Conjunctival Epithelium in Healing of Corneal Epithelial Wounds

Shinji Danjo,* Judith Friend,† and Richard A. Thoft†

The mitotic rate and goblet cell content of conjunctival epithelium following total or central corneal epithelial removal using n-heptanol was measured to determine how the conjunctival epithelium responds to injury and whether conjunctiva responds to central corneal epithelial loss. One day following a wound that removed corneal, limbal, and 1–2 mm of bulbar conjunctival epithelium, the mitotic rate of the remaining conjunctival epithelium was ten times normal \( (P < 0.001) \), proving that the conjunctiva responds to injury by cellular proliferation. At 1 and 2 days following a limited 10 mm diameter central corneal wound, the mitotic rate of peri-limbal conjunctival epithelium was three to four times normal \( (P < 0.01) \), and even following a 5 mm diameter central wound, it was three to four times normal on day 1 \( (P < 0.05) \). Goblet cell frequency was a less reliable indicator of conjunctival response to corneal injury: it was decreased following the largest and smallest wounds but not affected by the 10 mm diameter wound. These studies demonstrate that conjunctival epithelium peripheral to the cornea is affected by small central corneal wounds, and may therefore play a role in corneal epithelium healing. Invest Ophthalmol Vis Sci 28:1445–1449, 1987

The role of peripheral ocular surface epithelium in the maintenance of corneal epithelium and in the repair of corneal epithelial wounds has been the subject of considerable speculation. Studies of epithelial graft rejection,1 of sex chromatin in donor corneal epithelium,2 and of hemidesmosome patterns3 all support the concept that the peripheral corneal epithelium is involved in normal maintenance as well as healing of central epithelial wounds.4 However, Buck5 was unable to demonstrate a centripetal movement onto the cornea of conjunctival epithelium in non-wounded mouse eyes. Furthermore, while a drop-out of conjunctival goblet cells peripheral to conjunctival epithelial defects and to large corneal epithelial defects has been shown in rabbits,6,7 no increase in the conjunctival epithelial mitotic rate was seen 4 days after a 10 mm diameter central corneal epithelial defect was made in rabbit eyes.7

The purpose of this study was to determine whether conjunctival epithelium is capable of developing an increased mitotic rate in response to injury, and whether it is involved in healing acute corneal epithelial wounds. To do so, the mitotic rates and goblet cell frequency of ocular surface epithelium were determined in normal eyes and at intervals after various-sized ocular surface epithelial defects were made in rabbit eyes.

Materials and Methods

Animal and Tissue Preparation

All investigations described in this manuscript conform to the ARVO Resolution on the Use of Animals in Research.

New Zealand white rabbits, weighing 1.5 to 2.0 kg, were anesthetized by intramuscular injection of 1 ml chlorpromazine hydrochloride (25 mg/ml) and 2 ml ketamine hydrochloride (100 mg/ml) supplemented by topical proparacaine. The eyes were proptosed, and a surgical microscope was used during wounding. Three types of epithelial wounds were made using
n-heptanol. In Group 1, the total corneal, limbal and a 2–3 mm ring of conjunctival epithelium were removed. To do so, a cotton swab soaked in n-heptanol and blotted was applied to the surface for 30 sec, following which the area was rinsed thoroughly with 200 ml isotonic saline. For removal of 10 mm diameter (Group 2) or 5 mm diameter (Group 3) areas of corneal epithelium, filter paper discs of the appropriate diameter were soaked in n-heptanol, then blotted and applied to the central cornea for 10–15 sec, following which the area was gently rubbed with a moist cotton swab and rinsed thoroughly with 200 ml isotonic saline. Following rinsing, the eyes were stained with Richardson's stain to confirm that the desired area had been debrided of epithelium. It is known that repeated applications of Richardson's stain delay rabbit corneal epithelial healing. However, the stain was applied only once in this study and would not be expected to affect the outcome. If necessary, the above steps were repeated. Erythromycin antibiotic ointment, which is not known to have any effect on epithelial healing, was applied once daily.

The rabbits were sacrificed by an overdose of intravenous sodium pentobarbital. For Group 1, eyes were collected at 1, 2, 3 and 4 days after injury and at 0–1, 7, 14, 21, and 28 days after healing. For Group 2, eyes were taken at days 1, 2, 3, and 4 after injury and for Group 3, eyes were taken at days 1, 2, and 3 after injury. Eight eyes from six rabbits were enucleated for each of the experimental groups at each time period. Four eyes from two rabbits which received the same treatment in each eye (n = 2) and four eyes from four rabbits which had different treatment in each eye (n = 4) for a total of N = 6 in Groups 1, 2, and 3 were used. Both the uninjured eyes from three non-injured rabbits (n = 3) plus 5 eyes from 5 other rabbits (n = 5) for a total of N = 8 control eyes were also prepared.

The eyes were enucleated carefully, retaining as much as possible of the bulbar conjunctiva. The eye was then dissected and 2 strips removed (Fig. 1). One strip was a 4 mm wide and 10 mm long conjunctival-scleral section taken from the limbus toward the lower nasal section of the eye (A in Fig. 1); the other was a 4 mm wide strip through the center of the cornea (B in Fig. 1). These were incubated in tissue culture medium (Medium 199, Gibco, Grand Island, NY) with 10 μCi per ml tritiated thymidine (20 Ci/nmol, New England Nuclear, Boston, MA) at 37°C for 2½ hr, followed by ½ hr incubation in isotope-free medium. After incubation, the samples were fixed in 10% buffered formalin, dehydrated, embedded in plastic, and cut into 4-μm sections. The sections were dipped in 0.1% periodic acid for 10 min, rinsed in water, then dipped in Kodak NTB-2 nuclear track emulsion (Kodak, Rochester, NY), stored at −20°C for 2 weeks, then developed with Kodak D19 developer, fixed, and stained with Schiff reagent (for a PAS stain) and hematoxylin.

**Analysis**

The number of s-phase cells (those which took up tritiated thymidine) and the total number of epithelial cells were counted in 1 mm fields using the surgical limbus as the starting point. Strip A (Fig. 1) was used for counting the limbal and conjunctival labeled and unlabeled cells in normal and experimental animals. The epithelium in the first 1.5 mm outside the corneal limbal junction was defined as limbal; the epithelium extending beyond that over the sclera was defined as conjunctiva. Conneal cells in normal animals were counted on Strip B (Fig. 1). Previous studies had shown that the tritiated thymidine uptake was the same across all meridians of the cornea (Kimoshita S, personal communication).

The number of s-phase cells was used as the mitotic rate and is expressed as the number of tritiated thymidine labeled cells per 100 epithelial cells per 1 mm field in Figure 2. To obtain the average number of labeled cells per field (mitotic rate = % labeled cells) in the conjunctiva (Figs. 3, 4, and 5), the percentages of labeled cells per mm field of all the fields were averaged since there was no difference in that percentage over the 2–4 mm of conjunctiva which was available for study.

Goblet cell frequency was determined by counting the number of PAS positive goblet cells per field and expressing the results as described above. Since goblet cell content close to the limbus was significantly less than that further away (see Fig. 2 and Results), only goblet cell frequency in the fields 2 mm or more from the limbus was used to calculate the average conjunctival goblet cell content.

![Diagram](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933134/ on 09/23/2017)

**Fig. 1.** Ocular Surface Epithelial Samples. Two strips (A and B) of ocular surface epithelium were removed from each enucleated eye and used for analysis of mitotic rates and goblet cell contents.
The $P$ values were obtained by comparing values using the student t-test.

**Results**

**Controls**

*Mitotic rate:* The mitotic rate (MR) of the normal corneal epithelium was $1.0 \pm 0.1\%$ ($n = 8$ eyes) tritiated thymidine labeled cells per 100 epithelial cells in 2½ hr across the entire cornea. That of the limbal epithelium was $1.4 \pm 0.2\%$ ($n = 8$ eyes), while that of the conjunctival epithelium was $0.9 \pm 0.1\%$ ($n = 8$ eyes) on average and did not vary with the distance from the limbus (Fig. 2).

*Goblet cells:* The number of goblet cells was higher in more peripheral conjunctival epithelium than in epithelium closer to the limbus. For example, in the 1 mm area of conjunctiva immediately adjacent to the limbus, $2.5 \pm 0.2\%$ ($n = 8$ eyes) goblet cells per 100 epithelial cells were present while there were $5.6 \pm 0.7\%$ ($n = 8$ eyes) goblet cells per 100 epithelial cells in the 1 mm field from 2 to 3 mm removed from the limbus ($P < 0.01$) (Fig. 2). As there were no significant differences in goblet cell percentage among the more peripheral values, it was possible to average those values from fields 2 mm or more from the limbus, giving an average goblet cell frequency of $4.8 \pm 0.3\%$ ($n = 8$) goblet cells per 100 epithelial cells in the peripheral conjunctival epithelium (Fig. 2).

**Group 1**

Removal of total corneal, total limbal and 2–3 mm of conjunctival epithelium.

*Mitotic rate:* One day after injury, the average MR of the conjunctival epithelium (the leading edge of healing epithelium at that time) was almost ten times normal ($P < 0.001$). By day 3, by which time the leading edge of the healing epithelium had moved onto the cornea, the conjunctival epithelium had a normal mitotic rate which it retained for the duration of the study (Fig. 3).

*Goblet cells:* The goblet cell frequency of the epithelium overlying the sclera (the conjunctival epithelium) was below normal at days 1, 2, 3, and 4 post injury. After wound closure, the goblet cell content of the conjunctival epithelium was higher than normal for at least 28 days. For example, the average goblet cell content of normal conjunctival epithelium was $4.8 \pm 0.3\%$ ($n = 8$ eyes) goblet cells per 100 epithelial cells while that of the conjunctival epithelium from eyes 28 days post healing was $11.7 \pm 0.7\%$ ($n = 6$ eyes) ($P < 0.001$) (Figs. 2 and 3).

**Group 2**

Ten mm diameter area of central corneal epithelium removed.

*Mitotic rate:* Two days after injury, the mitotic rate in the conjunctival epithelium was significantly higher than normal ($P < 0.01$). By day 3, the conjunctival epithelial MR was normal and it remained normal at day 4 when the epithelial defects had healed in half of the eyes and were only 0.5 to 1.0 mm in diameter in the rest (Fig. 4).

*Goblet cells:* Goblet cell density in the conjunctival epithelium was below normal at days 1, 2, 3, and 4 post injury. After wound closure, the goblet cell content of the conjunctival epithelium was higher than normal for at least 28 days. For example, the average goblet cell content of normal conjunctival epithelium was $4.8 \pm 0.3\%$ ($n = 8$ eyes) goblet cells per 100 epithelial cells while that of the conjunctival epithelium from eyes 28 days post healing was $11.7 \pm 0.7\%$ ($n = 6$ eyes) ($P < 0.001$) (Figs. 2 and 3).
MITOTIC RATES AND GOBLET CELL FREQUENCY
IN OCULAR SURFACE EPITHELIUM

Mitotic rate: In these rabbits, the MR of the conjunctival epithelium was higher than normal at day 1 \((P < 0.05)\), but was normal at day 3 when the eyes were healed (Fig. 5).

Goblet cells: The goblet cell frequency of conjunctival epithelium was statistically significantly below normal at day 2 \((P < 0.05)\) (Fig. 5).

Discussion

This study defines the mitotic rate and goblet cell content of the ocular surface epithelium of the rabbit under resting conditions and that of the conjunctival portion of the ocular surface epithelium in response to surface injury. The control mitotic rates of corneal, limbal, and conjunctival epithelia were \(1.0 \pm 0.1\) \((n = 8)\), \(1.4 \pm 0.2\) \((n = 8)\) and \(0.9 \pm 0.1\) \((n = 8)\) tritiated thymidine labeled cells per 100 epithelial cells per 2½ hr respectively, showing that there are no significant differences in mitotic activity among the three regions. The goblet cell frequency as defined by PAS staining was 4.8% of the epithelial cells in this study, which is less than the 7-8% reported PAS-positive earlier, and probably reflects a loss of goblet cells or goblet cell contents during the 3 hr incubation time.

Of importance to us were the demonstrations that the conjunctival epithelium is capable of mitosis in response to injury, and that it does participate in healing acute corneal epithelial wounds as reflected by changes in the mitotic rate of the epithelium and by alterations in the goblet cell content. Predictably, the total corneal, limbal and partial bulbar conjunctival epithelial wound resulted in an increase in the MR of the peripheral epithelium. This study also confirmed that there is reduction in the number of PAS-positive conjunctival goblet cells when that tissue migrates.

More interesting, however, was the response of the conjunctival epithelium to wounds which involved only the central corneal epithelium. These smaller wounds also resulted in an early rise in the conjunctival epithelial mitotic rate. These studies, therefore, demonstrate that conjunctival epithelium responds to acute wound healing of the corneal epithelium even after small central injuries.

This observation further strengthens the hypothesis that the corneal epithelial mass may, in part, be maintained by the very slow centripetal migration of epithelial cells from the periphery of the cornea, and possibly even the conjunctiva. This hypothesis was suggested to us by the observations of Alldredge and Krachmer who noted that epithelial rejection did not occur more than 1 year after corneal grafting, implying that by that time, the host peripheral epithe-
lium had replaced the donor epithelium. In support of this observation, Kinoshita et al\textsuperscript{2} demonstrated a slow replacement of donor by host epithelium following penetrating keratoplasty in rabbits. In studies of mouse epithelium, while Buck\textsuperscript{4} was not able to demonstrate a centripetal migration of conjunctival epithelial cells in mouse eyes, he did demonstrate such a movement of peripheral corneal cells. However, he followed the animals for only 1 week. The studies by Alldredge and Krachmer\textsuperscript{1} and Kinoshita et al\textsuperscript{2} suggest that the centripetal movement in non-injured eyes is too slow to be observed in only 1 week, since it requires almost 1 year for the entire donor epithelium to be replaced by host epithelium.\textsuperscript{4} What is certain from the current studies is that the conjunctiva responds by cellular multiplication after even relatively small, central corneal wounds.

**Key words:** ocular surface epithelium, mitotic rate, goblet cell content

**References**