The Inhibitory Effects of Bevacizumab Eye Drops on NGF Expression and Corneal Wound Healing in Rats

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PURPOSE. To investigate the effect of bevacizumab eye drops on corneal epithelial wound healing in rats.

METHODS. One hundred twenty Sprague-Dawley male rat corneas were divided into two groups and de-epithelialized with a microblade. The bevacizumab group was treated with 5% bevacizumab and antibiotic 0.5% levofloxacin eye drops four times daily and the control group with antibiotic eye drops only. Corneal wound healing was evaluated by fluorescein staining at initial wounding and 24, 48, and 72 hours after epithelial debridement. Nerve growth factor (NGF) and vascular endothelial growth factor (VEGF) proteins were measured in rat corneas by ELISA. Immunofluorescent staining for NGF and VEGF was performed in rat corneas. NGF mRNA and VEGF mRNA was measured in rat corneas by real-time PCR.

RESULTS. The corneal wound healing rate was decreased in the bevacizumab group compared with that in the control group. Twenty-four, 48, and 72 hours after epithelial debridement, corneal NGF and VEGF proteins in the bevacizumab group were lower than that in the control group (P < 0.05). Immunofluorescent staining showed that NGF and VEGF expression were stronger in the control group than in the bevacizumab group. NGF mRNA and VEGF mRNA levels in the bevacizumab group were also lower than in the control group (P < 0.05).

CONCLUSIONS. After corneal epithelial damage, VEGF and NGF increased normally in the rat corneas. In contrast, when VEGF was inhibited by bevacizumab eye drops, the wound healing rate was decreased, and NGF was downregulated. Bevacizumab eye drops have an inhibitory effect on corneal wound healing in rats. (Invest Ophthalmol Vis Sci. 2010;51:4569 – 4573) DOI:10.1167/iovs.09-4937

Several growth factor receptors, including epidermal growth factor, transforming growth factor-β, keratinocyte growth factor, hepatocyte growth factor, fibroblast growth factor, and platelet-derived growth factor play important roles in corneal wound healing, by mediating the proliferation of epithelial and stromal tissue and inducing the remodeling of the extracellular matrix (ECM).¹

Nerve growth factor (NGF) is a polypeptide identified in the early 1950s by R. Levi-Montalcini. It is essential for the survival and growth of sympathetic and sensory neurons and for differentiation of neurons in the central nervous system.² It has been found in ocular surfaces such as cornea, conjunctiva, tear film, and lacrimal glands.³ ⁴ In rabbit cells in vitro, it promotes the proliferation of corneal epithelial cells and accelerates the rate of epithelial wound healing.⁵ Recent studies have shown that the topical application of NGF leads to the repair of neurotrophic corneal ulcers, with the recovery of corneal surface sensitivity.⁶ ⁷

Vascular endothelial growth factor (VEGF) has long been known to be a potent stimulator of the proliferation of blood vessels. It is an endothelial cell mitogen and angiogenic factor and a potent mediator of vascular permeability.⁸ It has also been implicated as an important player in nerve growth. In vitro experiments have shown that VEGF and its receptors are expressed by neurons and stimulate proliferation of cortical neurons, protect central and peripheral neurons from hypoxia-induced death, and promote axonal outgrowth in peripheral neurons.⁹ Results of studies indicated that VEGF plays a role in mediating corneal nerve repair¹⁰ and that anti-VEGF delays corneal epithelial wound healing in rabbits.¹¹

However, implications for anti-VEGF therapy for corneal wound healing leading to NGF expression have not been explored. The objective of this study was to investigate whether anti-VEGF antibodies such as bevacizumab have inhibitory effects on corneal epithelial wound healing and NGF expression in rats.

MATERIALS AND METHODS

Animals

For the wound-healing and eye drop application assay, 120 male Sprague-Dawley rats (weight range, −250–300 g) were used. The animals were divided into two groups of 60 rats each. The animal care committee of Pucheon St. Mary’s Hospital in the Catholic University Medical Center, in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, approved all animal protocols.

In Vivo Animal Model of Corneal Epithelial Wound Healing

Rats were deeply anesthetized by intraperitoneal injection of 50 mg/kg tiletamine plus zolazepam (Zoletil; Virbac, Carros, France) and 15 mg/kg xylazine hydrochloride (Rompun; Bayer, Leverkusen, Germany). The 5-mm trephine demarcated the central corneal region of the rat eye but did not encroach on the limbus or conjunctiva. The eye was stabilized by grasping the conjunctiva with forceps, and the corneal epithelium was scraped with a microblade (Sharpoint 78-6900; Surgical Specialties, Reading, PA) under a microscope.

The bevacizumab group was treated with 5% bevacizumab (Avastin; Hoffmann LaRoche, Basel, Switzerland) and antibiotic (0.5% levofloxacin; Cravit; Daiichi Pharmaceutical Co. Ltd., Tokyo, Japan) eye drops four times daily and the control group with antibiotic eye drops only; both regimens lasted for 3 days. Bevacizumab eye drops were prepared in the hospital pharmacy from the standard solution diluted in 0.9% saline to a concentration of 5 mg/mL. The eye drops were...
stored at −20°C for up to 14 days; after opening, they were used within 1 day and kept at 4°C during that time. Wound size was determined by staining the corneas with 1% fluorescein and photographing them at 0, 24, 48, and 72 hours after debridement. The area of the corneal scrape wound was quantified from the photographs by using a computer-assisted image analyzer (Cell^F; Olympus GmbH, Mainz, Germany). We calculated the healing rate by the following formula: initial wound size minus wound area at each time point.

**Protein Extraction and ELISA for VEGF and NGF Detection from Cornea**

The cornea was trephined with a 4.0-mm-diameter trephine before epithelial debridement and at 24, 48, and 72 hours after wounding (n = 20 per group). The excised cornea was frozen at −80°C and homogenized by thawing and freezing three times in lysis buffer consisting of 50 mM PIPES/KOH (pH 6.5), 2 mM EDTA, 0.1% CHAPS, 20 mg/mL leupeptin, 10 mg/mL pepstatin, 10 mg/mL aprotonin, 5 mM DTT, and the protease inhibitors. Homogenates were centrifuged at 14,000g for 30 minutes. The resultant supernatants were used for the VEGF and NGF assay. VEGF and NGF levels were measured before wounding and 24, 48, and 72 hours after wounding, by an ELISA development system (Duoset; R&D Systems, Minneapolis, MN) for rats. Before ELISA, total protein was measured by using the Bradford method.13 The ratio of VEGF and NGF to total protein (picograms per milligram) was calculated.

**Immunofluorescence Staining**

Twenty rats in each group were used for immunofluorescent staining. Rat corneas before epithelial debridement and at 24, 48, and 72 hours after wounding were embedded in optical cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA), frozen, and cut into cryo-sections (6 μm). The frozen sections were thawed, dehydrated, and fixed in cold methanol/acetone (1:1) at −30°C for 10 minutes.

The sections were incubated with blocking solution (normal goat serum; Zymed, South San Francisco, CA) for 30 minutes. Immunofluorescent staining was performed with affinity-purified mouse monoclonal antibodies against VEGF (C-1; Santa Cruz Biotechnology, Santa Cruz, CA) and NGF (M20; Santa Cruz Biotechnology), according to a previously reported method.14,15 Primary antibodies against VEGF and NGF were used at a dilution of 1:50 and were incubated for 1 hour at room temperature. Secondary antibodies (goat anti-mouse IgG-FITC [1:100]; Santa Cruz Biotechnology) were then applied and incubated in a dark chamber for 45 minutes, followed by counterstaining with DAPI (4,6-diamidino-2-phenylindole, dihydrochloride; 1:1000) for 5 minutes. After the sections were washed with PBS, antifade mounting medium (Gel Mount; Biomedical Corp., Foster City, CA) and a coverslip were applied. Staining was evaluated by fluorescence microscope (BX50; Olympus, Tokyo, Japan) and photographed with a digital camera (DP70-Set2; Olympus).

**Real-Time Polymerase Chain Reaction**

Real-time polymerase chain reaction (PCR) amplification and relative quantification of VEGF and NGF were performed with gene expression assays (TaqMan master mix; Roche), and 10 ng cDNA was used in each reaction. Glyceraldehyde-3-phosphate dehydrogenase (GADPH) served as an endogenous control. A no-template control was included in each quantitative real-time PCR experiment to confirm the absence of DNA contamination in the reagents used for amplification. The results were analyzed on the thermal cycler instrument software (Light Cycler ver. 1.2; Roche, Germany) and were presented as expression relative to the average in a healthy cornea. Statistical analysis of quantitative real-time PCR data were performed with repeated-measures ANOVA (StatCrunch online statistical software http://www.statcrunch.com/ Pearson Education Core Technology Group, Boston, MA). P ≤ 0.05 was the level of significance. All samples were analyzed separately, and the average gene expression value (geometric mean) of each tissue type is presented. Data are expressed as RQ (relative quantity), and differences are shown in the figures as the expression ratio of the normalized target gene, according to the software-obtained results.

**Statistical Analysis**

Data were compared between groups and are expressed as the mean ± SD. Statistical analysis was performed with repeated-measures ANOVA, to determine whether there were significant changes in the corneal wound-healing rate, corneal NGF and VEGF levels, or NGF and VEGF mRNA levels after epithelial debridement; the significance level was P < 0.05.

**RESULTS**

**Effect of Bevacizumab on Corneal Epithelial Wound Healing**

The initial area of the abrasion ranged from 19.1 to 20.3 mm² and corresponded to corneal injuries 5.0 mm in diameter. Quantitative analysis revealed that the healed area in the bevacizumab group (5.25 ± 1.1 mm²) was significantly smaller than in the control group after 24, 48, and 72 hours after debridement, respectively (P < 0.05; n = 20 per group; repeated-measures ANOVA). Data represent the mean ± SD.

![Figure 1](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932963/ on 11/25/2018)
than that in the control group (8.01 ± 2.0 mm²) and the defect area in the bevacizumab group (14.35 ± 2.3 mm²) was significantly larger than that in the control group (11.75 ± 1.9 mm²) at 24 hours after epithelial debridement (P < 0.05).

At 48 hours, the healed area in the bevacizumab group (8.75 ± 2.8 mm²) was significantly smaller than that in the control group (13.29 ± 3.1 mm²), and the defect area in the bevacizumab group (11.53 ± 2.1 mm²) was significantly larger than that in the control group (5.25 ± 1.3 mm²; P < 0.05).

At 72 hours, the healed area in the bevacizumab group (13.97 ± 2.9 mm²) was significantly smaller than that in the control group (17.28 ± 2.1 mm²), and the defect area in the bevacizumab group (6.32 ± 1.6 mm²) was significantly larger than that in the control group (1.09 ± 0.5 mm²; P < 0.05).

Conical photographs in the bevacizumab group showed subepithelial haze at 24 hours, subepithelial fibrosis at 48 hours, and some stromal necrosis at 72 hours after debridement. In the control group, however, epithelial defects were healed without any haze or opacity during the healing period (Fig. 3).

**VEGF and NGF Protein in Cornea**

VEGF levels (152.26 ± 22.7, 113.37 ± 18.6, and 159.68 ± 26.3 pg/mL) in the bevacizumab group were significantly lower than in the control group (289.25 ± 38.9, 404.75 ± 47.1, and 237.89 ± 25.3 pg/mL) at 24, 48, and 72 hours after debridement, respectively (P < 0.05; Fig. 4A). The NGF levels (3.24 ± 0.5, 6.15 ± 1.1, and 9.52 ± 2.7 pg/mL) in the bevacizumab group were also significantly lower than in the control group (7.97 ± 0.7, 45.91 ± 3.8, and 54.53 ± 4.5 pg/mL) at 24, 48, and 72 hours after debridement, respectively (P < 0.05; Fig. 4B).

**Immunofluorescent Staining**

Immunofluorescent stain showed that VEGF and NGF staining was stronger in the control cornea than in the bevacizumab cornea at 24, 48, and 72 hours after debridement. At 48 hours, immunofluorescent staining of VEGF and NGF was intense in the control corneas (Fig. 5).

**Real-Time Polymerase Chain Reaction**

Real-time PCR showed that levels of VEGF and NGF mRNA transcripts were 2- to 6-fold higher in the control group than in the bevacizumab group. VEGF and NGF mRNA expression in the bevacizumab group was significantly less than in the control group at 24, 48, and 72 hours after debridement (P < 0.05).

**DISCUSSION**

Bevacizumab (Avastin; Hoffman LaRoche), a full-length recombinant humanized monoclonal antibody against VEGF, has been approved for systemic treatment of colorectal cancers. It now is widely used to treat age-related maculopathy, proliferative retinopathy, and neovascular glaucoma.

Topical bevacizumab has successfully reduced corneal neovascularization in animal models. Subsequently, a reduction in corneal neovascularization with topical and subconjunctival bevacizumab in human eyes has been reported.

Several studies have demonstrated that administration of bevacizumab to the cornea is relatively safe. Chalam et al. demonstrated that 0.1, 1, and 2 mg/mL bevacizumab was not cytotoxic to epithelial and fibroblast cell lines in cell culture. Other studies have also demonstrated that topical bevacizumab on the corneal surface does not have significant adverse effects on normal corneal epithelial wound healing, normal corneal integrity, or normal nerve fiber density.

However, Kim et al. have reported that bevacizumab delays corneal epithelial wound healing and inhibits integrin expression. When bevacizumab is used to reduce the development of new corneal vessels, slight delays in epithelial wound healing are possible, and cellular proliferation is to be expected. Also, a spontaneous epithelial defect in the central
Recent studies have demonstrated that capillary sprouting is associated with an increased expression of VEGF/VEGFR-2 in the brain of mdx mice.\(^{21}\)

NGF accelerates the corneal epithelial proliferation through its high-affinity receptor, TrkA.\(^{22}\) Corneal NGF levels were also shown to increase after corneal epithelial injury in the rat.\(^{23}\)

It is possible that endogenous NGF plays an important role in epithelial healing by modulating the proliferation and differentiation of epithelial cells in rabbit corneal epithelium.\(^{6}\)

We hypothesized that bevacizumab would inhibit NGF expression after corneal epithelial injury, and wound healing would be delayed because of low NGF levels.

In our study, the healed area in the bevacizumab group was significantly smaller than that in the control group at 24, 48, and 72 hours after epithelial debridement (Fig. 1 and 2). Kim et al.\(^{12}\) also demonstrated that bevacizumab topical eye drops at 1.0, 1.5, 2.5, or 5 mg/mL delayed rabbit corneal epithelial healing. Corneal photographs in the bevacizumab group in our study showed subepithelial haze and fibrosis and some stromal thinning (Fig. 3). Kim et al.\(^{19}\) reported similar phenomena.

In ELISA, corneal NGF and VEGF proteins in the control group were higher than that in the bevacizumab group (\(P < 0.05\); Figs. 4A, 4B). Immunofluorescent staining showed that NGF and VEGF staining was stronger in the control group than in the bevacizumab group (Fig. 5). Expression of NGF and VEGF in the bevacizumab group was also downregulated compared to the control (\(P < 0.05\); Fig. 6).

In this study, we demonstrated an increased expression of NGF and VEGF after corneal epithelial debridement in control rats, and NGF expression was downregulated by VEGF inhibition in bevacizumab-treated rats. The corneal defect with subepithelial haze in the bevacizumab group was similar to that in neurotrophic keratitis. Bonini et al.\(^{7}\) reported that nerve growth factor eye drops improved corneal sensitivity and promoted corneal epithelial healing in both moderate and severe neurotrophic keratitis. We suggest that NGF depletion by bevacizumab eye drops may cause neurotrophic keratitis-like ulcer.

Recent studies have demonstrated that capillary sprouting is promoted by NGF via the release of VEGF\(^{24}\) and that overexpression of NGF in brown adipose tissue of NGF-transgenic mice elevated both mRNA and protein levels of VEGF and VEGFRs.\(^{25}\) The activated VEGF system stimulated angiogenesis, and this effect was blocked by NGF neutralizing antibodies.\(^{25}\)

**FIGURE 4.** Corneal VEGF (A) and NGF (B) concentrations after epithelial debridement in the bevacizumab and control groups. Corneal VEGF and NGF concentrations in the bevacizumab group were decreased compared with the levels in the control group at 24, 48, and 72 hours after debridement (\(P < 0.05\); \(n = 20\) per group; repeated-measures ANOVA). Data represent the mean ± SD.

**FIGURE 5.** Immunofluorescent stain of VEGF and NGF in bevacizumab and control cornea 48 hours after epithelial debridement. DAPI was used as a nuclear stain (blue). At 48 hours, immunofluorescent stains of VEGF and NGF were intense in the control corneas, but no stain was present in the bevacizumab-treated corneas. Original magnification, ×200.

**FIGURE 6.** Real-time PCR of the relative expression of VEGF and NGF mRNA in the bevacizumab group compared with the control group. The control VEGF and NGF were regarded as the standards (RQ = 1). VEGF and NGF mRNA expression in the bevacizumab group was significantly decreased compared with that in the control group at 24, 48, and 72 hours after debridement (\(P < 0.05\); \(n = 20\) per group; repeated-measures ANOVA). Data represent the mean ± SD.
Even without a mechanistic demonstration supplied herein, these data suggest the existence of a paracrine loop between NGF and VEGF, as has been demonstrated by other groups.\textsuperscript{20,21}

Bock et al.\textsuperscript{14} demonstrated no difference in corneal nerve density of corneas treated with anti-VEGF antibodies, but Yu et al.\textsuperscript{11} reported that blockade of VEGF signaling with anti-VEGF antibody reduced the growth of cultured neurons by 17\% and regeneration of subbasal neurons by 23\% in living mice. This result was similar to that in our study in which NGF expression was downregulated by inhibiting VEGF in rats.

Bevacizumab may be nontoxic to human corneal epithelial and fibroblast cells in cell culture,\textsuperscript{18} but bevacizumab delayed corneal epithelial wound healing and inhibited integrin expression in the rabbit.\textsuperscript{12} This result was similar to that in our study in which wound healing was delayed by bevacizumab in the rat.

To the best of our knowledge, our study is the first to demonstrate that corneal wound healing may be delayed by downregulated NGF, caused by VEGF inhibition in rats.

In conclusion, VEGF and NGF are normally increased in rat corneas after corneal epithelial defect. In contrast, wound-healing rates are decreased, and NGF is downregulated when VEGF is inhibited by bevacizumab eye drops. NGF depletion could cause neurotrophic keratitis-like ulcer to develop in the bevacizumab-treated eye. Bevacizumab eye drops thus have an inhibitory effect on corneal wound healing in rats. We hypothesize that VEGF has a positive effect on corneal wound healing through a paracrine loop between NGF and VEGF. Therefore, further study of the effects of VEGF on wound healing is necessary.

\textbf{References}