Wounding Induces Motility in Sheets of Corneal Epithelial Cells through Loss of Spatial Constraints

ROLE OF HEPARIN-BINDING EPIDERMAL GROWTH FACTOR-LIKE GROWTH FACTOR SIGNALING*

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Cellular responses to wounding have often been studied at a molecular level after disrupting cell layers by mechanical means. This invariably results in damage to cells at the edges of the wounds, which has been suggested to be instrumental for initiating wound healing. To test this, we devised an alternative procedure to introduce gaps in layers of corneal epithelial cells by casting agarose strips on tissue culture plates. In contrast to mechanical wounding, removal of the strips did not lead to detectable membrane leakage or to activation of the stress-activated kinase JNK. Nonetheless, cells at the edge underwent the typical morphological transition to a highly motile phenotype, and the gaps closed at rates similar to those of mechanically induced wounds. To allow biochemical analysis of cell extracts, a procedure was devised that makes cell-free surface area acutely available to a large proportion of cells in culture. Rapid activation of the epidermal growth factor receptor (EGFR) was detected by immunoblotting, and the addition of an EGFR-blocking antibody completely abolished wound healing. In addition, wound healing was inhibited by agents that block signaling by the heparin-binding epidermal growth factor-like growth factor (HB-EGF). Cells stimulated with cell-free tissue culture surface released a soluble factor that induced activation of the EGFR, which was distinct from HB-EGF. These studies suggest that the triggering event for the induction of motility in corneal epithelial cells is related to the sudden availability of permissive surface area rather than to mechanical damage, and they demonstrate a central role of signaling through HB-EGF.

Epithelia serve to physically separate various compartments of organisms from each other and from the outside world. In wounding, this separation breaks down, and powerful mechanisms have evolved to re-establish the barrier function quickly after damage. Accidentally inflicted wounds in the corneal epithelium are commonly seen in clinical practice, and refractive surgery is increasingly popular. Defects in corneal epithelial healing are often the consequences of viral or bacterial infections or are due to compromised innervation and can be very serious and may ultimately lead to blindness (1). These are compelling reasons for studying wound healing in the cornea. In addition, the cornea is an attractive experimental model because of its simple structure; it consists of only three tissue layers, contains no lymph or blood vessels under normal conditions, and is very accessible for experimental manipulations.

The human corneal epithelium is stratified squamous and consists of 5–7 cell layers. As in other epithelia, corneal epithelial wounds initially heal by cells migrating to cover the lesion, and only later are lost cells replenished by accelerated mitosis (for reviews, see Refs. 2–7). The cells move after a lag phase, during which protein synthesis is redirected (8). In addition, the cells at the leading edge typically undergo marked changes such as dissolution of cortical actin filaments, formation of stress fibers and lamellipodia, and loosening or loss of intercellular adhesion structures. These are profound changes in the cellular phenotype and are reminiscent of the epithelial to mesenchymal transitions that occur during development and malignant progression (9, 10).

Wounding is often studied in tissue culture after inflicting wounds by mechanical means. This invariably induces damage to the cells at the edges of the wounds and has been suggested as triggering initiation of movement, for instance by the release of intracellular components to stimulate neighboring cells (11). Alternatively, this initiation of movement could result from the sudden availability of a permissive surface area after wounding. The purpose of the present work was to evaluate these possibilities and to clarify the signaling processes that initiate epithelial cell movement.

EXPERIMENTAL PROCEDURES

Materials—The antibody against E-cadherin was from Transduction Laboratories, antibodies against the epidermal growth factor receptor (EGFR),1 the EGFR phosphorylated on tyrosine 1173 (pEGFR/Tyr-1173), and JNK phosphorylated on Thr-183 and Tyr-185 were from Santa Cruz Biotechnology, the EGFR neutralizing antibody LA1 was from Upstate Biotechnologies, antibodies against epidermal growth factor (EGF) and heparin-binding epidermal growth factor-like growth factor (HB-EGF) were from R&D Systems, and the antibody against HB-EGF, membrane-bound HB-EGF precursor; TGFα, transforming growth factor-α; JNK, cJun NH2-terminal kinase; PBS, phosphate-buffered saline.

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with adjacent limbs were excised and grown as explants according to Ebato et al. (12) in SHEM medium consisting of base medium (Ham’s F12 and Dulbecco’s modified Eagle’s medium (1:1), 0.5% Na2HPO4, 50 units/ml penicillin, and 50 µg/ml streptomycin) supplemented with 15% fetal calf serum, 0.1 µg/ml cholera toxin, 10 ng/ml human recombinant EGF, 5 µg/ml insulin, and 40 µg/ml gentamicin. A431 cells were from the American Type Culture Collection and were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Human corneal rims were obtained from Center for Organ Recovery and Education (CORE), Pittsburgh, PA, and explants were prepared as described above.

Preparation of Cell Cultures Containing Agarose Strips and Mechanical Wounding—Molds for casting agarose strips were made of plastic vehicle lettering (Chroma International Inc.). 6 × 17-mm rectangles were cut, and 1.0 × 11-mm strips were removed from the centers of these by means of two razor blades separated by five layers of laboratory tape. The rectangles were attached to the bottom of tissue culture plates, and 2.5% agarose with 1% glycerol was pipetted in the opening and allowed to dry. The plastic was removed, and cell suspensions were added according to standard tissue culture procedures. Mechanical wounds were inflicted by scraping with 1-ml pipette tips. For quantification of the widths of the gaps, the cells were fixed in 3.7% formaldehyde and 0.5% Triton X-100 in PBS and stained with 0.05% gentian violet. The stained cell layers were displayed on a screen with an overhead projector at 10-fold enlargement, 10 measurements of the width of the gaps were taken at equally spaced intervals, and averages were calculated for each data point. To analyze cell stress and membrane damage, propidium iodide (10 µg/ml) was included in the medium, and cells were fixed 3 min after the infliction of wounds and stained with anti-phospho-JNK antibodies. Neutralizing antibodies against TGFRα, EGF, HB-EGF, and EGFR were added 1 h before removal of the strips at 20 µg/ml. Cells were preincubated for 1 h with tyrophostin AG 1478, GM 6001, and CRM 197 at 10 µM, 50 µM, and 50 µg/ml, respectively. All experiments were performed at least three times with similar results.

Preparation of Extracts of Cells Acutely Presented with Increased Culture Area and Immunoblotting—0.25% agarose with 0.85% glycerol was applied to tissue culture dishes on ice/water baths using an atomizer that generated a fine mist. Inclusion of glycerol was found to be necessary to reduce the adhesion of the agarose droplets to the tissue culture plastic so that they could be subsequently removed. The dishes were dried at 65 °C for 10 min, and a ring of 1% agarose was cast around the periphery to prevent cells from growing up the sides of the dishes. After drying the plates for an additional 2 h, cells were plated by standard procedures. When the cells had grown to generate an epithelial phenotype, they were serum-starved for 3 h and then fixed with 4% paraformaldehyde in phosphate-buffered saline (171 mm NaCl, 10.1 mm Na2HPO4, 3.35 mm KCl, and 1.84 mm KH2PO4, pH 7.2). After one wash with PBS, the cells were permeabilized with 0.5% Triton X-100 in PBS with 10% fetal calf serum (blocking buffer) for 1 h, washed once with PBS, and incubated for 2 h with a 1:30 dilution of anti-E-cadherin antibody in blocking buffer. To visualize F-actin, the cells were washed with PBS and incubated with Alexa Fluor® 546-conjugated phalloidin in 3 units/ml. The cells were washed three times with PBS and then incubated in a 1:2,000 dilution of the secondary antibody conjugated to Alexa Fluor® 488. The plates were washed again three times with PBS, and coverslips were applied with 90% glycerol in PBS plus 2.5% (2,2,2)-octane. Images were captured on an Olympus 1 × 70 inverted microscope with a Bio-Rad Radiance 2000® confocal system and Lasersharp® software.

RESULTS

Induction of Cell Movement in the Absence of Detectable Cell Damage—To test whether the processes related to cell damage are important for inducing motility upon the wounding of rabbit corneal epithelial cells in tissue culture, we devised a method to introduce gaps that results in minimal perturbation of the cells. Small blocks of agarose were cast on tissue culture dishes, and rabbit corneal epithelial cells from primary explants were seeded on the plates. The cells grew to form confluent sheets interrupted by the agarose blocks and acquired a distinct epithelial phenotype even at the edges bordering the agarose; the cells had the typical “cobblestone” epithelial morphology and contained cortical F-actin, rather than stress fibers, that extended to parts of the cells that directly bordered the agarose (Fig. 1A). Also, E-cadherin, which is a component of adherens junctions, was found at cell-cell junctions, but it was absent from the parts of the membrane of cells that were contiguous with the agarose (Fig. 1A). The absence likely reflects a requirement of E-cadherin to interact with E-cadherin on adjacent cells in order to concentrate in areas of the membrane (13). Gaps were then introduced by removing the agarose carefully with a forceps. To assess possible membrane damage, we included a small membrane-impermeable marker molecule, propidium iodide, in the tissue culture medium before removal of the agarose or scrape wounding. As is shown in Fig. 1B, staining was undetectable in cells at the edges of the gaps after removal of the agarose. In contrast, cells bordering mechanical wounds exhibited strong staining with propidium iodide. Cell stress commonly leads to strong activation of the JNK class of protein kinases, and we found that mechanical scrape wounding leads to robust JNK activation as determined by staining with an antibody that specifically recognizes the activated form of JNK. This response was absent in the agarose strip technique. We conclude that removal of agarose strips constitutes a means of introducing a gap in a cell layer that causes minimal cell stress.

We next determined whether cells move to cover the gaps introduced by removal of the agarose strips. Lamellipodia were evident on the cells at the wound edge after ~30 min, cell-cell contacts were loosened as evidenced by the internalization of E-cadherin, and F-actin was re-organized from a cortical localization to form stress fibers (Fig. 1A). After a lag phase of 3–4 h, the cell sheet started to migrate, and the gaps closed after 10–14 h (Fig. 1C). Control experiments using mitomycin C revealed that healing of wounds was not dependent on cell division (data not shown). These observations are very similar to those seen after mechanical wounding, suggesting that cell damage is not necessary for the induction of motility, but rather that the induction of movement is the result of the sudden availability of appropriate tissue culture surface area on which the cells can move.

Activation of the EGFR after the Release of Cells from Spatial Constraints—Analysis of signaling pathways often involves the addition of hormones or growth factors to cells in tissue culture and the subsequent preparation of cell extracts. A presumption
is that a majority, if not all, of the cells in the cultures respond to the treatment. To examine the response to sudden release of spatial constraints, it was necessary to devise a procedure that acutely subjects a large fraction of the cells in a culture to increased surface area. Agarose was sprayed on tissue culture plates, and cells were seeded. The cells grew to form a reticulum, the strands of which were a few cell diameters wide (Fig. 2A). They had a distinctly epithelial morphology, and staining with Alexa Fluor®-conjugated phalloidin revealed that F-actin was localized in the cortical regions of the cells and that E-cadherin was present in cell-cell contacts (data not shown). The data shown in this and the following figures are the means of four determinations, and the error bars are S.D.

The EGFR has been implicated in inducing cell migration in wounds in corneal and other epithelia (14–18), and we therefore examined whether the EGFR is activated when corneal epithelial cells are presented with additional culture space. The cells were briefly serum-starved, and extracts were prepared at various times after removal of the agarose droplets. The extracts were then immunoblotted with an antibody that recognizes the Tyr-1173 phosphorylated form of the EGFR, which is a major autophosphorylation product of the receptor stimulated with extracellular ligands (19). As seen in Fig. 2B, the EGFR was rapidly activated with a peak at ~5 min. As a control, which is depicted as time 0, no top agarose was added, and the cells were lysed directly on the plates. As an additional control, top agarose was added to confluent cells in the absence of agarose droplets and processed as described above. No activation of the EGFR was detectable under these conditions, indicating that activation is related to the sudden availability of increased culture space rather than to a possible direct mechanical stimulation of the cells caused by removal of the top agarose.

Fig. 2. Activation of the EGFR upon release of spatial constraints. A, phase contrast images of cells grown on plates containing agarose droplets before and 14 h after removal of the agarose droplets. Note the appearance of prominent lamellipodia (indicated by arrows) and fibroblast-like morphology. The magnification at 14 h is twice that at 0 h. B, tyrosine phosphorylation of the EGFR at various times after removal of the agarose droplets. Control is a sample in which cells had been grown to confluence on plates in the absence of agarose droplets and analyzed 5 min after the removal of top agarose. The 0 point is a sample that was lysed directly without the addition of top agarose. For comparison, cells were stimulated with HB-EGF. C, the samples shown in panel B were blotted with an antibody that detects the EGFR irrespective of phosphorylation state.

Signaling through the EGFR Is Necessary for Closing of the Gaps—We initially examined whether stimulation of the EGFR enhances closure of gaps in cultures of corneal epithelial cells. Cells in serum-free conditions were found to heal, but the addition of either EGF or HB-EGF significantly enhanced the rate of healing (Fig. 3A). To examine whether activation of the receptor is necessary for the healing of wounds, an inhibitor of the EGFR kinase, tyrphostin AG 1478, was added, and it was found to completely inhibit closure of the gaps (Fig. 3B). This is in agreement with observations using mechanical wounding (14, 15). As a more specific test of the involvement of the EGFR, we also utilized the neutralizing antibody LA1, which binds to the extracellular domain of the EGFR molecule and blocks binding of ligands without activating the receptor kinase. Because this antibody is human-specific, we used corneal epithelial cells from human donors. As is illustrated in Fig. 3C, addition of the antibody completely blocked the healing of gaps.
in the cell layers. Examination of extracts after removal of the top agarose showed that phosphorylation of Tyr-1173 of the receptor was completely abolished by the antibody (Fig. 3D). The antibody suppressed formation of lamellipodia and stress fibers at the wound edge, and internalization of E-cadherin in cells at the edge was also inhibited (Fig. 3E). Taken together, these data strongly suggest that activation of the EGFR is central in regulating the processes that induce cell movement upon the release of spatial constraints.

Healing Occurs through HB-EGF Signaling—The observation that the LA1 antibody blocked activation of the EGFR and healing indicated that transactivation of the EGFR occurs through stimulation by an extracellular ligand rather than by an intracellular mechanism. We therefore tested the effect of neutralizing antibodies against several EGFR ligands. As is shown in Fig. 4A, the addition of an antibody to HB-EGF blocked closure of the gaps by 70%, whereas the addition of anti-EGF or anti-TGFα antibodies was without effect. Similarly, as with the anti-EGFR antibody, the anti-HB-EGF antibody also suppressed the formation of lamellipodia, re-organization of the actin cytoskeleton, and internalization of E-cadherin (data not shown). ProHB-EGF serves as the receptor for diphtheria toxin, and a non-toxic analog of this toxin, CRM 197, inhibits the biological activity of HB-EGF but not the activities of several other ligands for the EGFR (20, 21). The addition of this compound similarly inhibited healing of gaps in the cell layers (Fig. 4A).

Like other EGFR ligands, HB-EGF is released from a larger membrane-bound precursor, proHB-EGF, by the activities of Zn2+ proteases of the ADAM (a disintegrin and metalloproteinase) or matrix metalloprotease families (22). We therefore analyzed the effect of GM 6001, which is a general inhibitor of these proteases, and found that the inclusion of 50 μM GM 6001 inhibited the closure of gaps in monolayers of primary rabbit corneal epithelial cells in accordance with the notion that cleavage of proHB-EGF is necessary for the process (Fig. 4B). Importantly, this block was overridden by the inclusion of 10 ng/ml HB-EGF, indicating that the inhibition of migration was not due to some unspecific toxic effect of the inhibitor on the cells.

Also, it was important to confirm that the inhibition of HB-EGF signaling resulted in the expected reduction of EGFR activation. As is seen in Fig. 4C, incubation with the anti-HB-EGF antibody prior to and during removal of the top agarose inhibited activation of the receptor, as did incubation with the HB-EGF-neutralizing agent CRM 197. Also, activation of the receptor was inhibited in the presence of the protease inhibitor GM 6001. Importantly, the addition of GM 6001 did not block the ability of exogenously added HB-EGF to stimulate the
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Cell damage has been suggested to be a prerequisite for the initiation of cell movement (11). Proposed mechanisms have included the release of intracellular growth factors or other messengers, such as ATP from damaged cells, that could induce adjacent cells to migrate. In the present paper we report that the typical wound healing response can be induced in layers of corneal epithelial cells by removing strips or droplets of agarose with little evidence of cell damage. This is in accord with the proposal that the movement of epithelial sheets during dorsal closure in Drosophila melanogaster development is mechanistically similar to wound healing (23) because dorsal closure is not associated with any obvious mechanical damage to cells. JNK signaling is clearly important for dorsal closure (24, 25), and the lack of detectable JNK activation by immunostaining after the removal of agarose strips does not necessarily signify that JNK has no role in wound healing in corneal epithelial cells. Signaling by JNK is extraordinarily complicated (26–28).

In general, stress induces a stronger activation than what is seen upon stimulation with growth factors (29), and a lower level of activation may have been missed by our procedures. Also, basal rather than activated JNK is necessary for wound healing in monolayers of fibroblasts (30).

An often used procedure for studying wound healing in tissue culture is to inflict a wound by some mechanical means, for instance by scraping a cell layer with a pipette tip, and then biochemical analysis can be performed on extracts from cultures in which multiple scrape wounds have been inflicted. However, there are problems with this approach. Ideally, the experimental stimulation of the cells should only induce cell migration, but mechanical wounding induces other processes as well. For instance, some cells are detached from the tissue culture plates and may undergo cell death by anoikis (31). Mechanical wounding invariably damages cells physically, which either stimulates membrane repair processes (32, 33) or induces cell death. In addition, many epithelial cells, including corneal epithelial cells, are highly phagocytic, and the sudden appearance of cell debris stimulates phagocytosis and cytokine production (34). These processes constitute experimental “noise” that is minimized by the present procedure. We therefore believe that the procedures described here will be generally useful in studies on cell movement.

Like other in vitro models of wound healing, the procedures described in this paper have limitations. Clearly, cell stress or damage may have consequences in the context of the whole animal that are not observable in cultures of cells, such as induction of inflammation or angiogenesis (6, 35). One mechanism that presumably contributes to wound healing in vivo in the cornea and certain other tissues is contraction of an actin/myosin cable that is connected from cell to cell at the wound edge (the “purse string” mechanism) (23, 36, 37). However, we and others (36) have, to date, been unable to observe this structure ex vivo in corneal epithelial cells.

It is plausible that the initiating event for the induction of cell movement in our system is the interaction of cell surface receptors with elements of the extracellular matrix in the vicinity of the cells. This interaction is expected to be blocked by the presence of neighboring cells, or, in our system, by agarose on the tissue culture surface. Integrins are obvious candidates for receptors that may sense the extracellular matrix in this system (38, 39). However, our attempts to block activation of the EGFR by RGD-containing peptides have, to date, been
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unsuccessful, and it is noteworthy that integrin-mediated EGF activation was reported to occur through an intracellular mechanism that does not involve the production of extra-cellular ligands (40), a mechanism that is clearly different from the one reported here. A different initiating mechanism was demonstrated in a recent elegant study in airway epithelium (41). Disruption of the compartmentalization of heregulin-α in the fluid surrounding the apical parts of the cells and its receptors in the basolateral membranes is responsible for activation of the erbB2–4 receptors upon wounding. This mechanism is unlikely to apply to our system, because removal of the agarose strips does not break cell-cell contacts. Furthermore, in contrast to the study of Vermeer et al. (41), we find that the disruption of cell junctions by Ca2+ chelation cannot account for the observed activation of the EGF receptor and that the addition of HB-EGF results in robust activation of the EGFr, which is not increased by disruption of cell junctions (data not shown). Also, the cessation of negative cues from neighboring cells has been proposed to constitute a triggering event for the wound-healing response (42). However, because initiation of the movement of cells in the immediate leading edge after the removal of the agarose strips cannot be ascribed to the termination of negative signals from cells ahead of them, such a mechanism can be discounted in this system. Our observations are in accord with previous reports suggesting a role for the EGF receptor in the healing of mechanically induced wounds (14, 15). We here report that activation occurs though signaling by HB-EGF. This ligand appears to have a significant role in wound healing in vivo, because it is a major growth factor in fluids from excisional and burn wounds (43, 44), and it is therefore of considerable interest to understand how HB-EGF is released. In one model, mechanical scraping of an epithelial cell line caused increased production of HB-EGF mRNA near wounds, and increased transcripts have been observed in skin organ culture (45, 46). However, the rapid onset of EGF activation does not suggest a role for transcription in the system described in this communication. Tokumaru et al. (47) reported the release of HB-EGF by proteolysis upon mechanical wounding of human keratinocytes. We believe that this reflects a different underlying mechanism because subconfluent cells were used in that study, and the release may be caused by mechanical trauma rather than the sudden availability of extracellular space. In addition, the EGF receptor was activated 90 min after the wounding in contrast to the rapid activation reported to occur in this study. Presumably, there are several mechanisms that can contribute to HB-EGF signaling.

The lack of detectable HB-EGF activity in the cell culture supernatants has clear precedents in a number of systems demonstrating G protein-mediated transactivation of the EGFr. It is likely that the high affinity of HB-EGF for the heparan sulfate proteoglycan cell coat leads to its removal from the tissue culture supernatants (Refs. 48–50; for reviews, see Refs. 51 and 52). More surprising was the appearance of a factor in the supernatants from stimulated cells that caused activation of the EGFr, the properties of which differed markedly from HB-EGF and therefore likely represents a different molecule. It is noteworthy that the production of this factor was strictly dependent on HB-EGF signaling, and it could have a role in propagating EGF-inducing signals in adjacent cells. Establishing its identity is clearly an interesting topic for future studies.

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Note Added in Proof—A recent report has demonstrated a role for HB-EGF signaling in the healing of mechanically inclined wounds in corneal epithelial cells (Xu, K.-P., Ding, Y., Ling, J., Dong, Z., and Yu, F.-S. Invest. Ophthalmol. Vis. Sci. (2004) 45, 813–820).
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