Morphological and Functional Changes in the Rat Cornea with an Ethanol-Mediated Epithelial Flap

In-Kyung Song and Choun-Ki Joo

PURPOSE. To establish morphologic and functional changes in the rat cornea after 20% ethanol-mediated epithelial flap creation.

METHODS. The epithelial flap was detached using 20% ethanol and then repositioned. On the other eye, the corneal epithelium was mechanically scraped. The morphologic changes were examined by light and transmission electron microscopy (LM and TEM). Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assays and proliferative cell nuclear antigen (PCNA) staining were performed to detect apoptosis and proliferation, respectively. The number of central stromal keratocytes was counted during wound-healing periods. A Y-chromosome–specific gene (SRY) was detected in genomic DNA obtained from female epithelial cells with male epithelial flap. Expressions of matrix metalloproteinase (MMP)-9, aquaporine5 (AQP5), and mucin1 (MUC1) mRNAs were examined by reverse transcription-polymerase chain reaction.

RESULTS. After flap repositioning, damaged basal cells were observed until day 1, and the death of epithelial cells and stromal keratocytes loss peaked at day 1. Cell proliferation and MMP-9 mRNA levels peaked from days 1 to 3. By contrast, after mechanical scraping, the denuded stromal surface was covered with multilayer epithelial cells at day 3, cell death peaked at 4 hours, cell proliferation peaked from 12 hours to day 1, stromal keratocytes loss peaked at 8 hours, and MMP-9 mRNA was widely expressed from 12 hours to day 3. In both corneas, hemidesmosomes were not observed until day 1, and they continued to be present at day 3. The SRY gene was detected in female epithelial cells at day 1 after transplantation, but not at day 3. Expressions of AQP5 and MUC1 mRNAs were unchanged.

CONCLUSIONS. Ethanol-mediated flap creation induces less keratocyte loss and a slower wound-healing process than mechanical scraping. The flap protecting the underlying stromal surface may have epithelial function. In addition, this Sprague-Dawley rat model may be useful in the study of the wound-healing response after LASEK. (Invest Ophthalmol Vis Sci. 2004;45:423–428) DOI:10.1167/iovs.03-0947

Laser subepithelial keratomileusis (LASEK) is a relatively new refractive surgical technique that makes up for the disadvantages of laser in situ keratomileusis (LASIK) and photorefractive keratectomy (PRK). LASEK theoretically produces faster visual recovery, less postoperative pain, reduced stromal haze, and faster epithelial healing than PRK. It also offers the advantages of avoiding the flap- and interface-related complications of LASIK, the difficulty in predicting flap thickness and ablation depth, and posterior ectasia. Ethanol was initially used in refractive surgery to assist in the removal of epithelium before PRK and has recently been shown to enhance epithelial flap creation without a significant loss of flap viability in LASEK. Exposure times to ethanol of more than 1 minute result in significant damage to superficial corneal epithelium and treatment with 20% alcohol for 30 seconds results in reproducible epithelial flap creation in the chick cornea and in relatively low levels of stromal and epithelial cell death after surgery. Alcoholic delamination of corneal epithelium results in a very smooth cleavage at the level of the hemidesmosomal attachments, including the superficial lamina lucida.

The cornea is covered by nonkeratinized, stratified epithelium that is responsible for maintaining ocular surface integrity and is essential for vision. This provides an initial barrier to tears and to the intraocular environment. The integrity of the epithelium can be monitored from the presence of aquaporins (AQPs), a family of water channels, which are water-selective transporting proteins. A special role of AQPs in water transport is to prevent dehydration or to secrete watery products, and the epithelium thus contributes to corneal hydration homeostasis. On the ocular surface, mucin (MUC) is the main component of the innermost layer of the tear film. Mucin1 (MUC1) mRNA has been detected in all cell layers of the corneal epithelium, and MUC1 plays a protective role against the adherence of pathogens.

After PRK or LASEK surgery, the anterior stroma including Bowman’s layer is removed, and an ethanol-induced epithelial flap is replaced on the ablated stromal layer. After surgery, the corneal epithelial flap is attached to the stroma and undergoes wound healing. We developed an available animal model for LASEK study using the eyes of Sprague-Dawley rat (although rat cornea does not have Bowman’s layer). In the present study, corneal morphologic, biochemical, and functional changes were studied after 20% ethanol-induced epithelial flap creation and reposition and compared with mechanical scraped cornea.

MATERIALS AND METHODS

Animals and Procedures

Adult Sprague-Dawley rats (250–280 g) were used for this study. All animal procedures conformed to Institutional Guidelines and to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The rats were anesthetized with an intramuscular mixture of ketamine hydrochloride (20 mg/kg) and xylazine hydrochloride (5 mg/kg). A total of 252 eyes were divided into four groups: group 1 for paraffin block (n = 5 at each time point); group 2 for Epon block (n = 5 at each time point); group 3 for RNA extraction (n = 6 at each time point); and group 4 for genomic DNA extraction (six male eyes and six female eyes).

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Electron Microscopy 

Light Microscopy and Transmission Electron Microscopy 

Cell Death Detection by TUNEL Assay 

Reverse Transcription–Polymerase Chain Reaction 

Immunohistochemistry
Gaithersburg, MD). A 5-μg portion of total RNA was used for reverse-transcription (RT) with reverse transcriptase (SuperScript II; Invitrogen-Gibco). Then, 50 ng of reverse-transcribed DNA and 80 ng of genomic DNA were amplified using PCR with 1.0 U of Taq DNA polymerase (Perkin Elmer, Boston, MA) in a mixture containing 0.4 μM of each primer, 0.2 μM dNTPs, and 1.5 mM MgCl₂. Primer sequences were as follows: MMP-9 (380 bp) sense, 5'-AAC TCA GCC TTT GAG GAT GC C-3' and antisense, 5'-CAG TAT CCA GTG CAT CCG GT-3'; SRY (351 bp) sense, 5'-TGG CTC AAG AAG AGC TCA GC C-3' and antisense, 5'-TGG GGA TGT CCA GAC GCT GT-3'; AQP5 (730 bp) sense, 5'-C AGG GCC GTG TTC GCA GAG TCC C-3' and antisense, 5'-GTC TCG ATG ATC TTC CCA GTC C-3'; MUC1 (286 bp) sense, 5'-TCG ACA GGC AAT GGC AGT AG-3' and antisense, 5'-TCT TGG AGG AGC CAC CAC TAC CC-3'; and β-actin (350 bp) sense, 5'-AGG CCA ACC GGC AGA AGA TGA CC C-3' and antisense, 5'-GAA GTC CAG GGC GAC GTA GCA C-3'. β-Actin was used to control for RNA quality. The amplification reaction was performed in a thermal cycler (PTC-100; MJ Research, Watertown, MA). The conditions consisted of an initial denaturation for 5 minutes at 94°C, followed by 30 cycles (25 cycles for AQP5) of denaturation for 30 seconds at 94°C, amplification for 1 minute at 58°C (at 55°C for AQP5), and extension for 1 minute at 72°C. A final extension was performed for 10 minutes at 72°C. The PCR products were analyzed by electrophoresis on a 1% agarose gel and viewed using ethidium bromide staining.

RESULTS

Light Microscopic Analysis of Corneas after 20% Ethanol-Detached Epithelial Flap Repositioning and Mechanical Scraping

The cornea is composed of the epithelium, stroma with resident keratocytes, and endothelium. The normal corneal epithelium has five to seven cell layers, including the superficial squamous layer, the wing layer, and a single basal layer of columnar cells. The corneal stroma forms approximately 90% of the cornea (Fig. 2A).

After ethanol-detached epithelial flap repositioning, damaged corneal basal cells were observed, and superficial cells were observed peeling off gradually through day 1. The epithelial cell nuclei were vacuolated. At day 3, damaged basal cells and peeling superficial cells were not observed, and swollen basal epithelial cells and death of stromal keratocytes were evident. At day 7, epithelial and stromal morphology were normal (Figs. 2B–H).

After mechanical scraping, corneal epithelial cells proliferated and migrated over the denuded stroma. Basal epithelial cells were swollen by day 1 and a stratified epithelium, five to seven layers thick, covered the stroma by day 3 (Figs. 2I–K).

In the corneas that were exposed only to 20% ethanol, no significant changes were seen (Figs. 2L, 2M).

Death of Epithelial Cells and Stromal Keratocytes

After ethanol-detached flap repositioning, TUNEL-positive cells were detected in the superficial cells and the wound-edge epithelial cells by 4 hours and in the central corneal basal epithelial cells by day 1 (Figs. 3A–D). After mechanical scraping, TUNEL-positive cells were detected in the central anterior stromal keratocytes and in the migratory edge of epithelial cells at 4 hours, but were not detected at day 1 (Figs. 3E–H).

The number of central stromal keratocytes during the wound-healing period after each surgery decreased maximally (5.65 ± 1.67 cells) at day 1 after ethanol-detached epithelial flap repositioning and at 8 hours (3.27 ± 1.87 cells) after mechanical scraping. There were fewer corneal keratocytes after mechanical scraping than after ethanol-detached flap repositioning (P < 0.05; Fig. 4).

Proliferation of Epithelial Cells

PCNA staining was performed to identify the proliferation of epithelial cells. After ethanol-detached epithelial flap repositioning, proliferation peaked in the central cornea at day 3 and in the peripheral cornea at day 1, continuing in the peripheral cornea until day 7 (Figs. 5A–J). After mechanical scraping, cell proliferation peaked in the peripheral cornea at 12 hours. In the unwounded cornea, PCNA-positive cells were rarely detected in basal cells, and PCNA-positive cells were not detected in the negative control (Figs. 5K–T).

After each surgery, mRNA levels of MMP-9, which is synthesized and secreted by basal corneal epithelial cells migrating to resurface a wound, were examined using RT-PCR. MMP-9 mRNA levels in the corneas after ethanol-detached flap repositioning peaked at days 1 to 3, and in corneas subjected to mechanical scraping, they were widely expressed from 4 hours to 3 days (Fig. 6).

Attachment of Corneal Epithelial Flap after Each Surgery

One day after both types of surgery, the basal lamina was not well defined, there was no space between the basal lamina and
the basal epithelial cell layer, and there were no hemidesmosomes present in the cell membranes of basal epithelial cells (Figs. 7A, 7C). In the basal epithelial layer of flap after ethanol-detached flap repositioning, surviving basal epithelial cells had migrated to the position of dead cells at day 1 (Fig. 7A, inset). At day 3, the basal lamina was well defined, and hemidesmosomes were present (Figs. 7B, 7D). In ethanol-only-treated corneas, the basal lamina was well defined, and hemidesmosomes were detected at all time points (Figs. 7E, 7F).

One day after transplantation, the SRY gene was detected in the female recipient corneal epithelium after attachment of the donor ethanol-detached male epithelial flap, but it was not detected at day 3 (Fig. 8).

**DISCUSSION**

Surgery for the correction of refractive errors has been performed for many years, and its use accelerated with the development of laser-assisted in situ keratomileusis (LASEK). However, surgical outcomes and potential complications associated with this technique have raised concerns about the safety and efficacy of this procedure. Our study aimed to investigate the functional capacity of corneal epithelium after ethanol detachment and to assess the potential for using this technique in clinical practice.

**Functional Capacity of Corneal Epithelium after Ethanol Detachment**

The mRNA levels of AQP5 and MUC1, which are related to normal corneal epithelial function, were unchanged in both epithelial cells in the flap after ethanol-exposed flap repositioning and regenerated corneal epithelial cells after mechanical scraping (Fig. 9).

**Surgery**

Surgery for the correction of refractive errors has been performed for many years, and its use accelerated with the development of laser-assisted in situ keratomileusis (LASEK). However, surgical outcomes and potential complications associated with this technique have raised concerns about the safety and efficacy of this procedure. Our study aimed to investigate the functional capacity of corneal epithelium after ethanol detachment and to assess the potential for using this technique in clinical practice.

**Figure 3.** Apoptosis of epithelial cells (A–J) and stromal keratocytes (K). After ethanol-detached flap repositioning, TUNEL-positive cells were detected in the superficial epithelial cell (A) and the edge (B) at 4 hours and in basal epithelial cells at day 1 (C, D). After mechanical scraping, TUNEL-positive cells were detected in the central anterior stromal keratocytes (E) and in the migratory epithelial cells of the edge (F) at 4 hours and are not detected at day 1 (G, H). Similar results were observed in at least three other corneas. Arrows: edges of the flaps. Bar, 50 μm.

**Figure 4.** The number of central stromal keratocytes. During wound healing, anterior keratocytes decreased maximally at day 1 after ethanol-detached epithelial flap repositioning and at 8 hours after mechanical scraping. The number of keratocytes at 8 hours after scraping decreased more than that of keratocytes at day 1 after flap repositioning. *P < 0.05.

**Figure 5.** Proliferation of epithelial cells. After ethanol-detached flap repositioning (A–J), cell proliferation peaked in the central cornea at day 3 and in the peripheral cornea at day 1. Proliferation continued in the peripheral cornea until day 7. After mechanical scraping, cell proliferation peaked in the peripheral cornea at 12 hours. Similar results were observed in at least three other corneas. Arrows: edges of the flaps. Bar, 50 μm.

**Figure 6.** Expression of MMP-9 mRNA in corneal epithelial cells. In the corneas after ethanol-mediated flap repositioning, MMP-9 mRNA levels peaked between days 1 to 3, and after mechanical scraping, MMP-9 was widely expressed in the corneas from 4 hours to day 3. The presented data are from one of four independent experiments with similar results.
opment of the excimer laser. However, one problem with the study of LASEK has been the lack of a suitable experimental animal model in which to create reproducible epithelial flaps. Many studies have therefore evaluated the toxic effects of dilute ethanol on the corneal surface. These have demonstrated that treatment with 20% ethanol for less than 1 minute results in effective epithelial flap creation with decreased cell damage. Lee et al. have developed an animal model for LASEK in the eyes of White Leghorn chicks (which have a Bowman's layer similar to that of humans). The animal used in the current study of LASEK has been the lack of a suitable experimental animal model with similar results.

FIGURE 7. Transmission electron micrographs of rat central corneal basement membrane. These micrographs demonstrate results at day 1 (A) and day 3 (B) after ethanol-mediated flap repositioning, day 1 (C) and day 3 (D) after mechanical scraping, and 0 hours (E) and 4 hours (F) after 20% ethanol treatment for 30 seconds. After both types of surgery, the basal lamina was not well defined: There was no space between the basal lamina and the basal epithelial layer and no hemidesmosomes in the cell membrane of the basal epithelial cell at day 1 (A, C). In the basal epithelial layer of the ethanol-mediated flap, surviving basal epithelial cells were seen migrating to the position of dead cells at day 1 (A, inset). At day 3, the basal lamina is defined well, and hemidesmosomes are seen at all time points (E, F). Similar results were observed in at least three other corneas. Arrows: hemidesmosomes. Bar, 1 μm.

FIGURE 8. Detection of SRY gene in female corneal epithelium after receiving an ethanol-mediated male epithelial flap. The SRY gene was detected in female corneal epithelial cells at day 1 after transplantation but was not detected at day 3.

FIGURE 9. Expression of AQP-5 and MUC1. There were no changes in the mRNA levels of AQP5 and MUC1 in epithelial cells with ethanol-mediated flap or regenerated corneal epithelial cells after mechanical scraping. The presented data are from one of four independent experiments with similar results.

model was in Sprague-Dawley rats, which have no Bowman’s layer. After LASEK surgery in humans, the anterior stroma including Bowman’s layer is removed with excimer laser ablation, the ethanol-induced epithelial flap is replaced on the stroma, and wound-healing occurs between epithelial flap and stroma. Although the Sprague-Dawley rat eye has no Bowman’s layer, it seems to be a useful model for examining the wound-healing process after LASEK surgery.

Ethanol is frequently used during PRK to facilitate removal of the epithelium, and removal with ethanol is faster to perform and provides sharper edges and a smoother Bowman’s layer surface than mechanical scraping. Dilute ethanol is also used in LASEK to make a corneal epithelial flap effectively. Espona et al. reported that the cleavage plane of the ethanol-induced corneal epithelial flap is located between the lamina lucida and the lamina densa of the basement membrane, where integrin α6 interacts with laminin 5 to form hemidesmosomes. Corneal reepithelialization involves necessary basement membrane remodeling and cell migration. MMP-9 is the primary MMP synthesized and secreted by basal corneal epithelial cells migrating to resurface a wound. The pattern of MMP-9 synthesis is consistent with the timing of basement membrane degradation—a rapid increase in expression within a day of wounding.

The present study compared the wound-healing process between ethanol-mediated flap repositioning and mechanical scraping. After epithelial flap repositioning, damaged basal epithelial cells and detachment of superficial cells were observed until day 1, and the death rate of epithelial cells and loss of stromal keratocytes peaked at day 1. Epithelial cell proliferation peaked at day 5, and MMP-9 mRNA was also expressed from days 1 to 3. By contrast, in the mechanically scraped cornea, the denuded stromal surface was covered with multilayers of epithelial cells at day 3, cell death peaked at 4 hours, cell proliferation peaked from 12 hours to day 1, and MMP-9 mRNA was widely expressed from 12 hours to day 3. Thus, the cornea with ethanol-mediated flap repositioning had a slower wound-healing process and less keratocyte loss than the mechanically scraped cornea.

Furthermore, epithelial cells in the repositioned flap protecting the denuded stromal surface were alive, proliferated, and proceeded to a wound-healing response. Through sex cross-transplantation, we demonstrated that the epithelial cells in the repositioned flap regenerated within 3 days, similar to the renewal time of normal corneal epithelium: 5 to 7 days.

The corneal epithelium forms a barrier between the environment and the stroma of the cornea, and through its interaction with the tear film, forms a smooth refractive surface on the cornea. In this study, AQP5 and MUC1 mRNA expression was used to demonstrate corneal epithelial functions after ethanol-mediated flap repositioning. AQP5 is one of a family of proteins that regulate water permeability and protect against water loss and dehydration.
water channel proteins, selectively localized on the surface of corneal epithelium in the eye. Its mRNA expression is enriched in the cornea and a major role of AQP5 in the corneal epithelium is to prevent dehydration or to secrete watery products and the epithelium can contribute to corneal hydration homeostasis.\(^{15,25}\) In this study, AQP5 and MUC1 mRNA expression levels were unchanged in corneas with ethanol-induced epithelial flap repositioning and in mechanically scraped corneas. Consequently, corneal epithelium may maintain its function after both types of surgery.

In conclusion, extrapolating from the results of this study, it appears that ethanol-mediated epithelial flap repositioning during LASEK induces less keratocyte loss and a slower wound-healing process than mechanical scraping in PRK. The repositioned epithelial flap protecting the underlying stromal surface may have an epithelial function and allow for slow replacement of damaged epithelial cells by peripheral epithelial cells during the wound healing process. In addition, this Sprague-Dawley rat eye model may be useful to study the wound-healing response after LASEK.

References