Changes in Corneal Morphology Associated with Chronic Epithelial Injury

Woo-Jung Kim,1,3 Marco C. Helena,1 Rahul R. Mohan,1,2 and Steven E. Wilson1,2

PURPOSE. The purpose of this study was to evaluate the effect of chronic epithelial scrape injury on corneal morphology.

METHODS. The corneal epithelia in one eye of 8-week-old New Zealand White rabbits were scraped at weekly intervals. Central corneal thickness was measured by ultrasonic pachymetry before epithelial scrape each week. Control never wounded (C), chronic wounded with scrape the last week (W), and chronic wounded without scrape the last week (WW) corneas were processed for histologic analysis and transmission electron microscopy (TEM). The time intervals for histologic analysis were 4 (4 C, 2 W, 2 WW), 8 (4 C, 2 W, 2 WW), and 16 (7 C, 2 W, 5 WW) weeks. Histologic findings were monitored using hematoxylin and eosin staining, the TdT-dUTP terminal nick-end labeling (TUNEL) assay, and TEM.

RESULTS. Chronic wounded corneas developed marked epithelial hyperplasia and a subepithelial acellular zone. Keratocytes undergoing apoptosis were primarily detected adjacent to the acellular zone by TUNEL assay and TEM. Total central corneal thickness measured by ultrasonic pachymetry (n = 7) was significantly thinner in chronically scraped eyes compared with control eyes after 8, 12, and 16 weeks (P < 0.05). Control corneas increased in total thickness over the 16 weeks of the study, but there was no significant change in total thickness of the corneas that had chronic epithelial scrape injury over this time interval. Two scraped corneas had marked decreases in total corneal thickness relative to the corneal thickness at the beginning of the study. Epithelial hyperplasia developed in all scraped corneas examined histologically after 4, 8, or 16 weeks of scraping. When central epithelial thickness measured on hematoxylin and eosin-stained sections was subtracted from the total pachymetric corneal thickness to give approximate stromal thickness, the stromal thickness was 23% lower in the chronic wounded (277 ± 15 μm) compared with the unwounded (356 ± 6 μm) corneas (P = 0.0008) after 16 weeks of wounding.

CONCLUSIONS. Chronic epithelial injury induces stromal thinning and epithelial hyperplasia. These changes in cornea structure associated with chronic epithelial injury may have relevance to the pathophysiology of keratoconus. (Invest Ophtalmol Vis Sci. 1999;40:35-42)

It has been demonstrated that the disappearance of keratocytes after corneal epithelial injury is mediated by programmed cell death (apoptosis).1 The apoptosis process is important in development, wound healing, immune response, and tissue homeostasis. Recent studies have suggested that keratocyte apoptosis triggered by epithelial injury has a role in the response to corneal infections due to viruses such as herpes simplex2 and in initiation of the corneal wound healing cascade that occurs after corneal surgery.3,4 We have also hypothesized that keratocyte apoptosis has a role in maintaining tissue organization (i.e., modulating localization of keratocytes within the stroma) and that an imbalance in the normal homeostatic balance between keratocyte apoptosis and proliferation could underlie the pathogenesis of keratoconus.1,5 Keratoconus is a chronic, noninflammatory, corneal disease in which stromal thinning progresses over a period of decades.6 The pathophysiology of the disease is unknown, but keratoconus has been associated with chronic eye rubbing, poorly fit contact lenses, and atopic diseases.7-11 Each of these factors may result in chronic injury to the corneal epithelium. The purpose of this study was to examine the effect of chronic corneal epithelial scrape injury on keratocyte apoptosis, keratocyte localization, and corneal morphology in the rabbit.

MATERIALS AND METHODS

Chronic Epithelial Scrape Injury

Twenty 8-week-old male New Zealand White rabbits weighing 2.5 kg to 3.0 kg were used in this study. Anesthesia was accomplished by intramuscular injection of ketamine hydrochloride (30 mg/kg) and xylazine hydrochloride (5 mg/kg). One drop of 0.5% proparacaine hydrochloride was instilled...
into each eye immediately before measurement of corneal thickness and epithelial scraping. The corneal epithelium was removed at weekly intervals by scraping in one eye with a No. 64 Beaver blade (Becton-Dickinson, Franklin Lake, NJ). The entire central epithelium extending to basement membrane was removed, sparing approximately 1 mm of epithelium at the limbus. Three drops of ciprofloxacin (Ciloxan; Alcon, Fort Worth, TX) was instilled immediately after epithelial scrape. Tape was applied to close the lid for 2 hours to prevent epithelial thickness measured on hematoxylin and eosin-stained 7-μm sections with a micrometer in a X4()O Optiphot-2 microscope ocular lens (Nikon, Melville, NY) was performed as previously described.15

**Histologic Analysis**

Corneas never wounded (C), those chronically wounded with scrape the last week (W), or those chronically wounded without scrape the last week (WW) were processed for histologic analysis and transmission electron microscopy (TEM). The time intervals were 4 (4 C, 2 W, 2 WW), 8 (4 C, 2 W, 2 WW), and 16 (7 C, 2 W, 5 WW) weeks. Tarsoorrhaphy was performed on each eye for 4 hours whether or not scrape was performed. The animal was killed with an intravenous injection of 100 mg/kg pentobarbital. The tarsorrhaphy was removed, and the corneoscleral rim was excised with surgical scissors and forceps.

Corneoscleral rims were immediately embedded in orinithine carbamoyltransferase (Sakura Fineteck, Torrance, CA) and frozen in liquid nitrogen. Seven-micrometer-thick sections that extended transversely across the central cornea were prepared. Histology was monitored with hematoxylin and eosin staining. To detect fragmentation of DNA associated with apoptosis, peroxidase-based TUNEL assay was performed according to the manufacturer’s instructions (ApopTag assay; Oncor, Gaithersburg, MD).

Both corneas from 4-week-scrape and 8-week-scrape rabbits (one cornea scraped the last week and one not scraped the last week for each time point) were fixed for TEM at 4 hours after the scrape. Two corneas from rabbits that had chronic scrape in one eye for 16 weeks (including the final week) and no scrape in the opposite eye were also fixed for TEM at 4 hours after the scrape. Corneas were fixed for 48 hours in 3% glutaraldehyde and 1% paraformaldehyde. TEM (Jem 1200EX; Joel, New York, NY) was performed as previously described.15

**Measurement of Corneal Thickness**

The corneal thickness in the geometric center of both corneas was measured with an ultrasonic pachymeter (Kerasonix KSP1000 Ultrasonic Pachymeter; OTI Medical Service, Sacramento, CA) before epithelial scraping at weekly intervals. The ultrasonic pachymeter was calibrated according to the manufacturer’s instructions each week before beginning measurements. Five measurements were performed on each cornea, and the average was recorded. Rabbits remaining at 16 weeks were killed, and the stromal thickness calculated in corneas not wounded the final week by subtracting the average central epithelial thickness measured on hematoxylin and eosin-stained 7-μm sections with a micrometer in a X400 Optiphot-2 microscope ocular lens (Nikon, Melville, NY) from the average ultrasonic pachymetric measurement of total corneal thickness for that cornea at week 16. The epithelial thickness was determined from the mean of five measurements performed on separate 400× fields within the central cornea.

**Statistical Analysis**

Variations were expressed as SEM. ANOVA was used to test the significance of differences between chronically scraped and control corneas at 0, 4, 8, 12, and 16 weeks. The difference in calculated stromal thickness at 16 weeks was determined with the Mann-Whitney U test. A probability value <0.05 was considered statistically significant.

**RESULTS**

The epithelium of the scraped corneas normally healed within 4 days. Examinations were performed with the operating microscope at the time of weekly scrape. No opacity or other abnormality of the corneas was noted in the majority of eyes that had a weekly scrape. However, two eyes that had epithelial scrape for 16 weeks developed limbal vessels localized outside of the scrape zone after week 12. The vessels did not progress beyond the limbal region in one of the rabbits with neovascularization, and this animal was included in the 16-week analysis. In the other rabbit, the vessels progressed into the central scrape zone, and data from this rabbit were excluded from the 16-week analysis. Three eyes developed epithelial and stromal edema with limbal injection at 10, 13, and 14 weeks. Pachymetry data from these eyes were included up to the time point at which the cornea became abnormal. One rabbit died of an anesthesia overdose at 1 week and was excluded from the study.

**Corneal Thickness Determined by Pachymetry**

There was a significant increase in the thickness of the control unwounded corneas measured by ultrasonic pachymetry (Table 1) between 0 and 16 weeks (389 ± 3 μm and 412 ± 5 μm, respectively; P = 0.001). There was no significant change in the mean total thickness of the chronically scraped corneas measured with ultrasonic pachymetry between 0 and 16 weeks (387 ± 3 μm and 384 ± 8 μm, respectively; P = 0.5). The corneal thickness of chronically scraped corneas measured with ultrasonic pachymetry (Table 1) was significantly thinner.
FIGURE 1. Hematoxylin and eosin-stained central sections of chronically scraped corneas after 4 (A and B are adjacent fields of the same cornea), 8 (C), or 16 (D) weeks of chronic epithelial scrape. Epithelial hyperplasia (compared with unwounded control corneas) was present in all corneas examined with 4 to 16 weeks of chronic epithelial scrape at weekly intervals. Each cornea with 4, 8, or 16 weeks of chronic epithelial scrape injury also developed a subepithelial zone with decreased keratocyte density (above the arrows in each panel). Magnification, ×120.

than control corneas at 8 weeks (379 ± 4 µm and 396 ± 4 µm; respectively; P = 0.01), 12 weeks (392 ± 6 µm and 422 ± 4 µm, respectively; P = 0.001), and 16 weeks (384 ± 8 µm and 412 ± 9 µm, respectively; P = 0.01). Two chronically scraped corneas had marked changes in total corneal thickness, although they appeared normal on gross visual inspection. In one of these animals that was killed at 8 weeks, the corneal thickness measured by ultrasonic pachymetry in the cornea that had chronic scrape had decreased from 398 µm at the beginning of the study to 357 µm at week 8, a decrease of 41 µm. The contralateral control eye changed from 394 µm to 372 µm, a decrease of only 22 µm. In the other animal that was killed at 16 weeks, the corneal thickness in the cornea that had chronic scrapes decreased from 376 µm at the beginning of the study to 345 µm at week 16, a decrease of 31 µm. The contralateral control eye changed from 374 µm to 404 µm, an increase of 30 µm.

Histologic Findings

Epithelial hyperplasia developed in the central cornea of eyes that were scraped at weekly intervals. All eyes examined after 4, 8, or 16 weeks of epithelial scrape injury had epithelial hyperplasia (Figs. 1, 2). Epithelial hyperplasia (compared with unwounded control corneas) was prominent after 16 weeks of scrape injury (Fig. 2). The average thickness of the central epithelium was approximately two times thicker in the wounded corneas than in the unwounded control corneas at 16 weeks (epithelial thickness in chronically scraped corneas: 113, 91, 104, and 98 µm, mean 101 ± 5 µm; epithelial thickness in control corneas:
Figure 2. Hematoxylin and eosin-stained central sections of chronically scraped (A, C, E, G) and contralateral control (B, D, F, H) corneas after 16 weeks of scrape injury. Note that epithelial hyperplasia and a thick subepithelial acellular zone (arrowheads) are present in each of the chronically scraped corneas. Note the relative uniformity of epithelial thickness between the different control corneas (B, D, F, H). Arrow in (A) indicates a keratinocyte cell that was present within the acellular zone in a chronically scraped cornea. Magnification, X100.

Histologic data were available from four rabbits after 15 weeks of chronic scrape with no scrape on week 16, with the other eye serving as an unwounded control. There was a 23% difference in calculated stromal thickness (total corneal thickness measured with ultrasonic pachymetry — epithelial thickness measured on hematoxylin and eosin-stained sections) between the eyes that had been chronically scraped and eyes that were controls (273 ± 12 μm and 356 ± 6 μm, respectively; P = 0.0008).

TUNEL-positive keratocytes were noted in eyes that had chronic scraping at each time of examination. The location of TUNEL-stained keratocytes was limited to the anterior half of the stroma at each time point. TUNEL-positive keratocytes were typically found in a linear pattern below the anterior acellular zone (Fig. 3A) but were occasionally detected within the acellular zone (Fig. 3B). There tended to be a decrease in the number of TUNEL-positive keratocytes detected as more weeks of chronic epithelial scrape were performed (Fig. 3).

TEM (Fig. 4) showed disorganized collagen fibers of different sizes in anterior stromal acellular zone. TUNEL-positive cells underlying the acellular zone had morphologic changes consistent with apoptosis (Fig. 4), including cell shrinkage, chromatin condensation, chromatin fragmentation, and blebbing.

Discussion

The results of this study demonstrate that weekly scrape injury to the corneal epithelium causes changes in corneal morphology and epithelial thickness in rabbits. Interestingly, the difference in mean total corneal thickness measured by ultrasonic pachymetry between wounded and control corneas was for the most part attributable to an increase in total thickness of unwounded control corneas over time. In contrast, the mean total corneal thickness in the chronically wounded corneas remained unchanged over 16 weeks. Two chronically scraped corneas, however, had marked decreases in total thickness beginning at 8 weeks. The increase in thickness in the control corneas over time may be attributable to normal growth of the rabbit corneas with age. It is also possible that chronic loss of anterior keratocytes through apoptosis due to chronic epithelial injury leads to a decrease in synthesis and deposition of extracellular matrix components that are needed if the
FIGURE 3. TdT-dUTP terminal nick-end labeling (TUNEL) assay to detect DNA fragmentation characteristic of apoptosis. Stained keratocytes (arrows) with fragmented DNA were typically found deeper in the stroma after weekly scrape injury than in corneas that were acutely wounded for the first time, which always have keratocytes with fragmented DNA in the most anterior stroma. Arrowheads indicate the stromal surface. Shown are corneas after 4 (A, B), 8 (C), and 16 (D) weeks of weekly epithelial scrape injury. Note that fewer cells that stain with the TUNEL assay are detected at later time points, probably because there has been insufficient time to allow for full recovery of keratocyte density with weekly epithelial injury and associated keratocyte apoptosis. Few keratocytes that stain are noted in the acellular zone that develops in this chronic scrape model (Figs. 2 and 3). Magnification, ×400.

Ultrasonic pachymetry measures the combined thickness of the corneal epithelium, stroma, and endothelium. The marked change in epithelial thickness in chronically wounded corneas confounds interpretation of the ultrasonic pachymetric measurements. Unfortunately, consistent measurements of stromal thickness cannot be made directly on the basis of histologic sections because the stromal thickness varies from section to section cut with a keratome, even with consecutive sections from the same specimen (SE Wilson, unpublished data, 1997). This is attributable to the lamellar structure of the stroma and its tendency to be compressed or stretched during

Downloaded From: https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933210/ on 11/14/2018
FIGURE 4. Transmission electron microscopy of the stroma after chronic scraping of the corneal epithelium. (A, B) Cornneas after 16 weeks of chronic scraping, with the final scrape 4 hours before euthanatizing the animal, removing the eye, and fixing for electron microscopy. Chromatin condensation (c) consistent with apoptosis is noted in a keratocyte at approximately 100 μm in depth in (A). (B) shows a keratocyte with more normal-appearing chromatin (n), although there is a hint of condensation, and marked rough endoplasmic reticulum (small arrows) is present. A normal-appearing keratocyte with marked rough endoplasmic reticulum is also present in the middle of (A). Throughout both fields there are numerous small structures (large arrows) that are likely apoptotic bodies from keratocytes that have previously undergone apoptosis in response to ongoing epithelial injury. Some of these bodies contain visible cellular organelles. (C, D) Cornneas after 15 weeks of chronic scrape with no scrape the 16th week. Again, a keratocyte with chromatin condensation (c) suggestive of early apoptosis is detected in (C), even though the last scrape occurred 1 week earlier. A keratocyte with normal-appearing chromatin (n) is seen in (D). Note that numerous small structures (arrows) that are likely apoptotic bodies are also detected in (C) and (D). (E) A cornea that never had epithelial scrape shows a normal keratocyte (arrowhead) and no membrane-bound structures in the surrounding stroma. Note the regular organization of the collagen lamellae (arrows). (F) shows the anterior stroma in a cornea that had epithelial scrape for 15 weeks with no scrape the 16th week. A keratocyte that appears to have undergone apoptosis and only consists of a large number of membrane-bound structures (apoptotic bodies) is shown (arrowheads). The collagen lamellae (arrows) between the epithelium and the dead keratocyte cell are irregular and poorly organized compared with those shown in the unwounded cornea in (E). Magnification, (A, B, C, D) ×4000; (E, F) ×10,000.
HGF is also produced by the lacrimal gland and released in the tear film. Tear HGF bioavailability increases after corneal injury. Therefore, it is also possible that increased tear HGF from the lacrimal gland contributed to the corneal epithelial hyperplasia. Epidermal growth factor and transforming growth factor-α released by epithelial cells and keratocytes could also promote epithelial hyperplasia noted in this model.23,25

Keratoconus is an ectatic corneal dystrophy in humans associated with slow thinning of the corneal stroma occurring over a period of years or decades. Keratoconus has been associated with chronic eye rubbing,7 poorly fit contact lenses8,9 and atopy.10,11 Each of these conditions would also be associated with chronic injury to the corneal epithelium, albeit at a much lower level than that associated with scrape removal of the epithelium at weekly intervals in this rabbit model. The stromal thinning we observed in this rabbit model provides evidence that there could be an association between chronic epithelial injury and stromal thinning in humans. Factors other than direct epithelial injury could also have a role. For example, Pouliquen and coworkers,26,27 have demonstrated that keratocytes of keratoconus patients express 400% more interleukin-1 receptors than normal keratocytes. Because previous studies have indicated that interleukin-1 can directly or indirectly trigger corneal fibroblast apoptosis, these observations could suggest a genetic susceptibility of keratocytes to the cytokines released from the injured epithelium in keratoconus patients. Interestingly, we have recently noted that keratocytes with fragmented DNA suggestive of apoptosis are frequently detectable in the stroma of corneas removed at the time of corneal transplantation for keratoconus.5 Apoptosis is usually thought of as a gentle controlled process used to eliminate cells with minimal release of degradative enzymes and other components that may damage the surrounding cells and tissues.28,29 It is possible, however, that the apoptosis process itself could be imperfect and that chronic apoptotic cell death might lead to an abnormal release of degradative enzymes or other biochemical alterations noted by previous investigators.30-42 Clearly, the pathophysiology of keratoconus is complex. Although ongoing epithelial injury may be an important inciting event in this disease, a cascade of factors may ultimately be involved in producing the typical pathologic changes.

References


