in all corneas at 1 month after -9 D PRK compared to corneas at 1 month in the -4.5 D PRK group.

CONCLUSIONS. The epithelial basement membrane acts as a critical modulator of corneal wound healing. Structural and functional defects in the epithelial basement membrane correlate to both stromal myofibroblast development from precursor cells and continued myofibroblast viability, likely through the modulation of epithelial–stromal interactions mediated by cytokines. Prolonged stromal haze in the cornea is associated with abnormal regeneration of the epithelial basement membrane.

Keywords: basement membrane, corneal epithelium, myofibroblasts, haze, corneal wound healing

Corneal myofibroblast generation and associated abnormal extracellular matrix deposition have been identified as important biological events that lead to the generation of corneal opacity or haze.^{1–4} Prior studies have suggested that the structural integrity of the regenerated epithelial basement membrane plays a critical role in determining whether a particular cornea develops haze by limiting the access of epithelium-derived growth factors, such as transforming growth factor beta, to the stroma that modulate myofibroblast development from precursor cells and block myofibroblast apoptosis.^{5,6} The purpose of this study was to assess the ultrastructure of the cornea epithelial basement membrane in rabbit corneas with and without subepithelial stromal opacity (haze) using transmission electron microscopy.

MATERIALS AND METHODS

Animals and Surgery

The Animal Control Committee at the Cleveland Clinic Foundation approved all animal studies described in this work. All animals were treated in accordance with the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Anesthesia was obtained by intramuscular

injection of ketamine hydrochloride (30 mg/kg) and xylazine hydrochloride (5 mg/kg). In addition, topical proparacaine hydrochloride 1% (Alcon, Fort Worth, TX) was applied to each eye just prior to surgery. Euthanasia was performed using an intravenous injection of 100 mg/kg pentobarbital while the animal was under general anesthesia.

Female New Zealand White rabbits 12 to 15 weeks old, weighing 2.5 to 3.0 kg each, were included in this study. One eye of each rabbit was selected at random to have photorefractive keratectomy (PRK) surgery, and eight eyes were included in each PRK and control group. Contralateral eyes were used in the control groups since we have not identified any contralateral eye effects on haze in previous studies of haze in PRK.⁵ The two groups underwent PRK with 6.0 mm ablation zones using a VISX Star S4 IR excimer laser (Abbott Medical Optics, Irvine, CA). The first PRK group had a -4.5 -diopter (-4.5 D) ablation and the second group had a -9.0 D ablation, according to a previously published method.⁷ Briefly, with the animal under general and topical anesthesia, a wire lid speculum was positioned within the interpalpebral fissure. A 7 mm diameter area of central epithelium overlying the pupil was removed by scraping with a No. 64 Beaver blade (Becton-Dickinson, Franklin Lake, NJ). A spherical ablation was then performed on the exposed stroma with the excimer laser. No

FIGURE 1. Slit-lamp photo of rabbit cornea at 1 month after surgery. (A) Dense subepithelial haze restricted to the area of corneal ablation (arrows) was noted in all corneas after -9.0D PRK. (B) Mild haze (arrows) was noted in one cornea after -4.5D PRK, but the remaining corneas in that group were clear (not shown). (C) Control corneas were clear without any haze. Magnification: $\times 20$.

corticosteroids were applied. One drop of Vigamox (Alcon) was applied to the PRK and control cornea four times a day until the epithelium healed (4–6 days in all corneas and no significant difference between the -9D and -4.5D groups).

Tissue Fixation

At 4 weeks after PRK (the time point at which haze and myofibroblast density were shown to peak after PRK in previous studies⁷), the rabbits were euthanized and the corneal scleral rims of treated and untreated contralateral eyes were removed without manipulation of the cornea using 0.12 mm forceps and sharp Westcott scissors (Fairfield, CT).

For immunohistologic analyses to confirm myofibroblasts, two corneas of each group were immediately embedded in liquid Optimal cutting temperature (OCT) compound (Sakura FineTek, Torrance, CA) within a $24 \times 24 \times 5$ mm mold (Fisher Scientific, Pittsburgh, PA). Corneas were centered within the mold so that the block could be bisected and transverse sections cut from the center of the cornea. The frozen tissue blocks were stored at -80°C until sectioning was performed. Central corneal sections ($7\text{ }\mu\text{m}$ thick) were cut with a cryostat (HM 505M; Micron GmbH, Walldorf, Germany). Sections were placed on $25 \times 75 \times 1$ mm microscope slides (Superfrost Plus; Fisher Scientific) and maintained frozen at -80°C until staining was performed.⁸

For electron microscopy, six corneas of the -9D PRK and control groups and five corneas of the -4.5D PRK group (one rabbit in this group developed a gastrointestinal infection and had to be euthanized at 1 week after surgery) were stored in 2.5% glutaraldehyde and 4% paraformaldehyde with 0.2 M cacodylate buffer at 4°C . Sections were prepared for transmission electron microscopy by cutting small blocks of tissue from the treated central cornea in corneas that had PRK or the untreated central cornea of controls. The excised blocks were placed into the electron microscopy (EM) fixative and stored at 4°C until sectioned.

Immunocytochemistry

Immunohistochemistry was performed as previously described⁹ in order to identify myofibroblast cells in the stroma. Briefly, immunofluorescent staining for α -smooth muscle actin (SMA) was performed using a mouse monoclonal anti-human α -SMA clone 1A4 (Dako, Carpinteria, CA). The slides were washed twice with PBS and incubated for 90 minutes at room temperature with anti- α -SMA antibody at 1:50 dilution in 1% BSA. Slides were then incubated at room temperature with secondary antibody, Alexa Fluor 488 (Invitrogen, Carlsbad, CA)

goat antimouse IgG at a dilution of 1:100 in 1% BSA for 1 hour. Slides were rinsed with PBS, and cover slips were mounted with Vectashield containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc., Burlingame, CA) to allow visualization of all nuclei in the tissue sections. The sections were viewed and photographed with a Leica DM5000 microscope (Leica, Buffalo Grove, IL) equipped with Q-imaging Retiga 4000RV (Surrey, BC, Canada) camera and ImagePro software (Leica).

Electron Microscopy

EM samples were prepared according to the protocol described by Fantes et al.¹⁰ Briefly, specimens were placed in 2.5% glutaraldehyde and 4% paraformaldehyde with 0.2 M cacodylate buffer immediately after removal of the cornea and scleral rim, and then the specimens were left in the fixative for a minimum of 24 hours. Corneas were then rinsed with 0.2 M cacodylate buffer three times for 5 minutes each, postfixed in 1% osmium tetroxide for 60 minutes at 4°C , and dehydrated in increasing concentrations of ethanol from 30% to 95% for 5 minutes each at 4°C . Finally, dehydration was performed using three 10-minute rinses in 100% ethanol at room temperature and three 15-minute rinses with propylene oxide at room temperature. Excised blocks of central anterior cornea were then embedded in epoxy resin medium. One-micrometer-thick sections were stained with toluidine blue for orientation and light microscopy. Ultrathin 85 nm thick sections were cut with a diamond knife, stained with 5% uranyl acetate and lead citrate, and then observed using a Philips CM12 transmission electron microscope operated at 60 kV (FEI Company, Hillsboro, OR).

RESULTS

Cornea Evaluation by Slit-Lamp Biomicroscopy

At 4 weeks after surgery, dense corneal haze was noted in all -9.0D PRK corneas (Fig. 1A). In contrast, each -4.5D PRK cornea had trace haze or was completely clear (Fig. 1B). Untreated control rabbit corneas were uniformly clear (Fig. 1C).

α -SMA+ Myofibroblasts in Anterior Corneal Stroma

No α -SMA-positive myofibroblasts were observed in the control corneas (Fig. 2A). High densities of α -SMA-positive myofibroblasts were observed in the subepithelial stroma of the ablated

FIGURE 2. Immunocytochemistry for α -smooth muscle actin (SMA)+ myofibroblast cells at 1 month after PRK in rabbits. (A) Control cornea without PRK. No α -SMA+ cells (red) were detected in any control corneas. Blue represents DAPI staining of cell nuclei. (B) All sections of corneas that had $-9D$ PRK had a high density of α -SMA+ myofibroblasts (arrows) in the subepithelial stroma underlying the excimer laser ablation zone. (C) Few, if any, α -SMA+ myofibroblasts were detected at 1 month after PRK in rabbit corneas that had $-4.5D$ PRK. Shown is one section from a $-4.5D$ PRK where a few α -SMA+ myofibroblasts (arrows) were detected in the subepithelial stroma. This likely corresponds to a region like that shown in Figure 4E on $-4.5D$ PRK transmission electron microscopy. Magnifications: $\times 400$.

zone in all sections of rabbit eyes that had $-9.0D$ PRK at 1 month after surgery (Fig. 2B). In the $-4.5D$ PRK group, few, if any, α -SMA-positive cells were noted in the subepithelial stroma (Fig. 2C).

Transmission Electron Microscopy

Untreated control rabbit corneas had the normal ultrastructure found in rabbits and other species.^{11,12} The lamina lucida, lamina densa, and lamina reticularis of the epithelial basement membrane appeared as continuous layers between basal epithelial cells and the anterior stroma (Fig. 3), and scattered keratocytes were observed in the anterior stroma.

At 1 month after $-4.5D$ PRK (Fig. 4), all five corneas showed normal basement membrane structure with a continuous lamina densa and associated structures of the mature normal basement membrane (Figs. 4A–C) in most areas examined by transmission electron microscopy. In one cornea, however (Fig. 4D), in an area of the cornea with a patch of anterior stromal haze noted at the slit lamp prior to euthanasia, there was no evidence of regeneration of normal basement membrane; and several cells in the subepithelial stroma had features suggestive of myofibroblast cells, such as large amounts of rough endoplasmic reticulum at higher magnification (not shown). Light microscopy immunohistochemistry for α -SMA on this same $-4.5D$ PRK cornea shows a small number of α -SMA+ myofibroblasts in the anterior stroma (Fig. 2C).

In all six corneas 1 month after $-9D$ PRK, there was no evidence of a normally regenerated epithelial basement membrane (Fig. 5). Each of these corneas had large numbers of cells in the subepithelial stroma that had the ultrastructural appearance of myofibroblasts (Figs. 5A–D), with prominent rough endoplasmic reticulum, which were embedded in disorganized extracellular matrix. These cells were often multiple and stacked one on top of the other with large amounts of disorganized extracellular matrix between the cells (Figs. 5B–D). Light microscopy immunohistochemistry for α -SMA in all these $-9.0D$ PRK corneas showed large numbers of α -SMA+ myofibroblasts in the anterior stroma (Fig. 2B).

DISCUSSION

Prior studies have demonstrated that rabbit corneas with high $-9D$ PRK corrections develop dense slit-lamp haze and high density of subepithelial myofibroblasts, similar to some human corneas that generate haze after PRK for high myopia when mitomycin C prophylaxis is not used,¹³ whereas most rabbit and human corneas with lower PRK corrections for myopia

($-4.5D$ PRK in rabbits) do not.^{7,13} In a study of haze generation after irregular PTK in mice,⁵ which is the method used to generate haze in this normally haze-resistant species, disruptions in epithelial basement membrane were noted in areas where myofibroblasts were present. In the current study, a near absence of basement membrane regeneration was noted in all rabbit corneas at 1 month after $-9D$ PRK (Fig. 5). Conversely, in rabbit corneas that had moderate $-4.5D$ PRK, all corneas had basement membrane regenerated with morphology similar to that in unwounded corneas, except in one cornea where localized areas of haze lacked normal epithelial basement membrane and had associated myofibroblasts (Fig. 4D).

Abnormalities in the epithelial basement membrane have been noted previously in monkey or rabbit models at 4 weeks after PRK.^{10,14,15} In another study, an irregular basement membrane was associated with late corneal epithelial healing defects and altered corneal wound healing.¹¹

Basement membranes are specialized extracellular matrices that underlie cells and separate them from, and adhere them to, connective tissues. They have been shown to be important for cell adhesion, migration, differentiation, and signal transduction.^{16–18} A wide array of human disorders result from, or are associated with, defects in basement membrane assembly or composition, such as Alport syndrome, epidermolysis bullosa, and Fraser syndrome.¹⁹ Moreover, basement membranes are involved in embryonic development,^{20,21} remodeling of tissues,²² and wound healing.²³ At the light microscopic level, matrix molecules have been localized in a linear staining pattern noted beneath epithelial cells in the basement membrane zone. Basement membrane components such as laminins, nidogens, collagen type IV, and perlecan have been localized to basement membranes in immunohistologic and electron microscopic ultrastructural studies.²⁴ Epithelial basement membranes range from approximately 50 to 100 nm in thickness²⁵ and typically display three layers at the EM level—lamina lucida, lamina densa, and lamina fibroreticularis.²⁶

The importance of the integrity of the regenerating epithelial basement membrane as a factor in the development of haze has been suggested by prior studies.^{5,6} Late haze that occurs after PRK is localized to the anterior subepithelial stroma on slit-lamp examination.⁷ This proximity of the haze and the associated myofibroblasts to the epithelium suggested that epithelial-stromal interactions are involved in myofibroblast development.³ Other work suggested that normally functioning epithelial basement membrane modulates myofibroblast development through barrier function limiting penetration of epithelium-derived TGF beta and platelet-derived growth factor (PDGF) into

FIGURE 3. Control rabbit cornea transmission electron microscopy. Central sections from three different control corneas (e, epithelium; s, stroma). (A) A representative image from a control cornea at magnification of $\times 30,000$. *Arrows* indicate the lamina densa. The lamina lucida is the less dense band between the lamina densa and the epithelium. (B) (Magnification: $\times 13,000$) and (C) (magnification: $\times 6800$) show control corneas at lower magnification, with the area where the lamina densa is located indicated by *black arrows*. *Black arrowheads* indicate keratocytes in (C). Note the stromal zone beneath the lamina densa in (B, C) where anchoring fibrils (*white arrowheads*) are located.

the stroma at sufficient levels to drive myfibroblast development and maintain myfibroblast viability once the mature cells are generated.⁴ Corneal myfibroblasts may be generated from either keratocyte-derived or bone marrow-derived precursor cells.²⁷

Many corneas that develop haze return to transparency over a period of months to years. This disappearance of haze is associated with apoptosis of myfibroblasts, movement of keratocytes back into the subepithelial stroma, and reabsorption of abnormal extracellular matrix produced by the myfibro-

FIGURE 4. Transmission electron microscopy of sections from the central corneas of rabbits at 1 month after -4.5D PRK . (A) Representative high-magnification ($\times 30,000$) image of a -4.5D PRK cornea with a regenerated basement membrane. Arrows indicate the regenerated lamina densa of the epithelial basement membrane. The less dense layer between the epithelium (E in [A], e in [B–E]) and the lamina densa is the lamina lucida. S, stroma. Note that the ultrastructure in (A) is very similar to that in the control cornea in Figure 3A. Representative lower-magnification images are shown in (B) $\times 13,000$, (C) $\times 13,000$, (D) $\times 6800$, and (E) $\times 6800$. Arrows in (B–D) from different corneas without haze are the area where the lamina densa-like layer regenerated. In (B), anchoring fibril structures (white arrowheads) similar to those in control corneas in Figure 3 can be seen. (E) An area of one cornea from this group (same cornea shown in [B]) with localized mild haze where no lamina densa-like structure could be detected even at higher $\times 30,000$ magnification (not shown). In this cornea, myofibroblasts (arrowheads) were detected in the anterior stroma of the area with haze.

blasts.⁴ Studies have shown that interleukin-1 (IL-1) produced by the myofibroblasts (autocrine) or surrounding stromal cells (paracrine) triggers apoptosis of the myofibroblasts when TGF beta levels decline in the stroma.^{9,28} The return of normal epithelial basement membrane structure and function is

thought to lead to a drop in the penetration of epithelium-derived TGF beta in the anterior stroma. The abnormalities of the basement membrane noted at 1 month after -9D PRK in the present study are consistent with this hypothesis. Conversely, in corneas showing a rapid restoration of basement

FIGURE 5. Transmission electron microscopy of sections from the central cornea of rabbits at 1 month after -9.0 D PRK. (A) is a representative higher-magnification ($\times 30,000$) image of the junction between the epithelium (e) and stroma (s). Note that no lamina densa-like or lamina lucida-like structures suggestive of regenerated basement membrane can be identified beneath the epithelium. X, disorganized extracellular matrix. Black arrowheads are myofibroblasts with large amounts of rough endoplasmic reticulum. (B–E) Images from different -9.0 D PRK corneas at lower magnifications (all $\times 6800$). Note that all of these sections from corneas with dense haze had numerous cells with high levels of rough endoplasmic reticulum (arrowheads) typical of myofibroblasts and large amounts of disorganized extracellular matrix (X) in the stroma beneath the epithelium (e). As in (A), no evidence of lamina densa-like or lamina lucida-like structures or anchoring fibrils was noted in any of the corneas that had -9.0 D PRK within the 6 mm diameter zone ablated by the excimer laser, even at higher magnifications (not shown).

membrane structure, for example as noted at 1 month after –4.5D PRK in the present study, apoptosis of myofibroblast precursors and mature myofibroblasts likely outstrips myofibroblast generation from precursor cells since epithelial cell-derived TGF beta penetration into the anterior cornea stroma is rapidly reduced after the injury.

Epithelial and other basement membranes regenerate after injury primarily through self-assembly on cell surfaces²⁵—in the case of corneal epithelial basement membrane, this would occur on the basal epithelial surface. In vivo and culture studies have suggested that laminins are principally responsible for initial organizing of basement membrane assembly since they are uniquely able to self-assemble into sheet-like structures on cell surfaces without the contribution of other components required for the assembly of a fully functional basement membrane, such as type IV collagens bound to nidogen-1 and nidogen-2, heparan sulfate proteoglycans agrin and perlecan, and many other components.²⁹ Collagen type IV can also self-assemble but is usually not present until later in the basement membrane regeneration process.³⁰ Although corneal epithelial cells can produce most of these basement membrane components, some may be contributed by normal stromal cells (keratocytes in the cornea), and not by other stromal cell types such as stromal fibroblasts and myofibroblasts.³¹ Also, components such as enzymes and signaling molecules provided by stromal cells likely contribute to formation of the basement membrane and associated structures such as the anchoring fibrils. For example, bone morphogenic protein (BMP) 1 produced by keratocytes is a procollagen protease that converts procollagen VII to produce mature anchoring fibril collagen.³²

It has long been known that higher-correction PRK produces a greater early wave of keratocyte apoptosis/necrosis, and greater and more prolonged acellularity of the anterior stroma, than lower-correction PRK.⁷ Our working hypothesis, based on the results of the current study, is that the prolonged recovery of the basement membrane is attributable to a deficiency of components and/or signaling from keratocytes, and that becomes especially limiting in the higher PRK corrections. Surface irregularity can also contribute to difficulty in regenerating the basement membrane.⁵ The delay in restoration of a fully functional basement membrane then allows prolonged penetration of high levels of TGF beta and possibly other factors into the stroma from the regenerated epithelium to drive myofibroblast generation from precursor cells.²⁷ Presumably myofibroblasts do not contribute the necessary components to complete assembly of the basement membrane; and their presence, plus the large amount of random extracellular matrix they produce, further hinders keratocytes from reoccupying a position in the anterior stroma. Therefore, in a cornea with persistent haze, a vicious cycle of sorts is set up whereby myofibroblasts are maintained by the epithelium in the absence of a functional basement membrane; and myofibroblasts themselves, along with the disorganized matrix they produce, provide a barrier against keratocytes that are present deeper in the stroma moving into a subepithelial position to contribute to basement membrane regeneration. In some corneas, over a period of many months to years, this cycle is somehow interrupted and the basement membrane is regenerated; myofibroblasts deprived of TGF beta undergo apoptosis; abnormal extracellular matrix is reabsorbed; keratocytes reoccupy the anterior stroma; and the cornea is returned to transparency. Further work is needed to delineate the specific mechanisms involved in these processes leading to corneal scarring and recovery after scarring is produced.

Acknowledgments

The authors thank Mei Yin from the image core facility at the Lerner Research Institute for assistance in preparation and analysis of electron microscopic samples and Mary Ann Stepp, PhD, for valuable input on the results of this study.

Supported in part by US Public Health Service Grants EY10056 and EY015638 from the National Eye Institute, National Institutes of Health, Bethesda, Maryland, and Research to Prevent Blindness, New York, New York. The authors alone are responsible for the content and writing of the paper.

Disclosure: **A.A.M. Torricelli**, None; **V. Singh**, None; **V. Agrawal**, None; **M.R. Santhiago**, None; **S.E. Wilson**, None

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