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Epithelial basement membrane injury and regeneration modulates corneal fibrosis after pseudomonas corneal ulcers in rabbits

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ABSTRACT

The purpose of this study was to investigate whether myofibroblast-related fibrosis (scarring) after microbial keratitis was modulated by the epithelial basement membrane (EBM) injury and regeneration. Rabbits were infected with *Pseudomonas aeruginosa* after epithelial scrape injury and the resultant severe keratitis was treated with topical tobramycin. Corneas were analyzed from one to four months after keratitis with slit lamp photos, immunohistochemistry for alpha-smooth muscle actin (α-SMA) and monocyte lineage marker CD11b, and transmission electron microscopy. At one month after keratitis, corneas had no detectible EBM lamina lucida or lamina densa, and the central stroma was packed with myofibroblasts that in some eyes extended to the posterior corneal surface with damage to Descemet's membrane and the endothelium. At one month, a nest of stromal cells in the midst of the SMA + myofibroblasts in the stroma that were CD11b+ may be fibrocyte precursors to myofibroblasts. At two to four months after keratitis, the EBM fully-regenerated and myofibroblasts disappeared from the anterior 60–90% of the stroma of all corneas, except for one four-month post-keratitis cornea where anterior myofibroblasts were still present in one localized pocket in the cornea. The organization of the stromal extracellular matrix also became less disorganized from two to four months after keratitis but remained abnormal compared to controls at the last time point. Myofibroblasts persisted in the posterior 10%–20% of posterior stroma even at four months after keratitis in the central cornea where Descemet's membrane and the endothelium were damaged. This study suggests that the EBM has a critical role in modulating myofibroblast development and fibrosis after keratitis—similar to the role of EBM in fibrosis after photorefractive keratectomy. Damage to EBM likely allows epithelium-derived transforming growth factor beta (TGFβ) to penetrate the stroma and drive development and persistence of myofibroblasts. Eventual repair of EBM leads to myofibroblast apoptosis when the cells are deprived of requisite TGFβ to maintain viability. The endothelium and Descemet's membrane may serve a similar function modulating TGFβ penetration into the posterior stroma—with the source of TGFβ likely being the aqueous humor.

Stromal fibrosis (scarring) commonly occurs after bacterial keratitis and often causes severe loss in visual function (Gopinathan et al., 2009). Regeneration of the epithelial basement membrane (EBM) is a key determinant of regenerative (transparent) versus fibrotic (scarred) corneal healing after photorefractive keratectomy (Torricelli et al., 2013a, 2013b, 2016; Marino, et al., 2016). The structural and functional regeneration of the EBM is critical because of its important function in regulating the bidirectional passage of cytokines, chemokines and growth factors—including transforming growth factor (TGF) β and platelet-derived growth factor (PDGF), between the epithelium or the tear film and the stroma—where TGFβ and PDGF regulate the development and persistence of fibrosis-associated myofibroblasts from precursor cells (Singh et al., 2011). Myofibroblasts produce fibrosis (scarring) of the corneal stroma because they are opaque due to diminished crystallin protein production relative to keratocytes (Jester et al., 1999) and they secrete disordered extracellular matrix that alters the

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precise distribution of the collagen fibers that is essential for corneal transparency (Hassell and Birk, 2010). The aim of the present study was to determine 1) whether corneal stromal fibrosis that occurs after a severe bacterial corneal ulcer is associated with EBM injury and 2) whether eventual EBM regeneration is associated with the disappearance of stromal myofibroblasts.

All animals were treated in accordance with the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The Animal Control Committee and Institutional Biosafety Committee at the Cleveland Clinic approved this animal study.

Twelve-to 15-week-old female New Zealand white rabbits weighing 2.5–3.0 kg each were included in this study. General anesthesia was obtained by intramuscular injection of ketamine hydrochloride (30 mg/kg) and xylazine hydrochloride (5 mg/kg). In addition, topical proparacaine hydrochloride 0.5% (Akorn Inc., Lake Forest, IL) was applied to both eyes at the time of the procedure. Rabbits were also treated with acetaminophen 20 mg per 100 ml of drinking water for five days after infection and buprenorphine 0.05 mg/kg by subcutaneous injection twice a day for up to 10 days after infection, as needed if there were signs of discomfort.

One eye was randomly selected to be infected, while the opposite eye was included as an uninfected control. A wire lid speculum was positioned in the eye followed by the production of a small central epithelial defect with a #64 Beaver blade (BD Beaver, Waltham, MA). The epithelial defect was inoculated with *Pseudomonas aeruginosa* (Hazlett, 2007; Fleiszig and Evans, 2002) at a concentration of 20 CFU/μL in balanced salt solution. The strain of bacteria used named PA13346SW and was a clinical isolate from a patient with a corneal ulcer that was highly sensitive to tobramycin. The strain was genotyped for ExoS, ExoU, ExoT and ExoY using previously published PCR primers and methods (Ledbetter et al., 2009) and was found to be ExoS+, ExoT+ and ExoY+, and ExoU-.

The eyes were monitored untreated for 24–36 h until severe keratitis developed (Fig. 1A) and then were treated with topical 13.5 mg/ml tobramycin (Sigma-Aldrich, St. Louis, MO) every hour for two days and then every 2 h for five days. Corticosteroids were not applied to avoid the potential confounding effects on the corneal wound healing response. Epithelial defects in all eyes were found to heal by 7–10 days after the initial infection.

Rabbits were anesthetized with ketamine and xylazine for slit lamp photographs and then euthanized with an intravenous injection of 100 mg/kg pentobarbital. The corneoscleral rims of infected and uninfected control eyes were removed without manipulation of the cornea using 0.12 mm forceps and sharp Westcott scissors (Fairfield, CT). The corneas were cut into two equal halves with a sharp straight-edged razor blade and one half was embedded in liquid optimal cutting temperature (OCT) compound (Sakura FineTek, Torrance, CA) within a 24 × 24 × 5 mm mold (Fisher Scientific, Pittsburgh, PA) and quickly frozen on a block of dry ice and stored at −80 °C until sectioning was performed for immunohistological evaluation. The second corneal half was immediately stored in 2.5% glutaraldehyde and 4% paraformaldehyde in 0.2 M cacodylate buffer at 4 °C for transmission electron microscopy (TEM) evaluation. Two ulcer and two uninfected control corneas were analyzed at each time point (one, two, three and four months) after the pseudomonas infection using immunohistochemistry and TEM.

Corneal sections (7 μm thick) were cut within the central cornea pseudomonas infected and uninfected control corneas with a cryostat (HM 505M; Micron GmbH, Walldorf, Germany) and placed on 25 × 75 × 1 mm microscope slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA) and maintained at −80 °C until used for immunohistochemical analysis. Immunohistochemistry was performed (Torricelli et al., 2013b) to detect the alpha-smooth muscle actin (α-SMA) marker for myofibroblasts or the CF11b for monocyte lineage cells. Briefly, for α-SMA a mouse monoclonal anti-human α-SMA clone 1A4 (Cat# M-0851, Dako, Carpinteria, CA) was used as the primary antibody. The slides were washed with PBS and incubated for 90 min at room temperature with anti−α-SMA antibody at 1:50 dilution in 1% BSA. Slides were washed twice with phosphate buffered saline (PBS) and then incubated at room temperature with a secondary antibody, Alexa Fluor 568 goat anti-mouse IgG (Cat# A-11004, ThermoFisher Scientific, Rockford, IL) at a dilution of 1:100 in 1% BSA for 1 h. For CD11b, monoclonal antibody (Cat# MA1-80091, Invitrogen, Thermo-Fisher, Grand Island, NY) was used as the primary antibody. The slides were washed with PBS and incubated for 90 min at room temperature with anti-CD11b antibody at 1:25 dilution in 1% BSA. The slides were then washed twice with PBS and incubated at room temperature with secondary goat anti-rat IgG (H + L), Alexa Fluor 488 (Cat# A-11006, Invitrogen (Thermo-Fisher) at 1:100 dilution for 60 min. Coverslips 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. Negative controls for α-SMA or CD11b included a non-specific antibody of the same isotype since no antigen was available for pre-absorption. The sections were analyzed and photographed with a Leica DM5000 microscope (Leica, Buffalo Grove, IL) equipped with Q-imaging Retiga 4000RV (Surrey, BC, Canada) camera and Image-Pro software (MediaCybernetics, Inc. Bethesda, MD).

TEM samples were prepared as previously described (Fantes et al., 1990). Briefly, 1 mm wide, full-thickness blocks of central cornea were cut perpendicular to the epithelial surface from both the pseudomonas ulcer and control corneas, and were fixed in the 2.5% glutaraldehyde and 4% paraformaldehyde with 0.2 M cacodylate buffer solution for a minimum of 24 h. The excised blocks of central corneas were then rinsed with 0.2 M cacodylate buffer three times for 5 min, post-fixed in 1% osmium tetroxide for 60 min at 4 °C, and dehydrated in increasing concentrations of ethanol from 30% to 95% for 5 min each at 4 °C. Finally, dehydration was performed using three 10-min rinses in 100% ethanol at room temperature and three 15-min rinses with propylene oxide at room temperature. Samples were then embedded in epoxy resin medium. One-micrometer-thick sections were stained with toluidine blue for light microscopy. Ultrathin 85 nm thick sections were cut with a diamond knife and stained with 5% uranyl acetate and lead citrate. Sections were analyzed and photographed using a Philips CM12 transmission electron microscope operated at 60 kV (FEI Company, Hillsboro, OR).

Control corneas had no α-SMA+ myofibroblasts detected by immunohistochemistry analysis (Fig. 2A). TEM of control corneas revealed the normal EBM lamina lucida and lamina densa characteristic of rabbit corneas (Fig. 2B) (Sta Iglesia and Stepp, 2000; Torricelli et al., 2013b).

All pseudomonas-infected corneas healed with closure of the epithelial defects over seven to ten days after infection (no fluorescein staining) and developed dense stromal scarring with vascularization. All eight infected corneas had 4+ stromal scarring at the slit lamp with no view of the iris at one month after infection (Fig. 1B). There was a definite decrease in opacity at the slit lamp in the four corneas that reached three months and the two corneas that reached four-months post infection (Fig. 1C–E), although iris details were still obscured at these time points.

At one month keratitis, immunohistochemistry showed nearly full stromal thickness high density α-SMA+ myofibroblasts in one cornea (Fig. 2C) and full stromal thickness high density α-SMA+ myofibroblasts in the other cornea removed at this time point (not shown). At two months after keratitis, α-SMA+ myofibroblasts had substantially disappeared in the anterior