Subconjunctival Injection of Bevacizumab (Avastin) on Corneal Neovascularization in Different Rabbit Models of Corneal Angiogenesis

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PURPOSE. Bevacizumab is a potent recombinant humanized monoclonal antibody directed against vascular endothelial growth factor (VEGF). The purpose of this study was to evaluate the therapeutic effect of subconjunctival injection of bevacizumab on corneal neovascularization (NV) in different rabbit models.

METHODS. Several rabbit models of corneal NV were used, including (1) a corneal micropocket assay with VEGF pellet, (2) a corneal micropocket assay with basic fibroblast growth factor (b-FGF) pellets, (3) mechanical limbal injury–induced corneal NV, and (4) an alkali-induced model of corneal NV. Subconjunctival injections of bevacizumab (0.25–2.5 mg) were applied twice per week for 2 to 8 weeks. Digital photographs of the cornea were analyzed to determine the length of corneal NV and the area of cornea covered by NV as a percentage of the total corneal area. Immunohistochemical staining with anti-human IgG antibody labeled with Cy3 was used to determine the detection of intracorneal distribution of bevacizumab after injection.

RESULTS. Subconjunctival injection of bevacizumab caused significant inhibition of corneal NV formation as measured by length or surface area in all animal models (P < 0.05). No significant ocular complications were found. Staining of bevacizumab was found in the corneal stroma for 3 to at least 14 days in the different rabbit models.

CONCLUSIONS. Subconjunctival injection of bevacizumab is effective in inhibiting corneal NV in several rabbit models. Bevacizumab may diffuse into the corneal stroma and persist for a few days after injection. It may be useful in preventing corneal NV in the acute phase of various kinds of corneal inflammation. (Invest Ophthalmol Vis Sci. 2009;50:1659–1665) DOI:10.1167/iovs.08-1997

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Corneal neovascularization (NV), a sequela of anterior segment inflammation, may lead to corneal opacity, reduced visual acuity, graft rejection after penetrating keratoplasty, and unwanted bleeding during creation of the flap in laser in situ keratomileusis (LASIK). Although various compounds, including steroids, cyclosporin, indomethacin, methotrexate, rapamycin, low-molecular-weight heparin sulfate, and thalidomide, have been shown to inhibit corneal NV in clinical and experimental situations, they may induce complications and are not always effective.

During the formation of corneal NV, an upregulation of angiogenic factors in association with the downregulation of antiangiogenic factors is usually found. Among the angiogenic factors, vascular endothelial growth factor (VEGF) is well known for its potency. It has been shown to stimulate choroidal NV in pathologic myopia, and exudative age-related macular degeneration (ARMD). Recently, the effects of subconjunctival injection of bevacizumab in the inhibition of corneal NV have been reported. In this study, we tested the effects of subconjunctival injection of bevacizumab in different rabbit models of corneal NV. We also used immunohistochemical staining to show the intracorneal diffusion of bevacizumab after subconjunctival injection, which can explain the effectiveness of this treatment strategy.

MATERIALS AND METHODS

Chemicals and Antibodies

Bevacizumab (Avastin, 100 mg/4 mL) was purchased from Roche Pharmaceutical (Welwyn Garden City, UK). Human recombinant basic fibroblast growth factor (bFGF), human recombinant VEGF, sucrafate, and hydron polymer (poly-heme) were purchased from Sigma-Aldrich (St. Louis, MO). Mouse anti-human CD31, ready to use for staining of vascular endothelium, was purchased from Dako (Carpinteria, CA). Donkey anti-human IgG labeled with Cy3, which can bind to the heavy and light chains of the humanized IgG and was used for the detection of bevacizumab, was purchased from Jackson Immunoresearch (West Grove, PA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Animals

A total of 120 female New Zealand albino rabbits (3.0–3.5 kg, 6 months of age) were used. Use, care, and treatment of all animals were in strict agreement with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All experimental procedures were approved by the Committee for Animal Research of the National Taiwan
University Hospital. All procedures were performed with animals under general anesthesia induced by intramuscular injection of ketamine hydrochloride (35 mg/kg) and xylazine hydrochloride (5 mg/kg), and the eyes were topically anesthetized with 0.5% proparacaine hydrochloride (Alcain; Alcon, Fort Worth, TX) before manipulation. The right eye of each animal was used for the experiments, whereas the left eyes were not treated. Six eyes were included in each group. To increase the reproducibility of the surgeries to induce corneal NV, all procedures were performed in the same manner in all eyes by a single investigator.

The corneas were inspected under the microscope, and observations were recorded daily after induction of corneal NV until the completion of observation (2 weeks in the corneal micropocket NV models, 4 weeks in the alkali-induced NV model, and 8 weeks in the limbal injury-induced NV model).

**Corneal Micropocket NV Models**

For assessment of NV caused by VEGF and bFGF in vivo, we used a corneal micropocket assay. In brief, an aliquot of bFGF or VEGF was added to 2 μL of 12% (wt/vol) hydron polymer (poly-HEME) in ethanol. Four-microliter aliquots of the mixture were placed on parafilm (American National Can, Greenwich, CT) and allowed to dry to produce pellets, each containing 500 ng of bFGF or 100 ng of VEGF. A partial corneal incision was made at the limbus, and dissection to the corneal stroma was performed up to 4 mm from the limbus. The pellet was placed in this pocket (referred to as day 0).

**Alkali Induced-Corneal NV Model**

Induction of the alkali-induced corneal NV model was performed as described by Ormerod et al. with some modifications. In brief, a 5.5-mm-diameter circular filter disc was incubated with 20 μL of 1 N NaOH for 60 seconds. The filter disc was then placed on the cornea surface of anesthetized rabbits under the surgical microscope for 30 seconds. The ocular surface was then irrigated with 25 mL physiological saline.

**Limbal Injury–induced Corneal NV Model**

The limbal injury–induced corneal NV model was produced by surgical removal of the circumferential limbal tissue 2 mm into the cornea and 3 mm into the conjunctiva. Superficial lamellar keratectomy was also performed to remove the entire corneal epithelium. In this model, corneal NV occurred within 2 weeks after surgery.

**Subconjunctival Injection of Bevacizumab**

With the animal under systemic anesthesia, subconjunctival injection of bevacizumab into the upper and lower bulbar conjunctiva was performed twice per week until the completion of the observation (2 weeks in the corneal micropocket NV models, 4 weeks in the alkali-induced corneal NV model, and 8 weeks in the limbal injury-induced corneal NV model). In the high-dose group, 1.25 mg each in the upper and lower subconjunctiva (total dose, 2.5 mg, 0.1 mL) was injected. In the low-dose group, 0.125 mg each in the upper and lower subconjunctiva (total dose, 0.25 mg, diluted with normal saline to 0.1 mL) was injected. In the control group, an equal amount of normal saline was injected in the same manner.

For detection of the intracorneal diffusion of bevacizumab after subconjunctival injection, a single dose of 0.05 mL (1.25 mg) of bevacizumab was injected into the upper subconjunctival space. At postoperative days 1, 2, 3, 7, and 14, the eyes were enucleated for further immunohistochemical studies.

**Record of NV or Corneal Epithelial Defects and Statistical Analysis**

Corneal NV and epithelial defects were photographed with an operative microscope (OPMI Pico; Carl Zeiss Meditec, Jena, Germany) every day after the induction of corneal NV. Fluorescein staining was used to measure corneal epithelial defects. Measurements of the length and the area of corneal NV/corneal epithelial defects were made by an image processing and analysis software program (Image J ver. 1.37; http://rsb.info.nih.gov/ij/index.html) developed by Wayne Rasband, the Research Services Branch, National Institutes of Health, Bethesda, MD). The area of neovascularization and epithelial defects was measured in pixels, and its ratio to the entire corneal area was determined as the percentage of corneal NV and epithelial defects. The length of corneal NV was considered the distance from the limbal vascular plexus to the leading point of the new vessel. This image analysis system is modified from previous studies. All photographs were evaluated by two blinded observers. Experimental data were analyzed using one-way analysis of variance (ANOVA); the Dunnett multiple comparison test, and Student’s paired t test. The results were expressed as the mean ± SEM. *P* < 0.05 was considered to be statistically significant.

**Immunohistochemistry**

For immunohistochemistry staining, rabbit eyeballs were cryopreserved in OCT embedding medium, and 8-μm cryosections were obtained. Sections were air dried at room temperature for 30 minutes and then fixed in cold acetone for 15 minutes. Sections were then permeabilized with 0.4% Triton X-100 for 10 minutes and were blocked by 1% fetal bovine serum (FBS) at 37°C for 1 hour. For visualization of vascular endothelial cells, sections were incubated with primary antibody (CD31) at room temperature for 2 hours. After incubation with the primary antibody, tissue sections were incubated for with fluorescein isothiocyanate (FITC)–conjugated secondary antibody for 1 hour at 37°C. Tissue sections were then washed and mounted in medium containing propidium iodide (PI; Vector Laboratories, Burlingame, CA) for visualization of nuclei. For localization of bevacizumab, donkey anti-human IgG antibody conjugated with fluorescein isothiocyanate Cy3 was added onto tissues and incubated at 4°C overnight. This polyclonal antibody binds to many epitopes of both Fc and Fab portions of human IgG. The nuclei were double-stained by 4′,6-diamidino-2-phenylindole (DAPI). The normal cornea without the induction of NV but with the subconjunctival injection of bevacizumab and the negative control cornea without the induction of NV or the subconjunctival injection of bevacizumab were both used in control experiments. All experiments were repeated three times to ensure consistent results. The staining pattern of the tissue sections was observed by conventional fluorescence microscopy (Eclipse E800 Microscope with a VFM Epi-Fluorescence Attachment; Nikon, Melville, NY, equipped with a Spot Digital Camera and Spot version 1.1 CE software; Diagnostic Instruments, Sterling Heights, MI).

**Western Blot Analysis**

*Escherichia coli*–expressed recombinant human VEGF-A (hVEGF-A; 165-amino-acid isoform; R&D, Inc., South San Francisco, CA) or *E. coli*–expressed recombinant rabbit VEGF-A (rVEGF-A; 164-amino-acid isoform with GST tag; Abnova, Walnut, CA) were reduced with 50 mM dithiothreitol and loaded on a 15% SDS-PAGE (Invitrogen, Carlsbad, CA). After completion of electrophoresis, transfer (2 hours) was performed by semidy transfer cell (Bio-Rad, Hercules, CA). Polyvinylidene difluoride (PVDF) membranes were incubated overnight with bevacizumab at a final concentration of 10 μg/mL in blocking buffer (5% skim milk in PBST). After three washes in 0.2% skim milk in PBST, the blots were incubated with peroxidase-conjugated, Fab-specific goat anti-human IgG (Immunopure; Pierce, Rockford, IL) for 1 hour at a dilution of 1:10,000 and were developed using enhanced chemiluminescence (ECL Plus Western Blotting Detection System; GE Healthcare BioSciences, Little Chalfont, UK) after 1-minute exposure of the film.

**RESULTS**

In all animals tested in this study, no ocular adverse complications, such as corneal epithelial defect, corneal ulcer, corneal edema, conjunctival necrosis, anterior uveitis or infectious
endophthalmitis related to the subconjunctival injection, were found.

Effect of Subconjunctival Injection of Bevacizumab on Corneal NV in the Corneal Micropocket NV Models

No matter whether the corneal micropocket NV model was induced by bFGF or VEGF, the ingrowth of corneal NV was found at day 2, peaked at day 12, and decreased from day 14 (Figs. 1A, 1B, 2). Corneal NV emerged uniformly in a wedge shaped pattern from the limbus to the implanted pellets. No corneal epithelial defect was seen in this model. This result showed that the corneal micropocket NV models are simple and useful animal models for studying corneal NV. Subconjunctival injection of bevacizumab inhibited the formation of corneal NV in corneal micropocket NV models induced by bFGF and VEGF, and the time-dependent effect persisted for at least 2 weeks after treatment (Figs. 1A, 1B, 2). There was minimal variation among individual animals in the progression of NV in the experimental groups. In the bevacizumab-treated groups, almost no growth of NV was found (Fig. 1A, 1B; P < 0.05 from days 6–14 in both groups).

Effect of Subconjunctival Injection of Bevacizumab on Corneal NV in the Alkali-Induced NV Model

After removal of the alkali-immersed disc from the eye, the injured central cornea appeared opaque, with a clear margin. The alkali-injured areas could be identified until 4 weeks after damage. The very first clinical finding of NV ingrowth toward the central corneas occurred before day 7. The corneal NV reached the central injured corneas by day 21, persisted until at least day 28 (Fig. 3) and regressed after day 28 (data not shown). The corneal epithelial defects healed within 7 days. There was minimal variation among individual animals. This result showed that alkali-induced corneal NV was a simple and useful animal model system for the study of NV. As shown in Figures 1C, 1D, and 3, subconjunctival injection of bevacizumab inhibited the formation of corneal NV in alkali-induced corneal NV, and the dose- and time-dependent effect persisted until at least 4 weeks after treatment. A dose-dependent effect was seen when corneal NV was measured by length (P < 0.05 from days 10 to 24) or surface area (P < 0.01 from days 14 to 24; Figs. 1C, 1D).
Effect of Subconjunctival Injection of Bevacizumab on Corneal NV in the Limbal Injury–Induced Corneal NV Model

At 2 weeks after keratectomy, considerable corneal NV emerging from the whole circumference of the limbus was noted (Fig. 4). The area of the corneal surface covered by NV peaked at week 3 and slowly decreased until week 8. At postoperative week 8, 39% ± 11% of the corneal surface area was covered by corneal NV in the control group. In the bevacizumab-treated corneas, the corneal surface areas covered by NV significantly decreased from weeks 2 to 8 (P < 0.05 at week 2, P < 0.01 from weeks 3 to 8). A dose-dependent effect of bevacizumab in inhibiting corneal NV was found. Bevacizumab also promoted corneal epithelial wound healing during weeks 2 and 3 (Fig. 5, P < 0.05). In addition, less corneal haziness was found in the bevacizumab-treated group between weeks 2 and 4 (Fig. 4). In this model, the corneal NV and corneal epithelial defects remained stationary without significant change from postoperative week 8 to month 6 after surgery in both the control group and the bevacizumab-treated group.

Immunohistochemistry

Immunohistochemical staining by CD31 clearly showed the growth of vascular endothelial cells in the corneal micropocket NV model induced by bFGF and the alkali-induced cornea NV model (Fig. 6). Treatment with bevacizumab inhibited the CD31 staining in both experimental groups. One day after the subconjunctival injection, staining of bevacizumab was found in the whole corneal stromal thickness adjacent to the injection site in the alkali-induced corneal NV model, the micropocket NV model induced by bFGF, and the normal cornea without induction of corneal NV. The distribution of bevacizumab staining moved from the peripheral cornea to the more central cornea on days 2 and 3 in all three groups. On day 7, the staining of bevacizumab decreased dramatically in the two experimental groups, whereas strong bevacizumab staining was still found in normal corneas without induction of corneal NV (Fig. 7). No specific staining was found in the negative control cornea in which subconjunctival injection of normal saline instead of bevacizumab was performed. This demonstrated that the anti-IgG antibody used in our study indeed recognized the injected bevacizumab molecule.

Western Blot Analysis

We performed Western blot analysis of rVEGF-A and hVEGF-A with bevacizumab as the primary antibody using conditions similar to those reported by Bock et al.25 and Yu et al.31 As
shown in Figure 8, after a 1-minute exposure of the film, the bevacizumab-probed blot revealed immunoreactive bands of the size expected for rVEGF-A and hVEGF-A. The interaction of bevacizumab with rVEGF-A was weaker than that with hVEGF-A, as evaluated by Western blot.

**DISCUSSION**

During the formation of corneal NV, upregulation of angiogenic factors such as VEGF has been demonstrated.\textsuperscript{1,7–9,32,33} VEGF is an important signaling protein that promotes several steps of angiogenesis, including proteolytic activities, proliferation, migration of endothelial cells, and formation of capillaries.\textsuperscript{1} Treating corneal NV by inhibiting VEGF activity with a neutralizing anti-VEGF antibody is a promising method that has drawn much attention recently and deserves to be evaluated. Bevacizumab has been used recently by ophthalmologists as an intravitreous agent in the treatment of proliferative (neovascular) eye diseases, particularly for choroidal NV in age-related macular degeneration (ARMD).\textsuperscript{15–17} Many retinal specialists also noted impressive results in the settings of proliferative diabetic retinopathy,\textsuperscript{12,13} neovascular glaucoma,\textsuperscript{13,34} diabetic macular edema,\textsuperscript{12,13} and macular edema secondary to retinal vein occlusions.\textsuperscript{35} The injection of 1.25 to 2.5 mg of bevacizumab into the vitreous cavity of rabbit eyes has been shown to have no significant intraocular toxicity.\textsuperscript{36,37}

Recently, several studies demonstrated the effects of topical or subconjunctival injection of bevacizumab in the inhibition of corneal NV.\textsuperscript{18–25} Similarly, our experimental results demonstrated that subconjunctival injection of bevacizumab inhibited corneal NV in rabbit eyes. The inhibition of corneal NV was almost complete in the VEGF and bFGF corneal micropocket...
of the most potent angiogenic agents in addition to VEGF.\textsuperscript{1,38} bFGF-induced corneal NV remain to be investigated. FGF is one of the most potent angiogenic agents in addition to VEGF.\textsuperscript{1,38} FGF-2 and VEGF are known to have synergistic effects, and intimate cross-talk exists among FGF and different members of the VEGF family during angiogenesis, lymphangiogenesis, and vasculogenesis.\textsuperscript{39,40} Several experimental findings point to the possibility that FGF2 induces NV indirectly by activation of the VEGF/VEGFR system.\textsuperscript{40–42} Administration of anti-VEGF neutralizing antibodies, VEGFR blocking antibodies, or the expression of a dominant-negative VEGFR resulted in a significant reduction of FGF2-induced endothelial cell extensions and capillary morphogenesis.\textsuperscript{41,42} Although we did not investigate the direct interaction of bFGF, VEGF, and their receptors after the administration of bevacizumab in this study, our study demonstrated the effects of anti-VEGF antibody in inhibiting bFGF-induced corneal NV. Further studies should be performed to explore the underlying mechanisms.

Bevacizumab was found to have a partial but significant effect in inhibiting corneal NV in the limbal injury-induced corneal NV model in this study. Our result is similar to that reported by Bock et al.,\textsuperscript{43} who demonstrated that topical bevacizumab eye drops (5 mg/mL, 0.5–6 months) inhibited corneal NV in patients with limbal stem cell deficiency. Although the exact mechanism controlling the corneal NV formation after injury to the limbal structure remained to be evaluated, VEGF had been shown to play a role.\textsuperscript{44} Amano et al.\textsuperscript{45} examined VEGF expression in limbal deficiency–induced corneal NV models. The inhibition was incomplete, but significant in the alkali-induced and limbal injury–induced corneal NV models. It was not surprising that bevacizumab inhibited the corneal angiogenesis caused by VEGF and alkali, because VEGF was expected to increase after the surgical manipulations;\textsuperscript{50} however, the mechanisms controlling the inhibitory effects in bFGF-induced corneal NV remain to be investigated. FGF is one of the most potent angiogenic agents in addition to VEGF.\textsuperscript{1,38} FGF-2 and VEGF are known to have synergistic effects, and intimate cross-talk exists among FGF and different members of the VEGF family during angiogenesis, lymphangiogenesis, and vasculogenesis.\textsuperscript{39,40} Several experimental findings point to the possibility that FGF2 induces NV indirectly by activation of the VEGF/VEGFR system.\textsuperscript{40–42} Administration of anti-VEGF neutralizing antibodies, VEGFR blocking antibodies, or the expression of a dominant-negative VEGFR resulted in a significant reduction of FGF2-induced endothelial cell extensions and capillary morphogenesis.\textsuperscript{41,42} Although we did not investigate the direct interaction of bFGF, VEGF, and their receptors after the administration of bevacizumab in this study, our study demonstrated the effects of anti-VEGF antibody in inhibiting bFGF-induced corneal NV. Further studies should be performed to explore the underlying mechanisms.

In conclusion, we showed that subconjunctival injection of bevacizumab in this study. However, the safety of bevacizumab on corneal cells is still an issue that needed to be considered. Yoeruek et al.\textsuperscript{45} demonstrated that bevacizumab is not toxic to corneal cells of human origin in in vitro at doses normally used for treatment of corneal NV (up to 5.0 mg/mL), which is 20-fold higher than the dose used for intravitreal application. Kim et al.\textsuperscript{46} showed that topical application of bevacizumab causes spontaneous loss of corneal epithelial integrity and progression of stromal thinning. However, our study and others seem to support the conclusion that the subconjunctival delivery method is a good option for inhibiting corneal NV. The delivery method is easy and simple to perform and leads to minimal related complications.

Heiduschka et al.\textsuperscript{26} demonstrated that bevacizumab can penetrate the retina through the retinal pigment epithelium, choroid, and photoreceptors after intravitreal injection using an immunohistochemical staining assay with donkey anti-human IgG. In that study, the antibody used for the detection of bevacizumab recognized the injected bevacizumab molecule specifically. The observed fluorescence did not arise from any other sources except the lipofuscin signal in the retinal pigment epithelium. Using the same antibody, we found a time-dependent pattern of intracorneal diffusion of bevacizumab after subconjunctival injection (Fig. 7). To our knowledge, this is the first time that the diffusion of bevacizumab through the subconjunctival site to the corneal stroma, its site of action, has been demonstrated. The strong intracorneal bevacizumab staining could persist for at least 5 days in the VEGF micropocket and alkali-induced corneal NV models after a single subconjunctival injection. The distribution of intracorneal bevacizumab could persist at least up to 7 days in the normal corneas. The reason for the different durations of intracorneal diffusion remains to be evaluated. Although not proven, the shorter duration of bevacizumab distribution in the vascularized corneas may be caused by the rapid absorption through the corneal vessels. Because some studies have demonstrated that bevacizumab eye drops inhibit corneal NV,\textsuperscript{25–25} it remains to be evaluated whether bevacizumab would diffuse into the cornea by topical administration. Comparison of the durations of intracorneal bevacizumab staining between subconjunctival injection and topical use is also important before choosing the treatment strategy.

In conclusion, we showed that subconjunctival injection of bevacizumab is effective in controlling corneal NV in several rabbit corneal NV models. Bevacizumab staining was found in the corneal stroma for 5 to at least 4 days after the injection. Complications were observed in this study. Controlled clinical trials should be performed to demonstrate the efficiency of bevacizumab in the treatment of acute or chronic human corneal NV. The possible systemic absorption and extracocular side effects must be adequately addressed to avoid potential complications.

References


