Movements of Cultured Corneal Epithelial Cells and Stromal Fibroblasts in Electric Fields

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The effects of an externally applied direct-current electric field on the movement of cultured rabbit corneal epithelial cells and stromal fibroblasts were studied. After a latency of approximately 20 minutes in an electric field, both epithelial cells and stromal fibroblasts became spindle shaped and underwent galvanotropism by aligning their long axes perpendicular to the applied electric field. The electric field stimulus thresholds for galvanotropic movements in epithelial cells and stromal fibroblasts were 4 V/cm and 6 V/cm, respectively. After an additional latency of 30 minutes, both cell types manifested galvanotaxic movements: epithelial cells commenced migration in the cathodal (downfield) direction and stromal fibroblasts in the anodal (upfield) direction. For both types of cells, ruffled membranes and lamellipodia were abundant at the leading edges of migrating cells, and cell processes underwent retraction at the trailing edges. At field strengths of above 10 V/cm, evidence of cellular damage (manifested by cellular rounding and detachment), attributable to the electric field treatment, was observed after 4 hours. These preliminary results suggest that galvanotaxic responses could be exploited clinically in the enhancement of corneal wound healing.

Several extrinsic factors are involved in the control of directed cell-shape changes and movement during cell migration. These include contact inhibition, chemotaxis, haptotaxis, and contact guidance. Recently, the influence of physiologic electric (E) fields on the control of directional cell movements has attracted the widespread attention of cell biologists in the areas of developmental biology and wound healing. It is now known that many types of cells respond to weak direct-current (DC) E-fields with a galvanotropic response (the polarized shifting of cellular orientation in an E-field), followed by a galvanotaxic response (the migration of a cell in an E-field). Biologically generated E-fields are ubiquitous in nature and are particularly significant in embryonic development and wound healing, during which strong extracellular ionic currents are produced by the living cells. These ionic currents constitute a relatively slow electrical phenomenon and are distinct from the faster, pulsatile electrical events associated with action potentials in nerve and muscle cells. During embryogenesis, such E-fields are generated by many different varieties of embryonic tissues.

Materials and Methods

Cell Cultures

Adult New Zealand albino rabbits were killed with intravenous pentobarbital. Use and maintenance of the animals were in accordance with the ARVO Resolution on the Use of Animals in Research. Corneal epithelial cultures were established according to the method of Gipson and Grill. Epithelial sheets were
placed onto plastic dishes and cultured at 37°C and 5% CO₂ in Eagle’s minimum essential medium (MEM) with nonessential amino acids, L-glutamine, penicillin, streptomycin, amphotericin B, epidermal growth factor (EGF) (10 ng/ml), and 10% fetal calf serum. For stromal fibroblast cultures, Descemet’s membrane and endothelium were stripped mechanically with jewelers’ forceps from the epithelium-free corneal buttons. The remaining stroma was cut into small pieces, explanted onto plastic culture dishes, and incubated at 37°C in 5% CO₂. Except for the absence of EGF, the culture medium used for corneal fibroblasts was identical to that used for corneal epithelial cultures. After establishment of preconfluent epithelial cell and fibroblast cultures (approximately 5–7 days), the monolayer cell cultures were then detached from the dishes with trypsin-ethylenediaminetetraacetic acid treatment and seeded onto acid-washed 22 × 30-mm (#1½ glass coverslips placed on the bottoms of plastic culture dishes. The cells were allowed to spread on the coverslips for 48 hr more at 37°C and 5% CO₂ (in the same MEM formulation, but without serum or EGF) before being placed in the experimental (galvanotaxis) chamber for study. Confluence was avoided.

**Experimental Chamber and Quantitation of Cell Movement**

Galvanotaxis chambers were constructed by attaching two parallel (0.50-cm apart) strips of glass coverslips (180-μm thickness) onto the coverslip with attached cells. The parallel strips thus formed 180-μm-high side walls of the galvanotaxis channel, while the cell-covered coverslip formed the floor (Fig. 1). The channel was then covered with another coverslip, thus producing a flat, closed channel with the approximate dimensions of 0.018 × 0.5 × 3.0 cm. This dimensional configuration maximized the surface area-to-volume ratio, thus reducing joule heating effects. A watertight seal between the coverslips was produced with sterile petrolatum, and the channel was filled with serum-free MEM. Agar-bridge electrodes, filled with 2% agarose in serum-free MEM, were assembled into a culture medium reservoir at both ends of the galvanotaxis channel (Fig. 1). The other ends of the agar-bridge electrodes were immersed in culture medium. Agar–saline bridges were used to isolate the cells from electrolysis products generated by the silver chloride (Ag–AgCl) electrodes. Electrical current was applied via a current-regulating circuit powered by a DC voltage supply. Voltage and current in the chamber were constantly monitored with a pair of miniature AgCl electrodes, the voltage via a high-impedance digital voltmeter, and the current via a low-loss digital milliammeter. The chamber assembly was placed in a custom-made incubator constructed around an inverted microscope. The temperature in the incubator was maintained at 37°C with a thermistor-controlled heater system. In addition, the temperature in the immediate vicinity of the

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Fig. 1. Schematic diagram of galvanotaxis chamber setup.
microscope objective lens was constantly monitored with a separate temperature-sensitive probe. A maximum of 0.02°C rise was observed during E-field stimulation. The atmosphere in the chamber was maintained at 5% CO₂. Under inverted microscopy, cells were photographed on ASA-400 movie film at automatically preset time intervals (usually 5–10 min apart) for up to 5 hr. No color changes in the culture medium (phenol-red indicator for pH) were noted at any time during E-field application. Stimulus field strengths of 2–13 V/cm were used in this study. Movements recorded on film were traced on an optical analyzer which plotted pivotal and displacement movements.

Results

In both epithelial cells and fibroblasts, at E-fields of weaker than 4 V/cm, little or no changes in cellular shape or orientation were observed compared with field-free controls. Controls showed only random cellular movements and no net directional cell translocation. Epithelial cells underwent galvanotropism within 20 min at a threshold stimulus field intensity of 4 V/cm. The stimulus threshold for galvanotropism of stromal fibroblasts was slightly higher (6 V/cm). Galvanotropism was manifested in both cell types by elongation of the cell bodies into spindle-shaped somata that became oriented orthogonally to the E-field lines (for brevity, only epithelial cells are shown, Fig. 2). Cells extended ruffled membranes, lamellipodia, and filopodia preferentially in the direction of anticipated galvanic migration and concomitantly retracted membrane extensions from the trailing edges.

Galvanotaxic movements were seen as early as 30 min after the onset of galvanotropism. Epithelial cells migrated to the cathode at a displacement rate of 20–30 μm/hr (Fig. 2), and fibroblasts migrated to the anode at a rate of 10–20 μm/hr. Migration rates of both cell types remained constant throughout the field strengths used (ie, from the respective thresholds of each cell to the highest field strength used [13 V/cm]). Cells did not migrate in absolutely straight lines along the E-field, but instead migrated approximately along the E-field with the major vectorial component aligned toward the appropriate destination electrode. Some cells with prominent ruffled lamellipodia before E-field stimulation were observed to reverse their lamellipodial polarity, and hence the direction of migration, as much as 180° in response to the applied E-field stimulus (Fig. 2). Reversing the polarity of the E-field stimulus caused both epithelial cells and fibroblasts to reverse the direction of migration. Field reversal constituted an experimental control to ensure that the cells were not following some external guidance other than the E-field.

After 4 hr under experimental conditions, cells which were exposed to E-fields and control cells that were not both showed morphologic evidence of cellular damage by inverted microscopy. This was manifested by intracytoplasmic vacuoles, cellular rounding, and cellular detachment from the substrate. These damaged cells often did not migrate (even if they remained firmly attached to the substrate) and showed no movements even after being allowed to recover for 2 hr in E-field free culture. At stimulus intensities of less than 10 V/cm, the degree of cellular damage after 4 hr was indistinguishable between E-field-stimulated cells and unstimulated control cells. However, above 10 V/cm, morphologic evidence of cellular damage after 4 hr was noticeably more prominent in the cells exposed to the E-field than in the control cells.
Discussion

The development of the vibrating probe, capable of measuring extracellular voltage gradients at spatial intervals as little as 10-μm apart, led to the mapping of net ionic fluxes surrounding cells in embryonic development and wound healing. Through this technique, we now know that cells are naturally surrounded by a blanket of "physiologic" E-fields. For instance, polarized epithelial monolayers usually produce a trans-layer voltage drop of up to 100 mV, which in turn, can move substantial secondary currents out of discontinuities in the monolayer that occur after wounding (ie, injury currents). Similar potentials have been measured across the cornea. Sizable extracellular electric currents are also produced endogenously by fibroblasts migrating into a wound, and these currents are directed inward at the leading edge. Injury currents diminish with progressive coverage of the cellular discontinuity during wound healing.

Although galvanotaxis in most cells is toward the cathode, a few cells (eg, macrophages and osteoclasts) migrate toward the anode. We observed that corneal fibroblasts and epithelial cells migrate in mutually opposite directions. Similarly in bone, osteoclasts migrate toward the anode, while osteoblasts migrate toward the cathode. It has been postulated that peculiar differences in Ca²⁺, Na⁺, and K⁺ channels, surface charge, and specific membrane resistance may contribute to the inherent direction of galvanotaxis. Both perpendicular alignment and directed migration of cells in an E-field appear to be Ca²⁺-dependent, blocked by Ca²⁺ antagonists, and are influenced by extracellular Ca²⁺ levels. Local perturbations in the cell-membrane potential produced by the external E-field produce an intracellular voltage gradient, which may affect the orientation and function of integral membrane proteins that control the voltage-sensitive ionic channels. It is possible that this gradient may be affecting other intrinsic cellular processes that control directed cell movements.

Cellular damage in the corneal cells after 4 hr at weaker stimuli (<10 V/cm) is probably not due to the E-field, since it was observed in both stimulated and unstimulated cells. It is more likely that this nonspecific damage may have resulted from the less than ideal conditions associated with the relatively simple and rudimentary incubator built around the microscope, repeated exposure of the cells to the microscope light during time-sequence photography, and manipulation of the cells during the process of mounting into the galvanotaxis chamber. Morphologic evidence of cellular damage after 4 hr at high stimulus intensities (>10 V/cm), on the other hand, appears to be directly related to E-field effects, as it was more prominent in the stimulated cells than in the unstimulated control cells. This stimulus-specific damage may be a result of joule heating effects or other yet unidentified electrochemical alterations. Cells which appeared damaged by morphologic criteria did not regain their original shapes or migrate when allowed in recover in field-free culture. As a part of a future study, we are planning to investigate further the viability of these cells using supravital dyes (eg, trypan blue) and measuring lactic dehydrogenase levels.

We believe that the corneal cells are migrating in response to the E-field rather than some other external guidance cue for several reasons: (1) in the absence of an E-field, both cell types showed random movements rather than directional tropism or translocation; (2) some cells underwent as much as a full 180° flip in their directional polarity (ie, ruffled membranes and lamellipodia) after they were exposed to a field; (3) the serum-free condition reduces the effects of an external protein gradient in the culture medium resulting from electrophoresis, and only live, healthy cells underwent galvanotaxis, while dead or damaged cells did not show translocation; (4) by placing the cells onto a collagen gel, thereby reducing electroosmotic fluid flow, others have found that particle movement had very little effect on the electrical orientation response; and (5) galvanotropic cellular elongation at right angles with respect to the field cannot be explained on the basis of electrophoresis alone.

Investigations of galvanic responses of corneal cells to physiologic E-fields may open new vistas in the understanding of biologic controls involved in corneal wound healing. Although our findings are still preliminary and rudimentary, it is nevertheless attractive to conjecture that galvanotropism and galvanotaxis in the enhancement of corneal wound healing may constitute a future clinical modality.

Key words: corneal wound healing, cell migration, corneal epithelium, corneal stroma, fibroblasts, electric field, galvanotropism, galvanotaxis

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References


