Characterization of Wound Reepithelialization Using a New Human Tissue-Engineered Corneal Wound Healing Model

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PURPOSE. The reepithelialization of the corneal surface is an important process for restoring the imaging properties of this tissue. The purpose of the present study was to characterize and validate a new human in vitro three-dimensional corneal wound healing model by studying the expression of basement membrane components and integrin subunits that play important roles during epithelial cell migration and to verify whether the presence of exogenous factors could accelerate the reepithelialization.

METHODS. Tissue-engineered human cornea was wounded with a 6-mm biopsy punch, and the reepithelialization from the surrounding margins was studied. Biopsy samples of the reepithelialized surface were harvested 3 days after wounding and were processed for histologic, electron microscopic, and immunofluorescence analyses. The effects of fibrin and epithelial growth factor (EGF) on wound reepithelialization were also studied.

RESULTS. Results demonstrated that this in vitro model allowed the migration of human corneal epithelial cells on a natural extracellular matrix. During reepithelialization, epithelial cell migration followed a consistent wavelike pattern similar to that reported for human corneal wound healing in vivo. This model showed a histologic appearance similar to that of native tissue as well as expression and modulation of basement membrane components and the integrin subunits known to be main actors during the wound healing process. It also allowed quantification of the reepithelialization rate, which was significantly accelerated in the presence of fibrin or EGF. The results indicated that α5β1 integrin expression was increased in the migrating epithelial cells compared with the surrounding corneal tissue.

CONCLUSIONS. The similarity observed with the in vivo wound healing process supports the use of this tissue-engineered model for investigating basic mechanisms involved in corneal reepithelialization. Moreover, this model may also be used as a tool to screen agents that affect reepithelialization or to evaluate the effect of growth factors before animal testing.

The cornea is a highly specialized and unique organ in the human body. Because of its anatomic location, the cornea is continually subjected to abrasive forces and occasional mechanical trauma. Damage to the cornea can result in scarring or opacification, causing visual defects that compromise transparency and that can even lead to complete loss of vision. Many of these wound healing problems are associated with the inability to reorganize a complete and mature smooth epithelium, which is essential in restoring the imaging properties of the tissue. Therefore, to improve wound management and to develop new therapeutic approaches, it is essential to better understand the mechanisms of corneal wound healing.

Epithelial wound healing involves complex epithelial-stromal interactions mediated by growth factors and extracellular matrix (ECM) components. Cell-cell and cell-matrix interactions play important roles in maintaining the stratified structure of the corneal epithelium. Cell adhesion and cell migration depend on the synthesis and assembly of the extracellular matrix, including the basement membrane at the epithelium-stroma junction (ESJ). During wound healing, the regeneration of a functional corneal epithelium depends on epithelial migration and the reconstitution of the ESJ, which anchors the epithelium to the stroma.

Several models of wound healing have been developed to investigate the corneal mechanisms of reepithelialization and to screen growth factors that may stimulate a healthy healing response. However, the in vitro models of wounding cell monolayers are limited by the absence of multiple epithelial cell layers and the lack of epithelial-mesenchymal interactions taking place in vivo. Significant advances have also been made by studying corneal wound healing with in vivo models such as rabbits, rodents, and horses. However, these studies are expensive and difficult because of the complexity of the in vivo experiments and the well-known variability among animals.

Corneal wound healing has also been studied using an ex vivo organ culture model. The availability of healthy human donor cornea, however, is limited, and the delay between donor death and delivery of the cornea to the laboratory (2–6 days) could negatively influence results. Reductions in the number of epithelial cell layers, stromal edema, and incomplete reepithelialization have occasionally been reported. There is a need for a new human in vitro wound healing model closely mimicking the cornea in vivo that may represent a more appropriate alternative to the use of...
monolayer cultures, organ culture models, and, to a certain extent, animal experimentation.

Although several three-dimensional bioengineered corneal models have been generated with human (Germain L, et al. IOVS 1999;40:ARVO Abstract 1745) and animal31-36 cells, they were not used to study corneal wound healing. We previously reported the engineering of the first corneal model reconstructed from a collagen gel with untransformed human corneal fibroblasts and epithelial cells.37 More recently, using the self-assembly approach,38,39 we developed a completely natural tissue-engineered cornea without the addition of exogenous extracellular matrix proteins (Germain L, et al. IOVS 1999;40:ARVO Abstract 1745; Carrier P, et al. IOVS 2002;43: ARVO EAbstract 4166). In the skin, a tissue-engineered wound healing construct has been produced to study the mechanisms of skin reepithelialization in vitro.40

In the present study, a new human three-dimensional corneal wound healing model comprising living fibroblasts and epithelial cells was used in vitro to study reepithelialization. The purpose of the study was to determine whether the pattern of epithelial cell migration (typical wavelike pattern) and the acceleration of reepithelialization by exogenous factors could be detected with this in vitro model. Modulation in the expression of basement membrane components and integrin subunits that play important roles during epithelial cell migration was also evaluated.

METHODS

This study was conducted in accordance with our institution’s guidelines and with the Declaration of Helsinki. The protocols were approved by the institution’s Committee for the Protection of Human Subjects.

Tissue Extraction and Cell Culture

Human corneal epithelial cells (HCECs) were isolated and cultured from postmortem donor corneas unsuitable for transplantation (Banque Nationale d’Yeux du CHUQ), as described previously.37 Briefly, corneas were dissected from the ocular globes with curved scissors (Storz, St. Louis, MO), and the limbus was separated from the central cornea with a 7.5-mm diameter trephine (Pilling Weck, Markham, ON, Canada). The limbal ring was incubated in 2 mg/mL dispase II (Roche Diagnostics, Laval, QC, Canada) in HEPEs buffer (MD Biomedicals, Montreal, QC, Canada) for 18 hours at 4°C. The epithelium was mechanically removed from the stroma with forceps under a dissecting microscope (model SMZ2T, Nikon, Montreal, QC, Canada), cut into small pieces with a scalpel, and centrifuged for 10 minutes (200g) at room temperature. The HCEC pellet was then seeded in tissue culture flasks along with irradiated (60 Gy) murine Swiss-3T3 fibroblasts (ATCC, Rockville, MD). Primary cultures (P0) were subcultured up to the fourth passage (P4). Human corneal fibroblasts were isolated from the stromal portion of the cornea left after dispase digestion and removal of the epithelium and endothelium. Briefly, the stroma was incubated for 3 hours at 37°C in a collagenase H solution (0.125 U/mL Roche Diagnostics). After a 10-minute centrifugation (room temperature), cells were seeded in 25-cm² tissue culture flasks and cultured, as previously described.37 Fibroblasts were used in all experiments between the fifth and seventh passages. Human skin fibroblasts were obtained from the dermal portion of adult breast skin and were cultured, as described previously.40,41 Each cell type was grown under 8% CO2 at 37°C, and the culture medium was changed three times a week.

Human Tissue–Engineered Corneas

The self-assembly approach was used to produce human tissue-engineered corneas (hTECs). Fibroblasts were cultured for 40 days in Dulbecco–Vogt modification of Eagle medium (DME), supplemented with 10% fetal calf serum (HyClone, Logan, UT), 100 IU/mL penicillin G (Sigma, Oakville, ON, Canada), and 25 µg/mL gentamicin (Schering Canada, Pointe-Claire, QC, Canada) containing 50 µg/mL ascorbate (Sigma) that supports extracellular matrix production and allows thick fibrous sheet formation in plastic culture flasks. After they were peeled from the dishes, one dermal and one corneal fibroblast sheet were superimposed and cultured for an additional week; this resulted in human tissue-engineered stroma (hTES). For this study, dermal and corneal fibroblasts were used to produce the hTES. Preliminary studies have led to the conclusion that the combination of these two types of fibroblasts allowed the formation of a well-differentiated corneal epithelium histologically resembling that of a human cornea and resulting in higher reepithelialization rates than the use of corneal fibroblasts alone. HCECs were seeded on the surface of hTES and were cultured in submerged conditions in complete epithelial cells medium supplemented with 50 µg/mL ascorbate, as previously described.39 After 7 days, hTECs were raised at the air–liquid interface and cultured for another week in the same medium without human epidermal growth factor (EGF) to induce the differentiation of epithelial cells. Media were changed three times a week.

Wound Healing Model

After 1 week of epithelial maturation at the air–liquid interface, hTECs were wounded with a 6-mm diameter biopsy punch (Laboratoire Stiefel, Nanterre, France) and placed over a second hTES devoid of epithelium to allow reepithelialization by epithelial cells onto this natural matrix. This human tissue-engineered corneal wound healing model (hTECWH) was cultured at the air–liquid interface for 3 days, at the end of which the reepithelialized surface was photographed before biopsy. Biopsy samples were processed for histologic, electron microscopic, and immunofluorescence analyses. All experiments were repeated four times.

Wound Treatments and Measurement of the Reepithelialization Rate

To study the effect of EGF on wound reepithelialization, 20 µL of a 25-ng/mL EGF solution (Austral Biological, San Ramon, CA) in culture medium (treated), or culture medium alone (control), was dropped on the wound twice daily for 3 days after wounding. In another set of experiments, the effect of fibrin clot was evaluated by adding or not adding 20 µL of a mixture of 10 mg/mL fibrinogen (Sigma) with 50 IU/mL thrombin (Sigma) at a 10:1 ratio immediately after wounding.

The progression of reepithelialization was calculated for each treatment (n = 4) using photographed surfaces (Figs. 1B, 1D) and National Institutes of Health Image software (Bethesda, MD) on the third day after wounding. Results were expressed as surface percentage of wound closure on the third day. Differences in relative reepithelialization between each treatment and controls were tested for statistical significance with the paired unilateral Student’s t-test. P < 0.05 was considered statistically significant.

Histologic Analysis

Biopsy specimens from the tissue-engineered wound healing model were fixed with Bouin solution (Produits Chimiques ACP, St-Leonard, QC, Canada) and embedded in paraffin. Five-micrometer-thick microtome sections were stained with Masson trichrome for histologic analysis.

Immunofluorescence Staining

Samples were embedded in optimal cutting temperature compound (Somagen, Edmonton, AB, Canada), frozen in liquid nitrogen, and stored at −70°C until use. An indirect immunofluorescence assay was performed on acetone-fixed (10 minutes at −20°C) cryosections (5 µm), as previously reported.32 Sections were incubated for 45 minutes with mouse monoclonal primary antibodies used at optimal dilution in phosphate-buffered saline containing 1% bovine serum albumin: anti-
human collagen VII (Chemicon, Temecula, CA), laminin V chain γ2 (Chemicon), AE5 (keratin 3; MD Biomedicals), Ki67 (BD PharMingen, Mississauga, ON, Canada), integrin subunit α4 (Chemicon), α3 (ATCC), α6 (Chemicon), and α-smooth muscle actin (Dako, Mississauga, ON, Canada). The appropriate conjugated secondary antibody was incubated for 30 minutes (goat anti-mouse IgG-IgM conjugated with rhodamine [Chemicon]). Cell nuclei were also labeled with reagent (Hoechst 33258; Sigma) after immunofluorescence staining. Samples were then observed with an epifluorescence microscope (Eclipse E600; Nikon) and were photographed with a numeric charge-coupled device camera (Sensys; Roper Scientific, Trenton, NJ). Negligible background was observed for controls (primary antibodies omitted).

Transmission Electron Microscopy

Samples were fixed in 2.5% glutaraldehyde and processed for electron microscopy, as previously described.40

RESULTS

Sensitivity of the In Vitro Human Corneal Wound Healing Model to Evaluate the Pattern and Rate of Reepithelialization

To study the reepithelialization of corneal wounds in an environment close to the in vivo conditions, hTEC comprising an epithelium and a stroma was produced by the self-assembly approach. After wounding with a 6-mm biopsy punch (Figs. 1A, 1C), epithelial cell migration onto the natural matrix secreted by fibroblasts was evaluated. This hTECWH model allowed us to follow the wound closure macroscopically by observing the ring of reepithelialization that progressed toward the wound center (Figs. 1B, 1D). Macroscopic observations after wounding revealed that the corneal epithelium migrated after a specific pattern observed in the four independent experiments performed. The progression of the epithelium was not uniform; it occurred more rapidly in some areas than in others. Indeed, three or more convex leading fronts from different regions of the surrounding intact epithelium advanced toward the center (Figs. 1B, 1D). These migrating fronts developed within 12 to 24 hours after wounding and continued to advance for the period studied (72 hours). Therefore, this model allowed us to follow the wound closure macroscopically by observing convex fronts of reepithelialization that progressed toward the wound center in a pattern similar to the one reported for human corneal wound healing in vivo.44

EGF and Fibrin Induced Faster Reepithelialization of Corneal Wounds

To quantify the effect of fibrin or EGF on wound healing, the reepithelialized surface was measured 3 days after wounding. The rate of wound reepithelialization was significantly faster for hTECs treated with EGF than for control wounds treated with culture medium without EGF (Fig. 2). Fibrin clot was also a potent factor because it increased the surface reepithelialized after 3 days by twofold (Fig. 2). These results indicated that EGF and fibrin clot accelerated reepithelialization.

Histologic Analysis of Human Tissue–Engineered Cornea before and after Wounding

Histologic observations of unwounded hTEC revealed a normal epithelial architecture after 10 days of maturation at the air-liquid interface. As seen in Figures 3A and 3C, corneal epithelium...
Lial cells stratified uniformly over the hTES to form a well-differentiated epithelium composed of five or six cell layers. Basal cells were cuboidal with round nuclei. The superficial layers showed spindle winglike cells and flat squamous cells that did not undergo cornification, a phenotype similar to that of normal human cornea in situ. The human tissue-engineered stroma, supporting the epithelium, was composed of two fibroblast sheets comprising abundant natural collagen matrix secreted and assembled by human fibroblasts (F1 and F2 on Figs. 3A, 3C). After wounding, the hTECs were placed over two supplementary fibroblast sheets (F3 and F4 on Figs. 3A, 3C) to allow reepithelialization over a natural matrix. The cut produced in the two initial fibroblast sheets could be used as a landmark for the location of the wound margin. The fibroblast sheets placed underneath, after wounding, served as a natural matrix for epithelial cell migration from the surrounding uninjured epithelium. The 3-day neoepithelium was separated into three regions (Fig. 3A), as follows: the “near the wound margin” region, proximal to the wound margin (cut made by the biopsy punch); the middle of the neoepithelium; the migrating epithelial tongue (MET), consisting of epithelial cells forming the tip of the neoepithelium. Three days after wounding, epithelial cells migrated to regenerate a neoepithelium (Fig. 3B). Suprabasal epithelial cells at the tip of the MET migrated over the basal layer to make contact with the hTES (not shown) or the fibrin matrix (Fig. 3D). Moreover, at the tip of the leading edge, the migrating basal cells were not columnar but presented a more flattened morphology (Fig. 3D).

Taken together, these results indicated that tissue-engineered cornea produced using the self-assembly approach allows the formation of a well-differentiated epithelium capable of responding to an injury by restoring the epithelium.

**Basement Membrane Components and Integrin Expression during Wound Healing**

The deposition of basement membrane components, collagen VII (Figs. 4A, 4B), and laminin V (Figs. 4C, 4D) formed a continuous line along the ESJ (Figs. 4A, 4C) of the unwounded hTEC. Three days after wounding, increased expression of collagen VII and laminin V was detected in the cytoplasm of the migrating epithelial cells at the tip of the MET (Figs. 4B, 4D). Closer to the wound margin, the laminin V labeling became strong and almost continuous whereas collagen VII was weaker and patchy at the ESJ, indicating that as soon as epithelial cells settled, they initiated extracellular deposition of laminin V, which occurred earlier than deposition of collagen VII. Fibroblasts were present in the stromal portion of the hTEC and hTECWH. Myofibroblasts were detected using antibodies directed against α-smooth muscle actin. They repre-
sented a proportion of 10.88% ± 0.71% of the mesenchymal cells.

To further characterize the reepithelialization process occurring after wounding of the hTECs, we studied the expression of integrins likely to be involved in cell migration processes during remodeling of the healing epithelium. The β4 subunit (Fig. 4E) and α6 (Fig. 4J) integrin subunits, both components of the hemidesmosomes, were present at the ESJ in the unwounded cornea. In contrast, in the MET, the β4 integrin subunit was present on the entire cytoplasmic membrane surrounding the basal cells, though the staining of the basal side was weaker than that of the unwounded area (Figs. 4E, 4F). Near the wound margin, the staining of the β4 integrin subunit was almost continuous and restricted to the basal membrane of the neoeipithelium (Fig. 4E). The distribution of the α6 integrin subunit resembled that of β4, though it was not restricted to the basal membrane but rather extended to the lateral membrane of basal cells of the unwounded cornea (Fig. 4J) and the
wounded area with a continuous staining of the basal membrane in the neoe epithelium (Fig. 4K) and MET (Fig. 4L). The expression of αv, αv, and β1 subunit proteins was detected around the basolateral cells of the intact epithelium (Figs. 4M, 4P, 4S). For the β1 subunit, a slight staining of the superficial cells was also observed (Fig. 4M). A brighter staining of these integrins was detected at the tip of the MET (Figs. 4O, 4R, 4U) and in the middle of the neoe epithelium (Figs. 4N, 4Q, 4T). In addition, the staining intensity of the β1 subunit increased in the suprabasal cells of the neoe epithelium (Figs. 4N, 4O). The distribution of the β1 integrin subunit suggested the presence of αvβ1 complexes in the newly forming epithelium of the MET because the β1 subunit staining was diminished. Of particular interest was the change noted in αvβ1 integrin during wound healing. This integrin was not observed in the unwounded hTECs (Fig. 4I). In contrast, strong αvβ1 staining was detected all along the basal cells of the neoe epithelium (Fig. 4I), indicating that epithelial cells induced the synthesis of αvβ1 during migration.

To determine how differentiated were the intact epithelium and the neoe epithelium, we probed the cornea-specific differentiation marker keratin 3 (K3)45 by immunofluorescence microscopy. In the neoe epithelium, the pattern of K3 expression resumed progressively in the most superficial epithelial cells from the MET to the wound margin (Fig. 4G), indicating that the differentiated epithelium progressively regenerated during reepithelialization. The basal cells of unwounded and wounded epithelium did not express K3.

The evaluation of the proliferative activity using Ki67 revealed positive labeling in two regions of the epithelium during wound healing (Fig. 4H). A great number of proliferating cells were closely located to the unwounded side of the wound margin. Moreover, the proliferation was also high in the middle of the neoe epithelium. It is likely that this pool of proliferating epithelial cells could supply cells for migration and stratification over the surface of the wound as the neoe epithelium elongated and the wound margin became distant.

Thus, labeling the corneal constructs for basement membrane components and a differentiation marker showed that these proteins gradually recovered their normal expression pattern, similar to that of the unwounded epithelium. In addition, the expression of some integrin subunits in the regenerating epithelium was different from the intact surrounding epithelium.

Ultrastructural Analysis of the Human Tissue-Engineered Corneal Wound Healing Model

To extend our study on the basement membrane components expressed under the neoe epithelium, the structure of the basement membrane was then examined using electron microscopy. An organized ESJ was observed in the unwounded side of the hTECWH. Many hemidesmosomes that attached basal cells to the underlying fibroblast sheets were present along the continuous basement membrane (Fig. 5B). Higher magnification showed anchoring filaments, which crossed the lamina lucida to form a subbasal dense plate and connect hemidesmosomes to the lamina densa (Fig. 5B'). Electron microscopy of the superficial layer of the epithelium revealed numerous projections, the typical structures known to contribute to the maintenance of the tear film (Fig. 5B').

No basement membrane was observed at the tip of the MET, where cells were migrating to reepithelialize the wound (Fig. 5G). Behind the MET, foci of lamina densa in reorganization were observed under the neoe epithelium (Figs. 5D-F). Toward the wound edge, the ESJ was progressively more continuous. Near the wound margin, the ESJ was comparable to that of the adjacent unwounded side in terms of continuity, but hemidesmosomes were less frequent (Fig. 5C). The stromal compartment underneath the epithelium was composed of numerous collagen fibers. Thus, these results indicated that the formation of a continuous and organized ESJ with characteristic ultrastructural features resumed after reepithelialization.

DISCUSSION

Because a smooth and intact corneal epithelium is essential for the maintenance of good vision and to protect the cornea against infection,46 improvements in the understanding of the mechanisms regulating reepithelialization after epithelial defects are required. Several corneal reepithelialization models that contain only epithelial cells have been described. We developed a new model that includes mesenchymal cells, which are essential for basement membrane formation. Thus, we think our model mimics many aspects of the reepithelialization in vivo. To the best of our knowledge, this is the first description of an in vitro human tissue-engineered cornea comprising an epithelium and of a natural stroma comprising living fibroblasts to study corneal wound healing. The described model offers several advantages. This in vitro system is a three-dimensional and completely biological cellular environment; comprises living fibroblasts and epithelial cells, thus allowing epithelial–mesenchymal interactions; exhibits the major histologic, immunohistologic, and ultrastructural properties of the human corneal epithelium; allows the study of a single parameter at a time as a result of controlled and reproducible conditions; and mimics many aspects of the reepithelialization, including cell migration, proliferation, and the restoration of a stratified epithelium. This model reproduced the peculiar migration pattern observed in corneal wound healing. Our results show that EGF and fibrin significantly increased the reepithelialization rate. Finally, the study of integrins indicated that αvβ1 was expressed de novo in the neoe epithelium during wound healing.

Interestingly, in our hTECWH model, reepithelialization occurred as convex fronts of migrating cells, a particular pattern observed in vivo in corneal healing,44 ex vivo in human17 and animal models,47 and in vitro after wounding of confluent epithelial cell cultures.48 In patients, all corneal abrasions, irrespective of the nature of the injury, follow this consistent pattern during reepithelialization.44 It has been proposed that these convex fronts result from areas of delayed healing, mixed with areas of faster cell migration/multiplication along the circumference of an abrasion. The present model offers a tool to further study this hypothesis with human cells in a natural environment.

Our results on the acceleration of wound closure by EGF or fibrin clot are consistent with previous studies performed in other systems.40,49-50 EGF is known to increase the migration of skin epithelial cells.51-53 In vivo, the temporary scaffold formed by fibrin plays a role in epithelial migration and transitory adhesion to the substrate during corneal wound healing at a time when normal anchoring mechanisms are lost. The importance of fibrin in wound healing in vivo is suggested by the successful reepithelialization over corneal ulcers previously resistant to therapy after topical inhibition of plasmin,54 a serine protease capable of degrading fibrin and fibronectin, using aprotinin. Taken together with our results, it could be suggested that topical application of fibrin could have a potential therapeutic effect in some ocular disorders involving a loss of epithelium.

Therefore, this model offers a tool to compare and evaluate, under standard conditions, the effect of various exogenous factors on the rate and quality of reepithelialization. In addition, our method offers other considerable benefits over in vivo
animal models. Not only does it reduce animal use and ocular discomfort associated with in vivo corneal wounding, it overcomes the inherent interindividual variability associated with animal models. Overall, modulation of corneal reepithelialization by the addition of exogenous factors can be studied and easily quantified in vitro with this wound healing model, indicating its potential use for drug screening in the pharmaceutical industry.

The stability of our corneal wound healing model allows the study of reepithelialization over several days. Hence, as observed during in vivo studies, cells actively divided and migrated in our in vitro model. This contrasted with other in vitro models in which cells migrate but rarely divide.55 It is likely that the two clusters of proliferating cells have distinct functions. The first, just behind the leading edge, provides a mass of cells ready to migrate,56 whereas the second, in the unwounded epithelium proximal to the wound bed,57 generates cells for stratification and differentiation.

Our model allows the study of healing in a three-dimensional context. As occurred in vivo, basal cells of the human tissue-engineered cornea formed hemidesmosomes and lay on a continuous basement membrane. After wounding, cells migrated over the natural matrix provided by the fibroblast sheet. This migration from the intact edge toward the wound center is possible through the disassembly of hemidesmosomes in MET and their replacement by focal adhesion. The integrins,
which link the cells to the ECM, undergo changes. We described that αβ integrins, initially present in the hemidesmosomes as in native cornea, redistributed to lateral and apical membranes in MET, as also observed in other models.55–61 The increased expression of α2, α4, and β1 subunits that we observed in the neoeppithelium is consistent with the presence of α2β1 and α4β1, in focal contacts,52–65 the temporary adhesion involved in epithelial cell migration that is well described in skin wound healing.66–67 This difference reinforces the hypothesis of a role for these integrins in corneal epithelial cell adhesion to basement membrane and adjacent cells during wound healing.68–70 The distribution of the α1 integrin subunit in the migrating epithelial cells was different from that of β1, suggesting that it could form a heterodimer with β1. This is consistent with the role of αβ integrin in epithelial cell migration proposed after studies with corneal organ culture models.56–60

The most striking modification in integrin expression was the upregulation of the αβ integrin in the basal cells of the migrating neoeppithelium. αβ was not detected in the unwounded adjacent epithelium, as it was in the corneal epithelium in situ (data not shown). This first report of αβ in corneal healing is consistent with previous studies in skin and oral mucosa reporting the induction of this integrin during wound healing.70,71 Taken together with the fact that the αβ integrin mediates adhesion to fibronectin and tenasin,72–75 our results strongly suggest that αβ may play a primary role in epithelial cell spreading and migration during corneal wound healing.

The reformation of the stratified epithelium after migration can be visualized in our model near the initial wound edge. Interestingly, the β1 integrin subunit returned to its normal localization at the basal aspect of the basal cells, presumably in hemidesmosomes, in the middle of the neoeppithelium. Moreover, in this region, ultrastructural studies showed that hemidesmosomes reappeared and the basement membrane reorganized in a more continuous structure as observed under the unwounded tissue-engineered cornea. This reorganization occurred by foci, where we and others41,74 have observed precursors of hemidesmosomes. This progressive reestablishment of the epithelial-stroma junction at the wound margin preceded the complete epithelial wound closure. It was accompanied by the deposition of laminin V followed by collagen VII, a sequence similar to that occurring in skin.75,76 Moreover, K3 reexpression indicates a progressive redifferentiation of the suprabasal cells of the neoeppithelium toward a phenotype observed in the adjacent unwounded tissue-engineered cornea.

In summary, we developed a fully human in vitro tissue-engineered corneal wound healing model devoid of any synthetic material that allows reepithelialization over a natural matrix after wounding. This model is produced from living fibroblasts and untransformed human corneal epithelial cells. Thus, given that fibroblasts can produce cytokines and growth factors in three-dimensional substitutes, it is likely that epithelial-mesenchymal interactions are present. Our model also shows appropriate histology, expression of basement membrane components, and integrins. However, it presents some limitations, such as differences in the method of wounding compared to the production of wounds in vivo. Reepithelialization occurs without the mechanical shear stress of the eyelids and without growth factors in the tear film that could modulate cell migration. In addition, the absence of the corneal endothelial cells in our model might have changed the metabolism of the other corneal cells (epithelial and fibroblasts) and thereby influenced processes such as cell proliferation, migration, and differentiation. Because the duration of the healing period studied was 3 days, this model resembled an acute small wound more than a large wound. Indeed, although some myofibroblasts were observed, the increase in the number of myofibroblasts, occurring 10 days after wounding in vivo, was not present in our model. Similarly, the increase in the density of stromal cells and the fibrotic stromal scar formation with disorganized collagen fibers were not reproduced in our model. Nonetheless, this biological, three-dimensional model is promising for further study of the mechanisms involved in the corneal reepithelialization process. Moreover, this model could be used for toxicologic and pharmacologic studies such as the screening and evaluation of potential agents affecting reepithelialization. The use of our model will also enable investigation of the expression, distribution, and characterization of the role of growth factors, their receptors, and extracellular matrix proteins during corneal wound healing.

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