Reassembly of the Anchoring Structures of the Corneal Epithelium during Wound Repair in the Rabbit

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Reappearance of the structures involved in adhesion of the corneal epithelium to the stroma was studied in healing 7 mm keratectomy wounds in rabbit corneas. Corneas were taken at 48 and 66 hr, 1, 2, 3, 4, 6 and 8 weeks, and 4, 6 and 12 months post-wounding. Immunolocalization of bullous pemphigoid antigen (BPA), laminin and type VII collagen was used to determine time and sequence of appearance of hemidesmosomes, basement membrane and anchoring fibrils, respectively. Electron micrographs from three regions in the wound were used to correlate the immunohistochemical data and to quantitate the increase in basal cell membrane occupied by hemidesmosomes and the increase in basement membrane over healing time. Evidence of resynthesis of the adhesion structures was present at the wound margin before epithelial wound closure (48 hr). BPA, laminin and type VII collagen co-localized, indicating that hemidesmosomes, basement membrane and anchoring fibrils reappeared synchronously. Reappearance of the structures proceeded from wound margin to the center, and by 1 week BPA, laminin, and type VII collagen were present in discontinuous segments across the wound. From 2 weeks to 6 months, the segments became more continuous, and anchoring fibril networks were discerned at 4 weeks. Strata of type VII collagen and laminin were present within the newly synthesized stromal matrix at wound margin at 1 week, continuous across the wound bed by 2–4 weeks, and still present at 6 months; however, at 12 months, only a few strata of type VII collagen were present below the basement membrane at wound center. The amount of basement membrane increased steadily over time; duplications and discontinuities were present at 6 and 12 months. The amount of basement membrane present at 6 and 12 months was greater than in unwounded controls. The percentage of basal cell membrane occupied by hemidesmosomes increased until 6 weeks, then decreased and remained constant. At no time point did hemidesmosome values reach that found in unwounded controls. These data indicate that reassembly of the adhesion structures does not reach the normal unwounded state by 12 months post-wounding. Invest Ophthalmol Vis Sci 30:425–434, 1989

Wounds on the ocular surface can result in recurrent epithelial erosions and persistent epithelial defects. The fact that initial epithelial wound closure often occurs in such wounds, with subsequent sloughing of the epithelium from the underlying stroma, suggests that repair of the adhesion structures may be at fault. Few of the studies of corneal epithelial wound healing focus on repair of the adhesion structures. Khodadoust et al in 1968 described the adhesion of regenerating epithelium on experimental scrape and keratectomy wounds in rabbits. This study demonstrated that reuse of the denuded basement membrane by epithelial healing of a scrape wound resulted in more rapid tight adhesion of the epithelium as compared with healing on a keratectomy wound bed. This study also was one of the first to publish electron micrographs demonstrating segmental reappearance of the basement membrane on the keratectomy wound surface. The authors reported that “months” were required for total reappearance of the basement membrane. More recently, Fujikawa et al studied by electron microscopic and immunohistochemical techniques the basement membrane components in healing scrape and keratectomy wounds on rabbit corneas, and Hirst et al compared aspects of basement membrane healing in various wound types and species.

Since these studies, information has been obtained about the ultrastructural arrangement, linkage, development, and composition of the anchoring structures of the corneal and epidermal epithelium. These structures include: (1) hemidesmosomes, which are the cellular portion of the adhesion complex; (2) anchoring filaments, which extend from the hemidesmosome site on the cell membrane through the lam-
ina lucida to the lamina densa of the basement membrane; (3) the basement membrane; (4) on the opposite side of the basement membrane from the basal cells of the epithelium, the anchoring fibril network; and (5) anchoring plaques. Anchoring fibrils insert into the basement membrane directly opposite from hemidesmosomes, and they terminate in the anterior stroma distal from the basement membrane in anchoring plaques. Recent immunohistochemical data indicate that the bullous pemphigoid antigen is localized intracellularly in the hemidesmosome plaque, and that anchoring fibrils are composed of type VII collagen.

In light of the more recent information on adhesion structures of the corneal epithelium, it seems appropriate to reexamine the repair of anchoring structures of rabbit corneal epithelium in keratectomy wounds. We used morphometric analysis of electron micrographs and immunohistochemical markers of hemidesmosomes (bullous pemphigoid antisera), basement membrane (laminin antibody), and anchoring fibrils (type VII collagen antibody) to study the temporal appearance and pattern of reassembly of the hemidesmosome, basement membrane and anchoring fibril network from 48 hr to 1 year post-wounding.

Materials and Methods

Animals and Wounding

All investigations involving animals reported in this study conform to the ARVO Resolution on the Use of Animals in Research. Adult New Zealand White rabbits were deeply anesthetized using 30 mg of chlorpromazine hydrochloride followed by 200 mg of ketamine hydrochloride. Topical proparacaine was applied to corneas, and a 7 mm superficial keratectomy was performed on one eye of each rabbit. A 7 mm trephine was placed on the cornea to demarcate the wound, and a jeweler’s forceps was used to peel back the anterior stroma inside the wound area. Thus, the epithelium, basal lamina, and a superficial layer of stroma were removed (Fig. 1). The depth of the keratectomy wound varies slightly depending on how deeply the trephine is placed, but the approximate depth is 0.3 mm. Erythromycin ophthalmic ointment (E. Fougera, Melville, NY) was applied immediately following wounding and daily for 5 days thereafter. The corneas were allowed to heal for 48 and 66 hr, 1, 2, 3, 4, 6 and 8 weeks, and 4, 6 and 12 months. N = 2 for each time point except for 12 months where N = 1 (the second animal died at 9 months). The animals were followed by gross or slit-lamp examination before sacrifice and daily for 2–3 weeks. The animals were sacrificed with an intravenous overdose of sodium pentobarbital. Healing corneas were excised and bisected. One-half was frozen in Tissue Tek II O.C.T. compound (Lab Tek Products, Naperville, IL) for immunohistochemistry, and the other half was fixed for transmission electron microscopy either in 2% glutaraldehyde with 0.5% H2O2 in 0.1 M cacodylate buffer, pH 7.4 (modification of Perrachia and Mittler) or in 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, and postfixed in 2% OsO4 in 0.1 M cacodylate buffer, pH 7.4.

Electron Microscopy and Morphometric Analysis

Following fixation and en bloc staining with aqueous 0.5% uranyl acetate, the tissue was dehydrated in a graded acetone series followed by propylene oxide and embedded in Epon-Araldite. Sections 1 µm thick were stained with toluidine blue for orientation. Thin sections were taken from three sampling areas: immediately inside the wound margin, wound center, and an area midway between the two. The sections were stained with 2% uranyl acetate and lead citrate and photographed using a Philips 410 electron microscope (Lico, Bedford, MA). Ten micrographs of the basal cell membrane-basal lamina region of each of the three sampling areas of each specimen were taken at a standard magnification of ×10,400 (printed to a final magnification of ×31,200) for morphometric analyses of hemidesmosomes and basal lamina. A Zeiss Videoplan Image Analysis System (Rainin Instruments, Woburn, MA) was used to measure the percentage of basal cell membrane occupied by hemidesmosomes as well as the area of basal lamina present per 100 µm of basal cell membrane. Values from the three sampling areas were pooled to obtain an average value for each specimen.

Immunohistochemistry

Cryostat sections of 6 µm placed on gelatin-coated slides were dried overnight at 37°C. The sections were rehydrated in phosphate-buffered saline (PBS), pH 7.2, and washed in PBS with 1% bovine serum albumin (BSA) for 10 min. Primary antibody was applied for 1 hr at room temperature in a moist chamber. The slides were rinsed with PBS followed by a 10 min incubation in PBS with 1% BSA. The secondary antibody was applied similarly for 1 hr at room temperature. After a PBS wash, coverslips were mounted with a medium consisting of PBS, glycerol and paraphenylene diamine. Negative control tissue sections (primary antibody omitted) were routinely run with every antibody-binding study. The sections were viewed and photographed on a Zeiss photomicroscope III equipped for epi-illumination. The primary antibody used was either mouse anti-human type VII collagen, clone NP76 or 185 (obtained from...
Dr. Robert E. Burgeson, Shriner’s Hospital for Crippled Children, Portland, OR), human bullous pemphigoid antisera (obtained from Dr. John Stanley, National Cancer Institute, NIH, Bethesda, MD), or an affinity-purified sheep anti-mouse laminin produced in our laboratory (see below). The specificity of the anti-type VII collagen antibodies was determined previously.\textsuperscript{16} The secondary antibody used was either fluorescein isothiocyanate (FITC)- or rhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG, FITC-conjugated goat anti-human IgG, or FITC-conjugated rabbit anti-sheep IgG (all from Organon Teknika, West Chester, PA). For the double labeling studies, a mixture of anti-type VII collagen antibodies and anti-BPA or anti-laminin antibodies was applied and incubated as usual, followed by a mixture of TRITC-goat anti-mouse IgG and either FITC-goat anti-human IgG or FITC-rabbit anti-sheep IgG.

Laminin Antibody Production

Polyclonal antibodies to laminin were raised by standard procedures. Briefly, 1 mg of laminin derived from EHS sarcoma (Gibco, Grand Island, NY) mixed with an equal volume of Complete Freund’s Adjuvant (Sigma, St. Louis, MO) was injected subcutaneously into a sheep. Two booster injections at 2 week intervals followed, using 0.5 mg laminin/ml buffer in an equal volume of Incomplete Freund’s Adjuvant (Difco, Detroit, MI). The laminin antiserum was purified by applying it to a laminin Sepharose 4B affinity column (Pharmacia Fine Chemicals, Piscataway, NJ) after it had been fractionated with 45% saturated ammonium sulfate. Preadsorption of the antibody with 100 μg laminin/ml eliminated all antibody binding to cryostat sections of cornea.

Results

Epithelial wound closure of the 7 mm diameter keratectomy wounds occurred at 66-72 hr. In four of 21 eyes, a small central epithelial defect occurred 8-14 days post-wounding. Reepithelialization in these four eyes occurred within 1-2 days, and the data from these specimens and from eyes that had no secondary epithelial sloughing did not differ.

Corneas at 48 hr to 1 week appeared clear and uninflamed but with a rough surface over the wound bed. From 2 weeks through 6 months, the wound area had a ground-glass or hazy appearance. These corneas did not appear inflamed, and polymorphonuclear neutrophils were not present in the stroma of any of the corneas.

The immunohistochemical localization of hemidesmosomes with bullous pemphigoid antibodies, basement membrane with antibodies to laminin, and anchoring fibrils with antibodies to type VII collagen in normal unwounded rabbit appears as a single band of intense antibody binding at the basal cell-basement membrane zone.

Early Repair: 48–66 hr, 1 Week

At 48 hr, before epithelial wound closure, evidence of reassembly of the adhesion structures was already present at the wound periphery. BPA, laminin, and type VII collagen were immunolocalized as small segments along the base of the stratified epithelium at the wound margin (Fig. 2a–c). At this early stage of repair, all three of the antigens were co-localized (co-localization of laminin and type VII collagen shown). Electron microscopy of the wound margin demonstrated small hemidesmosome plaques exactly adjacent to small segments of basement membrane (Fig. 2d). We could not detect cross-banded anchoring fibrils even though type VII collagen was demonstrated by immunohistochemistry to be present. There was no evidence of any of the antigens or structures under the leading edge of the migrating epithelium. By 66 hr post-wounding, epithelial wound closure had occurred or was about to occur. Localization of BPA, laminin and type VII collagen extended farther toward the wound center than at 48 hr, and as at 48 hr the three antigens co-localized (data not shown). Forming hemidesmosomes were present opposite small segments of basement membrane in the zone halfway between the wound margin and the wound center.

At 1 week post-wounding, localization of the adhesion structure antigens extended all across the wound bed (Fig. 3). The immunolocalization appeared as a discontinuous line decreasing in intensity at wound center (Fig. 3a). At the wound margin, strata of laminin and type VII collagen were present down into the
Fig. 2. Wound margin 48 hr after wounding but before epithelial wound closure. (a) Bullous pemphigoid antiserum binding at wound margin. Note intense binding at unwounded area at far right, and faint patchy binding at the base of the epithelium at wound margin (arrows). (b, c) Co-localization (arrows) of laminin (b) and type VII collagen (c) under the epithelium on the same section of wound margin. Unwounded basement membrane is at the top of both micrographs. These antigens, as well as BPA, reappear synchronously. (d, e) Electron micrographs of wound margin area demonstrating small segments of basement membrane (arrows) underlying hemidesmosome plaques. Bars: (a-c) 26 μm; (d, e) 0.64 μm.

stromal matrix under the more intense localization directly under the epithelium (Fig. 3b). Bullous pemphigoid antigen was present only at the level of the basal cells of the epithelium (data not shown). Electron microscopy showed longer segments of basement membrane with hemidesmosomes located along these segments (Fig. 3c). No cross-banded anchoring fibrils were evident, but filamentous material extended toward thestromal from the segments of basement membrane.

Repair: 2-8 Weeks

Intensity of binding of antibodies to BPA, laminin, and type VII collagen increased directly under the epithelium during 2-8 weeks post-wounding. Although discontinuities still existed in the fluorescent bands, they were fewer and not as long (Fig. 4a-d). Strata of laminin and type VII collagen in the wound bed under the epithelium extended all the way from the wound margin to wound center (Fig. 4b-d), but over time the intensity of laminin localization in these strata appeared to decrease. Compared with laminin and type VII collagen, BPA was present only in the epithelium (Fig. 4a). The stromal laminin and type VII collagen often appeared as lines or bands (strata) parallel to the more intense line directly under the epithelium (Fig. 4b). Networks of cross-banded anchoring fibrils were present in electron micrographs of sections of wounds taken from 4 weeks on (Fig. 4e). Discontinuities in the basal lamina were found at all time points (Fig. 4c).
Fig. 3. Wound bed 1 week after wounding. (a, b) Type VII collagen was present as a discontinuous band all the way across the wound including wound center (a). Laminin and bullous pemphigoid antigen were similarly localized (data not shown). At the wound margin (b), layers of type VII collagen were found in the stromal matrix (presumably newly synthesized) below the epithelium. At this time point, an abrupt stromal wound edge was not discernible. (c) Electron micrograph of region from wound center. Discontinuous segments of basement membrane with associated hemidesmosomes are longer than at previous time points. Cross-banded anchoring fibrils were not clearly evident by electron microscopy even though type VII collagen could be localized to this region. Bars: (a, b) 50 μm; (c) 0.64 μm.

Repair: 4, 6 and 12 Months

Immunofluorescent localization of antibodies to BPA, laminin and type VII collagen was present as a very bright line at the epithelial stromal junction. Some irregularities in the line persisted, and laminin localization within the stromal wound bed had disappeared. Type VII collagen still persisted as lamellar bands at 6 months (Fig. 5), but was mostly gone by 1 year (Fig. 6). By electron microscopy, discontinuities and duplication were found at all three time points [6 month (Fig. 5) and 1 year (Fig. 6) micrographs shown]. Areas of massive duplication were evident in the 1 year post-wounding eye.

Morphometric analyses of the percentage of basal cell membrane occupied by hemidesmosomes and the area of basement membrane per 100 μm of basal cell membrane are summarized in Figure 7. The percentage of basal cell membrane occupied by hemidesmosomes reached a maximum at 6 weeks, then declined; the percentage was less than in unwounded eyes. The area of basement membrane present under basal cell membrane reached normal values by 4 weeks and was greater than normal values after 6 months.

Discussion

Data from this study indicate that return of the structures of the adhesion "complex" to the normal
Fig. 4. 4 weeks after wounding. (a, b) Co-localization of bullous pemphigoid antigen (a) and type VII (b) collagen on a section from the wound margin. Bullous pemphigoid antigen is restricted to basal cells of the epithelium, but strata of type VII collagen are present in the new stromal matrix of the wound bed. (c, d) Co-localization of laminin (c) and type VII collagen (d) on a section from wound center. Each antigen is found within the wound bed matrix. (e) Electron micrograph of section of wound bed, demonstrating cross-banded anchoring fibrils arranged in a network beneath the basal lamina (arrows). Discontinuities in the basal lamina were present (far left and right). Bars: (a, b) 67 μm; (c, d) 26 μm; (e) 0.4 μm.

prewounding assembly does not occur within 1 year of mechanical keratectomy in the rabbit. Although there were areas of duplication, regions in which basement membrane was absent, and an incomplete return of percentage of basal cell membrane occupied by hemidesmosomes, we detected no sign of epithe-
Fig. 5. 6 months after wounding. (a, b) Type VII collagen localization from two different regions in the wound area. Strata of type VII collagen are still present in the wound matrix (three arrows), and irregularities in the basement membrane zone localization are present (arrows). (c-e) Electron micrographs of wound areas, demonstrating discontinuities adjoining unilaminar (c), moderately duplicated (arrows) (d), and extensively duplicated (e) basement membrane. Bars: (a, b) 26 µm; (c-e) 0.64 µm.

Halo erosion. It is also not clear how these eyes would respond to additional trauma and why the structural assembly of the repaired adhesion structures is abnormal. Unlike the developing state, in which the basement membrane is laid down prior to the anchoring fibril network and appearance of hemidesmosomes, in the wound-healing state the basement membrane is replaced in segments at the same time as anchoring fibril collagen and hemidesmosomes appear. It may be that in “segmental repair” the coor-
Fig. 6. 12 months after wounding (a–d) and unwounded contralateral eye (e). (a) Type VII collagen localization at wound center shows primarily basement membrane localization; only a few patches of type VII collagen remain in the wound bed matrix. (b–d) Electron micrographs demonstrating unilaminar basement membrane adjacent to a region lacking in adhesion structures (b) and two other regions (c, d) of the same wounded eye, demonstrating massive reduplication of the basement membrane. Pockets within the basement membrane contain anchoring fibrils (arrows). Electron micrograph of unwounded contralateral eye (e) shows the unilaminar basement membrane and normal hemidesmosome distribution. Bars: (a) 42 μm; (b–e) 0.64 μm.
Fig. 7. Percentages of basal cell membrane occupied by hemidesmosomes (--- O ---) and area of basement membrane per 100 μm basal cell membrane (--- ○ ---) at times after wounding. N = 2 animals, three areas sampled per animal, except 12 months N = 1. N = 9 for unwounded controls; three of these nine eyes were contralateral unwounded eyes of 4 month and 1 year animals. Because the data from these three eyes did not differ from the other six eyes of non-age-matched controls, the data were pooled.

Regeneration required for assembly of the adhesion structures is lost. In fact, a recent report indicates that there is a coordinated expression of genes for basement membrane proteins in differentiating F9 cells (a tetracarcinoma cell line which under appropriate conditions differentiates into endoderm), but not in normal adult tissues.17

Data from the earliest time point examined, 48 hr, indicate that resynthesis of all three adhesion structures examined begins at the margin of the wound under stratified epithelia even before wound closure. Reassembly of the structures then proceeds from wound periphery to wound center. Our data agree with those of Fujikawa et al,2 who detected laminin at wound periphery 1–2 days post-wounding. Both our immunofluorescence data and EM data from these early time points indicate that the assembly of hemidesmosomes, basement membrane, and anchoring fibrils occurs synchronously in corneal wound healing. Co-localization of BPA, laminin and type VII collagen occurred at the early time points and, by electron microscopy, hemidesmosomes always accompanied segments of new basal lamina. Although we could localize type VII collagen at the early stages of wound healing, we could not demonstrate cross-banded anchoring fibrils at these stages. Similarly, in studies of developing ocular surface, type VII collagen could be localized before cross-banded anchoring fibrils were discerned in electron micrographs.6 Networks of anchoring fibrils were observed first at 2–4 weeks. Perhaps secondary sloughing of epithelium, which occurred up to 2 weeks post-wounding in four of our animals, is correlated to time of reappearance of the anchoring fibril network.

The reports of sequence of reappearance of basement membrane components following wounding in other stratified squamous epithelia vary. In pig epidermis, BPA is the first component present after wounding,18 whereas in primate epidermis, laminin appeared first, followed 2 days later by BPA.19 In rat oral mucosal epithelium, mature hemidesmosomes and basal lamina appeared at the same time followed by cross-banded anchoring fibrils,20 and in human skin in vitro, BPA was deposited first, followed by type IV collagen (for review, see Woodley et al21). Corneal epithelium may be unique in its synchronous resynthesis of the adhesion structures. Even conjunctival epithelium apparently has BPA binding that extends to the tip of the leading edge of migrating epithelium in corneal wounds being covered by conjunctiva.2 The conjunctival epithelium moves more slowly and may resynthesize adhesion structures as it moves.

We were surprised to find lamellae or strata of type VII collagen and laminin within the newly synthesized matrix that was laid down to fill the keratectomy wound bed. These strata appear first at the wound bed margin and then spread to the wound center. Our data indicate that these strata are retained within the wound bed at least 6 months, with discernible laminin localization to the strata disappearing before type VII collagen localization. Since it is...
known that type VII collagen and laminin are products of the epithelium, we interpret the presence of the strata to be layers of adhesion complex components laid down by the epithelium when it was at deeper positions in the wound bed. As new stromal matrix and collagen were synthesized and laid down by the stromal keratocytes, the epithelium was displaced upward where it was required to yet again lay down a new adhesion complex. Thus, strata of type VII and laminin in the wound bed are perhaps not unlike an archeologic dig, with the deepest strata the product of the earliest position of the epithelium in the wound bed. The ground-glass, hazy appearance seen grossly in the wound area of the rabbits through 6 months may be a result of these strata.

A comparison of healing of keratectomy wounds in the rabbit and in animals that have a Bowman’s layer occurs in rabbits, but such data on primate wounds would be of interest. It is apparent from our data and those of Davison and Galbavy that fill-in of stroma occurs in rabbits, but such data on primate wounds are not available.

Key words: basement membrane, corneal wound healing, epithelial adhesion, hemidesmosome, type VII collagen, wound healing

References