Basement Membrane Components in Healing Rabbit Corneal Epithelial Wounds: Immunofluorescence and Ultrastructural Studies

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ABSTRACT The nature of the substrate that supports epithelial migration in vivo is of interest, particularly with respect to mechanisms of wound healing. Immunofluorescence and electron microscopy were used to search for common substrate components in prototype rabbit corneal wounds: epithelial scrape wounds, in which the corneal or conjunctival epithelium migrated over the denuded lamina densa of the corneal basement membrane (CBM), and superficial keratectomy, in which the corneal epithelium migrated over a bare stroma without CBM. The corneal epithelium moved rapidly over the CBM or stroma to cover the defect within 2–3 d, whereas the conjunctival epithelium required 1–2 wk. In all wounds, fibronectin and fibrin/fibrinogen were deposited onto the bare surface within 8 h after wounding and persisted under the migrating epithelium until migration was complete. Bullous pemphigoid antigen (BPA), a normal component of the CBM, was removed with the epithelium upon scrape wounding and reappeared in the CBM after migration was completed. In contrast, the conjunctival epithelium had a continuous subepithelial band of BPA out to the migrating tip. Laminin, also a normal component of the CBM, was not removed in the scrape wounds, indicating that the region of least resistance to shear stress was between the BPA and laminin layers. Laminin was removed by superficial keratectomy and was not detectable under the leading edge of the migrating cells. Laminin and BPA were restored in the CBM by 2–4 wk. Type IV collagen could not be detected in normal CBM, but was conspicuously present in conjunctival basement membrane and in blood vessels. Focal bands of type IV collagen did appear in the newly synthesized CBM 2–4 wk after keratectomy. These results argue that BPA, laminin, and type IV collagen are not essential for the migration of corneal epithelium during wound healing and support the hypothesis that fibronectin and fibrin/fibrinogen are the common, perhaps the essential, components of the provisional matrix that serves as a substrate until the permanent attachment components are regenerated.

In normal corneal epithelium, the anchoring mechanism lies in the corneal basement membrane (CBM) where the basal epithelial cells contact the stromal substratum. This is analogous to the epidermal basement membrane, in which several components, including bullous pemphigoid antigen (BPA), laminin, and type IV collagen, have been found. Ultrastructural studies have localized BPA, a disulfide-linked, 220,000-dalton glycoprotein (1), to the lamina lucida, the electron-lucent region just below the basal cell plasma membrane (2). Type IV collagen has been localized to the lamina densa of the basal lamina (3), the electron-dense band below the lamina lucida that is closest to the underlying connective tissue. The localization of laminin is controversial; immunohistostructural studies have described laminin predominantly in the lamina lucida (4–6) or densa (7, 8). These three structural components are believed to be important in stable epithelial

Abbreviations used in this paper: BPA, bullous pemphigoid antigen; CBM, corneal basement membrane; Fg, fibrin/fibrinogen; Fn, fibronectin.
cell-substrate interactions because laminin promotes attachment of epithelial cells to type IV collagen in vitro (9), and patients with autoantibodies to BPA have detachment of basal epithelial cells from the lamina densa (1, 2). It has been suggested that BPA is important in the adherence of migrating skin epithelium when neither laminin nor type IV collagen is detectable (10–12).

We have recently shown by immunofluorescence that BPA is lost from the rabbit corneal wound surface when the epithelium is gently removed (13). Fibronectin (Fn) and fibrin/fibrinogen (Fg), which are not detectable in the normal CBM, deposit rapidly as a linear band on the wound surface. The epithelium migrated over this Fn/Fg surface in the absence of BPA, and we proposed that Fn and Fg serve as a provisional matrix that temporarily substitutes for other basement membrane components (reviewed in 14). The remaining CBM components, however, were not established.

We have, therefore, analyzed this and two other prototype corneal wounds for laminin and type IV collagen as well as BPA, Fn, and Fg, and correlated immunofluorescence observations with the ultrastructural features of the epithelial substrate.

MATERIALS AND METHODS

Rabbit Corneal Epithelial Wounds: 85 normal New Zealand albino rabbits weighing 2.5–3.0 kg were anesthetized intravenously with sodium pentobarbital and their eyes anesthetized topically with 1% proparacaine drops. Both corneas in each of 80 rabbits received one of three wounds (diagrammed in Fig. 1): (a) A partial corneal epithelial scrape wound was made in 70 proptosed eyes by scalpel debridement of an 8.5-mm-diam disc, demarcated in the central cornea by a trephine visualized through an operating microscope (Fig. 1a). (In this wound, the corneal epithelium migrated over an intact lamina densa; see Results.) (b) A complete corneal epithelial scrape wound was made in 45 eyes by scalpel debridement of the entire corneal epithelium including ~1 mm of the limbal conjunctival epithelium (Fig. 1a). In this wound, conjunctival epithelium migrated over the preserved lamina densa; (c) A partial superficial keratectomy wound was made on 45 eyes by surgical removal of the anterior third of the corneal stroma with attached epithelium in an 8.5-mm-diam disc initially demarcated by a trephine (Fig. 1c). (This wound resurfaced by corneal epithelial migrating over the bare stroma without a lamina densa; see Results.) Epithelial healing was documented by 1% methylene blue staining of the nonhealed area and by routine histology. Serial photography was performed on each animal.

Corneas were removed from rabbits killed at 1, 8, 22–24, 36, 52, and 72 h, and at 1, 2, and 4 wk after wounding. Ten normal corneas served as controls. Corneas for immunofluorescence studies were excised, frozen immediately, and quick-frozen in OCT compound (Ames Company, Elkhart, IN) for cryostat sectioning. Normal guinea pig corneas were similarly processed. Corneas for electron microscopic studies were fixed in situ with several drops of 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, on the ocular surface. The corneas were then excised and placed into this same fixative, then postfixed in 1% osmium tetroxide and embedded in Epon-Araldite.

Antisera: Anti-Fn antibodies were obtained from guinea pigs immunized with purified rabbit Fn and conjugated with fluorescein as described (13). Reactivity with Fn could be abolished by prior absorption of the antibodies with purified rabbit Fn, but not with Fn-free rabbit plasma. The fluoresceinated antibody did not stain cryostat sections of a rabbit fibrin clot.

Autoantibodies in the serum from a patient with bullous pemphigoid were used to detect BPA. This serum had a titer of 1:10,000 by indirect immunofluorescence against the basement membrane of normal human skin and cross-reacts with the other species tested (rabbit, guinea pig; 13).

Affinity-purified antibodies to laminin and to type IV collagen were generously supplied by Drs. J. R. Stanley and G. R. Martin (National Institutes of Health, Bethesda, MD), who had established antibody specificity using immunoelectron microscopy and the enzyme-linked immunosorbent assay. Fluoresceinated antibodies to rabbit Fg and IgG and albumin, guinea pig, sheep, and human IgG were obtained commercially (Cappel Laboratories, Cochransville, PA), and specificity was confirmed by Ouchterlony analysis and absorption studies (13).

Fluorescent Antibody Technique: 4-μm cryostat sections, used in all immunofluorescence studies, were washed with PBS and incubated in a moist chamber for 30 min at room temperature with one of the following fluoresceinated antibodies or antigen:

- Anti-Fn antibodies (1:8)
- Anti-BPA antibodies (1:10)
- Sheep antialbumin (1:160)
- Anti- Fn antibodies
- Human anti-Fn antibodies
- Anti-BPA antibodies

Sections were incubated for 30 min at room temperature, followed by fluoresceinated goat anti-rabbit IgG (1:10); and rhodamine-conjugated goat anti-human IgG (1:10). Sections were then washed in phosphate-buffered saline and mounted in glycerol (1:1) with 1% bovine serum albumin for examination by epifluorescence microscopy.

RESULTS

Ultrastucture of CBM and Epithelial Cells in Normal and Wounded Corneas

The CBM of the normal rabbit cornea is shown in Fig. 2a. After an epithelial scrape wound, the lamina densa (or basal
Figure 2 (a) CBM of normal rabbit. Note hemidesmosomes (H), lamina lucida (L), lamina densa (D), and anchoring fibrils (AF).

(b) Surface of a fresh corneal epithelial scrape wound. The lamina densa remains and is similar in structure to the unabraded cornea shown above, except that fewer anchoring fibrils (AF) are present in this section. × 43,920.

Laminin

Laminin was present in the normal rabbit cornea as a thin linear band in the epithelial basement membrane of the cornea and conjunctiva (Fig. 5a), in rare isolated strands throughout the stroma, and in Descemet's membrane. These stromal strands may be remnants of vessels left from embryogenesis (L. S. Fujikawa et al., unpublished results). The linear pattern of laminin staining in the CBM was indistinguishable from that of the BPA (Fig. 5b). Laminin was not removed in the scrape wounds (Fig. 6b). The wounded surface showed a delicate linear band of laminin that was continuous with the CBM of the peripheral intact epithelium and just as intense. As the epithelium migrated over the laminin layer during healing, the pattern of fluorescence remained unchanged. There was no difference between wounds healed by corneal or conjunctival epithelium.

Laminin was removed with the CBM by superficial keratectomy, as expected. Laminin retained its normal pattern in the CBM of the surrounding intact epithelium. As the epithelium migrated over the wound, no laminin was detectable under the leading edge or on the wound surface (Fig. 7a). Laminin first reappeared under the migrating cells at the wound margin behind the leading edge at 1–2 d, and became desmosomes (Fig. 4b). Even at 1 mo, however, segments of lamina densa were still missing.
Figure 3  (a) Migrating epithelium of conjunctival origin. Cells are from the leading edge of a cornea 36 h after complete scrape wound (total epithelial defect). Cells have characteristics of conjunctival cells, including undulating superficial surface and more rounded appearance, when compared with corneal epithelium. Note absence of hemidesmosomes under migrating cells. (b) Section through central cornea 2 wk after complete epithelial scrape wound. Note goblet cell and its granules (G), another indication that cells covering the wound are of conjunctival origin. Cells on the CBM have re-established hemidesmosomes (H). \( \times 9,390 \) (a); \( \times 23,925 \) (b).
FIGURE 4  (a) Cell migrating over keratectomized cornea. Note the absence of basal lamina. (b) Surface 1 wk after keratectomy. Note segments of newly synthesized lamina densa (arrows). (c) 2 wk after keratectomy. Epithelium has more prominent segments of lamina densa (arrows) and more hemidesmosomes (H). Fibroblasts with abundant rough endoplasmic reticulum are located below the epithelium in the zone of stromal restoration. \( \times 43,920 \) (a); \( \times 36,612 \) (b); \( \times 15,840 \) (c).
almost continuous over the wound surface several days after closure (Fig. 7c).

**Type IV Collagen**

Antibody to type IV collagen produced only faint (or no) fluorescence staining in the CBM of the normal rabbit corneas (Fig. 5c). Within the same tissue section, limbal blood vessels stained intensely for type IV collagen, and conjunctival basement membrane and Descemet's membrane stained moderately (Fig. 5c). These structures served as positive controls for each tissue section, and in each the CBM was negative or equivocably positive for type IV collagen. Similar results were obtained in normal guinea pig corneas. No change in type IV collagen occurred in any of the wounds, with one exception: samples taken 2–4 wk after keratectomy showed discontinuous, linear patches of type IV collagen in 10 to 20% of the cross-sectional length of CBM of the central healed wound (Fig. 8).

To determine whether the type IV antigens were masked in normal corneas by glycoproteins or glycosaminoglycans, the tissue sections were treated with dilute solutions of chondroitinase AC and/or trypsin, and then stained for type IV
collagen. No increase in the staining of the epithelial CBM was detectable.

**BPA**

In the normal rabbit cornea, BPA was present as a delicate linear band under the epithelium in the CBM (Fig. 5b) as previously reported (13). BPA was present in the normal conjunctival epithelial basement membrane in a similar pattern. In contrast to laminin, no BPA remained on the bare corneal surface after wounding (Fig. 6a), but the delicate linear band persisted, unchanged, beneath the surrounding intact epithelium. BPA was not detectable under the migrating corneal epithelium, although weak cytoplasmic staining was evident. BPA reappeared in the CBM after wound closure in

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**FIGURE 6** Surface of scrape wound at 22 h stained for BPA (a) or laminin (b). The scalpel scrape removes the BPA and leaves the laminin (and lamina densa) behind (compare with Fig. 2b). (c) Phase-contrast photograph of b; no cells are left. × 230.

**FIGURE 7** Superficial keratectomy wound at 48 h (a and b) and 4 wk (c and d) stained for laminin (a and c) and BPA (b and d). Laminin and BPA are not present on the bare stromal surface or under the migrating tip of epithelium at 48 h (a and b) but have both reappeared after migration is complete (c and d). × 230 (a and b); × 92 (c and d).
FIGURE 9  Total scrape at 48 h. A tongue of migrating conjunctival epithelium is seen that stains for BPA, both within the cytoplasm and under the cell (a). Same field, phase contrast (b). × 230.

FIGURE 8  Healed superficial keratectomy at 4 wk. Patchy bands of type IV collagen are seen in the CBM. These are not detected in the normal cornea (Fig. 5c). × 230.

Fg
Fibrinogen antigen, presumably in the form of fibrin, deposited on the surface of all types of wounds in a pattern similar to that of Fn (Fig. 10c). Within 1 h of wounding, a prominent layer of Fg appeared on the corneal surface and stained more intensely than Fn. The Fg layer remained until the epithelium had covered the wound, and then diminished, somewhat more rapidly than the Fn layer.

DISCUSSION
BPA, laminin, and type IV collagen have been implicated as attachment factors for sessile epithelial cells in vivo and in vitro (9, 10, 16). Our results indicate that corneal epithelium can migrate during wound healing without detectable BPA, laminin, or type IV collagen in the CBM (Table I). BPA was
Corneal wounds stained for fibronectin (Fn) (a, d, and e) or Fg (c). Superficial keratectomy at 48 h (a–c), and at 2 wk (e), and scrape wound at 22 h (d). The Fn is a fine band on the scrape surface (d) but extends more diffusively into the bare stroma (a), which is superficially coated with Fg (c). The epithelium migrates across this surface and can be seen on the right in b, a phase-contrast micrograph of the same field as a. After the epithelium covers the surface in 2–3 d, Fn accumulates in the stroma around the proliferating fibroblasts that fill in the stroma defect over the next several weeks (e). × 230 (a–c); × 92 (d and e).

**TABLE I**

Substrate of Normal and Wounded Corneal Epithelium

|                     | Fibronectin | Fibrin* | BPA | Laminin | Type IV collagen |
|---------------------|------------|---------|-----|---------|-----------------|
| Normal CBM          | —          | —       | +   | +       | —               |
| Wound Surface (1–8 h) |           |         |     |         |                 |
| Scrape              | +          | +       |     | +       |                 |
| Keratectomy         | +          | +       |     | —       |                 |
| Substrate under migrating epithelium (1–2 d) |         |         |     |         |                 |
| Scrape              |            |         |     |         |                 |
| Corneal (partial)   | +          | +       |     | —       | —               |
| Conjunctiva (total) | +          | +       |     | +       |                 |
| Keratectomy         | +          | +       |     | —       | —               |
| Restored CBM (2–4 wk) |           |         |     |         |                 |
| Scrape              | —          | —       | +   | +       | —               |
| Keratectomy         | +          | +       |     | +       | —               |

* Fibrinogen-related antigen, in the form of fibrin, fibrinogen, or large degradation products.
removed in all wounds, and, when corneally-derived epithelium healed the wound, BPA reappeared in the CBM several days after wound closure. Laminin was removed by keratectomy and was not detectable on the bare surface or under the migrating tongue of epithelium. These results suggest that BPA and laminin are regenerated in the basement membrane after epithelial cell migration is complete. We cannot exclude the possibility that these components are present in amounts too low to detect. Even with this reservation, our results show that the amount under the migrating cells is appreciably less than that of the normal CBM. BPA and laminin did return to normal in the basement membrane of healed corneas, and these glycoproteins probably assist in the adhesion of the relatively stationary epithelial layer to its normal substratum.

The diminished or undetectable levels of BPA, laminin, and type IV collagen in the epithelial CBM during wound healing imply that the factors responsible for adhesion of an epithelial cell layer to its substratum may be very different during wound healing, compared with the “normal” state. In all three types of corneal wounds studied, the epithelium migrated over a matrix of Fn/Fg deposited on the bare surface after wounding. This finding extends our earlier work (13) that identified Fn/Fg as components of a provisional matrix upon which the corneal epithelium traveled in vivo during healing of scrape wounds. Fn has also been noted under corneal epithelium in scrape wounds by others (17) and under healing rabbit (18) and guinea pig epidermis (12).

Fn enhances the migration of normal and transformed fibroblasts in vitro, presumably by increasing adhesion to the substratum (19). Whether this applies to epithelial cells on a collagenous extracellular matrix in vivo is unknown. There are conflicting data on whether Fn does (20, 21) or does not (9, 22, 23) promote epithelial cell attachment. Some of the variation in results may be related to the state of differentiation or conditions of isolation of the epithelial cells or the form of the Fn. In an elegant study of corneal epithelium in vitro, Fn in solution diminished basal blebs on corneal epithelium, as did type IV collagen and laminin (24).

The presence of Fn in all three types of wounds in our study, as well as under migrating epidermis (12, 18), suggests that, together with fibrin, Fn forms a provisional matrix to provide a temporary scaffolding that promotes epithelial migration and adhesion. The provisional substrate presumably corresponds to the amorphous, irregular coating on the lamina densa or bare stroma. Immunoelectron microscopic studies are underway to determine the relationship between the Fn/fibrin and the migrating cells. The molecular form of the Fn and the fibrinogen was not established by these studies. The fibrinogen is likely to be in the form of fibrin, although fibrils with characteristic periodicity, as seen in fibrin clots, were not found. The persistence of Fg and Fn on a tear-bathed surface suggests insolvency. Further work is required to determine whether this is due to cross-linking, multimer formation, or noncovalent binding with matrix components.

The distribution of BPA and laminin in the corneal epithelial scrape wounds illustrates their relationship within the CBM. Whereas laminin remained on the wounded surface with the lamina densa, BPA was removed with the epithelial cells. This indicates that the basement membrane is split at the level of the lamina lucida between the laminin and BPA. The BPA appears to be more associated with the cell layer and the laminin more firmly linked to the lamina densa. Whether these relative strengths of attachment relate to different planes in the CBM is not known. As noted above, laminin has been localized to the lamina lucida in some studies (4–6) and to the lamina densa in others (7–8). Our data do not discriminate between these possibilities, because the split could be within the lamina lucida itself. Immunoelectron microscopy studies in progress may clarify this relationship.

Our results do indicate that the region of the CBM of greatest sensitivity to shear stress lies between the BPA and laminin layers. This finding is consistent with earlier work in which the application of continuous suction to the skin produced epidermal-dermal separation in the lamina lucida (25, 26). A similar phenomenon is seen in treatment of skin in vitro with cold trypsinization (27, 28) and in epidermolysis bullosa latalis (29). In either case the epidermis separates from the dermis at the level of the lamina lucida; in epidermolysis bullosa latalis the split also is between the BPA and laminin layers (30).

Whether BPA is involved in cell attachment has not been proved, although it has been proposed, because antibodies to BPA cause detachment of epidermal cells in vitro (31), and detachment occurs in vivo in patients with these antibodies. The lack of BPA under the leading edge of corneal epithelium or as a continuous subepithelial band immediately after wound closure may be unique to corneal epithelium. Conjunctival-derived epithelium did demonstrate BPA in the basement membrane out to the migrating tip. In organ culture and in wounded skin samples processed the same way as the corneal samples, the leading edge of healing epidermis has BPA (10–12). This difference may be due to intrinsic differences in rates of BPA synthesis or cell migration. Corneal epithelium does migrate more rapidly than conjunctiva or epidermis, and this may exceed the ability of the cell to synthesize BPA.

Type IV collagen antigens were not detected in the CBM of the normal rabbit corneal epithelium or under the migrating epithelium. Ultrastructural studies have localized type IV collagen to the lamina densa of the epidermal basement membrane (3). Since the basement membrane of the cornea is similar ultrastructurally to that of the epidermis, we expected that the two would contain similar amounts of type IV collagen. However, little or no type IV collagen could be detected by immunofluorescence in normal CBM, whereas the same sections had bright staining for type IV collagen in conjunctival and vascular basement membranes. This does not appear to be restricted to the rabbit, since guinea pig and chicken (32) corneas show the same pattern, the latter with monoclonal antibodies. Human corneas have some, but less intense, staining for type IV collagen in the CBM compared with conjunctival basement membrane (33, 34). These observations suggest that the corneal lamina densa is different from the lamina densa of conjunctiva or skin. Type IV collagen was expressed in keratectomy wounds in newly synthesized CBM. Even though normal conjunctival epithelium displayed type IV collagen at the basement membrane, wounds that healed with conjunctival epithelium over a corneal lamina densa did not later acquire type IV collagen. This may indicate that the epithelium does not modify the lamina densa, at least with respect to type IV collagen. In contrast, the expression of type IV collagen was apparently stimulated by contact with the underlying bare stroma. Further study is needed to determine whether type IV collagen molecules are indeed absent from adult CBM or whether the antigenic sites are altered or

FUJIKAWA ET AL. Epithelial Substrate in Corneal Wounds 137
concealed by other molecules.

Migration of cells is a complex process, requiring both adhesion and controlled detachment as the cells move (35). The permanent basement membrane components may produce less reversible, firmer adhesion that is not optimal for cell movement. Although our studies do not rule out participation of other plasma (36, 37) or cellular adhesive molecules (38), they do provide strong circumstantial evidence that Fn and Fg are important, perhaps essential, components of the substrate used by migrating epithelial cells in vivo.

This research was supported in part by grants from the National Institutes of Health (CA-20822 and EY-03063) and Fight for Sight (G-658). Dr. Fujikawa was a recipient of a National Eye Institute Fellowship (F32-EY-05405).

Received for publication 20 May 1982, and in revised form 19 July 1983.

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