Biosynthetic Responses of the Rabbit Cornea to a Keratectomy Wound

James D. Zieske,*† Sheila C. Higashijima,* Sandra J. Spurr-Michaud,* and Ilene K. Gipson*t

Following a corneal wound involving removal of the epithelium and basement membrane, the epithelium must migrate across bare stroma. To examine the effect of the removal of the basement membrane on protein and glycoprotein synthesis in both the epithelium and stroma, we performed superficial keratectomies on rabbits and allowed the corneas to heal in organ culture. We then analyzed the following parameters: (1) rate of epithelial wound closure; (2) proteins synthesized during epithelial wound closure in both the epithelium and stroma using SDS-PAGE; and (3) presence of fibronectin in the epithelium and stroma using immunodot blots and immunofluorescence. We found that: (1) a 7 mm keratectomy wound heals in 66 hr with a maximal rate of epithelial migration of 0.83 mm²/hr; (2) four proteins, 400+K, 220K, 70K, and 58K, are present in the epithelium migrating to close the wound that are not seen in the control epithelium; (3) a 220K band is seen in the wounded stroma but not in control stroma; and (4) fibronectin represents 2% of the total protein in the stroma 66 hr post-keratectomy but less than 0.02% in wounded epithelium, unwounded epithelium, and unwounded stroma. Invest Ophthalmol Vis Sci 28:1668–1677, 1987

Following a corneal wound involving removal of epithelium, leaving the basement membrane intact, the wound is healed by the sliding of adjacent cells into the wound area followed by cell mitosis. The adhesion of the epithelium to the underlying stroma regains much of its normal strength within 1 week after wound closure. In contrast, following a superficial keratectomy in which the basement membrane zone is removed, 2 months or more are required before normal adhesive strength is regained. The epithelial cells must migrate to cover the injured area and then remain adherent to an abnormal surface during resynthesis of the components of the basement membrane zone.

The objective of our current study was to determine the initial biosynthetic responses of the epithelium and stromal cells following a wound involving removal of both epithelium and basement membrane where the epithelium must migrate over bare stroma to cover the wound. Previous studies have shown that keratins are synthesized in the rabbit epithelium following scrape waves and penetrating wounds. In addition, we have shown that a 110K and a 70K cell surface protein are synthesized in response to a scrape wound in the rat. Fujikawa et al. found, in rabbit, that fibronectin (FN) is deposited following both scrape and keratectomy wounds in vivo, and that laminin and bullous pemphigoid antigen were deposited following epithelial wound closure of keratectomies. FN has recently received much attention for its role in wound healing. It is a large glycoprotein (440,000 MW) found in both blood plasma and on the surface of certain cell types. FN is of particular interest because it has been shown to contain both a haptotactic domain and an adhesive domain. Thus, it could stimulate wound healing either by providing a matrix for the cells to adhere to after migration, or by actively being involved during migration as a haptotactic agent. FN has been shown to promote adhesion of human keratinocytes and neutrophils in vitro. It has also been reported to be involved in the migration of newt epidermis, mouse germ cells, chick neural crest cells, and sea urchin mesenchymal cells. In organ culture of rabbit corneal explants, FN is deposited on the cut stromal surface and enhances epithelial migration. FN does not appear under rabbit scrape wounds in vitro. In epidermis, Clark et al. using cross species transplants and species-specific antibodies to FN, demonstrated that following a wound FN is initially derived from the plasma and that the injured tissue synthesizes FN several days later.

We asked the questions: How do the corneal epithelial and stromal cells respond biosynthetically to a keratectomy wound where the epithelium migrates
over bare stroma? And, is FN synthesized in situ? To answer them, we performed superficial keratectomies, allowed the corneas to heal in culture, and examined healing rates, proteins synthesized following keratectomy, and dot blot analysis of FN in the epithelium and stroma. Our results indicated that: (1) the maximal healing rate is 0.83 mm²/hr over bare stroma in comparison to 0.86 mm²/hr over a scrape wound; (2) a 220K protein is present in the stroma; (3) four proteins are synthesized following wounding that are apparmounts than in unwounded epithelium.

Materials and Methods

Organ Culture

New Zealand rabbits were killed by action of sodium pentobarbital. Surgeries were then performed in situ. The cornea was demarcated with a trephine, the circular area was traced with a marker, and the stroma was cut with a microtome. The anterior portion of the stroma within the area was removed by pulling with the forceps, and the stroma was cut with a small scalpel. The resulting wound leaves a bare stroma with basement membrane removed, and the size created was 43.0 ± 1.02 mm². Wounds were made by demarcating an area with a 7 mm trephine and then scraping off the epithelium (loosely adherent during migration) within the area. The original 7 mm wound area was concentric to the defect, and the epithelium (loosely adherent during migration) was removed. The epithelial proteins were precipitated in 7.5% TCA and washed three times in 7.5% TCA. The TCA-precipitable material was then digested in 0.2 N NaOH, radioactive content was determined using Aquasol II in a Beckman (Palo Alto, CA) scintillation counter, and protein was determined by the method of Lowry.

Protein Radiolabeling

Cell surface epithelial proteins were radiolabeled using lactoperoxidase-catalyzed iodination. The epithelial sheets, removed with a small scalpel as described previously, were placed in 0.5 ml of phosphate-buffered saline (PBS). The following additions were then made: lactoperoxidase (40 U/ml), glucose oxidase (20 U/ml), glucose (5 mM), and NaI (400 μCi/ml) (ICN Radiochemical, Irvine, CA). The tissues were incubated for 30 min on ice with occasional shaking. The reaction was stopped by the addition of phosphate-buffered iodine (PBI). The epithelial sheets were washed five times in PBI and then pelleted through PBI containing 15% glycerol and 0.1 mM phenylmethyl sulfonyl fluoride. Omission of lactoperoxidase from the reaction mix resulted in incorporation of less than 5% as much 125I into the pellet compared with the amount when the enzyme was added. Sheets of control epithelium could not be obtained without disrupting the tissue since unwounded epithelium is tightly adherent.

Total cellular proteins were radiolabeled by adding [35S]-methionine (10 μCi/ml) (Amersham, Chicago, IL) to the culture medium 18 hr before harvesting of the epithelium. Stromal proteins were not isolated at a specific activity high enough to be used for fluorography.

SDS-PAGE and Fluorography

Stromal and epithelial proteins were prepared for electrophoresis by digesting the tissue in reaction mix
containing 16 mM Tris-HCl, pH 8.9, 25% glycerol, 1% SDS, and 91 mM dithiothreitol and heating at 80°C for 15 min. The samples were then applied to linear polyacrylamide gradient gels, 5–15%. The gels were run using the buffer system described by Can-alc06 and stained in 0.1% Coomassie blue R-250 in 25% methanol, 7.5% acetic acid.

Proteins radiolabeled with 35S were detected using EN3HANCE and fluorography as described by the manufacturer.27 Cell surface proteins labeled with 125I were detected using autoradiography. In both fluorography and autoradiography the dried gels were placed against Kodak X-omat X-ray film, exposed at –80°C for up to 1 week, and developed in Kodak GBX X-ray film developer.

Detection of FN on Nitrocellulose Paper

FN was assayed in stromal and epithelial protein samples by reacting goat anti-rabbit FN, IgG fraction (Cooper Biomedical, Malvern, PA) with protein samples immobilized on nitrocellulose paper (Bio-Rad, Richmond, CA). In this procedure,28 protein samples solubilized in SDS-PAGE reaction mix were applied to nitrocellulose paper using a Bio-dot apparatus (Bio-Rad). Up to 50 µg of total protein was applied in 100 µl of total volume. After the liquid filtered through the nitrocellulose paper, 100 µl of 1% BSA was added to each well to block nonspecific protein binding, followed by two washes with 200 µl of PBS. The Bio-dot apparatus was then taken apart and the nitrocellulose paper removed. The paper was then placed in a solution containing 5% BSA and agitated for 1 hr at room temperature. The BSA solution was removed, and the paper was incubated in 1% BSA containing anti-FN at a 1:200 dilution overnight at room temperature. The antibody solution was removed, and the nitrocellulose paper was incubated for 1 hr in a moist chamber. After a PBS wash, coverslips were mounted with a medium consisting of PBS, glycerol, and paraphenylenediamine.30 Negative control tissue sections (primary antibody omitted) were routinely run with every antibody-binding study. To ensure specificity, the antibody was preabsorbed as described earlier for nitrocellulose paper. The sections were viewed and photographed using a Zeiss photomicroscope III equipped for epi-illumination.

Electron Microscope Techniques

Rabbit corneas 30 or 66 hr post-keratectomy were fixed in half-strength Karnovsky’s fixative and processed routinely for transmission electron microscopy. All experimental techniques adhered to the ARVO Resolution on the Use of Animals in Research.

Results

Following keratectomy, the epithelial cells migrated into the wound area, moving first downward along the side of the wound and then outward into the 7 mm circular wound area (Fig. 1A). As the epithelial sheet migrated, the cells flattened and enlarged with the leading edge in most cases tapering down to a single cell layer similar to migration over a basement membrane. However, the shape of the leading edge showed greater variability than epithelium mi-
micrographs of cornea 66 hr after keratectomy. (A) Note that epithelium must migrate down the side of the wound before moving into the original 7 mm wound area (original magnification X220). (B) Note fibrillar bundles indicated by arrows (original magnification, X31,200.) (C) Note blebbing and extensive amounts of rough endoplasmic reticulum and polysomes. Arrows indicate amorphous material (original magnification, X31,200).

To examine the rate of epithelial migration over denuded stroma, we followed wound closure of a 7 mm keratectomy wound. The original size of the epithelial defect created was 43.0 ± 1.0 mm². The wound closed in approximately 66 hr (Fig. 2). In comparison, a 7 mm scrape wound, in which the basement membrane is left intact, closed in approximately 48 hr (Fig. 2). The overall rate of migration in keratectomized rabbit corneas was 0.65 mm²/hr with a rate of 0.83 mm²/hr from 18-48 hr post-wounding. The time period 0-18 hr post-wounding healed at a rate of 0.58 mm²/hr. In comparison, the overall rate of migration over a scrape wound was 0.79 mm²/hr with a rate of 0.86 mm²/hr 18-48 hr post-wounding.

Rates of protein and glycoprotein synthesis in the
Fig. 3. Incorporation of [14C]-leucine (black bars) and [3H]-glucosamine (white bars) by the epithelium migrating over keratectomy wound into TCA-precipitable material. DPM, disintegrations per min; Con, unwounded cornea maintained in culture 48 hr.

Fig. 4. 5–15% PAGE of proteins solubilized from control (C) and keratectomized stroma (66) 66 hr post-wounding. Control corneas were cultured 66 hr with no wound. Arrow indicates 220K band. Stain, Coomassie blue; 20 µg of protein/lane. Left, molecular weights (K daltons) determined from the following standards: myosin (200K daltons); β-galactosidase (116K daltons); phosphorylase B (92K daltons); bovine serum albumin (67K daltons); ovalbumin (43K daltons).

Fig. 5. 5–15% PAGE of proteins of control (C) epithelium and epithelium removed 30, 48, and 66 hr following keratectomy. 100,000 DPM of [35S]-labeled material were applied to each lane, representing 20–30 µg of total protein for the keratectomized samples and 100 µg of total protein in the control lane. Stain, Coomassie blue. Left, molecular weights determined from standard proteins (K daltons). DPM, disintegrations per minute.

Epithelium migrating over a keratectomy were monitored by assaying [14C]-leucine and [3H]-glucosamine incorporation into TCA-precipitable material (Fig. 3). Maximal incorporation occurred 30 hr after wounding; leucine incorporation was 16.6-fold higher than control, and glucosamine was 47.3-fold higher.

SDS-PAGE was run on stromal and epithelial digests to determine if polypeptide bands not present in control tissue were present following keratectomy. In the control stroma (Fig. 4) the two most prominent bands were ~100K and 120K. These bands co-migrated with purified type I collagen (not shown). The 100K and 120K bands were also seen in stroma isolated from corneas 66 hr post-keratectomy. In addition, a band at slightly over 200K appeared in increased amounts in the keratectomized corneas. This
molecular weight region is where FN would be expected, but other proteins such as dimers of type I collagen would also migrate here. In the epithelium a complex mixture of bands was seen. Only one band, at 58K, was easily detected in migrating epithelium 30, 48, and 66 hr post-keratectomy that was not seen in control epithelium (Fig. 5). Newly synthesized bands were identified in the epithelium using fluorography following [35S]-methionine labeling. In this procedure, four bands, ~400K, 220K, 70K, and 58K (Fig. 6), showed increased labeling in the migrating epithelium. Fluorography was performed on the stromal samples, but the specific activities were too low to give useful results.

Epithelial cell surface proteins were labeled using lactoperoxidase-catalyzed iodination (Fig. 7). At least ten distinct polypeptide bands were detected in epithelium 30 hr post-keratectomy. The bands showing the greatest radiolabel were 220K, 150K, and 55K. At 48 and 66 hr post-keratectomy, the band at 220K increased in relative amount to become the major radiolabeled polypeptide. Thus, polypeptide bands that run on SDS-PAGE to 220K were seen in both the epithelium and the stroma following keratectomy. Since FN, 220K in SDS-PAGE, has been reported as playing a role in the wound-healing process, we attempted to determine if the 220K band was FN.

To determine if FN was present in the epithelium or stroma, protein digests were applied to nitrocelullose paper and reacted with anti-FN (Fig. 8). The amount of samples applied is indicated in Table 1. No reaction with wound or control epithelium was seen even with the application of 50 μg of total protein, indicating that FN was present in the epithelium at a level of less than 0.02% of total protein. In the stroma, FN was present 18 hr after keratectomy at a level of ~0.05% of total protein. At 66 hr after keratectomy, the level of FN increased dramatically to ~2% of the total protein. Only minimal FN was present in unwounded control stroma samples.
Anti-FN was preabsorbed with FN and then reacted with immobilized samples on nitrocellulose paper (Fig. 9). No reaction was seen with FN or epithelial proteins.

Table 1. Samples applied to immunodot blot in Figure 8

As a control to ensure that epithelial proteins were not binding to FN and blocking antibody-binding sites, 0.5 μg of FN was added to a sample of epithelial proteins and then assayed for FN (Table 2). The presence of epithelial proteins did not decrease the intensity of the reaction of FN with anti-FN (Fig. 9). As a second control to ensure that epithelial proteins were not masking the presence of FN in the epithelium, Western immunoblots were done. Epithelial protein samples (66 hr post-keratectomy) with or without the addition of 0.2 μg of FN were separated with SDS-PAGE, transferred to nitrocellulose paper, and assayed for FN. No FN was detected in the epithelial sample (Fig. 10). The presence of epithelial proteins did not decrease the intensity of the reaction of FN with anti-FN (Fig. 10).

To further localize FN, anti-FN was applied to tissue sections. Large amounts of FN were detected in the stroma just beneath the migrating epithelium. This immunofluorescence began abruptly at the edge of the keratectomy wound (Fig. 11). No immunofluorescence was present in the epithelium. The most intense staining appeared about one-half of the distance from the leading edge to the original wound. FN was also present at 30 hr, but in lesser amounts.

Discussion

Following a keratectomy wound the epithelium migrates across bare stroma to close the wound. Our objective was to determine how the epithelial and stromal cells respond biosynthetically to this type of wound. We examined the time period from initial wounding to epithelial wound closure (66 hr).

Perhaps our most provocative finding is that FN is present in the stroma but is not detectable in the epithelium following keratectomy. Plasma, stromal cells, and epithelium have all been suggested as possible sites of supply or synthesis of FN post-wounding. Our findings strongly indicate that FN is synthesized in situ and suggest that the source is the keratocytes. This is based on immunofluorescence data (Fig. 11).

Table 1. Samples applied to immunodot blot in Figure 8

<table>
<thead>
<tr>
<th>Lane</th>
<th>Str</th>
<th>W</th>
<th>WP</th>
<th>Anti-FN 1:200 (μl)</th>
<th>FN (μg)</th>
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<tr>
<td>1</td>
<td>18 hr/10 μg</td>
<td>18 hr/50 μg</td>
<td>18 hr/50 μg</td>
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<td>1.00</td>
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<td>2</td>
<td>66 hr/10 μg</td>
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<td>30 hr/50 μg</td>
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<tr>
<td>3</td>
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<td>48 hr/50 μg</td>
<td>48 hr/50 μg</td>
<td>40</td>
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</tr>
<tr>
<td>4</td>
<td>18 hr/25 μg</td>
<td>66 hr/50 μg</td>
<td>66 hr/50 μg</td>
<td>30</td>
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<tr>
<td>5</td>
<td>66 hr/25 μg</td>
<td>Con/50 μg</td>
<td>Con/50 μg</td>
<td>20</td>
<td>0.20</td>
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<tr>
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<td>Blank</td>
<td>15</td>
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</tr>
<tr>
<td>7</td>
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<td>Blank</td>
<td>10</td>
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<tr>
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<td>Con/50 μg</td>
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<td>Blank</td>
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Str, stromal proteins; W, epithelial proteins from inside the 7 mm wound area; WP, epithelial proteins peripheral to the 7 mm wound area; FN, fibronectin; Con, control (66 hr, unwounded epithelium).
Table 2. Samples applied to immunodot blot in Figure 9

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<th>F</th>
</tr>
</thead>
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<tr>
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<td>50 µg Con</td>
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<td>1.00</td>
<td>50 µg Con</td>
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</tr>
<tr>
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<td>0.80</td>
<td>50 µg Con</td>
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<td>50 µg Con</td>
<td>1.00</td>
</tr>
<tr>
<td>3</td>
<td>0.60</td>
<td>0.5 µg FN</td>
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<td>0.5 µg FN</td>
<td>1.00</td>
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<tr>
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FN, human fibronectin; Con, control (66 hr unwounded epithelial tissue); BSA, bovine serum albumin.

FN is localized in the stroma with no epithelial staining above the basement membrane epithelial border, and immunodot blot data (Fig. 8) showing no detectable FN in the epithelium and FN making up at least 2% of the total protein in the stroma. Since FN binds to various proteins that could mask antigenic sites, two controls were done to determine whether FN was present in the epithelium but not detectable as a result of antigenic masking. First, FN was added to epithelial samples and compared to FN alone in immunodot blots (Fig. 9). The presence of epithelial proteins did not alter the intensity of the reaction of FN and anti-FN. Second, Western immunoblots demonstrated that FN could not be detected in epithelial samples (Fig. 10). Since SDS-PAGE should disrupt any binding of proteins to FN, antigenic masking should not occur. Both controls indicated that FN is not present at a detectable level in the epithelium. It is conceivable that FN could be synthesized in the epithelium and then rapidly moved into the stroma, but FN is present in over a 100-fold higher amount in the stroma than in the epithelium. Thus, the transport mechanism would have to be extremely efficient. Phan et al. reported that in transplant experiments of guinea pig epithelium on to rabbit stroma, only rabbit FN is produced. This agrees with our hypothesis that the stromal cells are the synthetic source in organ culture. In vivo, where the cornea is in contact with the tear film and can receive metabolites from plasma, the source of FN could be different. However, our results demonstrate that in controlled conditions the injured tissue itself is capable of synthesizing FN.

It has been suggested that the involvement of FN in the migration process lies in providing a track along which cells, via a FN receptor, can bind and move. Our data is in agreement with this hypothesis. After a wound, the stromal cells synthesize FN for the epithelial cells to bind as they migrate to cover the wound. However, our data indicate that FN is present at a much higher level at wound closure (66 hr) than at 18 hr, when rapid migration is occurring. This suggests that FN may also play a role in the adhesion of epithelium to the stroma during resynthesis of basement membrane components. It is also possible that in vivo plasma FN may be used for initial migration of the epithelium and that cellular FN is more involved in adhesion or migration of the fibroblasts.

Comparison of healing rates over intact basement membrane versus bare stroma indicates that the overall rate of wound closure is slower over a keratectomy wound. However, at 18–48 hr, the most linear portion of both healing curves, the migration rates are nearly identical over scrape and keratectomy wounds: 0.86 versus 0.83 mm²/hr. We could not de-
Fig. 10. (A) Blot of 5-15% PAGE: (W & FN) 25 μg of protein from epithelium removed 66 hr following keratectomy plus 0.2 μg of FN; (W) 25 μg of protein from epithelium removed 66 hr following keratectomy; and (FN) 0.2 μg of FN. Blot was stained with 0.1% naphthol blue-black in 45% methanol and 10% acetic acid to reveal total proteins present. Left, molecular weights determined from standard proteins (K daltons). (B) Immunoblot of 5-15% PAGE identical to A. Blot was reacted with anti-FN as described in Methods. Note: anti-FN is reactive against breakdown products of FN as well as 220K intact protein. Left, molecular weights determined from standard proteins (K daltons).

Fig. 11. Micrograph showing immunofluorescence of anti-FN on tissue section 66 hr after keratectomy. Large arrows indicate top of epithelium; small arrows, basement membrane (original magnification ×480). Unwounded cornea extends to left of open arrow with keratectomy area extending down and to the right.
we detected four polypeptides appearing in greater amounts in the keratectomized cornea than in control tissue (Fig. 6). None of these molecules has been positively identified, although the band at 58K is most likely a keratin, which agrees with Kinoshita's findings. The band at 70K is also seen in rat superficial scrape wounds and may be generally important in migration. The two large-MW bands are unidentified; the band around 220K is especially interesting because it becomes the major cell-surface molecule detected with the lactoperoxidase technique. Possible identities under examination are laminin and bullous pemphigoid antigen.

In summary, the rabbit cornea responds to a keratectomy wound by increasing protein and glycoprotein synthesis (Fig. 3), synthesizing FN in the stroma (Figs. 4, 8), and synthesizing four polypeptide bands in the epithelium, ~400K, 220K, 70K, and 58K.

**Key words:** biosynthesis, cornea, keratectomy, wound healing

**References**


