Roles of wound geometry, wound size, and extracellular matrix in the healing response of bovine corneal endothelial cells in culture

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Grasso S, Hernández JA, Chifflet S. Roles of wound geometry, wound size, and extracellular matrix in the healing response of bovine corneal endothelial cells in culture. Am J Physiol Cell Physiol 293: C1327–C1337, 2007. First published August 8, 2007; doi:10.1152/ajpcell.00001.2007.—It has classically been accepted that the healing of narrow wounds in epithelia occurs by the formation of a contractile actin cable, while wide wounds are resurfaced by lamellipodia-dependent migration of border cells into the denuded area. To further investigate the general validity of this idea, we performed systematic experiments of the roles of wound geometry, wound size, and extracellular matrix (ECM) in wound healing in monolayers of bovine corneal endothelial cells, a system shown here to predominantly display any of the two healing mechanisms according to the experimental conditions. We found that, in this system, it is the absence or presence of the ECM on the wound surface that determines the specific healing mode. Our observations demonstrate that, independent of their size and geometry, wounds created maintaining the ECM heal by migration of cells into the wound area, while ECM removal from the wound surface determines the predominant formation of an actin cable. While the latter mechanism is slower, the actin cable permits the maintenance of the epithelial phenotype to a larger extent during the healing process, as also confirmed by our finding of a more conserved localization of cadherin and vinculin. We also introduce a model that simulates experimental findings about the dynamics of healing mechanisms, both for the maintenance or removal of the ECM on the wound surface. The findings of this study may contribute to the understanding of physiological and pathological aspects of epithelial wound healing and to the design of therapeutic strategies.

epithelial wound healing; actin cable; lamellipodial crawling; dynamic model

THE FUNCTION of an epithelial sheet depends on its integrity. Thus, any discontinuity must be rapidly overcome. Upon injury, the first response of an epithelial layer is to move forward to cover the denuded area. Epithelia display two main mechanisms of motility during the wound healing response: the so-called purse-string closure and cell migration by lamellipodial crawling (18, 45, 48). In the first mechanism, an actomyosin cable develops at the free border of the leading cells (2). This actin cable is connected from cell to cell via insertion to the adherens junctions (5) and, in this way, constitutes a continuous functional unit along the wound border. Thus, for the case of small circular wounds, the contraction of this ring can rapidly close the gap, similarly to the pulling of a purse string. This type of wound closure is typical of embryonic tissues (36, 48), although it has also been observed in several adult tissues both in vivo and in culture (5, 46). The second mechanism, observed in adult epithelia in vivo (16–18), consists of the emission of lamellipodia by the cells at the wound border followed by the migration of the cell towards the center of the wound, utilizing similar cellular and molecular mechanisms to those of single migrating cells. Whatever the mechanism, not only the cells at the wound border exhibit motile activity. Indeed, it has been recently demonstrated that several rows of cells back from the leading edge spread and migrate in concert toward the denuded area (5, 7, 8, 26), in an attempt to quickly cover as much bare area as possible. Depending on the size of the wound, these migrating cells can separate from the monolayer and enter the mitotic cell cycle.

The majority of the studies about epithelial wound healing have been performed on cultured monolayers. Among other advantages, the employment of cultured systems allows the control of diverse factors involved in the wound healing response. Most epithelia in culture display a mixed healing phenotype (21, 46), where portions of the wound border exhibit actin cable and others show lamellipodial activity. Since the pioneering work of Bement and coworkers (2), it has generally been accepted that the healing phenotype adopted by an injured monolayer depends on the size of the wound, with small wounds (<10 cells in diameter) healing by purse string closure and large wounds by lamellipodial crawling. The development of a purse string actin cable represents a rapid and efficient mechanism to repair single cell epithelial losses (9, 38). However, for the case of larger wounds, no systematic studies have been performed to establish a clear relation between the size and geometry of the wound and the particular healing mechanism. The general objective of this work was to contribute to the understanding of the factors that determine the selection of a particular mechanism of healing in epithelia. For this purpose, we performed experiments on the effects of the size and geometry of the wounds on the healing responses of confluent monolayers of bovine corneal endothelial (BCE) cells in culture, a system shown here to be capable of displaying all the healing phenotypes depending on the experimental conditions. Also, we investigated the role of the extracellular matrix (ECM) on the acquisition of specific healing mechanisms. Although the influence of the ECM on cell migration and motility has been extensively studied (11, 10, 23, 31, 39), its participation in the selection of a particular healing mechanism in epithelia has not, to our best knowledge, been thoroughly addressed. In this work, we provide with evidence showing that, in BCE monolayers, it is not the wound size and geometry...
that play a role in the acquisition of the healing mechanism but the absence or presence of the ECM. We also introduce here a dynamic model that reproduces our experimental findings about the time course of actin cable and lamellipodial formations in the absence or presence of the ECM. The fact that BCE cells in culture permit the experimental induction of a predominant healing mechanism makes them a most convenient system for the studying of the molecular events of the different healing mechanisms in the same cellular type.

MATERIALS AND METHODS

Cell culture. BCE cells were obtained from fresh bovine eyes and cultured as previously described (4). Under these conditions, when they reach confluence, cultured cells exhibit the morphology of a highly contact-inhibited cell monolayer composed of flattened and closely apposed cells and show a polarized distribution of F-actin. Before the wounds were made, confluent monolayers grown on glass coverslips were transferred to 35-mm petri dishes containing MEM without serum. Only cells from the first to fifth passages were used.

Wounds. Wounds of different sizes (narrow and wide) and geometries (circular and linear) were performed. Linear narrow wounds were ~150 μm wide, linear wide wounds were 2 mm wide, and small circular wounds were <10 cells in diameter. When cultured BCE cells reach confluence, they produce a thick ECM that covers the whole tissue culture dish (15). Confluent monolayers of BCE cells were wounded with different sterile instruments depending on whether maintenance or removal of the ECM was desired. To remove the ECM, a piece of razor blade (for linear wide wounds) or a 21-gauge syringe needle (for linear narrow and circular wounds) was used. To maintain the ECM, a piece of silicon (for linear wide and large circular wounds) or silicon-coated wire (for linear and circular narrow wounds) was used. For immunoblot analysis, confluent cell monolayers grown on 35-mm tissue culture plates were wounded using homemade combs of silicon-coated or uncoated syringe needles to maintain or remove the ECM, respectively. Once wounds had been performed, monolayers were kept in culture media for the corresponding times at 37°C in a tissue culture incubator.

Crystal violet staining. To confirm that the ECM had been either removed or maintained after the procedures described in Wounds, monolayers were stained with crystal violet. Immediately after being wounded, cells were fixed in 4% paraformaldehyde for 15 min at room temperature, washed three times in PBS, and stained with crystal violet [5 mg/ml in MetOH (20%)] for 2 min. Next, cells were rinsed thoroughly in PBS and distilled water to obtain the desired staining. Cells were air dried and observed as described in Cell images. Figure 1 shows typical bright-field images of wounds at time 0. It is important to note that in wounds performed by removing the ECM, a band of ECM always remains at the wound border (Fig. 1B, asterisk). This band of ECM results from the detachment of the dead cells from the monolayer, which leaves the underlying ECM exposed.

Fluorescence microscopy. After wounds had been performed, monolayers were maintained in culture medium without PBS at 37°C. After an incubation for the corresponding time periods, cells were fixed in 4% paraformaldehyde in Dulbecco’s PBS (Ca²⁺ and Mg²⁺ were added immediately before use) for 15 min at room temperature, washed three times with PBS, and permeabilized with 0.1% (vol/vol) Triton X-100 in PBS for 15 min. For actin localization, coverslips were incubated with FITC-conjugated phalloidin in PBS and 1% BSA (Molecular Probes) at a 1:100 dilution for 20 min at room temperature. For cadherin visualization, cells were incubated with a 1:1,000 dilution of anti-pancadherin polyclonal antibody (Sigma, St. Louis, MO). After an incubation with the primary antibody, coverslips were washed three times in PBS and incubated with a 1:1,000 dilution of Cy3-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA). For vinculin staining, cells were incubated with an anti-vinculin monoclonal antibody (Sigma clone VIN-11-5) at a 1:50 dilution, washed three times with PBS, and incubated with an Alexa Fluor 488 Signal Amplification Kit (Molecular Probes) at a 1:200 dilution. All primary and secondary antibodies were diluted with PBS containing 1% BSA and incubated for 60 min at 37°C in a humid chamber. After an incubation with the corresponding probes, coverslips were washed three times with PBS, rinsed with distilled water, and mounted in glycerol-Tris (1.5 M, pH 8.8; 1:5).

Cell images. Crystal violet staining of the ECM was observed on a Nikon Optiphoto epifluorescence microscope using bright field with a ×20 objective. For fluorescence visualization, cells were observed using a fluorescein filter set with a ×20 Planneofluor objective. Images were captured with a Kodak MDS120 digital camera coupled to the microscope using MDS120 (Kodak Digital Science) and Ulead Photoimpact (Ulead Systems) Imaging software as parent applications. Confocal images were acquired with an Olympus Fluoview FV300 confocal laser scanning microscope mounted on an Olympus BX61 upright microscope. Projections of confocal z-stacks were obtained utilizing ImageJ software (National Institutes of Health; http://rsb.info.nih.gov/ij/).

Wound healing velocity. To determine the rate of wound closure, wounded monolayers were examined every 2 h under phase optics on an inverted microscope with a ×10 ocular lens equipped with a Kodak MDS120 digital camera. The resulting images were processed with Adobe Photoshop software (Adobe Systems, Mountain View, CA) as follows. First, the denuded area was painted with the “Bucket” tool. Second, this area was then selected with the “Magic Wand” tool, and the numbers of pixels contained in it were determined using the histogram command. Third, the wound length was obtained using the “Measure” tool, employing “pixel” as the unit. Fourth, the quotient between these two values was determined and considered to represent the average width of the wound. Finally, the width value (expressed in pixels) was converted into micrometers using the micrometric scale of the corresponding image. Three independent experiments were processed in duplicate.
Detergent extraction and Western blot analysis. Confluent cell monolayers grown on 35-mm tissue culture plates were wounded by employing the comb instrument as described in Wounds. After being wounded, monolayers were solubilized in 200 μl of 0.5% Triton X-100 in Dulbecco’s PBS for 5 min on ice; 70 μl of 4× Laemmli sample buffer (19) were immediately added to the soluble material. The insoluble fraction was resuspended in 270 μl of 4× Laemmli sample buffer using a cell scraper. For each fraction, 50 μl were loaded on a 4% stacking-7% running SDS-PAGE gel. Following electrophoresis, proteins were transferred to an Immobilon P membrane (Millipore, Bedford, MA). Nonspecific binding sites of the membrane were blocked with 5% fat-free milk in PBS for 30 min at 37°C and incubated with the anti-pancadherin polyclonal antibody at a 1:10,000 dilution for 1 h. After being washed, the membrane was incubated with a horseradish peroxidase-coupled secondary antibody (Sigma) at a 1:10,000 dilution. The blot was blotted and developed using an enhanced chemiluminescence kit (Amersham, Arlington Heights, IL) and exposed to XAR5 film (Kodak). Quantification of the blots was performed using ImageJ 1.32 software.

Actin cable quantification. The length of the actin cable at the wound border was measured on images taken after cells had been stained with FITC-phalloidin (see above) by employing Adobe Photoshop software (Adobe Systems) as follows. First, the wound border was marked using the “Pencil” tool with a width of 1 pixel (total wound length). Second, the delineated border was selected with the “Magic Wand” tool, and the total numbers of pixels contained in it were determined using the histogram command. Third, the numbers of pixels contained in the actin cable area were determined following the same procedure described above in Wound healing velocity. Finally, the quotient between this value and the total wound length (expressed in pixels) was determined and considered to represent the percentage of actin cable in an image. Three independent experiments were performed for each type of wound. Each one of these experiments was performed in duplicate. Between 7 and 9 images/experiment were processed to obtain the percentage of actin cable.

RESULTS

Mechanisms of wound healing in BCE monolayers. According to the characteristic organization adopted by the actin cytoskeleton, three mechanisms of wound healing could be observed in cultured BCE monolayers: lamellipodia-dependent crawling (Fig. 2A), whole sheet displacement via actin cable formation (Fig. 2B), and a mixed mechanism (Fig. 2C). As shown below in Influence of the ECM on the healing mechanism, the expression of each mechanism depends on the presence of the underlying substratum on the wound surface, a property that can be employed to experimentally induce a predominant mechanism of healing (see MATERIALS AND METHODS). Below, we describe some of the basic cytoskeletal modifications that take place in the two elementary mechanisms (actin cable formation and lamellipodia-dependent crawling) and the influence of wound size and geometry and of the ECM on the selection of these mechanisms.

The adoption of one of the two basic healing mechanisms, lamellipodial crawling or actin cable formation, may have profound consequences on the maintenance of the epithelial phenotype and, consequently, on its function. Thus, it has been shown that during wound healing the formation of an actin cable contributes to the maintenance of many aspects of the phenotype of normal confluent epithelia (2, 34). To further characterize the cellular modifications that occur during the healing response, we explored whether other components of the intercellular junction complexes experience different types of alterations in the two elementary mechanisms of wound healing, in correspondence with those experienced by the actin cytoskeleton. For this purpose, we studied the reorganization undergone by cadherin and vinculin during the healing process. In confluent uninjured BCE monolayers, cadherin is typically localized at cell-cell junctions (Fig. 3A). As shown in Fig. 3B, in monolayers that healed by lamellipodial crawling, cadherin experienced a noticeable reorganization in cells at the wound margin. Thus, 4 h after monolayers had been wounded, cadherin fluorescence decreased in the cell membrane (Fig. 3B, arrowheads), and a highly pronounced vesicular staining of this protein appeared in many cells at the wound border (Fig. 3B, arrow). This pattern could also be observed in some cells a few rows away from the wound margin (not shown). The presence of intracellular staining suggests that cadherin may be internalized from the cell surface, disrupting cell-cell adhesion. Six hours after monolayers had been wounded, an attenuation of the fluorescence could be appreciated in many cells lining the wound, while the vesicular staining could no longer be observed (Fig. 3B). Simultaneously, the F-actin distribution dramatically changed its localization from the cell-cell border (Fig. 3A) toward a typical migratory phenotype (Fig. 3B, arrow). In contrast, in monolayers that healed by actin cable formation, the distribution of cadherin remained almost unchanged in cells at the wound border (Fig. 3C). In this case, no apparent internalization of the protein seemed to occur, and no vesicular staining could be observed. It has been shown that the stability of cadherin-based junctions is correlated with the

![Fig. 2. Different modes of wound closure adopted by BCE monolayers. Shown are wounded BCE monolayers stained to reveal F-actin (see MATERIALS AND METHODS). BCE monolayers repaired wounds by means of lamellipodial crawling (A) or actin cable formation (B) or by a mixed mechanism (C). The prevailing mechanism depended on the presence or absence of the ECM on the wound surface (cf. Fig. 5), a property employed to select the predominant mechanism shown in A and B. During the initial stages of healing, the mixed mechanism (C) predominated. Bar = 30 μm.](http://ajpcell.physiology.org/content/293/10/C1329/F2.large.jpg)
amount of cadherin present in the Triton X-100-insoluble fraction (1, 27). Figure 4 shows the Western blot of Triton X-100-soluble and -insoluble fractions of cadherin of monolayers that healed by acting cable and lamellipodial crawling (A) and a bar diagram of the ratio between both fractions (B). Consistent with the results shown in Fig. 3, Fig. 4B reveals that in monolayers that healed by lamellipodia-dependent crawling, cadherin significantly redistributed from the Triton X-100-

Fig. 3. Actin, vinculin, and cadherin in confluent uninjured and injured BCE monolayers. Uninjured (A) and wounded (B–D) BCE monolayers were stained to reveal modifications undergone by actin, cadherin, and vinculin (see MATERIALS AND METHODS). Times after injury are shown. In uninjured confluent BCE monolayers (A), the three proteins displayed the characteristic epithelial organization. In wounds performed with maintenance of the ECM (B), the lamellipodia-dependent mechanism predominated, and cadherin experienced dramatic modifications. The arrowheads indicate cells where the cadherin fluorescence significantly decreased, and the arrow points to a cell with highly pronounced cadherin vesicular staining. When the ECM was removed (C), the actin cable mechanism became predominant, and cadherin conserved the epithelial localization. Correspondingly, vinculin (D) remained more conserved when the ECM was removed than when it was maintained. The arrows point to vinculin localized at the level of focal adhesions. D shows full stack projections of confocal images; the time after injury was 6 h. Bar = 30 μm.
insoluble fraction to the Triton X-100-soluble fraction. In monolayers that healed by actin cable formation, a shift between the Triton X-100-soluble and insoluble pools could also be observed, although to a considerably lower extent than in the former case. Correspondingly, no significant statistical difference was found in this later case (Fig. 4B). This is in agreement with the observation that cadherin distribution remains practically undisturbed in monolayers that healed by actin cable formation (Fig. 3C).

Similarly to cadherin, vinculin also experienced distinctive modifications according to the healing mechanism adopted. In normal uninjured BCE monolayers, vinculin demarcates cell-cell boundaries, appearing as typical continuous lines at the level of the adherens junctions (Fig. 3A). Figure 3D shows the distribution of actin and vinculin in BCE monolayers that healed by lamellipodial crawling and by actin cable formation. It can be observed that in monolayers that healed by the former mechanism, vinculin experienced a noticeable redistribution. As shown, vinculin was strongly displaced from the lateral plasma membrane and expressed at the level of focal adhesions in many cells (Fig. 3D, vinculin, arrows). In monolayers that healed by actin cable formation, vinculin conserved its typical belt-like localization at the cell periphery in most of the cells near the wound margin. Also in these monolayers, the actin bundles remained mostly at the periphery (Fig. 3D, actin).

Taken together, the results shown in Figs. 3 and 4 support the idea that the mechanism of wound healing entailing the formation of an actin cable contributes to the conservation of the epithelial phenotype during the closure process. In contrast, healing by lamellipodia-dependent cell crawling involves dramatic modifications of the cells at the wound border, i.e., they lose their intercellular contacts and, consequently, the characteristic cobblestone-like morphology.

**Influence of the ECM on the healing mechanism.** As advanced in *Mechanisms of wound healing in BCE monolayers*, the presence or absence of the ECM on the wound surface is critical for the induction of a specific healing mechanism. In this section, we detail the results of systematic experiments in this respect. Figure 5A, left, shows the actin reorganization during the healing of a linear wide wound performed with maintenance of the ECM intact (see MATERIALS AND METHODS). Immediately after injury (*time 0*), cells at the wound border exhibited a cortical distribution of actin, typical of a resting, differentiated cell (not shown). Two hours after monolayers had been wounded (Fig. 5A), some of the adjacent cells around the denuded area became elongated and extended lamellae (arrowhead), whereas others formed an actin cable that spanned several cells along the wound border (arrow). Some cells simultaneously developed an actin cable and extended a lamellipodia beneath it (Fig. 5A, asterisk). Approximately 24 h postinjury, most of the endothelial cells at the leading edge extended lamellipodia into the open wound area (Fig. 5A). By this time, significant cell movement had occurred, and some cells had lost their contacts with neighboring cells (see also Fig. 6A). By 48 h, some areas of the wound had already been totally resurfaced, while others had only partially healed, whereas by 72 h, cells had completely repopulated the once-denuded region (not shown). Figure 5B, top, shows the quantification of the actin cable length during the time course of the healing process of linear wide wounds performed with maintenance of the ECM intact. It can be observed that, 2 h after monolayers had been wounded, between 40% and 50% of the cells presented an actin cable. By this time, an approximately similar percentage of the cells had formed lamellipodia (not shown). By 6 h, the percentage of actin cable diminished to ~25%, reaching a percentage of 0% after 24 h. Similar results for the time course of the actin cable percentage were obtained for narrow linear wounds under the same experimental conditions (data not shown).

To investigate if the ECM affects the wound healing phenotype, linear wide wounds were made with removal of the ECM (see MATERIALS AND METHODS). Under this condition, 2 h after being injured, BCE monolayers showed a mixed mode of closure (Fig. 5A, right): some cells at the leading edge extended lamellipodia (arrowhead), while others formed an actin cable (arrow), similarly to the case of ECM maintenance. To be noted, since the cells are still displacing on the ECM surface (cf. Fig. 1), it is not possible to evaluate the influence of the ECM on the healing mechanism at these initial stages. By 24 h, cells had moved into the wound area originally deprived of the ECM. At this time, most of the cells at the wound margin had developed an actin cable (Fig. 5A). In general, after 48 h, the denuded area still was not completely covered by endothelial cells, whereas by 72 h, the wound was almost fully resurfaced (not shown). Figure 5B, bottom, shows the quantification of the actin cable during the time course of the healing process of
linear wide wounds performed with removal of the ECM. It can be observed that, similarly to the case of ECM conservation (cf. Fig. 5B, top), 2 h postinjury, between 40% and 50% of the cells presented an actin cable, whereas after 6 h, the percentage increased up to nearly 80%, reaching a value of ~95% 24 h postinjury. A similar time course of actin cable formation was observed for linear narrow wounds made with removal of the ECM (not shown).

As mentioned above, the formation of an actin cable during the healing mechanism may play an important role in the maintenance of the epithelial phenotype during the closure event. Despite this, monolayers that employed this mode healed more slowly than monolayers that underwent closure by lamellipodial crawling. In this respect, Fig. 6A shows the degree of closure achieved 24 h postinjury by monolayers that healed by actin cable (i.e., removal of the ECM) and by lamella formation (i.e., maintenance of the ECM). It can be observed that, by this time, the healing process had progressed considerably, both in the presence and absence of the ECM, although for the former case, it occurred significantly faster. In the light of these observations and to further characterize the dynamics of the two healing mechanisms, we determined the time course of the distance traversed by the wound border (obtained from measurements of the wound width, see MATERIALS AND METHODS) in wounds performed with maintenance and removal of the ECM. As shown in Fig. 6B, during the first 2 h postinjury, similar results were obtained for the two experimental conditions. After ~2 h, the distance traversed by the wound border per unit time became progressively larger for the case of wounds performed with maintenance of the ECM. The mean healing rates determined were 0.1 and 0.2 μm/min for wounds made with removal and maintenance of the ECM, respectively. From these results and from the microscopic observations of the evolution of the healing process (not shown), we may conclude that, in BCE wounds performed with removal of the ECM, the leading cells reach the border of the remaining ECM (cf. Fig. 1) ~2 h postinjury.

Influence of wound size and geometry on the healing mechanism. As described above in Influence of the ECM on the healing mechanism, we showed that linear wounds in BCE monolayers healed by lamellipodial cell crawling when the ECM was conserved and by actin cable formation when the ECM was removed, independent of the wound size, according to our observations. To evaluate if the geometry of the wound influences the healing mechanism, small and large circular wounds were created with both maintenance and removal of the ECM (Fig. 7). As shown in Fig. 7, analogous to the case of linear wounds, small and large circular wounds made with conservation of the ECM healed by lamellipodial crawling, whereas those made with removal of the ECM healed by actin cable formation, independent of their sizes. Taken together, the results shown here and in Influence of the ECM on the healing mechanism are highly suggestive that the mechanism of wound healing adopted by BCE cells in culture does not depend on the size or geometry of the wound but on the presence or absence of the ECM on the wound surface.

DISCUSSION

Epithelial wound healing is a complex process developed by epithelial sheets to promptly restore their integrity and thus their function. To this aim, cells at the wound border develop specific mechanisms of healing and, to variable extents according to the particular epithelia, increase their mitotic rate. For the case of BCE monolayers, until the first 24 h of wound healing, no significantly increased rates of mitotic activity of
cells migrating into the wound area can be observed (29). Although it has been known for some time that two basic cellular mechanisms of wound healing can develop in epithelia, actin cable formation and lamellipodia-dependent cell migration, the stimuli for the adoption of either mechanism have not yet been fully recognized. While it has been generally accepted that the size of the wound is a determining factor in this decision, this has only been unequivocally established for the case of wounds consisting of single cell ablations, where the actin cable mechanism always seems to be induced (9, 28, 38, 42). In this work, we have shown that, for the case of BCE monolayers, neither the size nor geometry of the wound are determinants of the particular mechanism selected for the healing process. Instead, we have provided evidence that the presence or absence of the ECM on the wound surface determines the particular cellular mechanism chosen for tissue restitution.

This novel finding is, however, not surprising since, among other properties, the ECM is an intricate mixture of proteins that acts as a reservoir for signaling factors for a variety of processes (30), such as cell proliferation, inflammation, the immune response, angiogenesis, cell migration, and, in particular, wound healing (10, 11, 23, 24, 31, 39). BCE cells secrete a very thick basement membrane-like ECM that has been shown to contain collagen types III, IV, and V (43), fibronectin (15), laminin (14), and proteoglycans (37). In an attempt to explore the possibility that one or more of the components of the ECM is the main determinant of the healing mode adopted by BCE cells, we cultured cells seeded at twice the confluence density on laminin, fibronectin, collagen type I, and glass. Even under these conditions, cells did not reach confluence until 48 h after being seeded, in agreement with previously reported evidence (44). During this period, the cells, independently of

Fig. 6. Evolution of the healing process and closure rate of linear wide wounds performed on BCE monolayers with removal or maintenance of the ECM. A: bright-field images of BCE monolayer wounds performed with preservation or removal of the ECM. Monolayers were stained with crystal violet. Bar = 200 μm. B: time courses of the distance displaced by the border in BCE monolayer wounds made with preservation or removal of the ECM (see MATERIALS AND METHODS). Data are means ± SD of 3 independent experiments. There was a significant difference between the values obtained for the two conditions at 8 h of healing (P < 0.02). The final values of velocity of healing achieved in the two conditions were 21.6 (with ECM) and 6.3 (without ECM) μm/h, determined by considering the distances traversed between 4 and 8 h.

AJP-Cell Physiol • VOL 293 • OCTOBER 2007 • www.ajpcell.org
the substrate, synthesized and deposited their own ECM, as also reported by these authors (44). We confirmed this by laminin immunofluorescence on BCE cells cultured on fibronectin, collagen, and glass (not shown). This characteristic of BCE cells in culture precluded the performance of experiments to identify ECM components involved in the adoption of a particular healing mechanism.

In their vast majority, studies developed to comprehend the healing processes in corneal endothelia in situ have been performed on wounds made with preservation of the totality of the underlying ECM (i.e., Descemet’s membrane). It is to be noted that under this condition, wound healing was found to occur by lamellipodia-dependent cell migration into the wounded area (12, 13, 17, 12, 33, 34), similar to that observed in the present study for the case of cultured BCE layers. This strengthens the possibility that the findings of this work could contribute to the understanding of wound healing in corneal endothelia in situ and, consequently, to the design of strategies to overcome wound-associated pathologies in this tissue. In this respect, it must be emphasized that, although with a lesser rate than corneal epithelium, corneal endothelium is subject to wounding to significant extents in the course of surgical maneuvers and transplants (22, 41) and during hypothermic storage (35).

By creating wounds under different conditions in BCE monolayers, we were able to observe distinct wound healing modes in the same cellular type. Whenever an injury was performed with preservation of the ECM, the healing process predominantly entailed the displacement of the endothelial sheet by spreading and crawling of cells at the leading edge. In contrast, when an injury was created with removal of the ECM, we could observe the progressive production of an actin cable running in the front row of the cells at the wound margin. This highly conserved structure was originally described in embryonic tissues (25) and has been shown to contain myosin II, villin, and zonula occludens-1 (2, 3). The force of contraction across cells is transmitted via adherens junctions, which link the segments of actin cable of each cell to its neighbors (3, 5, 47). For the case of circular wounds, the actin cable undergoes concerted contraction similarly to a purse string (25), which can effectively and rapidly recover tissue integrity. In our work, both circular and lineal wounds were performed. In the latter case, the actin cable-dependent wound closure could be mediated by a few leader cells that extrude lamellae and drag the rest of the monolayer into the denuded area, as previously described for rat liver cells in culture (32). In this work, we have also contributed to the concept that the formation of an actin cable better preserves the epithelial phenotype (2), by showing that this type of closure is more conservative of the typical epithelial distribution of cadherin and vinculin than lamellipodial crawling.

Although the formation of an actin cable represents an advantageous condition from the point of view of the preservation of the epithelial phenotype during wound healing, we could nevertheless observe that actin cable-dependent wound closure was significantly slower than healing via lamellar extension (Fig. 6B). According to our results, this slower rate may be related to the absence of the ECM on the wound bed. There is a substantial body of evidence that supports the concept that the ECM and growth factors are the two major stimuli pro cellular motility (6, 40). Based on our evidence, we can postulate that in the absence of the ECM, growth factors released from the damaged cells represent the only motogens for cell movement, leading to a slower healing rate compared with monolayers that heal by lamellar extension, where both stimuli are permanently present. In this respect, Li et al. (20) have shown that growth factors cannot stimulate the migration of human keratinocytes per se, but can instead augment and refine ECM-initiated migration. If this is the case, what would be the stimulus for movement in monolayers that heal in the...
absence of the ECM? For our case, we can hypothesize that in experiments with removal of the ECM, endothelial cells can synthesize and deposit their own matrix during wound healing. This newly synthesized ECM could hence act as a motogen for motility.

The results obtained in this work permit an outline of the evolution of the cellular events that take place during the healing process of BCE monolayers under the experimental conditions of this study. For the case of wounds made with removal of the ECM, the procedures employed nevertheless conserve a portion of the matrix at the wound borders (Fig. 1). Therefore, under the two experimental conditions, the healing process always starts in the presence of an underlying ECM. As a consequence, in the early stages, cells at the wound border exhibit the same behavior. It is interesting to note that during these initial stages, border cells develop two basic structural modifications, actin cable formation and lamellipodial protrusions, to approximately similar extents. From a physiological perspective, this mixed initial response may constitute an advantageous property, since it prepares the healing cells for any ulterior contingency, i.e., absence or presence of the ECM on the wound surface. For the case that the totality of the ECM has been conserved on the wound bed, the healing process occurs with a gradual predominance of the lamellipodia-dependent mechanism. For the case that most of the ECM has been removed, when border cells encounter the ECM-deprived region, the mechanism entailing an actin cable progressively prevails. As suggested above, this could be a consequence of the gradual loss of ECM-dependent signaling factors. The sequence of events under the two experimental conditions can be described by a simple dynamic model of actin cable and lamellipodia formation at the wound border (see the APPENDIX). As can be seen (compare Figs. 5B and 6B with Fig. 8), this model represents a good approximation to experimental results. The fact that this rather straightforward model easily reproduces the results for the two experimental conditions without the introduction of elaborated assumptions further supports the concept that the two mechanisms are initially developed by border cells and are later selected according to the ulterior presence or absence of the ECM on the wound surface. As mentioned, the occurrence of a similar response at the beginning of the healing process for the two experimental conditions is related to the fact that the removal of the ECM nevertheless conserves a portion of the matrix near border cells (Fig. 1).

In summary, in this work, we have shown that BCE cells in culture represent a good model for the study of the factors affecting wound healing since it permits the induction of a predominant cellular mechanism of healing, actin cable formation or lamellipodia-dependent cell crawling, in a single cellular system. In particular, we employed this system to determine that the absence or presence of the underlying ECM represents a critical factor to determine the selection of a predominant mechanism of healing. This finding may thus contribute to the elucidation of the cues that regulate the different modes of motility adopted by an epithelial cell at a free wound edge and, consequently, permit a better understanding of the physiological and pathological processes where cell and tissue motility play a pivotal role, including normal development, inflammatory responses, angiogenesis, and tumor invasion.

Fig. 8. Numerical simulation of the dynamic model of the healing mechanisms of BCE monolayers. A: time course of the actin cable formation for the cases of ECM removal and maintenance. Eqs. A2 and A3 were integrated numerically by employing the Runge-Kutta fourth-order method and the parameters listed below. For the case of ECM maintenance, rate constants of formation of actin cables and lamellipodia (α and β) and rate constants of destruction of actin cables and lamellipodia (γ and δ) remained unchanged throughout the simulation and equal to α0, β0, γ0, and δ0 (see below). For the case of ECM removal, at time = 2 h, parameters α, β, γ, and δ were gradually modified from the initial values of \( α_0, β_0, γ_0, δ_0 \) to the final values of \( α_1, β_1, γ_1, δ_1 \) (see below), following the time course given by Eq. A7. B: time course of the distance traversed by the healing border under the two experimental conditions. Equation A6 was employed, using the values of the variables resulting from the integration of Eqs. A2 and A3 and the parameter values listed below. \( t_{act} = 2 \times 10^{-4} \text{s}^{-1}; β_0 = 1 \times 10^{-3}, γ_0 = 1.5 \times 10^{-4} \text{s}^{-1}; δ_0 = 1 \times 10^{-3} \text{s}^{-1}; \text{ characteristic parameter } A = 2 \times 10^{-3} \mu \text{m}; \text{ characteristic parameter } B = 6 \times 10^{-3} \mu \text{m}; \text{ and time constant } T = 10^3 \text{s}. \)

APPENDIX: A DYNAMIC MODEL OF THE HEALING MECHANISM

We introduce here a model to describe the dynamics of formation of the actin cable and lamellipodial protrusions at the border of an epithelial wound. At any time \( t \), the wound border displays zones with actin cable, lamellipodial protrusions, or none of these structures, such that

\[
L_{AC} + L_L + L_T = 1
\]

(\( A1 \))

where \( L_{AC} \), \( L_L \), and \( L_T \) represent the fractions of wound border
displaying actin cable, lamellipodial protrusions, and none of these structures, respectively. The total relative length of the wound border \((L_E)\) equals 1.

We assumed that the dynamic model governing the velocity of change of \(L_{AC}\) and \(L_E\)

\[
\frac{dL_{AC}}{dt} = \alpha L_E - \gamma L_{AC} \quad (A2)
\]

\[
\frac{dL_E}{dt} = \beta L_E - \delta L_E \quad (A3)
\]

where \(\alpha\) and \(\beta\) are the rate constants of formation of actin cable and lamellipodia and \(\gamma\) and \(\delta\) are the rate constants of destruction of actin cable and lamellipodia, respectively.

From Eqs. A1, A2, and A3, the steady-state values of \(L_{AC}(L_{AC(ss)})\) and \(L_E(L_E(ss))\) are

\[
L_{AC}(ss) = \alpha \delta L_E/D \quad (A4)
\]

\[
L_E(ss) = \beta \gamma L_E/D \quad (A5)
\]

where \(D\) equals \(\alpha \delta + \beta \gamma + \delta \beta\).

For simulation purposes, Eqs. A2 and A3 were integrated numerically by employing the Runge-Kutta fourth-order method and the numerical values of the parameters shown in Fig. 8. These values were determined in a trial and error fashion so as to obtain good approximations to the experimental results. We assumed that, for any time step (i.e., 1 s), the distance traversed by the wound border in the course of the healing process \((\Delta l)\) is

\[
\Delta l = AL_{AC} + BL_E \quad (A6)
\]

where \(A\) and \(B\) are characteristic parameters (numerical values employed are also shown in Fig. 8).

According to the experimental results, the presence or absence of the ECM determines differences both in the rate of production of the actin cable and in the overall velocity of healing. As can be observed in Fig. 6B, the differences start to be evident approximately after 2 h postinjury. For the simulations, we therefore assumed that \(\alpha, \beta, \gamma, \) and \(\delta\) were the same, both in the presence and absence of the ECM, for the first 2 h of the healing process (parameters \(\alpha_0, \beta_0, \gamma_0, \) and \(\delta_0\)). For all rate constants, we assumed that this modification did not take place abruptly but followed instead a time course given by

\[
\eta = \eta_0[1 - \exp(-\tau/\theta)] + \eta_1[\exp(-\tau/\theta)] \quad (A7)
\]

where \(\eta\) stands for \(\alpha, \beta, \gamma,\) or \(\delta; \tau\) is the new time variable \((\tau = t - 2 h; \text{in s})\); and \(\theta\) is the time constant (Fig. 8). The ~2-h delay observed may be related to the fact that the manipulations to detach the ECM nevertheless conserve a portion of the matrix close to the wound border (see DISCUSSION).

For the example shown in Fig. 8, we actually considered one particular case, where only \(\gamma\) and \(\delta\) experience alterations in the absence of the ECM. As can be seen from the numerical values employed (Fig. 8), we particularly considered the case where the removal of the ECM determines a decrease in \(\gamma\) and an increase in \(\delta\). Although, as mentioned, the numerical values for the parameters were determined in a trial and error fashion, the criteria for the final choices were based on the biochemical and physiological plausibility of the elected values. Thus, for instance, under initial conditions \(L_{AC} = 1\).

Since, at these initial conditions, no destruction of actin cable and lamellipodial protrusions occurs at the wound border, the relative rates of formation of these structures are directly given by parameters \(\alpha\) and \(\beta\), respectively. These parameters therefore represent the maximum relative rates of formation of the corresponding structures. As another example, the significance of parameters \(A\) and \(B\) can be put into evidence at the final steady-state conditions of the healing process. For the case of the ECM maintenance, the steady-state condition implies that the major part of the wound border displays lamellipodial protrusions (cf. Fig. 5). Under this condition, only the term containing \(L_{AC}\) affects Eq. A6. Since \(L_{AC}(ss)\) approximately equals 1 (cf. Eqs. A4 and A5 and the values listed in Fig. 8), parameter \(B\) directly yields the stationary velocity of healing (i.e., \(6 \times 10^{-3}\) mm/s; see Fig. 8). Analogously, for the case of ECM removal, the final values of \(L_{AC}(ss)\) and \(L_E(ss)\) approach 0.8 and 0.02, respectively (cf. Eqs. A4 and A5 and the values listed in Fig. 8). Hence, the steady-state velocity of healing under this condition is approximately given by 0.8 A mm/s (i.e., 1.6 \(\times 10^{-3}\) mm/s; see Fig. 8). These values are also plausible from a physiological point of view and consistent with those experimentally determined (see Fig. 6).

Other particular situations mimicking the possible signaling effects of the ECM on cellular structures, such as modifications on \(\alpha\) and \(\beta\), were not considered in this study.

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REFERENCES


