

HHS Public Access

Author manuscript *Exp Eye Res.* Author manuscript; available in PMC 2017 May 01.

Published in final edited form as:

Exp Eye Res. 2016 May ; 146: 361-369. doi:10.1016/j.exer.2015.08.023.

Topical Mitomycin-C enhances subbasal nerve regeneration and reduces erosion frequency in the debridement wounded mouse cornea

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Abstract

Corneal epithelial basement membrane dystrophies and superficial injuries caused by scratches can lead to recurrent corneal erosion syndrome (RCES). Patients and animals with reduced corneal sensory nerve innervation can also develop recurrent erosions. Multiple wild-type mouse strains will spontaneously develop recurrent corneal erosions after single 1.5 mm debridement wounds. Here we show that this wound is accompanied by an increase in corneal epithelial cell proliferation after wound closure but without a commensurate increase in corneal epithelial thickness. We investigated whether excess corneal epithelial cell proliferation contributes to erosion formation. We found that topical application of Mitomycin C (MMC), a drug used clinically to improve healing after glaucoma and refractive surgery, reduces erosion frequency, enhances subbasal axon density to levels seen in unwounded corneas, and prevents excess epithelial cell proliferation after PRK, may also function to enhance subbasal nerve regeneration and epithelial adhesion when used to treat RCES.

Introduction

Corneal epithelial thickness (3–5 layers), unlike stromal thickness, is well conserved during mammalian evolution and is typically restored soon after small corneal epithelial injuries as

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first observed by Friedenwald and Buschke (1944). Injury to the corneal epithelium induces a decrease in cell proliferation during active cell migration followed by an increase in proliferation to restore normal epithelial thickness (Stepp, et al., 2014). If cell proliferation rates remain elevated after wounding, an increase in the turnover rate of basal and suprabasal cells must take place to maintain epithelial thickness at 3–5 layers. As basal cells leave the basement membrane and detach from subbasal axons, they lose integrin expression. When this occurs at a steady state level, the corneal epithelium remains intact, but if clusters of adjacent basal cells leave the basement membrane and detach from subbasal axons simultaneously, the risk of developing erosions and focal sites of denervation will be increased. A recent study using a mouse model for recurrent erosions showed that 7 days after debridement wounds, clusters of corneal epithelial cells at the corneal center stop proliferating and begin to undergo apoptosis before erosions form (Pajoohesh-Ganji, et al., 2015). The studies presented here ask whether excess corneal epithelial cell proliferation leads to subbasal nerve denervation and erosion formation.

Corneal erosions are a common clinical complaint. An article by Mencucci and Favuzza (2014) states that corneal surface defects were first described in 1872 and clinical signs of recurrent epithelial erosion syndrome (RCES) have been actively studied since the 1940's. Corneal epithelial basement membrane dystrophies and superficial injuries caused by scratches can lead to RCES. Patients and animals with reduced corneal sensory nerve innervation also develop corneal erosions (Ferrari, et al., 2013a; Ferrari, et al., 2013b; Sacchetti and Lambiase, 2014; Shaheen, et al., 2014), suggesting defective reinnervation may also play a role in erosion formation.

Treatments for RCES have evolved over the years (Mencucci and Favuzza, 2014) and were reviewed in a Cochrane Collabaoration report in 2012 (Watson, et al., 2012). Superficial keratectomy and alcohol delamination have been used successfully to polish the corneal surface and improve epithelial adhesion. Peripheral erosions can be treated by superficial stromal puncture. Since this condition occurs most frequently between 30–40 years of age (Mencucci and Favuzza, 2014), when treatment fails, patients experience a significant loss of productivity and quality of life.

We investigate here whether excess corneal epithelial cell proliferation within the periphery after superficial injury contributes to erosion formation. We study cell proliferation by assessing the numbers of Ki-67+ cells as a function of time after 1.5 mm debridement wounds. While Ki-67 expression does not directly assess cell proliferation, its expression has been shown to be a reliable marker of cell proliferation (^{Brown}, et al., 2014) and is routinely used to assess responses to chemotherapy in the clinic (Cattoretti, et al., 1992). After showing increased numbers of corneal epithelial cells express Ki-67 between 7–28 days after 1.5 mm debridement wounding, we determine whether topical application of MMC, a drug used clinically to improve healing after glaucoma and refractive surgery (^{Chen} and Chang, 2010; Santhiago, et al., 2012; Majmudar, et al., 2015), enhances wound resolution.

Materials and Methods

Corneal wounding

All studies performed comply with the George Washington University Medical Center Institutional Animal Care and Use Committee guidelines and with the ARVO Statement for the Use of Animals in Vision Research. Male BALB/c mice (NCI, Frederick, MD, USA) between the ages of 7 and 8 weeks were used for all of the experiments described. Mice were anesthetized with ketamine/xylazine and a topical anesthetic applied to their ocular surface. Unless otherwise indicated, a 1.5-mm trephine was used to demarcate the wound area and the epithelial tissues within the area removed using a dulled blade. Manual superficial keratectomy (1.5 mm) experiments were performed as described in Blanco-Mezquita, et al., (2011). After wounding, erythromycin ophthalmic ointment was applied to the injured cornea and mice were allowed to heal for different time points after which they were euthanized. Tissues were fixed immediately for immunofluorescence (IF) studies in fix (1x PBS, 1% formaldehyde, 2mM Mgcl2, 5mM EGTA, 0.02% NP-40) for 1 hr and 15 min at 4°C followed by 2 washes for 10 min each in PBS with 0.02%NP40 at room temperature. Tissues were then placed in 4:1 Methanol:DMSO at -20° C for 2 hours and then in 100% methanol for long term storage at -20° C.

Mitomycin C treatment

For the MMC experiments, 1ml of sterile water was added to 10 mg MMC vial to generate a 1%MMC stock solution, which was aliquoted and stored frozen. For each application, 1% MMC solution was diluted in PBS to a concentration of 0.02%. For vehicle, the same volume of water was added to PBS. A schematic representation of the design of the experiments performed is seen in Figure 4. MMC solution or vehicle (20 µl) was applied to corneas either on days 4 and 7 (Experiment 1) or on days 3, 7, and 10 (Experiment 2) after wounding. Animals were sacrificed at days 11, 14, and 28. Tissues were stored as mentioned above.

Microscopy

For Sholl analysis, images were acquired using the Zeiss Cell Observer Z1 spinning disk confocal microscope (Carl Zeiss, Inc., Thornwood, NY, USA), equipped with ASI MS-2000 (Applied Scientific Instrumentation, Eugene, OR, USA) scanning stage with z-galvo motor, and Yokogawa CSU-X1 spinning disk. A multi-immersion $\times 25/0.8$ objective lens, LCI Plan-Neofluar, was used for imaging, with oil immersion. Evolve Delta (Photometrics, Tucson, AZ, USA) 512 \times 512 EM-CCD camera was used as detector (80-msec exposure time). A diode laser emitting at 594 nm was used for excitation (54% power). Zen Blue software (Carl Zeiss, Inc.) was used to acquire the images, fuse the adjacent tiles, and produce maximum intensity projections. The adjacent image tiles were captured with overlap to ensure proper tiling. Sholl analysis was performed using ImageJ as described in Pajoohesh-Ganji, et al. (2015).

Confocal microscopy was performed at the Center for Microscopy and Image Analysis (CMIA) at the George Washington University Medical Center. A confocal laser-scanning microscope (Zeiss 710) equipped with a krypton-argon laser was used to image the

localization of Alexa Fluor 488 (488 nm laser line excitation; 522/35 emission filter), and Alexa Fluor 594 (568 nm excitation; 605/32 emission filter). Optical sections (z=0.5 µm) were acquired sequentially with a 20x objective lens and images were generated using Volocity software (Version 5.0, Perkin Elmer).

Ki-67 Analysis

20x images were taken with a Nikon Eclipse 600 fluorescent microscope using a SPOT RT Slider cooled CCD camera (Diagnostic Instruments, Inc). Eyes were stained with Ki-67 antibody (Abcam; #ab16667) and flat mounted by making four cuts in the tissue. 2 images were taken from each quadrant and 2 from the central cornea for each eye (total of 10 images per eye). Supplemental Figure 1 shows schematically where images were obtained for Ki-67 stained flat mounted corneas. At times when erosions form, images from the corneal center yield no Ki-67+ cells. To reduce bias due to erosion formation, data from the 8 images taken in the periphery were used at all time points assessed. Counting of Ki-67 positive cells was performed using Image J version1.46r.

Antibodies used

Corneas were stained with the following antibodies: rat anti mouse $\beta4$ integrin (BD Pharmingen, #346-11A), rabbit monoclonal against Ki-67 (Abcam, #ab16667), β III tubulin (Covance; #MMS-435P-100). Appropriate secondary DyLite 488 or 594 antibodies from Jackson Immunobiologicals were used for immunolabeling. Corneas were stained with DAPI (Thermo Fisher; #46190) before flat mounting to reveal nuclei. To achieve the best flattening, the corneas were placed epithelial side-up with mounting medium and coverslipped.

Statistics

Data are presented using one-way ANOVA or Student's *t*-test using Graphpad Prism Program, Version 6 (GraphPad Software, Inc. San Diego, CA). Data are considered significant if p values are less than 0.05.

Results

Corneal erosion formation is accompanied by an elevation in the numbers of Ki-67+ cells on the corneal surface

RCES spontaneously develops in multiple wild-type mouse strains after a single 1.5 mm debridement wound (Pal-Ghosh, et al., 2008, Pal-Ghosh, et al., 2011, Stepp, et al., 2014). The typical appearance of an erosion site on the surface of the mouse cornea after debridement wounding stained with antibodies against β III tubulin and β 4 integrin is shown in Figure 1A–C. Areas where the epithelial sheet expressing β 4 integrin has rolled up leaving an erosion are seen (arrows) (Figure 1A). Also seen are sites where epithelial cells are migrating from the margin of the erosion into the denuded area (asterisks). When the epithelial sheet rolls up, basal cells detach leaving bare stroma (arrowheads); however, some basal cells appear to remain attached to the basement membrane and stroma where they spread and upregulate β 4 integrin. Circling the erosion site at the margin are the blunt tips of

the subbasal axons (arrows) (Figure 1B). In the merged image (Figure 1C), axons appear to be attempting to extend beneath the rolled up epithelial sheet (asterisks).

To assess cell proliferation, corneas were wounded by 1.5 mm debridement and animals sacrificed at 2, 7, 14, and 28 days after injury and their corneas assessed for the numbers of Ki-67+ corneal epithelial cells. Images representing 8 different fields on the ocular surface at the mid-periphery of each cornea were used (See Supplemental Figure 1).

Figure 2A shows that by 7d after wounding, the numbers of Ki-67+ cells are significantly elevated compared to those in unwounded corneas and remain elevated for 14 and 28 days. The thickness of the corneal epithelium was assessed 28 days after wounding at four different sites at the corneal mid-periphery away from potential erosion sites using confocal imaging of DAPI stained flat mounted corneas; data are shown in Table 1. No difference is seen in the thickness of the corneal epithelium between unwounded and wounded corneas. By contrast, stromal thickness is increased significantly 28 days after wounding. If more corneal epithelial cells are being generated on the ocular surface at days 7 and 14 when erosion frequency is low and hyperplasia is not observed, an increase in the extrusion and desquamation rate of corneal epithelial cells must take place.

To determine whether the size or type of wound contributes to elevated Ki-67 expression in corneal epithelial cells, 1 mm debridement wounds and 1.5 mm manual keratectomy (MK) are assessed after 28 days. As indicated in Figure 2B, the numbers of Ki-67+ cells on the corneal surface were similar to controls 28 days after 1.5 mm MK and 1 mm debridement wounds.

To determine whether 1.5 mm manual keratectomy and 1 mm debridement wounds reinnervate adequately after wounding, mice were allowed to heal for 28 days and whole mount corneas were stained with an antibody against β III tubulin to reveal subbasal axons. Axon density was quantified as described in the methods section by Sholl analysis. Representative images are shown in Figure 3A and quantification is presented in Figure 3B. There are no significant differences between subbasal axon density at the corneal center after MK and 1mm debridement wounds compared to controls. The data in Figures 2 and 3, along with data showing that subbasal axon density is significantly reduced at the corneal center 28 days after 1.5 mm debridement wounds (Pajoohesh-Ganji, et al., 2015), suggest that chronically elevated corneal epithelial cell proliferation contributes to the inability of the subbasal axons to reinnervate the corneal center after debridement wounding.

MMC treatment reduces erosion formation

We next designed experiments to determine whether treating the mouse cornea with 0.02% MMC, the concentration used clinically to reduce scar formation, would improve corneal wound healing after 1.5 mm debridement wounds. Two different experimental designs were used as indicated schematically in Figure 4. Since we did not know whether MMC might impact epithelial cell migration and sheet movement in vivo in mice, we decided to treat the corneas after reepithelialization was complete but before corneal epithelial cells at the center begin to show pathology (^{Pajoohesh-Ganji, et al., 2015}). Experiment 1 involved treating the corneas topically with vehicle alone or vehicle plus 0.02% MMC on days 4 and 7 after

wounding; mice were sacrificed at 14 and 28 days. Experiment 2 involved treating mice with vehicle alone or vehicle plus 0.02% MMC on days 3, 7, and 10. After the last treatment, mice were either sacrificed on day 11, 24 hours after the final MMC treatment, or allowed to heal until 14 or 28 days after wounding. After sacrifice, corneas were stained with Richardson stain to determine whether their corneal wounds were open or closed. Unwounded vehicle and MMC treated corneas for both experiments did not develop erosions (data not shown). The percentages of the 1.5 mm debridement wounded corneas that were considered open and had erosions are shown in Table 2. No differences between wounded vehicle and MMC treated corneas are seen at 14 days. However, corneas treated with MMC in Experiments 1 and 2 show fewer open wounds (30% and 20%) 28 days after injury compared to vehicle treated corneas (75% and 100%).

In Experiment 2 we also assessed corneas 24 hours following the last vehicle or MMC treatment (day 10) on day 11 after wounding. The percentage of corneas with open wounds increases from 8% to 60% between days 11 and 14 following the final MMC treatment. Yet, by day 28, only 20% of the MMC treated corneas in Experiment 2 have open wounds. For vehicle treated corneas, erosion frequency decreases from 42% at day 11 to 20% at day 14 followed by a dramatic increase (100%) between day 14 and 28. By 14 days after wounding, 60% of the wounded corneas treated 3x with MMC had erosions but only 10% had erosions after being treated twice.

MMC treatment significantly improves reinnervation of subbasal nerves after debridement wounds

After assessment for erosions, corneas from Experiments 1 and 2 were fixed and whole mount immunofluorescent staining experiments were performed to quantify subbasal nerve density using Sholl analyses of images obtained by confocal imaging. Representative confocal images for 3 of the corneas assessed are presented in Figure 5 and quantitation shown in Figure 6; the total number of corneas assessed is indicated on each bar. The vortex is present in unwounded corneas after vehicle or MMC treatment. By 28 days after treatment with vehicle, wounded corneas show incomplete reinnervation at the corneal center as shown in Figure 5. At 14 days after injury, both vehicle and MMC treated wounded corneas had significantly reduced subbasal axon density in the corneal center compared to unwounded corneas. However, both Experiments 1 and 2 show significantly improved subbasal axon density at 28 compared to 14 days after MMC treatment.

MMC treatment suppresses the number of Ki-67+ corneal epithelial cells in unwounded corneas for 3 days after the last treatment

To determine how MMC treatment decreases erosion formation and increases reinnervation, we assessed the numbers of Ki-67+ cells on vehicle and MMC treated unwounded and wounded corneas 24 hours after the final MMC treatment (11 days after wounding). Data presented in Figure 7A show that MMC treatment significantly reduces cell proliferation in unwounded mouse corneas by 17% compared to vehicle alone. There are no significant differences in Ki-67+ cells between unwounded and wounded vehicle treated or wounded vehicle and MMC treated corneas. Yet Ki-67+ cells are significantly elevated in the wounded MMC treated when compared to unwounded MMC treated corneas.

To understand the significance of these data, we compared vehicle and MMC treated unwounded corneas at days 11, 14, and 28 (Figure 7B). Vehicle treated corneas show significantly elevated numbers of Ki-67+ cells at day 11 compared to days 14 and 28. By contrast, MMC treated corneas have significantly reduced numbers of Ki-67+ cells at days 11 and 14 compared to day 28. Cell proliferation rates at 11 days after wounding are not significantly elevated after vehicle treatment, in part, because vehicle treatment raises proliferation rates in unwounded corneas. Likewise, there are more Ki-67+ cells 11 days after wounding in MMC treated corneas, in part, because cell proliferation has been suppressed in the MMC-treated unwounded corneas. More Ki-67+ cells are present at day 28 in the vehicle treated wounded compared to unwounded corneas but the difference is not statistically significant as shown in Figure 7C.

Discussion

The data presented show that after reepithelialization is complete, treating debridement wounded mouse corneas with 0.02% MMC improves the quality of the healed corneal epithelial surface by 28 days after wounding. After MMC treatment, erosion frequency is reduced and subbasal axon density is significantly improved. While there is a trend for 3 MMC treatments to result in improved healing outcomes at 28 days (20% erosions) compared to 2 treatments (30% erosions); subbasal axon density is similar. By 14 days after wounding, corneas treated 3 times with MMC have significantly more (Chi= 10; p=0.0016) erosions (60%) compared to vehicle treated corneas (20%). Corneal epithelial cell apoptosis, subbasal nerve retraction, and erosions develop after 1.5 mm debridement wounds 7 to 14 days after wounding (Pajoohesh-Ganji, et al., 2015). We hypothesize that adhesion of the corneal epithelium is destabilized by the application of vehicle or MMC at 10 days after wounding. We do not know whether a single treatment with MMC between 3–7 days after wounding would reduce erosion formation and enhance reinnervation but treatment before day 10 minimizes the risk of disrupting the ocular surface.

After 1.5 mm debridement wounds, the mouse corneal epithelial basal cells proliferate more than cells in unwounded corneas for prolonged times starting 7 days after injury before erosions form and continuing for 28 days. Smaller 1.0 mm debridement wounds and 1.5.mm wounds that completely remove the basement membrane (manual keratectomy wounds) do not show persistent increases in cell proliferation, have improved reinnervation, and do not develop erosions. Despite a prolonged increase in corneal epithelial cell proliferation after 1.5 mm debridement wounds, the overall thickness of the corneal epithelium remains the same as unwounded corneas implicating an increase in the rate of turnover of basal and suprabasal cells.

Studies of crowding-induced-extrusion of living cells in simple epithelia have shown that increased cell density stimulates stretch activated ion channels which leads to reduced cell volume and fewer basement membrane and cell:cell contacts (Eisenhoffer and Rosenblatt, ²⁰¹³). These events are followed by secretion of sphingosine-1 phosphate, contraction of an actin/myosin ring at the basal aspect of the cell, and extrusion (Eisenhoffer and Rosenblatt, ²⁰¹³). Whether basal cell extrusion in stratified epithelia involves similar mechanisms is not clear. Increases in the mechanical forces exerted during extrusion of more basal cells would

be transmitted to both subbasal axons and to the reassembling basement membrane. Reducing corneal epithelial cell proliferation rates before erosions form would be predicted to improve wound resolution by maximizing adhesion between corneal epithelial cells, the basement membrane, and the subbasal axons.

A clinical study done in 2012 on 1520 eyes of 760 patients showed that applying 0.02% MMC to the ocular surface after PRK "delayed corneal wound healing" (Kremer, et al., ²⁰¹²). In the study, 2% (30 out of 1520) of the MMC treated corneas showed evidence of an epithelial defect 12-14 days after PRK was performed compared to 0.8% (4 out of 500) of the non-MMC treated corneas, a difference which was significant. Yet, the same study concluded that eyes treated with MMC had better visual outcomes, significantly lower rates of best corrected visual acuity loss and retreatment compared to non-MMC treated eyes. A study performed using human corneas in organ culture also showed that MMC treatment of PRK wounded corneas delayed reepithelialization (Rajan, et al., 2006). Another study. however, showed that MMC used in vitro in cell migration studies of human organ cultured corneas did not alter epithelial cell migration rates (Yamamoto, et al., 1990). Treating corneas after initial wounds have closed raises concerns that MMC may not penetrate the corneal epithelium. Yet, data show that MMC treatment of unwounded corneas reduces the numbers of Ki-67+ cells significantly by 17% compared to vehicle treated eyes 24 hours after the final MMC treatment. The reduction in cell proliferation in unwounded corneas due to MMC lasted through day 14 but was no longer apparent by day 28. Vehicle treated unwounded corneas also showed changes in cell proliferation rates over time; by day 14, the cell proliferation rate dropped 14.5% compared to day 11; no further change was seen at day 28. These data indicate that vehicle treatment of the unwounded mouse cornea three times increases corneal epithelial cell proliferation. However, vehicle treatment alone does not impact subbasal axon density compared to that seen in untreated unwounded corneas (Pajoohesh-Ganji, et al., 2015). The acute impact of MMC on cell proliferation after wounding is subtle. No differences are observed between MMC and vehicle treated corneas 24 hr after MMC treatment 10 days after wounding.

Refractive surgeons limit MMC treatment to the wound bed and take care to avoid getting MMC on the limbus to prevent damage to limbal stem cells (Rajan, et al., 2006, Santhiago, et al., 2012. Majmudar, et al., 2015). Here we apply MMC topically which gives the drug access to corneal and limbal epithelial cells raising concern over potential toxicity to the limbal stem cells. Treatment of conjunctival and eyelid neoplastic lesions involves surgical removal of the tumor followed by treatment of the ocular surface topically with one or more of the following drugs: 5-fluorouracil, MMC (0.04%), or interferon a2b (Adler, et al., 2013: Bahrami et al., 2014, Yin, et al., 2015). Topical MMC is also used to treat patients after pterygium removal (Fakhry, 2011). 0.04% MMC eye drops are applied several times a day for a week. Treatment is followed by a week of no treatment; this cycle is repeated 2-3 times until there is no evidence of tumor regrowth (Bahrami, et al., 2014. Kashkouli, et al., 2012) A retrospective study of 58 patients indicates that although MMC is effective at managing ocular surface neoplasia, it induces limbal stem cell deficiency with an incidence of 12% assessed 36 months following initial treatment (Russell, et al., 2010). However, another study of 153 eyes reported no significant long-term complications over an average of 33.6 months for patients treated with either MMC or 5-fluorouracil (Bahrami, et al., 2014).

Although the frequency of limbal stem cell deficiency after several rounds of multiple daily topical MMC treatments remains unknown, the majority of patients do not develop this pathology. The fact that 28 days after 2 or 3 topical applications of MMC, the mouse ocular surface remains clear and the corneal epithelial thickness remains similar to that of unwounded corneas indicates minimal impact of topical MMC used as described on limbal stem cell viability. However, we did not re-injure the corneas near the limbus to induce stem cell proliferation.

Despite its use in the clinic, MMC is a cytotoxic agent that can bind covalently to DNA (^{Paz, et al., 2012}). It is inert in its original form but is converted into an extremely reactive electrophile after its quinone ring is reduced. Activation of MMC is mediated by several different reductases (^{Paz, et al., 2012}) and activated MMC can alkylate DNA, RNA, and proteins affecting their activity. MMC is an irreversible inhibitor of the activity of the selenoprotein thioredoxin reductase (TrxR) (^{Arnér} and Holmgren, 2006). Interestingly, selenoprotein containing compounds have also been shown to mediate oxidative stress and tear secretion in dry eye (Higuchi et al., 2010; Higuchi, et al., 2012).

Additional studies are needed to determine whether MMC facilitates wound resolution after debridement wounds by reducing corneal epithelial cell proliferation alone or by altering other cell signaling pathways. While treating the ocular surface with 0.02% MMC reduced erosion frequency and allowed subbasal axon density to recover to levels similar to those seen in unwounded cornea, the subbasal vortex did not reform suggesting that the healing outcome could be improved further. Using the same study design presented here with 3 treatments, 0.05% MMC improved healing to a similar extent as 0.02% MMC at 28 days; further, improved reinnervation was stabile for 42 days after wounding (data not shown). These data suggest the potential of a commonly used ophthalmic drug, MMC, to improve reinnervation in the peripheral nervous system and reduce erosion formation after corneal injuries and infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded by NIH grants EY08512, EY021784, and EY023106

We would like to thank Dr. Anastas Popratiloff, the director of center for microscopy and image analysis (CMIA) at the George Washington University, for his help with confocal microscopy. We also acknowledge and appreciate the valuable assistance of Audrey Hutcheon and Philip Seifert at the Massachusetts Eye and Ear Infirmary and Harvard Medical School.

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Highlights

- **1.** Treating the mouse cornea topically with MMC following reepithelialization reduces erosion frequency and enhances reinnervation.
- **2.** Corneal epithelial cell proliferation rates remain elevated after 1.5 mm debridement wounds.
- **3.** Corneal epithelial thickness does not increase despite increased epithelial cell proliferation indicating increased cell turnover.



Figure 1. Subbasal axons terminate at the periphery of the erosion sites beneath corneal epithelial cells showing a range of distinct morphologies

Whole mount immunofluorescence staining was performed to visualize the localization of $\beta4$ integrin (**A**) and β III tubulin within subbasal axons (**B**); the merged image is also shown (**C**). Arrows in **A** indicate corneal epithelial cells within the epithelial sheet that have rolled up; arrows in **B** highlight the termini of subbasal axons. Asterisks in **A** highlight corneal epithelial cells that appear to be migrating into the erosion site and in **C** indicate subbasal nerves present beneath the migrating epithelial cells. Arrowheads indicate bare stroma between strands of $\beta4+$ corneal epithelial cells that remain on the stroma when the sheet retracts. These cells flatten on the basement membrane; do not express Ki-67 (^{Pajoohesh-Ganji, et al., 2015}). Bar = 60 µm



Figure 2. Cell proliferation remains elevated compared to controls for several weeks after 1.5 mm debridement wounding but not after 1 mm debridement or 1.5 mm manual keratectomy wounding

1.5 mm debridement wounds were made on corneas and allowed to heal for times ranging from 2–28 days. The n values shown on each bar indicate the numbers of corneas assessed for each variable. Data show that cell proliferation increases significantly compared to controls at 7 days and remains elevated at 14 and 28 days. By contrast, 1 mm debridement and manual keratectomy wounded corneas do not show elevated cell proliferation 28 days after wounding. Asterisks indicate significance below p= 0.05 determined by ANOVA.



Figure 3. Axon density is restored to levels similar to control 28 days after 1 mm debridement and 1.5 mm manual keratectomy (MK) wounds

Corneas were wounded as indicated and allowed to heal for 28 days. The corneas were stained with an antibody against β III tubulin and axon density was quantified using Sholl analysis as described in the methods section. **A** shows three representative images for the subbasal axons for control, 1 mm debridement wounded, and 1.5 mm MK wounding 28 days after injury and **B** shows the axon densities assessed at the center of the injured corneas by Sholl analysis. The n values shown indicate the numbers of corneas assessed for each variable. No significant differences are seen in the axon densities after 1 mm debridement and 1.5 mm MK wounding compared to controls.





Figure 4. Schematic representation of the treatment schemes tested in the studies presented The treatment times and dates of sacrifice for Experiments 1 and 2 are indicated in red and black fonts respectively. The treatments tested were vehicle or vehicle plus 0.02% MMC applied topically. For both experiments unwounded and wounded mice were assessed.



Figure 5. Subbasal axon density is improved by MMC treatment

Shown are three representative results obtained from confocal imaging of flat mounted corneas stained with an antibody against β III tubulin to reveal subbasal axons. The corneas shown are from Experiment 2. The unwounded and wounded corneas shown in **A** and **B** respectively were treated with vehicle (left) or MMC (right). Subbasal axon density was assessed for each cornea by Sholl analysis and results are presented in Figure 6.



Figure 6. Experiments 1 and 2 both show significant improvements in subbasal axon density 28 days after MMC treatment

Shown are the axon density data obtained for unwounded and wounded and for vehicle and MMC treated corneas. The numbers of corneas assessed are indicated by the n values in each bar. At 14 days after wounding in both Experiment 1 and 2, axon density is significantly lower for vehicle and MMC treated wounded corneas compared to vehicle and MMC treated controls. In Experiment 1, at 28 days, there is no significant difference in axon density in the wounded vehicle treated compared to the unwounded vehicle treated cornea but the difference seen at day 14 remains in Experiment 2. Wounded MMC treated corneas significantly increase their axon density between 14 and 28 days in both Experiments 1 and 2. Asterisks indicate significance below p=0.05 by ANOVA for comparisons involving more than 2 groups; asterisks indicate significance by student t test for comparisons involving 2 groups.



Figure 7. MMC treatment eliminates the chronic elevation in cell proliferation

A. Unwounded and wounded vehicle treated corneas sacrificed at day 11 (Experiment 2) show no differences in corneal epithelial cell proliferation. However, unwounded MMC treated corneas sacrificed at day 11 show a significant decrease compared to vehicle treated unwounded or wounded corneas. In addition, there is also an increase in cell proliferation after wounding for MMC treated corneas. **B.** Shown are data Ki-67+ cells in unwounded corneas to determine whether cell proliferation rates change over time following the last treatment with vehicle or MMC. Vehicle treated corneas reduce their proliferation rates at 14 and 28 days compared to day 11. MMC treated corneas increase their proliferation rate at 28 days compared to days 11 and 14. **C.** When Ki-67+cells are quantified after 3 MMC treatments, there are no significant differences seen between unwounded and wounded corneas.

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Table 1

Corneal thickness 28d after 1.5 mm debridement wounding

| | Unwounded (n=7) (µm) | Wounded (n=6) (µm) | |
|--------------------|----------------------|--------------------|--|
| Epithelium: | 13.14 | 12.42 | |
| Stroma: | 22.41 | 27.54 * | |
| Overall thickness: | 34.12 | 39.96 * | |

 * indicates a p value less than 0.05 when wounded is compared to control.

Table 2

MMC treatment and Erosion Formation After Debridement

| Expt. 1 | open | closed | % open |
|----------|------|--------|--------|
| Vehicle: | - | | |
| 14d | 1 | 7 | 13% |
| 28d | 6 | 2 | 75% |
| MMC: | | | |
| 14d | 1 | 9 | 10% |
| 28d | 3 | 7 | 30% |
| | | | |
| Expt. 2 | open | closed | % open |
| Vehicle: | | | |
| 11d | 5 | 7 | 42% |
| 14d | 2 | 8 | 20% |
| 28d | 10 | 0 | 100% |
| MMC: | | | |
| 11d | 1 | 11 | 8% |
| 14d | 6 | 4 | 60% |
| | | | |