Effects of Keratinocyte Growth Factor-2 on Corneal Epithelial Wound Healing in a Rabbit Model of Carbon Dioxide Laser Injury

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Keratinocyte growth factor-2 (KGF-2), also called fibroblast growth factor-10 (FGF-10), is a member of the fibroblast growth factor family. It plays a critical role in epithelial development and exerts its biological activities in a paracrine manner on the receptor FGFR2-IIIb. This study examined the function of topically applied KGF-2 in vivo on wound healing using a CO2 laser, corneal epithelial wounded, rabbit model. Topically applied 25 μg/ml KGF-2 accelerated corneal epithelial wound healing, in contrast to the control, and reduced inflammation, stromal edema, and fibrosis. In addition, this factor also exhibited significant inhibition of corneal neovascularization. KGF-2 appears to be another important growth factor in the regulation of corneal epithelial wound healing.

Key words keratinocyte growth factor-2; corneal; epithelium; wound healing

The cornea is directly exposed to the external environment and the corneal epithelium functions as a barrier in maintaining the integrity of the ocular surface. However, corneal epithelium is very vulnerable to injury and the persistence of corneal epithelial defects often results in the development of corneal ulcers, which can lead to corneal perforation and loss of vision. Proper healing of corneal wounds is thus vital for maintaining a clear, healthy cornea and preserving vision.

Successful wound healing involves a number of processes including cell migration, cell proliferation, matrix deposition, and tissue remodeling.1) The most critical of these processes are cell migration and proliferation, which are driven by growth factors and cytokines released coordinately into the injured tissue bed. Thus, growth factor mediated communication is important for corneal wound healing.2) Corneal epithelial cells express receptors for several families of growth factors including epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), fibroblast growth factors (FGFs), and transforming growth factor beta (TGF-β).3) Inhibition of the EGF receptor (EGFR) influences corneal wound healing as EGF stimulates the proliferation of epithelial cells. Cetuximab (an EGFR antibody) can cause persistent epithelial defects, and patients with an impairment of corneal wound healing under cetuximab treatment can benefit from the topical application of human EGF.5) EGF and TGF-α are synthesized by the lacrimal gland and are present in physiologically significant concentrations in tears.5) Corneal epithelial cells also synthesize mRNA for EGF and TGF-α protein.6) Furthermore, concentrations of EGF in the tears of patients with chronic epithelial defects are significantly decreased,5) and mice that genetically lack TGF-α production have chronic corneal erosions.7) EGF and FGF-2 stimulate mitosis and chemotactic migration in epithelial cell cultures.8) After epithelial debridement wounding, TGF-β expression is high in the Bowman’s layer (BL), whereas normal corneas show low-level TGF-β staining.9) FGF-2 initiates the hypercellular phase of corneal wound healing and insulin-like growth factor-1 (IGF-I) and platelet-derived growth factor (PDGF) are involved in the restoration of a normal extracellular matrix.10) Thus, there are substantial data to suggest that endogenous growth factors and their receptors are important regulators of spontaneous epithelial wound healing.

Keratinocyte growth factor-2 (KGF-2), also known as fibroblast growth factor-10 (FGF-10) and a member of the fibroblast growth factor family, appears to be an important growth factor in the regulation of corneal epithelial wound healing. KGF-2 has been employed in several animal models to improve wound healing, wound strength, and time to closure. In 1999, Xia and Colleagues used an ischemic, rabbit ear, ulcer model, published earlier in the decade by Ahn and Mustee, to study the effects of KGF-2 on ischemic ulcers in both young and aged rabbits.11) This study supports the use of KGF-2 in the healing of ischemic wounds. Soler and colleagues found that KGF-2 may be useful in accelerating healing, mainly through epithelialization, in various wound types, including venous stasis ulcers, partial thickness burn wounds, and skin graft donor sites.12) The findings in these two studies support the hypothesis that the regulated expression of KGF-2, as with other growth factors, is vital to the event sequence that produces tissue healing. Moreover, Jimenez and Rampy showed statistically significant increases in breaking strength, epidermal thickness, and wound collagen content in KGF-2 treated incisional wounds in rats and concluded that KGF-2 could enhance surgical wound healing.13) Recently, Liu et al. reported that KGF-2 provided a beneficial effect in the treatment of alkali burns of the cornea.14,15) The results of epithelial stem cell analysis demonstrated that KGF-2 accelerated the corneal epithelial healing by markedly stimulating epithelial stem cells proliferation. This study suggests that KGF-2 may be useful in corneal epithelial disorders. In current study, we examined the ability of the topical application of KGF-2 to enhance corneal epithelial healing in vivo using a rabbit model of CO2
laser injury. We further examined corneal opacity healing rate, cornea neovascularity and corneal epithelial cell proliferation and viability.

MATERIALS AND METHODS

Reagents and Instruments  Restriction enzymes, DNA polymerase, were purchased from Dalian Takara Co. (Dalian, China). Primers were synthesized by Shanghai Sangon (Shanghai, China). CM-Sepharose Fast F, Heparin–Sepharose CL-6B, and Sephadex G-25 were obtained from Amersham Pharmacia (Piscataway, U.S.A.). Methylthiazolletetrazolium (MTT) and Dulbecco’s modified Eagle’s medium (DMEM) was from Sigma-Aldrich (St. Louis, U.S.A.). Fetal bovine serum was purchased from Lanzhou National Hyclone Bio-Engineering Co., Ltd. (Lanzhou, China). Slit lamp (SLM-4, Kanghua Science & Technology Co., Ltd., China) CO2 Incubator (Shellab 2300, Sheldon Manufacturing Inc. U.S.A.) Carbon-dioxide laser (IZ30GZ, Beijing Research Institute of Electro-Optical, China).

Purification of KGF-2 from Escherichia coli Cells  Polymerase chain reaction (PCR) was applied to amplify the fragments encoding human KGF-2 (GenBank accession number AB002907) with the forward and reverse primers (forward, 5'-GGA ATT CCA TAT GCA AGC CCT TGG TCA-3'; reverse, 5'-CGG GAT CCT TAT GAG TGT ACC-3') including Ndel and BamH I sites respectively from the pET3c vector containing human KGF-2 coding sequence. KGF-2 was expressed in strain BL21 (DE3)/pLysS and purified using standard column chromatography techniques, and all isolation and purification performed at 4 °C. The frozen cell paste was thawed and resuspended in 20 mM Tris–HCl buffer (pH 7.2) containing 1 mM ethylenediaminetetraacetic acid (EDTA)–2Na and 0.05% Tween 80 at a ratio of 1 g cell paste to 8 ml Tris–HCl buffer, the cell suspension sonicated in an ice bath, and the insoluble paste removed by high speed centrifugation for 10 min. The supernatant was filtered through 1 μm membrane, loaded onto a preequilibrated CM–Sepharose column (5.0 × 50 cm, 500 ml bed volume), and the loaded column washed with three bed volumes of 20 mM PBS (sodium phosphate buffer, pH 7.2) containing 0.3 M NaCl until the eluent 280 nm absorbance was near zero. Proteins were then eluted with two bed volumes of 20 mM PBS (pH 7.2) buffer with step gradients of 0.8 and 1.2 M NaCl and the pooled fractions diluted with 20 mM PBS (pH 7.2) and applied to a Heparin–Sepharose column (3.5 × 40 cm, 200 ml of bed volume.). This column was washed with three bed volumes of 20 mM PBS (pH 7.2) containing 0.6 M NaCl and the bound proteins eluted with two bed volumes of buffer with step gradients as above. The purification process was monitored at 280 nm and the purified proteins finally desalted by passage through a Sephadex G-25 column (5.0 × 50 cm, 500 ml bed volume). This protein was then tested for purity sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, showed in Fig. 1), and for biological activity (NIH 3T3 cell proliferation assay by MTT, showed in Fig. 2).

Animals Protocols  Japanese White rabbits (2.5—3.0 kg, Laboratory Animal Center of Academy of Military Medical Science, Beijing, China) were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and all procedures were performed under deep anesthesia using 80 mg of ketamine. Anesthetized animals were placed in a conventional holder and positioned with the aid of a He–Ne alignment laser whose beam was colinear with a CO2 laser’s output. The incident radiation (28 W, 15 mA) was aligned perpendicular to the cornea surface which was then irradiated for 0.5 s, causing a 6 mm diameter defect. Experimentally treated eyes (n=40) received 25 μg/ml of human recombinant KGF-2 in PBS, and control eyes (n=40) received PBS only, three times a day immediately after CO2 laser injury. The corneas were then evaluated by slit lamp biomicroscopy and scored, as detailed below, on days 7 and 14. Photographs of the corneas were stored for masked measurement of the epithelial defect areas.

Biomicroscopic Examination  Corneal opacity was classified as follows: 0, no opacity; 1, less than one third of the corneal surface was clouded; 2, less than two-thirds clouded; 3, more than two-thirds clouded; and 4, almost all the corneal surface clouded and the opacity prevented visualization of the pupil margins. Corneal opacity healing rate was calculated as: (opacity score at 0 d—opacity score at 7 d or 14 d)/ (opacity score at 0 d), expressed as a percentage. Additional observation criteria included corneal and conjunctival edema, hyphema, conjunctival secretions, and cornea neovascularity (CNV).

Histopathological Evaluation  For histologic evaluation, six eyes in each group were randomly selected and enucleated on day 7 and 14. After enucleation, the cornea were fixed in a neutral formaldehyde (10%). Then the fixed eye
was dehydrated through ascending ethanol concentrations, embedded in paraffin, cut into 4 \( \mu m \) sections, stained with hematoxylin–eosin for light microscopic examination.

**Evaluation of Epithelial Wound Healing** Corneal photographs were digitized, imported to a computer, corneal defect variations between the central and peripheral cornea eliminated by conversion of the curved surface to flat, and the corneal defect area surveyed. The epithelial healing rate on day 7 and 14 after wounding was calculated for each eye, and the control and treated healing rates compared by a paired \( t \)-test and \( p < 0.05 \) considered significant.

**Evaluation of Epithelial Cell Proliferation and Viability** The viability of cornea epithelial cells was tested through killing the rabbits with an overdose of intravenous pentobarbital sodium at day 7 and 14 and enucleation of both eyes. The lamellar cornea were cut and each corneal block placed epithelial side down in a well of a 24-well tissue culture plate containing 2 ml DMEM with 10% fetal bovine serum (FBS) and cultured in 5% CO\(_2\) at 37 °C for 1 h. Then, 200 \( \mu l \) of a tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay solution was added to the culture, incubated for 4 h, the media removed, the formazan precipitate dissolved in dimethyl sulfoxide, and the 570 nm absorbance measured, with cell viability expressed as the absorbance. Each experiment was performed at least in triplicate with 12 replicates in each treatment. The control and treated absorbance were compared by a paired \( t \)-test.

**Statistical Analysis** All data were statistically analyzed using a statistical analysis system and the results expressed as mean ± standard deviation (SD). Groups were compared using SAS Software 8.0, depending on the variable type. Significance was assumed at \( p < 0.05 \).

**RESULTS**

**Biomicroscopic Examination** The observed corneal opacity healing rates showed that, in KGF-2-treated group, the corneal opacity gradually decreased from day 1 to 14 (Fig. 3). Although the corneal opacity also decreased in the control group, there were statistically significant differences in the mean opacity healing rates of days 7 and 14. The gross appearance of the KGF-2-treated eyes on day 14 was almost clear (Fig. 4F), whereas severe opacity and CNV remained in the control rabbits (Fig. 4C). The differences in CNV between the KGF-2 and control eyes was also evident on days 7 and 14, with most of the control eyes showing CNV invading the central cornea and not so in the KGF-2-treated eyes. The average percentage of CNV in KGF-2-treated eyes was 7.5% (3 of 40) on day 7 and 30.0% (6 of 20) on day 14, whereas the control rates were 17.5% (7 of 40) and 42.1% (8 of 19) respectively. Additional observational criteria included corneal and conjunctival edema and conjunctival secretions; corneal edema, judged by the thickness of the slit lamp beam on the stroma, in the control group eyes was greater than in the KGF-2-treated eyes. Clinically significant secretions appeared by day 1 in the control group. However, we could not establish a precise scoring system to quantify these differences.

**Histopathological Evaluation** Histological examination showed that the corneal lesions consisted of stromal edema, keratocyte loss, inflammatory cell infiltration, and CNV (Fig. 5). Cornea epithelium cell desquamation, stromal edema, and numerous neutrophil infiltrations were present in the cornea stroma in the control group (Figs. 5D—F). In contrast, the KGF-2-treated group exhibited no corneal stroma inflammation. Only mild stromal edema remained in KGF-2 corneas on day 14 (Fig. 5C) and significant changes were observed in the re-epithelialization of KGF-2-treated group.

**Evaluation of Epithelial Wound Healing** The degree of healing of CO\(_2\) laser-burned corneas were compared between the KGF-2-treated and control groups, with the KGF-2-treated group showing accelerated rates of re-epithelialization. There were statistically significant differences in the epithelial defect area on days 7 and 14. The defect areas of the two groups on days 0, 7, and 14 are summarized in Fig. 6.

**Cornea Epithelial Cell Viability Was Assessed by the MTT Assay** On days 7 and 14 after wounding, the mean...
570 nm absorbances in the KGF-2-treated group were 0.785 \pm 0.013 and 0.877 \pm 0.013, respectively, while they were 0.670 \pm 0.007 and 0.850 \pm 0.036 in the control group, respectively (Fig. 7).

**DISCUSSION**

Epithelial defects of the cornea frequently occur and are difficult to treat, possibly resulting in significant ocular morbidity and vision loss. Numerous measures to promote healing in corneal epithelium have been explored, and the clinical efficacy of exogenous growth factors used for the treatment of nonhealing epithelial defects has been examined. Among the numerous known growth factors, EGF, NGF, and IGF-1 have been tested clinically for enhancing healing of corneal epithelial defects.\(^4,17,18\)

Growth factors such as KGF-2 play a critical role in the epithelial development\(^9,20\) and KGF-2, specifically, exerts its biological activities in a paracrine manner on FGFR2-IIIb, a
receptor exclusively expressed on epithelial cells.\textsuperscript{21} KGF-2 is abundantly released into wounds following injury,\textsuperscript{12} and it is very likely an essential growth factor in this process. Treatment of wounds with KGF-2 has produced improvements in incisional wound healing, as characterized by an increase in breaking strength, collagen content, and epidermal thickness.\textsuperscript{13} KGF-2 and specific structural elements of dermatan sulfate, such as size and sulfation, promote maximal keratinocyte migration and cellular proliferation.\textsuperscript{22} Over expression of KGF-2 can attenuate bleomycin-induced pulmonary fibrosis in mice.\textsuperscript{23} This factor is an important cellular mediator responsible for the initiation and acceleration of wound healing and may enhance the healing of surgical wounds.\textsuperscript{13}

In this study, the effect of KGF-2 on the corneal wound healing was examined using a CO\textsubscript{2} laser injury rabbit model and it was observed that topically applied KGF-2 accelerated corneal epithelial wound healing, in contrast to the controls, and reduced inflammation, stromal edema, and fibrosis. In addition, KGF-2 also produced a significant inhibition of CNV.

The obtained data showed that exogenously added KGF-2 decreased epithelial wound areas such that, in the KGF-2-treated group, the corneal surface was smooth and epithelial defects were not recurrent throughout the observation period. KGF-2-treated rabbits also showed increased corneal epithelial cell viability, exhibiting statistically significant differences in the quantity of corneal epithelial cell at days 7 and 14. In contrast with the controls, the KGF-2-treated group had decreased corneal opacity, with corneas becoming almost clear by day 14. The controls showed severe opacity and CNV at this time, indicating corneal scarring.

Histologic examination showed massive infiltration of inflammatory cells in the corneal stroma in the control group beginning on day 1, becoming most severe by days 7 and 14. In contrast to the controls, the KGF-2-treated group had minimal inflammation at these times. In addition, stromal edema, keratocyte loss, fibrosis, and CNV was observed in the control group, and significant changes in re-epithelialization was detected in the KGF-2-treated rabbits.

Corneal wound healing is an important function of the cornea following injury. Corneal epithelium must rapidly be resurfaced to avoid microbial infection and further damage to the underlying stroma and, thus, corneal epithelial renewal is essential to this process and serves to further facilitate the maintenance of the smooth optical surface of the cornea. This epithelialization results in reestablishment of the protective barrier that separates living organisms from their external environment, making re-epithelialization a prerequisite for successful corneal stromal healing. Several studies have demonstrated that activation of stromal keratocytes at an ulcer margin can not occur in the absence of overlying epithelium.\textsuperscript{24,25} Re-epithelialization is believed to occur with reduction in proteolytic activity produced by inflammatory cells and the controlled expression of metalloproteinases.\textsuperscript{26} Several growth factors and cell adhesion molecules are believed to facilitate epithelial migration; EGF, bFGF, heparin-binding growth factor (HGF), and keratinocyte growth factor (KGF) have been implicated in epithelial healing.\textsuperscript{27—30} Interestingly, HGF and KGF, which promote epithelial healing, are produced by stromal keratocytes in response to epithelial injury. Fibronectin, an important extracellular matrix protein, is also found coating the stromal ulcer bed.\textsuperscript{31}

Inflammation is an essential response to injury and infection and surface tissue, this damage and scarring are acceptable outcomes as they do not interfere with the primary function of the tissue, but, in the cornea, these changes dramatically reduce the optical function of this tissue. This outcome occurs because the normal inflammatory processes, responsible for reestablishing the integrity of damaged tissue, depend on degradation of the damaged tissue, proliferation of the vasculature, fibroblast activity to replace damaged tissue, and wound contraction and tissue remodeling to restore strength to the injury site. In the cornea, these processes can result in refractive distortion and opacity, significantly reducing visual function.

The present results, taken together, indicate that the observed accelerated corneal wound healing in KGF-2-treated rabbits was associated with rapid re-epithelialization, reduced inflammation, and reduced fibrosis. KGF-2 did not cause or allow scar formation and CNV, normal side effects in normal corneal wound healing, making it an important growth factor for successful corneal wound healing.

REFERENCES