Migration of Human Keratinocytes in Electric Fields Requires Growth Factors and Extracellular Calcium

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Currents that leak out of wounds generate electric fields lateral to the wound. These fields induce directional locomotion of human keratinocytes *in vitro* and may promote wound healing *in vivo*. We have examined the effects of growth factors and calcium, normally present in culture medium and the wound fluid, on the directional migration of human keratinocytes in culture. In electric fields of physiologic strength (100 mV per mm), keratinocytes migrated directionally towards the cathode at a rate of about 1 μm per min. This directional migration requires several growth factors. In the absence of these growth factors, the cell migration rate decreased but directionality was maintained. Epidermal growth factor alone restored cell migration rates at concentrations as low as 0.2 ng per ml. Insulin at 5–100 μg per ml or bovine pituitary extract at 0.2%–2% vol/vol also stimulated keratinocyte motility but was not sufficient to fully restore the migration rate. Keratinocyte migration in electric fields requires extracellular calcium. Changes in calcium concentrations from 3 μM to 3.3 mM did not significantly change keratinocyte migration rate nor directionality in electric fields; however, addition of the chelator ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid to migration medium reduced, and eventually abolished, keratinocyte motility. Our results show that (i) growth factors and extracellular calcium are required for electric field-induced directional migration of human keratinocytes, and (ii) keratinocytes migrate equally well in low and high calcium media. *Key words: bovine pituitary extract/epidermal growth factor/galvanotaxis/motility/wound current/wound healing. J Invest Dermatol 111:751–756, 1998*

Keratinocyte proliferation and migration into wounds are essential for cutaneous wound healing (Krawczyk, 1971; Clark, 1985). At a wound site, the coordinated actions of several factors are required to produce a normal epithelial response to an injury. These include growth factors, appropriate ion concentrations, and extracellular matrix components. Growth factors present at wound sites include epidermal growth factor (EGF), transforming growth factor (TGF)-α, and heparin-binding-EGF: each one a ligand for the EGF receptor. Also present within the wound are keratinocyte growth factor (KGF), basic fibroblast growth factor, insulin-like growth factor I, and TGF-β (McNeil *et al*, 1989; Sarret *et al*, 1992; Martin, 1997). All these factors are known to have some or all of the following functions: (i) enhancing keratinocyte proliferation (Rheinwald and Green, 1977; Barrandon and Green, 1987; DeLapp and Dieckman, 1990; Krane *et al*, 1991); (ii) promoting keratinocyte migration *in vitro* (Ando and Jensen, 1993; Ju *et al*, 1993; Taylor *et al*, 1993; Cha *et al*, 1996; McCawley *et al*, 1997); and (iii) accelerating wound healing following topical application *in vivo* (Schultz *et al*, 1987, 1991; Brown *et al*, 1989; Lynch *et al*, 1989; Greenhalgh *et al*, 1990). Whether these growth factors act synergistically or sequentially is still unknown.

Cell migration also involves a dynamic interaction between cells and the extracellular matrix. Integrins localized to the leading edges of migratory cells bind matrix proteins and the dissociation of these proteins at trailing edges is necessary for cell movement (Lawson and Maxfield, 1995). The divalent cations, Ca^{++} and Mg^{++}, are required for integrin function (Lange *et al*, 1994; Clark and Brugge, 1995), and changes in their concentrations have been noted in the wound fluid (Grzesiak and Pierschbacher, 1995b). Normally, extracellular Ca^{++} is 2.5–5 mM and Mg^{++} is 1.0 mM (Alberts *et al*, 1989). Early in the wound healing process, coincident with the phase of cell migration at wound edges, Ca^{++} falls below 1 mM and Mg^{++} rises to 1.5 mM in the wound fluid (Grzesiak and Pierschbacher, 1995b), which leads to the speculation that alterations of Ca^{++} and Mg^{++} levels may activate and regulate cell locomotion.

Another wound-related event is the emergence of a current from the wound edge with the generation of a lateral electric field. In mammals, lateral fields of 10–100 mV per mm have been measured near the edge of the wounds (Illingworth and Barker, 1980; Barker *et al*, 1982). These wound-induced electric fields and currents may promote cell migration during wound healing. We have shown previously that human keratinocytes *in vitro* migrate to the cathode in direct current (DC) electric fields of physiologic magnitudes, 100 mV per mm ("galvanotaxis") (Nishimura *et al*, 1996). We have also shown that galvanotaxis of human keratinocytes in physiologic electric fields is strongest on collagen types I and IV (Sheridan *et al*, 1996). Those galvanotaxis experiments were conducted in a commonly used serum-free keratinocyte growth medium (Boyce and Ham, 1983; Johnson *et al*, 1992), supplemented with 2 mM calcium. Keratinocyte migration studies in other systems, however, have been performed in low Ca^{++} media, 0.05–0.1 mM (Bussolino *et al*, 1992; Tsuboi *et al*, 1993; Chen *et al*, 1994b; Grzesiak and Pierschbacher, 1995a; Cha *et al*, 1996). Incubation in media containing higher than 1 mM Ca^{++} has been shown to suppress migration (Nickoloff *et al*, 1988), presumably due

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Abbreviations: BPE, bovine pituitary factor; HKGS, human keratinocyte growth supplement; KMM, keratinocyte migration medium.

to production of matrix proteins and increased cell–matrix or cell–cell interactions (O'Keefe *et al*, 1987; Kim *et al*, 1992; Woodley *et al*, 1994). It is also well known that keratinocytes in culture proliferate when Ca^{++} levels are less than 0.2 mM, and differentiate when Ca^{++} rises above 1 mM (Hennings *et al*, 1980a, b; Yuspa *et al*, 1989; Fuchs, 1990; Johnson *et al*, 1992; Leigh and Watt, 1994). In this study we have examined the effects of high (>1 mM) and low (<0.2 mM) extracellular Ca^{++} levels on keratinocyte galvanotaxis. Because we subjected cells to electric fields several hours after plating, we concentrated on the short-term direct effects of Ca^{++} levels on keratinocyte galvanotaxis rather than the longer-term Ca^{++}-mediated effects, e.g., on gene expression or cell differentiation, which could also alter the migratory response. In addition, we have also examined the effects on keratinocyte galvanotaxis of growth factors normally present in the culture medium and the wound fluid.

MATERIALS AND METHODS

Cells and cell culture Normal human keratinocytes from neonatal foreskin epidermis were prepared and cultured following the methods described previously (Isseroff *et al*, 1987). Aliquots of cells derived from a single donor were cryopreserved. Cells were cultured in the serum-free keratinocyte growth medium that was composed of M154 medium (Cascade Biologics, Portland, OR), 10 mM HEPES (pH 7.4), antibiotics/antimycotics (penicillin, streptomycin, and amphotericin), and human keratinocyte growth supplement (HKGS) that includes 0.18% hydrocortisone, 5 μg transferrin per ml, 0.2% vol/vol bovine pituitary extract (BPE), 0.2 ng EGF per ml, and 5 μg insulin per ml. Cultures were kept at 37°C in a humidified atmosphere of 5% CO_2. Cells from two donors were used in this work, and passage 2–5 cells were used for experiments.

Galvanotaxis Coverslips and cells were prepared following a procedure described previously (Sheridan *et al*, 1996). Briefly, 12 mm glass coverslips were coated with bovine collagen I by soaking in calcium and magnesium-free phosphate-buffered saline containing 2% Vitrogen 100 (60 μg per ml) (Collagen, Palo Alto, CA) for at least 1 h at 37°C. Coverslips were rinsed three times with phosphate-buffered saline and air-dried for 5–10 min before cells were plated. Normal human keratinocytes were plated on the collagen-coated coverslips at a density of $4–6 \times 10^4$ in a 35 mm plate in keratinocyte migration medium (KMM), which is keratinocyte growth medium supplemented with 1.8 mM calcium chloride (a final Ca^{++} concentration is 2 mM). After 2–6 h to allow attachment, coverslips were rinsed with medium to remove unattached cells and placed in a galvanotaxis chamber. Galvanotaxis experiments were conducted for 1 h in DC electric fields of physiologic strength (100 mV per mm). If an additional factor or unique condition was being tested, coverslips were rinsed with phosphate-buffered saline twice, incubated in the medium containing the indicated factor or condition for 1 h at 37°C, then subjected to the fields in the same medium. Recombinant human EGF was obtained from Gibco Life Technologies (Grand Island, NY), human insulin from Sigma (St. Louis, MO), and bovine pituitary extract from Cascade Biologics. The free calcium concentrations in all media were later verified by using atomic absorption by Perkin-Elmer Analyst 300.

The galvanotaxis chamber and apparatus for applying DC electric fields have been described previously (Erickson and Nuccitelli, 1984; Nishimura *et al*, 1996; Sheridan *et al*, 1996). KMM, or modifications as otherwise stated, was used for galvanotaxis. A physiologic electric field was applied with a constant voltage at 100 mV per mm and a current at 0.1–0.6 mA. The galvanotaxis was performed at 37 ± 2°C in room air.

Recording and data analysis Cells were observed with phase contrast optics on inverted microscopes, and video images of cells were digitally captured every 10 min for 1 h to an image analysis program on a Power Macintosh 8500 using a modified version of NIH Image 1.60 and File Maker Pro 3.0. At the end of 1 h, the center of each cell was identified manually on each image. The translocation distance and directionality of migration of each imaged cell were analyzed. The translocation distance covered by each cell was measured in μm per h, and migration rate was expressed as μm per min. The directionality of the cell translocation was indicated by an average cosine, cos ϕ, of the angles that the path of each cell made with respect to the electric field direction, $\langle\cos\phi\rangle = \Sigma_i \cos\phi i/N$ (Nishimura *et al*, 1995). The value of cosine ϕ equals minus one (cosine ϕ = −1) if the cell moves towards the anode; cosine ϕ = +1 if the cell moves directly towards the cathode; and is zero if the cell moves perpendicular to the field direction or randomly. For any given condition, a compilation of average cosine ϕ from 65 to 150 cells (collected from four to eight experiments) is presented. Also for any given condition, the migration characteristics of control cells exposed to the electric fields in the presence of unmodified KMM are included (**Tables I** and **II**). Statistical analysis was performed by Student t test on all experimental data, using the Instat program.

RESULTS

Requirement of HKGS for keratinocyte migration in physiologic electric fields In KMM, human keratinocytes migrated at an average rate of 1.0 ± 0.05 μm per min (ranging from 0.8 to 1.1 μm per min) and migrated towards the cathode with an average cosine ϕ of 0.41 ± 0.07 (ranging from 0.30 to 0.55, n = 102) over 1 h exposure to a physiologic electric field of 100 mV per mm (**Table I**, **Fig 1*a***). The track cosine, an indicator of the directional movement at a given time interval (every 10 min in our case), showed that cells were less cathodally directed within the first 10–20 min of the field exposure, with cosine ϕ averaging 0.1–0.2 (**Fig 1*b***, *open bars*). Keratinocytes became more cathodally directed thereafter, suggesting that cells need a latent time period to sense and respond to the field. The net cosine ϕ, measured as an average cosine ϕ from time zero to the indicated time point, increased steadily over the 1 h course of exposure (**Fig 1*b***, *hatched bars*). On the other hand, the rate of cell migration remained unchanged throughout the entire 1 h exposure, 0.9–1.0 μm per min (data not shown). In the absence of electric fields, human keratinocytes migrated randomly (cosine ϕ = 0.06 ± 0.10), yet migrated at the same rate as cells exposed to the fields (1.0 ± 0.08 μm per min, n = 119, **Table I**, **Fig 1*a***). Data collected from 1 h field exposures are consistent with previous work from our laboratory in which human keratinocytes were exposed to the fields for 2–2.5 h (Nishimura *et al*, 1996; Sheridan *et al*, 1996), and collectively demonstrate that physiologic electric fields affect migration directionality but have no effect on migration rate. The greater cosine ϕ value (0.7–0.8) obtained in the earlier work is probably due to longer exposure to the fields, because the migration directionality (cosine ϕ) of keratinocytes increases gradually over the time, and/or due to the variation in cell donors.

Growth factors are known to be important for cell proliferation and locomotion. To test if they are essential for keratinocyte galvanotaxis, all growth factors (HKGS) were withdrawn from KMM. In the absence of HKGS, human keratinocytes migrated poorly at a rate of 0.5 ± 0.03 μm per min (n = 128), 50% slower than in the presence of HKGS; however, cells moved directionally towards the cathode with a cosine ϕ of 0.50 ± 0.06, comparable with those cells in the presence of HKGS (**Table I**, **Fig 1*a***). Therefore, HKGS appears to contribute directly to the overall motility of keratinocytes but not to the translocation directionality in the electric fields.

Effects of EGF on keratinocyte galvanotaxis HKGS contains EGF, insulin, and BPE. To determine which growth factor in HKGS is required for the galvanotaxis response in physiologic electric fields, we examined the effect of individual components by adding each factor back to the medium devoid of HKGS. Human keratinocytes were plated in KMM as described in *Materials and Methods*. After 2 h, cells were rinsed twice with phosphate-buffered saline and were then incubated for 1 h in the medium without HKGS but containing the indicated growth factor and 50 μg bovine serum albumin per ml as carrier protein, before subjecting them to galvanotaxis in the same medium. Fifty micrograms bovine serum albumin per ml alone in the medium, with or without growth factors, did not affect cell motility (data not shown), but its presence was required for any added growth factor to exert an effect. In the absence of HKGS, human EGF at 0.2 ng per ml provided a migration rate of human keratinocytes at 0.9 ± 0.06 μm per min (n = 84), 90% of the control (KMM contains 0.2 ng EGF per ml). At 1 or 10 ng EGF per ml, keratinocytes migrated at ≈1.1 μm per min (n = 73 and 82, respectively), as fast as control cells in the medium containing HKGS (**Table I**, **Fig 2*a***). These data show that EGF alone fully restored the migration rate of human keratinocytes in physiologic electric fields. Under conditions where EGF was the only added growth factor, keratinocytes migrated towards the cathode as well as control cells. Also, in the medium supplemented only with EGF, human keratinocytes appeared somewhat elongated.

Effects of insulin or BPE on keratinocyte galvanotaxis The effect of insulin on the migration of human keratinocytes in physiologic

Table I. Effects of growth factors on keratinocyte migration in DC electric fields

Reagents	Rate (μm per min) (%)[a]	Average cosine ϕ	Cell no. (n)
no electric field	1.0 ± 0.08 (100)[d]	0.06 ± 0.10[b]	119
control (+HKGS)	1.0 ± 0.05 (100)	0.41 ± 0.07	102
–HKGS	0.5 ± 0.03 (50)[b]	0.50 ± 0.06[d]	128
control	1.0 ± 0.06 (100)	0.53 ± 0.08	71
–HKGS + 0.2 ng EGF per ml	0.9 ± 0.06 (90)[d]	0.62 ± 0.08[d]	84
–HKGS + 1 ng EGF per ml	1.1 ± 0.07 (110)[d]	0.64 ± 0.06[d]	73
–HKGS + 10 ng EGF per ml	1.1 ± 0.06 (110)[d]	0.61 ± 0.05[d]	82
control	1.0 ± 0.05 (100)	0.51 ± 0.08	54
–HKGS + 5 μg insulin per ml	0.7 ± 0.02 (70)[b]	0.47 ± 0.06[d]	102
–HKGS + 10 μg insulin per ml	0.8 ± 0.05 (80)[c]	0.51 ± 0.07[d]	70
–HKGS + 100 μg insulin per ml	0.6 ± 0.03 (60)[b]	0.45 ± 0.07[d]	85
control	0.9 ± 0.04 (100)	0.73 ± 0.05	80
–HKGS + 0.2% vol/vol BPE	0.7 ± 0.04 (78)[b]	0.45 ± 0.07[c]	65
–HKGS + 2.0% vol/vol BPE	0.6 ± 0.03 (67)[b]	0.67 ± 0.05[d]	71

[a]Cell migration rate under the indicated conditions is compared in percentage with the rate of control cells.
[b]p value <0.005 compared with control of each set.
[c]p value <0.01 compared with control of each set.
[d]Not significant at all compared with control of each set.

Table II. Effects of calcium and EGTA on keratinocyte migration in DC electric fields

Reagents (presumed $[Ca^{++}]$)	Measured $[Ca^{++}]$	Rate (μm per min) (%)	Average cosine ϕ	Cell no. (n)
calcium-free (0 mM)	2.9 μM	0.7 ± 0.03 (86)[h]	0.43 ± 0.06[i]	89
KGM[a] (0.2 mM)	0.19 mM	0.8 ± 0.03 (100)[i]	0.26 ± 0.06[i]	152
KMM[a] (2.0 mM)	3.3 mM	0.8 ± 0.03 (100)	0.29 ± 0.06	136
"calcium-free" (0 mM)	2.9 μM	0.8 ± 0.04 (100)	0.29 ± 0.07	79
+ 1 mM EGTA[c]		0.5 ± 0.02 (63)[f]	0.26 ± 0.07[i]	98
+ 2 mM EGTA[c]		0.5 ± 0.02 (63)[f]	0.25 ± 0.07[i]	102
+ 4 mM EGTA[c]		0.3 ± 0.02[f]	NV[b]	89
+ 2 mM EGTA (1 h)[d]		0.4 ± 0.02 (50)[f]	–0.04 ± 0.08[g]	88

[a]KGM, keratinocyte growth medium; KMM, keratinocyte migration medium.
[b]V, not valid. Cells that moved at a rate of 0.3 μm per min or less are considered as no movement.
[c]EGTA was added to the "calcium-free" medium immediately before set-up for galvanotaxis, which took about 10–15 min.
[d]EGTA was added to the "calcium-free" medium 1 h before set-up for galvanotaxis.
[e]Cell migration rate under the indicated conditions is compared in percentage with the rate of control cells.
[f]p value <0.005 compared with control of each set.
[g]p value <0.01 compared with control of each set.
[h]p value <0.05 compared with control of each set.
[i]Not significant at all compared with control of each set.

electric fields was examined at concentrations of 5, 10, and 100 μg per ml in the media without HKGS (KMM contains 5 μg insulin per ml). At all three concentrations, keratinocytes migrated at a rate of 0.6–0.8 μm per min (n = 102, 70, and 85, respectively), better than those in the medium without HKGS but more slowly than cells in medium containing HKGS (1.0 ± 0.05 μm per min, n = 54) or EGF alone (**Table I**, **Fig 2*b***). BPE is an undefined tissue extract and is commonly used in keratinocyte cultures. It surely contains other unknown components, but basic fibroblast growth factor is probably the major factor in the extract (Gospodarowicz *et al*, 1986; DeLapp and Dieckman, 1990). When in the medium containing 0.2% or 2% BPE only, human keratinocytes also migrated at a rate of 0.6–0.7 μm per min (n = 65 and 71, respectively), also faster than cells without HKGS but slower than cells in the presence of HKGS (0.9 ± 0.04, n = 80, KMM contains 0.2% BPE) or EGF alone (**Fig 2*c***, **Table I**). Increasing the concentrations of insulin from 5 to 100 μg per ml or BPE from 0.2 to 2% vol/vol did not further improve the keratinocyte motility. At all levels of insulin or BPE, human keratinocytes migrated directionally to the cathode (**Table I**). These data show that insulin or BPE alone promotes human keratinocyte motility, but is not sufficient to fully support the degree of cell motility provided by HKGS or EGF. It is noted that at 0.2% vol/vol BPE, the cosine ϕ was statistically significantly smaller than the control (**Table I**), but the cosine ϕ at 2% vol/vol BPE was similar to the control. Therefore, the difference in cosine values was probably caused by experimental discrepancy.

Effect of extracellular calcium on keratinocyte galvanotaxis Extracellular calcium is needed for cell migration in many cell types. Here we investigated the effect of calcium on human keratinocyte locomotion in physiologic electric fields. The control KMM contains 2 mM calcium. When the calcium concentration was reduced to 0.2 mM as in keratinocyte growth medium, or when commercially available calcium-free medium (Cascade Biologics) was used, human keratinocytes migrated to the cathode at rates and directionality similar to those in the control KMM, i.e., at average rates of 0.8 μm per min and with an average cosine ϕ of 0.3–0.5 (**Table II**, **Fig 3*a***). To verify the calcium levels in these commercially available media, we measured the free calcium concentrations using the atomic absorption method. As indicated in **Table II**, the control KMM actually contained 3.3 mM calcium, keratinocyte growth medium contained 0.19 mM, and the commercially available calcium-free medium contained 2.9 μM calcium, which rose to 3.17 μM if a collagen-coated coverslip was immersed in a total of 3 ml medium. Therefore, the commercially available "calcium-free" medium actually contains about 3 μM calcium.

To further establish the role of extracellular calcium in keratinocyte galvanotaxis, ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) was added to the commercially available "calcium-free" medium (which actually contains about 3 μM calcium) immediately before cells were exposed to the fields. **Table II** shows that the addition of EGTA reduced keratinocyte motility. In the media containing 1–2 mM EGTA, human keratinocytes migrated at a rate of

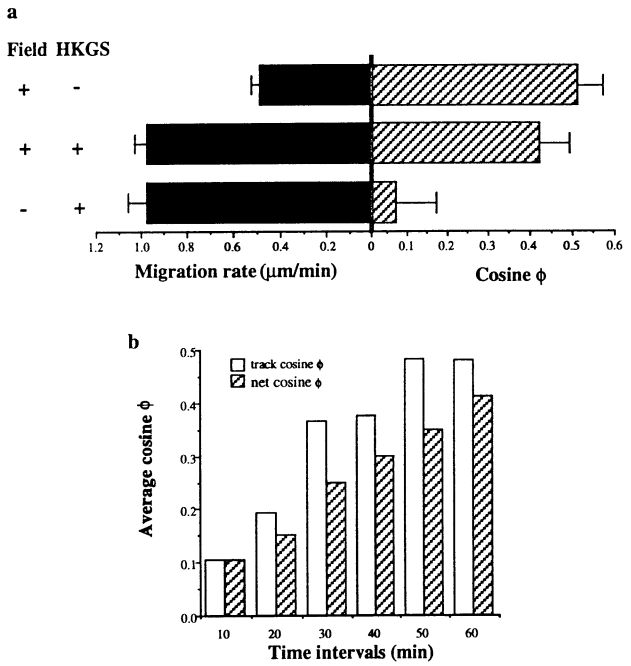

Figure 1. Human keratinocyte migration in DC electric fields requires growth supplement. Normal human keratinocytes were plated on collagen-coated coverslips in KMM (*Materials and Methods*). (*a*) Before galvanotaxis, cells were incubated for 1 h in either KMM (middle and bottom bars) or the medium devoid of HKGS (top bars), and then exposed to DC electric fields of 100 mV per mm (top and middle bars) or no field for 1 h (bottom bars). Video images of cells were captured every 10 min for 1 h, digitized, and analyzed by a Power Macintosh computer (see *Materials and Methods*). Solid bars on the left represent the cell migration rate, and the hatched bars on the right represent the cell migration directionality (cosine $\phi = \Sigma_i \cos\phi i/N$). (*b*) Track cosine ϕ (open bars) indicates the cosine ϕ at the indicated time intervals (10 min each); total cosine ϕ (hatched bars) indicates the cosine ϕ from time zero to the indicated time point.

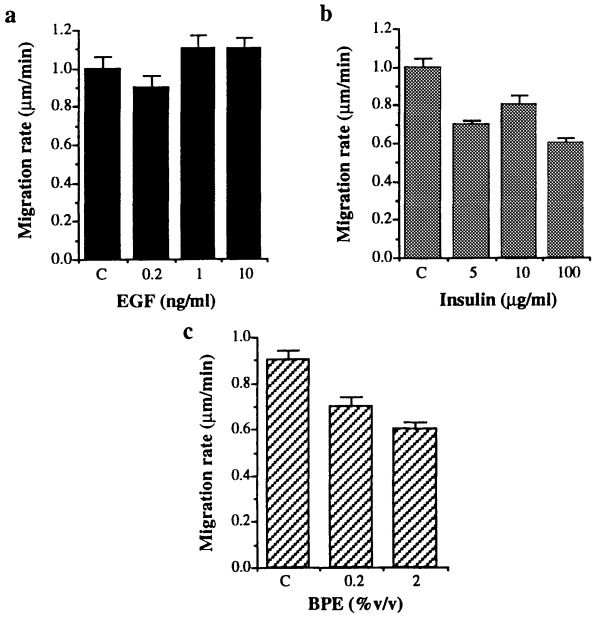

Figure 2. Growth factors promote human keratinocyte migration in DC electric fields. Normal human keratinocytes were plated as described in **Fig 1**. After 2–6 h, cells were rinsed twice with phospahte-buffered saline and incubated in the medium devoid of HKGS but supplemented with various concentrations of EGF (*a*, solid bars) or insulin (*b*, gray bars) or BPE (c, hatched bars), and 50 mg bovine serum albumin per ml for another 1 h, then exposed to DC electric fields in the same media. Control cells (C) migrated in KMM.

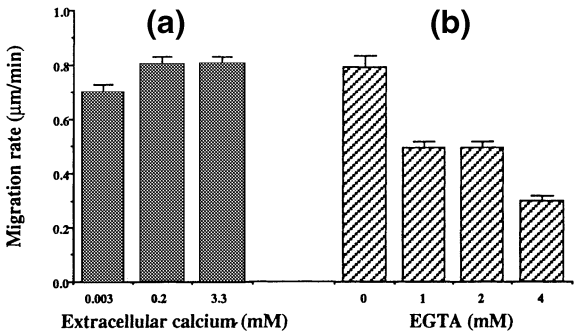

Figure 3. Calcium is required for keratinocyte migration in DC electric fields. (*a*) Normal human keratinocytes were plated in the medium containing measured concentrations of calcium for 2–6 h, and then exposed to DC electric fields in the same medium for 1 h; (*b*) normal human keratinocytes were plated in the commercially available "calcium-free" medium (measured $[Ca^{++}]$ was 0.003 mM), and exposed to DC electric fields in the same medium including various concentrations of EGTA.

0.5 ± 0.02 μm per min (n = 98 and 102, respectively), and the migration directionality was not affected. In the medium containing 4 mM EGTA, keratinocytes rounded up within 10–15 min without further changes in shape and position (n = 89). In addition to the dose dependence (**Fig 3*b***), the EGTA effect on keratinocyte motility also exhibited a time-dependent component. When incubated in 2 mM EGTA for a longer period of time up to 1 h before exposure to the fields in the same medium, keratinocyte migration rate was further reduced to 0.4 ± 0.02 μm per min (n = 88), slower than the cells treated with 2 mM EGTA immediately before the field exposure (**Table II**). Cells in the 2 mM EGTA medium for a longer time gradually elongated and then rounded up after 1.5 h. So the measured change in the migration rate represents more of a change in cell shape rather than true cell translocation. Therefore, the average cosine ϕ was near zero (–0.04 ± 0.08). The time- and dose-dependent effect of EGTA on motility and morphology of human keratinocytes suggested that EGTA acted specifically by removing extracellular calcium rather than via cell toxicity. A trypan blue exclusion test confirmed that cell viability in the so-called "calcium-free" medium and the media containing EGTA was similar to control cells (data not shown).

DISCUSSION

We have demonstrated that electric fields of physiologic magnitudes induce directional migration of human keratinocytes to the cathode as early as 10–20 min after exposure to the fields, and that their directionality improves steadily over the course of 1 h. We have shown that growth factors are required for keratinocyte locomotion, and that EGF alone at concentrations as low as 0.2 ng per ml is sufficient to maintain high keratinocyte migration rates. Extracellular Ca^{++} is required for keratinocyte migration in electric fields. Most interestingly, the keratinocyte migration rate and directionality remain similar in low and in high Ca^{++} media.

A number of systems have been employed in the study of cell migration, including modified Boyden chamber assays (Grzesiak and Pierschbacher, 1995a), phagokinetic assay (Ando and Jensen, 1993; Chen *et al*, 1994a; Albrecht-Buehler, 1997), cell dispersion assays (Tsuboi *et al*, 1993; Cha *et al*, 1996), and colony scattering assays (Bussolino *et al*, 1992; Giordano *et al*, 1993; Chen *et al*, 1994b). All of these systems measure mainly the net displacement, without quantitating directionality and the actual migration rate, which are better indicators of cell motility. Using electric fields coupled with time-lapse video images, we are uniquely able to direct keratinocyte migration and to quantitate migration rates and directionality. We have measured the difference in cell migration rate and directionality in the presence and in the absence of electric fields or growth factors (**Table I**, **Fig 1*a***). Our measurements suggest that migration *rate* and migration *directionality* are distinct properties of cell locomotion and are potentially subject to different regulatory mechanisms. In contrast to other assay systems where cell migration is observed over a long time period, we exposed

cells to the electric fields for only 1 h. Because significant cell division for human keratinocytes in culture occurs over 18 h (Tsuboi *et al*, 1993; Breitkreutz *et al*, 1993), our procedure allows us to measure cell locomotion unconfounded by cell division.

In cell culture medium devoid of exogenous growth factors (no HKGS), human keratinocytes still move towards the cathode, although at a much lower rate (**Table I**, **Fig 1*a***). This is probably due to autocrine secretion of TGF-α, amphiregulin, and interleukin-1α. All of these factors have been reported to stimulate keratinocyte migration (Schultz *et al*, 1991; Bennett and Schultz, 1993; Chen *et al*, 1995). At the low cell densities employed in our experiments, however, it is likely that concentrations of these autocrine factors are not high enough to fully stimulate cell locomotion. Addition of EGF to the medium fully restored the maximal keratinocyte motility and galvanotaxis, suggesting EGF and its receptor play a role in directional migration of keratinocytes. On the other hand, insulin and BPE, two other commonly used mitogens, also stimulate keratinocyte locomotion in physiologic electric fields, although less effectively than EGF (**Table I**, **Fig 2**). These results are consistent with previous reports (Sarret *et al*, 1992; Ando and Jensen, 1993; Ju *et al*, 1993). In culture and *in vivo*, EGF, insulin, and BPE can work together to create optimal conditions for keratinocyte survival, proliferation, and locomotion.

We have demonstrated that extracellular Ca^{++} is required for keratinocyte migration in electric fields, although concentrations as low as 3 μM seem sufficient to support motility. Ca^{++} levels from 3 μM to 3.3 mM supported keratinocyte migration at a similar rate and with similar directionality. The intracellular Ca^{++} concentration of cultured human keratinocytes is ≈100 nM when the extracellular Ca^{++} is 0.07 mM, and it rises to 130 nM within 2 h when extracellular Ca^{++} increases to 1–2 mM (Sharpe *et al*, 1989; Li *et al*, 1993; Sharpe *et al*, 1993). Because the keratinocytes used in our galvanotaxis experiments were incubated in a given medium for 2–6 h prior to imposition of the electric field, cells in 3.3 mM Ca^{++} medium must have higher intracellular Ca^{++} concentrations than cells in 3 μM Ca^{++} medium. This suggests that an overall elevation of extracellular Ca^{++} and subsequent intracellular Ca^{++} itself does not directly affect cell motility. A longer time of exposure to high Ca^{++}, however, could induce keratinocyte differentiation, which in turn could reduce cell motility. Indeed, if keratinocytes are incubated in high Ca^{++} medium for 8 h or longer, thymidine uptake decreases, differentiation markers are expressed, and cell migration rate reduces (Nickoloff *et al*, 1988; Yuspa *et al*, 1989; Li *et al*, 1993; Sharpe *et al*, 1993).

In contrast to our findings, Grzesiak and Pierschbacher reported that changes in calcium concentration alters human keratinocyte motility on collagen type I (Grzesiak and Pierschbacher, 1995a), while in our study, varying the extracellular Ca^{++} concentration from 3 μM to 3.3 mM did not significantly affect keratinocyte motility. This discrepancy is probably due to differences in cell type and/or in the assay system. Here we examine the migratory response of normal human keratinocytes, whereas Grzesiak and Pierschbacher utilized the spontaneously transformed keratinocyte line, HaCaT, whose migratory response differs from that of normal keratinocytes (Pilcher *et al*, 1997). Additionally, in their studies, Grzesiak and Pierschbacher placed a cell suspension in the upper chamber of a modified Boyden chamber, so that the time attributed to cell migration to the lower chamber actually includes the time for cell settling, cell adhesion, and cell translocation. Changes in cation concentration can be affecting processes other than translocation. In our assay system, all cells were adhered to the substratum before exposure to the electric fields, so that only cell translocation was measured. The requirement of extracellular Ca^{++} for keratinocyte migration in electric fields reported here is also different from that reported by Brown and Loew (1994), who did not find any change in cell motility during galvanotaxis of NIH3T3 cells and their transformants in the presence of EGTA. There are several explanations for this difference. First, their studies employed mouse fibroblast cell lines, NIH3T3, and its transformants. Second, they used an extracellular matrix biosynthesized by fibroblasts during 1–3 d culture, whereas our studies employed type I collagen and our previous studies have demonstrated that the nature of the underlying substrate can alter the galvanotaxic response (Sheridan *et al*, 1996). The different cation requirements of the specific integrins attaching to the different matrices may also contribute to the different findings (Grzesiak *et al*, 1992; Lange *et al*, 1994). Third, the field strengths employed in their study were significantly higher than those used here (400 mV per mm *versus* 100 mV per mm). Using Fura-2 dextran, Brown and Lowe did not observe any Ca^{++} influx in fibroblasts induced by electric fields. Because we know that extracellular Ca^{++} is necessary for keratinocyte galvanotaxis, it will be interesting to measure the Ca^{++} influx in keratinocytes. These studies are currently underway.

The mechanism by which electric fields and growth factors induce keratinocytes to migrate towards the cathode is not known. One possibility is that directionality arises from a redistribution of growth factor receptors and/or adhesion molecules induced by electric fields. Such a distribution difference has been observed on motile neutrophils in the presence of calcium (Lawson and Maxfield, 1995). Electric fields and growth factors may also act synergistically to activate localized ion channels, especially calcium channels. Increase of intracellular calcium has been associated with reorganization of actin and cell motility (Lawson and Maxfield, 1995; Doong *et al*, 1996). Electric fields and growth factors can also influence cell movements through tyrosine kinase activation and downstream phosphorylation (Nuccitelli, 1988; Nuccitelli *et al*, 1993; Chen *et al*, 1994a; Williams *et al*, 1994).

The presence of endogenous electric potentials across intact human skin (Barker *et al*, 1982; Foulds and Barker, 1983) and the existence of electric fields in the vicinity of wounds (Illingworth and Barker, 1980) make our studies relevant to wound healing. In wounded skin, electric fields, of 10–100 mV per mm have been measured in mammals with the more negative regions towards the center of the wounded area (Illingworth and Barker, 1980; Barker *et al*, 1982). In the glabrous epidermis of guinea pig skin, fields of 100–200 mV per mm have been documented (Barker *et al*, 1982). Wound healing in newt skin can be interrupted by disrupting the naturally occurring, laterally oriented electric fields, and application of an exogenous current that mimics the lateral electric fields restores the normal rate of re-epithelialization (Chiang *et al*, 1991). Many growth factors are also naturally present in a wound, including EGF, TGF-α, fibroblast growth factor, platelet-derived growth factor, TGF-β, and interleukin 1 (Steenfos, 1994; Martin, 1997). Applications of these growth factors to wounded areas or partial thickness burns enhance wound healing (Brown *et al*, 1989; Lynch *et al*, 1989; Greenhalgh *et al*, 1990; Schultz *et al*, 1991). It is tempting to hypothesize that application of electric fields and growth factors together – as occurs naturally following wounding – would promote directional cell migration and re-epithelialization, thus accelerating wound healing.

In conclusion, we have demonstrated that human keratinocytes migrate to the cathode at the physiologic level of DC electric fields, and that growth factors, especially EGF, enhance and maintain keratinocyte locomotion. Furthermore, we have identified directionality as a cell motility variable independent of migration rate, which can be independently regulated. We find that low levels of extracellular calcium are required for human keratinocyte motility. Our data provide an experimental basis for future applications of electric fields in promoting wound healing.

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