Change in Epithelial Keratin Expression during Healing of Rabbit Corneal Wounds

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Corneal epithelial wound healing following full-thickness trephination and transcorneal freeze injury was studied by electron microscopy and immunofluorescent microscopy using monoclonal antibodies AE1, AE2, and AE3 to human epithelial keratin. Wounds were evaluated at various time intervals between 4 hr and 2 mo after injury. By scanning and transmission electron microscopy, epithelial migration was evident 4 hr after injury and was characterized by thinning of the epithelium and extension of filopodial processes. AE1 monoclonal antibody, which stains specifically the superficial cells of normal corneal epithelium, reacted to cells at the leading edge of the migrating epithelium. By 24 hr, all cells migrating over the wound displayed positive fluorescence with AE1 while the epithelium over the undamaged cornea exhibited normal fluorescence limited to the superficial epithelial cells. In full-thickness corneal wounds, reepithelialization was complete by 1–2 wk; however, all epithelial cells covering the wound remained positive for the AE1 antikeratin antibody. By 2 mo, the AE1 fluorescence returned to normal. In transcorneal freeze injuries, reepithelialization was complete by 4 to 7 days after injury, with all cells overlying the wound reacting with the AE1 antibody. By 2 wk after freeze injury, all epithelial cells appeared to express a normal AE1 staining pattern. No change was noted in the fluorescent distribution of either AE2 antibody, which did not react with the corneal epithelium, or AE3, which reacts with all corneal epithelial cells. These results suggest that healing of corneal epithelial wounds involves changes in keratin expression of the corneal epithelium.


Regeneration of an intact corneal epithelial surface following corneal injury involves initial migration of the epithelium followed by reattachment to the underlying stroma.1 Depending on the nature and size of the injury, this process may require days to months in order to complete. The specific process of corneal reepithelialization has been extensively studied both morphologically and biochemically.

In general, epithelial injury appears to result in a retraction of the corneal epithelium away from the wound during the first hour after injury.2 This retraction is followed by extension of filopodia and lamellipodia out onto the bare corneal surface.2-4 Active cell migration is evident by 4 hr after wounding and progresses at a rate of 26–60 μm/hr until wound closure is complete.5,6 During reepithelialization, small defects are covered by migration of adjacent epithelial cells rather than cell division; this results in a thinning of the epithelial layer to 1–2 cells. Biochemically, actin appears to be an important intracellular constituent necessary for cell migration as well as a functioning glycolytic pathway.5-10 On the other hand, microtubule assembly and cell division appear not to be necessary for epithelial migration.7,9 Although the exact messenger which triggers epithelial migration is unknown, it appears to generate cAMP as a secondary messenger.11-12

In those situations where the original basement membrane is lost or damaged,13-15 wound closure appears to be followed by the synthesis of a new basement membrane. Concurrent with basement membrane synthesis is formation of new hemidesmosomal junctions between the basal epithelial cell plasma membrane and the basement membrane. Complete reepithelialization is characterized by a return of the epithelial layer to its normal 5–7 cell thickness combined with a normal distribution of hemidesmosomes that attach the epithelium to the underlying stroma.

The process of corneal epithelial regeneration almost certainly involves a change in the state of epithelial growth and differentiation. This may be particularly evident during the later phases of epithelial wound healing when cell division and basement membrane synthesis are the predominant cellular activities. Expression of keratin, important cytoskeletal structural proteins of all epithelial cells,16-19 is known to be regulated depending on the growth and differ-
Corneal injuries

Twenty albino rabbits weighing 2-4 kg, were used, in procedures conforming to the ARVO Resolution on the Use of Animals in Research. Of the 40 eyes, 36 eyes underwent standardized corneal injury consisting of either a full-thickness trephination wound (20 eyes) or a transcorneal freeze injury (16 eyes). The remaining 4 eyes were used as a control. All experimental animals were initially sedated with intramuscular ketamine/Rompun (1 mg/kg) and Rompun (1 mg/kg). Topical opthaine was instilled in each experimental eye to reduce ocular sensitivity prior to corneal injury. All injuries were carried out using sterile techniques in an operating room equipped with a Zeiss operating microscope (Zeiss; New York, NY).

Trehpination wound: Central corneal full-thickness trephination wounds were made using a 3-mm diameter Roboz microdissecting trephine (Roboz Surgical Instruments; Washington, DC). Following penetration of the trephine into the anterior chamber, the 3-mm corneal button was completely removed using corneal scissors. After injury, all eyes received topical neosporin drops.

Transcorneal freeze injury: Central transcorneal freeze injuries were induced by applying the flat end of a 6-mm diameter steel rod previously cooled in liquid nitrogen. The rod tip was held firmly to the cornea for 30 sec and then removed. This procedure resulted in the formation of an ice ball (not involving the limbus) extending to the anterior lens capsule. This indicated that all layers of the cornea had been frozen (confirmed by histopathology). After freeze injury, topical neosporin was applied.

Materials and Methods

Animal Sacrifice and Tissue Preparation

Animals were killed at various intervals from 4 hr to 2 mo after injury. At the time of death, animals were sedated with intramuscular ketamine/Rompun, and deep anesthesia was titrated using sodium pentobarbital administered through the marginal ear vein. The corneal specimen, excluding the limbus, was then obtained either fresh for immunofluorescence or after anterior chamber perfusion with 2.5% glutaraldehyde in 200 mM sodium phosphate buffer (pH 7.4) at 10-20 mmHg for scanning and transmission electron microscopy. In selected cases, specimens for transmission electron microscopy were obtained from fresh corneas used for immunofluorescence to more accurately compare the ultrastructural and immunohistochemical data. After removal of the cornea, rabbits were killed by intracardiac injection of concentrated sodium pentobarbital.

Immunofluorescence: Fresh corneas were immediately embedded in O.C.T. Compound (Lab-Tek Products; Naperville, IL), quick frozen in liquid nitrogen, and stored at −80°C. Cryostat sections (6 μm) were air dried (5-10 min) and fixed in cold (−20°C) acetone for 10 min. Sections were then rehydrated in phosphate buffered saline (PBS; pH 7.4) for 5 min and preincubated with normal goat serum (1:10) (Cappel Laboratories; Cochranville, PA) for 30 min at 35°C in a humidified chamber. Mouse monoclonal antibodies AE1, AE2, and AE3, prepared against human keratin proteins that crossreact with keratins of rabbit and many other animal species, were then applied to each section and incubated for 1 hr in a humidified chamber. Control sections were similarly incubated with control culture media from parent myeloma cultures. Sections were then washed three times in PBS for 10 min followed by incubation with fluorescein conjugated F(ab')2 fragment goat antimouse IgG (heavy and light chain) (1:20) (Cappel Laboratories) for 30 min in a humidified chamber. Sections were then washed three times in PBS for 10 min, mounted using Elvanol, and evaluated using a Zeiss Photomicroscope III with epifluorescence.

Electron microscopy: Corneal specimens were fixed for 4-12 hr in 2.5% glutaraldehyde in 200 mM sodium phosphate buffer, pH 7.4. Tissues were postfixed in buffered 1% OsO4 for 1 hr and then dehydrated in ethanol. Specimens for transmission electron microscopy were further dehydrated in propylene oxide and embedded in Epon 812 (Structure Probe; West Chester, PA). Ultrathin sections were cut using an LKB Ultramicrotome (LKB Instruments; Rockville, MD) and Diatome (MJO Diatome; Fort Washington, PA) diamond knife. Sections were mounted onto 200 mesh copper grids, stained with uranyl acetate and lead citrate, and viewed on a Phillips EM 400 electron microscope (Phillips Electronics; Mahwah, NJ). Specimens for scanning electron microscopy were dehydrated ending in amylacetate and critical point dried using a Denton DCP-1 Critical Point Drying Apparatus. Specimens were coated with gold-palladium using a Denton DESK-1 sputter coater (Denton Vacuum; Cherry Hill, NJ) and viewed in a...
Fig. 1. Immunofluorescent staining of normal rabbit corneal epithelium with (A) AE1 and (B) AE3 monoclonal antikeratin antibodies. Note in A that only superficial corneal epithelial cells (arrow) are AE1-positive. Inset. Control section incubated with parent P3 myeloma media. Note in B that all cells are AE3-positive (original magnification, ×375).

Results

Normal Rabbit Corneal Epithelium

Immunohistochemical staining and biochemical data have established that AE1, AE2, and AE3 mouse monoclonal antibodies recognize specific subsets of keratin proteins and stain different epidermal cell layers.24 Similar results were obtained with normal rabbit corneal epithelium. AE1 antibody, which recognizes a 40-kd and a 50-kd keratin in this epithelium, stains only occasional superficial cells (Fig. 1A). AE3 antibody, which recognizes a 58-kd and a 64-kd keratin in cornea, stained all corneal epithelial cells layers (Fig. 1B). By contrast, AE2 antibody, which recognizes 56.5-kd and 65–67-kd keratins in keratinized epidermis, did not react with any corneal epithelial layers. Control supernatants from parent myeloma cultures were negative (Fig. 1A, inset).

Trephination Wound

0–4 Hours: By 4 hr after injury, a fibrin meshwork formed a tight plug at the wound site, resulting in retention of aqueous fluid and partial reformation of the anterior chamber. Scanning electron microscopy of the wound revealed a leading edge of migrating epithelium located 0–50 μm from the wound edge (Fig. 2A). This finding, similar to that previously reported by Haik and Zimny during rabbit corneal epithelial wound healing,2 suggests that the epithelium had retracted away from the wound. However, it is also possible that the trephine damage to the epithelium extended beyond the cut edge of the stroma or that shrinkage artifact caused by specimen preparation occurred. Presence of cell migration was suggested by the finding of filopodial and lamellipodial processes extending from cells at the leading edge of the epithelium (Fig. 2B). This cellular activity has been previously linked to substrate recognition by migrating cells.25 Microplicae and microvilli covered the surface of the cells at the leading edge of the migrating epithelium, which was consistent with the morphology of superficial cells. By light and transmission electron microscopy, the epithelium appeared thinned near the wound site and the hemidesmosomal attachments were absent. At this stage of epithelial regeneration, AE1 antibody stained the superficial epithelial cells up to the leading edge of the migrating epithelium (Fig. 3). Assuming that these AE1-positive cells are derived from superficial layers, this finding suggests that the superficial cells may participate in epithelial migration. There was no change in the reaction of AE2 and AE3 antibodies.

1 Day to 1 week: One day to 1 wk after trephination
Fig. 2. Scanning electron micrograph of corneal wounds 4 hr after injury. A, Low power micrograph demonstrating retraction of epithelium (arrow) away from wound margin and the cut edge of the corneal stroma (original magnification, ×120). B, High power micrograph of cells at the leading edge from which prominent filopodial processes (arrows) appear to extend out onto the stromal surface (original magnification, ×1,600).

Injury, the corneal wound was characterized by continued migration of the epithelial cells over the fibrin clot (Fig. 4A). The leading edge of the epithelium displayed prominent filopodial processes that extended some distance away from the epithelial cells. These processes have been previously reported by Pfister to extend up to 100 μm in length. AE1 antibody reacted with all epithelial cells migrating over the fibrin plug (Fig. 4B) suggesting that migrating epithelium expresses AE1-specific keratin proteins detectable by immunofluorescence. Again, there was no change in the reaction of AE2 and AE3 antibodies.

1 Week to 1 month: By 1 wk after injury, the fibrin plug had been completely re-epithelialized. From 1 wk to 1 mo, fibroblasts migrated into the wound area and there was complete replacement of the fibrin with newly synthesized connective tissue. At this stage of wound healing, the regenerating corneal epithelial layer appeared to increase in thickness and lay down a new basement membrane (Fig. 5A). Basal epithelial cells appeared to be actively secreting material, as there were numerous vesicles fused to the basal cell plasma membrane (Fig. 5A, inset). Reformation of the basement membrane, however, remained incomplete by one month, with areas of the basal cell plasma membrane still devoid of basal lamina. At this stage of wound healing, AE1 antibody continued to react with all epithelial cells overlying the wound.

Fig. 3. Immunofluorescent staining of corneal epithelium 4 hr after injury using AE1 antikeratin antibody. Superficial epithelial cells as well as cells at the leading edge of the migrating epithelial sheet (arrow) are AE1-positive (×300).
Fig. 4. Morphology and AEI staining pattern of 48-hr wound. A, Morphology. By 48 hr epithelial cells have migrated along the cut surface of the cornea and out onto the central fibrin clot (asterisk) (toluidine blue, X190). B, Immunofluorescent staining showing that cells overlying the fibrin clot are AEI-positive. Arrow: leading edge (X300).

(Fig. 5B, open arrow). Away from the wound edge, AEI antikeratin staining appeared to be localized to only the superficial epithelial cells. However, basal epithelial cells adjacent to the wound edge appeared to react positively to the AEI antibody (Fig. 5B, arrows and inset). These findings suggest that regen-
Fig. 5. Morphology and AE1 staining pattern of 1-mo wound. A, Morphology. One month after injury the fibrin clot had been replaced by densely cellular connective tissue. The epithelium overlying the wound (arrow) appeared to have returned to a normal thickness. Transmission electron microscopy of basal epithelial cells overlying the wound revealed partial formation of basal lamina (inset) (toluidine blue, ×190; inset, ×29,000). B, AE1 staining. All cells overlying the densely cellular wound connective tissue (open arrow) were AE1-positive. Cells adjacent to the wound margin (between arrows) exhibited a transition from AE1-negative to AE1-positive with AE1-fluorescence first appearing in the basal epithelial cells (left arrow and inset) (×300; inset, ×740).
erating epithelial cells, although morphologically normal, still have an altered state of differentiation as defined by AE1 antibody staining.

2 Months: By 2 mo after trephination injury, epithelial cells over the wound reacted normally to AE1 antibody, exhibiting positive immunofluorescence over only the superficial epithelial cells (Fig. 6). At this stage, the regenerated corneal epithelium appeared normal with a complete basal lamina and a normal distribution of hemidesmosomal junctions. This indicates that by 2 mo after injury, regenerating corneal epithelium expresses a normal distribution of keratin proteins as revealed by antikeratin staining.

Freeze Injury

Corneal epithelial cells regenerating following freeze injury exhibited a similar change in their AE1 staining patterns. However, the time necessary to complete corneal reepithelialization and the establishment of a normal expression of keratin proteins was much shorter for freeze-injured corneas than for trephine wounded corneas. Freeze injury appears to result in the immediate loss of epithelial cells within the freeze zone with a concomitant loss of both corneal fibroblasts and corneal endothelial cells. Freeze injury, however, does not disrupt the basal lamina of the corneal epithelial cells; and, therefore, basement membrane synthesis by the regenerating epithelial cells is not required.

One day after freeze injury, all cells migrating over the denuded cornea exhibited a positive reaction with AE1 antibody (Fig. 7A). This change was correlated with thinning of the epithelial layer and absence of hemidesmosomal attachments to the underlying basal lamina (Fig. 7A, inset). One week after injury, the cornea was completely reepithelialized. However, at this stage many of the corneal epithelial basal cells continued to be AE1-positive (Fig. 7B, arrow). Although the epithelial layer was intact, transmission electron microscopy revealed incomplete reattachment of the basal epithelial cells to the underlying basal lamina with an abnormal distribution of hemidesmosomal junctions. By 2 wk after injury, the regenerated corneal epithelium appeared to express a normal distribution of keratin proteins with AE1 staining localized to the superficial layer of the cornea (Fig. 7C). There was also a normal distribution of hemidesmosomal junctions.

Discussion

The in vivo expression of keratin proteins appears to be dependent on several factors that may include the type epithelia, the stage of cell differentiation, and the period of embryonic development. Disease states may also influence the expression of keratin proteins as well as the particular growth state and cellular growth environment associated with epithelial cells. In our studies, regenerating corneal
epithelial cells appeared to exhibit a change in the expression of keratin proteins as revealed by immunofluorescence of AE1 mouse monoclonal antibody specific for human keratin proteins.

The AE1 antibody, which recognizes a 40-kd and 50-kd keratin in normal corneal epithelium, reacts with only the superficial cells of the epithelium. Due to the possibility of antigen masking, however, this staining pattern does not rule out the presence of AE1-reactive keratins in other cell layers. Following either trephination wound or freeze injury to the cornea, all migrating epithelial cells become AE1-positive as early as 1 day after injury. Although the role of superficial cells in epithelial wound healing is unclear, it is generally agreed that the basal and wing epithelial cell layers play a role in corneal reepithelialization. Since it is unlikely that all migrating epithelial cells are derived from the superficial epithelial cell layer, the data suggest that a change in the expression of keratin proteins in the basal and wing epithelial cells must have occurred by 1 day postinjury.

There are at least two possible explanations for this observed change in keratin expression. First, there may be an unmasking of keratin proteins in the basal and wing epithelial cells during epithelial cell migration. Woodcock-Mitchell et al have shown...
that AE1 antibody selectively stains basal cells of normal human epidermis. Direct biochemical analyses have established, however, that the lack of AE1 staining of suprabasal cells is due to antigenic masking. Franke et al. have described another monoclonal antikeratin antibody, Kc8.13, which stains the keratin fibers of mitotic PtK2 cells but not those of interphase cells due to the masking of the antigenic determinant. These results clearly demonstrate that although positive staining of a cell by a monoclonal antibody establishes the presence of at least one antigen as defined by the antibody, the lack of staining does not prove the absence of the antigen(s) in question.

A second explanation of the altered AE1 staining in regenerating corneal epithelium involves the appearance of new AE1-positive keratin antigen(s). In a recent study, Weiss et al. have established the new appearance of an AE1-positive, acidic 48-kd keratin and an AE3-positive, basic 56-kd keratin in a variety of hyperproliferative epidermal diseases including psoriasis, actinic keratosis, keratoacanthoma, and basal and squamous cell carcinomas. Moreover, these two keratins have also been found in cultured skin, corneal and conjunctival epithelial cells. These results suggest that the 48-kd/56-kd keratins may be regarded as markers for hyperproliferative keratinocytes.

In a recent study, Kinoshita et al. have analyzed the keratins of regenerating rabbit corneal epithelia by SDS gel electrophoresis and have found that such cells appear to express new keratins. Whether these regeneration-associated keratins correspond to the 48-kd and 56-kd keratins of humans is not yet clear.

The new AE1 staining phenotype is most likely related to the cells being in a hyperproliferative state. However, it should be noted that the alteration in AE1 staining precedes cell division and the generation of new corneal epithelial cells. Based on a report by Hanna, the epithelial proliferative response to corneal injury occurs 18-24 hr after injury, at which time increased uptake of H3-thymidine can be measured. Both Hanna and Kuwabara et al. have also noted that the area of epithelial proliferation appears to be located at a site removed from the initial injury and may be up to 120 cells away from the wound edge. Since the change in keratin protein expression by 24 hr after injury involves all cells overlying the wound, it appears most likely that the original epithelial cells differentiate to this new keratin phenotype. Based on experiments utilizing tritium-labeled leucine and uridine uptake in injured corneas, Bracher concluded that corneal cells exhibit a very early increase in RNA and protein synthesis occurring 90 min to 6 hr after injury. Such an increase in protein synthesis may explain the observed change in keratin protein expression.

The change in keratin expression persisted until the epithelial cells reattached to the underlying cornea and formed a normal distribution of hemidesmosomal junctions. The duration of the healing process was quite different following the two types of corneal injuries. In trephination wounds, which require synthesis of a new basal lamina, the change in keratin expression persisted for 1.5 mo after surgery. By contrast, in freeze injury, which does not damage the basal lamina, keratin expression returned to normal by 2 wk after injury. Since firm adhesion of the epithelium to the cornea is dependent on both an intact basal lamina and an appropriate number of hemidesmosomal junctions, expression of the wound healing keratin-phenotype may be somehow related to the attachment of epithelial cells to the basement membrane. Although cellular proliferation may play a major role in the expression of keratin during epithelial regeneration, 1 mo after trephine injury a greatly reduced proliferative response would be expected. By contrast, the reaction of the regenerating epithelium to AE1 was still abnormal 1 mo after injury, suggesting that the cell–matrix interactions and the immediate cell environment also play an important role in expression of keratin proteins in this system.

Further study is necessary to define the role of cell adhesion versus cellular proliferation in the expression of these keratins. Of particular interest would be the study of wound healing following alkali burns, where adhesion of the epithelium to the burned cornea presents a chronic problem that may later lead to corneal ulceration.

Key words: keratin expression, cornea, reepithelialization, wound healing

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References