Adhesion Complex Formation After Small Keratectomy Wounds in the Cornea

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The adhesion complex of the corneal epithelium consists of the hemidesmosome and its associated structures, such as anchoring filaments, lamina densa of the basement membrane, and anchoring fibrils. It contributes to the adhesion of the corneal epithelium to Bowman’s layer. To understand the adhesion complex better, an electron microscopic and immunofluorescence analysis was done of the reformation of the adhesion complex in small (1 mm) keratectomy wounds in the guinea pig cornea. In these wounds, the epithelium, hemidesmosomes, basal lamina, anchoring fibrils, and anterior stroma were removed. The wound bed was epithelialized completely by 24 hr after wounding. Immunofluorescence analyses involved the use of antibodies against plaque components of the hemidesmosome, an antibody against laminin, and an antibody against the collagen VII component of anchoring fibrils. At 18 hr after wounding, there was no morphologic evidence of hemidesmosomes at the epithelial-stromal interface. At 24 hr, hemidesmosomes were observed, with or without subjacent lamina densa. Furthermore, plaque components were detected by immunofluorescence in those cells in contact with the wound bed. In contrast, no type VII collagen was detected. On day 7, collagen VII, laminin, and bullous pemphigoid autoantibody markers colocalized along the wound bed as determined by immunofluorescence. However, at the ultrastructural level, even though the lamina densa of the basal lamina was observed primarily where hemidesmosomes were present, it remained incomplete. In this study, the precise temporal sequence in which components are incorporated into the assembling adhesion complex was described during wound healing. Furthermore, the possibility that the hemidesmosomal plaque nucleates the formation of the underlying basal lamina was discussed. Invest Ophthalmol Vis Sci 33:304-313, 1992

The corneal epithelium adheres to the underlying stroma in part by the adhesion complex, consisting of a hemidesmosome, anchoring filaments that traverse the lamina lucida region of the basal lamina, and anchoring fibrils that arise at the lamina densa of the basal lamina and splay out into the stroma. On its cytoplasmic side, the hemidesmosome possesses a tripartite plaque. This plaque acts as the anchorage site of bundles of intermediate filaments (IF). The biochemical nature of the adhesion complex is beginning to be understood. For example, recent studies have revealed that two high molecular weight polypeptides (180 and 230 kD) recognized by autoantibodies in the serum of patients with the blistering disease bullous pemphigoid (BP) are located in the plaque of the hemidesmosome. Furthermore, both in the skin and cornea, it has been shown that the major component of anchoring fibrils is type VII collagen.

To study the assembly of hemidesmosomes, several experimental systems have been used. For example, subepidermal blisters were created and hemidesmosome reformation was monitored as epithelial cells repopulated the denuded basal lamina. Others used an in vitro system in which epithelial cell sheets were added back to denuded basal lamina. In this system, hemidesmosome reformation is rapid, and it has been proposed that hemidesmosome assembly is nucleated by the preexisting anchoring fibrils associated with the basal lamina. In models where epithelial cells repopulate connective tissue in the absence of basal lamina, there are conflicting reports concerning the temporal sequence of the reformation of the complete adhesion complex. Some authors found that hemidesmosomes, basal lamina, and anchoring fibrils appear concomitantly. Others report that the hemidesmosome plaque assem-
bles before the appearance of the lamina densa region of the basal lamina.\(^8\)

In previous studies, it has been shown that, in an in vitro model of wound healing, the plaque components of the hemidesmosome appear, in most instances, before the appearance of collagen VII.\(^9,10\) Furthermore, the lamina densa region of the basal lamina appears to form subsequently and immediately subjacent to forming hemidesmosomal plaque structures.\(^9\)

We therefore wished to determine whether this is also the case in vivo. We analyzed the reformation of hemidesmosomes, anchoring fibrils, and basal lamina in small (1 mm) keratectomy wounds in vivo in the guinea pig cornea by electron microscopy and immunofluorescence analysis using BP autoantibodies as markers for the hemidesmosome plaque, a monoclonal antibody against collagen VII, and an antibody against laminin.

Fig. 1. (A) Light micrograph of keratectomy wound immediately after wounding. (B) Light micrograph of keratectomy wound 24 hr after wounding (×1400).
Materials and Methods

Keratectomies

All guinea pigs were anesthetized with xylazine HCl (8 mg/kg) and ketamine HCl (120 mg/kg), in accordance with the ARVO Resolution on the Use of Animals in Research. Topical tetracaine 0.5% (four drops) was used to supplement the anesthetic. A 1-mm Elliot trephine (Storz, St. Louis, MO) was used for the keratotomy, and the keratectomy was completed with a sharp blade. Chloramphenicol ophthalmic ointment (0.5%) was placed on the eye, and the animals were allowed to recover. The animals were killed by an overdose of sodium pentobarbital at 18, 24, and 48 hr or 7 days, and the corneas were removed. The corneas were either frozen solid in liquid nitrogen, then embedded in OCT compound (Miles, Inc., Elkhart, IN) for immunofluorescence microscopic analysis, or fixed in 1.0% glutaraldehyde for electron microscopy.

Antibodies

Serum samples from patients with BP were provided by Nancy Furey, MD, Northwestern University Medical School (Chicago, IL). The anticollagen VII monoclonal antibody was characterized previously. Antilaminin antibody was purchased from Telios (San Diego, CA).

Light Microscopic Analysis

Cryostat sections were cut at 6 μm and placed on polylysine-coated (Sigma, St. Louis, MO) slides. Double-label immunofluorescence was done as detailed previously. In brief, sections on slides were fixed for 5 min in −20°C acetone and then air dried. A mixture of primary antibodies diluted in phosphate-buffered saline (PBS) was overlaid on the sections. The slides were incubated 1 hr at 37°C, washed in PBS, and overlaid with a mixture of appropriate secondary fluorochrome-conjugated antibodies. After washing in PBS, cover slips were placed over sections which were viewed on a Leitz Diaplan microscope (Ernst Leitz, Wetzlar GMBH, Germany) with epifluorescence.

Electron Microscopy

After fixation in 1.0% glutaraldehyde for at least 2 hr, the tissue was washed six times in PBS, placed in 1% osmium tetroxide in PBS for 90 min at room temp-
perature, and rinsed in distilled water three times for 1 minute each. The tissue was dehydrated in graded ethanols, and two 15-min changes of propylene oxide were completed. It then was placed in 1:1 propylene oxide–Epon/Araldite resin mix (Electron Microscopy Sciences, Fort Washington, PA) for 2 days at room temperature for infiltration. On day 2, the cap was loosened to allow propylene oxide to evaporate from the mixture. Final embedding was accomplished in 100% Epon/Araldite resin, and the tissue was placed in a 60°C dry oven for 48 hr.

Sections were cut at 1 μm on a Reichert-Jung Ultra-cut E microtome (Reichert, Buffalo, NY) and stained with toluidine blue. The wound was localized in the 1-μm sections and the block trimmed for thin-section localization. Ultrathin sections were cut at 80 nm, placed on 300-mesh copper grids, stained with uranyl acetate for 20 min and lead citrate for 1 min, rinsed in filtered distilled water, and viewed on a JEOL 100CX (Peabody, MA) electron microscope at 60 kV.

Results
Keratectomy wounds of 1 mm resulting in the removal of both epithelium and basal lamina were made in the cornea. At various times after wounding, 1-μm sections of Epon-embedded corneas were processed for light microscopic observation. Immediately after wounding, the normal corneal epithelium ends abruptly on either side of the wound site (Fig. 1A). However, within 24 hr, epithelial cells have repopulated the wound bed completely, and the cells already are stratified (Fig. 1B). There is some evidence of inflammation in the wound site (Fig. 1B). No obvious differences were seen between wounds at 24 hr and 7 days at the light microscopic level of resolution (results not shown).

Wound Healing at 18 Hr
At 18 hr after wounding, where epithelial cells had moved over the wound bed, no obvious hemidesmosomes or basal lamina were observed along the area of

Fig. 3. Immunofluorescence analyses of cryostat sections of wounded corneas 18 hr postkeratectomy. (A) and (B) are double-labeled with collagen VII antibodies and BP autoantibodies, respectively. (C) and (D) are double-labeled with laminin antibodies and BP autoantibodies, respectively. No staining is observed along the wound bed with collagen type VII antibody preparations (A), or laminin (C), and very little staining is seen with BP (B, D, closed arrows). However, these antibodies generate linear staining along the epithelial–stromal (E = epithelial, S = stroma) interface of the unwounded ocular surface (open arrows) (original magnification ×1200).
epithelial–stromal interface as determined by electron microscopy (Fig. 2). The IF appear disorganized and did not associate obviously with the basal cell surface (Fig. 2). Those collagen fibers in the stroma that abutted the basal surface of the epithelial cell were aligned perpendicular to the epithelial–stromal interface (Fig. 2). Double-label immunofluorescence analyses of cryostat sections of wounded corneas show that, at 18 hr postkeratectomy, little staining was observed along the wound bed with BP, laminin, and collagen type VII antibody preparations, although these antibodies caused linear staining along the epithelial–stromal interface of the unwounded ocular surface (Fig. 3).

Wound Healing at 24 Hr

Hemidesmosomal plaque structures were present along the epithelial–stromal interface of the wound at 24 hr postkeratectomy (Fig. 4). The IF were associated with these electron-dense plaques (Fig. 4A). In some instances, collagen fibers in the stroma were aligned perpendicular to the hemidesmosomes in the epithelial cells in the absence of any obvious basal lamina (Fig. 4A). However, we also have observed lamina densa immediately subjacent to hemidesmosomes in other areas of the same wound (Fig. 4B). In the latter, the hemidesmosomes were similar in morphology to those seen in normal areas; they possessed a tripartite plaque to which organized bundles of IF were attached (Fig. 4B).

Double-label immunofluorescence observations of corneas at 24 hr after wounding revealed that BP antigens occurred along the wound bed; no staining was detected in this region using the collagen VII or laminin antibody preparations (Fig. 5).

![Fig. 4. Transmission electron micrograph of the wound bed at the epithelial–stromal interface 24 hr after wounding. (A) Hemidesmosomes are present with their sub-basal dense plate, but there is no obvious basal lamina (arrows) IF, intermediate filaments (original magnification X63,000).](downloaded from iovs.arvojournals.org on 07/30/2019)
Fig. 4. (B). The lamina densa of the basal lamina is immediately subjacent to each hemidesmosome (arrows). Note that there are no areas of lamina densa without overlying hemidesmosomes. The hemidesmosomes have a tripartite plaque structure to which intermediate filaments (IF) attach (original magnification X30,000).

Wound Healing at 48 Hr

Hemidesmosomes with an underlying lamina densa were observed along the region of epithelial-stromal interface at 48 hr after wounding (results not shown); this was similar to that observed at 24 hr (Fig. 4B). Double-label immunofluorescence analyses of wounded corneas revealed that, along the epithelial-stromal interface of the wound bed, both BP and laminin antibodies colocalized. No staining was observed using the collagen type VII antibody preparation (Fig. 6).

Wound Healing at 7 Days

The epithelial–stromal interface 7 days after keratectomy did not differ obviously from that observed in corneas 24 hr after wounding (Fig. 4B). There were many areas where the lamina densa region of the basal lamina was discontinuous and only occurred immediately underlying hemidesmosomes (results not shown).

Double-label immunofluorescence of the wound site on day 7 revealed that BP autoantibodies and collagen VII antibodies now caused similar punctate staining patterns along the wound bed (Figs. 7A–B). By contrast, although laminin colocalized with BP antigens along the epithelial–stromal interface of the wound bed, the laminin antibodies also caused a diffuse stain extending into the stroma immediately underlying the wound bed (Figs. 7C–D).

Discussion

We did a detailed immunofluorescence and electron microscopic analysis of assembly of the adhesion complex in small (1 mm) keratectomy wounds in the guinea pig cornea. In such small wounds, reepithelialization occurs much more rapidly than in the large (7 mm) keratectomy wounds in rabbit cornea used by other workers in comparable studies.7 In the larger wounds, reepithelialization is not complete at 48 hr even though hemidesmosome assembly has begun.
Analyses of adhesion complex formation in such wounds is complicated by the possibility that epithelial cells are still migratory. In our study, we were able to correlate precisely the appearance of adhesion complex components immunocytochemically with the appearance of hemidesmosomes at the ultrastructural level in cells that were not migratory because the wound site was covered completely.

Our results showed that, during the reepithelialization of 1-mm keratectomy wounds in vivo, hemidesmosomal plaque components appeared at the epithelial-stromal interface of the wound within 24 hr. At approximately the same time, hemidesmosomal plaques could be observed ultrastructurally simultaneously with the appearance of BP antigen. Laminin occurs along the epithelial-stromal interface of the wound bed at 24–48 hr. This contrasts with the results obtained recently using an in vitro model of corneal wound healing in the cow where the appearance of laminin preceded that of hemidesmosomal plaque components. It is possible that in vitro models may not simulate entirely the in vivo situation. For example, differences between in vivo and in vitro models may reflect the inflammatory response seen in vivo. The latter may result in degradation of certain extracellular matrix proteins by specific metalloproteinases. Alternatively, there may be differences between wound healing in guinea pig compared with cow ocular tissue (which are distinct morphologically).

Our immunocytochemical results show that hemidesmosomal plaque components appear before collagen VII during adhesion complex formation. These results differ from those presented for wound healing in the rabbit cornea where hemidesmosomal plaque components are claimed to appear simultaneously.
with collagen VII. However, these authors did not study the earliest stages of hemidesmosome assembly (ie, 18-24 hr after wounding) as we did but rather presented data for adhesion complex formation at 48 hr, a time when newly formed hemidesmosomes and underlying basal lamina already can be observed ultrastructurally.

Others suggested that anchoring fibrils appear to nucleate the assembly of the hemidesmosome in a recombination model where corneal epithelium is reassociated with denuded stroma with an intact basal lamina. However, in our study, the appearance of collagen type VII, an anchoring fibril component, followed the appearance of both plaque components of the hemidesmosome and laminin. Therefore, we found no evidence that anchoring fibrils nucleate hemidesmosome assembly. We are tempted to explain these results by hypothesizing that, if the basal lamina is present, hemidesmosome reformation can be nucleated by preexisting anchoring fibrils. However, if the basal lamina (and anchoring fibrils) is absent, as is the case in a keratectomy wound, hemidesmosomes form first with later anchoring fibril formation. Recent evidence suggests that collagen VII is not required for the development of hemidesmosomes. It was shown that sheep with recessive dystrophic epidermolysis bullosa do not possess collagen VII, yet still have morphologically intact hemidesmosomes. The possibility of hemidesmosomes being laid down first, followed by basal lamina, is attractive because our ultrastructural studies revealed that the lamina densa region of the basal lamina appeared immediately underlying hemidesmosomal plaques during wound healing. We suggest a role for hemidesmosomes in organizing or nucleating formation of the basal lamina and its associated structures, such as anchoring fibrils. This hypothesis is supported by several reports in the literature. For example, it was found...
Fig. 7. Immunofluorescence analysis of the wound at 7 days. (A) and (B) are double-labeled with collagen VII antibodies and BP autoantibodies, respectively. (C) and (D) are double-labeled with laminin antibodies and BP autoantibodies, respectively. In (A), collagen VII antibodies generate intermittent staining along the interface between the stroma (S) and the epithelium (E) in the region of the wound (closed arrows). In the same section shown in (B), BP autoantibodies show intense staining all along the same interface (solid arrows). (C) Laminin contrasts with the other antibodies since it is deposited diffusely in the stroma underneath the wound bed (solid arrows). Compare this with the linear deposition of BP antigens shown in the same section (D, solid arrows) (original magnification ×1200).

that basal lamina formed under the hemidesmosomes when human epidermis was recombined with human dermis devoid of its basal lamina. This sequence does not occur during development; therefore, there are differences between development and healing.

In summary, we described the sequence of incorporation of three components into the assembling adhesion complex. Because recent studies have shown that some extracellular matrix receptors belonging to the integrin family occur in the hemidesmosome, it will be interesting to assess the role these receptors play in hemidesmosome assembly.

Key words: cornea, wound healing, hemidesmosome, adhesion complex

References


