Continuous Epidermal Growth Factor Delivery in Corneal Epithelial Wound Healing

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Purpose. To investigate the effects of a single prolonged exposure to recombinant epidermal growth factor on the healing of anterior keratotomy wounds in New Zealand white rabbits.

Methods. After wounding, eyes were perfused for 1, 2, 4, and 8 hours with either epidermal growth factor solution at a concentration of 50 µg/ml or balanced saline solution using a Morgan therapeutic lens (Mortan Inc, Missoula MT) and a syringe pump. Furthermore, concentration response was evaluated by perfusing with epidermal growth factor solutions at concentrations of 5, 50, 100 and 500 µg/ml for 4 hours. Wound healing rates were determined by quantitative morphometry of the wound area. The ratio of healing rates of eyes perfused with epidermal growth factor and control eyes provided a measure of the effect of epidermal growth factor on wound healing, and was defined as the epidermal growth factor enhancement factor.

Results. The enhancement factor was found to be 1.04 ± 0.08, 1.17 ± 0.07, 1.43 ± 0.09, and 1.59 ± 0.07 for perfusion times of 1, 2, 4, and 8 hours, respectively. The concentration response enhancement factors were 0.99 ± 0.08, 1.43 ± 0.09, 1.21 ± 0.09, and 0.95 ± 0.07 for the 5, 50, 100, and 500 µg/ml 4-hour perfusions, respectively.

Conclusion. The results indicated that continuous epidermal growth factor exposures of as few as 2 hours produced a significant increase in healing rates (P < 0.05); increasing the time of exposure further increases the rate of wound healing. Results from the concentration response experiments showed that the optimum epidermal growth factor concentration for enhancing epithelial wound healing is approximately 50 µg/ml. Invest Ophthalmol Vis Sci. 1993;34:3593-3600.
Several factors combine to suggest that a controlled-release delivery system of EGF would enhance its effectiveness. The literature indicates that at least 6 hours of continuous exposure to human EGF is required to enhance DNA synthesis and that repeated and prolonged administration is necessary for effective therapy. Like most peptides, EGF is unstable in physiological fluids and has a very short half-life. Additionally it has been hypothesized that high concentrations of EGF may induce receptor downregulation and hence result in reduced therapeutic effect. Therefore we hypothesized that more effective therapy may be achieved with a drug delivery system capable of administering EGF directly to the wound site with the optimal concentration maintained for a prolonged duration.

The development of such a delivery system requires knowledge of the effect of concentration and duration of continuous and prolonged EGF exposure on corneal wound healing. The current study was undertaken to evaluate these effects and to provide a better understanding of the mechanism of action of EGF. The results of this study will aid in the specification of the target release rate profile for the EGF delivery systems currently under development in our laboratory.

**MATERIALS AND METHODS**

Recombinant human EGF (95% pure) was produced and donated by Allelix Biopharmaceuticals Inc. (Mississauga, Ontario). It was stored frozen as a lyophilized powder and was reconstituted with sterile balanced saline solution (BSS) before each study.

**Wound Healing Studies**

All procedures conformed to the regulations of the Canadian Council on Animal Care and the ARVO Resolution on the Use of Animals in Research.

New Zealand white rabbits weighing 2 to 2.5 kg were anesthetized by a 2 ml intramuscular injection of a 4:1 mixture of ketamine, (100 mg/ml Rogarsetic; Rogar/Stb, London Ontario) and xylazine, (20 mg/ml Rompum; Haver, Etobicoke, Ontario). One drop of proparacaine (0.5%) was instilled into the study eye. A 7.5 mm vacuum trephine (Hessburg-Barron) was used to create a shallow anterior keratotomy into the anterior stroma. The epithelium, basement membrane, and anterior stroma within this circle were removed using forceps to create a uniform stromal wound. After wounding, fluorescein (Fluor-I-Strip, Ayerst Laboratories, New York, NY) was applied to the cornea to delineate the wounded area. The eyes were photographed using a 60 mm macro lens and a cobalt blue filtered flash.

A Morgan therapeutic contact lens (Morton Inc, Missoula MT) was then placed on the eye and attached to a syringe pump. The eye was perfused with either human EGF in BSS or with BSS at a rate of 2 ml/hr. At this flow rate the eye was completely bathed in the perfusate with minimal fluid overflow. To determine the effect of perfusion duration, eyes were perfused with EGF at a concentration of 50 μg/ml for 1, 2, 4, or 8 hours with general unaesthetic maintained by intermittent intramuscular injections of the ketamine/xylazine mixture. For the concentration response experiments, a perfusion duration of 4 hours was chosen and EGF concentrations of 5, 50, 100, and 500 μg/ml in BSS were evaluated. Six eyes in each of the control and experimental groups were assessed for healing at 1, 2, and 4 hours and at all concentrations; at 8 hours, nine eyes were assessed in each group.

All of the eyes were stained and photographed immediately after the perfusion, and at regular intervals of approximately 24 hours thereafter until reepithelialization had occurred as evidenced by the absence of stromal fluorescein staining. The slides of the wounded corneas at each time were projected and the wound area was traced on paper. The wound outlines were digitized using a bit pad and the wound areas were measured by a morphometric analysis program (The Morphometer, Woodshole, MA). The radius of an equivalent-sized circle was used to determine the wound healing rate according to the method of Brazzell et al. The wound radius versus time data for the control and experimental groups were analyzed using a regression analysis for each perfusion duration and perfusion concentration assuming independence of each of the data points in the study. Differences between perfusion durations and experimental and control groups were assessed by comparing the slopes using an analysis of covariance. The slopes can be taken to be a measure of the rate of healing, r_EGF or r_c for EGF and control groups, respectively. Using the mean of the healing rates in each of the eyes, an EGF wound healing enhancement factor can then be calculated using the mean healing rate for each of the experimental groups using this equation:

\[ k^* = \frac{r_{EGF}}{r_c} \]

where \( k^* \) is the ratio of the rate of wound healing for the EGF-treated groups relative to the rate for the controls at the same perfusion duration. By normalizing the wound healing parameter in this manner it is possible to isolate the effect of EGF treatment from other experimental factors such as saline perfusion.
Histologic Studies

Histologic analysis was performed to assess the effects of EGF perfusion on cellular organization in corneal wound healing. Four eyes were wounded as described. The eyes were perfused for 4 hours with either EGF in BSS at a concentration of 50 μg/ml or with BSS at a flow rate of 2 ml/hr. From each group, one eye was removed at 2 days and one at 5 days. The eyes were fixed in 10% neutral-buffered formalin, cut into 6 μm sections, stained with hematoxylin and eosin, and examined under light microscopy.

RESULTS

Perfusion Results

The results of the perfusion duration study are presented in Figure 1. For each perfusion duration, wound radius is plotted against time from wounding. At 2, 4, and 8 hours of EGF perfusion, a significant increase in the rate of wound healing compared with controls (P < 0.05) was found. The 1-hour control and EGF groups were not significantly different. The enhancement factor is plotted against perfusion duration in Figure 2. After 1 hour of EGF perfusion there was no enhancement of healing with EGF compared with saline perfusion. Figure 2 illustrates that, as the duration of EGF perfusion increases, rate of wound healing increases. Enhancement factor for the 2-, 4-, and 8-hour perfusions are significantly greater than unity based on a 95% confidence interval.

The results of the concentration response experiments are plotted in Figure 3. EGF at concentrations of 50 and 100 μg/ml resulted in significant increases in the healing rate compared with control eyes (P < 0.05). Eyes perfused with EGF at concentrations of 5 and 500 μg/ml for 4 hours did not heal significantly faster than controls. The enhancement factor is plotted against perfusion concentration in Figure 4. This graph illustrates that an EGF concentration of 50 μg/ml resulted in maximal stimulation of healing. A summary of the wound healing results in both groups is presented in Table 1. The time to 100% healing is calculated based on the regression lines determined for each of the experimental conditions.

**FIGURE 1.** EGF perfusion duration effect on wound healing rate. EGF concentration was 50 μg/ml and the perfusate flow rate was 2 ml/hr. Best-fit linear regression lines for the EGF data are dotted; the saline controls are dashed. Significant increases in healing rate are seen with 2, 4, and 8 hours of EGF treatment (P < 0.05).
Histologic Results

Histologic examination of both the BSS- and EGF-perfused corneas after 2 days of healing showed complete reepithelialization of the wounds, despite the presence of persistent defects on fluorescein staining. The wounds were covered by a thin attenuated epithelial layer of one to two cells thick. Both groups showed mild basal corneal edema and an inflammatory infiltrate. The infiltrate consisted largely of eosinophils. The degree of inflammatory infiltrate was greater and extended farther into the corneal stroma in the BSS group (Fig. 5) than in the EGF-perfused eye (Fig. 6). At day 2 no histologic alterations were evident in the endothelial layer of either group. The difference in stromal thicknesses is indicative of rabbit to rabbit variations, and nonuniformity in the wounding process.

After 5 days of healing, both the saline and EGF-perfused corneas showed a mild fibroblastic proliferation in the superficial stroma with early scar formation. The two groups differed, however, in the degree...
of inflammatory infiltrate and edema. The cornea of the EGF-perfused eye (Fig. 7) showed moderate basal intracellular edema and severe subepithelial edema with bullous formation. The degree of inflammatory infiltrate was greater than in the day 2 corneas and was also greater than that of the saline perfused eye at 5 days. The infiltrate extended throughout the corneal stroma and infiltrated under the endothelial layer. The inflammatory cells were largely eosinophils but included occasional neutrophils and chronic inflammatory cells. In comparison, the saline-perfused cornea at 5 days (Fig. 8) showed moderate basal intracellular edema but no bullous formation. Only an occasional eosinophil was evident.

**TABLE 1. Summary of Wound Healing Results**

<table>
<thead>
<tr>
<th>Perfusate EGF Concentration</th>
<th>Perfusion Duration (hr)</th>
<th>Healing Rate (mm/hr)</th>
<th>Enhancement Factor</th>
<th>Time to 100% Healing (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 µg/ml</td>
<td>1</td>
<td>0.0260 ± 0.0014</td>
<td>1.04 ± 0.08</td>
<td>166</td>
</tr>
<tr>
<td>Saline</td>
<td>1</td>
<td>0.0250 ± 0.0014</td>
<td></td>
<td>172</td>
</tr>
<tr>
<td>50 µg/ml</td>
<td>2</td>
<td>0.0298 ± 0.0014</td>
<td>1.14 ± 0.07</td>
<td>137</td>
</tr>
<tr>
<td>Saline</td>
<td>2</td>
<td>0.0261 ± 0.0011</td>
<td></td>
<td>153</td>
</tr>
<tr>
<td>50 µg/ml</td>
<td>4</td>
<td>0.0308 ± 0.0013</td>
<td>1.43 ± 0.09</td>
<td>158</td>
</tr>
<tr>
<td>Saline</td>
<td>4</td>
<td>0.0229 ± 0.0011</td>
<td></td>
<td>184</td>
</tr>
<tr>
<td>50 µg/ml</td>
<td>8</td>
<td>0.0368 ± 0.0013</td>
<td>1.59 ± 0.07</td>
<td>118</td>
</tr>
<tr>
<td>Saline</td>
<td>8</td>
<td>0.0254 ± 0.0008</td>
<td></td>
<td>172</td>
</tr>
<tr>
<td>5 µg/ml</td>
<td>4</td>
<td>0.0249 ± 0.0014</td>
<td>0.99 ± 0.08</td>
<td>158</td>
</tr>
<tr>
<td>Saline</td>
<td>4</td>
<td>0.0253 ± 0.0014</td>
<td></td>
<td>160</td>
</tr>
<tr>
<td>100 µg/ml</td>
<td>4</td>
<td>0.0277 ± 0.0011</td>
<td>1.21 ± 0.09</td>
<td>146</td>
</tr>
<tr>
<td>Saline</td>
<td>4</td>
<td>0.0230 ± 0.0014</td>
<td></td>
<td>176</td>
</tr>
<tr>
<td>500 µg/ml</td>
<td>4</td>
<td>0.0251 ± 0.0013</td>
<td>0.95 ± 0.07</td>
<td>160</td>
</tr>
<tr>
<td>Saline</td>
<td>4</td>
<td>0.0265 ± 0.0013</td>
<td></td>
<td>156</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The results of this study indicate that a single but prolonged dose of EGF produces a dramatic increase in the rate of corneal reepithelialization in the rabbit anterior keratotomy model. In all previous work, EGF has been administered in the form of topical eyedrops. No one, to our knowledge, has delivered the peptide for a prolonged period in an in vivo corneal wound model. The application of topical drops containing human EGF four times daily for the entire healing duration in the same wound model has been shown to enhance the rate of healing.\(^5\)\(^6\) Although several concentrations of drops were evaluated in these studies, only EGF at 500 µg/ml was found to provide any significant enhancement of the wound healing rate when compared with the vehicle only. A 28% increase in the rate of healing of the wounds was noted at this concentration. In the current study, a single 2-hour perfusion of EGF at a concentration of 50 µg/ml, one tenth the concentration of topical eyedrops, at the beginning of wound healing resulted in a lower, but still significant, \((P = 0.05)\) 17% increase in the wound closure rate. Increasing the perfusion duration to 8 hours further increased the enhancement factor to 59%, when compared with controls. This corresponds to a decrease in the time to wound closure of approximately 50 hours in the eyes perfused for 8 hours. It seems remarkable that as few as 2 hours of continuous treatment with human EGF at the beginning of wound healing can result in an effect that lasts for more than 5 days, an effect similar to that noted with 1 week of treatment with EGF drops four times daily.

It has been suggested that a minimum of 5 to 6 hours of EGF exposure is required to commit the majority of cells to DNA synthesis.\(^15\) However, our in vivo perfusion results demonstrate that whereas 1 hour of
EGF perfusion did not result in a significant increase in the wound healing rate, as few as 2 hours of constantly delivered EGF caused significantly enhanced healing; increasing the perfusion time to 4 and 8 hours further increased the enhancement factor and decreased the average time to wound healing.

It is possible that commitment to DNA synthesis requires shorter exposure to EGF than previously reported. Alternatively, continuous perfusion of EGF for 2 to 4 hours could result in the saturation of available EGF cell receptors. After EGF receptor binding the complexes migrate to protein coated pits in the cell membrane, where further binding between the ligand receptor complexes and the protein occurs before internalization.\(^\text{16,17}\) The rates at which these processes
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occur in corneal epithelial cells are, to our knowledge, unknown. Furthermore, reported values for the half-lives of EGF receptors are between 1 and 20 hours depending on cell type and quantity of EGF present. Hence, it is possible that EGF remains bound to the receptors after the latent phase, again promoting mitotic activity in the healing phase. A third interpretation is that EGF may affect the wound healing processes during the latent phase in some as yet unknown fashion. Finally, it is possible that short-term EGF exposure could result in de novo synthesis of EGF or other growth factors as suggested by Coffey et al.

This could in turn stimulate proliferation of corneal epithelial cells. In vitro cell culture results show that cell proliferation can be further enhanced if the exposure time is increased to 12 to 16 hours; hence, it is reasonable to hypothesize that longer than 8-hour durations of EGF exposure may result in a further enhancement in the epithelial wound healing rate.

Based on our initial perfusion duration results, 4 hours of perfusion with EGF at various concentrations was evaluated to determine the concentration response. In the range of 5 to 500 μg/ml, the greatest wound healing enhancement was found with EGF at a concentration of 50 μg/ml. Doubling the perfusate concentration to 100 μg/ml decreased this effect. EGF concentrations of 5 and 500 μg/ml did not result in an enhancement of wound healing. These results suggest that an optimum tear fluid EGF concentration exists and that downregulation occurs at higher concentrations.

The histology results show complete reepithelialization of the wounds after 2 days of healing, despite the continued presence of a defect as indicated by fluorescein staining. This suggests the possibility that the epithelium initially covering the wound area is immature, and fluorescein stain may still infiltrate this thin epithelial layer to the underlying stroma. After 2 days both the BSS and EGF perfused groups show similar histologic changes, although there is less inflammation in the EGF-perfused cornea. After 5 days of healing, however, the EGF-treated cornea shows greater corneal edema with bullous formation and a greater inflammatory response, compared to the BSS-perfused cornea. It is unknown if this result is related to EGF or if it is caused by greater trauma at the time of wound formation. At 5 days both corneas showed a mild degree of stromal scarring. Further histologic studies on a greater number of cases are warranted to more clearly define the role of EGF in the healing process at the cellular level.

In summary, enhancement of epithelial wound healing was noted after a minimum of 2 hours of perfusion; further increasing the perfusion duration increased the rate of wound healing. Perfusion with EGF at a concentration of 50 μg/ml resulted in maximum stimulation of corneal epithelial wound healing; higher EGF concentrations resulted in downregulation of the EGF receptors.

Key Words
human epidermal growth factor, cornea, wound healing, prolonged delivery, anterior keratotomy model, reepithelialization

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References


