Limbal epithelium in ocular surface wound healing

Shigeru Kinoshita, Timothy C. Kiorpes, Judith Friend, and Richard A. Thoft

The regenerated epithelium derived from limbal epithelium was histologically and biochemically compared with epithelia regenerated from corneal and bulbar conjunctival epithelia. The histologic results indicated that regenerated epithelium of limbal origin increased in thickness with time after healing and showed no goblet cell appearance on the cornea. This suggests that regenerated epithelium from the limbus is more like regenerated epithelium of corneal origin than that of bulbar conjunctival origin. However, the glycogen content and protein pattern profile showed that regenerated epithelium of limbal origin had characteristics intermediate between those of corneal and bulbar conjunctival origin. Thus it is proposed that there are three distinct types of ocular surface epithelia—corneal, bulbar conjunctival, and limbal—and that limbal epithelium behaves differently from corneal and conjunctival epithelia in ocular surface wound healing. (INVEST OPHTHALMOL VIS SCI 23:73-80, 1982.)

Key words: limbal epithelium, glycogen, goblet cell, regenerated ocular surface epithelium, gel-electrophoresis

The characteristics of epithelium regenerating from cornea and bulbar conjunctiva after mechanical or chemical removal of corneal epithelium have been well documented and suggest that there are marked differences between those two types of regenerating ocular surface epithelia.1,2,5 For example, most glycolytic enzyme activities are normal immediately after healing in epithelium regenerated from corneal epithelium but remain low for 2 to 3 weeks after healing in epithelium regenerated from bulbar conjunctiva.2 Histologically, there is a goblet cell appearance on the cornea in epithelium regenerated from bulbar conjunctiva but not in regenerated corneal epithelium.1,5,6

In addition to these two sources of epithelium for resurfacing the cornea, there is a narrow transitional zone of epithelium between corneal and bulbar conjunctival epithelium, the "limbal" epithelium, which usually remains after debridement of the corneal epithelium from limbus to limbus. This epithelium is histologically different from bulbar conjunctival epithelium in that it lacks goblet cells, and from corneal epithelium in that it has Langerhans' cells, melanocytes, and subjacent blood vessels.7 Mau-menee8 has suggested that even a small amount of limbal epithelium can resurface the cornea and that epithelium regenerated from limbal epithelium appears histologically normal within 2 to 3 weeks, implying that this epithelium may behave more like corneal than conjunctival epithelium when resurfacing the cornea. However, no detailed

From the Department of Cornea Research, Eye Research Institute of Retina Foundation, and Department of Ophthalmology, Harvard Medical School, Boston, Mass. Supported in part by research grants EY01830, EY00601, and Eye Research Core Services Support p30EY01784 from the National Eye Institute, National Institutes of Health, and in part by The Lions Eye Research Fund, Inc.

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Reprint requests: Shigeru Kinoshita, M.D., Eye Research Institute of Retina Foundation, 20 Staniford St., Boston, Mass. 02114.

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study of regeneration of corneal epithelium from limbal epithelium has been undertaken. The purpose of the work reported here is to compare the histologic and biochemical differences between regenerated epithelia from three types of ocular surface epithelia—corneal, limbal, and bulbar conjunctival.

Materials and methods

Animal preparation. Albino rabbits weighing 2 to 3 kg were anesthetized intramuscularly with chlorpromazine hydrochloride (25 mg) and ketamine hydrochloride (250 mg), and anesthesia was maintained with ether inhalation. Epithelial defects were made by scraping with a Bard-Parker No. 15 blade under a surgical microscope. The defect area was assessed by sodium fluorescein staining, rescraped if necessary, and re-examined with Richardson stain.9 The area scraped was about 2 mm beyond the limbo-corneal junction (wound type A), the entire corneal epithelium to just beyond the limbo-corneal junction (wound type B), or the entire corneal epithelium plus limbal and bulbar conjunctival epithelium up to about 2 mm beyond the limbo-corneal junction (wound type C). In some cases of wound type C, n-heptanol epithelial debridement was also performed using the method described previously.5 The n-heptanol–debrided eyes were used only for histologic analysis. The limbo-corneal junction is clinically defined as the peripheral edge of semi-translucent corneal stroma where the micro-capillaries from conjunctival vessels are seen in the underlying tissue. The extent of the different epithelial defects was clinically and histologically monitored in three eyes of each category immediately after wounding (Fig. 1). Antibiotic ointment and 1% atropine sulfate were used immediately after and then for 1 to 2 days after wounding. Only eyes that healed without edema, vascularization, or delays in healing were used in the experiments.

Histologic analysis. After rabbits were killed by an overdose of intravenous pentobarbital, eyes were enucleated, fixed in 10% buffered neutral formalin for at least 24 hr, dehydrated, embedded in paraffin, cut into 7 μm sections through the greatest diameter of the cornea, and stained with periodic acid–Schiff (PAS) and hematoxylin. The goblet cells in the regenerated epithelium on the cornea (limbus to limbus) were counted in four adjacent sections. The mean value of the four counts was used as the number of goblet cells in the regenerated epithelium on the cornea.

Biochemical analyses. After animals were killed, 10 mm diameter areas of regenerated or normal corneal epithelium were mechanically scraped off and immediately frozen in liquid nitrogen. Samples for glycogen measurements were lyophilized, weighed, and stored at −80° C until used. Other samples were immediately dispersed into 100 μl 2% (w/v) sodium dodecyl sulfate (SDS) in 10 mM sodium phosphate, pH 7.0, by sonification and then boiled for 5 min as described by Hassell et al.10 These samples were stored at −80° C until slab gel electrophoresis was performed.

Glycogen. Dried epithelial samples were extracted in 20% (v/v) sodium hydroxide. Glycogen was then precipitated with iced absolute ethanol and hydrolyzed in 2N sulfuric acid for 90 min. Glucose was measured by the hexokinase reaction. Glycogen is expressed as micromoles glucose per gram dry weight of tissue.11

Protein. Protein of the solubilized samples was measured by the Lowry method,12 with bovine serum albumin as the standard.

SDS-slab gel electrophoresis. Solubilized proteins from epithelial samples underwent electrophoresis by a modification of the techniques of Laemmli.13 Vertical polyacrylamide slab gels were constructed in a Bio-Rad Model 220 apparatus to contain 3.0% (w/v) acrylamide in the stacking gels and 8.0% (w/v) acrylamide in the running gels. Samples for electrophoresis were prepared to contain 75 μg of protein and were reduced with 500 μM dithiothreitol at 37° C for 10 min before application to the well.10 Electrophoresis was achieved with a pulsed constant power supply (Ortec 4100). After electrophoresis, the gel was fixed with 50% (v/v) trichloroacetic acid for 30 min, stained overnight with 0.1% (w/v) Coomassie blue in water/methanol/acetic acid (53:40:7), and destained with the same solution without dye.

Results

Observation of eyes immediately after surgery showed that eyes with wound type A (Fig. 1, A) had an approximately 1 mm-wide zone of goblet cell–free epithelium at the limbus, plus a ring of corneal epithelium about 2 mm wide remaining. Eyes that received wound type B (Fig. 1, B) had a 0.5 to 1 mm-wide area of goblet cell–free epithelium on the limbus, but no corneal epithelium. Eyes with wound type C (Fig. 1, C) had no epithelial cells on the corneal or limbal area. The epithelia that regenerated after the three dif-
Fig. 1. Clinical and histologic appearance of ocular surface immediately after mechanical scraping. The following epithelial defects had been made: 10 mm diameter central area of corneal epithelium (wound type A), the entire corneal epithelium up to just beyond the limboocorneal junction (wound type B), or the entire corneal epithelium plus limbal and bulbar conjunctival epithelium up to about 2 mm beyond the limboocorneal junction (wound type C). The dark area on the cornea in the clinical pictures (A1, B1, and C1) shows the epithelial defects stained with Richardson stain. The broken line in C1 demarcates the limboocorneal junction. The histologic pictures (A2, B2, and C2) are stained with PAS and hematoxylin. In A2, the arrow points to the beginning of the limbal epithelium. Goblet cells (gc) indicate the end of limbal epithelium and the beginning of bulbar conjunctival epithelium. The figure shows that there is about 1.0 mm goblet cell–free limbal epithelium in wound type A. In B2, the arrow points to the edge of the 0.5 to 1.0 mm goblet cell–free limbal epithelium remaining in wound type B. No epithelium is present on the limbus in wound type C. v, Vessels (In A2, B2, and C2, bar = 0.5 mm.)

Different wounds were defined as being of corneal, limbal, or bulbar conjunctival origin (Fig. 1). Usually epithelia regenerating from corneal, limbal, and bulbar conjunctival epithelia had completely covered the denuded cornea 2 to 3 days, 4 to 6 days, and 5 to 7 days after wounding, respectively.

Histologic analysis. Epithelia regenerated from corneal and limbal epithelia healed initially with one to three cell layers, then gradually and continuously increased in thickness to five to six cell layers and became light-microscopically normal. These epithelia developed no goblet cell appearance on the cornea during or after healing. On the other hand, as has been shown previously, epithe-
Hum derived from the bulbar conjunctiva was one to three cell layers thick immediately after healing and remained so until about 3 weeks after healing. Concurrently, there was a prominent goblet cell bloom between 5 days and 3 weeks after healing (Fig. 2). Fig. 3 shows the goblet cell counts of the regenerated epithelia on the cornea at intervals up to 21 days after healing. In epithelium regenerated from bulbar conjunctiva after both mechanical scraping and heptanol debridement, goblet cells appeared on the cornea 5 days after healing and gradually increased in frequency to a maximum density approximately 14 days after healing. Epithelium regenerated from limbal epithelium showed only zero to three goblet cells on the cornea at any time up to 17 days after healing (Fig. 3). Regenerated epithelium of corneal origin showed no goblet cells on the cornea at any time after healing.

**Biochemical analyses: glycogen.** At 2 weeks after healing there was no statistically significant difference in glycogen content between regenerated and normal corneal epithelium. Regenerated limbal epithelium had slightly less glycogen than did regenerated corneal epithelium (p < 0.05) or normal corneal epithelium (p < 0.005). Epithelium regenerated from bulbar conjunctiva also had less glycogen content than epithelia from cornea (p < 0.001) and limbus (p < 0.02) or than normal corneal epithelium (p < 0.001) (Table I).

**Protein profiles.** Fig. 4 shows the protein pattern of normal corneal epithelium and of...
epithelia regenerated from corneal, limbal, and bulbar conjunctival epithelia 10 days after healing. Regenerated epithelium of corneal origin had a protein pattern identical to that of normal corneal epithelium (tracks N and A). On the other hand, regenerated epithelium of bulbar conjunctival origin (Track C) showed two major bands (69,000 and 66,000 M.W.) and two minor bands (52,000 and 47,000 M.W.) that were not present in normal or regenerated corneal epithelium. In addition, there were significant changes in the intensity of three other bands (63,000, 58,000, and 55,000 M.W.). Epithelium regenerated from limbal epithelium was distinguishable from both corneal epithelium and bulbar conjunctival epithelium and showed most of the characteristic bands of both (Track B). That this epithelium was simply a mixture of corneal and bulbar conjunctival cells was ruled out by the absence of the 69,000 and 66,000 M.W. bands.

**Discussion**

This study clearly demonstrates that there are three distinct types of ocular surface epithelium and that there are marked differences between epithelia regenerating from these three types when they resurface a totally denuded cornea. Regenerating limbal epithelium acts more like regenerated corneal epithelium than bulbar conjunctival epithelium. Like corneal epithelium, regenerated limbal epithelium increases continuously in thickness after healing and shows no goblet cells at any healing stage. However, our biochemical analyses indicate that regenerated limbal epithelium is not identical to regenerated corneal epithelium and that it in fact has characteristics intermediate between corneal and bulbar conjunctival regenerated epithelia. For example, the glycogen content in epithelium regenerated from limbal cells was intermediate between that in epithelium regenerated from corneal and bulbar conjunctival cells. The protein pattern analysis also showed that the intensity of staining of the protein bands that fall between 47,000 and 69,000 M.W. (the bands that include keratin proteins) is significantly different in the three types of regenerated epithelia.

One question that arises, however, is whether the limbal epithelium is sufficient to cover the whole cornea or whether bulbar conjunctival cells contribute to the epithelial

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**Table I.** Glycogen content in regenerated ocular surface epithelium 14 days after healing

<table>
<thead>
<tr>
<th>Source of regenerated epithelium</th>
<th>No. of samples</th>
<th>Glycogen*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corneal epithelium</td>
<td>6</td>
<td>271.0 ± 8.7</td>
</tr>
<tr>
<td>Limbal epithelium</td>
<td>4</td>
<td>199.7 ± 31.3</td>
</tr>
<tr>
<td>Bulbar conjunctival epithelium</td>
<td>8</td>
<td>116.1 ± 12.1</td>
</tr>
<tr>
<td>Normal corneal epithelium</td>
<td>14</td>
<td>273.9 ± 6.1</td>
</tr>
</tbody>
</table>

*Average μM glucose per gram dry weight of epithelium ± S.E.M.

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**Fig. 3.** Goblet cell counts of the regenerated ocular surface epithelia on the cornea after healing. Number of goblet cells is the total number of goblet cells from limbus to limbus on a section of cornea taken at the greatest diameter of the sample. Regenerated epithelium of limbal origin after mechanical scraping (●), and regenerated epithelium of bulbar conjunctival origin after mechanical scraping (●) and after n-heptanol debridement (○) are shown. Each point is an average bracketed by the standard error of the mean. The number of samples is in parentheses.
Fig. 4. Protein pattern profile in regenerated epithelia 10 days after healing. The direction of electrophoresis was from top to bottom. Samples in the different tracks were: Std, standard proteins, including phosphorylase B (92,500 M.W.), bovine serum albumin (66,200 M.W.), ovalbumin (45,000 M.W.), and carbonic anhydrase (31,000 M.W.); N, normal corneal epithelium; A, regenerated epithelium of corneal origin; B, regenerated epithelium of limbal origin; C, regenerated epithelium of bulbar conjunctival origin. The reproducibility of these protein patterns was established by analyzing a minimum of three different tissue samples for each category. The migration of the standard proteins was analyzed by linear regression and the result was used to estimate the molecular weights as indicated at the right.

regeneration. By calculating the area of the whole convex corneal surface, and a 1 mm-wide limbal zone, one finds that the area of the limbal epithelium is only about 24% that of the whole cornea (Fig. 5 and Appendix). However, total coverage might still be possible, since migrating epithelial cells flatten and expand to many times their original size and length.

The fact that regeneration from the limbal epithelium with minimal or no conjunctival participation did occur is supported by two observations. First, there were no goblet cells in the limbal regenerated epithelium, and there probably would have been had bulbar conjunctival epithelium been responsible for re-epithelialization. Second, there are two major protein bands (69,000 and 66,000 M.W.) present in regenerated epithelium of bulbar conjunctival origin that are missing in regenerated epithelium of limbal origin. Although it is possible that these two bands might be serum contaminants, not true epithelial components, it is very unlikely when one considers the method of sample preparation, the reproducibility, and the absence of a gamma globulin band in the gel electrophoresis results.

Previous reports support our proposal that epithelium regenerated from limbus is different from that derived from cornea or conjunctiva. Thus Maumenee found that limbal epithelium healed like corneal epithelium. However, an electron microscopic study of regenerated limbal epithelium 4 to 6 days after denudation showed that the regenerating epithelial cells had some "conjunctival features." In addition, a recent report indicated that limbal, but not corneal, regenerated epithelial migration was specifically inhibited by steroids, showing differences in pharmacologic response between the regenerated epithelia.

Clinically, the experiments suggest that loss of the entire corneal epithelium may not be as devastating as previously thought, since regeneration from limbal cells seems to approximate regeneration from corneal cells. Loss of the limbal epithelium, on the other
hand, forcing healing from conjunctiva, introduces a different epithelial wound healing process. Several studies have shown that there are problems associated with healing cornea using conjunctival epithelium that do not exist if the cornea is covered with cells of corneal origin. It may be that the prognosis after chemical or thermal burn depends on the severity of damage not only to the corneal and conjunctival but also to the limbal epithelium.

There are clinical examples of corneal lesions that require a major contribution from limbal epithelium in re-epithelialization. Limbus-to-limbus epithelial debridement is now advocated in epikeratophakia. In these cases, epithelial abnormalities are frequent and require constant attention. Limbus-to-limbus keratectomy has recently been proposed as a surgical treatment for corneal epithelial basement-membrane dystrophy, again leaving the limbal area responsible for corneal coverage. The distinction between different types of ocular surface epithelium is therefore becoming increasingly important clinically, and one must be aware of the unique role of the limbal epithelium in superficial corneal wound healing.

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Appendix

Fig. 5 shows the schematic diagram that was used to estimate the convex area of the whole corneal epithelium (Ac) and that of the 1 mm-wide zone of limbal epithelium (Al). In this model, it is assumed that the radius of curvature of the limbus is the same as that of the cornea. The formula used to calculate the area of convex surface is

$$Ac = 2\pi r h_1$$
$$Al = 2\pi r h_2$$

From Fig. 5, B, it can be calculated that

$$h_1 = r (1 - \cos \theta_1)$$
$$h_2 = r (\cos \theta_1 - \cos \theta_2)$$

and

$$\theta_1 = \sin^{-1}a/r$$
$$\theta_2 = \theta_1 + b/r$$

Therefore, the area of the convex surface of the cornea (Ac) and of the limbus (Al) is

$$Ac = 2\pi r^2 (1 - \cos \theta_1)$$
$$Al = 2\pi r^2 (\cos \theta_1 - \cos \theta_2)$$

For a typical rabbit eye, $$r = 7.5 \text{ mm}$$, $$a = 6.5 \text{ mm}$$, and $$b = 1.0 \text{ mm}$$. Thus

$$Ac = 177.1 \text{ mm}^2$$
$$Al = 42.3 \text{ mm}^2$$

$$Al/Ac = 0.24$$

REFERENCES

4. Thoft RA, Friend J, and Murphy HS: Ocular surface


