Effects of EGF and Indomethacin on Rabbit Corneal Endothelial Wound Closure in Excised Corneas

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Corneal endothelial cells of rabbit corneas stored in M-K medium at 37°C were wounded by touching them lightly with a micropipet under video specular microscope observation. Three groups were studied: control, with EGF, and with EGF + indomethacin. The wound closure process (initial wound area about 8500 μm²) was observed and recorded with time-lapse videography for 6 hr. The recorded video images were digitized and computer assisted morphometric analysis was performed. (1) Addition of either EGF (10 ng/ml) + indomethacin (1 μM), or EGF (10 ng/ml) alone to the M-K medium statistically significantly shortened the wound closure time as compared with the control group. (2) Both EGF + indomethacin and EGF alone resulted in a greater average percent relative change of the shape factor, more than three times greater with EGF + indomethacin and more than two times greater with EGF alone, than in the control group 150 min after wounding. (3) The maximum cell shape change occurred at about 150 min after wounding in the groups EGF + indomethacin and EGF alone, and at about 200 min in the control group. After this time in all three groups the cells began to approach a normal shape. (4) The cells near the wound boundary moved faster in the EGF + indomethacin and the EGF groups as compared with the control group. These results suggest that EGF and indomethacin may be of therapeutic value in promoting closure of traumatized human corneal endothelium.

The effects of epidermal growth factor (EGF) on corneal tissues have recently attracted considerable attention by ophthalmologists, because of the possibility that EGF may stimulate regeneration of injured or diseased tissue in vivo. EGF is an acidic, low-molecular-weight protein purified from mouse submaxillary gland which was initially identified because of its ectodermal effects on newborn mice to cause premature eyelid opening and incisor eruption. EGF is a 53-residue polypeptide of 6045 Daltons, with three cysteine–cysteine linkages. Three amino acids are completely absent from its structure: lysine, alanine and phenylalanine.

EGF has been shown to stimulate the proliferation of chick and rabbit corneal epithelium in organ culture and in vivo. EGF has also been found to be biologically active in human and primate tissue, stimulating corneal epithelial growth in culture. Several recent studies have demonstrated the ability of EGF to stimulate proliferation of cultured corneal endothelial cells. Although there are a few reports on the effects of EGF on intact corneal endothelial cells in organ culture, there have been no reports on the effects of EGF on either endothelial wound closure rate or the morphometric changes of cells near a wound boundary in excised corneas. Indomethacin, a cyclooxygenase inhibitor, causes marked elongation of cultured corneal endothelial cell shape and this change is greatly enhanced by EGF.

When a wound or discontinuity occurs in the endothelial cell layer, the cells bordering the defect become elongated and also become migratory. These events have been studied both in vivo and in vitro. The purpose of the present study was to investigate whether EGF or EGF + indomethacin could influence endothelial mobility and morphology in an intact cornea, as Neufeld et al have reported in cultured corneal endothelial cells. This article is the first to describe the effects of EGF and indomethacin on both endothelial wound closure rate and morphometric changes of cells near the wound boundary in corneal endothelium.

Materials and Methods

Corneal Preparation

New Zealand albino rabbits, weighing 2–3 kg, were anesthetized with an intramuscular injection of keta-
mine and xylazine and then were sacrificed with an overdose of intravenously administered phenobarbital sodium. The eyes were immediately enucleated. For isolated cornea measurements, corneoscleral buttons were made using standard eye bank procedures. These buttons were then placed endothelial side up in medium in a specular microscope examination chamber. Three media were prepared; M-K medium with EGF (10 ng/ml), M-K medium with EGF (10 ng/ml) and indomethacin (1 μM), and M-K medium alone. Five eyes from different animals were selected in each group; the fellow eyes of each rabbit were used by other investigators. Corneas were incubated in media for at least 1 hr prior to wounding. Commercial M-K medium (Aurora Biologicals, Aurora, IL), EGF (murine EGF from Collaborative Research, Bedford, MA) and indomethacin (Sigma Chemical Co., St. Louis, MO) were used. The animals were handled in accordance with the ARVO Resolution on the Use of Animals in Research.

**Wounding of the Endothelium**

The examination chamber was placed under an eye bank specular microscope (Bio-Optics LSM 2100C; Arlington, MA) for observation. During the observation period the chamber was placed on a warm water circulating plate through which a heated bath (WESLAB RTE-8; Portsmouth, NH) circulated warm water that maintained the medium at a constant 37°C. The touch method used to produce the mechanical wounds has been previously described. In brief, a 27-gauge stainless steel needle was bent using pliers so that the needle could be inserted at an appropriate angle along the side of the objective lens. A fine glass needle with a tip diameter of 150 μm was made by a specially designed micropipette puller and affixed onto the tip of the stainless steel needle. The glass tip was inserted into the chamber and positioned under the objective lens. Using a micromanipulator (Stoelting), the needle tip was carefully guided under the specular microscope. An endothelial wound was made with the tip of glass by a gentle and controlled touch to the endothelium. The wound closure process (initial wound area about 8500 μm²) was observed and continuously recorded with time-lapse videography for 6 hr.

**Analysis**

**Wound area:** To quantitate the rate of wound closure and changes in the shape of cells around the wound area, the recorded video images were digitized and computer-assisted morphometric analysis was performed with a Video Digitization Image Analysis System (Bio-Optics ECC-2000-2). Each wound area that gave a dark appearance in the specular image was digitized at 1, 5, 10, 20, 40, 60, 90, 120, 150, 180, 210, 240, 300, 330 and 360 min after wounding. Within each of the three groups, the mean wound area for each time point was calculated and used for comparisons.

**Shape of the cells near the wound boundary:** The shape of the first row of visible cells around each wound area was analyzed at 1, 50, 100, 150, 200 and 250 min after wounding. Seven to 17 cells surrounding the wound area were arbitrarily chosen and the apices of these cells were digitized. The computer calculated the area, shape factor and other morphologic parameters of each cell automatically. Each cell group was analyzed at 1 and 150 min after wounding to compare the rate of shape change in each of the three groups. The relative shape change was calculated as:

\[
\text{shape factor at 1 min} = \frac{\text{shape factor at 150 min} - \text{shape factor at 1 min}}{\text{shape factor at 1 min}} \times 100 (1)
\]

where \(4\pi A/P^2\) (\(P = \text{perimeter}, A = \text{area}\)) was used as the shape factor. A decreasing shape factor denotes increasing cell elongation.

**Cell migration near the wound boundary:** Under each condition in two corneas, the migration distance of the cells near the initial wound border was calculated. We initially identified a cell in the field that was located as far as possible from the wound as a reference cell; this cell was digitized as the origin of the x-y coordinate system. The apices of several cells near the wound boundary at 1 min and at 150 min after wounding were then digitized. The computer calculated the x-y coordinates of the center of gravity of each cell and measured the distance of cell migration between each cell at 1 min and 150 min after wounding by using the following equation:

\[
\text{cell migration distance} = \sqrt{(X_{(150)} - X_{(1)})^2 + (Y_{(150)} - Y_{(1)})^2} \quad (2)
\]

**Statistical analysis:** The student t-test for unpaired data was used to compare the values between groups for statistical significance.

**Results**

**Wound Area**

The specular microscopic cell image was clear and distinct for 240 min after wounding. After this time the specular microscopic image rapidly degraded and it became difficult to perform the cell analysis. The initial wound area for each of the 15 corneas studied
was approximately 8500 μm², roughly round in shape, corresponding to an area of about 35 cells (Fig. 1). In all cases, the wound area increased immediately after wounding, reaching a maximum value at 10 min after wounding for the EGF + indomethacin group and the EGF group and at 20 min after wounding for the control group (Fig. 2). The wound area decreased to 50% of the original size within 100 min in the EGF + indomethacin treated group, within 120 min in the EGF-treated group, whereas the wounded area of the control group decreased to 50% of the original size in about 240 min (Fig. 2). During the entire experiment there was no significant difference between the EGF + indomethacin and the EGF group in the wound closure time. However, after 90 min each of these groups showed a significant difference from the control group ($P < 0.005$). In the EGF group 80% of the wound was closed within 250 min. The initial wound areas were highly reproducible and had an average value of $8535 ± 190 \mu m^2$ (mean ± SEM).

To compare the wound closure rates between the various groups, the data from the peak of the area vs. time curve were plotted on a semilogarithmic scale and a linear regression of log (area) vs. time was performed for each of the three groups of data (Fig. 3). The closure rates were then taken as the slope of these linear regression curves. The closure rates for the treated corneas ($30.13 \mu m^2/min$) were significantly larger than that for the control corneas ($17.45 \mu m^2/min$) ($P < 0.01$), while there was no statistically significant difference between the two treated groups ($P = 0.4$).

**Shape of the Cells near the Wound Boundary**

We measured the cell shape and other morphometric parameters at 1, 50, 100 and 150 min after wounding. The relative average percent change of the shape factor increased with time in all groups (Fig. 4). At 150 min after wounding, both EGF + indomethacin and EGF alone resulted in a greater average percent change of the shape factor, more than three times greater in EGF + indomethacin and more than two times greater in EGF alone, than in the control group.
Fig. 3. Semilog plot of the change in endothelial wound area with time during the period when the wound area is decreasing. The equation of the linear regression lines shown are log(area) = -0.00180 t + 4.01 for the control group (C), log(area) = -0.00395 t + 4.08 for the EGF group (Δ) and log(area) = -0.00381 t + 4.03 for the EGF + indomethacin group (O).

One cornea of each group was followed longer than 150 min (Fig. 5). The peak of the shape change occurred at 150 min in the EGF + indomethacin group and in the EGF group and at 200 min in the control group. Following these times the rate of shape change decreased in all cases.

Mitotic figures were not found in any of the video images.

**Cell Migration near the Wound Boundary**

Cellular pattern and cell movement for each group at 150 min after wounding are shown in Figure 6. In each group, most of the cells migrated toward the wound center but, as seen, there were dramatic changes of shape for individual cells as they migrated. Furthermore, not all the cells migrated toward the wound center with the same speed and in the same direction. The average displacement of cells near the wound boundary 150 min after wounding is shown in Figure 7. The cells near the wound boundary in the EGF + indomethacin and the EGF groups migrated significantly more than those in the control group (P < 0.005). However, there was no significant difference between the EGF + indomethacin and the EGF groups.

**Discussion**

The possible beneficial effects of EGF on the wound healing process in the intact corneal endothelium have not been previously reported. Neufeld et al have shown that EGF and indomethacin promote mitosis and cell elongation in cultured rabbit corneal endothelial cells but it was not known if these effects also would be present in the intact corneal endothelium. The purpose of the present study was to investigate whether EGF or EGF + indomethacin could influence endothelial mobility and morphology in an intact cornea during the endothelial wound closure process.

EGF interacts with specific, high-affinity cell surface receptors that are present in a wide variety of cultured cells, including corneal cells, human fibroblasts, lens cells, human glial cells, human epidermoid carcinoma cells, 3T3 cells and granulosa cells. Fabricant et al have shown that the time course of association of radiolabeled EGF to cat corneal endothelium was complete after approximately 120 min at 22°C, and that there were 4000 receptors...
per cell. In the present study, a sclerocorneal button was maintained in medium containing EGF at 37°C from 1 hr before wounding until the completion of the experiments. Thus we can assume binding of EGF to the receptor and activation during this period.

Neufeld et al studied the effects of EGF and indomethacin on corneal endothelial cell shape.6,7 They theorized that endogenous synthesis of prostaglandin E2 (PGE2) is an important factor for the maintenance of the normal, polygonal endothelial cell shape and that if the synthesis of PGE2 is blocked, an extraordinary change in the cell shape occurs that is potenti ated by EGF. Thus, the cells become even more fibroblast-like in culture.8 They reported that EGF promotes mitosis and elongation in cultured rabbit corneal endothelial cells, and when cells are cultured in the presence of EGF (10 ng/ml) and indomethacin (1 μM), elongation was more pronounced with maximum axes of 60 μm, compared with 33 μm for untreated cells.6

For the first 20 min after producing the wound in the control corneas and 10 min for the EGF and EGF + indomethacin corneas the wound area increased in size. This phenomenon was also reported by Fukami et al,19 using a similar method, and is presumably due to an alteration of the surface structure of endothelial cells that neighbor those that were touched. The nature of this communication process is not presently understood.

In the present study, the average shape change was 3 times greater in the EGF + indomethacin group and 2 times greater in the EGF alone group than in the control group at 150 min after wounding. This demonstrates that the combination of EGF and indomethacin enhanced more marked elongation of corneal endothelial cell shape near the wound boundary. In addition, EGF-treated cells became more elongated than control cells. Thus, we have confirmed the findings of Neufeld et al in corneal endothelium in intact corneas.6,7

The presence of either EGF (10 ng/ml) + indomethacin (1 μM) or EGF (10 ng/ml) alone in the M-K medium significantly increased migration of cells near the wound boundary and shortened the wound closure time as compared with the control group. However, there was no significant difference between the EGF + indomethacin group and the EGF group in either wound closure time or the average rate of the migratory cells. Therefore, the effect on cell migration must primarily be due to EGF.

Matsuda et al30 demonstrated that in vivo the cells surrounding the wound border showed remarkable elongation toward the center of the wound between 3 and 12 hr after wounding. This elongation decreased considerably by 24 hr and progressively decreased with time. In our experiment, the peak of the cell shape change occurred between 150 to 200 min. Following these times the cell shape change progressively decreased and the cells approached their original shapes. The difference in the time to reach the maximum rate of change of cell shape between in vivo and excised cornea is probably due to the difference in the
size of the original wound produced. They made wounds between 0.7 and 2.0 \times 10^5 \mu m^2 in area by scraping the endothelium with a length of 4-0 nylon monofilament. In our study, much smaller wounds, about 8500 \mu m^2 in area, were made reproducibly by a controlled touch method.

In most cell culture systems, increased DNA synthesis begins approximately 15 hr after the addition of EGF and reaches a maximum at about 22 hr.\textsuperscript{31} In our study, endothelial wounds treated with EGF were closed to 80% of the original size within 250 min after wounding. Therefore, it is unlikely that mitosis has contributed to the wound closure process in our study. The present study demonstrates the closure process with elongation and migration of cells, without cell division.

Our results demonstrate that EGF and indomethacin have beneficial effects on the intact rabbit corneal endothelium. EGF enhances the migration of endothelial cells near a wound boundary and shortens the wound closure time while indomethacin in combination with EGF enhances cell elongation without shortening the wound closure time. These results suggest that epidermal growth factor and indomethacin may be of therapeutic value in promoting healing of traumatized human corneal endothelium.

Key words: corneal endothelium, EGF, indomethacin, wound closure, wound healing

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