HGF, MAPK, and a Small Physiological Electric Field Interact during Corneal Epithelial Cell Migration

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PURPOSE. To investigate the effects of hepatocyte growth factor (HGF) and an applied electric field (EF) on corneal epithelial cell (CEC) migration.

METHODS. Primary cultures of bovine CECs were exposed to an EF (25–250 mV/mm) in the presence or absence of HGF (100 ng/mL). The rate and directionality of CEC migration were quantified. The expression of HGF receptors (HGFRs), p42/44 mitogen-activated protein kinase (MAPK) and the time-course of activation of p42/44 MAPK were investigated by confocal microscopy and Western blot analysis.

RESULTS. CECs migrated significantly faster in the presence of HGF, EF, or HGF and an EF combined. The distribution of HGFRs was intracellular and in the presence of an EF was concentrated in the cathode-facing side. This EF-induced asymmetrical accumulation of HGFRs correlated with the direction of CEC migration. The application of HGF or an EF led to the activation of the MAPK signaling pathway and in the presence of an EF, activation of MAPK was greater in the cathode-facing half of the CECs. Inhibition of the MAPK signaling pathway by PD98059 (100 μM) reduced the ability of HGF and an EF to enhance the rate of CEC migration, but did not alter EF-induced cathodal directionality.

CONCLUSIONS. These data suggest that both HGF and an EF augment the rate of CEC migration through activation of p42/44 MAPK. Moreover, EF-induced redistribution of HGFRs and asymmetry of MAPK signaling, although not instrumental in directing CEC migration cathodally, may be important for the signaling and maintenance of migration. (Invest Ophtalmol Vis Sci. 2003;44:540–547) DOI:10.1167/iovs.02-0570

Wounding the cornea generates steady DC electric fields (EFs) directed toward the wound’s edge and initiates the expression of hepatocyte growth factor (HGF). Both biologically generated EFs and endogenous growth factors (GFs) are significant during wound healing. Wound healing involves cell adhesion, migration, and proliferation, which are modulated by factors such as extracellular matrix (ECM) proteins, GFs, and EFs. The vibrating probe technique and traditional glass microelectrodes placed in varying locations have enabled the measurement of physiological, self-generating EFs at the wound site and with progressive closure of a wound, the endogenously produced EFs gradually diminish. In vitro, a variety of cells respond to EFs with directed migration (galvanotaxis) or directed growth (galvanotropism). These include neural crest cells, fibroblasts, neurons, and corneal epithelial cells (CECs). Applying an EF to cultured CECs imposes a cathodal directionality and perpendicular orientation on cell migration. The mechanisms underlying this behavior are unclear but may involve the redistribution of charged plasma membrane glycoproteins such as GF receptors. Whether an EF enhances the rate of CEC migration is unclear.

HGF is structurally distinct from other GFs and elicits a pleiotropy of behavior. In vitro HGF acts as a motogen, mitogen, and morphogen on a variety of epithelial cells, including CECs. Therefore, in vivo HGF may stimulate migration of CECs to cover the denuded area of a wound and stimulate proliferation of CECs to restore corneal integrity. The signal transduction pathways involved in HGF-stimulated cell migration, proliferation, and differentiation are well studied and involve an extended repertoire of cytoplasmic transducers. However, the mechanism by which the transduced HGF signal can trigger motility changes through the actin cytoskeleton remains unclear. Several protein families are engaged downstream of HGF activation and include the membrane-cytoskeletal protein ezrin, the Rho family of small GTP-binding proteins, and the integrin superfam.

We sought to investigate the MAPK signaling pathway as a potential common downstream target of HGF and an EF in CEC migration. We also investigated the expression of HGF and p42/44 MAPK in EF-treated CEC cultures. The results show that both HGF and an EF enhanced the rate of CEC migration and that both activated MAPK signaling. Inhibition of MAPK signaling reduced the ability of HGF and an EF to augment CEC migration. These data strongly suggest that HGF and an EF use the MAPK signaling pathway to augment the rate of CEC migration. An applied EF also induced an asymmetric accumulation of HGFRs and of p42/44 MAPK activation in the cathode-facing half of the CEC body. Although this may not be instrumental in directing CEC migration cathodally, it may be important in signaling and maintaining directed cell movement.

MATERIALS AND METHODS

Cell Culture

CECs were isolated as described previously, but with the following modifications. Dispase II (2 U/mL) was diluted in Ca2+ and Mg2+ Hanks’ balanced salt solution containing penicillin-streptomycin (1000 U/mL-1000 μg/mL), amphotericin B (12.5 μg/mL), and kanamycin (200 μg/mL). The medium used for all the experiments was buffered for room air and consisted of minimal essential medium (MEM) containing 1-glutamine (2 mM), 10% fetal bovine serum (γ-irradiated), amino acid supplement (7.5–14.7 g/L), amphotericin B (2.5 μg/mL), penicillin (100 U/mL), and streptomycin (100 μg/mL). The CECs were seeded and incubated for 24 hours at 37°C before experimentation.

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For immunofluorescence experiments, the base of the chamber was an acid-washed glass microscope slide (BDH, Poole, UK).

**EF Stimulation**

EFs were applied as described previously. Briefly, two chambers were constructed in 100 × 20-mm tissue culture dishes (Falcon; BD Biosciences, Plymouth, UK) using glass coverslips (No. 1) fixed to the base with silicon rubber (BDH). A coverglass roof was applied to give final chamber dimensions of 22 × 11 × 0.1 mm. Agar salt bridges 15 cm long were used to connect Ag/AgCl electrodes in beakers of Steinberg solution (sodium chloride, 58 mM; potassium chloride, 0.67 mM; calcium nitrate, 0.44 mM; magnesium sulfate, 1.3 mM; and a buffer base, 4.6 mM [Trizma: Sigma-Aldrich, Poole, UK]), to pools of excess medium at either side of the chamber. This prevented diffusion of electrode products into the culture medium. Field strengths were measured directly throughout the experiment. Medium, with or without HGF (100 ng/mL) or PD98059 (100 μM), was applied with a Pasteur pipette and a push-pull technique. HGF concentrations of 25, 50, and 100 ng/mL were tested initially, and 25ng/mL did not affect migration rates, but both 50 and 100 ng/mL increased control migration rates. All subsequent experiments were performed using HGF at a concentration of 100 ng/mL.

**Quantification of Cell Behavior**

Cells were monitored and analyzed using an image analyzer (Q500mc; Leica, Heidelberg, Germany). Mean migration rates and directedness were quantitated hourly. The rate of cell migration was measured using the distances from a fixed point (a scratch on the chamber base) and the angle between the two cell positions. The method of Gruler and Nuccitelli was used to quantify directionality and, in short, used the cosine angle (θ) that each cell moved in relation to the imposed EF vector. If cosine θ was 1 the cell was moving cathodally, if cosine θ was 0 the cell was moving perpendicular to the EF, and if cosine θ was −1 the cell was moving anodally. Averaging the cosine θ values gives a mean directionality of cell movement.

**Western Blot Analysis**

Western blot analysis investigating the level of total ERK 1/2 and pERK 1/2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in CECs were performed as follows. CECs were cultured as previously and then exposed to either a 150-mV/mm EF or 100 ng/mL HGF for 15, 30, and 60 minutes. Cells were harvested using a lysis buffer (2% SDS, 70 mM Tris!HCl [pH 6.8]), aspirated, and boiled for 20 minutes. Cell extracts were normalized for total protein with a protein assay (Bio-Rad Laboratories, Pasteur pipette and a push-pull technique. HGF concentrations of 25, 50, and 100 ng/mL were tested initially, and 25ng/mL did not affect migration rates, but both 50 and 100 ng/mL increased control migration rates. All subsequent experiments were performed using HGF at a concentration of 100 ng/mL.

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**Confocal Microscopy**

Cultured cells were exposed to an EF (150 mV/mm) or HGF (100 ng/mL) for 15 minutes, washed with PBS, fixed in formaldehyde (4% vol/vol in PBS-T) for 1 hour, and washed again with PBS-T before blocking in BSA (10 mg/mL in PBS-T) for 1 hour. All antibodies were prepared in 1 mg/mL BSA in PBS-T and used at the following concentrations: m-Met rabbit primary, 1:100; p-ERK1/2 mouse primary, 1:100 (Santa Cruz Biotechnology, Inc.), FITC anti-rabbit secondary, 1:50 (Jackson Immunoresearch, West Grove, PA), and FITC anti-mouse secondary 1:100 (SAPU). For negative control staining, only the secondary antibodies were incubated with the cells. No positive staining was observed with secondary antibodies alone. Cells were mounted in antifade medium (Vectashield; Vector Laboratories, Peterborough, UK) and viewed with a confocal microscope (MRC 1024; BioRad, Hercules, CA).

For quantification of asymmetric distribution of HGFRs, the fluorescence intensity (FI) of FITC-labeled cell body HGF was measured in the cathode- and anode-facing halves. Ten CECs were selected randomly by using the rhodamine-phalloidin fluorescence (i.e., F-actin) to ensure that there was a mix of cell morphologies. A z-series (1-μm horizontal sections through an entire CEC), was obtained and then projected vertically using an image-processing package (2.1a Lasersharp; BioRad) to give a collapsed image of total cell fluorescence. The freehand polygon function was used to measure the mean FI in the cathodal and anodal half of each CEC body. The corresponding area of each half was measured to ensure equality. A background mean FI for each cell was also obtained and subtracted from each measurement. The asymmetry index (Ai) was calculated for each CEC as: \( C_i = (C_{an} - A_i)/(C_{an} + A_i) \), where \( C_i \) indicates mean cathodal FI, and \( A_i \) indicates mean anodal FI. A value of 1 indicates fluorescence asymmetry entirely localized to the cathode-facing side, −1 indicates fluorescence asymmetry entirely localized to the anode-facing side, and 0 indicates no fluorescence asymmetry.

The extent of correlation between the location of cell body HGFRs and the direction of CEC migration was determined in CEC displaying prominent leading edge lamellipodia (i.e., migratory morphology). The individual z-series were viewed three dimensionally (3-D) on computer (2.1a Lasersharp; BioRad). The number of HGFR stacks with staining located toward the leading edge were used as an indication of whether the location of HGFR and the direction of CEC migration were correlated. Each cell was designated as having no correlation, a little correlation, some correlation, and a striking correlation.

Quantification of pERK 1/2 staining was performed by using the line intensity profile (LIP) tool in the image-processing software (2.1a Lasersharp; BioRad). The average LIP measured across the midline of 10 CECs gave the mean FI for each experiment, and the data were normalized by dividing the mean treated FI by the mean control FI. To quantify the \( A_i \) for p-ERK 1/2 staining in the CEC body, the FI in the first and last 2 μm (i.e., the cathode- and anode-facing sides) of each mean LIP was measured, and the corresponding \( A_i \) calculated.

**Statistical Analysis**

All experiments were repeated at least three times on separate primary cell cultures. Data are expressed as the mean of total results in all experiments ± SEM. Student’s t-test or the Welch t-test, when the standard deviations were significantly different, were used, and the level of significance expressed as \( P < 0.01 \), \( P < 0.001 \), or \( P < 0.0001 \).

**RESULTS**

**HGF- and EF-Enhanced Migration**

The effect of HGF and an EF on rate of migration (Fig. 1A) and directionality (Fig. 1B) in CECs was determined. HGF (100 ng/mL) or an EF (150 mV/mm) significantly enhanced the rate of CEC migration by 57% compared with control (\( P < 0.001 \); Fig. 1A). Coaplication of HGF with an EF further enhanced the rate of CEC migration by 62% compared with the control (\( P < 0.0001 \), Fig. 1A).

In control cultures, CECs exhibited random, nondirectional migration, with a directionality close to zero (Fig. 1B). A small physiological EF (150 mV/mm) significantly directed the migration of CECs toward the cathode, with a mean directionality
between the cell body distribution of HGFRs and the direction of We also determined whether there was any correlation between the accumulation of HGFRs in the presence of an EF was striking in the cathode-facing half of the CECs. HGFRs accumulated asymmetrically in the cathode-facing half of each CEC body (20 per variable) was measured, and the A5 were calculated. An EF of 250 mV/mm resulted in a significant accumulation of HGFR-associated fluorescence in the cathode-facing half of the CECs (P < 0.001; Fig. 2C). The accumulation of HGFRs in the presence of an EF was striking and appeared as stacks of receptors viewed in 3-D (not shown).

**Distribution of p42/44 MAPK in EF-Treated CECs**

CECs exposed to an EF also demonstrated asymmetric activation of p42/44 MAPK in the cathode-facing side of the cell body (Fig. 4A; white arrows). The level of p42/44 MAPK activation was almost 50% greater in the first few micrometers on the cathode-facing side of the CEC (Fig. 4B). As were calculated from the mean FI in the first and last 2 µm of each LIP (see the Materials and Methods section). No p42/44 MAPK fluorescence asymmetry was observed in the control or HGF-treated (100 ng/mL) CECs, with A5 close to 0 (P > 0.05; Fig. 4C). However, EF-treated (150 mV/mm) CECs displayed significant cathodal fluorescence asymmetry, with an A5 close to 0.4 (P < 0.0001; Fig. 4C).

**Inhibition of HGF- and EF-Induced p42/44 MAPK Activity**

PD98059, a selective inhibitor of MEK 1/2, significantly reduced the mean migration rate of control CEC by 28% (P < 0.01; Fig. 5A). The methanol vehicle had no effect (not shown). The presence of PD98059 prevented HGF from increasing the rate of CEC migration (P < 0.001; Fig. 5A), reduced the extent of EF-enhanced migration by 19% (P < 0.01; Fig. 5A) and reduced the combined HGF and EF enhancement of CEC migration rate by 30% (P < 0.0001, Fig. 5A). PD98059 did not affect CEC directionality (Fig. 5B). In the presence of 100 mM PD98059 plus an applied EF, CEC cathodal directionality was also seen in EF-treated CECs (Fig. 5B; lane 5). PD98059 inhibited HGF- and EF-stimulated migration, but did not affect EF-induced cathodal directionality; which indicated that EF-induced directionality and enhancement of migration rate may operate through separate and parallel mechanisms.

**DISCUSSION**

Endogenous EFs and GFs such as HGF are present during wound healing in the cornea. We have shown that the MAPK signaling pathway was activated in CECs treated with HGF or a physiological EF. Both HGF and an EF enhanced the rate of CEC migration but only EF-treated CECs displayed directional migration, migrating cathodally. HGF- and EF-enhanced migration was reduced in the presence of PD98059, an MAPK inhibitor.
EF-induced, cathode-directed migration was unaffected, although, both HGFRs and activated MAPK accumulated asymmetrically in the cathode-facing side of CEC bodies.

**EFs, GFs, and CEC Migration**

An exogenous EF enhances migration rates of several cell types—for example, *Xenopus* neural crest cells,12 avian fibroblasts,13 keratocytes,44 and *Xenopus* neurites14—and stimulates the rate of epithelialization during wound healing in vivo.2,45,46 However, the EF-enhanced migration rate of 37% (Fig. 1A) reported herein was greater than found previously in CECs.16,17 and keratinocytes,48 perhaps due to differences in the serum used. EF-directed migration had been demonstrated in CECs16,17 and keratinocytes,48 and is similar to that reported in the current study (Fig. 1B).

GFs regulate migration, mitosis, and differentiation. EGF enhances the rate of migration of cultured bovine CECs,17 human keratinocytes,46 and cells from a human corneal epithelial cell (HCEC) line.6 The level of expression of HGF and HGFR mRNAs are low in the unwounded cornea; however, a marked upregulation occurs after wounding.4 Applying HGF in culture, significantly stimulated bovine CEC migration (Fig. 1A). HGF also stimulates migration in MDCK cells15 and in HCECs.45 Coapplication of HGF with an EF, as occurs at a wound, enhanced the rate of CEC migration over that seen with GF or EF alone (Fig. 1A). The enhancement was not fully additive, suggesting that HGF and an EF may operate through common downstream pathways capable of saturation.

HGFR did not affect the extent of EF-induced cathodal directionality (Fig. 1B). Coapplication of EGF plus an EF enhances cathode-directed migration17 and EGF partially restores EF-induced cathode-directed migration in CECs and keratinocytes in the absence of serum.17,49 Activation of EGFR may be crucial in EF-induced cathode-directed migration of CECs and keratinocytes, but HGF, although present in serum, was not involved in initiating cathode-directed migration.

**HGFR and CEC Behavior**

EGFRs accumulate at the leading edge of the cathode-facing side of cells in an EF and may be instrumental in directing migration.4,19,42 HGFR staining was mostly intracellular in control and field-treated CECs (Fig. 2A). The absence of HGFR staining in the leading lamellae of field-treated CECs also indicates that redistribution of HGFRs to the leading edge of the cathodal lamellae was not instrumental in directed migration. In EF-treated CECs, HGFRs accumulated asymmetrically within 15 minutes, but in the cathode-facing half of the CEC bodies rather than in the leading lamellae (Fig. 1). This observation raises three questions. Where was this asymmetric accumulation of HGFRs located? How did the HGFRs accumulate there? What was the purpose of this asymmetric location of HGFRs?

The stacks of HGFRs spanning the cell body of the CEC may indicate that the HGFRs had been internalized into the endosomal compartments. Both EGF and EGFRs have been detected in the early endosomal compartment within 2 to 5 minutes after receptor-mediated internalization at 37°C.49 A recent report of a colocalization study noted that a non-Golgi compartment, termed the subapical compartment (SAC), was involved in redirecting apical and basolateral membrane components in polarized and semipolarized cells.50 There was a striking sim-
EF treatment significantly reduced EGF-enhanced and EF-directed cell migration. However, the asymmetric accumulation of HGFRs was correlated with the direction of migration. The Golgi apparatus and microtubule organizing center (MTOC) redistribute to the exposed side of cells at a wound’s edge within minutes, before any apparent leading-edge extensions, which indicates a role for these organelles in directing cell movement. Perhaps the EF-induced redistribution of HGFRs represents a similar event. The EF-induced cathodal accumulation of cell body HGFRs in CECs was also rapid (<15 minutes; McBain VA, unpublished data, 1999) and could indicate that the internalized HGFRs were involved in intracellular signaling of directed migration. It has been shown that internalized EGF remains associated with the EGFRs and that, in the early endosomal compartments, signaling may continue by phosphorylating endogenous substrates intracellularly.

**p42/44 MAPK and CEC Behavior**

EFs and GFs share common signaling pathways because EFs influence cathode-directed migration through activation of tyrosine kinase and downstream phosphorylation events. MAPK is activated after stimulation of EGFRs to induce cell migration. In that PD98059, the selective MEK 1/2 inhibitor significantly reduces EGF-enhanced and EF-directed cell migration.

HGF induced a rapid and sustained activation of ERK 2 (Fig. 3B), also shown in other cell types, and this may underpin enhancement of cell motility in epidermal keratinocytes. An EF also induced activation of p42/44 MAPK in CECs, perhaps as early as 15 minutes. We do not know whether the applied EF directly activated p42/44 MAPK or induced GF and/or integrin receptor clustering that indirectly activated p42/44 MAPK. PD98059, the selective MEK 1/2 inhibitor, significantly reduced both the EF- and HGF-enhanced rate of CEC migration (Fig. 5A), but neither was inhibited completely. Other second-messenger cascades may be involved in cell migration. For example, ligand binding to the HGFR also phosphorylates P13K and PLCγ, both of which are involved in HGFR’s enhancement of cell migration. HGF also directly phosphorylates components of the cytoskeletal signaling complexes—for example, focal adhesion kinase (FAK) and the small GTPase proteins Rho and Rac. In addition, ezrin, a protein that acts as a membrane–cytoskeleton linker, is activated after activation of HGFR. These represent alternate pathways for HGF-enhancement of CEC migration besides activation of MAPK. Similar to HGF, the EF may stimulate other second-messenger pathways capable of enhancing CEC migration, especially if the EF indirectly activates p42/44 MAPK through GF and/or integrin receptor clustering. PD98059, however, did not affect EF-induced cathodal directionality, suggesting that the upstream asymmetric activation of EGFR for example, engages pathways other than the MAPK pathway to effect directed migration.

**Proposed Mechanisms for HGF- and EF-Enhanced CEC Migration**

The application of an EF and HGF may converge at the level of p42/44 MAPK activation to enhance the rate of CEC migration. Two downstream signaling cascades are involved in the enhancement of the cell migration rate after EGF- and extracellular matrix (ECM)-stimulated activation of p42/44 MAPK. First, myosin light chain kinase (MLCK), a key regulator in cell

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**Figure 3.** p42/44 MAPK activation with HGF and an EF. (A) Mean normalized phospho-p42/44 MAPK LIP FIs for control CECs and those treated with HGF (100 ng/mL) and an EF (150 mV/mm). CECs were cultured, treated, fixed, stained, and imaged and the image subjected to computer-assisted analysis. Mean FIs were obtained from the LIP for each CEC, and then the data from individual experiments normalized. Application of either HGF or an EF significantly increased activation of phospho-p42/44 MAPK, compared with the control. (B) Time course of phospho-p42/44 MAPK activation in CECs after application of HGF (100 ng/mL) and EF (150 mV/mm). Anti-phospho-p42/44 MAPK and total p42/44 MAPK were detected by Western blot analysis (10 mg soluble protein extract for each treatment was loaded). HGF-treated CECs demonstrated p42 MAPK phosphorylation within 5 minutes (lane 2), which peaked at 30 minutes (lane 4) and remained activated at 60 minutes (lane 5). EF-treated CECs, in contrast, only displayed p42 phosphorylation after 60 minutes of EF exposure (lane 5). Lane 1: baseline; lane 3: 15 minutes. The experiment was repeated with identical results.
motility and contraction,\textsuperscript{62,63} is a substrate of p42/44 MAPK after the activation of integrins and EGFRs.\textsuperscript{56} Second, EGF-stimulated p42/44 MAPK activates cPLA\textsubscript{2}, which in turn increases release of arachidonic acid from phospholipids, with consequent production of leukotrienes.\textsuperscript{64,65} Both of these signaling cascades converge at the level of the actin cytoskeleton, where the leukotrienes stimulate actin polymerization and phosphorylation of MLCK results in actin contraction.\textsuperscript{62–64} Therefore EF- and HGF-stimulated activation of p42/44 MAPK may also converge on these signaling cascades.
In conclusion, corneal wound healing may involve a temporal and spatial interplay of GFs and EFs as well as ECM proteins. A wound-induced EF may direct and stimulate cell migration, while GFs such as HGF further enhance activated migration. These observations indicate potential therapeutic strategies that combine treatment with an EF and GFs.

References