Protein Synthesis During Corneal Epithelial Wound Healing

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Previous investigations have shown that corneal epithelium, migrating to cover a wound, synthesizes protein and glycoprotein at a faster rate than does normal stratified epithelium. The authors have found that the maximal rate of synthesis, as indicated by the incorporation of leucine and glucosamine, occurs 16 hr after wounding, 6 hr before wound closure. A comparison of total protein and protein synthesized during migration indicates that the increased synthesis is the result of the enhanced synthesis of many of the proteins present in unwounded epithelia. However, one protein band with a molecular weight of 110 K daltons was present to a much greater extent in migrating tissue than in normal epithelium. A time course analysis indicates that this band is apparent during migration and is not present either before wounding or 24 hr after wound closure. Invest Ophthalmol Vis Sci 27:1-7, 1986

Epithelial sheet migration is a component of wound healing, morphogenesis, and regeneration. Until recently, little information beyond morphologic description has been reported about the movement of an epithelial sheet. Gipson and Kiorpes devised an in vitro system that facilitates the analysis of biochemical events occurring during the migration of corneal epithelium. Using this system, they reported that (1) protein and glycoprotein synthesis as measured by [14C]leucine and [3H]glucosamine incorporation shows a large increase during epithelial sheet migration as compared to normal epithelium; (2) tunicamycin, an inhibitor of N-linked glycoprotein synthesis, slows wound healing and prevents complete wound closure; and (3) the cell surfaces of migrating epithelium bind more Concanavalin A and wheat germ agglutinin than do normal stratified epithelium, indicating that cell surface changes occur as the cells become migratory.

Using this in vitro system, we have attempted to ascertain the molecular nature of these changes in synthetic rates. Increased rates of protein and glycoprotein synthesis could presumably result from the synthesis of specific molecules necessary for migration, or could be the result of a higher rate of synthesis of the proteins found in normal stratified tissue. Only a few studies of the biosynthesis of proteins during epithelial migration have been reported. Kinoshita et al detected a 58 K dalton keratin-like protein in the epithelium that was apparent 1 day after wounding in rabbit corneas. Also, Jester et al noted that a keratin normally found in superficial cells was present in all cell layers following wounding. Their immunofluorescent study suggests that the basal cells may synthesize this keratin during migration. In this study we have found that while there is one distinct difference in the proteins present in normal versus migratory epithelium as determined by SDS-PAGE, the large increase in leucine and glucosamine incorporation is the result of the increased synthesis of many of the proteins found in normal stratified corneal epithelium.

Materials and Methods
The organ culture system used in this investigation has been described. Briefly, Sprague-Dawley rats

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with clear, healthy corneas were killed with an overdose of sodium pentobarbital. An epithelial wound 3 mm in diameter was created in situ by demarcating an area on the cornea with a 3-mm trephine and removing the epithelium within the circle with a small Bard-Parker blade scalpel. The eyes were then excised, and the corneas were removed and cultured. Unwounded corneas served as controls. Four corneas were cultured per 60 × 15 mm petri dish. For support during harvesting of epithelium, the corneas were pinned to rounded paraffin posts. All experimental techniques adhered to the ARVO Resolution on the Use of Animals in Research.

The corneas were cultured for time periods varying from 4 to 48 hr. Radiolabeled precursors were present for the final 3 hr of culture at a concentration of 2 μCi/ml for either D-[6-3H]glucosamine HCl or L-[4,5-3H]leucine (New England Nuclear, Boston, MA). When fluorography was performed, a concentration of 10 μCi/ml of radiolabel was used. Corneas were stained with Richardson's stain, which revealed the remaining defect. To harvest migrating epithelium, the 3-mm trephine was placed on the cornea concentric to the defect, and the migrating epithelium was removed with the small scalpel. For controls, epithelium was removed from a central area of unwounded corneas. The epithelium was washed in 7.5% TCA and digested in 0.2 N NaOH. Aliquots were counted for radioactivity in Aquasol II (New England Nuclear) using a Beckman LS-8100 (Beckman Instruments; Palo Alto, CA 94304). Protein was determined by the Bradford dye-binding method using bovine serum albumin as the standard.

To determine if cell proliferation had an influence on the incorporation of [3H]leucine, corneas were cultured in the presence of 400 μg/ml of colchicine (Sigma Chemical; St. Louis, MO 63178). Colchicine inhibits the formation of microtubules, which are necessary for mitosis. At this concentration Gipson et al. found that epithelial migration was slightly slowed. Corneas were wounded and placed into culture as described above with the addition of colchicine to the medium. Three hours before harvesting [3H]leucine (2 μCi/ml) was

**Fig. 1.** Incorporation of [3H]glucosamine or [3H]leucine into TCA-precipitable material. Migrating epithelium — ● — control — ○ — (unwounded corneas). Results presented as mean ± standard error of mean. N > 4 for all time points.
added to the medium. The epithelium was harvested 16 hr after wounding and processed.

Epithelium analyzed by SDS-polyacrylamide gel electrophoresis was digested in a reaction mix containing 91 mM dithiothreitol, 2 M Urea, 1% SDS and 16 mM Tris-HCl, pH 8.3. The digested tissue was electrophoresed using the buffer system described by Miles Laboratories. The gels were fixed in 50% methanol, and protein was detected with ammonium silver reagent as described by Wray et al., except that incubation times were doubled. To determine which proteins were newly synthesized, fluorography was employed as described by New England Nuclear. Slab gels were fixed in 10% TCA, 10% glacial acetic acid and 30% methanol, incubated in 100 ml of EN3HANCE® (NEN), precipitated in cold water and dried with a slab gel dryer (Biorad Laboratories; Richmond, CA). The dried gels were placed against Kodak XAR-5 film (Eastman Kodak; Rochester, NY), which was exposed at -80°C for 7 or 14 days. The x-ray film was subsequently developed using Kodak GBX developer (Eastman Kodak; Rochester, NY). Standard proteins used to determine molecular weights of unknown components were myosin 200,000 daltons, β-galactosidase 116,000, phosphorylase B 93,000, bovine serum albumin 67,000, and ovalbumin 43,000.

Autoradiography was conducted to determine the site of leucine incorporation. Corneas were wounded as described earlier and allowed to heal for 13 hr in culture. L-[4,5-3H]leucine was added to a final concentration of 2 μCi/ml, and corneas were incubated for an additional 3 hr. After 16 hr total incubation time, the corneas were washed extensively with Ringer's salt solution and fixed for 1 hr in 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). The tissues were dehydrated and embedded in methacrylate, as described by DuPont. Tissue sections, 3 μm thick, were dipped in Kodak NTB-2 emulsion, dried, and exposed at -20°C for 7 or 14 days. The slides were developed for 4 min in Kodak D-19 and post-stained with hematoxylin and eosin. Unwounded corneas were processed identically and served as controls.

**Table 1. Effect of colchicine on the incorporation of [3H]leucine into TCA-precipitable protein**

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>Colchicine-treated</th>
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<tr>
<td>Wound</td>
<td>1312 ± 72</td>
<td>1305 ± 290</td>
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<tr>
<td>Wound periphery†</td>
<td>374 ± 57</td>
<td>414 ± 33</td>
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<tr>
<td>Normal</td>
<td>88 ± 7</td>
<td>124 ± 16</td>
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* Values are indicated as xSEM, N = 4.
† Wound periphery = epithelium harvested from outside original 3-mm wound area.

closure. After 16 hr the levels of incorporation gradually decrease.

To determine if the increase in incorporation was the result of synthesis by new cells, colchicine was added to the culture medium to block mitosis. At the concentration of 400 μg/ml, colchicine did not appear to affect the incorporation of [3H]leucine (Table 1). No differences in incorporation were seen between colchicine treated and untreated epithelium.

**Fig. 4. SDS-polyacrylamide gel of corneal epithelial proteins. (A)** Original epithelium removed to create wound. (B) Epithelium removed after 16 hr in culture (no wound). (C) Migrating epithelium harvested 16 hr after wounding. Arrow indicates 110 K polypeptide. Each lane represents approximately 10 μg of protein. Gels were stained for protein as described in methods. Standard molecular weights are indicated as 10^3 daltons.
To determine if the peak in incorporation was the result of the synthesis of new proteins involved in migration or the increase in the synthesis of the proteins normally found in stratified corneal epithelium, an analysis was made using SDS-gel electrophoresis. The results appear in Figure 2. One difference was apparent between the migrating tissue and the controls: a protein band present at 110 K daltons appeared in the migrating epithelium that did not appear in either normal epithelium or unwounded epithelium maintained in culture.

Since the silver stain did not specifically reveal proteins synthesized during migration, newly synthesized molecules were detected with fluorography following radiolabeling with leucine. When migrating epithelium was analyzed 16 hr after wounding, many proteins had incorporated leucine (Fig. 3). The bands incorporating the majority of the radiolabel were present in the 40-70-K area. The 110-K band detected using silver stain was radiolabeled at 16 hr. A time course analysis of migrating tissue indicated that this 110 K protein was not apparent 4 hr after wounding, but was present at 8, 12, 16, 20, and 24 hr after wounding (Fig. 4). The band again decreased in labeling at 48 hr, one day after wound closure.

In addition, migrating and normal stratified epithelium were radiolabeled with glucosamine (Fig. 5). Approximately seven glycoprotein bands were detected in normal stratified tissue in this analysis. A band present between 130-140 K daltons incorporated the majority of the $[^{3}H]$glucosamine. Migrating epithelium also incorporated radiolabel into seven bands. In addition, radiolabel was incorporated into a band migrating to approximately 110 K daltons.

Autoradiographic analysis of leucine incorporation during migration revealed a uniform distribution of silver grains over all the cells (Fig. 6A, B). Corneal epithelium migrates in a wedge-like configuration; the tip of the sheet is a single cell in thickness (Fig. 6A).
thickness of the sheet increases gradually to 2-3 cells (Fig. 6B) and finally to the normal 5-7-cell thickness. There was no discernible trend in the amount of silver grains present over the area of the sheets 1-3 cells thick; the number of grains seemed to decrease slightly in cells further away from the tip of the migrating sheet. Unwounded corneas did not contain as many silver grains as did healing corneas (Fig. 6C), which agrees with the biochemical analysis indicating a greater level of incorporation in migrating tissue.

Discussion

The technique for harvesting migrating corneal epithelium devised by Gipson and Kiorpes\textsuperscript{7} has allowed biochemical analysis of proteins synthesized during epithelial migration. Although Gibbins\textsuperscript{20} and DiPasquale\textsuperscript{21} argued that protein synthesis is not necessary for the initiation of cell migration, Gibbins found that cycloheximide blocked continued migration of an epithelial sheet. It seems reasonable that protein synthesis is necessary for extended migration. Indeed, protein and glycoprotein synthesis, as indicated by leucine and glucosamine incorporation, increase dramatically during wound healing. Also, tunicamycin blocks wound closure,\textsuperscript{8} indicating that asparagine-linked glycoprotein synthesis is necessary for the continued migration of corneal epithelium.

The maximum increase in leucine and glucosamine incorporation occurred 16 hr after wounding, and 6 hr before wound closure. It is not known why the peak in incorporation occurs at this point; it is possible, however, that the need for new proteins is high, and the wound size has not decreased enough to increase the level of inhibition of synthesis that may occur with contact inhibition. It is interesting that the N-linked glycoprotein synthesis inhibitor, tunicamycin, blocks wound healing at about this same time point,\textsuperscript{8} indicating that asparagine-linked glycoprotein synthesis is necessary for the continued migration of corneal epithelium.

The increase in protein and glycoprotein synthesis in migrating epithelium is apparently the result of the de novo synthesis of many of the molecules found in normal, unwounded stratified corneal epithelium. A comparison of total protein (Fig. 2) and newly synthesized protein (Fig. 3) indicates that proteins in the 40-70 K dalton range form the major portion of both total and newly synthesized molecules. The separation technique used does not allow the assaying of changes other than molecular weight. To determine charge or carbohydrate alteration, more sensitive techniques, such as two-dimensional gels, must be used. Although the 110-K band is more apparent during migration (Fig. 4), its relative intensity suggests that it alone cannot cause the dramatic increase in leucine incorporation. The cells may require more of the proteins found in normal stratified epithelium for the migration process, or migration may be a period of rapid protein turnover with a subsequent rise in biosynthesis.

Radiolabelling with glucosamine reveals results similar to those of the leucine experiments. Again, the only major difference is that the 110-K band is present to a greater extent in migrating tissue. Because of this band's intensity, it is unlikely that it can account for the total increase in glucosamine incorporation. The increase may be the result of normal glycoproteins incorporating more glucosamine moieties during migration. This is consistent with the finding that Concanavalin A and wheat germ agglutinin show greater affinity for cell surfaces of migrating epithelium than do normal stratified tissue.\textsuperscript{9} However, the increase in incorporation could be the result of the increased synthesis of glycoproteins found in stratified tissue.

An increase in protein and glycoprotein synthesis could reflect contributions by pre-existing migrating cells and/or synthesis by new cells moving into the wound area. Two lines of evidence suggest that cell proliferation plays only a minimal role in the increased
incorporation of leucine and glucosamine. First, colchicine did not appear to lower the amount of \[^3\text{H}\]leucine incorporation (Table 1), as would be expected if extensive mitosis were occurring. Second, the autoradiographic data suggest that the protein synthesis is not the result of cell division (Fig. 6). These results show a fairly uniform incorporation of leucine over the migrating cells. If the increase in leucine incorporation were the result of mitosis, a preferential labelling of the basal cells would be observed. The \[^3\text{H}\]leucine is only present for 3 hr, which is not enough time for the cells to move extensively out of the basal layer. This observation supports other work demonstrating that colchicine does not inhibit movement of the epithelial sheet.\textsuperscript{15,21,22}

One of the most intriguing results of this study is the appearance of a band migrating to approximately 110 K daltons, which was labeled with both leucine and glucosamine, suggesting a glycoprotein structure. Although the identity of this molecule is unknown, an appealing possibility is that the glycoprotein is associated with the migratory process. Another possibility is that the 110-K dalton glycoprotein could be a molecule normally secreted into the underlying stroma, which accumulated during migration when the epithelium was not in close contact with the basement...
membrane. This possibility is suggested by the findings of Alper,23 who isolated a glycoprotein complex from bovine corneal stroma and found several components, one of which was approximately 110 K. A molecule was found in rat stroma that comigrated with the band seen in migrating epithelium (unpublished results).

Kinoshita et al10 reported that a 58-K keratin-like protein was present in migrating rabbit corneal epithelium which was not present in stationary epithelium. In these experiments, a 10-mm wound was created with n-heptanol. We did not detect any changes in the keratin region (40–70 K) in our experiments using a 3-mm wound in rats. This contradiction might be explained by species variation. Another possibility is that the conjunctival epithelium may migrate into the wound area when a large wound is created. Kinoshita et al10 report that a major band was present at 58 K in rabbit conjunctival epithelium. Whereas we did not detect any new bands between 40 and 70 K in the wound area, a new band was detected when epithelium from the wound periphery was analyzed. This band at 48 K was similar to a major keratin band in the rat conjunctival epithelium. These data suggest that conjunctival epithelium may migrate into the corneal epithelial area following a wound.

Key words: corneal epithelium, protein synthesis, migration, wound healing, 110 K dalton glycoprotein

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References