Directed Migration of Corneal Epithelial Sheets in Physiological Electric Fields

Min Zhao,* Adriana Agius-Fernandez,† John V. Forrester,‡ and Colin D. McCaig*

Purpose. To characterize the effects of small applied electric fields (EFs) (100 to 250 mV/mm) on cultured bovine corneal epithelial cell (CEC) sheets and to determine how EFs interact with other environmental cues in directing CEC sheet migration.

Methods. Primary cultures of bovine CECs were exposed to EFs in medium with or without serum, epithelial growth factor, basic fibroblast growth factor, or transforming growth factor-β1. Cell sheet migration was traced using an image analyzer.

Results. Cell sheets migrated toward the cathode (negative pole). The directional migration was voltage dependent, and, at low field strength (up to 200 mV/mm), it required serum in the medium. Sheets showed no migration responses up to 200 mV/mm in serum-free medium, whereas those in medium with serum showed evident migration toward the cathode, at an average rate of approximately 15 μm/h (n = 15 ~ 20) at 150 mV/mm. When serum was present, the threshold was below 100 mV/mm, very close to the measured wound field strength (~42 mV/mm). After supplementing serum-free medium with individual growth factors or with combinations of epithelial growth factor, basic fibroblast growth factor, and transforming growth factor-β1, significant restoration of cathode-directed migration occurred at 150 mV/mm. Lamellipodia were abundant at the leading edges of migrating sheets, extending the area of sheets covered. The extension of cell membranes toward the cathode was more prominent in cell sheets than in single cells.

Conclusions. The endogenous EFs generated by wounded cornea could play an important role by interacting with other environmental factors to promote changes in shape and in directed migration of CEC sheets. Invest Ophthalmol Vis Sci. 1996;37:2548-2558.

Corneal epithelial cells respond rapidly to injury by changing shape and by migrating to cover the wound and restore the barrier function. Corneal epithelial wound healing occurs predominantly by the integrated centripetal migration of sheets of cells.1-5 Successive tiers of cells move as a continuous sheet in a unified, coordinated manner while maintaining intercellular linkages. Migration is unidirectional, toward the wound center.2 Contact inhibition, chemotaxis, haptotaxis, and contact guidance have been shown to underlie directed cell movement. Additionally, electric fields (EFs) may play an important role in directing cell migration during development and wound healing because endogenous DC electric fields exist in vivo, and physiological electric fields direct cell movements in culture.6,7 In the eye, the wounded corneal epithelium generates a small DC electric field,8 whereas single corneal epithelial cells reorient and migrate directionally when cultured in a DC field.9,10 We reported recently that single bovine corneal epithelial cells responded to EFs at a field strength close to that which exists in vivo.10 Most in vitro experiments with EFs are performed with single cells for ease of analysis. However, cells coupled by gap junctions react as a single unit to an applied electric field, and gap junctional coupling may increase the sensitivity of cells to weak EFs because the perturbations caused by an applied field are greater for electrically coupled cell ensembles than for single cells11 (see Discussion). Importantly therefore in this regard, two distinct gap junction proteins (connexins) have been demonstrated recently in rat corneal epithelium.12

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For the Proprietary interest category: N. Referent requests: Colin D. McCaig, Department of Biomedical Sciences, Marischal College, Aberdeen AB9 1AS, Scotland.
We tested whether cultured bovine corneal epithelial cell sheets respond to EFs in a manner similar to single cells. Cell sheets migrated cathodally in EFs, and directional migration was voltage and serum dependent. Possible mechanisms underlying the serum dependency also were tested by adding epithelial growth factor (EGF), basic fibroblast growth factor (bFGF), and transforming growth factor (TGF)-β1 into serum-free medium. Additionally, this allowed a test of the interactions between an EF and several growth factors, all of which will be present at a lesion in the corneal epithelium. Part of the results have been published in abstract form.[13,14]

**MATERIALS AND METHODS**

**Materials**

Human recombinant EGF (expressed in *Saccharomyces cerevisiae*), bFGF (from bovine pituitary glands), and TGF-β1 (from human platelets) were from Sigma (St. Louis, MO). Anti-bovine bFGF monoclonal antibody (type I, monoclonal IgG1K) was from TCS Biologicals (Upstate Biotechnology, NY). Stock solutions of EGF and bFGF were reconstituted with 5 ml of serum-free Dulbecco’s modified Eagle’s medium. Stock solution of TGF-β1 was made by the addition of 4 mM HCl containing 1 mg/ml bovine serum albumin. Aliquots were stored at -70°C and diluted to the final working concentration immediately before use.

**Cell Cultures**

Corneal epithelial cells were obtained by methods modified from Gipson and Grill.[15] The epithelial cells obtained with this or similar methods[16,17] have been demonstrated to be largely free of contamination from other cell types. Details of the methods are given in Zhao et al.[10] In brief, fresh bovine eyes were obtained from a local abattoir immediately after death and were refrigerated until used (usually within 2 hours). Blocks of the limbus corneal epithelial layer next to the conjunctiva (approximately 3 x 5 mm², with part of the stroma) were removed under sterile conditions and transferred to a dish containing Dispase II (1.2 U/ml, Boehringer Mannheim, Mannheim, Germany) in calcium- and magnesium-free phosphate-buffered saline with antibiotics and incubated at 37°C for 1.5 hours. The epithelial layer was peeled off in saline under a dissection microscope and transferred to saline containing 0.25% trypsin for 5 to 7 minutes at 37°C with intermittent gentle shaking. Digestion was stopped by adding 10 to 15 ml Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (FBS) to deactivate the protease. The cell pellets collected after centrifugation at 1000 rpm for 5 minutes, twice in the same media, were resuspended to a cell density of 14 to 24 x 10⁶ cells/ml and were seeded in a specially made trough[18] formed by two parallel strips of glass coverslip (no. 1; length, 6.4 or 2.2 cm) adhered to the base of the dish (Falcon 3003; Becton Dickinson Labware, Plymouth, UK) with silicone grease (MS4; Dow Corning GMBH, Munchen, Germany) and separated by a gap of 1 cm. Observation immediately after seeding of the cells in the chamber showed primarily single cells and small clumps of cells. After a 24-hour incubation at 37°C, large cell sheets formed in the center of the chamber, where cell density was higher, whereas single cells or small colonies congregated and grew most commonly in the peripheries. Before seeding cells, scratch lines were made on the substratum, perpendicular to the long axis of the chambers, with a fine sterile needle and were used as reference marks for directed cell migration. The cells were incubated for 24 to 48 hours (37°C; 5% CO₂), which allowed them to settle and adhere to the base of the dish, before a roof of no. 1 coverslip was applied and sealed with silicone grease. The final dimensions of the chamber, through which current was passed, were 64 x 10 x 0.5 mm or 2.2 x 10 x 0.5 mm (Fig. 1 in ref. 10). Cells grown this way developed as monolayer sheets of different sizes, but rarely did we note cells piling up to form multiple layers.

**Electric Field Stimulation**

Electric field exposure was started 24–48 hours after seeding of the cells. Agar-salt bridges at least 15 cm long were used to connect silver–silver chloride electrodes in beakers of Steinberg’s solution to pools of excess culture medium at either side of the chamber. This prevents diffusion of electrode products into the cultures. Field strengths were measured directly at the beginning and end of the observation period. No fluctuations in field strength were observed. Immediately before field application, the original FBS containing medium was replaced with Dulbecco’s modified Eagle’s medium with or without 10% FBS and growth factors using a push–pull technique and handheld Pasteur pipettes. In some cases, the replacement medium contained bFGF or 10% FBS with antibodies to bFGF. This medium was incubated for 30 minutes at 37°C with intermittent shaking before it was added to the culture chamber. For the experimental design, see Figure 1 of reference 10. During EF exposure, the culture dishes were set in room air; therefore, the carbon dioxide concentration and the temperature were those of room air. Our previous experiments using the same culture chambers showed that EF exposure (185 mV/mm) caused no detectable changes in temperature within the culture medium. Phenol in the medium was used as an indicator of culture medium pH level. In most cultures, this remained sta-
Directional translocation rate

\[ \text{directional translocation rate} = \frac{[(d_1 + d_2)/2]}{5} \]

FIGURE 1. Translocations of both the leading and the trailing edges of cell sheets relative to the electric field vector (indicated) were measured and averaged as translocation along the x-axis over a 5-hour period. Measurements were made with reference to a static scratch on the substratum, indicated at the right. A representative cell sheet is indicated as a circle at time 0 hour and after translocation at time 5 hours.

RESULTS

Effects of Electric Fields on Cell Sheets

Cell sheets cultured in a control chamber (no EFs) showed little obvious migration during the 5-hour observation period and no preferentially directed extension of the cell membrane. In a DC electric field, cell sheets migrated toward the cathode while they change shape (Fig. 2). Shortly after the onset of the applied EF (20 ~ 30 minutes), cell sheets began to retract membrane extensions from the anode-facing side and to extend lamellipodia toward the cathode. During
FIGURE 3. Reversing the polarity of the electric field (EF) reversed the direction of migration of cornea epithelial cells (CEC) sheets. (top) A CEC sheet initially attached to a scratch at the right margin migrated away from the scratch, toward the cathode at the left. (bottom) Two hours after the EF was reversed, membranes extended toward the new cathode (now to the right), and the cell sheet eventually merged again with the static scratch on the substratum in Dulbecco’s modified Eagle’s medium with serum at 150 mV/mm.

The rate of cathode-directed migration was quantified as the speed along the x-axis, in the direction of the electric field vector, toward the cathode (Fig. 1). Among the sheets we followed, there was no significant correlation between migration rates and sheet size, although sheets moved at variable rates (Fig. 2). The sheets we followed comprised approximately 50 cells (ranging from 3 to approximately 100 cells). As for single cells, the directional migration of cell sheets in EFs showed voltage dependence and serum dependence (see next section).

field application (5 hours), there was no habituation to the field, and cell sheets migrated toward the cathode persistently and steadily. The leading edge of the sheets extended ruffled membranes and lamellipodia in the direction of migration and retracted membrane extensions from the trailing edges. These events were much more obvious for cell sheets than for dissociated cells. Many single cells extended lamellipodia at the ends of the long axis of the cells during field exposure—that is, in a direction perpendicular to the field vector. When the polarity of EFs was reversed, directed movement of sheets also reversed (Fig. 3). Reversal of sheet movement occurred through the retraction of protrusions facing the anodes and by the production of new lamellipodia on the side of the sheets facing the cathodes. Large cell sheets also migrated toward the cathodes; the sheets even became confluent (Fig. 4) at one point. The largest sheets, observed showing cathodal migration, were approximately 0.5 ~ 1 x 5 ~ 7 mm and became confluent between two reference scratches. Cathode-directed migration of such sheets was indicated by a jamming of cells against the scratch to the cathode side and detachment from the scratch at the anode side (not shown). Sheets of corneal epithelial cells of varying size responded to EFs in a unified and coordinated way and appeared to maintain intercellular linkages. In other words, they responded in a manner similar to wound healing after corneal epithelium injury in vivo and in vitro.

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Mean translocation rate of cell sheets to the cathode

![Graph showing mean translocation rate of cell sheets to the cathode](image)

**FIGURE 5.** Directional translocation rate of cell sheets to the cathode in electric fields. Cells were cultured in Dulbecco's modified Eagle's medium with serum (●), without serum (■), with 10% fetal bovine serum (▲), and with 10% fetal bovine serum and 25 ng/ml epithelial growth factor. The directional translocation rate of cell sheets is both voltage dependent and serum dependent. *P < 0.01 when compared to serum-free control; \( P < 0.05 \) when compared to 150 mV/mm serum-free control.

Prolonged exposure of cultured cells to 150 mV/mm for 48 to 72 hours showed no evidence of cell damage, whereas cathode-directed migration persisted throughout this period (not shown). Figure 3 shows that active responses of cells to EFs persisted during a 10-hour exposure period. To quantify the cathode-directed migration of cells after long exposure to EFs, we exposed cultures to EFs of 150 mV/mm in serum containing medium for 24 hours, then replaced the medium with fresh medium and followed the cells for another 5 hours at the same EF strength. Cell sheets showed cathode-directed migration at a directional translocation rate of 8.09 ± 2.12 μm/hour (n = 16), which is approximately half the rate at which they migrate cathodally in an initial 5-hour period (17 ± 1.9 μm/hour; n = 15) (Fig. 5 and next section).

### Effect of Serum on Electric Field-Directed Migration of Cell Sheets

No net directional movement of cell sheets was found in control dishes (no EF) (Table 1). Random movement of sheets was extremely rare in either serum-free medium or medium with serum. No significant directional migration of cell sheets was found up to 250 mV/mm in serum-free medium (Fig. 5). At 250 mV/mm, cell sheets showed signs of contraction (not shown). In medium containing serum or in serum with added EGF, cell sheet migration was voltage dependent. Sheets showed significant cathodal migration at 100 mV/mm, with peak migration rates occurring at 150 mV/mm (Fig. 5). Additionally, supplementing serum containing medium with EGF enhanced cathode-directed migration rates at 100 mV/mm (Fig. 5).

Sheets moved more uniformly than did dissociated cells. Single corneal epithelial cells (CECs) moved to the cathode in a zigzag pattern. In most cultures, one or two dissociated cells moved anodally during EF application (albeit very slowly; for details, see Fig. 3 in ref. 10). Sheets of cells never migrated anodally, although shrinkage of cell sheets in serum-free medium at higher voltages sometimes resulted in retraction of the leading edge. At higher voltages (200 and 250 mV/mm), a few cell sheets showed signs of shrinkage. This was more severe in serum-free medium.

Field-directed migration of cell sheets was faster than that for dissociated cells. At 100 mV/mm, the translocation rate toward the cathode in medium with serum and EGF for cell sheets was 7.3 ± 1.2 μm/hour for the leading edge and 7.6 ± 1.5 μm/hour for the trailing edge; for dissociated cells, it was 3.9 ± 1.4 μm/hour, a significant difference (\( P < 0.05 \)). The directional translocation rate of cell sheets in medium with serum was also markedly higher than that in serum-free medium at 150 and 200 mV/mm (\( P < 0.01 \)) (Fig. 5). At 150 mV/mm, cell sheets in both media

### TABLE 1. Directional Translocation Rate (μm/hour; mean ± SEM) of Control Epithelial Cells* (No Electric Field, 5 Hours)

<table>
<thead>
<tr>
<th>Epidermal Growth Factor (ng/ml)</th>
<th>0</th>
<th>12.5</th>
<th>25</th>
<th>50</th>
<th>100</th>
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</thead>
<tbody>
<tr>
<td>Dissociated cells</td>
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<tr>
<td>(n = 16)</td>
<td></td>
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<tr>
<td>Leading edge</td>
<td>1.30 ± 1.90</td>
<td>-1.70 ± 1.60</td>
<td>2.67 ± 1.75</td>
<td>0.87 ± 0.86</td>
<td>-1.40 ± 1.40</td>
</tr>
<tr>
<td>(n = 13)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Trailing edge</td>
<td>-1.00 ± 1.60</td>
<td>-1.20 ± 2.10</td>
<td>1.35 ± 1.47</td>
<td>0.58 ± 1.33</td>
<td>3.10 ± 1.20</td>
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<tr>
<td>(n = 9)</td>
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* Values are given as μm/hour; mean ± SEM; medium contains 10% fetal bovine serum plus EGF as indicated.

* = movement to left of chamber; − = movement to right of chamber.
Addition of Growth Factors Partially Restored Electric Field-Directed Migration of Sheets

In serum-free medium, cathodal migration of cell sheets was restored by the addition of specific growth factors (Fig. 6). We have demonstrated this also for single cells.10 With optimal concentrations of single growth factors in serum-free medium, cathodal migration of cell sheets was restored to a greater extent for cell sheets than for single cells measured with the same method (Fig. 6).

The leading edge of sheets extended ruffled membranes and lamellipodia preferentially in the direction of field-stimulated migration. In medium supplemented with bFGF or TGF-β1, cell sheets expanded to cover a greater area by extending membrane dramatically and preferentially toward the cathode while moving in the same direction (Fig. 7). This expansion was most apparent at 1.5 pg/ml for TGF-β1 and 200 ng/ml for bFGF, as shown by the separation of the directional translocation rates of the leading edge and trailing edge of cell sheets (see Materials and Methods; directional translocation rates for the leading edges were 8.0 ± 1.1 and 7.0 ± 1.3 μm/hour for TGF and bFGF, respectively, whereas those for the trailing edges were 2.1 ± 0.7 and 1.0 ± 0.6 μm/hour, respectively).

Combinations of two and three growth factors in the medium yielded higher directional translocation rates (Fig. 8) than those in single growth factor-supplemented medium. The mean migration rate in medium with EGF + TGF (13.4 ± 2.1 μm/hour) was
significantly higher ($P < 0.05$) than the highest rate obtained with a single growth factor, TGF 1 pg/ml (7.1 ± 1.6 μm/hour).

We used the current method of measuring directional translocation rates to compare the effects of single growth factors on cell sheets and on single cells. Directional translocation rates of single cells or of cell sheets in serum-free medium supplemented with single growth factors did not differ. However, in medium supplemented with combinations of growth factors, cell sheets had higher directional translocation rates than single cells. At 150 mV/mm, directional translocation rates of cell sheets in serum-free medium, with the following combined supplements, were (in μm/hour):

- EGF (25 ng/ml) + TGF-β1 (1 pg/ml) 13.4 ± 2.1 ($n = 27$);
- *EGF (25 ng/ml) + bFGF (100 ng/ml) 7.3 ± 1.5 ($n = 20$);
- *bFGF (100 ng/ml) + TGF-β1 (1 pg/ml) 9.8 ± 1.9 ($n = 19$).

The respective values for single cells under the same conditions were 8.9 ± 1.2 ($n = 37$; $P = 0.06$); 3.7 ± 0.8 ($n = 42$; $P = 0.04$); and 3.9 ± 1.5 ($n = 26$; $P = 0.02$).

To test whether field-induced directional migration in bFGF-containing medium could be abolished by neutralization of bFGF by its antibody, monoclonal anti-bovine bFGF antibodies were added to medium containing 100 ng/ml bFGF (antibody final concentration: 30 μg/ml). For single cells, the directional translocation rate was reduced by 65%, from 4.6 ± 0.8 μm/hour (without neutralization of bFGF; $n = 23$) to 1.6 ± 1.0 μm/hour ($n = 44$; $P < 0.05$). For cell sheets, the directional translocation rate after neutralization also was reduced slightly (5.0 ± 1.3 μm/hour, $n = 19$, compared to 3.0 ± 1.4 μm/hour, $n = 13$). Addition of the antibody to medium containing 10% FBS failed to reduce significantly the directional migration rates toward the cathode of either single cells or cell sheets. Directional translocation rates without and with antibody were:

- single cells, 9.1 ± 2.0 μm/hour ($n = 21$) and 7.7 ± 1.8 μm/hour ($n = 21$);
- *cell sheets, 17.0 ± 1.9 μm/hour ($n = 15$) and 12.7 ± 1.8 μm/hour ($n = 14$).

**DISCUSSION**

**Electric Fields and Wound Healing**

Cell migration is critical in wound healing. Commonly, groups of cells move together.\(^1\)\(^2\)\(^3\) During reepithelization of a corneal wound, for example, sheets of epithelial cells migrate in a unified manner to the wound. A possible role for endogenous DC electric fields in directing this migration has been proposed\(^9\)\(^10\)\(^19\) on the following grounds: A potential difference exists across the entire cornea in frog, rabbit, and cattle\(^8\)\(^20\)\(^21\) with most of the potential drop across the corneal epithelium. Lesion-induced disruption to this transepithelial potential difference instantaneously results in a steep, laterally oriented voltage gradient close to the newly created current sink. In bovine cornea, DC electric fields of 42 mV/mm close to a wound have been measured and represent a lower limit.\(^8\) Recent work also indicates the importance of endogenous and applied EFs in directing cell growth, in both embryonic development\(^22\)\(^23\) and wound healing.\(^19\) In newts, there is direct evidence for the involvement of wound currents in reepithelization. Disruption of the naturally occurring wound current of the skin inhibits wound healing, whereas normal rates of epithelization are restored by passing exogenous currents to mimic the inhibited wound current.\(^19\) A wounded cornea, covered by a tear film that keeps the outer surface moist, resembles amphibian skin in which electric fields have been shown to play an important role.\(^10\)\(^20\) Soong et al\(^9\) showed that single rabbit CECs change shape to lie perpendicular to the applied field and to migrate toward the cathode. We have confirmed and extended this work by showing that single bovine CECs respond similarly to small EFs, that responses are serum dependent, and that the addition of EGF, bFGF, and TGF-β1 at certain concentrations to serum-free medium restores EF-directed cell migration.\(^10\)

However, experiments on single cells may not replicate adequately the in vivo situation, in which healing occurs through the migration of integrated cell sheets. It is important, therefore, to know whether sheets of CECs also respond to electric fields. We have demonstrated, for bovine cells, that large sheets of cells respond to electric fields by migrating directionally; that cell sheets respond more readily to smaller electric fields than do single cells and, indeed, to field strengths very close to the measured field strength in lesioned bovine cornea; that, as with single cells, the field-directed migration of cells sheets is serum dependent; that field-directed translocation of cell sheets in serum-free medium is restored partially by the addition of EGF, bFGF, and TGF-β1, singly or in combination; and that cell sheets expand to cover greater areas by extending lamellipodia in the direction of the field vector.

**Corneal Epithelial Sheets Respond to Small Electric Fields**

Although single CECs and cell sheets responded to small EFs applied in vitro, there were distinct differ-
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Change. Cell sheets migrated toward the cathodes more smoothly and uniformly; the directional translocation rate (see Materials and Methods) was faster under conditions that may mimic more closely the in vivo situation (lower field strength, serum presence); and cell sheets showed more dramatic spreading of lamellipodia, extending toward the cathodes, than did single CECs (Figs. 3, 7).

Fish epidermal cell sheets have been shown to migrate directionally in an EF at very high speed: ~20 μm/m. 16 How do cell sheets respond to EFs as a unified unit? Cells coupled by gap junctions will react as a single unit to an applied EF. Cooper 11 suggested with a theoretical model that the membrane potentials of gap junction-coupled cells facing the anode or cathode would be substantially more hyperpolarized or depolarized, respectively, than those of uncoupled cells in a given electric field. This may enhance cellular responses to a weak electric field. Gap junctions are widespread in corneal epithelium, 12 and CEC coupling may underlie the enhanced responses of sheets over single cells. Sheets moved faster than isolated cells—100 mV/mm 7.5 μm/hour versus 3.9 μm/hour (P < 0.05). However, although sheets moved faster than isolated cells, we did not see an obvious correlation between the size of a sheet and the migration rate toward the cathode, as this model predicts. Further experiments with pharmacologic agents affecting gap junction function may determine the extent of gap junction involvement in the field-induced responses of corneal epithelial sheets. In animal models 27,28 and from clinical observations, 29,30 movement of cell sheets is critical in corneal wound healing. Our observation that CEC sheets do respond to low EFs (and to added EGF) may be important in the context of wound healing.

The highest directional migration rate (17 μm/hour) in this in vitro model lies at the lower end for reported CEC migration rates. Migration rates of 60 to 70 μm/hour commonly are reported. 29,30 Both the measuring and the culturing methods may contribute to a reduced migration rate. The migration rate was measured by averaging the speed of the leading and trailing edges of a whole moving sheet (see Materials and Methods and Fig. 1), whereas most previous reports traced the leading edge only. Migration rates in vivo are fastest at the leading edge, closest to the wound margin. 2 That, however, does not fully explain our slower rates because the fastest leading edge in this study was approximately ~30 μm/hour. Additionally, most previous experiments have been conducted in vivo or with organ culture models, both of which involved a multilayered corneal epithelium. 29,30 The current study used a cell monolayer experimental model. In vivo and in organ culture, cell migration and cell spreading occur during healing. Cells migrate to the wound center, and they flatten and extend lamellipodia toward the wound. 2,28,29,30 Spreading of cells could contribute markedly to the migration rate of the leading edge of a wound, where the cells are denser. We saw a dramatic spreading out of monolayer sheets at the leading edges (Fig. 7). However, in stratified cell sheets, reorganization at the wound margin caused by cells flattening and spreading, with a resultant thinning of the epithelium, might produce substantially greater rates of epithelial migration. 2,28,29,31

Another possible factor affecting migration rate might be the temperature; our experiments were conducted at room temperature (18°C to 25°C), whereas the fastest migration rates observed were in experiments conducted at 37°C. (Our culture system did not induce a detectable temperature change at field strengths of 185 mV/mm, but in a substantially deeper chamber [1 mm], a small temperature rise of only 0.7°C occurred at 198 mV/mm. 18 )

Serum, Growth Factors, and Electric Field Interact in Promoting Migration of Sheets

Endogenous EFs are unlikely to operate in isolation. Serum and plasma induced significantly greater epithelial growth in a wound than in serum-free medium. 32,33 Serum elements improved the maintenance of cornea in air–liquid corneal organ culture. Interactions might be expected between EFs in wounded cornea and serum–growth factor elements, which gain access to the corneal epithelium from stroma or from the tear film.

Sheets show little migration in control chambers (without EFs) (Table 1). Cathodal migration of cell sheets was dependent on serum, as we have shown previously for single cells. 10 Migration of sheets was robust at 150 mV/mm in serum-containing medium, but it was absent or extremely weak in serum-free medium, even at 250 mV/mm. Thus, serum markedly reduced the threshold level at which migration occurred. The threshold to respond with directed migration may be reduced further by the addition of EGF to serum-containing medium. Under these conditions, cell sheets migrated faster cathodally at 100 mV/mm than they did without the added EGF (Fig. 5). We do not know the absolute threshold, but clearly it is less than 100 mV/mm. These data have a twofold importance. First, they indicate that in cultures that may mimic more closely the in vivo situation (added multiple environmental cues and most cell linkages remaining), field-directed migration of CEC sheets is enhanced. Second, they offer pointers to underlying mechanisms. Shortly after injury, the basal cells of the corneal epithelium at the wound margin begin to lose their hemidesmosome attachment sites, change their shape from columnar to elongated, and project lamellipodia in the direction of migration. This is similar to
our observations that cell sheets expand by extending huge lamellipodia (Figs. 3, 7) to cover more area while migrating cathodally.

Individual growth factors only partially restored serum-induced cathodal migration. Even at optimal concentrations, single growth factors only restored cathodal migration rates to a maximum of 38% of the serum-supplemented rate. Combinations of growth factors, however, had additive effects on directional migration of cell sheets. EGF + TGF or EGF + bFGF + TGF significantly increased cathodal migration rates of cell sheets, at best restoring migration to 68% of serum-supplemented values (Figs. 6, 8). Interactions between growth factors may arise. TGF-β1 modulates the effect of EGF on corneal cells. Exposure of corneal keratocytes to TGF-β1 increased the number of high-affinity receptors for EGF as much as tenfold, although there was no effect on corneal epithelial cells. Additionally, TGF-β1 binding to its receptors activates different kinases (serine–threonine kinases) from those activated by EGF and bFGF (tyrosine kinases) and, in this way, may be capable of recruiting different, parallel signaling pathways, involved in directed cytoskeleton reorganization.

Mechanistically underpinning the cathode-directed migration of CEC sheets may be the demonstration that growth factor receptors (or other cell surface proteins) become redistributed and accumulate predominantly cathodally within the plasma membrane—for example, in fibroblasts. Electric fields and EGF both result in localized, spatial reorganization of the cytoskeleton, and together these events may drive the directed migration seen when EFs are presented, together with serum or with specific growth factors. A more detailed discussion of potential mechanisms underlying field-directed CEC migration is given elsewhere.

With serum present and at low voltages, or with certain combinations of growth factors replacing serum, cell sheets migrated faster cathodally than did isolated cells (Fig. 8). Cells within sheets frequently are coupled to each other electrically by gap junctions. Cellular assemblies thus become much longer than their electrical length constant, with the consequence that a growing proportion of an externally applied electric field could target intracellular, as well as extracellular, elements to induce directed migration.

Basic fibroblast growth factor antibodies prevented cathode-directed migration of CECs when bFGF replaced serum, but they did not prevent directed migration in serum-containing medium. This indicates that activation of a specific growth factor receptor is sufficient to permit some field-directed migration, but that other individual elements within serum also are capable of inducing a similar response.

In medium supplemented with 1.5 pg/ml TGF-β1 and 200 ng/ml bFGF, cell sheets expanded to cover a greater area by extending membrane preferentially toward the cathode (Fig. 7). Independent of either whole cell migration or cell proliferation, this would be of clinical significance if it reduced the area of wounded corneal surface lacking epithelial coverage.

An external gradient of protein–growth factors might arise in field-exposed cultures because of electrophoresis. Electric fields of similar size do create a gradient of fluorescently labeled albumin in an agarose gel. Negatively charged growth factor proteins, however, would be expected to accumulate anodally, and cell sheets migrated cathodally. Unless they grow away from such a gradient, we consider that the directed migration of cell sheets is most likely a direct effect of the EF. In addition, using similar culture systems, experiments with cross-perfused medium to disrupt chemical gradient formation demonstrate quantitatively similar responses to EF application with or without cross perfusion. Finally, what (if any) chemical gradients were established in culture would be established by endogenous wound currents

Physiological–Clinical Significance

Significantly, whole cell sheets migrate cathodally in an applied electric field and in the presence of serum and added EGF. The threshold for migration is <100 mV/mm, close to the EF values measured directly at wounds in bovine corneal epithelium (42 mV/mm was a low estimate). These responses implicate physiological wound currents in corneal wound healing, in which the wound becomes covered by the expansion of epithelial cells, by cell shape changes, by the extension of lamellipodia toward the wound center, and by migration as a unified sheet, in a coordinated manner, with intercellular linkages maintained. Mitosis is rare during migration.

To promote corneal wound healing, especially in clinical conditions when this is poor or absent, it will be critical to know how to guide epithelial cell migration. Strategies for enhancing overall rates of epithelial migration additionally may prove useful. The demonstration that an applied electric field alone can provide both these elements for cell sheets and that these
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can be enhanced by appropriate use of certain growth factors may be clinically relevant.

The manner in which these data relate to the highly ordered epithelial migrations that underlie "hurricane keratopathy" in humans, which produces a vortex pattern of healing, is unknown. Interestingly, Dua et al have proposed that this pattern results from the natural endogenous electromagnetic fields of the eye. We are dealing with natural, endogenous DC electric fields induced by epithelial disruption on wounding and with the application of DC fields to mimic this.

Key Words

cell sheet migration, corneal epithelial cells, electric fields, galvanotaxis, wound healing

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