Published in final edited form as:

Exp Eye Res. 2011 December ; 93(6): 927–936. doi:10.1016/j.exer.2011.10.012.

Removal of the basement membrane enhances corneal wound healing

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Abstract

Recurrent corneal erosions are painful and put patients' vision at risk. Treatment typically begins with debridement of the area around the erosion site followed by more aggressive treatments. An *in vivo* mouse model has been developed that reproducibly induces recurrent epithelial erosions in wild-type mice spontaneously within two weeks after a single 1.5 mm corneal debridement wound created using a dulled blade. This study was conducted to determine whether 1) inhibiting MMP function during healing after dulled-blade wounding impacts erosion development and 2) wounds made with a rotating burr heal without erosions. Oral or topical inhibition of MMPs after dulled-blade wounding does not improve healing. Wounds made by rotating burr heal with significantly fewer erosions than dulled-blade wounds. The localization of MMP9, $\beta 4$ integrin and basement membrane proteins (LN332 and type VII collagen), immune cell influx, and reinnervation of the corneal nerves were compared after both wound types. Rotating-burr wounds remove the anterior basement membrane centrally but not at the periphery near the wound margin, induce more apoptosis of corneal stromal cells, and damage more stromal nerve fibers. Despite the fact that rotating-burr wounds do more damage to the cornea, fewer immune cells are recruited and significantly more wounds resolve completely.

Keywords

mouse; cornea; wound healing; $\beta 4$ integrin; CD45; sub-basal nerves

Introduction

Due to its exposed site and sheerness, corneal surface injuries are the most common ophthalmic complaint and recurrent erosions develop in a sub-population of patients with surface injuries. Treatment for recurrent corneal erosions typically begins with debridement of the area around the erosion site. A combination of debridement and diamond burr polishing of the anterior epithelial basement membrane zone (BMZ), laser phototherapeutic keratectomy, or alcohol delamination are used for patients with recurrent erosions (Aldave, et al., 2009; Ewald and Hammersmith, 2009; Laibson, 2010; Mencucci, et al., 2010). Most

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erosions heal and the corneal surface remains intact but 5-15% of patients treated for recurrent erosions develop additional painful erosions that put their vision at risk (Laibson, 2010).

An *in vivo* mouse model has been developed that reproducibly induces recurrent epithelial erosions that form within weeks after a 1.5 mm corneal debridement wound made using a dulled blade (Pal-Ghosh, et al., 2004); this type of wound is referred to here as a dulled-blade wound. The frequency of erosions after 1.5 mm dulled-blade wounds in C57BL6 and BALB/c mouse strains varies ranging from 73-81% (Pal-Ghosh, et al., 2008). Erosions develop secondary to chronically elevated expression of matrix metalloprotein 9 (MMP9) in the corneal epithelium that degrades $\alpha 6\beta 4$ integrin leading to sloughing off of epithelial cells (Pal-Ghosh, et al., 2011). However, C57BL6 and BALB/c mice subjected to superficial keratectomy wounds do not develop erosions (Pal-Ghosh, et al., 2011).

Elevated levels of MMPs are seen in tears of people with ocular roseacea (Afonso, et al., 1999), dry eye (Chotikavanich, et al., 2009), corneal ulcers (Hadassah, et al., 2009), climatic droplet keratopathy (Holopainen, et al., 2011), and recurrent erosions (Afonso, et al., 1999; Garrana, et al., 1999; Ramamurthi, et al., 2006). The level of MMP9 in tears has been proposed as a tool for the clinical diagnosis of dry eye caused by defects in tear production secondary to persistent inflammatory conditions affecting the lacrimal glands (Pflugfelder, et al., 2000; Lemp, 2008; Jackson, 2009; Chotikavanich, et al., 2009). Determining the underlying cause of elevated MMP9 expression in the dulled-blade mouse model will provide insight not only into the etiology of recurrent erosions but also of other eye diseases.

Several hypotheses offer possible explanations for the chronic MMP expression seen within the corneal epithelium after dulled-blade wounds. Epithelial cells may secrete higher levels of MMPs to degrade BMZ proteins left behind after the removal of the corneal epithelial cells. Also residual BMZ proteins may delay signaling between the epithelial cells and the underlying corneal stromal cells (CSCs). Such a mechanism could operate through a reduced rate of diffusion of cytokines released by epithelial cells into the stroma or by the reduced diffusion of proteins from the tear film onto the exposed basement membrane. Studies by Stramer and colleagues (2003) support the idea that diffusion of cytokines secreted by epithelial cells is regulated by the presence of a basement membrane. Their study shows that corneal tissue constructs with corneal epithelial basement membranes show no diffusion of the epithelial cytokine TGF β 2 past the BMZ into the stroma. Corneal constructs lacking a basement membrane showed TGF β 2 throughout the stroma and this difference in diffusion induced differences in α SMA expression in corneal fibroblasts.

Chronic MMP9 expression after dulled-blade wounds could also be caused by differences in immune cell recruitment into the wound site. Keratectomy wounds damage fewer corneal epithelial cells; cells in the center of the corneal epithelial:stromal button remain intact. Differences in the exposure of resident immune cells to intracellular epithelial proteins and DNA or differences in mechano-sensing of resident stromal cells to stress placed on the cornea by keratectomy wounding could differentially stimulate chemokine release. Although in both dulled-blade and keratectomy wounds sheering forces are used, dulled-blade wounds leave epithelial cell debris and remnants of damaged sub-basal axons on the ocular surface. The presence of this debris could induce recruitment of different types or numbers of immune cells from the limbal vasculature. Another method of wounding the mouse and rat ocular surface involves the use of a rotating burr that is 0.5 mm in diameter; this tool is similar in design to the diamond burr used by clinicians. The studies that follow were conducted to evaluate the impact of MMP inhibition on corneal wound healing and to evaluate the healing that takes place when wounds are created using a rotating burr.

Materials and Methods

Manual Corneal Debridement

All experiments described in this article were conducted in voluntary compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all procedures were approved by the George Washington University Animal Care and Use Committee. Mice (BALB/c) were obtained from NCI-Frederick. 8- to 12-week-old BALB/c mice (22 to 25 g) were anesthetized with 250 μ l of a 1:10 dilution of a 1:1 mixture of ketamine (100 mg/mL; Aveco Co., Inc., Fort Dodge, IA) and xylazine (20 mg/mL; Miles Inc., Shawnee Mission, KS). Once anesthetized, a topical anesthetic (proparacaine ophthalmic solution, Butler Schein #NDC-24208-730-06) was applied to their ocular surface until the blink sensation was lost. A 1.5-mm central corneal area was demarcated with a trephine and corneal epithelium was removed manually by gentle scraping with either a dulled blade held by a blade breaker (dulled-blade wounds) or a rotating burr (rotating-burr wounds; Algerbrush II, Ambler Surgical, 404 Gordon Drive, Exton, PA 19341). After wounding, eyes were treated with erythromycin ophthalmic ointment (Butler Schein #NDC 0166-0070-38) to minimize inflammation and keep the ocular surface moist while the mice recovered from anesthesia. Corneas were allowed to heal *in vivo* for 6 hours and 4 weeks. After sacrifice, enucleated eyes were fixed for 2 hours in 1:4 dilution DMSO:Methanol at -20°C followed by 100% methanol at -20°C . For studies of corneas at time 0, mice were wounded within 5 minutes of sacrifice, eyes enucleated, and fixed immediately as described.

For studies in which MMP activity was inhibited, oxytetracycline (Sigma Aldrich) was administered orally in water at a concentration of 200 mg/L in water (Chandler, et al., 2007) beginning immediately after wounding and continued for 4 weeks. For studies using topical MMP inhibition, the broad spectrum MMP inhibitor GM6001 (Millipore/Chemicon, Billerica, MA 01821; CC1100) was applied 2 times per day for 5 days beginning 2 days after wounding. GM6001 was used at a concentration of 400 $\mu\text{g/mL}$ in 50 mM Hepes, 0.1% DMSO, and 1X Pen-Strep-Fungizone as described by Schultz and colleagues (1992). Control mice were treated with vehicle alone. Richardson stain was used to score the corneal wounds as open or closed. Closed eyes were assigned a score of 1 and open eyes a score of 0. Significance was determined with the average scores using a one way analysis of variance with the Tukey post test using Instat software from Graphpad, Inc.; p values less than 0.05 were considered significant. After erosion assessments were made, eyes were enucleated and corneas were fixed and used for whole mounts as described above. Experiments were repeated twice and data pooled.

Materials

Methanol (Fisher, #A454-4), Triton (Fisher, # AC21568-0010), 10X PBS (Fisher # 17-515Q), BSA (Jackson Immunoresearch #001-000-162), and horse serum (ThermoFisher # ICN2921249). Alexa 488 anti rabbit (A11008), Alexa 488 anti rat (A11006), and Alexa 594 anti-rat (A11007) secondary antibodies were purchased from Invitrogen/Molecular Probes (Eugene, OR, 97402). Nuclear stains including Propidium iodide (#P1304MP) and DAPI (#D21490) were also purchased from Invitrogen/Molecular Probes. Primary antibodies used for these studies were: polyclonal rabbit anti-mouse MMP-9 antibody (Abcam, Cambridge, MA 02139; #ab38898), Rat anti-mouse $\beta 4$ integrin (BD Pharmingen, San Diego, CA 92121; clone 346-11A; BD553745), rat anti-mouse CD45+ antibody against the common leukocyte antigen (BD Pharmingen; clone 30-F11; BD553076), and mouse anti-mammalian $\beta 3$ tubulin (Covance Research Products, Berkeley, CA, 94710; Tuj1; MMS-435P). The polyclonal antibody against LN332 was a generous gift of Jonathan C. Jones (J18; Northwestern University Medical School, Chicago, IL 60611).

Whole Mounts

The tissues stored in 100% methanol were treated with triton in a graded methanol series (70%, 50%, and 30% methanol:triton, 30 minutes each). All incubations were performed with gentle shaking and at room temperature, unless otherwise specified. Eyes were washed in PBS twice for 30 minutes each, followed by incubation with blocking buffer for 2 hours. Blocking buffer was 100 ml 1x PBS, 1 g BSA and 1 ml horse serum stirred for half an hour. The tissues were incubated overnight with primary antibody, at 4°C. The next day, tissues were washed five times with PBS and 0.02% Tween 20 (PBST) for 1 hour each, blocked for 2 hours, and incubated with Alexa secondary antibody overnight at 4°C. Eyes were washed three times with PBST for 1 hour each. Under a dissecting microscope (Model SZ40; Olympus, Lake Success, Melville, NY), the retina, lens, and iris were removed, and four incisions were made in the cornea. To achieve the best flattening, the corneas were placed epithelial-side-up; Flomount G mounting media (EMS, #179-84-25) was added and coverslipped. Images were acquired with the Zeiss LSM 710 (NIH 1S10RR025565-01; Popratiloff, P.I.) AXIO EXAMINER upright microscope and LSM 710 confocal system with 34 spectral detection channels and two transmitted light photomultipliers, 5 laser lines - 458, 488, 514, 561 & 633 nm, control electronics, ZEN 2009 software and x/y/z motorized scanning stage. Typically, eighteen to twenty optical sections were acquired sequentially (1µm steps in the z coordinate) with a 63x objective lens (NA=1.40). 3D images were obtained using Volocity (Version 5.0; Perkin Elmer). Images captured with the confocal were merged and presented *en face* and rotated at 90° for cross section images.

Results

Wounding with the rotating burr significantly improves healing

Wound closure was determined at the time of sacrifice using the vital dye Richardson stain and confirmed by immunofluorescence. Representative data obtained 4 weeks after wounding by dulled blade and rotating burr are shown in Figure 1A. Data showing the frequency of erosions 4 weeks after wounding are presented in Figure 1B. Inhibiting MMP activity either orally or topically did not reduce the formation of erosions but wounding mice with the rotating burr significantly reduced erosion frequency. Over 88% of rotating-burr wounded mouse corneas had closed wounds and only 22% of the corneas of mice wounded with the dulled blade had closed wounds 4 weeks after wounding.

Corneas were also evaluated for the localization of $\beta 4$ integrin, LN332, and MMP9 4 weeks after wounding and data are presented in Figures 2A and B. Rotating-burr wounded corneas have abundant $\beta 4$ integrin and laminin 332 (LN332) at the BMZ and no detectable MMP9. However, dulled-blade wounded corneas show reduced levels of $\beta 4$ integrin and the presence of MMP9 adjacent to and within erosions; LN332 was not be detected near erosions after dulled-blade wounds.

Rotating-burr wounds remove the basement membrane at the center of the wound

Dulled-blade wounds to the mouse cornea leave $\alpha 6\beta 4$ on the anterior stroma at the BMZ (Sta. Iglesia and Stepp, 2000) but it is not clear whether the BMZ is left intact after rotating-burr wounds. Mice were wounded within 5 minutes of sacrifice using the rotating burr or dulled blade and their eyes were fixed immediately (time 0) for the measurement of corneal stromal thickness and use in whole mount immunofluorescence. There is no evidence for $\beta 4$ integrin, LN332, or type VII collagen at the center of the wound area after rotating-burr wounds (Figure 3A); the basal cell basal surface and basement membrane are present at the site immediately adjacent to the wound margin. It is impossible to get closer to the wound edge with the rotating burr without removing additional epithelial tissue. As expected from previous studies, after dulled-blade wounding, the basement membrane is mainly left intact

and stained uniformly positive for $\beta 4$ integrin, LN332, and type VII collagen (Figure 3B). These data show that wounds created with the rotating burr remove the basement membrane.

The stromal surface is uneven after rotating-burr wounds compared to dulled-blade. At time 0, the nuclei of the CSCs located near the anterior stroma are visible with DAPI staining as shown in Figures 3A and 3B. The CSC nuclei are elongated and distorted after rotating-burr wounding whereas the CSC nuclei of dulled-blade wounded corneas appear morphologically normal as seen in the inset in Figure 3B, which shows DAPI alone.

The total stromal thickness was assessed at time 0 at three locations at the central cornea in four corneas from four different mice after both wound types. The stromal thickness is $56 \pm 1 \mu\text{m}$ in rotating-burr wounds and $55 \pm 3 \mu\text{m}$ in dulled-blade wounds. Even though Figures 3A and 3B show that rotating-burr wounding removes the BMZ centrally, the amount of tissue removed was less than could be detected using a method with a sensitivity of $1 \mu\text{m}$.

To determine whether remnants of basement membrane remain associated with the BMZ adjacent to the margin of the 1.5 mm wound after dulled-blade wounds, whole mounts of corneas from both wound models were obtained 6 hours after wounding. While there is less $\beta 4$ integrin present on the surface of the stroma at the margin of the rotating-burr wounds compared to 0 hr in Figure 3A, $\beta 4$ integrin is still present at this site (Figure 3C). In contrast, 6 hours after dulled-blade wounds (Figure 3D), $\beta 4$ integrin, LN332, and type VII collagen remain localized at the exposed stromal surface.

DAPI-stained nuclei are visible at the anterior aspect of the exposed stroma at time 0 (Figures 3A and B) and not 6 hours after wounding (Figures 3C and D). Anterior CSCs exposed to the tear film after removal of corneal epithelial cell barrier undergo apoptosis, a process that begins immediately upon wounding and can last for several hours (Wilson, et al., 2007). Using confocal imaging of DAPI stained nuclei, CSC apoptosis was quantified by measuring the depth of the nuclei-free zone present in the central exposed stroma 6 hours after wounding. Measurements were taken with a 63X objective at three different sites at the center of the wound site using $1 \mu\text{m}$ step-intervals from 10 dulled-blade wounded corneas and 8 rotating-burr wounded corneas. The nuclei-free zone is significantly larger for rotating-burr wounded corneas ($47 \pm 2 \mu\text{m}$ or 84% of the total stromal thickness) compared to the dulled blade ($32 \pm 2 \mu\text{m}$ or 58% of the total stromal thickness). More CSCs have undergone apoptosis 6 hours after rotating-burr wounds compared to dulled-blade wounds.

Fewer sub-basal nerves are present adjacent to erosion sites 4 weeks after wounding with the dulled blade

The cornea's sensory axons or nerve fibers originate from the trigeminal ganglion and enter the cornea at the limbus and travel through the stroma towards the center of the cornea (Muller, et al., 1996. 2003). A dense collection of axons, called the sub-basal nerve plexus, runs between the basal membrane of the corneal epithelial basal cells and the basement membrane; these nerve fibers are thinner than those in the stroma. They branch and some penetrate to the apical surface of the corneal epithelium. While the sub-basal nerves have a thin basement membrane, the larger nerve bundles found deep in the stroma and at the corneal periphery have a basal lamina ensheathing both Schwann cells and axons (Muller, et al., 1996. 2003).

Dulled-blade wounds leave the basement membrane behind but it is not known whether they damage all or only part of the sub-basal nerve plexus. In rotating-burr wounds, removal of the BMZ is expected to remove the sub-basal nerve plexus at the wound site. The impact of

these wounds on the deeper stromal nerve fibers was not known. The data presented in Figures 3B and 3D show more stromal nerves, revealed by the localization of $\beta 4$ integrin to the basal lamina of their axons, immediately and 6 hours after dulled-blade wounds. These results were confirmed by staining whole mount corneas with Tuj1, an antibody directed against neuronal-specific $\beta 3$ tubulin, 6 hours after rotating-burr and dulled-blade wounds (Figure 4). Dulled-blade wounds leave more nerves intact in the anterior stroma.

To determine whether there are similar numbers of nerve fibers near erosion sites, corneas were evaluated 4 weeks after dulled-blade wounding. Tuj1 was used for nerve staining and $\beta 4$ integrin to identify erosion sites. Unlike the data presented in Figure 4, this study looks at the sub-basal nerves and not the stromal nerves. Figure 5 shows *en face* confocal images of the mouse cornea near an erosion site. The lower magnification image (Figure 5A) allows identification of an erosion site by the loss of $\beta 4$ integrin 4 weeks after dulled-blade wounding. There is a reduction in the number of nerve fibers at these sites. There are fewer nerve fibers away from (Figure 5B; indicated by the white ^) and within (Figure 5C; indicated by the white *) erosions.

The sub-basal nerve fibers at the periphery shown in Figure 5B are organized to run under the cell membranes that join adjacent basal cells rather than under the basal cell nuclei. It appears that almost every basal cell is innervated by a sub-basal nerve running parallel to its basal surface and the BMZ. The fact that erosion sites have fewer nerves implicates failed reinnervation in the formation of erosions.

More CD45+ immune cells are present in the stroma near the limbus after dulled-blade wounding

The removal of the corneal epithelial cells and the damage these wounds do to the nerves induces an immune response. The cornea is avascular with angiogenic and lymphatic blood vessels located within the stroma at the corneal limbus. When a corneal infection or injury occurs, immune cells are attracted by cytokines and extravasate and migrate along a chemokine gradient established within the corneal stroma towards the infectious agent or the site of injury (Carlson, et al., 2007; 2010). We assessed the number of immune cells that had infiltrated the corneal stroma 6 hours following injury. Corneas were stained using an antibody against the common leukocyte antigen, the protein tyrosine phosphatase CD45, which is expressed on the surfaces of all leukocytes (Saunders and Johnson, 2010). Figure 6 shows data from whole mount corneas 6 hours after rotating-burr and dulled-blade wounding. Lower magnification *en face* images of regions adjacent to the limbus focus on the stromal compartment; high resolution (63X oil objective) 3D reconstructed images from the same locations show cross section views. These images show more CD45+ cells in the stroma near the limbus of the dulled-blade wounded corneas. The data indicate that the recruitment of immune cells into the cornea varies significantly in response to dulled-blade and rotating-burr wounds.

Figure 5 shows fewer nerves at erosion sites and Figure 6 shows more immune cells recruited within 6 hours after dulled-blade wounds. In Figure 7 the presence of both CD45+ immune cells and nerves at erosion sites was investigated. Shown are *en face* confocal images at high resolution of corneas whose wounds resolved (A) compared to those with erosions (B). Location of the sub-basal nerves are shown on the left and on the right, a region within the corneal stroma 2-3 μm below the sub-basal nerve is presented. The erosion sites have fewer sub-basal nerves; several CD45+ immune cells are present within the remaining epithelial cell layer. Below the erosion site, there are twice as many CD45+ immune cells compared to the healed cornea.

Discussion

In the mouse, when manual debridement wounds are made to the cornea, a portion of the basal surface of the epithelial cells remains with the stroma along with the lamina densa (Sta. Iglesia and Stepp, 2000) and data presented here show that $\alpha 6\beta 4$ integrin, LN332, and type VII collagen are removed from the BMZ by rotating-burr wounds. Studies of corneal re-epithelialization have been reported in the rat cornea; debridement wounds made in the rat using the dulled blade remove $\alpha 6\beta 4$ integrin (Stepp, et al., 1996) and studies of the rabbit cornea report findings similar to those in the rat (Latvala, et al., 1996). Human corneal epithelial sheets removed by debridement were studied by TEM (Mencucci, et al., 2010); $\alpha 6\beta 4$ -containing hemidesmosomes were removed with the corneal epithelial sheet from healthy corneas undergoing PRK but when removed as treatment for recurrent erosions, epithelial basal cells were damaged and missing their basal membrane (Mencucci, et al., 2010).

Taken together, these data indicate that mouse corneal epithelial basal cells adhere more tightly to their underlying BMZ compared to rats, rabbits, and humans. Another difference between mouse corneal wound healing and that observed in the rat, rabbit and human cornea, is that myofibroblasts are not generated in the stromas of Balb/c strains of mice in response to corneal wounds (Pal-Ghosh, et al., 2011 and data not shown). We have evaluated dulled-blade and wounds that remove the basement membrane in Balb/c mice and C57BL6 mice for α SMA positive cells and do not find any at any time point. Mohan and colleagues (2008) were able to demonstrate the formation of myofibroblasts in the C57BL6 mouse but only when corneas were wounded by a surgical blade followed by the excimer laser to create irregular photorefractive keratectomy wounds. It is not clear why mouse corneal stromal cells resist activation to the myofibroblast phenotype. Despite the unique properties of the mouse cornea, the mouse remains an essential model for the study of the reepithelialization and resolution phases of corneal wound healing. The availability of genetically modified mice to study corneal wound healing allows vision scientists the ability to ask targeted questions about specific molecules and their roles in corneal wound healing.

Efforts to reduce erosion size and frequency by inhibiting MMP activity using oral or topical application of MMP inhibitors failed; while not mathematically significant, there was a trend for the erosions to be larger and more frequent. Chronically elevated MMP9 levels and recurrent erosions are reduced significantly by wounding the cornea with the rotating burr. These data lead us to conclude that chronic MMP9 expression after dulled-blade wounds represents an attempt by the corneal epithelium to remodel damaged ECM in the stroma.

Experiments looking at K12 expression and corneal stem cell maturation indicate that the mouse cornea does not fully mature until 12 weeks after birth (Collinson, et al., 2002; Tanifuji-Terai, et al., 2006). To confirm that the erosions were not due to immature corneas, mice were wounded at 12 weeks instead of 8 weeks; no significant impact was observed in the frequency of erosions (data not shown).

In vivo studies show that mice with dry eye have enhanced corneal epithelial basal cell apoptosis, elevated MMP9 expression in their tears, and barrier function defects (Dursen, et al., 2002; Corrales, et al., 2006; Pflugfelder, et al., 2005; Chen, et al., 2008). Experiments to induce dry eye in MMP9 null mice failed when mice were subjected to the same experimental conditions as wild-type mice (Pflugfelder, et al., 2005). The clinical data on dry eye and other studies showing elevated MMP9 in mouse corneas with erosions (Pal-Ghosh, et al., 2011) together suggest that MMP9 activation within the cornea can lead to accelerated epithelial cell shedding and subsequent loss of barrier function.

High resolution confocal microscopy images presented in this paper show that CD45+ immune cells are present within the corneal epithelium adjacent to erosion sites. This raises the possibility that immune cells rather than corneal epithelial cells are the primary source of the MMP9 mRNA and protein seen in immunoblots of 4 week dulled-blade wounded corneal epithelial cell extracts (Pal-Ghosh, et al., 2011). Increased immune cell recruitment into the stromas of corneas after dulled-blade wounds is seen within 6 hours of wounding implicating differences in immune cell recruitment in the formation of erosions.

To differentiate among the various immune cell types present at erosion sites, it will be necessary to use seven or more different antibodies to identify neutrophils, T-cell receptor positive/MHC class 1, and APC/MHC class 2 cells. Further staining will be needed to determine the numbers of the $\gamma\delta$ T-cells, dendritic cells, and to classify macrophages as M1 or M2 types. Studies could be done using flow cytometry, but to localize specific immune cell types to erosion sites, the whole mount procedure has to be used. To determine the identity of the immune cells involved in the formation of recurrent erosions we are now optimizing whole mount staining using various immune cell markers.

The majority of the immune cells that initially migrate into the corneal stroma after debridement wounding have been reported to be neutrophils (Li, et al., 2006). These move in from the limbal vasculature in response to the chemokine CXCL1 released by both activated resident immune cells and damaged corneal cells. Neutrophils in the stroma peak in number between 12-24 hours after debridement wounding (Li, et al., 2006; Hayashida, et al., 2009). Large numbers of immune cells could damage the stromal ECM and maintain CSCs in their activated state to produce collagens and proteoglycans to replace those degraded by leukocyte-derived proteases. Since immune cells are recruited into the corneal stroma from the limbal vasculature by cytokines, we hypothesize that dulled-blade wounds induce different types and/or amounts of cytokines compared to rotating-burr wounds.

The exposed stroma in rotating-burr wounds showed more damage to the nerve fibers 6 hrs after wounding. The re-growth of the corneal sub-basal and stromal nerves in the mouse after debridement wounds has been the subject of several recent studies (Yu and Rosenblatt, 2007; Esquenazi, et al, 2007; Leiper, et al., 2009; McKenna and Lwigale, 2011; Li, et al., 2011). While fewer nerves are observed at erosion sites, it is not known whether this is the cause or the result of erosion formation. Erosions are not present initially; 1.5 mm dulled-blade wounds close completely at 24 hours; erosions begin to form between 1 and 2 weeks (Pal-Ghosh, et al., 2004). These erosions do not form uniformly on the mouse ocular surface after dulled-blade wounds but are more frequent in the nasal quadrant (Pal-Ghosh, et al, 2011). To determine the role of the sub-basal nerves in erosion formation, it will be necessary to evaluate reinnervation by quadrant after dulled-blade and rotating-burr wounds at time points prior to erosion development.

Studies by McKenna and Lwigale (2011) show that during development the nerves enter the inferior nasal region of the mouse cornea first; sub-basal nerves that branch from stromal nerves at the nasal quadrant innervate a larger portion of the mouse cornea than nerves from other quadrants. This anatomical difference could make the nasal region prone to reinnervation defects. According to studies by Ecoiffer and colleagues (2010), there are more blood vessels in the nasal region of the mouse cornea. Because immune cells emerge from the limbal vasculature, more immune cells may enter the stroma at the nasal region compared to other regions. If so, they may inadvertently do collateral damage to the nerves and stroma at this site. Finally, studies by Li and colleagues (2011) show that, after debridement wounding, reinnervation of the corneal sub-basal nerves is optimal in the presence of IL17A produced by activated $\gamma\delta$ T-cell, indicating the critical role of immune cell activation in reinnervation. The relationship between immune cell activation and

reinnervation after corneal injury is complex and understanding it will require analyses of nerves and immune cells during normal and pathological healing.

The events that occur within 6 hours after dulled-blade and rotating-burr injury to the mouse cornea are presented schematically in Figure 8. The *in vivo* data presented in this paper show that, after rotating-burr wounds, the basement membrane is removed, more sub-basal sensory nerve fibers are eliminated, more stromal cells undergo apoptosis, and significantly more corneal wounds resolve 4 weeks after wounding. After dulled-blade wounds, CD45+ immune cells are more rapidly recruited into the corneal stroma and are present within the corneal epithelium at erosion sites 4 weeks after dulled-blade wounding. Fewer sub-basal nerves are present within and adjacent to erosions. While additional experiments are needed, these studies will help to determine the mechanism underlying the chronic elevation of MMP9 in the mouse cornea after debridement wounding. This knowledge will in turn improve treatment options for patients with recurrent erosions and with inflammatory conditions of the cornea involving elevated MMP9 expression.

Acknowledgments

We would like to thank people who have provided assistance during the preparation of this manuscript including Dr. David Leitenberg, Dr. Vickery Trinkaus-Randall, and Dr. James Zieske. Confocal imaging was performed in the GWU Center for Microscopy and Image Analysis under the direction of Dr. Anastas Popratiloff and is supported by S10 RR023365 from NIH/NCRR. This work was supported by NIH grant EY08512 to MAS.

References

- Afonso AA, Sobrin L, Monroy DC, Selzer M, Lokeshwar B, Pflugfelder SC. Tear fluid gelatinase B activity correlates with IL-1 α concentration and fluorescein clearance in ocular rosacea. *Invest. Ophthalmol. Vis. Sci.* 1999; 40:2506–2512. [PubMed: 10509643]
- Aldave AJ, Kamal KM, Vo RC, Yu F. Epithelial debridement and Bowman's layer polishing for visually significant epithelial irregularity and recurrent corneal erosions. *Cornea.* 2009; 28:1085–1090. [PubMed: 19730089]
- Chandler HL, Colitz CM, Lu P, Saville WJ, Kusewitt DF. The role of the slug transcription factor in cell migration during corneal re-epithelialization in the dog. *Exp. Eye Res.* 2007; 84:400–411. [PubMed: 17196588]
- Chandler HL, Gemensky-Metzler AJ, Bras ID, Robbin-Webb TE, Saville WJ, Colitz CM. In vivo effects of adjunctive tetracycline treatment on refractory corneal ulcers in dogs. *J. Am. Vet. Med. Assoc.* 2010; 237:378–386. [PubMed: 20707747]
- Chen W, Zhang X, Zhang J, Chen J, Wang S, Wang Q, Qu J. A murine model of dry eye induced by an intelligently controlled environmental system. *Invest. Ophthalmol. Vis. Sci.* 2008; 49:1386–1391. [PubMed: 18385054]
- Chotikavanich S, de Paiva CS, Li de-Q, Chen JJ, Bian F, Farley WJ, Pflugfelder SC. Production and activity of matrix metalloproteinase-9 on the ocular surface increase in dysfunctional tear syndrome. *Invest. Ophthalmol. Vis. Sci.* 2009; 50:3203–3209. [PubMed: 19255163]
- Collinson JM, Morris L, Reid AI, Ramaesh T, Keighren MA, Flockhart JH, Hill RE, Tan SS, Ramaesh K, Dhillon B, West JD. Clonal analysis of patterns of growth, stem cell activity, and cell movement during the development and maintenance of the murine corneal epithelium. *Dev. Dyn.* 2002; 224:432–440. [PubMed: 12203735]
- Corrales RM, Stern ME, De Paiva CS, Welch J, Li de-Q, Pflugfelder SC. Desiccating stress stimulates expression of matrix metalloproteinases by the corneal epithelium. *Invest. Ophthalmol. Vis. Sci.* 2006; 47:3293–3302. [PubMed: 16877394]
- Dursun D, Wang M, Monroy D, Li de-Q, Lokeshwar BL, Stern M, Pflugfelder SC. Experimentally induced dry eye produces ocular surface inflammation and epithelial disease. *Adv. Exp. Med. Biol.* 2002; 506:647–655. [PubMed: 12613973]
- Ecoiffier T, Yuen D, Chen L. Differential distribution of blood and lymphatic vessels in the murine cornea. *Invest. Ophthalmol. Vis. Sci.* 2010; 51:2436–2440. [PubMed: 20019372]

- Esquenazi S, He J, Li N, Bazan NG, Esquenazi I, Bazan HE. Comparative in vivo high-resolution confocal microscopy of corneal epithelium, sub-basal nerves and stromal cells in mice with and without dry eye after photorefractive keratectomy. *Clin. Experiment. Ophthalmol.* 2007; 35:545–549. [PubMed: 17760637]
- Ewald M, Hammersmith KM. Review of diagnosis and management of recurrent erosion syndrome. *Curr. Opin. Ophthalmol.* 2009; 20:287–291. [PubMed: 19491682]
- Garrana RM, Zieske JD, Assouline M, Gipson IK. Matrix metalloproteinases in epithelia from human recurrent corneal erosion. *Invest. Ophthalmol. Vis. Sci.* 1999; 40:1266–1270. [PubMed: 10235563]
- Hadassah J, Bhuvaneshwari N, Rao U, Sehgal PK. Evaluation of succinylated collagen bandage lenses in corneal healing by the expression of matrix metalloproteinases (MMP-2 and MMP-9) in tear fluid. *Ophthalmic Res.* 2009; 42:64–72. [PubMed: 19478544]
- Hayashida K, Parks WC, Park PW. Syndecan-1 shedding facilitates the resolution of neutrophilic inflammation by removing sequestered CXC chemokines. *Blood.* 2009; 114:3033–3043. [PubMed: 19638625]
- Holopainen JM, Serra HM, Sánchez MC, Sorsa T, Zalentien WN, Barcelona PF, Moilanen JA, Tervahartiala T, Tervo TM, Cafaro TA, Virtanen I, Urrets-Zavalía EA, Bhattacharya SK, Urrets-Zavalía JA. Altered expression of matrix metalloproteinases and their tissue inhibitors as possible contributors to corneal droplet formation in climatic droplet keratopathy. *Acta Ophthalmol.* 2011; 89:569–574. [PubMed: 19900203]
- Jackson WB. Management of dysfunctional tear syndrome: a Canadian consensus. *Can. J. Ophthalmol.* 2009; 44:385–394. [PubMed: 19606158]
- Laibson PR. Recurrent corneal erosions and epithelial basement membrane dystrophy. *Eye Contact Lens.* 2010; 36:315–317. [PubMed: 20724847]
- Latvala T, Päällysaho T, Tervo K, Tervo T. Distribution of $\alpha 6$ and $\beta 4$ integrins following epithelial abrasion in the rabbit cornea. *Acta Ophthalmol. Scand.* 1996; 74:21–25. [PubMed: 8689474]
- Leiper LJ, Ou J, Walczysko P, Kucerova R, Lavery DN, West JD, Collinson JM. Control of patterns of corneal innervation by Pax6. *Invest. Ophthalmol. Vis. Sci.* 2009; 50:1122–1128. [PubMed: 19029029]
- Lemp MA. Advances in understanding and managing dry eye disease. *Am. J. Ophthalmol.* 2008; 146:350–356. [PubMed: 18599017]
- Li Z, Burns AR, Han L, Rumbaut RE, Smith CW. IL-17 and VEGF are necessary for efficient corneal nerve regeneration. *Am. J. Pathol.* 2011; 178:1106–1116. [PubMed: 21356362]
- Li Z, Burns AR, Smith CW. Two waves of neutrophil emigration in response to corneal epithelial abrasion: distinct adhesion molecule requirements. *Invest. Ophthalmol. Vis. Sci.* 2006; 47:1947–1955. [PubMed: 16639002]
- McKenna CC, Lwigale PY. Innervation of the mouse cornea during development. *Invest. Ophthalmol. Vis. Sci.* 2011; 52:30–35. [PubMed: 20811061]
- Mencucci R, Paladini I, Brahimi B, Menchini U, Dua HS, Romagnoli P. Alcohol delamination in the treatment of recurrent corneal erosion: an electron microscopic study. *Br. J. Ophthalmol.* 2010; 94:933–939. [PubMed: 20606026]
- Mohan RR, Stapleton WM, Sinha S, Netto MV, Wilson SE. A novel method for generating corneal haze in anterior stroma of the mouse eye with the excimer laser. *Exp. Eye Res.* 2008; 86:235–240. [PubMed: 18068702]
- Müller LJ, Marfurt CF, Kruse F, Tervo TM. Corneal nerves: structure, contents and function. *Exp Eye Res.* 2003; 76:521–542. [PubMed: 12697417]
- Müller LJ, Pels L, Vrensen GF. Ultrastructural organization of human corneal nerves. *Invest. Ophthalmol. Vis. Sci.* 1996; 37:476–488. [PubMed: 8595948]
- Pal-Ghosh S, Blanco T, Tadvalkar G, Pajoohesh-Ganji A, Parthasarathy A, Zieske JD, Stepp MA. MMP9 cleavage of the $\beta 4$ integrin ectodomain leads to recurrent epithelial erosions in mice. *J. Cell Sci.* 2011; 124:2666–2675. [PubMed: 21750188]
- Pal-Ghosh S, Pajoohesh-Ganji A, Brown M, Stepp MA. A mouse model for the study of recurrent corneal epithelial erosions: $\alpha 9\beta 1$ integrin implicated in progression of the disease. *Invest. Ophthalmol. Vis. Sci.* 2004; 45:1775–1788. [PubMed: 15161840]

- Pal-Ghosh S, Tadvalkar G, Jurjus RA, Zieske JD, Stepp MA. BALB/c and C57BL6 mouse strains vary in their ability to heal corneal epithelial debridement wounds. *Exp. Eye Res.* 2008; 87:478–486. [PubMed: 18809399]
- Pflugfelder SC, Solomon A, Stern ME. The Diagnosis and Management of Dry Eye: A Twenty-five-Year Review. *Cornea.* 2000; 19:644–649. [PubMed: 11009316]
- Pflugfelder SC, Farley W, Luo L, Chen LZ, de Paiva CS, Olmos LC, Li de-Q, Fini ME. Matrix metalloproteinase-9 knockout confers resistance to corneal epithelial barrier disruption in experimental dry eye. *Am. J. Pathol.* 2005; 166:61–71. [PubMed: 15632000]
- Ramamurthi S, Rahman MQ, Dutton GN, Ramaesh K. Pathogenesis, clinical features and management of recurrent corneal erosions. *Eye.* 2006; 20:635–644. [PubMed: 16021185]
- Saunders AE, Johnson P. Modulation of immune cell signalling by the leukocyte common tyrosine phosphatase, CD45. *Cell Signal.* 2010; 22:339–348. [PubMed: 19861160]
- Schultz GS, Strelow S, Stern GA, Chegini N, Grant MB, Galardy RE, Grobelny D, Rowsey JJ, Stonecipher K, Parmley V. Treatment of alkali-injured rabbit corneas with a synthetic inhibitor of matrix metalloproteinases. *Invest. Ophthalmol. Vis. Sci.* 1992; 33:3325–3331. [PubMed: 1385350]
- Sta Iglesia DD. Disruption of the basement membrane after corneal debridement. *Invest. Ophthalmol. Vis. Sci.* 2000; 41:1045–1053. [PubMed: 10752940]
- Stepp MA, Zhu L, Cranfill R. Changes in $\beta 4$ integrin expression and localization in vivo in response to corneal epithelial injury. *Invest. Ophthalmol. Vis. Sci.* 1996; 37:1593–1601. [PubMed: 8675402]
- Stramer BM, Zieske JD, Jung JC, Austin JS, Fini ME. Molecular mechanisms controlling the fibrotic repair phenotype in cornea: implications for surgical outcomes. *Invest. Ophthalmol. Vis. Sci.* 2003; 44:4237–4246. [PubMed: 14507867]
- Tanifuji-Terai N, Terai K, Hayashi Y, Chikama T, Kao WW. Expression of keratin 12 and maturation of corneal epithelium during development and postnatal growth. *Invest. Ophthalmol. Vis. Sci.* 2006; 47:545–551. [PubMed: 16431949]
- Wilson SE, Chaurasia SS, Medeiros FW. Apoptosis in the initiation, modulation and termination of the corneal wound healing response. *Exp. Eye Res.* 2007; 85:305–311. [PubMed: 17655845]
- Yu CQ, Rosenblatt MI. Transgenic corneal neurofluorescence in mice: a new model for in vivo investigation of nerve structure and regeneration. *Invest. Ophthalmol. Vis. Sci.* 2007; 48:1535–1542. [PubMed: 17389482]

Highlights

- * Using a rotating burr to make mouse debridement wounds reduces erosion frequency.
- * Dulled-blade wounds induce recruitment of more immune cells within 6 hours.
- * Fewer sub-basal nerves are present near erosion sites after dulled blade wounds.
- * CD45+ immune cells are present within and beneath erosion sites.

Figure 1. MMP-inhibition does not impact the frequency of erosions but wounding with the rotating burr significantly improves healing

Corneas were wounded by removal of 1.5 mm corneal epithelial tissue using either the dulled blade or rotating burr. Representative images of Richardson stained corneas are shown (A). Data from assessments of erosion frequency are shown (B). MMP-activity was reduced by treating corneas with GM6001 topically or orally with oxytetracycline. Erosions were assessed 4 weeks after wounding. Mice wounded with the rotating burr have significantly fewer open wounds compared to dulled-blade wounded corneas. The asterisk indicates that data are significantly different with a p value < 0.05 obtained using one-way Anova with post-tests. MMP inhibition had no impact on erosion formation.

Figure 2. Rotating-burr wounded corneas do not express MMP9 and rarely form erosions 4 weeks after wounding

Shown are corneas 4 weeks after wounding by rotating burr (A) and dulled blade (B) processed for whole mount confocal immunofluorescence to reveal the localization of $\beta 4$ integrin, LN332, and MMP9. Note that $\beta 4$ integrin and LN332 localize within basal cells at the BMZ after rotating-burr wounds. At erosion sites after dulled-blade wounding, $\beta 4$ integrin localization is irregular and discontinuous and LN332 is absent at the BMZ. For the cross sections, $\beta 4$ integrin is in red and MMP9 in green. The *en face* images are merged from 18 confocal layers taken at $1\mu\text{m}$ intervals; CS images are 3D reconstructions obtained by rotating 18 layers at a 90° angle to obtain cross sections (see Methods for details). For the dulled-blade MMP9 image, the dotted line indicates the region where the cross section was obtained. The bar in the *en face* and CS confocal images = $7\mu\text{m}$.

Figure 3. Rotating-burr wounds remove $\alpha 6\beta 4$ and BMZ proteins at the wound center but not at the periphery

On the left are 20x images of rotating-burr (A, C) and dulled-blade (B, D) wounded eyes at 0 (A, B) and 6 hour (C, D) after wounding. The arrows indicate the orientation of the wound relative to the wound margin. On the right are 63x images of the regions indicated by the asterisks to reveal the localization of $\beta 4$ integrin, LN332, and type VII collagen in green and DAPI in blue. The *en face* images are merged images obtained from 18 confocal layers taken at 1 μ m intervals; CS images are 3D reconstructions obtained by rotating 18 layers at a 90° angle to obtain cross sections (see Methods for details). Note the presence of basement membrane in dulled-blade wounded corneas and their absence in rotating-burr wounded corneas at 0 time and 6 hours after wounding and the distortion seen in the nuclei after rotating-burr wounds at time 0. Note also, the absence of nuclei at these sites 6 hours after wounding. Bar in 20X image = 50 μ m; bar in 63X image = 7 μ m.

Figure 4. More stromal nerves remain intact 6 hours after dulled-blade wounding compared to rotating-burr wounds

Shown are 63X *en face* images of rotating-burr (left) and dulled-blade (right) wounded corneas 6 hours after wounding and stained to reveal the localization of the nerve-specific $\beta 3$ tubulin. The arrow indicates the direction of migration. Note that there are several nerve branches within the stroma in front of the leading edge after dulled-blade wounds; no nerves are present in front of the leading edge after rotating-burr wounds. The bar = 7 μ m.

Figure 5. The region surrounding erosions 4 weeks after dulled-blade wounds shows a significant reduction in sub-basal nerve fibers

Shown are 20X *en face* images of dulled-blade wounded corneas 4 weeks after wounding and stained to reveal the localization of the nerve-specific $\beta 3$ tubulin (Tuj1; green), $\beta 4$ integrin (red), and corneal epithelial nuclei with DAPI (blue). Each antigen is shown separately on the left and merged along with DAPI on the right. The area indicated by the white ^ show nerve fibers peripheral to the erosion site; the area indicated by the white * show nerve fibers within an erosion. 20X images were digitally enlarged and shown below. Note that the sub-basal nerves are organized parallel to the basal surface of the basal cells in the region peripheral to the erosion site. Within the erosion, there are few nerves and those present are disordered and appear tangled. The bar in the 20X images = 50 μm ; the bar in the enlarged images = 10 μm .

Figure 6. CD45+ immune cells increase dramatically within the stroma near the limbus within 6 hours after dulled-blade wounds

On the left are 10x *en face* images of rotating-burr (A) and dulled-blade (B) corneas 6 hours after wounding stained to reveal the localization of immune cells using an antibody against the common leukocyte antigen, CD45. The arrows below the 10X images indicate the orientation of the limbus relative to the central cornea. Using a 63X oil immersion objective, confocal *en face* layers (18) were obtained at 1- μ m intervals from these corneas. 3D reconstructions were generated and rotated at a 90° angle to obtain cross sections (CS; see Methods for details). There are more CD45+ cells after dulled-blade compared to rotating-burr wounded corneas. The bar in the 10X images = 100 μ m and the bar in the 63X images = 7 μ m.

Figure 7. Erosions 4 weeks after dulled-blade wounds show CD45+ immune cells within the epithelium at sites where there are few nerve fibers

Shown are 63X *en face* images of corneas that have healed (A; rotating burr) or have developed erosions (B; dulled blade) after staining to reveal the localization of immune cells (CD45, green), nerve fibers (Tuj1; red), and nuclei (DAPI; blue). On the left, images focus on corneal epithelial cells and sub-basal nerves and on the right images show regions within the anterior stroma that are 2-3 μm below the areas shown on the left. Note the absence of CD45+ immune cells within the corneal epithelium in the healed cornea and the presence of nerve fibers. In the cornea with the erosion, CD45+ immune cells are present within the corneal epithelium adjacent to the erosion. Also, there are twice as many CD45+ immune cells in the stroma beneath the erosion site compared to beneath the healed cornea epithelium. The bar = 7 μm .

Figure 8. Comparison of the cornea after rotating-burr and dulled-blade wounds 6 hours after wounding