Electric Fields and MAP Kinase Signaling Can Regulate Early Wound Healing in Lens Epithelium

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PURPOSE. To use lens epithelial cell monolayer wounds as a model system for the aberrant cell migration underlying posterior capsule opacification (PCO) and to investigate the effects of an applied physiological electric field (EF) on monolayer wound healing.

METHODS. Scratch wounds were made in cultured bovine lens epithelial monolayers, and the wounds were exposed to an EF, with or without U0126 treatment (an inhibitor of active extracellular signal-regulated kinase [ERK 1/2]). Serial wound images were taken and wound areas were measured. Western blot analysis and immunocytochemical staining for ERK 1/2 in the wounded monolayers were performed.

RESULTS. An applied EF of a given polarity influenced the healing of lens epithelial monolayer wounds. Wounds facing the anode healed at normal rates, those facing the cathode closed much more slowly. U0126, an inhibitor of mitogen-activated protein kinase (MAPK) signaling, inhibited wound healing, with or without exposure to an EF. Western blot analysis showed that both wounding and application of an EF enhanced the activation of ERK 1/2 independently and that U0126 completely inhibited these activations of ERK 1/2 in monolayers. Immunocytochemical staining showed an asymmetric activation of ERK 1/2 in EF-exposed wounds, with much weaker fluorescence in cathode-facing wounds, which could contribute to differentially directed wound-healing rates in an EF.

CONCLUSIONS. Exposure to an EF inhibited the healing of lens epithelial monolayer wounds facing the cathode. ERK signaling pathways were involved in healing of lens epithelial monolayer wounds and in the EF-directed migration of the wound edge. It may be possible to use an applied EF to regulate the aberrant migration of lens epithelial cells that results in PCO after cataract surgery. (Invest Ophthalmol Vis Sci. 2003;44:244–249) DOI:10.1167/iovs.02-0456

Electric fields influence cell shape, directed cell motility, and tissue architecture and have been proposed for at least a century. More recently, physiological electric fields (EFs) have been measured in many developing and regenerating systems and are modulated spatially and temporally and in magnitude. Many types of cells, including cell sheets, migrate in specific directions when exposed to a small EF. An applied EF, similar in magnitude to those found endogenously, can direct migration of cells. A unique pattern of current, which creates endogenous EFs, exists in the vertebrate lens. Focused currents carried largely by K⁺ ions, flow out at the lens equator, where cells are migrating, dividing, and differentiating and return over the anterior central part of the lens epithelium. Controlling the directions of lens epithelial cell (LEC) migration is critical for normal lens physiology. LECs are found in a peripheral germinative zone close to the equator, migrate toward the equator and differentiate into lens fiber cells. We have shown that an applied EF of physiological strength can induce directed migration of LECs.1 However, the physiological importance of endogenous EFs in controlling the normal movements of LECs or their involvement in the pathologic migrations of LECs is unclear.

Cataract is the most common cause of blindness, and extra-capsular cataract extraction is the most efficient therapy. However, surgery is often followed by a high incidence of posterior capsule opacification (PCO), which occurs because LECs, which line the residual anterior and equatorial areas of the lens capsule, proliferate and migrate inappropriately onto the intact posterior capsule, where they compromise vision. The mechanisms underlying PCO are not yet understood. However, because cataract surgery completely abolishes the normal electric current pattern by removal of the biological lens, one explanation for aberrant proliferation and migration of LECs may involve the loss of a regulatory influence exerted by the lens currents. Currently, there are no good methods for preventing aberrant migration of LECs and PCO. Therefore understanding the mechanisms that control the migration of LECs and determining whether they include an electrical component could be important in preventing PCO. Because PCO is essentially a wound-healing response, we used a wound-healing assay to study the electrical controls of LEC migration. The underlying idea is that restoring the normal electrical controls in LECs may be of use in preventing aberrant cell migration.

MAP kinase (MAPK) signaling plays an important role in signal transduction during cell migration and is required for EF-directed migration of corneal epithelial cells. MAPK/extracellular signal-regulated kinase (ERK) levels are elevated rapidly after mechanical injury to epithelia and confluent monolayers and are essential for epithelial healing. Activation of ERK in lens cells is necessary and sufficient to increase gap junctional coupling and is required for FGF-induced proliferation and differentiation of LECs, which is blocked by U0126, a specific inhibitor of ERK 1/2 activation. In addition, altered MAPK activity in the lens is sufficient to cause formation of cataract.

We show that an EF of a given polarity can prevent directed migration of the leading edge of a wound in an LEC monolayer and that MAPK signals were involved in the healing of wounds in the LEC monolayer.

METHODS

Materials

Tissue culture plastic dishes, 35 × 10 mm (3001; Falcon, Wiesbaden, Germany) and 100 × 20 mm (3003; Falcon) were used. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, amphotericin-B, 2-glutamine, and Ca and Mg-free Dulbecco’s phosphate-buffered saline (PBS) were obtained from Gibco BRL (Paisley, Scotland, UK). Anti-active ERK 1/2 antibodies and anti-ERK 1/2 pan-antibodies for Western blot analysis, and ERK inhibitor

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Control wounds had no exposure to an EF.

Wounds with edges parallel to the EF vector, wounds with the edge of EFs on wound healing, three types of wounds were included: (1) wounds with the EF vector parallel to the long axis of the chamber and either peripheral (equatorial) LECs that migrate to create PCO after surgery, (2) wounds in which the anodal half was delineated by the wound scratch, the wound was measured with the image analyzer at various times after wounding. The initial enlargement response was much more striking than if the EF was applied in the opposite direction. (3) Wounds were created on a 10 × 22-mm monolayer in each chamber, with a mean width of 10 mm and length of 8 mm for each wound. Wound scratches were either perpendicular or parallel to the long axis of the culture chamber depending on the purpose of the experiment. Serial micrographs of wounds were taken immediately (0 hour), 1 hour, and 2 hours after wounding, respectively. Individual frames were stored by an image analyzer (Q500MC; Leica, Cambridge, UK). A wound was divided by the wounding scratch into two halves. For clarity, only one of these is shown in Figure 2A. (A wound, exposed to an EF perpendicular to the wound edges, therefore has both an anode-facing edge and a cathode-facing edge). The wound area (WA) of each half was measured with the image analyzer at various times after wounding. The WA of each half was delineated by the wound scratch, the wound edge, and two retaining scratches (perpendicular to both the wound scratch and the wound edge) made before seeding the cells.

Application of the EF

EFs were applied to LEC monolayers as before. A lid made of a coverslip was applied to the trough and sealed with silicone grease immediately after wounding. The final dimensions of the chamber through which the current was passed were 22 × 10 × 0.3 mm. Immediately after 60 minutes, wound images were obtained (generally finished within 5 minutes after wounding). The wounded monolayers were exposed to an EF of 200 mV/mm in a 5% CO₂ incubator at 37°C for 2 hours. EF strengths were measured directly at the beginning and end of the experiment. No fluctuation in field strength was observed. The EF vector was parallel to the long axis of the chamber and either perpendicular or parallel to the edge of the wound. To test the effects of EFs on wound healing, three types of wounds were included: wounds with edges parallel to the EF vector, wounds with the edge facing the anode, and wounds with the edge facing the cathode. Control wounds had no exposure to an EF.

Administration of U0126

In some experiments, peripheral LEC monolayers were preincubated with 20 μM U0126 in DMEM or 0.2% dimethyl sulfoxide (DMSO) vehicle for 30 minutes before they were wounded and/or exposed to an EF with a vector perpendicular to the wound edge.

Western Blot Analysis

After various treatments, peripheral monolayers were rinsed with cold PBS and lysed with lysis buffer (0.1% SDS, 1% igepal CA-630, 0.5% sodium deoxycholate, 0.01% phenylmethylsulfonyl fluoride [PMSF], 5% [vol/vol] aprotinin and 1 mM sodium orthovanadate). Identical amounts of protein lysates were resolved by 4% to 12% SDS-PAGE, followed by electroblot analysis onto nitrocellulose membrane (Invitrogen, San Diego, CA). The gels were stained for remaining proteins with Coomassie blue as a control of loading. The membrane was blocked with 5% skim milk PBS and then probed with anti-active ERK 1/2 antibody (1:5000). Donkey anti-rabbit secondary antibody with horseradish peroxidase (HRP: 1:10,000) was used, and the immunoblots were detected by an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, Amersham, UK).

Immunocytochemistry and Confocal Fluorescence Microscopy

At 15, 30, 60, and 120 minutes after wounding, the peripheral monolayers, with or without exposure to an EF, were fixed in 4% formaldehyde for 15 minutes at room temperature, permeabilized with 0.1% Triton X-100 in PBS for 3 minutes, blocked with 1% bovine serum albumin in PBS for 20 minutes, incubated with anti-active ERK 1/2 antibodies at 1:50 dilution and sheep anti-mouse antibodies conjugated with FITC at 1:100 dilution for 1 hour, respectively, and slides viewed with confocal microscopy (MRC-1240; Bio-Rad, Hercules, CA). Unwounded monolayers, stained by using the same procedure, and wounded monolayers stained with nonimmune mouse serum were used as the control.

To assess the distribution of active ERK 1/2 in monolayer wounds, fluorescence intensity was measured quantitatively by confocal microscopy, with a method similar to that used previously. Briefly, a monolayer wound was divided into cathodal and anodal halves by the wound gap. Polygons were drawn to determine equivalent cathodal and anodal areas of the wound. Mean fluorescence intensity on cathodal and anodal sides was quantified for each probe, by using the same areas. The acellular wound gap in the same image was used for background subtraction. An asymmetry index was calculated: 

\[ A_\Delta = \left(\frac{C_A - A_\Delta}{C_B + A_\Delta}\right) \]

where \( C_A \) represents mean fluorescence intensity on the cathodal side, and \( A_\Delta \) mean fluorescence intensity on the anodal side. A wound with uniform fluorescence staining has an \( A_\Delta \) of 0. If staining is restricted entirely to the cathodal half, the \( A_\Delta \) would be 1. Staining only on the anodal half would give an \( A_\Delta \) of −1. An \( A_\Delta \) greater than 0 therefore, indicates a stronger intensity of fluorescence on the cathodal side of a wound, which is the side moving toward the anode as wound closure occurs.

Statistics

Statistical analysis was performed with the unpaired, two-tailed Student’s t-test, or the Welch unpaired t-test, when the standard deviations were significantly different from each other. Data are expressed as the mean ± SEM, unless stated otherwise.

RESULTS

Central and Peripheral LEC Wounds Healed Differently

The healing of both central and peripheral LEC monolayer wounds occurred in two phases (Fig. 1A). WA increased initially before decreasing as the wound closed. The initial response involved wound edge retraction and a rounding up of some cells at the wound edge. In the closure phase, cells crawled into the void, extending substantial lamellipodia (Fig. 2A). The initial enlargement response was much more striking in central monolayer wounds, which showed faster wound
edge retraction and a more protracted wound-enlargement phase. Wound closure by lamellipodial extension was evident within 15 minutes in peripheral monolayers, whereas in central monolayer wounds, this occurred within approximately 20 minutes. Although at 0 hour the mean WA of central and peripheral wounds was the same, the central WA thereafter was always greater than the peripheral WA, indicating delayed closure (Fig. 1A).

Effect of Polarity of the EF

Wound healing of both central and peripheral LEC monolayers was influenced by the applied EF, but the difference in speed of healing of central and peripheral LEC monolayers was unaffected by the EF (Fig. 1B). Three configurations of wounds were exposed to an EF, as seen in Figure 2. When the EF vector was parallel to the leading wound edge, control and EF-exposed wounds healed at the same rate, by lamellipodial pro-

Figure 1. Central and peripheral LEC monolayer wounds heal differently. Monolayers, from central and peripheral regions of lens epithelium were wounded and wound images captured at intervals up to 2 hours after wounding. (A) In these control wounds (no EF), wounds opened up transiently before healing ensued. Wounds to monolayers of central cells showed a markedly enhanced wound-enlargement response which prolonged wound healing. *P < 0.01 compared with mean wound area of peripheral LEC monolayer wounds at the same time point. (B) In an EF of 200 mV/mm (with EF vector perpendicular to wound edges), significant directed wound edge migration occurred. Central wounds still were slower to heal than wounds in peripheral LEC monolayers and wound healing toward the anode was much faster than toward the cathode in both central and peripheral wounds. *P < 0.01 compared with the relative mean in central monolayer wounds and #P < 0.01 compared with the relative mean of the anode-facing wounds, at the same times.

Figure 2. The effects of EFs on the healing of LEC monolayer wounds. (A) Wounded LEC monolayers, originating from peripheral regions of lens epithelium, were exposed to EF stimulation of 200 mV/mm with polarity as shown. Four experimental configurations are illustrated: no exposure to an EF control; EF vector parallel to the wound edge. Again, wound healing was not influenced by this EF vector: wound-healing direction with cathode-facing wound edge. Wound healing was inhibited significantly by the EF vector, which prevented wound closure. A reference scratch is shown at the edge of each frame. (B) Quantitative comparison of wound healing in each experimental configuration. Control wounds, those with an EF applied parallel to the wound edge, and those with the wound edge facing the anode all healed at equivalent rates. Wounds facing the cathode opened up and did not close. Data in different experimental conditions were from two to three separate experiments. *P < 0.01 compared with control wounds with no exposure to an EF.
combination of three events: The wound enlargement response was greater, wound closure was delayed, and the speed of closure was reduced substantially. The lamellipodial extension of cells at the wound edge also was reduced (Fig. 2).

MAPK Signaling

U0126, an inhibitor of MAPK signaling, reduced the healing of wounds in the LEC monolayer (Fig. 3). DMSO (the vehicle for U0126) had no significant effect on the wound healing of LECs (not shown). U0126 (20 μM) inhibited control and EF-induced wound healing. The most prominent inhibition was evident in wounds attempting to heal by migrating toward the cathode (compare Figs. 3A and 3B). These enlarged substantially and did not close at all during 2 hours.

EF or Wounding Enhanced Activation of ERK 1/2 in Monolayers of LECs

Western blot analysis showed that the expression of active ERK 1/2 in unwounded LEC monolayers increased significantly within an applied EF, compared with the control (no EF). Wounding a monolayer also increased active expression of ERK 1/2. However the combination of wounding and application of an EF did not enhance the expression of active ERK 1/2, additively. The MAPK inhibitor U0126 completely prevented activation of ERK 1/2 induced either by an EF or by a wound (Fig. 4).

EF-Induced Asymmetric Distribution of ERK 1/2 in Monolayer Wounds

Immunocytochemical staining showed that in control wounds (no EF), staining with active ERK 1/2 antibodies was symmetrical (asymmetry index = 0.01 ± 0.05; n = 18). By contrast, a physiological EF induced an asymmetric distribution of active ERK 1/2, with weaker fluorescence at the wound edge that faced the cathode (Fig. 5). The mean asymmetry index was 0.25 ± 0.04 (n = 20), significantly higher than that in control wounds (P < 0.01). The asymmetric distribution of active ERK 1/2 between anodal and cathodal wound edges was already detectable at 15 minutes after wounding. Some cells in Fig. 5 give an impression of greater staining for pERK in the nucleus. However, we also studied pERK distribution in single LECs, both control cells and those exposed to an applied EF and find no difference between nuclear and cytoplasmic levels of staining.

Discussion

Differences in Wound Healing between Central and Peripheral LEC Monolayers

Lens epithelium has been arbitrarily divided into three regions: central, peripheral, or pre-equatorial; and equatorial. In this study, injuries to monolayers of LECs revealed an intrinsic difference between cells from different regions. Monolayers of central LECs were more sensitive to injury, with a stronger wound-enlargement response, and showed considerably delayed wound healing, compared with peripheral LEC monolayers. Although regional differences in cell morphology, proliferation, and the synthesis of crystalline have been described, this is the first indication that there are regional differences in the response of epithelial cells to a wound.

Effect of EF on Wound Healing

Lens epithelium generates endogenous EFs and we are interested in whether these may influence aberrant cell migration when a diseased lens is removed. An endogenous EF arises instantaneously in wounded epithelia and has been measured directly at wounds in skin, cornea, and lens. Wounded monolayer cultures also may produce endogenous EFs. There is convincing evidence that an endogenous EF is necessary for normal wound epithelialization, which does not occur at normal rates when the wound-induced EF is compromised. There are also several claims of enhanced wound healing by EF application to skin wounds. In this study, we have shown that an applied direct-current (DC) EF influenced the healing of LEC monolayer wounds and that this effect varied with the polarity of the EF. Wound healing was inhibited substantially in wounds facing a cathode. Because strips of cells several hundred micrometers wide were created by the scratch wounds, each wound had an anode- and a cathode-facing wound edge. Edges facing the anode closed much faster than edges facing the cathode. The latter close eventually, but only slowly, after initially enlarging (Fig. 2B). Anode-facing cells therefore could not simply have been induced to close the wound with cathode-facing cells drawn passively, or retracting actively in their wake. We think it more likely that there must be a separate mechanistic explanation for the much-inhibited closure of cathode-facing wounds. The asymmetry in distribution of ERK 1/2 staining between cathode- and anode-facing edges (described later) also supports this conclusion.
Mechanisms Underlying the EF-Mediated Wound Healing of LEC Monolayers

The MAPK pathway may mediate signal transduction during wounding. Mechanical injury to confluent monolayers causes rapid activation of ERK, although in addition to the mechanical injury, wounded monolayers also may produce endogenous EFs. In the present study, the healing of LEC monolayer wounds was inhibited by the MAPK inhibitor U0126 and expression of active ERK 1/2, an enzyme on the MAPK signaling pathway, was upregulated after wounding of LEC monolayers. Both results indicate that MAPK signaling was involved in this response. A physiological EF also stimulated expression of ERK 1/2 in unwounded peripheral monolayers, whereas U0126 inhibited both wound edge migration of monolayer wounds and activation of ERK 1/2 after wounding, irrespective of whether an EF was present. When wounds (which may generate their own EFs) were exposed to an applied EF, upregulation of ERK 1/2 was not additive, perhaps indicating that the wound-induced EF enhances expression of ERK 1/2 by itself and that an applied EF does not further enhance expression.

The distribution of activated ERK 1/2 was asymmetric at an EF-exposed wound edge with weaker fluorescence in wound edges facing the cathode. This may indicate that activation of ERK 1/2 is essential for directed wound healing and that the weaker expression in cathode-facing wounds is causal in the compromised wound healing that they show. MAPK signaling was a prominent element in the wound-healing response and the expression of active ERK 1/2 at wounds was inhibited completely with U0126. However, this did not completely suppress the ability to heal a wound, albeit slowly, indicating that other signal transduction pathways must be involved in the healing of control and EF-exposed wounds.

Clinical Importance

The currents generated by bovine, rat, and frog lenses have been measured directly and noninvasively with a vibrating probe. The currents generated by bovine, rat, and frog lenses have been measured directly and noninvasively with a vibrating probe. The currents generated by bovine, rat, and frog lenses have been measured directly and noninvasively with a vibrating probe. The currents generated by bovine, rat, and frog lenses have been measured directly and noninvasively with a vibrating probe. The currents generated by bovine, rat, and frog lenses have been measured directly and noninvasively with a vibrating probe. The currents generated by bovine, rat, and frog lenses have been measured directly and noninvasively with a vibrating probe. The currents generated by bovine, rat, and frog lenses have been measured directly and noninvasively with a vibrating probe. The currents generated by bovine, rat, and frog lenses have been measured directly and noninvasively with a vibrating probe.

References


Figure 4. Western blot analysis of active ERK 1/2 in LECs. Peripheral LEC monolayers were wounded (+) by scratching. An EF (200 mV/mm, 15 minutes) enhanced expression of active ERK 1/2 in the unwounded (−) control monolayer (lanes 1 and 2). Wounding increased expression of active ERK 1/2 and masked the effect of the EF in enhancing the expression (lanes 5 and 6). U0126 (20 μM) completely inhibited the expression of active ERK 1/2 regardless of treatments (lanes 3, 4, 7, and 8). Each membrane was a representative of three to four repeated experiments.

Figure 5. Immunohistochemical staining for active ERK 1/2 in LEC monolayer wounds. Wounded peripheral LEC monolayers were exposed to an EF (200 mV/mm) for 15 minutes and then fixed and stained for active ERK 1/2. Staining intensity was quantified by confocal microscopy. Control wounds (no EF) showed no asymmetry of active ERK 1/2 staining and the mean asymmetry index was 0.01 ± 0.05 (n = 18). Wounds exposed to a physiological EF showed an asymmetric distribution of active ERK 1/2, with weaker fluorescence on the side facing the cathode. The mean asymmetry index was 0.25 ± 0.04 (n = 20), significantly higher than control (no exposure to an EF), P < 0.01. FI, relative fluorescence intensity along a line drawn across the corresponding wound.


