Epithelial Regeneration After Limbus-to-Limbus Debridement
Expression of α-Enolase in Stem and Transient Amplifying Cells

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Purpose. To examine the expression of the glycolytic enzyme α-enolase after limbus-to-limbus epithelial debridement in the rabbit.

Methods. Corneas were debrided, leaving limbal epithelium intact, and were allowed to heal from 2 days to 8 weeks. Immunofluorescence microscopy was used to observe the expression of α-enolase. To quantitate changes in α-enolase levels 2 days to 4 weeks after wounding, epithelium was harvested, homogenized, and assayed using anti-α-enolase in immunoslot blots.

Results. Expression of α-enolase appeared to increase in the limbus and the central cornea during epithelial migration (2-day time point) with intense labeling of all basal cells. These levels were maintained until wound closure (1 week). By 2 weeks, expression in the limbal basal cells decreased to levels present in unwounded corneas. Expression in the corneal epithelium decreased after 2 weeks, progressing from central cornea to the periphery. At 4 weeks, antibody binding decreased concomitantly with a change in the shape of the basal cells from flattened or ovoid to columnar. At 8 weeks, expression of α-enolase was similar to that in control corneas. Immunoslot blot data indicated that α-enolase made up 0.28% of the total soluble protein in unwounded corneal epithelium and 0.73%, 1.22%, 0.96%, and 0.49% at 2 days, 1 week, 2 weeks, and 4 weeks after debridement, respectively.

Conclusions. These data indicate that expression of α-enolase is elevated during corneal epithelial migration initiating from the stem (limbal basal) cell population and that expression is linked to active migration. Furthermore, it appears that limbal basal cells are metabolically active during the period of epithelial sheet movement, whereas peripheral corneal basal cells remain activated as long as 4 weeks after wounding. Invest Ophthalmol Vis Sci. 1995; 36:1336–1343.
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Antibody Preparation

We have developed a monoclonal antibody, 4G10.3, that is specific for α-enolase and that binds to limbal basal cells in rat, rabbit, and human corneas. After central epithelial debridement, this antibody binds to an increased number of cells, which appear to extend toward the central cornea. Therefore, 4G10.3 can be regarded as a biochemical marker for corneal epithelial stem cells and daughter cells that still carry enhanced levels of α-enolase, making the antibody an excellent tool to examine differentiation after wounding. The antigen for 4G10.3 was identified as α-enolase using amino acid sequencing of the purified antigen. A glycolytic enzyme, α-enolase catalyzes conversion of 2-phosphoglyceric acid to phosphoenolpyruvic acid. Although the primary role of α-enolase is apparently in the glycolytic pathway, it has been shown to have other potential functions. In the lens, α-enolase is identical to γ-crystallin, a major lens component in birds and reptiles. It also has been localized to the cell surface, where it has been postulated to function as a plasminogen receptor. Markers of glycolytic enzymes may be useful particularly to examine wound healing because glycolysis has been shown in several systems to be necessary for epithelial migration.

In this study using 4G10.3, we observed the expression of α-enolase during epithelial migration and re-stratification of a limbus-to-limbus debridement wound. Because the corneal epithelial stem cells are restricted to the limbal zone, this model allowed us to follow the progression of stem cells to transient amplifying cells and then to terminally differentiated cells during the healing process. Changes in α-enolase expression during epithelial wound healing suggest α-enolase levels increase during epithelial re-stratification as well as during migration, and α-enolase expression is correlated with shape change and maturation of the basal cells.

METHODS

Antibody Preparation

Monoclonal antibody 4G10.5, specific for α-enolase, was prepared using standard hybridoma techniques described by Zieske et al. The original hybridoma was detected during production of monoclonal antibodies against migratory rat corneal epithelium. The source of immunogen was a 0.1 M ammonium acetate extract of limbus-to-limbus scrapes of rat corneal epithelium harvested 18 hours after a 3-mm central debridement wound. The antibody reacts with a single protein band in Western blots of rat and rabbit corneal epithelium.

Animal Model

All investigation described in this study conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. New Zealand Albino rabbits weighing 2.0 to 2.5 kg each were used in all studies. After tranquilization by intramuscular injection of sodium pentobarbital (25 mg/kg) and after topical administration of proparacaine hydrochloride to the cornea, an initial wound was made in one eye of each rabbit by scraping the corneal epithelium from limbus to limbus with a scalpel, leaving the basement membrane intact. The contralateral eye was used as a control. Wounds were allowed to heal in vivo, and rabbits were sacrificed at time points of zero hour, 2 days, 1, 2, 4, and 8 weeks after debridement with an intravenous injection of a lethal dose of sodium pentobarbital.

Immunofluorescent Staining

Corneas were excised and frozen in Tissue Tek II OCT Compound (Lab Tek Products, Naperville, IL), then 6-μm cryostat sections were placed on gelatin-coated slides and air dried overnight at 37°C. These slides were rehydrated in phosphate-buffered saline (PBS) and blocked in 1% bovine serum albumin for 10 minutes. The primary antibody, 4G10.3, was applied at a 1:10 dilution. The slides were incubated for 1 hour in a moist chamber at room temperature, then blocked with 1% bovine serum albumin for 10 minutes. Fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Jackson Immunoresearch, Avondale, PA) was applied as a secondary antibody, and the slides were incubated again for 1 hour in a moist chamber. After washing in PBS for 10 minutes, the sections were mounted with a medium consisting of PBS, glycerol, and paraphenylenediamine. Negative control tissues were prepared by omitting the primary antibody. The sections were then viewed and photographed using an Axiohot III microscope equipped for epi-illumination (Carl Zeiss, Thornwood, NY). Four locations were observed for alteration of binding intensity and basal cell shape change during all phases of wound healing: limbus, peripheral cornea, midcornea, and central cornea. Binding intensity was graded from 0 (no binding) to 4+ (most intense binding) directly from microscopic examination by one observer (E-HC) and confirmed from micrographs by another (JDZ).

Quantitation of α-enolase

Immunoslot blots were used to quantitate changes in α-enolase 2 days to 4 weeks after wounding. In these experiments, the epithelium was harvested by scraping the cornea from limbus to limbus as described earlier, homogenized in a ml of PBS in a glass–glass homogenizer, and centrifuged for 1 hour at 100,000g. Three
rabbits were used per time point. Protein levels in the supernatant were determined using the Pierce (Rockford, IL) bicinchoninic acid protein assay. Equal amounts of protein (50 µg) were applied to nitrocellulose paper using a slot blot apparatus, and the paper was incubated with anti-α-enolase (4G10.3). Assays were performed in triplicate. Levels of α-enolase were detected using a vectastain Avidin-Biotinylated enzyme Complex kit (Vector, Burlingame, CA) followed by quantitation with a Molecular Dynamics (Sunnyvale, CA) scanning densitometer. Purified liver α-enolase was used as a standard, with a linear response obtained over the range of 0.2 to 10 µg of purified enzyme.

RESULTS

In the mammalian eye, the limbal epithelium can be identified by the presence of the subjacent limbal blood vessels. Monoclonal antibody 4G10.3, specific against α-enolase, bound to basal cells above these blood vessels (Fig. 1A). In contrast to human and rat corneal epithelium, where little or no binding was observed, punctate membranous binding in the rabbit was observed in the corneal epithelial cells (Fig. 1B).

To examine the expression of α-enolase during epithelial migration and restratification, a limbus-to-limbus debridement was performed. The initial wound removed all the corneal epithelial cells, excluding limbal epithelium. The location of the limbus was confirmed by the subjacent blood vessels and expression of α-enolase (1 to 2+) in basal cells (Fig. 1C). The suprabasal cells in the limbus were not removed in this wound model. Wound closure occurred at approximately 1 week after debridement. No vascularization was observed during healing, except in inflamed eyes.

At 2 days after wounding, one layer of flat, elongated cells was seen to migrate on the peripheral cornea. 4G10.3 bound intensely (4+) from the limbal basal cells to the migratory leading edge (Fig. 2A). Expression of α-enolase increased from 1 to 2+ in the limbal basal cells of unwounded corneas (Fig. 1A) to 4+ after debridement (Fig. 1C). This increase in α-enolase expression was accompanied by a change in shape of the limbal basal cells from cuboidal to flattened. The increased expression of α-enolase did not occur in the conjunctival epithelium (Fig. 3B).

After 1 week, wound closure was complete or nearly complete and basal cells throughout the limbus bound anti-α-enolase intensely (3 to 4+) (Figs. 2B, 3C). Basal cells in the peripheral and midcornea were flat and elongated and expressed high levels of α-
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FIGURE 2. Immunolocalization of α-enolase (4G10.3) in rabbit corneas after epithelial debridement. (A) Two days after debridement. Note flattened shape of basal cells. (B) One week after debridement. Note intense binding of 4G10.3 to basal cells across cornea. (C) Two weeks after debridement. Note intense binding of 4G10.3 to majority of basal cells, except for those at extreme left (central cornea) and at extreme right (limbus). (D) Four weeks after debridement. Note that basal cells in central cornea have become columnar and have lost binding of 4G10.3. Arrow is in peripheral cornea. (E) Eight weeks after debridement. Note binding has essentially returned to control levels. Arrows indicate direction of central cornea. Bars = 50 μm.

Enolase (Fig. 2B). In the central cornea, however, binding intensity of 4G10.3 and the shape of basal cells varied according to the state of healing. Although some maintained intense binding of 4G10.3, others lost that binding (1 to 2+) with shape change. During epithelial migration until wound closure, limbal basal cells did not retain their small cuboidal shape but became flat and elongated, and they expressed the same intense binding of 4G10.3 (3 to 4+) as that of migrating cells (Fig. 3C).

At 2 weeks, basal cells in the limbus seemed to return to their small cuboidal shape, and the binding intensity dropped from 3 to 4+ to 1 to 2+ (Fig. 3D). Most basal cells in the peripheral cornea retained an intense binding of 4G10.3 (3 to 4+) with a flat, elongated form (Figs. 2C, 3E). In the central cornea, cellular maturation and rearrangement were more distinct than at 1 week. A few columnar cells began to appear in the central cornea, and large cuboidal basal cells began to appear in the midcornea.

At 4 weeks, limbal basal cells were cuboidal with 1 to 2+ binding intensity. Basal cells in the peripheral cornea remained flattened with intense binding of 4G10.3 (3 to 4+) (Fig. 2D). In the midcornea, they were large and cuboidal with weak binding (1 to 2+) (Fig. 3F), and in the central cornea they were tall and columnar with little or no binding (Fig. 3G).

After 8 weeks, cellular shape and binding pattern of 4G10.3 were nearly identical to the controls, with only limbal basal cells showing binding of 4G10.3 (1
FIGURE 3. Immunolocalization of α-enolase (4G10.3) in rabbit corneas after epithelial debridement. (A) Limbal region, 2 days debridement. Note flattened shape of basal cells and high levels (3 to 4+) of α-enolase expression. (B) Limbal–conjunctiva interphase 2 days after debridement. Note that binding of α-enolase drops dramatically in conjunctiva. Arrow indicates direction of limbus. (C) Limbal zone 1 week after debridement. Arrow indicates direction of central cornea. (D) Limbal zone 2 weeks after debridement. Note that many of the limbal basal cells have returned to control level (1 to 2+) expression of α-enolase accompanied by a return to cuboidal shape. (E) Midcornea 2 weeks after debridement. (F) Midcornea 4 weeks after debridement. Note transition from flattened to columnar cells. (G) Central cornea 4 weeks after debridement. Bars = 50 μm.

To quantitate alterations in α-enolase during epithelial migration, levels of the enzyme were assayed using immunoslot blots (Fig. 5). This assay indicated that α-enolase represented 0.28 ± 0.05% of the total soluble protein in unwounded rabbit corneal epithelium. After wounding, α-enolase represented 0.75 ± 0.10%, 1.22 ± 0.16%, 0.96 ± 0.14%, and 0.49 ± 0.11% of the total soluble protein at 2 days and 1, 2, and 4 weeks after debridement, respectively.

DISCUSSION
In the present study, we observed, through the change in expression of the glycolytic enzyme α-enolase, the...
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Figure 4. Summary of changes in α-enolase expression, stratification, and cell shape observed after epithelial debridement. Note level of 4G10.3 binding is indicated by intensity of shading in basal cells.

<table>
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<th>Time</th>
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<td>Control</td>
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In this article, we examined the localization of α-enolase after a total corneal epithelial debridement. At 2 days after the limbus-to-limbus wound, one layer of flat, elongated cells was seen to migrate onto the peripheral cornea. These migrating cells expressed high levels of α-enolase and apparently were derived from the basal cells of the limbal epithelium. At this 2-day time point, limbal basal cells changed their shape from small and cuboidal to flat and elongated. This change in shape coincided with an increased level of binding of 4G10.3, suggesting that the limbal basal cells have been metabolically activated. Until the time of wound closure, all basal cells, including the limbal basal cells, maintained their flat, elongated shape and intense binding of anti-α-enolase. This finding suggests that glycolysis is active in these cells and may be providing the energy required for migration. Unexpectedly, we had two rabbits that showed...
delayed healing along with inflammation. Interestingly, in inflamed corneas in which epithelial migration ceased, we observed very low levels of expression of α-enolase, suggesting that expression of α-enolase is linked to migration. This coincides well with previous reports that sliding cells maintain a high level of lactate dehydrogenase and that the migration of cells is prevented by the degradation of glycogen or administration of glycolytic enzyme inhibitors.

After wound closure, the basal cells in the limbus returned to their original small cuboidal shape, concurrently with a decrease in their binding of anti-α-enolase. In contrast to studies using carbonic anhydrase, where the enzyme level rapidly dropped in corneal epithelium after wound closure, we observed that the levels of α-enolase were maintained in peripheral and midcornea for up to 4 weeks. This was confirmed by immunoslot blot data indicating that α-enolase reached its maximal levels 1 week after wounding and was still elevated 1.75-fold even 4 weeks after wounding. Because α-enolase is a glycolytic enzyme and glucose metabolism is closely linked to the initiation of DNA synthesis, the data suggest that the basal cells in the peripheral cornea that express high levels of α-enolase are transient amplifying cells that play a crucial role in corneal epithelial reconstitution. These flat, elongated, intensely stained cells were still present in the peripheral cornea at 4 weeks after wounding, suggesting that cellular repopulation and reconstitution are accomplished mainly by transient amplifying cells and not by stem cells themselves.

At 4 weeks after wounding, cellular maturation, including change in shape, decreased expression of α-enolase, and epithelial reconstitution, which had already occurred in central cornea, became more distinct and progressed toward the periphery. At this time, the progression of stem cell differentiation from early transient amplifying cell to late transient amplifying cell to terminal differentiated cell could be observed easily through the dramatic change in cell shape and α-enolase expression. If the stem cells reside in the limbal basal layer, then the early transient amplifying cells are the flat, elongated cells with enhanced expression of α-enolase located in the peripheral cornea. The large cuboidal basal cells in the midcornea, with decreased binding of α-enolase, would correspond to late transient amplifying cells, and the tall columnar cells with little or no α-enolase expression in the central cornea would correspond to terminally differentiated cells.

Our observations of changes in cell shape and altered expression of α-enolase in the limbus-to-limbus wound model are remarkably similar to our previous observations in developing rat eyes. In developing eyes, before eyelid opening, basal cells across the corneal epithelium expressed high levels of α-enolase and had flat, elongated shapes. Immediately after eyelid opening, the basal cells changed in shape from flat to ovoid to cuboidal with a concurrent decrease in α-enolase expression. This maturation started in central cornea and progressed toward the periphery. These data suggest that wound repair mimics development. Interestingly, in both the developing rat model and in the adult rabbit, the expression of α-enolase appears to be membranous in some situations. It is unclear what role the enzyme might have when localized in or near the membrane. However, α-enolase has been suggested to have both a structural and a receptor function in addition to its glycolytic function.

In summary, we have observed that enhanced levels of α-enolase are expressed during cell migration, suggesting that α-enolase, and most likely glycolysis, are involved in epithelial wound closure. To our surprise, enhanced levels of α-enolase are maintained up to 4 weeks after wounding in the peripheral cornea, suggesting that transient amplifying cells located in the periphery play a major role in epithelial reconstitution. Finally, the limbus-to-limbus debridement wound provides an excellent model to observe the progression of stem cell differentiation from early transient amplifying cell to late transient amplifying cell to terminally differentiated cell.

**Key Words**

α-enolase, corneal epithelium, wound healing, stem cells, debridement
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References


