Effects of Human Epidermal Growth Factor on Endothelial Wound Healing of Human Corneas

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Paired human donor corneas (age, 73 ± 12 yr), preserved in organ culture medium, were used to evaluate the effect of human epidermal growth factor (hEGF) on endothelial wound closure rate (WCR), on morphometric parameters (cell size, shape, and density), and on cell division in the wound area. The endothelium of the corneas was mechanically wounded (area, 4.9 ± 0.9 mm²). For each pair, one cornea was treated with 10 ng/ml hEGF, while the mate served as control. WCR was assessed by daily staining of the corneas with trypan blue. Morphometric data were obtained after alizarin staining. Mitotic activity was assessed using 3H-thymidine autoradiography. Addition of hEGF significantly increased the WCR compared to the control group. In the closed wound (between 4-9 d), the mean cell size in the center averaged 1940 μm² in the control group and 1287 μm² in the hEGF-treated group (P < 0.01). Fifteen days after wounding, the mean cell sizes averaged 1910 μm² and 1427 μm² in the control and hEGF-treated group, respectively (P < 0.01). All corneas exposed to hEGF had higher endothelial cell densities than the control corneas. In the early stages of wound closure, the cells in the transitional zone in hEGF-treated corneas had a somewhat more elongated shape. However, hEGF did not affect the final cell shape within the closed wound. Autoradiographic results revealed that hEGF accelerated DNA-synthesis, although only to a limited extent. The results indicate that, in human corneas, hEGF promotes endothelial wound healing predominantly by cell migration, at least in corneas from senior donors. Invest Ophthalmol Vis Sci 33:1946-1957, 1992

The corneal endothelium is crucial for maintaining the transparency of the cornea. Clinically significant dysfunctions of the endothelium—resulting from cell loss associated with dystrophies or degenerations caused by ocular diseases, drugs, traumas, or surgery—can compromise the integrity of the monolayer, leading to endothelial decompensation, loss of transparency, and finally the need for corneal transplantation. Counteracting endothelial cell loss and dysfunction by growth factors may produce a clinically beneficial effect, but these possibilities are only beginning to be explored.1 The effects of epidermal growth factor (EGF) on corneal tissues have recently attracted considerable attention by ophthalmologists, because of the possibility that EGF also may stimulate the regeneration of the injured or diseased corneal endothelium in vivo.

EGF is a 53-amino acid monomeric polypeptide with a molecular weight of 6045 Daltons that was first isolated from the mouse submaxillary gland.2 Upon binding to the cell membrane receptor, it stimulates proliferation and differentiation of epidermal and many other cell types. Therefore, it seems to be involved in mammalian growth and development.4 The human EGF (hEGF) has been identified in various human tissues and fluids,4 including tear fluid5-7 and aqueous humor.8 The receptor for EGF is widely distributed,4,9 and among the cell types carrying the EGF receptor on their plasma membranes are the endothelial cells lining the inner aspect of the cornea.10 Several studies have demonstrated the ability of EGF to stimulate proliferation and elongation of cultured rabbit or bovine corneal endothelial cells.11-15

Human tissue and organ culture tests are ultimately required to analyze the effects of growth factors on endothelial cell growth. The purpose of the present study was to investigate whether hEGF influences the endothelial wound closure rate (WCR), morphometric parameters of endothelial cells (cell area, density, and shape), and cell division in the wound area of human corneas preserved in organ-culture medium.

Materials and Methods

Paired human corneas donated to the Eurotransplant Foundation (Leiden, The Netherlands) were...
used. They had been intended for transplantation but were discarded by the Cornea Bank (Amsterdam, The Netherlands) because of macula (scar), stromal opacities, low endothelial cell density (<2000 cells/mm²), or irregular endothelium (polymegathism, pleomorphism). In a pilot study, the interindividual variation in wound closure and morphometric aspects were high, so paired corneas were used. Seventy two pairs of corneas were used. Donor ages averaged 73 ± 12 yr (range 24–93 yr). The interval between death and enucleation varied from 0.5–11.25 hr (mean 6.4 ± 3.4), and the interval between enucleation and corneal dissection varied from 5.75–25.25 hr (15.5 ± 5.4 hr). The onset of the experiments was always within 24 hr after preparation of the cornea for the morphometric study and within 3 d for the autoradiographic study.

Thirty-five pairs were used to establish the effect of hEGF on corneal wound closure rate. They also were used to evaluate the effect of hEGF on cell area, density, and shape in the wound center at two times—the time of wound closure (24 pairs) and 15 d after infliction of the wound (11 pairs). In seven pairs, the morphometric parameters were determined 3 d after wounding, ie, before wound closure was completed. Autoradiography was performed on 30 paired corneas. The culture media, with or without growth factor, were replaced every day except for the experiment with 11 pairs of corneas, in which the medium was replaced every other day.

Wounding Procedure

In all corneas, a central endothelial lesion was made as described by Treffers.16 The cornea was placed on a concave block of sterile teflon with the endothelial side up. A metal rod with a rounded, smoothly polished tip was placed perpendicularly on the center of the cornea. The rod was rotated a few turns, creating a circular wound. With this method, the endothelium and none of the other corneal layers, including Descemet’s membrane (DM), were injured. Upon scanning electron microscopic inspection, an intact, uncovered DM in the wound area, a sharp wound margin, and a normal endothelial cell pattern outside the wound were clearly visible. The sizes of the wounds between pairs of corneas varied considerably from 3.3–6.9 mm² (mean 4.9 ± 0.9 mm²), probably because of a variation in curvature of the corneas. However, within each pair of corneas, the initial wound sizes hardly varied. The difference in initial wound size between the corneas of each pair was determined and related to the largest wound size of each pair. The variation in the initial wound sizes within pairs averaged 2.2%. The larger wound sizes were randomly distributed among the untreated and treated groups.

Following mechanical injury, the corneas were placed in 25 ml of sterile Eagle’s minimum essential medium (EMEM) supplemented with 5% Dextran T500 (Pharmacia, Uppsala, Sweden), 2% fetal bovine serum, and antibiotics at 31°C.17 Ten nanograms per milliliter recombinant hEGF was added to the culture medium of one cornea of each pair, while the mate, incubated in the absence of hEGF, served as control. Dose-response data indicated that approximately 10 ng/ml EGF maximally stimulates mitotic rate in rabbit13 and bovine endothelial cell cultures (authors’ observations). Recombinant hEGF was obtained commercially (Biomedical Technologies Inc., Stoughton, MA).

Longitudinal Study of Wound Closure Rate

At 24 hr intervals, the endothelial sides of the wounded corneas were stained with 0.3% trypan blue in phosphate buffered saline (PBS) for 1 min and rinsed with PBS. The cornea was placed endothelial side upward on a drop of PBS in a sterile Petri dish. The corneas were examined in bright field mode using a 3.2× long-distance objective and a 10× eyepiece. The wound margin was outlined directly with a drawing-tube (final magnification 36.2×). The wound area was measured using a MOP-VideoPlan Image Analysis System software package developed by Kontron Bildanalyse (Munich, Germany). The wound closure time was the day at which Descemet’s membrane no longer stained with trypan blue and was covered by endothelial cells. The possibility that wound closure was delayed by the daily handling of corneas, including staining with trypan blue, cannot be disregarded. However, untreated and treated corneas were handled in the same manner.

Morphometric Analysis

After the wounds had closed, the cell borders and nuclei of the endothelium of paired corneas were stained with alizarin red18 and trypan blue,19,20 respectively (Figs. 1a, b). Corneal flat mounts were obtained by placing four radial cuts to within 2–4 mm of the center of the stained cornea and placing the wet specimen, endothelial side upward, under a coverslide. A 30 g weight was placed on either side of the cover slide to obtain a flat endothelial preparation. In each cornea, the endothelial cells were photographed on two different spots. The negatives were printed to a final magnification of about 345×. The exact final magnification for each set of micrographs was calibrated using an object micrometer.

The procedure of counting in fixed frames described by Sperling and Gundersen21 was applied. From each cornea, the photographed cells were outlined in two randomly selected fields using a pen-sty-
Fig. 1. (A) Survey photograph of a flat preparation of a human cornea after the wound had closed. Alizarin/trypan blue staining. T = transitional zone. (B) Endothelial cells in the closed wound center. Alizarin/trypan blue staining.

lens. Each field encompassed approximately 50–55 cells. The counting frames were adjusted to yield approximately the same final cell counts per cornea (100–110 cells/cornea). The Videoplan software package was used to measure and calculate cell area, perimeter, maximal diameter, shape factor, and coefficients of variation.

The endothelial cell density (ECD), expressed as the number of cells per mm², was calculated from the mean cell area (μm²). The coefficient of variation of area (CV_area) was defined as the ratio of the standard deviation of area to its mean values and was expressed in percentage. The CV_area is a dimensionless index, independent of cell size and, therefore, provides an unbiased estimate of variation in cell area. A high CV_area will occur when cells vary greatly in size, indicating polymegethism. Cell shapes can be described by the shape factor PM/Dmax, where PM is the...
perimeter (in μm) and Dmax is the maximum diameter (in μm). This shape factor equals π (3.14) for a perfect circle and 3.0 for a perfect hexagon. A shape factor below 3.0 indicates an elongated cell shape.

Between the central wound area and the undamaged peripheral cells, a transitional zone 5-10 cells wide exists, in which a gradual change in cell shape was observed (Fig. 1a). In 24 paired corneas, the radius from the wound center to the inside edge of the transitional zone (C-Ti) as well as the width (distance) of the transitional zone itself (T) was measured from a survey photograph taken at the time of wound closure to determine differences in wound closure patterns that indicate migration characteristics (Fig. 2). Four sets of measurements were made per cornea. The mean of the distances was calculated for each cornea. The radius from wound center to the outside edge of the transitional zone (C-To) was calculated according to the formula (C-Ti) + T (Fig. 2).

Autoradiography

Thirty pairs of corneas were cultured for 6-7 d in medium with or without hEGF and supplemented with 3H-thymidine (specific activity 15 Ci/mmol, 1 μCi/ml medium; New England Nuclear Research, Boston, MA) at the onset of the experiment (continuous labeling). After this incubation period, the corneas were stained with alizarin red,18 fixed between two glass slides in formaldehyde 3.8% for 6 hr, dried for 12 hr, and mounted on gelatinized slides. The preparations were covered with Ilford (Ilford, England) K-2 nuclear emulsion, exposed for 7 d at 4-5°C, and developed at 20°C with Kodak (Rochester, NY) D-19F for 4 min. After photographic fixation, they were rinsed in tap water for 3-5 min, dehydrated in ethanol, cleared in carboxy-xylene, and mounted in Entellan (Merck, Darmstadt, Germany). The number of labeled nuclei in the wound center was counted for each specimen. The percent change in labeling between both groups was only calculated for corneas in which more than 10 labeled nuclei were observed.

Statistical Analysis

Unless stated otherwise, Wilcoxon’s signed-rank test (two-tailed) for paired data was used to compare the values between groups for statistical significance. Data given in the text are mean ± SD. For correlations, we used Spearman’s rank correlation coefficient (r, two-tailed).

Results

Wound Closure Rate

To evaluate the effect of hEGF treatment on WCR, the wound area was plotted versus time for each pair of corneas. Medium replacement, every day (24 pairs) or every other day (11 pairs), did not influence the course of wound closure and wound closure time. Figure 3, therefore, combines WCR data of both groups (35 pairs) and shows there is a large biological variation in the closing of the wounds among pairs of corneas as indicated by the considerable SD. Wound closure occurred between 4 and 9 d. When hEGF was present, mean estimated wound closure time was 5.4 ± 0.8 d, compared to 6.5 ± 1.4 d in untreated controls (P<0.01).

On all days investigated, the mean wound area was significantly lower in the hEGF-treated corneas than in the control corneas (P < 0.01). For each pair, we also determined the times at which 50% and 10% of the initial wound area was left (Fig. 3). Fifty and 10% of the initial wound area was reached earlier in the hEGF-treated corneas than in the control corneas (P < 0.01). The 50% level was reached after an average of 2.3 ± 0.4 d in the hEGF-treated group and after 2.7 ± 0.6 d in the control group. For the 10% level, values of 4.2 ± 0.7 d in the hEGF-treated group and 5.1 ± 1.0 d in the control group were found. The difference in time between both groups is larger at the 10% level than at the 50% level, 0.9 and 0.4 on average, respectively (P < 0.01; Fig. 3).

Morphometry

Morphometric analysis was performed 3 d after wounding, at the time of wound closure, and 15 d after wounding. The morphometric data are listed in Table 1.

Three days after wounding (before wound closure): The uncovered area was greatly reduced. The major-
Fig. 3. Wound closure rates of 35 paired human corneas. The mean wound area, expressed as percentage of the initial wound area, is plotted versus time for all control and hEGF-treated corneas. Mean initial wound area 4.9 ± 0.9 mm². Each point represents mean ± SD; \( P < 0.01 \) (Wilcoxon's signed-rank test).

Table 1. Morphometric analysis of human corneal endothelial cells after wounding: values are mean ± SD (range)

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>hEGF-Treated Group</th>
<th>P-value*</th>
</tr>
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<tbody>
<tr>
<td>3 days after wounding (7 pairs)</td>
<td></td>
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<tr>
<td><strong>Transitional zone</strong></td>
<td></td>
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<tr>
<td>ECD (cells/mm²)</td>
<td>973 ± 221 (719–1330)</td>
<td>1158 ± 175 (921–1422)</td>
<td>( P &lt; 0.05 )</td>
</tr>
<tr>
<td>Shape factor (PM/Dmax)</td>
<td>2.62 ± 0.03 (2.59–2.67)</td>
<td>2.52 ± 0.05 (2.42–2.56)</td>
<td>( P &lt; 0.05 )</td>
</tr>
<tr>
<td><strong>Leading edge of wound closure</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECD (cells/mm²)</td>
<td>519 ± 141 (402–752)</td>
<td>557 ± 152 (438–858)</td>
<td>( P &lt; 0.05 )</td>
</tr>
<tr>
<td>Shape factor (PM/Dmax)</td>
<td>2.67 ± 0.03 (2.62–2.72)</td>
<td>2.66 ± 0.03 (2.63–2.70)</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>At the time of wound closure</strong> (24 pairs)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECD (cells/mm²)</td>
<td>537 ± 109 (327–768)</td>
<td>794 ± 117 (580–1030)</td>
<td>( P &lt; 0.01 )</td>
</tr>
<tr>
<td>Shape factor (PM/Dmax)</td>
<td>2.61 ± 0.04 (2.52–2.70)</td>
<td>2.61 ± 0.04 (2.52–2.69)</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>15 days after wounding</strong> (11 pairs), media replaced every other day</td>
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<tr>
<td>ECD (cells/mm²)</td>
<td>539 ± 99 (410–714)</td>
<td>717 ± 120 (565–930)</td>
<td>( P &lt; 0.01 )</td>
</tr>
<tr>
<td>Shape factor (PM/Dmax)</td>
<td>2.67 ± 0.04 (2.61–2.73)</td>
<td>2.69 ± 0.03 (2.62–2.72)</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

* Wilcoxon’s signed-rank test (n.s. = not significant).
mean size of the endothelial cells was considerably smaller in all 24 corneas exposed to hEGF (1287 ± 201 µm²) compared to the control corneas (1940 ± 416 µm²; P < 0.01). The corresponding ECDs for the hEGF-treated group were greater than those for the control group (P < 0.01; Table 1). The increase in ECD in the treated corneas of each pair averaged 257 ± 119 cells/mm² (range 62–570) or, given as percentage of the control corneas, 52 ± 34% (range 9–175%). The coefficient of variation of mean cell area (CV_area) amounted to 45 ± 6% on average in the control group and 48 ± 9% on average in the treated group. There was no significant difference in this parameter between both groups. The mean shape factor of the cells in the wound center averaged 2.61 ± 0.04 in both groups.

In contrast to the control group, the mean ECD in the hEGF group at the time of wound closure was significantly higher than the ECD in the leading edge, studied at 3 d after wounding (Wilcoxon’s two-sample test P < 0.01). The radius from the wound center to the inside edge of the transitional zone (C-T₁), the width of the transitional zone itself (T), and the radius of the wound center to the outside edge of the transitional zone (C-T₀) did not significantly differ between the hEGF-treated and nontreated group (Table 2). Distance T averaged 0.34 ± 0.07 and 0.36 ± 0.08 mm in the control and hEGF group, respectively.

Fifteen days after wounding: Fifteen days after wounding, corresponding to about 7–10 d after wound closure, all 11 corneas cultured in the presence of hEGF had smaller mean cell areas and higher

Table 2. Wound closure pattern

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>hEGF-Treated group</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-T₁ (mm)</td>
<td>1.05 ± 0.15 (0.83–1.45)</td>
<td>1.01 ± 0.14 (0.72–1.29)</td>
</tr>
<tr>
<td>T (mm)</td>
<td>0.34 ± 0.07 (0.24–0.57)</td>
<td>0.36 ± 0.08 (0.26–0.58)</td>
</tr>
<tr>
<td>C-T₀ (mm)</td>
<td>1.39 ± 0.18 (1.10–1.79)</td>
<td>1.37 ± 0.19 (1.00–1.76)</td>
</tr>
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</table>

The radius from the wound center to the inside edge of the transitional zone (C-T₁), the distance of the transitional zone itself (T), and the radius from the wound center to the outside edge of the transitional zone (C-T₀) of the untreated and hEGF-treated group (n = 24 pairs) (see Fig. 2).
ECDs in the wound center than their mates cultured in the absence of hEGF (Table 1). The mean cell area was 1910 ± 326 μm² in the control group and 1427 ± 223 μm² in the hEGF-treated group. The differences between both groups were statistically significant (P < 0.01). The average increase in ECD in the treated group amounted to 179 ± 54 cells/mm² (range 106–273) or 34 ± 11% (range 18–53%). Treatment with hEGF did not affect the final shape of cells within the closed wound as indicated by the identical shape factor (Table 1). The values for CVarea were statistically equal (49 ± 9%, controls; 48 ± 9%, hEGF).

The mean ECDs in the center of the wound in corneas examined at the time of wound closure were not significantly different from the ECDs examined 15 d after wounding (Wilcoxon’s two-sample test). However, the transitional area in the former group—formed by cells at the original wound edges—was better delineated compared to the latter group.

**Morphology**

In the center of the wound, extremely large and also small cells were seen. Giant cells of 2000–10,000 μm², ie, 7–30 times the average size of cells of nondamaged endothelium, emerged in all specimens of the treated and nontreated group. At the time of wound closure and 15 d post-wounding, the mean number of multinuclear cells in the wound center proper was approximately 4–5% in both groups. The majority of all multinuclear cells contained two nuclei. These nuclei often were approximately identical in size (Fig. 5a). Some cells contained a practically normal-sized nucleus and one smaller nucleus (Fig. 5b). In some of these cells, a constriction between the two unequally distributed nuclear materials was still visible (amitosis). The variation in the nuclear size of the transformed cells in the wound area was greater than that in the peripheral cells of the cornea.

**Autoradiography**

The wounds of all but two (untreated) corneas appeared to be closed. Labeling of the endothelial cells with 3H-thymidine was observed in all 30 paired corneas (Fig. 6). However, the variation in thymidine incorporation between pairs of corneas was dramatic, varying from a few to hundreds of nuclei. Therefore, the autoradiographic technique was checked carefully. Corneas treated at the same moment, using the same solutions and handled simultaneously for autoradiography, already showed this variation. In addition, we found that in corneas with few labeled cells, the nuclei were heavily labeled, indicating a proper thymidine incorporation. This means that the variation in labeling is most likely not a result of technical artifacts but is inherent to the biological variation among pairs of corneas. The density of photographic

![Fig. 5. (A) Multinuclear cells in the center of the wound. Alizarin/trypan blue staining. (B) Images of amitotic nuclear divisions in wounded human corneal endothelium (partial amitosis or nuclear budding) (arrows). Alizarin/trypan blue staining.](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933168/)
Fig. 6. Autoradiography of human cornea 6–7 days after wounding. Tritiated thymidine was added to the medium at the start of the experiment (continuous labeling). Labeled nuclei were located in the wound area. The small scattered silver grains represent the background exposure. Arrows indicate labeled nuclei with an amitotic division. Alizarin staining of cell borders.

Tritiated thymidine incorporation also was observed in nuclei showing partial amitosis (Fig. 6).

Discussion

The main finding of the present study is that the mean ECD in the wound center is higher in the hEGF-treated corneas than in the control corneas studied at the time of wound closure and 15 days after wounding. Thus, the effect of hEGF on ECD was still present 8–10 d after wound closure. Pretreatment with EGF prior to wounding of organ-cultured human corneas did not appear to influence the cell areas in the wound center. In animal models, the effects of EGF on ECD were rather conflicting. Injection of EGF (5 μg) into the anterior chamber of four rhesus monkey eyes immediately after the denudation of the central corneal endothelium at autograft transplantation, and again 3 wk later, showed 67% greater repopulation with endothelial cells at 10 wk than did the nonpaired control eyes of four other monkeys. The ECD in wounded cat corneal endothelium increased...
A significant inverse correlation between the number of labeled cells in the closed wounds and donor age was detected. This suggests that there is more DNA synthesis in younger donors. The increase in labeling in the hEGF group was positively correlated with the number of labeled cells in the control corneas. Thus, hEGF has a more stimulating effect on corneas that have a potency to divide. In addition, the increase in labeling in the hEGF group was inversely correlated with the age ranges studied (51–91 yr). Therefore, the effect of hEGF on cell division may play a more important role in the younger age groups.

Independent of hEGF’s presence and in agreement with previous observations, incorporation of thymidine occurred in all of our donor corneas during endothelial wound healing. However, in the large majority of corneas, the number of labeled cells was too small to account for the coverage of the damaged area by cell multiplication. Therefore, wound healing is originating mainly from enlargement and migration of the remaining endothelial cells that cover denuded surfaces of Descemet’s membrane.

In the present human model of endothelial wound healing, addition of hEGF to the culture medium sig-
nificantly shortened the wound closure time compared to the control group. Because the wound area in the hEGF group already was significantly smaller from day 1 onward, this acceleration of wound closure starts directly after wounding. However, only a few studies investigated the wound closure time in animal models, with rather conflicting results. Joyce
reported on an in vitro model of rabbit corneal endothelial wound closure in which the amitotic state of the adult corneal endothelium was mimicked with the mitotic inhibitor 5-fluorouracil. Treatment with EGF or indomethacin at the time of wounding had little effect on wound closure rates in mitotically competent and inhibited cell cultures. Yoshida reported the effects of EGF and indomethacin on rabbit corneal wound closure rate in excised corneas after minor trauma and demonstrated that the presence of EGF + indomethacin, or EGF alone, significantly increased migration of cells near the wound boundary and shortened the wound closure time compared to the control group. EGF did not influence the wound closure time of damaged cat corneal endothelium in vivo.46

Because cell division was marginally stimulated by hEGF, cell migration induced by hEGF must play the most important role in the higher ECD of the hEGF-treated group. The migrating cells seem to move as one group into the wound area. This is sometimes called “spreading.” A few cells move as individuals into the wound area. The migration process may include a faster migration as well as an increased number of migrating cells. The shorter wound closure time in the treated group strongly favors a faster migration rate of cells near the wound boundary. The following arguments favor an increased number of migrating cells as an additional effect of hEGF. The mean ECD in the transitional zone and in the leading edge were higher in the hEGF-treated corneas than in the control corneas 3 d after wounding. Also, the mean ECD in the closed wound center in the hEGF group was significantly higher than the ECD in the leading edge, studied 3 d after wounding. This was not the case in the control group. Finally, a higher ECD in the treated corneas still was present 8–10 d after wounding. If migration was only faster, a smaller difference or no difference in ECD would be expected between untreated and treated groups 8–10 d after coverage of the wound. Contrary to expectations, the width of the transitional zone was not greater in the hEGF-treated group than in the control group.

In agreement with our study, Yoshida reported that rabbit endothelial cells near the wound boundary moved faster in the EGF + indomethacin and EGF groups than in the control groups. Quantitative determination of the number of migrating cells in Giemsa-stained wounds and phase-contrast video microscopy of healing wounds of rabbit corneal endothelial cells indicated that the number of individual cells migrating from the leading edge was increased by exposure of cells to EGF. Exposure to indomethacin consistently suppressed this response. This increased migratory response did not appear to induce faster wound closure.

The experiments were performed with an organ culture medium that contained 2% fetal bovine serum (FBS). That the serum contained factors that enhanced the wound healing or the hEGF effect cannot be ruled out. However, control and hEGF-treated corneas had 2% FBS in the culture medium, and the differences in wound closure rate and ECD is a result of the (additional) effect of hEGF.

In the present study, hEGF did not affect the final endothelial cell shape within the closed wounds. However, it is possible that cell elongation as well as cell migration and division cease abruptly after initial coverage of the wound because of contact inhibition. Therefore, the cell shape was measured in the transitional zone and leading edge 3 d after wounding, ie, before wound closure occurred. In the transitional zone, more elongated cells were present in the hEGF group than in the control group, indicated by a lower shape factor. However, the differences between both groups were not striking. Noteworthy is that unlike the cells in the transitional zone, the cells in the leading edge showed minimal cell elongation despite the presence of hEGF.

EGF-induced cell elongation was more obvious in tissue cultures of animal endothelial cells. Cultured rabbit endothelial cells grown to confluency in the presence of EGF had elongated rather than normal polygonal shapes. The shape changes induced by EGF treatment resembled those observed during cell migration. By using specific modulators of intracellular messenger systems, two interdependent intracellular transduction pathways controlling the cell shape, mitotic activity, and presumably the state of differentiation in corneal endothelial cells have been identified—the protein kinases A- and C-dependent pathways. Available evidence indicates that the shape change induced by EGF is mediated via the phosphoinositol pathway and protein kinase-C activation.

Multinuclear cells represented about 5% of the cells in the wound center (Fig 5a). These multinuclear cells can be the result of incomplete mitosis, mitotic division, or cell coalescence (fusion). In some specimens, a few cells showed an unequal distribution of nuclear material, with or without thymidine labeling (Figs. 5b, 6). This suggests that partial amitosis or nuclear budding occurs in wounded human en-
endothelium. Amitotic nuclear division can be accompanied by DNA synthesis (Fig. 6). It is questionable whether DNA synthesis always implies nuclear or cellular division.

The results of this study demonstrate that hEGF promotes endothelial wound healing in corneas from senior donors, predominantly by cell migration, i.e., a faster migration rate and an increased number of migrating cells. Human EGF-induced cell division plays only a minor role in the healing of human corneal endothelium of senior donors. These results suggest that hEGF may be of therapeutic value in promoting wound healing in traumatized human corneal endothelium. While tissue and organ culture tests are necessary first steps in analyzing factors that influence cell growth, in vivo trials, essential for establishing efficacy in living systems, need to be done.

Key words: corneal endothelium, human epidermal growth factor, wound healing, organ culture, autoradiography, morphometry

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