Basic fibroblast growth factor stimulates epithelial cell growth and epithelial wound healing in canine corneas

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Abstract
Objective  To evaluate the effect of basic fibroblast growth factor (bFGF) on the proliferation of canine corneal epithelial cells and epithelial wound healing.
Animal studied  Canine corneal epithelial cells from the corneas of euthanized dogs and corneal epithelial wounds on one eye from each of 24 dogs.
Procedures  The proliferation of corneal epithelial cells in vitro was measured using the methylthiazolyl-tetrazolium (MTT) assay. A corneal wound on one eye of each dog was made with a corneal trephine (6 mm diameter). Four concentrations of bFGF, 0, 100, 500, and 1000 ng/mL, were applied to the affected eyes of dogs, t.i.d. Fluorescein staining was used to assess closure of the corneal epithelial wound.
Results  The addition of bFGF resulted in a significant increase in epithelial proliferation at 24 h after culture, except 1 ng/mL bFGF. Cells with all bFGF treatments proliferated significantly at 48 and 96 h compared to those in the non-bFGF group. bFGF at a concentration of 10 ng/mL promoted cell proliferation maximally. The wound healing rate in the bFGF-treated groups was greater than that in the control. All corneal wounds in bFGF-treated corneas closed by day 7, whereas two of six corneal wounds in the control showed poor healing. None of the eyes developed corneal clouding or neovascularization during the experiment.
Conclusions  Basic fibroblast growth factor accelerated the proliferation of canine epithelial cells and effectively promoted corneal epithelial wound healing.
Key Words: basic fibroblast growth factor, bFGF, cell proliferation, corneal epithelium, corneal epithelial wound healing, dogs

INTRODUCTION
The epithelial cells of the surface layer of the cornea form the first defense as a barrier against noxious agents. Corneal wounds caused by trauma, surgery, or disease are very common in dogs. Inadequate healing of epithelial injuries can lead to corneal haze, ulcers, perforations, or even blindness.

In recent years, growth factors have been found to accelerate wound healing in human and animal corneas. Fibroblast growth factors (FGFs) have attracted considerable attention because of their well-known mitogenic activity with various types of cells. FGFs were originally identified as an activity in extracts of pituitary and brain that stimulated the growth of 3T3 cells. Acidic and basic FGF (a/bFGF) were the first FGFs to be purified, sequenced, and cloned. Most of the activities of the FGFs in the eye can be attributed to bFGF, which is 10–100-fold more potent than aFGF, and a wide distribution of bFGF has been described in epithelium, keratocytes, endothelium, and the aqueous humor.

bFGF influences cell growth, migration, differentiation, and regeneration, and many studies have documented the ability of bFGF to stimulate the proliferation of corneal epithelial cells, stromal fibroblasts, and endothelial cells in vitro. bFGF also has been reported to enhance corneal epithelial wound healing in cattle, rabbits, and humans, in vitro and in vivo. However, Foreman reported that bFGF had no effect on re-epithelialization rates of bovine and human corneas in an organ culture model. This discrepancy may have been due to species variation, differences in methodology, or the absence of components (e.g. heparin or heparin sulfate) normally present in the tear film that may act synergistically with bFGF. To date, no experimental study on the effect of bFGF on canine corneal epithelial cell proliferation has been reported.

In this study, the direct actions of bFGF on canine corneal epithelial cell proliferation and its effectiveness in canine corneal wound healing were investigated to explore the therapeutic application of bFGF in dogs.
Materials and Methods

Canine corneal epithelial cell culture

Eyeballs were obtained from adult dogs that had been euthanized after severe traffic accidents and were washed with physiological saline. A small incision in the limbus cornea was created using a pair of corneal scissors, and an iris spatula was inserted into the incision to separate the corneal epithelium layer from the corneal stroma. The corneal epithelium layer was washed in D-Hanks with 100 U/mL penicillin and 50 μg/mL streptomycin three times, and then divided into four pieces. Each piece (about 1 mm²) was placed evenly in one well of a cell culture plate, with the epithelial surface down. After sticking to the well for about 15 min, the epithelial surface was submerged in HEPES-buffered Dulbecco's modified Eagle's medium/ Nutrient Mixture F-12 (DMEM/F-12; Gibco, Grand Island, NY, USA), with 20% (v/v) fetal bovine serum (FBS), 0.5% (w/v) insulin (Sigma, St. Louis, MO, USA), 100 U/mL penicillin, and 50 μg/mL streptomycin, and cultured at 37 °C in a humidified 5% CO₂ incubator. The culture medium was replaced at day 3 and every 2 days thereafter. The cells were washed with D-Hanks twice, and digested with trypsin- ethylenediaminetetraacetic acid (EDTA) (0.05%, 0.5 mM EDTA) after reaching 80–90% confluence. The digestion was terminated with D-MEM/F-12 medium containing 10% FBS when the cells were observed to be round or a few cells were coming off the well, as assessed by phase contrast microscopy. The cell suspension was collected and centrifuged (167 × g, 5 min). The cells were diluted to a concentration of 5 × 10⁵ cells/mL with D-MEM/F-12 containing 20% FBS, for subculture in flasks.

Immunohistochemistry using a mouse antihuman cytokeratin (Wuhan Boster Biological Technology Ltd, Wuhan, China) was used to confirm that the cells cultured were epithelial cells, as previously described. The positive and negative controls were embryonic human intestinal epithelium and mouse 3T3 feeder cells, respectively.

Cell proliferation assay

Cell proliferation was measured with methylthiazolyl-tetrazolium (MTT). The MTT cell proliferation assay is a colorimetric system that measures the reduction of a tetrazolium component into an insoluble formazan product by the mitochondria of viable cells. The third subcultures were suspended with D-MEM/F-12 containing 10% FBS. The cells were placed into a 96-well plate at 1 × 10⁵ cells/mL and incubated at 37 °C in a 5% CO₂ atmosphere for 24 h. The culture medium in each well was aspirated, and the cells were washed with D-MEM/F-12 three times. Then, culture media containing 1, 10, 50, or 100 ng/mL bFGF (PeproTech, Rocky Hill, NJ, USA, through Wuhan Boster Biological Technology Ltd) was added to the culture wells. The cells were continually cultured at 37 °C in a humidified 5% CO₂ incubator. One hour before cell harvest, the culture media were replaced with serum-free media, and 20 μL of MTT (5 mg/mL in PBS; Sigma) was added to each well. The cells were incubated for an additional 4 h, and the medium was replaced with acidic isopropyl alcohol (0.04 N HCl in absolute isopropyl alcohol) to dissolve the colored crystals. The MTT was changed to a blue compound by the activity of mitochondrial dehydrogenase. The samples were read using an enzyme-linked immunosorbent assay plate reader (Wellscan MK3; Labsystems, Vantaa, Finland) at a wavelength of 570 nm, with background subtraction at 650 nm. The amount of color produced, normalized with the background, was directly proportional to the number of viable cells, and is represented as the proliferation index. All assays were repeated four times, with six samples each.

Corneal epithelial wound

Twenty-four hybrid dogs (12 male, 12 female), about 1 year old, came from the experimental animal center, Huazhong Agricultural University. Before inclusion in the study, all dogs underwent a complete ophthalmic examination, including indirect ophthalmoscopy, slit-lamp biomicroscopy, Schirmer's tear test, and fluorescein staining. The dogs had no corneal epithelial abnormalities, lid conformational defects, distichiasis, ectopic cilia, or any clinical evidence of systemic disease. The experimental dogs were randomly allotted into four treatment groups of six dogs each. All the animals were dewormed and allowed to become accustomed to being approached for a week. Feed was withheld for 24 h before the start of the experiment. The animal use and experimental protocols were approved by the Animal Care and Use Committee of Huazhong Agricultural University.

A central corneal epithelial wound on one eye of each dog was made as previously described. Dogs were anesthetized by intramuscular injection of ketamine hydrochloride (10 mg/kg) and xylazine (6 mg/kg), and by topical administration of 1% lidocaine hydrochloride. The ocular surface was disinfected using a 1% povidine–iodine solution. Upper and lower eyelids were braced using an eyelid speculum. Excessive moisture on the corneal surface was absorbed using a sterile cotton swab, and two sterilized surgical sutures were sutured in the upper and lower cornea-scleral limbus for traction. A 6-mm-diameter corneal trephine was placed on the cornea with the pupil at the center, and the traction sutures were pulled by an assistant to make the corneal trephine touch the surface of the cornea. A circular incision was made on the cornea by turning the trephine to a depth of approximately one-third of the thickness of the cornea. An alcohol solution (20%, 0.15 mL) was added to the center hole of the corneal trephine and held there for 20 s for facilitating the dissection of the epithelium from its underlying stroma. After the ocular surface was washed with sterile saline and excessive fluid on it was absorbed by a sterile cotton swab, an excisional trephine wound was created by removing the epithelium and superficial stroma using a pair of corneal scissors and an iris spatula.

Topical application of bFGF

Four concentrations of bFGF, 0, 100, 500, and 1000 ng/mL, were dropped onto the affected eyes of the dogs in the four
Fluorescein staining was used to assess the closure of the corneal epithelial wound. A drop of 0.5% fluorescein sodium (Sigma) was added to the affected eye. The outline of the wound area clearly appeared because fluorescein adhered to the stromal tissue, and the eyes were photographed before and at 24, 72, 120, and 168 h after initial bFGF topical administration. Then, the wound area was measured using the Image Measurement Analysis System software package (Axioskop MOT, Carl Zeiss, Jena, Germany). Topical symptoms such as discharge, inflammation of eyes, and neovascularization in the cornea and conjunctiva were observed and documented every day. The wound healing rate was expressed as the healing area divided by the initial wound area.

Statistics
The arithmetic mean and standard error of the mean were calculated for each treatment. Data analysis was performed using analysis of variance (ANOVA) with the SAS software (SAS Institute Inc., Cary, NC, USA). Differences between treatment means were evaluated by Dunnett’s post hoc test, after a significant F-test. A P-value of < 0.05 was considered to be statistically significant.

RESULTS
Morphology of canine corneal epithelial cells in culture
Canine corneal epithelial cells floated out from and formed a halo around the corneal pieces at 1 day after culture. The cells became round or ellipse-shaped, with abundant cytoplasm. The cultured cells became confluent after 2 weeks of removal of the corneal pieces. In the subculture, canine corneal epithelial cells became irregular, and their arrangement showed a slab-stone-like pattern (Fig. 1).

Effects of bFGF on canine corneal cell proliferation
The results of the MTT cell proliferation assay are shown in Fig. 2. In corneal cell culture, bFGF treatment resulted in a significant increase (P < 0.01) in epithelial proliferation at 24 h after culture, except 1 ng/mL bFGF. Cell proliferation induced by bFGF at a concentration of 10 ng/mL was higher (P < 0.01) than that induced by bFGF at 1 or 50 ng/mL at 48 and 96 h.
Wound closure rates
To evaluate the effects of bFGF treatment on wound healing, the wound healing rate was plotted vs. time for each treatment (Fig. 3). The mean healing rate in bFGF-treated corneas was significantly greater (at days 1, 3 and 5, \( P < 0.01 \); at day 7, \( P < 0.05 \) ) than that in control corneas after bFGF topical application. Both 500 and 1000 ng/mL bFGF increased the wound healing rate significantly (at day 3, \( P < 0.01 \); at day 5, \( P < 0.05 \) ) compared to 100 ng/mL bFGF. All corneal wounds in the bFGF-treated groups closed within 7 days, whereas two cases in the controls showed poor healing until day 11 after the corneal wound. We also determined the times at which 50% of the initial wound area had healed. The 50% level was reached after an average of 2.1 ± 0.3, 1.8 ± 0.2, and 1.7 ± 0.2 days after treatment with bFGF 100, 500, and 1000 ng/mL, respectively, and after 2.7 ± 0.4 days in the control.

DISCUSSION
The effects of bFGF on corneal cell proliferation are still controversial. Although many studies have documented the ability of bFGF to stimulate the proliferation of corneal epithelial cells of cows, rabbits, and humans in vitro, \(^1\,^9\) a few studies have also shown no observable effect of bFGF on corneal cells from cattle and humans. \(^10\) The activity of an individual growth factor may depend on species, cell line (and age), state of development or differentiation of its target cells, cell density, concentration of the agents, presence or absence of other peptides or growth factors, and culture conditions. \(^4\) bFGF is thought to stimulate canine corneal epithelial cell activity, but data are limited data on this issue. In this study, bFGF promoted canine corneal epithelial cell proliferation significantly, and showed a maximal effect at 10 ng/mL. This optimal concentration in corneal epithelial cell culture is similar to that seen with some other tissues, such as the patellar tendon (10 ng/mL) and atrophic salivary glands. \(^16\,\,^17\) Note that in this study, the cell proliferating effect was diminished when bFGF was used at higher concentrations (50 and 100 ng/mL), which has also been reported in other tissues. \(^17\) Two possible explanations for this are the toxicity and reinforcement of a low-affinity receptor, which alters the cellular response. \(^7\,\,^16\)

Although in vitro experiments have demonstrated the direct activity of bFGF in promoting the proliferation of canine corneal epithelial cells, the role that bFGF plays in the in vivo situation is not fully understood. A conventional monolayer culture does not satisfactorily provide the component cells with a physiological environment because the corneal surface consists of a multilayered epithelium, rather than a single cell layer; corneal epithelial cells exist in close contact with underlying keratocytes, which have a great influence on the differentiation of epithelial cells; and epithelial centripetal migration is absent in a monolayer cell culture. \(^18–20\) This centripetal migration is characteristic of limbal basal epithelial cells that undergo cell division and migrate toward the central cornea and upward toward the corneal surface. Corneal organ culture has been used to investigate the involvement of growth factors in corneal wound healing. However, such submerged organ culture methods can suffer from a reduction in epithelial cell layers and epithelial and stromal edema, and endothelial and keratocyte deterioration. \(^10,\,^21\)

Excisional trephine wounds are considered to be the most appropriate model for studying the involvement of growth factors in vivo. Such wounds lead to the removal of all the corneal epithelial cell layers, along with a portion of the underlying stroma in the demarcated zone. Fluorescein staining is commonly used to assess the re-epithelialization of corneal wounds because it adheres to the stromal tissue, not to normal epithelial cells. In the present in vivo study, concentrations of bFGF in the topical application were far greater than those typically used in cell culture because the bFGF was diluted by tear fluid and cleared by eyelid blinking. bFGF at concentrations from 100 to 1000 ng/mL showed a dose-dependent effect on corneal re-epithelialization. Because some growth factors, such as epidermal growth factor (EGF), herpetic growth factor, and transforming growth factor (TGF) in tear and stromal tissue are mitogenic to epithelial cells, bFGF may promote canine corneal wound healing by accelerating epithelial proliferation, alone or in combination with other growth factors in tear and stroma.

FGFs are pleiotropic factors acting on different cell types, including vascular and capillary endothelial cells, and are capable of inducing an angiogenic response. \(^22,\,^23\) Neovascularization, the process of new blood vessel formation from preexisting ones, can reduce vision. In our study, no neovascularization in bFGF-treated corneas was observed. One possible explanation for this may be that the corneal re-epithelialization was terminated before neovascularization was initiated because the cellular repopulation process in the

Figure 3. Wound healing rate of canine corneas (mean ± SD). The mean wound healing rate is expressed as a percentage of the initial wound area. The mean healing rate in bFGF-treated corneas was significantly greater (at days 1, 3, and 5, \( P < 0.01 \); at day 7, \( P < 0.05 \) ) than that in the control corneas. Both 500 and 1000 ng/mL bFGF increased wound healing rates significantly (at day 3, \( P < 0.01 \); at day 5, \( P < 0.05 \) ) compared to 100 ng/mL bFGF.
corneal epithelium was much faster than that of corneal vascular endothelium. Additionally, neovascularization induced by bFGF would be affected by other growth factors and cytokines, such as transforming growth factor-beta (TGFβ), vascular endothelial growth factor (VEGF), and interleukin-4 (IL-4). Moreover, angiogenesis can be blocked by TGFβ and IL-4, but induced by VEGF. Although no angiogenic response was observed in our experiment, additional studies are required to determine the long-term consequences of administering bFGF to corneas.

Corneal wound healing is an essential prerequisite for restoring corneal integrity, preventing infection, and maintaining vision after injury. The healing process is believed to involve a variety of growth factors, and bFGF, EGF, and TGFβ-1 are the ones most commonly involved in wound healing. bFGF acts on a wide variety of cells of neuroectodermal and mesodermal origins and influences cell growth, migration, differentiation, and regeneration. EGF accelerated epithelial proliferation actively, but did not induce lamellar structural differentiation of the epithelium. TGFβ-1 affected corneal epithelial cells through keratocyte-mediated stimulation. Although our experiments demonstrated that bFGF was effective in promoting the healing of canine corneal epithelium wounds, a cocktail of growth factors looks more promising because it may more closely mimic the natural occurrence of growth factors in the natural healing process than any single substance. In addition, growth factors may interact in a complex way with each other, and combinations of growth factors may act additively, synergistically, or negatively, with respect to the activity of an individual growth factor. Considering its pleiotrophic functions, bFGF should be a main component of that cocktail.

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