Role of Tears in Keratocyte Loss after Epithelial Removal in Mouse Cornea

Jin Zhao, Takayuki Nagasaki, and David M. Maurice

PURPOSE. To study the role of tears in the death of keratocytes after epithelium removal in the mouse cornea.

METHODS. In anesthetized mice, an approximately 1-mm circle of epithelium was removed from the center of the cornea, exposing the underlying stroma. In one group of animals, access of tears to the bare stroma was allowed—in vivo, by closing the eyelids, or ex vivo, by dropping tears from another animal onto the denuded stroma of an enucleated eyeball. In another group, tear access was denied—in vivo, by bathing the cornea continuously in saline or by keeping the lids open, or ex vivo, by rinsing the denuded cornea before incubating the enucleated eyeball. In a separate group, corneal epithelial debris from another mouse was placed on the bare stroma of an enucleated eyeball. The corneas were isolated, stained with a fluorescent nuclear dye, and observed en face in a wholemount preparation under a fluorescence microscope, to evaluate the distribution of intact nuclei across the entire depth of the stroma.

RESULTS. Between 1.5 and 2 hours after exposure to tears, the nuclei of the anterior keratocytes under the area of epithelial debridement invariably degenerated. When they had been protected from the tears, however, no degeneration was observed. Epithelial debris applied on the bare stroma had no effect on the underlying keratocytes.


M echanical debridement of the corneal epithelium is common in the clinical management of various corneal conditions, such as recurrent epithelial erosions, epithelial herpes, intraoperative epithelial clouding, and photorefractive keratectomy. Underlying keratocyte loss within hours of this procedure has long been recognized by many investigators, and attempts have been made to explain this phenomenon and reverse its outcome. It has been suggested that factors released from epithelial cells after injury may be responsible for the initiation of cell death, and IL-1 and the Fas-Fas ligand system have been implicated in the process, which has been attributed to apoptosis.

However, keratocyte death or survival may be determined by chemical factors from the tears or leukocytes as well as from the injured epithelium, and it could be influenced by physical factors, such as corneal temperature and hydration or the mechanical consequences of blinking. How much these factors control the fate of the keratocytes could very well depend on how the cornea is treated after the epithelium is debrided, but this has been poorly documented in most investigations. Accordingly, in the context of a wider investigation into the reaction of corneal cells to injury, we performed a better controlled study into the mechanism of the death of underlying keratocytes after epithelial removal.

METHODS

In Vivo Studies

Animal studies adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the institutional animal care and use committee. Adult mice of the outbred strain ICR (Taconic Farms, Germantown, NY), were anesthetized by an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Tetracaine (0.5%) was instilled in one eye, which was then propided, and the cornea was rinsed with saline and the surface blotted to remove the tear film. The epithelium at the center of the cornea was removed within 30 seconds by repeated touching of the corneal surface with a dry glass slide coated with 10% gelatin. This created a roughly circular area of bare stroma with a diameter of 0.8 to 1.2 mm.

Tears. Pilot studies indicated that the presence of tears could be a major factor in determining cell death. Accordingly, the following four experiments were performed, in which the access of tears to the bare stroma was either allowed or denied after the epithelium was removed. Tear access was prevented as follows: (1) The corneal surface was continuously bathed in buffered saline at 37°C for 1 to 4 hours. The anesthetized mouse was placed on its side above a bath so that only the treated cornea was submerged. The head was supported with the nose raised, to prevent fluid inhalation. (2) The eye was kept open continuously. The anesthetized mouse does not blink spontaneously, and the eye naturally assumes a slightly protruded position, with the lid margins lying behind the cornea. This provided a simple situation in which contact of tears with the bare stroma in the debrided central area of the cornea was prevented. It is probable, however, that the cornea would become cold and would dry by evaporation if it remained out of contact with the conjunctiva. To avoid this, a humidifier was constructed (Fig. 1) that passed saturated air at 31°C to 35°C over the surface of the eye. The absence of any change in corneal gloss, as noted by gross observation, and the nonapparance of the cataractifier was constructed (Fig. 1) that passed saturated air at 31°C to 35°C over the surface of the eye. The absence of any change in corneal gloss, as noted by gross observation, and the nonappearance of the cataract were attributed to evaporation.

(3) The eyelids were closed with tape or sutures. (4) The eye was treated exactly as in (2), except that the eyelids were manually closed once every 15 minutes to gently bring the conjunctival surface into contact with the bare stroma. During all the procedures, anesthesia was maintained by periodic intraperitoneal injection of ketamine (100 mg/kg) and acepromazine (2.5 mg/kg), and the animal was warmed to maintain its normal body temperature, which was monitored by a thermocouple in its fur.

Cell Counting. Mice were killed by an intraperitoneal injection of pentobarbital at various times between 0 and 4 hours after the
DAPI staining. An uninjured cornea was fixed, and the epithelium was removed, stained with DAPI, and mounted whole. Microscopic images of the same region at three different focal planes are shown: (A) immediately below the epithelial surface, (B) half way through the stroma, (C) at the endothelium.

FIGURE 2. Distribution of nuclei in normal mouse cornea shown by DAPI staining. An uninjured cornea was fixed, and the epithelium was removed, stained with DAPI, and mounted whole. Microscopic images of the same region at three different focal planes are shown: (A) immediately below the epithelial surface, (B) half way through the stroma, (C) at the endothelium.

The opportunity was taken in the in vivo experiments to observe whether leukocytes, primarily polymorphonuclear neutrophils, were attracted into the debrided area, because they could be a source of cytotoxic factors. Their presence was monitored in DAPI-stained specimens by identifying their horseshoe-shaped nuclei, which can be easily distinguished from the roughly oval keratocyte nuclei.

Ex Vivo Studies

To eliminate uncontrolled systemic and local factors during the in vivo experiments, enucleated eyeballs were tested for the effect of short-term exposure to various conditions that might cause keratocyte death after epithelium debridement.

Immediately after an untouched mouse was euthanatized, both corneas underwent epithelial removal by lifting with gelatin-coated slides. Then, the eyes were rinsed with saline and enucleated, and each was mounted in a 35-mm petri dish with the cornea up. The sclera was in contact with a filter paper soaked in phosphate-buffered saline that covered the bottom of the dish, and the inner surface of the lid of the petri dish was wetted in the same way. The dishes were then kept in a humidified incubator at 37°C for 0.5 to 4 hours before the eyeball was fixed.

Tears. Tears were collected from one or both untouched eyes of a mouse under general, but no topical, anesthesia at approximately 2-minute intervals over 10 to 15 minutes. The end of a 1-μl micropipette (outer diameter 0.66 mm, inner diameter 0.20 mm; Drummond Scientific, Broomall, PA), which was flamed to make the surface smooth, was gently brought into contact with the surface of the inferior-temporal conjunctival sac, and the tears were taken up by capillarity until 0.2 to 0.5 μl was accumulated. Care was taken to minimize rubbing the surface cells during the tear collection. The tears were immediately delivered onto the freshly denuded cornea of an isolated eyeball from a different mouse, which was then incubated for 0.5 to 4 hours. The tears were viscous and ran slowly off the denuded area.

FIGURE 1. Device for generating a flow of warm humid air to maintain normal corneal hydration and temperature in situ. Air, heated by passing through a metal coil submerged in the water reservoir, bubbles through the reservoir to pick up water vapor, and is carried by way of an insulated copper tube toward the corneal surface, which is approximately 2 cm from the end of the tube. The light bulb heats the reservoir. The temperature of the air impinging on the cornea is monitored with a thermocouple and maintained at 31°C to 35°C by controlling the current through the bulb.

injury. The eyeballs were enucleated by evulsion and fixed either by formaldehyde (4% in phosphate-buffered saline) or by ethanol (70% in water or in 10% acetic acid). The whole cornea was dissected from the experimental eye and stained with 1 μM 4′,6-diamidino-2-phenylindole (DAPI) in H2O for 10 minutes. Radial cuts were made in the cornea so that it could be flattened by a coverslip, and it was mounted in glycerol (50% in phosphate-buffered saline with 0.1% n-propylgalacte) and examined en face under a fluorescence microscope (Axioskop2; Carl Zeiss, Oberkochen, Germany).

Routinely, images were recorded from the injured area with a digital camera (Orca; Hamamatsu, Hamamatsu City, Japan) at 5-μm intervals through the entire thickness of the cornea. Control images of the nearby uninjured area were taken after the overlying epithelium was removed. The digital images were processed with an image-processing program (MetaMorph; Universal Imaging Co., West Chester, PA) for analysis and documentation. The depth of keratocyte loss was expressed as a percentage of the entire thickness of the stroma occupied by the acellular zone. In some specimens, sharply focused cells within a 250 × 200-μm field of a single focal plane at one third the depth of the stroma were counted manually from the microscope image displayed on a computer monitor. To assess the counting error, normal areas from 10 corneas were chosen where fields at the same level had been counted on two different occasions. The root mean square of the difference between each pair of readings was 4.4 cells. In a few cases, the remaining epithelium near the wound margin was removed, and a cross-sectional view of the keratocytes in this area was constructed from serial optical sections recorded at intervals of 1 μm.

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**Figure 3.** Loss of keratocyte nuclei under the debrided area. The central epithelium was removed, and the lids were kept closed for 2 hours before enucleation, fixation, and staining. (A) A low-power image of the area of epithelial removal. The remaining epithelium is seen outside the bright ring of stained cells. Inside of this is a band of intact keratocyte nuclei. In the central zone the keratocyte nuclei have almost completely disappeared, allowing the endothelial nuclei to be seen. **Inset:** the entire cornea with the radial cuts that allowed flat mounting. (B) A cross-sectional view of the stromal nuclear distribution across the elongated box delineated in (C) and (D). The remaining epithelium was removed after photographing the image in (A), to avoid interfering fluorescence, and a cross-sectional view was constructed digitally from serial optical sections recorded at 1-μm intervals. Despite the out-of-focus haze, the distribution of the individual nuclei can be clearly discerned in the stroma and endothelium. Focal planes in (C) and (D) are indicated by the white arrows at the right. (C) A high-power image of the rectangular area in (A), in a plane immediately below the stromal surface, after removal of the remaining epithelium. The margin of the original epithelial injury is shown by the dotted line. Box: area of the cross-section view in (B). (D) The same as (C), but in a plane immediately above the endothelium, where keratocytes are uniformly distributed across the stroma. Out-of-focus endothelial nuclei can be seen in the background.

**Epithelium.** Two different experiments were performed to assess the cytotoxic potential of the epithelial cells of the cornea and conjunctiva that have an opportunity to release factors to the bare stroma. The first was intended to simulate the release of factors across the entire thickness of the epithelium from the ring of injured cells that surrounded the debrided zone. The central 2 mm of corneal epithelium was scraped from both eyes of a separate donor with a number 12 blade and applied to the cornea of an enucleated eye, to cover the entire thickness of the epithelium from the ring of injured cells that allowed flat mounting.

The second experiment tested the effect of the superficial cells that are normally desquamated into the tear film from the entire surface of the cornea and conjunctiva. After the conjunctival sac of a euthanized mouse was extensively rinsed with saline, the cornea and the conjunctiva were separately dissected, blotted, and spread out with the epithelial side up. They were then wetted with 10 ml phosphate-buffered saline and gently rubbed with a blunt spatula to harvest the superficial cells from each tissue. The cell density in an aliquot of the suspension was determined by Giemsa staining, and the remainder was assayed on an enucleated eye. For comparison, the cell density was determined in tears collected from the untouched mouse eyes, as already described.

**RESULTS**

The general appearance of the DAPI-stained nuclei of normal cells in the wholemount mouse corneal stroma is shown in Figure 2. Keratocyte nuclei have a somewhat irregular oval shape, with four to eight nucleoli.

**In Vivo Studies**

**Closed Eye.** In one group of experiments, the lids were kept closed after the removal of the epithelium, and the appearance of the stained stromal nuclei in the debrided zone was observed after various intervals of time. Essentially, no change was detected up to 1 hour after the injury, in seven corneas. At 1.5 hours, the nuclei of the keratocytes in the anterior layers of the debrided zone were found to be less brightly stained than those in the normal surrounding area, in three corneas. At 2 hours, all five eyes examined showed an almost complete disappearance of the nuclei in the anterior 30% or more of the stroma (Fig. 3). In three of these, the acellular zone extended across the anterior 80%. No further changes appeared to have occurred up to 4 hours in another 14 eyes. A quantitative measurement of randomly selected corneal sections showed that the total number of stromal cells in a plane at 30% of the stromal thickness remained unchanged for approximately 1.5 hours after epithelial removal, but decreased sharply thereafter (Fig. 4).

The zone of keratocyte loss was almost always slightly smaller than that of the bare stroma (Fig. 3C), and this was also revealed in the cross-sectional views of the stroma that were digitally constructed from the serial optical sections (Fig. 3B). Occasionally, the epithelium was not completely removed by lifting with the gelatin-coated slide, and islands of apparently intact basal epithelial cells remained inside the debrided zone (Fig. 5A). Directly beneath these islands, many keratocyte nuclei seem to have survived, even after the eyelids were closed for 2 hours (Fig. 5B).

**Fluid Immersion.** When the cornea was continuously immersed in a saline bath at 37°C in vivo for up to 4 hours after debridement, there was no sign of keratocyte loss in the bare area of the stroma in all four corneas examined at 2 to 4 hours (Fig. 4).

**Open Eye.** When, under controlled humidity and temperature, an eye was kept open for 2 to 4 hours, thus denying tear access to the cornea, no loss of keratocytes was detected in all five eyes examined (Fig. 4). Under the same conditions, except that the eyelids were briefly closed once in every 15 minutes to provide tears access to the
cornea, the keratocyte nuclei beneath the debrided area disappeared in all three corneas that were examined at 2.5 hours or longer after the injury (Fig. 4). One cornea that was fixed at 2 hours did not show a loss of stromal nuclei.

Leukocytes. In eyes when the lids were closed, there was no detectable leukocyte infiltration into the stroma at 2 to 4 hours after the epithelial debridement in 15 of 19 corneas, although there was always obvious keratocyte loss. In the other four, all at 4 hours, a few horseshoe-shaped nuclei corresponding to leukocytes could be identified, primarily at the outer edge of the injury margin in the anterior part of the stroma (not shown).

Ex Vivo Studies

Tears. When isolated eyeballs with central epithelial debridement were rinsed with saline and incubated in a humidified chamber, no keratocyte loss beneath the debrided zone was evident after 2 to 4 hours, in all 11 corneas tested (Figs. 6A, 7). When freshly collected tears from a donor mouse were placed on the surface of the bare stroma at the beginning of the incubation, keratocyte nuclei under the injury disappeared through almost the entire depth of the stroma by 2 to 4 hours in all seven experiments (Figs. 6B, 7). In two specimens, fixed after 0.5- and 1-hour incubations with the tears, many nuclei in the anterior to middle stroma were found to be only faintly stained, probably a sign of an early phase of cell disintegration (not shown). The cell disappearance zone was slightly smaller than the actual debridement area (Figs. 6B, 6C), as was the case in the in vivo cornea with closed eyelids (Fig. 5).

Epithelium. When freshly collected epithelial debris from a donor mouse was placed on the debrided area of an isolated eye, there was no change in the underlying cells after 2 to 4 hours of incubation, in all five eyes tested. The distribution and appearance of the keratocyte nuclei were identical with those under the untouched epithelium or in the bare area of denuded stroma that received no treatment other than its original saline wash (Fig. 7).

Similarly, no loss of keratocytes was observed when suspensions of the superficial epithelial cells of cornea or conjunctiva, collected by gentle rubbing, were incubated on debrided eyes. The concentrations of cells in these suspensions were much higher than those of desquamated cells in normal tears (Table 1), which suggests that these are unlikely to have any cytotoxic effect.

DISCUSSION

Tears

In our experiments, as summarized in Table 2, the most obvious difference between the conditions that cause or prevent keratocyte loss was the presence or absence of tears. It can be presumed that factors in the tears that are normally excluded from the stroma by the epithelial barrier are allowed access when it is removed. Procedures designed to promote this access resulted in the destruction of the keratocyte nuclei, whereas those designed to deny it left the nuclei unchanged. The former included closing the lids, occasional forced blinking while the eye was kept open, and placing tears on the cornea of the incubated globe. The latter included bathing the cornea in saline, avoiding lid...
contact while the eye was kept open and incubating the rinsed enucleated globe. It should be emphasized that nearly all the phenomena observed were unambiguously positive or negative. The results suggest, therefore, that tears are the major factor in the induction of keratocyte loss after de-epithelialization in the mouse cornea. We believe that our procedure for collecting tears was sufficiently gentle to prevent contamination with materials associated with surface cell injuries, and therefore our tear specimens corresponded to the fluid normally present in the untouched eye.

Keratocyte loss after epithelial debridement has been studied by many investigators, and some of the results are in accordance with the active role of tears. In one study in which the corneal epithelium was scraped from a rabbit eye under a physiological solution, the cornea was washed with the solution at 15-minute intervals for 16 hours, and the keratocytes were found to survive.4 In others, a collagen shield soaked in corneal preservative medium was demonstrated to reduce stroma cell loss, and topical application of culture medium alone helped preserve anterior stromal keratocytes after epithelial removal.12 It is possible that the loss of keratocytes around an incisional wound, that was described many years ago,13 may also be caused by the entry of tears into the cut.

On the contrary, Van Mellaert et al.14 reported that tears were not important in keratocyte death. In denuded areas of rabbit stroma that were covered with epithelial transplants from another animal, the keratocytes disappeared as in the uncovered control animals. These experiments have been presented only in an abstract, and it is difficult to assess the efficiency of the epithelial–stromal seal, among other details. Another finding contradictory to our conclusion was reported by Helena et al.15 who demonstrated that in the rabbit the exposure of stroma to the tears after lamellar keratectomy did not cause the death of underlying keratocytes. This may be because of a difference between the rabbit and the mouse, such as the anatomic structure of the cornea or the composition of the tears.

Table 1. Effect of Surface Epithelial Cells on Keratocyte Loss

<table>
<thead>
<tr>
<th>Specimens</th>
<th>Cell Concentration* (cells/µL)</th>
<th>Keratocyte Loss†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal tears</td>
<td>860 (410–1290)</td>
<td>Yes</td>
</tr>
<tr>
<td>Conjunctival surface cells</td>
<td>9900 (5100–18000)</td>
<td>No</td>
</tr>
<tr>
<td>Corneal surface cells</td>
<td>2500 (1470–3530)</td>
<td>No</td>
</tr>
</tbody>
</table>

* Mean (range).
† The specimens were assayed for their ability to trigger keratocyte loss by placing them on the denuded stroma of enucleated eyes and incubating for 2 to 3 hours.
TABLE 2. Summary of Keratocyte Loss at 2 to 4 Hours after Epithelium Removal under Various Postinjury Conditions

<table>
<thead>
<tr>
<th>Postinjury Conditions</th>
<th>Tear Access to Cornea</th>
<th>Corneas with Keratocyte Loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Closed eyelid</td>
<td>Yes</td>
<td>19/19</td>
</tr>
<tr>
<td>Open eye, bathed cornea</td>
<td>No</td>
<td>0/4</td>
</tr>
<tr>
<td>Open eye</td>
<td>No</td>
<td>0/5</td>
</tr>
<tr>
<td>Open eye, blinks</td>
<td>Yes</td>
<td>3/4</td>
</tr>
<tr>
<td>Enucleated eye</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>No</td>
<td>0/11</td>
</tr>
<tr>
<td>Tears</td>
<td>Yes</td>
<td>7/7</td>
</tr>
<tr>
<td>Corneal epithelial debris</td>
<td>No</td>
<td>0/5</td>
</tr>
<tr>
<td>Superficial epithelial cells*</td>
<td>No</td>
<td>0/5</td>
</tr>
</tbody>
</table>

Data are the number of corneas that exhibited substantial stromal cell loss/the number of corneas tested.

* Three specimens from conjunctiva and two from cornea.

Epithelium

A possible role of wounded epithelium in regulating the stromal cells was suggested by Weimar,10 but cell death was not discussed. More recently, it has been shown that the epithelial-stromal interactions can be mediated by apoptotic cytokines, such as IL-1 and soluble Fas ligand, which may be released by corneal epithelial cells in response to injury.6

Accordingly, it has been proposed that apoptotic stimulators released from the injured epithelial cells may be responsible for the death of underlying stromal cells after epithelial debridement.5,17,18

However, our results suggest that injured epithelial cells at the margin of the derinded area are not the source of the cytotoxic effect in the mouse cornea. First, in the absence of tears, there was no loss of keratocytes directly underneath the circle of damaged epithelium. This was demonstrated in the in vivo cornea where the eyelids were kept open, and also in the ex vivo cornea that was incubated at 37°C in the absence of tears. Even when the tears had access to the bare stroma, the margin of the cell death zone was usually several cell lengths within the margin of the epithelial debridement. Second, placing corneal epithelial debris on the bare stroma of an enucleated eye had no evident effect on the underlying keratocytes.

Similarly, the superficial epithelial cells that desquamate into the tear film do not seem to be a cause of keratocyte toxicity, because higher concentrations of cells rubbed off the cornea or conjunctival surfaces showed no such effect.

The reason for the discrepancy between our findings and those of Helena et al.15 is not clear, but it is possible that the mouse and the rabbit do not share the same mechanism of cell death. However, differences in the method of epithelial removal do not seem important, because gentle mechanical scraping with a blunt scalpel, similar to their procedure used in rabbits, gave results identical with gelatin lifting in virtually every case (data not shown).

The presence of leftover epithelial cells in the denuded area was almost always associated with the survival of underlying keratocytes (Fig. 5). This suggests that, apart from its barrier function, the epithelium may be a source of active molecules that protect the keratocytes, or it may serve as a neutralization site for the factors that would trigger keratocyte death.

Leukocytes

Normal human closed-eye tears contain various cytokines, such as IL-8, that would stimulate leukocyte recruitment, and, after a few hours of eye closure, a large number of polymorphonuclear leukocytes can be found in the tears.27 In rabbits, the number of leukocytes in the tears increases steeply 1 to 2 hours after a mild injury to the cornea.20,21 However, most workers have not found leukocyte infiltration into a small wound until at least 24 hours after epithelial debridement1,6,22—much later than the onset of keratocyte degeneration. In our experiments, infiltration of polymorphonuclear leukocytes into an injured zone was not observed in most corneas at 4 hours, even though the keratocyte loss was evident by 2 hours. Thus, leukocytes do not seem to play any direct role in the death of keratocytes after epithelial removal.

Other Mechanisms

Nakayasu1 suggested that the sudden deprivation of glucose may be a factor in stromal cell death after epithelial removal. However, the epithelium is not only a barrier to glucose uptake, but a site of much of its metabolism,23 and debridement should be expected to increase, not reduce, the availability of glucose in the stroma. Osmotic shock has also been suggested,4 but there is no evidence that it harms the cells, and the shock identified is only 60 mOsm/kg, whereas the cornea of the rabbit could be dried rapidly in vivo to one half its normal thickness without evident ill effect.24 An increase in anterior stromal lamellar tension as a result of swelling in the denuded area has also been proposed as the cause of keratocyte death.4 However, keratocyte death has not been reported in other cases in which increased lamellar tension would be expected, such as a partial-depth incision.

Source of the Cytotoxic Factors

Many soluble factors have been identified in human tears that may trigger keratocyte death, such as soluble Fas and Fas ligand,25 TGF-β,20–26 TNF-α,26 and IL-1α.30 In rabbits, the tears demonstrate prostaglandin E–type activity after corneal epithelial debridement.31 Because it is difficult to analyze the mouse tears because of their minuscule volume, it is planned to determine whether the tears from larger animals duplicate the effects described in the present study.

It is not evident from our experiments whether the cytotoxic factors are present in the freshly secreted lacrimal fluid, are released from the accessory glands, or are secreted from the normal surface cells of the cornea and conjunctiva. Superficial epithelial cells normally desquamate from the cornea and the conjunctiva and are present in the tear film of the mouse, but if they are separated mechanically, they do not exert a cytotoxic effect. However, it remains possible that over a long period there is an accumulation in the conjunctival sac of cytotoxic factors that have been released from either desquamated or intact epithelial cells. Further study will clarify the source and the identity of the tear factors that are toxic to the stromal cells.

References


