Lacrimal Gland HGF, KGF, and EGF mRNA Levels Increase after Corneal Epithelial Wounding

Steven E. Wilson,1,2 Qianwa Liang,2 and Woo Jung Kim2,3

PURPOSE. To evaluate the effect of corneal epithelial wounding on lacrimal gland expression of hepatocyte growth factor (HGF), keratinocyte growth factor (KGF), and epidermal growth factor (EGF) in the rabbit model.

METHODS. Rabbits had corneal epithelial scrape injuries, and the lacrimal gland was removed at different times after wounding. HGF, KGF, and EGF mRNA expression was examined by quantitative RNase protection assay. HGF, KGF, and EGF proteins were detected in rabbit lacrimal tissue using immunoprecipitation and western blot analysis.

RESULTS. HGF mRNA and EGF mRNA were significantly increased in rabbit lacrimal gland tissue within 8 hours after corneal epithelial injury. The increase in KGF mRNA expression was small and reached significance 1 day after corneal injury. Lacrimal gland expression peaked at 3 days after wounding for each growth factor mRNA, the same day, on average, that the epithelial defect healed. After the peak increase in expression, there was a progressive decline in expression of each growth factor mRNA, but production was still increased compared with prewound levels. HGF protein, KGF protein, and EGF proteins were detected in rabbit lacrimal gland tissue.

CONCLUSIONS. Levels of HGF, KGF, and EGF mRNAs increase in rabbit lacrimal gland tissue in response to corneal epithelial wounding. The results of this study are consistent with the existence of a cornea–nervous system–lacrimal gland regulatory loop modulating expression of these growth factor mRNAs. The lacrimal gland is a likely source of increased HGF and EGF proteins detected in tears in previous studies. (Invest Ophthalmol Vis Sci. 1999;40:2185–2190)

A number of studies performed over the past few years have suggested that the lacrimal gland produces growth factors that have a role in lacrimal gland physiology and maintenance of the ocular surface.1–17 Some of these investigations have noted increases in either tear growth factor levels or lacrimal gland growth factor production during the wound-healing response to ocular surface injury.7,15,17 For example, epidermal growth factor (EGF) expression in lacrimal gland has been shown to increase in response to corneal wounding.7 Cholinergic stimulation of the lacrimal gland increases EGF secretion, suggesting that upregulation of EGF in the lacrimal gland and tears in response to corneal epithelial wounding is controlled by a nervous system–mediated reflex arc.6,7 Corneal wounding also upregulates tumor necrosis factor (TNF)-α production by the lacrimal gland.15

HGF bioavailability in tears is also increased in response to corneal injury.17 Although HGF protein is probably produced by lacrimal gland interalveolar connective tissue cells or myoepithelial cells,15 it is not known whether HGF production in the lacrimal gland is upregulated in response to corneal injury, because both the lacrimal gland and keratocytes could be sources of increased tear HGF.13,17 Nothing is known about KGF production in the lacrimal gland or the presence of KGF in tears.

HGF, KGF, and EGF are growth factors that are thought to be important in modulating corneal epithelial homeostasis and wound healing. HGF and EGF have both been shown to stimulate corneal epithelial cell proliferation and migration while inhibiting corneal epithelial cell terminal differentiation.18,19 KGF has also been found to trigger corneal epithelial cell proliferation, but no KGF effects on migration or terminal differentiation of these cells have been noted.18,19

The present study was performed to examine lacrimal gland levels of HGF, KGF, and EGF mRNAs after corneal epithelial wounding using a sensitive quantitative technique. We also examined expression of HGF, KGF, and EGF proteins in rabbit lacrimal gland tissue using immunoprecipitation and western blot analysis.

METHODS

Animals and Tissues
Six-week-old New Zealand White rabbits were anesthetized with, ketamine (30 mg/kg weight intramuscular) and xylazine (2 mg/kg weight intramuscular). Corneal epithelial scrape
wounds were produced in one eye selected at random or in neither eye in the control animals. Wounding was performed using a scalpel blade to remove epithelium from the central cornea sparing approximately 1 mm at the limbus for 360°. Three drops of ciprofloxacin (Ciloxan; Alcon, Fort Worth, TX) were instilled immediately after epithelial scrape. Tape was applied to close the lid for 2 hours to prevent exposure while the animal was anesthetized. For the welfare of animals, only one eye of each rabbit was used in this study. If animals showed signs of having pain on awakening after corneal scrape injury, they were treated with oral acetaminophen, and patching was continued for another 12 to 24 hours. Animals were anesthetized again with ketamine and xylazine at the appropriate time after wounding, the lacrimal gland removed through the inferonasal conjunctival sack with forceps and sharp Wescott scissors, and the rabbit euthanatized with intravenous sodium pentobarbital (100 mg/kg). The lacrimal tissue was used for RNA extraction or protein isolation.

All animals were treated in accordance with the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**RNase Protection Assay**

Total cellular RNA was extracted from rabbit lacrimal gland tissue using TRIzol (Gibco BRL, Rockville, MD) according to the manufacturer’s instructions. RNA was dissolved in diethyl procarbonate water, and the concentrations were determined using a spectrophotometer. A cDNA probe for rabbit HGF, KGF, EGF, or β-actin was amplified using polymerase chain reaction (PCR) with the primers listed in Table 1. A melting temperature of 98°C, an annealing temperature of 55°C, and an extension temperature of 72°C were used in PCR amplifications, which were continued for 35 cycles. The amplification products were cloned into the pCR2.1 TA clone vector (Invitrogen, San Diego, CA) and sequenced using standard methods to confirm that the sequence was the corresponding rabbit sequences. Rabbit liver cDNA was used as target. All sequences were confirmed by DNA sequencing.

<table>
<thead>
<tr>
<th>Modulator</th>
<th>Size (bp)</th>
<th>Upstream Primer</th>
<th>Downstream Primer</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>β Actin</td>
<td>350</td>
<td>AGGCCAACCGGGAAGATGACC</td>
<td>GAAGTCAGGGGAGCTAGCAC</td>
<td>X00351</td>
</tr>
<tr>
<td>HGF</td>
<td>177</td>
<td>GATGTCTGCCGGTGCTCAGAA</td>
<td>CATGTCTGCTGTGAGGATA</td>
<td>X16323</td>
</tr>
<tr>
<td>KGF</td>
<td>322</td>
<td>GGGGATATAAGGGTGAGAAGA</td>
<td>GATTTAAGGCAACGAACATT</td>
<td>Z22703</td>
</tr>
<tr>
<td>EGF</td>
<td>394</td>
<td>TATGTCTGCAGGGGCTCAGAA</td>
<td>AGCGTGCGGAGTTCCACCA</td>
<td>X04571</td>
</tr>
</tbody>
</table>

Rabbit sequences were generated using PCR primers designed from the human or mouse sequence using rabbit liver cDNA as target. All sequences were confirmed by DNA sequencing.

The actual sizes of the protected RNA fragments were confirmed using 32P-labeled RNA ladder that was included on each gel. This ladder was prepared by using the full-length transcripts from the cloned cDNA templates for each of the growth factors and β-actin. The full-length RNA probe sizes (not protected sequence size) were 580 (HGF), 480 (β-actin), and 350 (EGF) nucleotides. These markers were revealed with a brief exposure, and then the marker lane was cut from the dried gel to prevent overexposure of adjacent RNase-protected lanes.

Quantitation of bands was performed by scanning the gels (Photoshop 4.0; Adobe, Seattle, WA) and using NIH Image 1.57 software to determine the density of both the growth factor and β-actin band in each lane. All lanes were run on gels simultaneously for a particular RNase-protection experiment. A uniform box size that enclosed the largest band in a series for a particular growth factor or β-actin was used for determining all the growth factor or β-actin band densities in a particular experiment. The relative intensity of each band was calculated as density units = (density of growth factor band/density of β-actin band) × 100. There were four different animals at each time point after wounding and in the control.

Statistical analyses were performed with analysis of variance using the Bonferroni–Dunn adjustment. P < 0.05 was considered statistically significant.
Immunoprecipitation and Western Blot Analysis

Rabbit lacrimal glands were homogenized and lysed in 5 ml lysis buffer (50 mM Tris Cl [pH 8.0], 0.5% Triton X-100, 10% glycerol, 0.2 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 3 μg/ml aprotonin, 2 μg/ml pepstatin, and 1 μg/ml leupeptin) on ice for 20 minutes. The extracts were centrifuged at 15,000 rpm in a microcentrifuge for 10 minutes in 4°C. Supernatants were decanted into a fresh tube, and the protein concentration of each extract was determined with a protein assay kit (Bio-Rad). Five hundred microliters of lysate at 1 mg/ml was incubated with preimmune serum (2.5 μl) containing protein A Sepharose 6MB (Pharmacia, Piscataway, NJ) for 1 hour, and the lysate was clarified by brief centrifugation in a microcentrifuge at 15,000 rpm. Sepharose beads were washed three times in cell lysis buffer, and the bound proteins were eluted in SDS gel loading buffer by boiling. SDS-PAGE was performed as previously described. Immunoblot analysis was performed by the chemiluminescence system (ECL; Amersham, Arlington Heights, IL) based on the recommended protocols supplied by the manufacturer, with minor modification described previously.

RESULTS

Corneal epithelial wounding resulted in increased levels of HGF mRNA, KGF mRNA, and EGF mRNA expression measured by RNase protection assay in the rabbit model (Figs. 1A, 1B, 1C). For HGF and KGF the increases in mRNA levels became significant at 8 hours after wounding (Fig. 1D). For KGF mRNA the increase was small and did not reach statistical significance until 1 day after wounding. There was a trend toward an increase in HGF mRNA levels as early as 1 hour after wounding (Fig. 1D). For each growth factor the peak in mRNA levels occurred at 3 days after wounding. Approximately 3 days are required for the epithelial defect to heal when injury is created by the method used in this study (2.8 ± 0.4 [SEM] days; S. E. Wilson, unpublished data, 1992). Each of the growth factor mRNAs had decreased at 7 days after wounding compared with the peak at 3 days after epithelial injury (Fig. 1D). In each case, however, the day 7 levels of mRNA were still significantly increased compared with those in the unwounded control corneas.

Proteins near the expected size for HGF, KGF, and EGF were detected in lacrimal glands from rabbits with normal control corneas using immunoprecipitation and western blot analysis (Fig. 2). The antibodies used for detection of HGF and KGF have been shown to detect the rabbit proteins. The detected proteins were 70 kDa for HGF, 28 kDa for KGF, and 17 kDa for EGF. The immunoprecipitation and western blot analysis technique was not used to attempt to measure changes in growth factor protein production after corneal epithelial wound healing, because, at best, the technique is semiquantitative.

DISCUSSION

The results of this study demonstrate that HGF mRNA, KGF mRNA, and EGF mRNA levels increased in the lacrimal gland after corneal epithelial injury. Thus, we conclude that there is a regulatory communication between the ocular surface and the lacrimal gland tissue that modulates the levels of the growth factor mRNAs. The route of communication is thought to be through the trigeminal sensory nerves of the cornea to the brain stem and subsequently to the lacrimal gland through parasympathetic nerves. Studies by Meneray et al. have demonstrated that secretory granules accumulate in the acinar cells after trigeminal denervation, providing evidence that trigeminal sensory nerve impulses contribute to the pathways mediating secretory release of components from the lacrimal gland. Yoshino et al. demonstrated that cholinergic stimulation of human lacrimal gland in vitro increases secretion of lactoferrin and EGF proteins. The results of these studies also support the hypothesis that the lacrimal gland responds to ocular surface stimulation by increasing secretion of growth factors and other tear components.

These results for EGF confirm previous results of other investigators reported in abstract form at the annual meeting of the Association for Research in Vision and Ophthalmology. No studies on changes in HGF or KGF production in the lacrimal gland that occur in response to corneal epithelial wounding had been reported before this study. Presumably, HGF, KGF, and EGF released from the lacrimal gland after epithelial injury to the cornea modulate corneal epithelial wound healing. The timing of increased expression relative to corneal epithelial wound closure supports this hypothesis. Basal HGF, KGF, and EGF released into the tears from the lacrimal gland may also have an important role in homeostasis of the ocular surface.

This study determined that the levels of HGF mRNA, KGF mRNA, and EGF mRNA increased in lacrimal gland after wounding but could not distinguish between increased transcription of mRNA and decreased degradation of mRNA. One or both of these processes could be involved in increases in tissue mRNA levels. In any case, it is likely that increased mRNA levels for each of these growth factors resulted in increased protein production within the lacrimal gland. HGF, KGF, and EGF proteins were detected in rabbit lacrimal gland tissue, but we could not quantitate changes in protein expression accurately because of the semiquantitative nature of the immunoprecipitation and western blot analysis technique. However, previous studies have shown that there is increased HGF in tears after corneal wounding. It is likely that this increased HGF in tears is, at least in part, attributable to increased lacrimal gland production of this growth factor. Studies are needed to examine tear EGF protein and KGF protein bioavailability in response to corneal injury.

The HGF and KGF proteins detected in the rabbit lacrimal gland are similar in size to those previously detected in rabbit tissues with the same antibodies. We know of no studies in which the size of rabbit EGF has been monitored. We detected only a 17-kDa protein in rabbit tissue, whereas the human EGF precursor is 120 kDa in size. Because this was the only protein band detected in the EGF immunoprecipitation and
western blot analysis, this is probably the size of the rabbit EGF precursor protein.

Our previous studies have demonstrated that HGF protein is expressed in interalveolar connective tissue cells or myoepithelial cells and that HGF receptor protein is expressed within acinar and ductal epithelial cells within human lacrimal gland tissue. In the present study HGF mRNA and protein were expressed in rabbit lacrimal tissue. Thus, in addition to being secreted into tears, HGF probably has localized effects on epithelial cell functions within the lacrimal gland. Nothing is known about which lacrimal epithelial cell functions are regulated by HGF. This is the first study to examine KGF expression in lacrimal tissue. Studies have yet to be performed evaluating KGF receptor expression in lacrimal tissue and it is therefore not known whether KGF regulates local functions in the lacrimal gland.

HGF and KGF are classic paracrine mediators of stromal–epithelial interactions that are produced by fibroblastic cells to promote epithelial cell growth and survival. HGF and KGF are involved in various biological processes, including wound healing, tissue regeneration, and tumor cell proliferation. The results of this study suggest that HGF and KGF might play important roles in the regulation of lacrimal gland function.
regulate the functions of epithelial cells in many organs. For example, HGF and KGF are produced by keratocytes in the cornea, and results of in vitro studies suggest that these cytokines regulate corneal epithelial cell proliferation, motility, and differentiation. This study shows that the lacrimal gland is also a probable source of HGF and KGF involved in homeostasis of the corneal epithelium and the wound-healing response after corneal epithelial injury.

Acknowledgment

The authors thank Ralph Schwall, of Genentech, Inc., South San Francisco, California, for providing the anti-HGF antibody.

References