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Human dermal fibroblasts do not exhibit directional migration on collagen I in direct-current electric fields of physiological strength

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Abstract: Endogenous electric fields are generated lateral to skin wounds, with the cathodal pole of the field residing in the center of the wound. These fields are thought to be an important mechanism in guiding the migration of keratinocytes and other cells into wounds to effect healing. In this work, human dermal fibroblasts were exposed to direct current electric fields of physiological strength, and their migrational behavior was quantitated. Only random migration of human dermal fibroblasts was observed in direct-current electric fields under conditions that support the directional migration of human epidermal keratinocytes. Additionally, neither the presence of serum nor serum plus additional Mg^{++} in the experimental medium supported directional migration. Migratory rates of fibroblasts varied depending on the experimental medium used: in serum-containing medium the average velocity was as low as 0.23 μm/min, while in serum-free keratinocyte medium the average velocity was as high as 0.36 μm/min. These studies suggest that dermal fibroblasts do not respond to the endogenous electric field of a wound, and use other migratory cues to direct their movement into the wound bed.

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Introduction

Wound healing is a complex process requiring the interaction of different mediators and cell types. Fibroblasts play an essential role in wound healing, migrating into the wound and depositing extracellular matrix (ECM) components that support migration of other cell types. Later in the healing process, fibroblasts generate traction to effect wound closure (1).

Fibroblasts adjacent to the wound are stimulated to migrate into the wound by chemotactic growth factors and cytokines. Human skin wound fluid contains many of these, including platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), interleukin-1α (IL-1α), and epidermal growth factor (EGF) (2). All of these have been shown to stimulate dermal fibroblast migration, either directly or indirectly (3–7), and would provide a potent signal for fibroblast migration into the wound bed.

In addition to chemotaxis, another form of directed cell migration is galvanotaxis, the directional migration of cells in response to an electric field. Lateral electric fields of approximately 100 mV/mm have been measured in mammalian skin wounds (8,9), and are thought to be an important mechanism in guiding cell migration into wounds. Indeed, our laboratory's work demonstrating that human epidermal keratinocytes migrate toward the negative pole of an applied direct-current electric field of physiological strength (10,11) is significant because, in a skin wound, the negative pole is located at the wound center. Thus, the wound field would provide guidance cues for keratinocyte migration to effect wound re-epithelialization. Furthermore, the significance of endogenous wound electric fields is underscored by the numerous clinical studies that demonstrate the efficacy of electrical stimulation to enhance healing in chronic skin wounds (12). Therefore, the endogenous wound electric field is thought to be an important component of the wound environment which may induce migration of various cell types into the wound and subsequently promote healing.

Despite the evidence suggesting that skin

wounds generate lateral electric fields of a magnitude capable of affecting cell migration (8,9), there has been no work examining the effect of electric fields on the migration of the dermal fibroblast, a key cell involved in cutaneous wound repair. Previous work has shown that murine fibroblast cell lines (13,14), embryonic chick heart fibroblasts (15), embryonic quail somite fibroblasts (16–18), and human gingival fibroblasts (19) respond to an electric field by reorienting their long axes perpendicular to the lines of an applied electric field and/or migrating towards the cathode. However, corneal stromal fibroblasts (20) have been reported to migrate towards the anode of an electric field, making the response of human dermal fibroblasts unpredictable. In order to broaden our understanding of the role that physiological electric fields might play in cutaneous wound healing, we examined the effect of electric fields on the migration of human dermal fibroblasts.

Methods

Cells and cell culture

Normal human fibroblasts were derived from a neonatal foreskin using a modification of the technique we have described for isolation of murine dermal fibroblasts (21). Briefly, neonatal foreskin was washed well with phosphate buffered saline (PBS, calcium and magnesium-free), minced finely with sterile scissors, and allowed to adhere to tissue culture dishes for 30 min in an incubator at 37°C in a humidified atmosphere of 5% CO_2. Fibroblast medium (FM) consisting of Dulbecco's modified growth medium (DMEM; Gibco-BRL, Gaithersburg, MD) supplemented with bovine calf serum (BCS; Hyclone Laboratories, Logan, UT; final concentration 10%) with 100 IU/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin-B was added to the tissue in the dishes. Fibroblasts emerging from the tissue adhered to the tissue culture dish and were grown to 50–70% confluence before passage or cryopreservation. Numerous aliquots of cells derived from a single donor were cryopreserved, and an aliquot was thawed and plated 3–5 days before experimentation. Passage 4 cells were used for all experiments. Normal human keratinocytes were derived and cultured using methods described previously (11). Briefly, neonatal foreskin tissue was washed extensively with PBS, cut into small pieces, and then trypsinized overnight. The epidermal layer was separated from the dermal layer, pipetted up and down to break up cell clumps, mixed with FM, and then centrifuged. The pellet was resuspended in DMEM containing 10% FBS, 1% ABAM, 2 mM L-glutamine, 10 ng/ml epidermal growth factor (EGF), 9 ng/ml cholera toxin, and 400 ng/ml hydrocortisone, and then plated onto tissue culture dishes containing mitomycin C-treated NIH 3T3 cells. When keratinocyte colonies containing 6–12 cells were observed, the culture medium was changed to keratinocyte growth medium (KGM), consisting of M154 (Cascade Biologics Inc., Portland, OR) supplemented with human keratinocyte growth supplement (HKGS, Cascade Biologics Inc. Portland, OR) to contain 0.18% hydrocortisone, 5 µg/ml transferrin, 0.2% (v/v) bovine pituitary extract (BPE), 0.2 ng/ml EGF, and 5 µg/ml insulin. Cells were trypsinized when they became 10–20% confluent and replated in KGM. When these secondary cultures reached 60–70% confluence, they were passaged or cryopreserved.

Experimental media

Three media were used for galvanotaxis experiments. Keratinocyte medium (KM) consisted of KGM supplemented with 10 mM HEPES buffer and Ca^{++} added to a final concentration of 2 mM. Serum-containing medium (SM) consisted of FM with 10 mM HEPES buffer added. High Mg^{++} medium (HM) consisted of FM with 10 mM HEPES buffer and Mg^{++} added to a final concentration of 2.6 mM. Both SM and HM contained 1.8 mM Ca^{++}.

Matrix and coverslip preparation

Glass coverslips were coated with bovine collagen I (Vitrogen, Celtrix Pharmaceuticals, Santa Clara, CA), 60 µg/ml in PBS for 24 h in an incubator at 37°C as previously described (11). Excess solution was then removed and the coverslips were allowed to air dry for 5–10 min. Fibroblasts were plated on the prepared coverslips and allowed to attach and spread on matrix for approximately 48 h. Coverslips were then placed in the galvanotaxis chamber. Keratinocytes were plated on prepared collagen I-coated coverslips as previously described (11).

Galvanotaxis chamber

Chambers used for applying an electric field to fibroblasts in culture were identical to those described previously (17). A constant DC voltage was applied across each chamber using Ag-$AgCl_2$ electrodes inserted into saline reservoirs on either side of the chamber. Reservoirs were connected to the wells of the chamber by means of agar-filled saline bridges. The current passing through the chamber was monitored continuously with an ammeter. The chamber temperature was measured with a thermocouple before each experiment, and was maintained between 35 and 39°C.

Imaging procedure

Cells were observed with phase-contrast optics on an inverted microscope, and their movements were recorded using time-lapse videography. Fields of cells to be studied were selected from those regions in which the cells were dispersed to reduce the influence of cell-cell interactions. Cells in groups were not used for subsequent analysis. Experiments were conducted for 1 h in the electric field. Images of cell positions were captured to a computer at 10-min intervals throughout the experiment. The positions of individual cells were recorded for each frame captured. Some keratinocytes exited the field of view during the experiment. The elimination of these cells from the translocation analysis would have caused a bias against the most responsive cells, so the final point of observation was taken as the point where they left the screen, even if this occurred before the end of the standard 1-h period. These cells, however, were not included in the average velocity analysis.

Data analysis

To quantitate the directedness of the average cellular translocation, we calculated the cosine of the angle at which each cell moved in relation to anodal-cathodal orientation. Specifically, a cosine value of 1 would indicate direct cellular movement toward the cathode (negative pole); a cosine value of 0 would indicate movement perpendicular to the field direction, and a cosine value of −1 would indicate direct cellular movement toward the anode (positive pole). The average directedness, $<\cos \phi>$, for each experiment was calculated from the formula: $<$

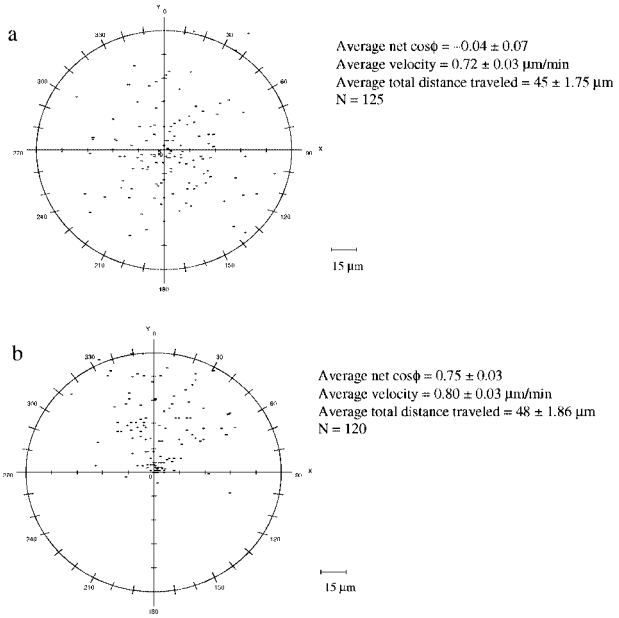

Figure 1. Migration of human epidermal keratinocytes in keratinocyte medium. Human epidermal keratinocytes were plated on collagen I-coated glass coverslips as described in Methods. Migration under non-field conditions *(a)* and in an applied electric field of 100 mV/mm *(b)* was quantitated as described in Methods. The starting position of each cell is located at the center of the circle graph, and the final position is plotted as a single point on the graph: 0° indicates cathodal direction; 180° indicates anodal direction. The average velocity, cosϕ, and total distance traveled ± SEM is indicated in the text at the upper right of each plot. The number of cells analyzed is indicated (*n*). Bars = 15 μm.

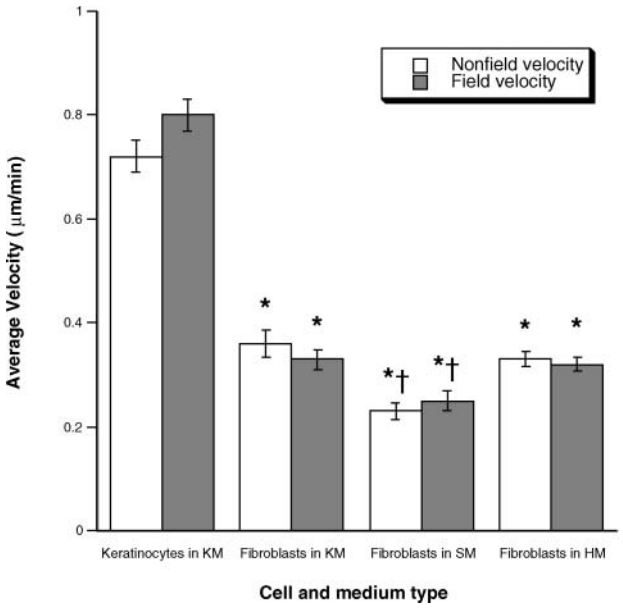

Figure 2. Average velocities of migration of keratinocytes and fibroblasts. Average migrational velocities (μm/min) are plotted. White solid bars represent velocities in the absence of an applied electric field; hatched bars represent velocities in the presence of an applied electric field of 100 mV/mm. Error bars represent ± SEM. *Significantly different compared with keratinocytes in KM; †Significantly different compared with fibroblasts in KM or HM.

$\cos\phi > = \Sigma_I \cos N_i/N$, where Σ_I is the summation of cosine values obtained from individual cells, ϕ is the angle between the field axis and the cellular translocation direction, and N is the total number of cells observed. Average velocities (μm/min) were calculated by dividing the sum of each 10-min translocation distance for each cell by the total time (60 min). Statistical significance was determined by Student's two-tailed *t*-test, with $P < 0.005$ considered significant.

Results

Human epidermal keratinocytes exhibit directed migration (galvanotaxis) toward the cathode

Human epidermal keratinocytes in KM demonstrated cathodally directed migration (average cosϕ of 0.75 ± 0.03) when exposed to a DC field of 100 mV/mm (Fig. 1b), although in the absence of an applied DC field their migration was random (average cosϕ of -0.04 ± 0.07) (Fig. 1a). Keratinocytes traveled an average distance of 45 ± 1.75 μm over a 1-h period, with an average velocity of 0.72 ± 0.03 μm/min in the absence of an applied DC field (Fig. 2). Figure 2 compares the average velocities of keratinocytes to those of dermal fibroblasts whose migration is described in Figures 3–5,

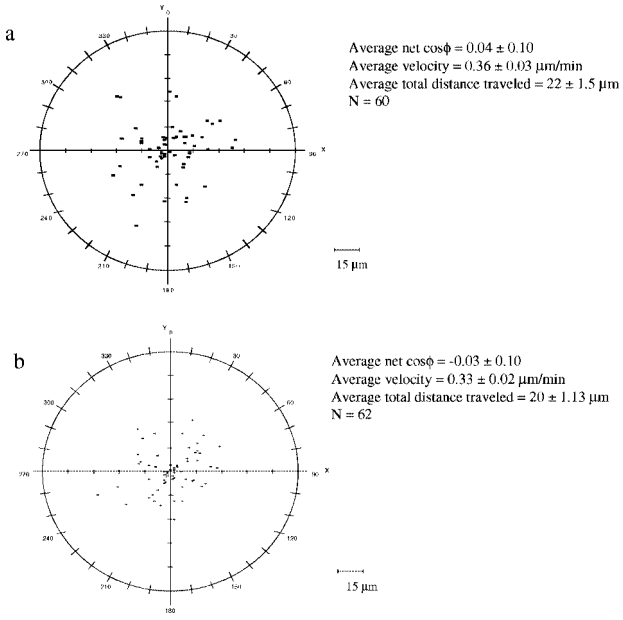

Figure 3. Migration of human dermal fibroblasts in keratinocyte medium. Human dermal fibroblasts were plated on collagen I-coated glass coverslips as described in Methods. Migration under non-field conditions *(a)* and in an applied electric field of 100 mV/mm *(b)* was quantitated as described in Methods, and was plotted as described in Figure 1. The average velocity, cosϕ, and total distance traveled ± SEM is indicated in the text at the upper right of each plot. The number of cells analyzed is indicated (*n*). Bars = 15 μm.

and demonstrates the more rapid basal migration velocity of keratinocytes compared to fibroblasts. Keratinocyte velocity was not significantly altered by the imposition of an electric field (Fig. 5). Keratinocytes in the field traveled an average distance of $48 \pm 1.86\,\mu m$ over a 1-h period, with an average velocity of $0.80 \pm 0.03\,\mu m/min$. Cathodal migration of human keratinocytes in DC electric fields of this magnitude has been previously reported by our laboratory (10,11,22,23). Thus, as we have previously reported, the response of keratinocytes to electric fields of this magnitude is to redirect their migration so that they move toward the cathodal pole of the field vector.

Human dermal fibroblasts do not exhibit galvanotaxis under conditions in which keratinocytes exhibit galvanotaxis. We evaluated the migratory response of skin-derived fibroblasts under experimental conditions identical to those used for keratinocytes. Human dermal fibroblasts in KM demonstrated random migration in an applied DC electric field of 100 mV/mm and under control (non-field) conditions (cosϕ of 0.04 ± 0.10 in the absence of a field, Fig. 3a; and cosϕ of -0.03 ± 0.10 in the presence of an electric field, Fig. 3b). The average distance of travel over the 1-h exposure time was $20 \pm 1.13\,\mu m$ with an average velocity of $0.33 \pm 0.02\,\mu m/min$, not statistically different from the average velocity or distance of travel of fibroblasts in the absence of an applied DC electric field ($0.36 \pm 0.03\,\mu m/min$, distance of travel was $22 \pm 1.5\,\mu m$). The average distance of travel and migratory velocity of the fibroblasts, however, are significantly slower than that of keratinocytes under these conditions (Fig. 2).

Human dermal fibroblasts do not exhibit galvanotaxis in serum-containing medium

Previous work in our laboratory has demonstrated that growth factors are required for the cathodal migration of keratinocytes in DC electric fields (22). Although KM contains BPE with a predominance of bFGF (24,25), as well as 0.2 ng/ml EGF, we suspected that other growth factors normally present in the wound environment might be absent in KM. We therefore assessed the migration of dermal fibroblasts in serum-containing medium (SM), as serum is normally exuded into the wound environment. Surprisingly, fibroblasts in SM demonstrated random migration both under non-field conditions (cosϕ of -0.09 ± 0.13, Fig. 4a) and in an applied DC electric field of 100 mV/mm (cosϕ of -0.17 ± 0.13, Fig. 4b). In SM, fibroblasts in the absence of an applied DC electric field traveled an average distance of $14 \pm 1.01\,\mu m$ with an average velocity of $0.23 \pm 0.02\,\mu m/min$ (Fig. 4a). Fibroblasts in SM traveled an average distance of $15 \pm 1.13\,\mu m$ with an average velocity of $0.25 \pm 0.02\,\mu m/min$ when a field of 100 mV/mm was present. Application of an electric field did not significantly alter the average velocities, average distances traveled, or cosines of fibroblasts in serum-containing media. The average velocity of migration in the presence or absence of an electric field was significantly slower for fibroblasts in SM than for fibroblasts in keratinocyte medium (Fig. 2).

Human dermal fibroblasts do not exhibit galvanotaxis in high-magnesium medium

Altering the Mg^{++} concentration of the experimental medium so that the $Ca^{++}:Mg^{++}$ ratio approaches 1 : 1 has been reported to increase the migration of skin fibroblasts in Boyden chamber assays (26). Therefore, we examined the migratory behavior of human dermal fibroblasts in high Mg^{++} media (HM). In the absence of an applied DC electric field, fibroblasts in HM migrated randomly, with a cosϕ of 0.03 ± 0.07, traveling an average distance of $20 \pm 0.91\,\mu m$ with an average velocity of $0.33 \pm 0.02\,\mu m/min$ during the 1 h time period (Fig. 5a). Human dermal fibroblasts in HM also demonstrated random migration in an applied

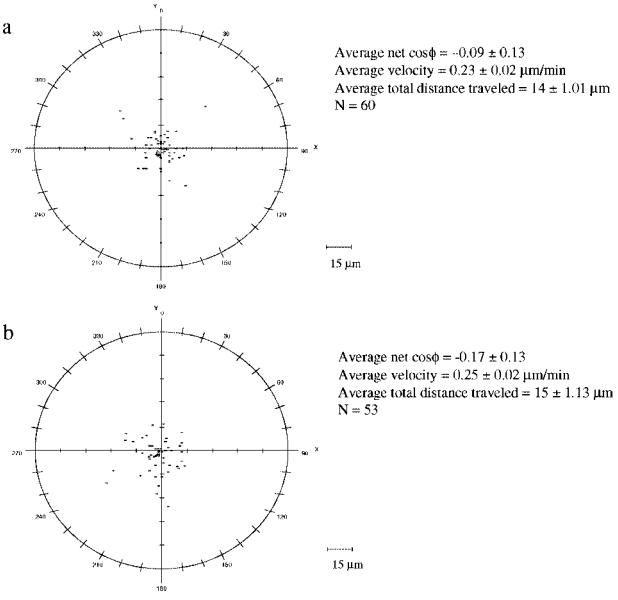

Figure 4. Migration of human dermal fibroblasts in serum-containing medium. Human dermal fibroblasts were plated on collagen I-coated glass coverslips as described in Methods. Migration under non-field conditions *(a)* and in an applied electric field of 100 mV/mm *(b)* was quantitated as described in Methods, and was plotted as described in Figure 1. The average velocity, cosϕ, and total distance traveled ± SEM is indicated in the text at the upper right of each plot. The number of cells analyzed is indicated (*n*). Bars = 15 μm.

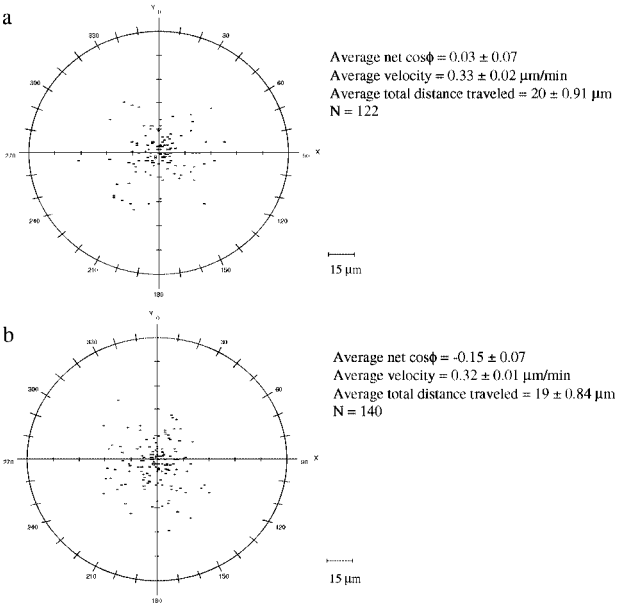

Figure 5. Migration of human dermal fibroblasts in high Mg^{++} medium. Human dermal fibroblasts were plated on collagen I-coated glass coverslips as described in Methods. Migration under non-field conditions *(a)* and in an applied electric field of 100 mV/mm *(b)* was quantitated as described in Methods, and was plotted as described in Figure 1. The average velocity, cosϕ, and total distance traveled ± SEM is indicated in the text at the upper right of each plot. The number of cells analyzed is indicated (*n*). Bars = 15 μm.

DC electric field of 100 mV/mm (Fig. 4b), with a cosϕ of -0.15 ± 0.07, traveling an average distance of 19 ± 0.84 μm with an average velocity of 0.32 ± 0.01 μm/min during the 1-h exposure period (Fig. 5b). The average migrational velocity and distance traveled was not statistically different between control and field-exposed fibroblasts in HM. However, the average migrational velocity and distance traveled in the absence of an electric field was significantly faster for fibroblasts in HM than for fibroblasts in SM (Fig. 2).

Discussion

When skin is wounded, a current emerges from the edge of the wound, and an electric field is generated laterally. In mammalian skin wounds, fields with a magnitude of 10–100 mV/mm have been measured near the edges of the wounds (8,9), with the negative pole residing inside the wound. Wound-associated electric fields may promote cell migration during wound healing. The vast majority of cells that exhibit galvanotaxis, including skin-derived keratinocytes, migrate cathodally (10,11). However, there are rare reports of some cell types exhibiting galvanotaxis toward the anode, such as rabbit osteoclasts (27), mouse peritoneal macrophages (28), and corneal stromal fibroblasts (20). As dermal fibroblasts are involved in skin wound healing, it is important to characterize their migratory behavior in physiological electric fields such as those found around skin wounds.

We have shown that normal human dermal fibroblasts exhibit neither cathodal nor anodal galvanotaxis under conditions that support keratinocyte galvanotaxis. The average velocity of fibroblasts was approximately half that of keratinocytes. The inclusion of bovine calf serum in the experimental media did not support directional migration of fibroblasts in the electric field (Fig. 4), although it did cause significantly slower migration compared with migration in KM (Fig. 2). Both skin-derived keratinocytes and corneal epithelial cells require growth factors or serum to mount a galvanotactic response (22,29,30). Therefore, if human dermal fibroblasts do require a growth factor to migrate directionally in an electric field, it is a factor that is not present in the serum or in the bovine pituitary extract used for these experiments. Of course, as this is an *in vitro* study, caution must be exercised when applying the results to the *in vivo* situation of the wound environment.

An increase in magnesium concentration and a decrease in calcium concentration relative to serum cation concentrations, so that the ratio of magnesium to calcium is 1 : 1, has been reported in porcine skin wound fluid (26). This change in ionic concentrations is thought to increase the motility of keratinocytes and fibroblasts at the wound site (26,31–33). Therefore, we tested whether increasing the Mg^{++} concentration of the medium so that the Mg^{++} : Ca^{++} ratio was closer to 1 : 1 would influence the migration of fibroblasts. Increasing the Mg^{++} concentration in the SM did not cause the fibroblasts to exhibit galvanotaxis (Fig. 5b), although it did increase the average velocity of migration compared with the fibroblasts in SM (Fig. 2), in agreement with the earlier report of Mg^{++}-induced enhancement of migration (26,31–33). Interestingly, migratory rates of fibroblasts showed a significant decrease in SM as compared to the serum-free, EGF- and BPE-containing KM (Fig. 2). This likely resulted from a 10-fold decrease in the concentration of growth factors contained in the serum, especially EGF and bFGF, by the dilution of serum in the medium to 10%.

The lack of a galvanotactic response has been reported previously for human gingival fibroblasts (19) and embryonic chick fibroblasts (15). These studies demonstrated fibroblast alignment perpendicular to the electric field, whereas we observed no such alignment of human dermal fibroblasts in an electric field. One reason for this difference in observations might be that we

studied the effects of a DC electric field of 100 mV/mm, a magnitude that approximates the field found at a wound site. Reorientation of human gingival fibroblasts and embryonic chick fibroblasts was observed in a 10-fold stronger DC electric field (1 V/mm) (15,19); in fact, human gingival fibroblasts did not reorient in the more physiological strength electric field of 100 mV/mm (19). Therefore, fibroblasts may in fact reorient in stronger electric fields, but not in fields that are similar in strength to those found in skin wounds. The experimental conditions used in the present study did not alter the general migrational velocity of dermal fibroblasts. The recorded velocities of 0.2–0.4 μm/min compare well with the rates of 0.2–0.5 μm/min reported for NIH 3T3 and SV101 cell lines (14), rabbit corneal stromal fibroblasts (20), and human dermal fibroblasts (7).

Presently, the mechanism by which cells sense and respond to a DC electric field has not been defined. The plasma membrane is thought to be the primary target of the electric field, as this part of the cell offers the highest electrical resistance. Our previous work in human keratinocytes has shown the necessity of epidermal growth factor receptor (EGFr) signaling for the galvanotactic response. Inhibiting the EGFr kinase activity blocks EGFr relocalization to the cathodal face of keratinocytes, and inhibits keratinocyte migration to the cathode, showing that the EGFr kinase activity is required for keratinocyte galvanotaxis (23). Zhao et al. showed that cathodally directed migration of corneal epithelial cells in a DC electric field was accompanied by a redistribution of the EGFr (34). This redistribution occurred in a time frame similar to that which we observed for keratinocytes. Additionally, cathodal migration of corneal epithelial cells in low strength electric fields requires growth factors such as EGF, bFGF, or TGF-β1 (29). EGF-induced migration of human dermal fibroblasts has been demonstrated *in vitro*, though at EGF concentrations at least 30-fold higher than is present in KM (5–7), and at least 100-fold higher than is present in human wound fluid (2). The lack of a galvanotactic response of dermal fibroblasts in the presence of EGF-containing KM suggests that if EGF is required it is not available in sufficient amounts to support directional migration in DC electric fields, if indeed dermal fibroblasts are capable of mounting a galvanotactic response at all. Another possible explanation is that dermal fibroblasts lack the specific signaling pathways used in a galvanotactic response.

In this study, we tested whether normal human dermal fibroblasts are able to respond with directional migration when exposed to a DC electric field of physiological strength. In all experimental systems tested (KM, SM, and HM), none supported a galvanotactic response in dermal fibroblasts. All of the systems tested did, however, support the random migration of dermal fibroblasts, at speeds that are in agreement with previous reports. We conclude that dermal fibroblasts are unable to exhibit galvanotaxis under conditions that model the wound environment and that support galvanotaxis in other cell types, including keratinocytes. Further investigation is necessary to determine whether an experimental system can be optimized to support human dermal fibroblast galvanotaxis. Translating those conditions into the *in vivo* wound would allow for more rapid recruitment of fibroblasts into the wound bed and enhancement of wound healing.

Acknowledgement

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