Enhanced Healing of Cat Corneal Endothelial Wounds by Epidermal Growth Factor

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Purpose. The authors investigated whether healing of cat corneal endothelial wounds could be enhanced in vivo by human epidermal growth factor (EGF).

Methods. EGF was administered in sodium hyaluronate to the anterior chamber of cats after an endothelial touch injury. Control contralateral eyes received sodium hyaluronate alone. At selected times after injury, the corneas were evaluated for thickness, the rate of endothelial wound closure, the endothelial cell density, any variation in cell size, the percentage of hexagonal cells, and endothelial cell mitosis.

Results. Two days after injury, endothelial wounds of eyes treated with EGF had healed an average of 65 ± 4% of the initial 38.5 mm² wound area; paired control eyes had healed an average of 59 ± 4% (P < 0.05). Both EGF-treated and control wounds had resurfaced over 90% of the initial wound area on day 4 after injury, and the wounds were completely resurfaced by 7 and 14 days after injury in both treatment groups. On days 4 and 7 after injury, the EGF-treated corneas were 5% and 8% thicker (835 versus 796 μm and 786 versus 728 μm, respectively) than the paired control corneas (P < 0.05). On days 10 and 14 after injury, both EGF-treated and control corneas were 19% and 12% thicker, respectively, than prewound the corneal thickness (621 μm). Seven days after injury, the corneas treated with EGF had an average of 76 ± 28% more (P < 0.05) endothelial cell nuclei labeled with tritiated thymidine compared with that of the paired control eyes (2472 versus 1543 labeled nuclei). Fourteen days after injury, the central endothelial cell density of EGF-treated corneas was an average of 38 ± 11% higher than that of the paired control eyes (P < 0.01). The percentage of hexagonal cells in the wound area was an average of 14 ± 4% higher (P < 0.01) than that of the paired control eyes (82% versus 69%), and the coefficient of variation of the cell size for EGF-treated corneas was an average of 31% (P < 0.05) smaller than that of the paired control corneas (0.21 versus 0.29 [standard deviation]/mean cell size).

Conclusions. A single intraocular application of EGF formulated in sodium hyaluronate after an endothelial cell injury significantly enhanced multiple parameters that are closely related to improved endothelial cell regeneration. Invest Ophthalmol Vis Sci. 1993;34:2305-2312.
Corneal endothelial cells vectorially pump ions from the stroma, thereby maintaining proper corneal hydration. Injuries to the endothelium can result in corneal edema and clouding if healing of the endothelial injury is insufficient. Extensive injuries to the endothelium of some animals, such as rabbits, heal well because of the ability of their endothelial cells to undergo mitosis extensively after an injury. By contrast, injuries to the corneal endothelium of adult humans, primates, and carnivores heal predominantly by migration and enlargement of endothelial cells rather than by mitosis, which limits the ability of the endothelium of these species to compensate adequately after large injuries or disease.

Various investigators have reported that peptide growth factors, including epidermal growth factor (EGF), enhance corneal endothelial cell proliferation in vitro. Several wound models have been used to investigate the endogenous response of corneal endothelium to wounding and to evaluate the effects of growth factors on endothelial wound healing. These include transcorneal freezing, mechanical injury, ultrasonic injury, and cytotoxic agents. Most of these methods inflict secondary damage to the stroma and Descemet's membrane; this can complicate studies designed to evaluate the effects of these agents on endothelial cell regeneration. The objective of the current study was to evaluate the effect of a single application of EGF in sodium hyaluronate on the healing of cat corneal endothelial wounds created by touching the endothelium with a plastic-tipped cannula.

MATERIALS AND METHODS

Endothelial Cell Injury Procedure

All investigations were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The endothelial cell injury procedure was performed on both eyes of a total of 44 adult random-bred cats of either sex weighing 2.4-4.5 kg. Thirty-six cats were randomly assigned to one of three groups consisting of 12 cats each. One eye of each cat received 500 μl of one of three concentrations of recombinant human EGF (Chiron, Emeryville, CA), which was administered to the anterior chamber through the stab incision. The concentrations of the EGF solutions were 10 μg/ml, 30 μg/ml, or 100 μg/ml, and they were dissolved in a phosphate-buffered sodium hyaluronate solution (Amvisc Plus, IOLAB, Claremont, CA). The contralateral eye of each cat received 500 μl of sodium hyaluronate alone and served as the paired control treatment eye.

Measurement of Corneal Thickness

One day before surgery and on days 4, 7, 10, and 14 after surgery, the corneal thickness of each eye was measured with an ultrasonic pachymeter. The thickness of each cornea was determined by averaging three measurements taken at the central region of the cornea. The difference in average corneal thickness for each pair of corneas was calculated, and the treatment groups were compared for statistical significance by a paired t-test. The average corneal thicknesses for the three EGF treatment groups were compared for differences by an analysis of variance (ANOVA) test.

Measurement of Endothelial Wound Closure

At 2, 4, 7, and 14 days after wounding, three cats from each of the three EGF treatment groups were killed. Their corneas were excised and placed in fixative (1% paraformaldehyde, 1% glutaraldehyde, 0.5% acrolein, and 4% sucrose in 0.1 mol/l sodium cacodylate buffer) overnight. Regions of Descemet's membrane that were not covered with a layer of endothelial cells were revealed by the staining corneas for 3 min with 2% osmium tetroxide; this stained the endothelial cells black but left bare Descemet's membrane unstained. The wound area of each cornea was photographed, and the area that lacked endothelial cells was measured by planimetry of enlarged photographs. These areas were expressed as a percent of the original wound area (38.5 mm²).

Beaver blade (Beaver, Inc., Waltham, MA) in clear cornea adjacent to the limbus through which a 25-gauge cannula with a rounded plastic tip was introduced into the anterior chamber. Infusion of balanced salt solution maintained the anterior chamber during the procedure. The central corneal endothelium within the trephination mark was removed by gently wiping the endothelium with the cannula tip using a spiral motion. Removal of the endothelium was confirmed by direct observation through the operating microscope. Both cannulas were removed, and 500 μl of sodium hyaluronate with or without EGF was injected into the anterior chamber.

After surgery, 36 cats were randomly assigned to one of three groups consisting of 12 cats each. One eye of each cat received 500 μl of one of three concentrations of recombinant human EGF (Chiron, Emeryville, CA), which was administered to the anterior chamber through the stab incision. The concentrations of the EGF solutions were 10 μg/ml, 30 μg/ml, or 100 μg/ml, and they were dissolved in a phosphate-buffered sodium hyaluronate solution (Amvisc Plus, IOLAB, Claremont, CA). The contralateral eye of each cat received 500 μl of sodium hyaluronate alone and served as the paired control treatment eye.

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EGF Enhancement of Endothelial Wound Healing

Measurement of Endothelial Cell Morphometry

After the wound closure measurements described were completed, the corneas from the nine cats that were killed 14 days after injury were further analyzed for their endothelial cell density, variation in cell size, and the percentage of hexagonal cells. Descemet's membrane with the endothelial attached was stripped off and flat mounted onto cover slips. Phase-contrast photomicrographs were taken of the central region of the endothelium wound, and three separate fields were digitized using a computerized image analysis system (BioOptics, Arlington, MA). The average endothelial cell size, coefficient of variation of cell size, and percentage of hexagonal cells were determined from an average of 90 cells per analysis field. The average endothelial cell density (in cells per millimeter squared) was calculated from the average endothelial cell size (1,000,000 cells/average cell area).

Pachymetry, wound area, and morphologic data measurements were analyzed for statistically significant differences between paired corneas using a two-tailed, paired t-test. In addition, the three EGF treatment groups and the three control groups were each compared for significant differences using ANOVA. If no statistically significant difference was found between the three EGF treatment groups or between the three control groups, the values were combined into one EGF treatment group and one control group, and the values were compared using a two-tailed, paired t-test.

Measurement of Corneal Endothelial Cell Mitosis

Eight cats had endothelial wounds created in both eyes as described. Then they were randomly assigned to one of two groups consisting of four cats each. One eye of each cat received 500 μl of EGF at a concentration of either 10 μg/ml or 30 μg/ml in sodium hyaluronate. In addition, 50 μl of sterile water containing 50 μCi of tritiated thymidine were made 24 and 48 hr after surgery. The fellow eye of each cat received a 500-μl injection of sodium hyaluronate and identical injections of tritiated thymidine to serve as a paired control for each EGF-treated eye.

Seven days after injury, the corneas of the eight cats were removed and placed in Carnoy's solution overnight. Descemet's membrane, with endothelial cells attached, was stripped off the stroma and flat mounted onto glass slides. The specimens were coated with Kodak NTB-2 emulsion (Eastman Kodak, Rochester NY), exposed for 2 weeks at −80°C, and then developed. The number of corneal endothelial cells that had undergone mitosis after wounding was measured by counting the number of nuclei with dense autoradiographic grains. The slides were projected at 20X with a Leitz projection microscope (Leitz, Inc., New York, NY), which permitted counting of nuclei over the entire field of the wound area. The number of labeled nuclei on EGF-treated or control paired corneas was compared for statistical significance using a paired t-test.

RESULTS

Corneal Thickness

The average central corneal thickness of both left and right eyes of the 36 cats 1 day before surgery was 620.8 ± 16.5 μm. ANOVA of the mean corneal thickness of the eyes treated with the three different concentrations of EGF showed that the three EGF-treated groups were not significantly different on days 4, 7, 10, and 14 after injury. Similarly, the three control groups were not significantly different on days 4, 7, 10, and 14 after injury. Therefore, the values for the corneal thicknesses of the three EGF treatment groups were combined into a single EGF treatment group on each day, as were those of the control groups. As shown in Table 1, the corneas treated with EGF were significantly thicker than the paired control corneas on days 4 and 7 after injury. Specifically, the average difference in corneal thickness between the pairs of corneas treated with EGF or sodium hyaluro-

<table>
<thead>
<tr>
<th>TABLE 1. Corneal Thickness of Cat Corneas</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Central Corneal Thickness (μm)</strong></td>
</tr>
<tr>
<td>Day 0          Day 4          Day 7          Day 10         Day 14</td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>EGF (10 + 30 + 100 μg/ml)</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Difference (mean ± SEM)</td>
</tr>
<tr>
<td>Probability</td>
</tr>
<tr>
<td>No. of paired eyes</td>
</tr>
</tbody>
</table>

* Mean difference in central corneal thickness between paired control and EGF-treated eyes.
Endothelial cell density between the paired eyes. By days 10 and 14 after injury, corneal edema had decreased substantially in both groups of corneas, such that the EGF-treated corneas were 12% thicker and the control corneas were 19% thicker than the corneas before the injury. The average difference in central corneal thickness of the paired corneas was not significantly different on days 10 and 14 after injury.

Endothelial Wound Area
On days 2, 4, 7, and 14 after injury, the average endothelial wound area of the corneas treated with different levels of EGF was not significantly different from any other by an ANOVA test nor were the three control groups different. The endothelial wound areas for the three EGF treatment groups were combined into one EGF treatment group, and likewise, the three control groups were also combined into one control group. On postinjury day 2, endothelial cells of the EGF-treated eyes had resurfaced 65 ± 4% of the original 7-mm diameter wound area, which was significantly (P < 0.05) higher than the 60 ± 4% area resurfaced for the vehicle-treated control eyes. Four days after surgery, the corneas treated with either EGF or vehicle had resurfaced greater than 90% of the original 7-mm wound area. At 7 and 14 days after surgery, the endothelial wounds were completely resurfaced in all cats.

Endothelial Cell Morphology

Endothelial Cell Density. Significant differences of endothelial cell morphology did not develop between the EGF-treated and control groups until 14 days after the injury when the endothelium had completely resurfaced. The effects of different concentrations of EGF on the central endothelial cell density at 14 days after injury are shown in Table 2. The difference in endothelial cell density between the paired eyes treated with EGF or vehicle was positive for all three concentrations of EGF (10, 30, and 100 μg/ml). However, only the highest concentration of EGF (100 μg/ml) produced a significant increase in the average endothelial cell density (P < 0.05). ANOVA did not detect a significant difference between the three EGF groups or between the three control groups on postoperative day 14. Combining the results of all EGF-treated corneas into one group and all control corneas into one control group, the average endothelial cell density of all corneas treated with EGF (1708 cells/mm²) was approximately 38% higher (P < 0.001) than that for paired control corneas (1235 cells/mm²).

Percent of Hexagonal Endothelial Cells. As shown in Table 3, the coefficients of variation of the endothelial cell size for each of the three EGF treatment groups decreased compared with that of the paired contralateral control corneas. As before, ANOVA indicated that the three EGF treatment groups were not significantly different from each other and the three control groups were not significantly different from each other. Combining all EGF-treated corneas into one group and combining all control corneas into one group, the coefficient of variation of the endothelial cell size (standard deviation/mean cell size) for all corneas treated with EGF was approximately 31% (P < 0.05) less than for all control corneas.

Endothelial Cell Mitosis. As shown in Table 5, both concentrations of EGF significantly (P < 0.025) in-

TABLE 2. Mean Central Endothelial Cell Density at Day 14 After Touch Injury

<table>
<thead>
<tr>
<th>EGF (μg/ml)</th>
<th>No. of Paired Eyes</th>
<th>ECD (cells/mm²)</th>
<th>Difference* (Mean ± SEM)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>EGF</td>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>2267</td>
<td>1627</td>
<td>640 ± 577</td>
</tr>
<tr>
<td>30</td>
<td>3</td>
<td>1281</td>
<td>898</td>
<td>385 ± 171</td>
</tr>
<tr>
<td>100</td>
<td>3</td>
<td>1762</td>
<td>1312</td>
<td>450 ± 89</td>
</tr>
<tr>
<td>10 + 30 + 100</td>
<td>8</td>
<td>1708</td>
<td>1235</td>
<td>473 ± 131</td>
</tr>
</tbody>
</table>

ECD = mean endothelial cell density.
* Mean difference in ECD between paired control and EGF-treated eyes.
TABLE 3. Mean Central Endothelial Coefficient of Variation of Cell Size at Day 14 After Scrape Injury

<table>
<thead>
<tr>
<th>EGF (μg/ml)</th>
<th>No. of Paired Eyes</th>
<th>CV (SD/Mean Cell Size)</th>
<th>Difference* (Mean ± SEM)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>2</td>
<td>0.17 0.27</td>
<td>-0.10 ± 0.06</td>
<td>0.31</td>
</tr>
<tr>
<td>30</td>
<td>3</td>
<td>0.21 0.35</td>
<td>-0.14 ± 0.08</td>
<td>0.22</td>
</tr>
<tr>
<td>100</td>
<td>3</td>
<td>0.22 0.25</td>
<td>-0.03 ± 0.05</td>
<td>0.67</td>
</tr>
<tr>
<td>10 + 30 + 100</td>
<td>8</td>
<td>0.21 0.29</td>
<td>-0.08 ± 0.04</td>
<td>0.05</td>
</tr>
</tbody>
</table>

CV = mean coefficient of variation of endothelial cells (SD of cell size/mean cell size).
* Mean difference in CV between paired control and EGF-treated eyes.

creased the number of labeled endothelial cell nuclei compared with the paired control eyes treated with sodium hyaluronate alone. The average percent increase in the number of labeled nuclei for all corneas treated with EGF was approximately 75% compared with vehicle treatment alone. The pattern of labeled endothelial cell nuclei is shown in Figure 1. Labeled nuclei occurred predominately at the edge of the touch injury and in the area of the wound. Very few labeled nuclei were observed in the nonwounded area adjacent to the injury.

DISCUSSION

In the current study, we investigated the effect of a single intraocular application of EGF in a sodium hyaluronate vehicle on several important parameters of corneal endothelial wound healing. These data indicate that a single injection of EGF into the anterior chamber of cats after a scrape injury enhanced corneal endothelial wound healing in an animal with a limited ability to regenerate corneal endothelium.

Our evaluation of different parameters of endothelial wound healing at various time points provided more detailed information about the complex response of the corneal endothelium to EGF. Two days after surgery, the endothelial wound areas of EGF-treated eyes were only slightly smaller than those of the vehicle-treated control eyes. On day 4 after injury, greater than 90% of the wound area had resurfaced, and by day 7, all wounds were resurfaced. The rapid rate of endothelial resurfacing of the wounded area that was observed in this model may have minimized the ability to demonstrate the effect of EGF to stimulate endothelial cell migration and enlargement. An effect of EGF on rabbit endothelial cell migration in vitro has been demonstrated previously by one group,27 who showed that EGF stimulated cells at the wound edge to loosen and begin to migrate as individual cells. These changes could cause additional breakdown of the endothelial cell barrier and may explain why, in the current study, the corneal thickness was slightly greater in the EGF-treated eyes compared with that in the vehicle-treated control eyes. The difference in corneal thickness diminished after 4 days, most likely because of reformation of cell-cell tight junctions and reestablishment of the barrier function.

A major objective of this study was to determine whether EGF had a beneficial effect on endothelial cell density and morphology in the wounded area. One week after injury, significantly more nuclei labeled with tritiated thymidine were detected in the corneas of eyes treated with EGF compared with those of control eyes treated with vehicle. In addition, 14 days after

TABLE 4. Mean Percent of Central Endothelial Cells With Hexagonal Shape at Day 14 After Scrape Injury

<table>
<thead>
<tr>
<th>EGF (μg/ml)</th>
<th>No. of Paired Eyes</th>
<th>Percent Hexagonal Cells*</th>
<th>Difference† (Mean ± SEM)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>2</td>
<td>90 68</td>
<td>22 ± 1</td>
<td>0.05</td>
</tr>
<tr>
<td>30</td>
<td>3</td>
<td>73 66</td>
<td>7 ± 3</td>
<td>0.12</td>
</tr>
<tr>
<td>100</td>
<td>3</td>
<td>86 72</td>
<td>14 ± 8</td>
<td>0.21</td>
</tr>
<tr>
<td>10 + 30 + 100</td>
<td>8</td>
<td>82 69</td>
<td>13 ± 3</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* Mean percent of central endothelial cells with hexagonal shape.
† Mean difference in percent hexagonal cells between paired control and EGF-treated eyes.
injury, the mean endothelial cell density in the central corneas of eyes treated with EGF was also significantly higher than that in control eyes. This increase in central endothelial cell density after a single injection of EGF in a hyaluronic acid vehicle was similar to that reported by others, who found an increased endothelial cell density after repeated injections of a solution of EGF into the anterior chamber of primates with endothelial wounds. Moreover, the increased numbers of nuclei labeled with tritiated thymidine observed in the current study suggests that the increased endothelial cell density was caused primarily by mitosis rather than redistribution of endothelial cells from peripheral areas. The pattern of labeled cell nuclei also indicates that endothelial cell division occurred primarily in the area adjacent to the wound area rather than in that occurring over the entire nonwounded area. This indicates that the endothelial cells that are in nonwounded, peripheral areas of the cornea remain contact inhibited and do not divide in response to distant endothelial cell injuries.

It is well established that corneal edema occurs when endothelial cell density falls below a critical level. Recently, endothelial cell size and shape have also been closely correlated with endothelial cell function. Clinical data have shown that endothelial dysfunction is associated more closely with variations in endothelial cell size than with cell density. Animal studies, using endothelial wound models in both rabbits and cats, have shown that recovery of corneal endothelial cell function is closely correlated with a gradual increase in hexagonality and a decrease in the coefficient of variation of the cell size. In the current study, the central corneal endothelium of the eyes treated with EGF demonstrated an increase in the percent of hexagonal endothelial cells and a decrease in the coefficient of variation of the cell size as early as 14 days after wounding. This would suggest that EGF at concentrations between 10–100 µg/ml stimulated an early restoration of cell size and shape in the wounded cat cornea.

The vehicle for EGF appears to be important in regard to its action on endothelial cells in vivo. One group injected 10 µg of EGF in water vehicle into the anterior chamber of cat eyes after an 8-mm transcorneal freezing and found no difference in corneal thickness or central endothelial cell density between EGF-treated corneas and control eyes. Others injected 100 µg of EGF in phosphate-buffered saline, methyl cellulose, or sodium hyaluronate into the anterior chamber of cat eyes after a 4-mm transcorneal

### TABLE 5. Average Number of Central Endothelial Cells With Nuclei Labeled With Tritiated Thymidine at Day 7 After Touch Injury

<table>
<thead>
<tr>
<th>EGF Concentration (µg/ml)</th>
<th>No. of Paired Eyes</th>
<th>EGF</th>
<th>Control</th>
<th>% Change*</th>
<th>Difference†</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>4</td>
<td>2901</td>
<td>2215</td>
<td>33</td>
<td>686</td>
<td>0.05</td>
</tr>
<tr>
<td>30</td>
<td>4</td>
<td>2038</td>
<td>870</td>
<td>119</td>
<td>1168</td>
<td>0.05</td>
</tr>
</tbody>
</table>

* Mean percent difference between paired eyes.
† Mean difference in number of labeled nuclei between each of the paired control and EGF-treated eyes.
EGF Enhancement of Endothelial Wound Healing

freezing. They found that the endothelial wounds of EGF-treated eyes closed significantly faster than those of vehicle-treated eyes only when EGF was formulated in sodium hyaluronate. The authors of both these studies suggested that EGF in water or phosphate-buffered saline may be cleared from the anterior chamber before sufficient binding occurred to the EGF receptors on the endothelial cell surface. However, the failure of EGF formulated in methyl cellulose suggests that additional properties besides the viscoelastic nature of the vehicle are important for EGF action. Corneal endothelial cells have been shown to express receptors for sodium hyaluronate. Binding of sodium hyaluronate to its receptor on the surface of endothelial cells may establish an environment that enhances binding of EGF to its receptor. Sodium hyaluronate may also enhance the effect of endogenously produced growth factors in the anterior chamber, which may explain the substantial number of labeled endothelial cell nuclei that were detected in the control eyes of the cats in this study.

In summary, these data demonstrate that a single treatment with EGF delivered in sodium hyaluronate stimulated endothelial cell mitosis, increased endothelial cell density, and improved restoration of cell shape and size in an animal with a limited ability to regenerate the corneal endothelium spontaneously. Although further studies are necessary to determine the effects of EGF on the corneal endothelium in human eyes, EGF may prove useful for maintaining corneal clarity in patients with an endothelial cell injury or disease.

Key Words

corneal endothelium, epidermal growth factor, wound healing, cat, sodium hyaluronate

References