Corneal Penetration of Topical and Subconjunctival Bevacizumab

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PURPOSE. To investigate the ability of bevacizumab to penetrate the cornea after topical application or subconjunctival injection.

METHODS. Bevacizumab 1% was topically applied three times a day to the corneas of mice (BALB/c) with intact corneas ($n = 14$), and with corneal neovascularization ($n = 14$). Animals were euthanized at 1, 6, 12, and 24 hours, and 2, 4, and 7 days for immunohistochemical analyses. Donkey anti-human IgG labeled with Cy3 was used for bevacizumab immunoreactivity detection. Additionally, one-time topical bevacizumab 1% was tested in corneas with denuded epithelium ($n = 16$). In another group ($n = 16$), a single dose of 0.5 mg bevacizumab was injected subconjunctivally. Animals were euthanized at 1, 6, and 24 hours, and 2, 4, 7, 14, and 21 days for immunohistochemical studies.

RESULTS. Bevacizumab was barely detected beyond the very superficial layer of the corneal epithelium in mice with intact corneas even after 7 days of topical administration. Application of bevacizumab in mice with corneal neovascularization; however, showed variable penetration into the corneal stroma. Experimentation with single application of topical bevacizumab in corneas with denuded epithelium or subconjunctivally injected bevacizumab showed intense staining for bevacizumab.

CONCLUSIONS. Topically applied bevacizumab has limited capacity to penetrate the corneas with intact epithelium. However, bevacizumab can penetrate the neovascularized cornea after topical application. This study demonstrates that subconjunctivally injected bevacizumab in eyes with an intact cornea penetrates well into the corneal stroma. (*Invest Ophthalmol Vis Sci.* 2011;52:8718–8723) DOI:10.1167/iops.11-7871

Topical application of drug is the preferred method of administration to the cornea, ocular surface, and anterior segment, because achieving a high therapeutic level of medicine in these tissues can often be feasible without imposing systemic side effects. However, topical treatment and periocular injections will only be effective if the drug can penetrate through the ocular barriers (e.g., corneal and conjunctival

epithelium for topical route; sclera for subconjunctival route) to reach the target tissues within a therapeutic level.¹

Recently, use of topical as well as subconjunctival bevacizumab, a recombinant humanized monoclonal IgG1 antibody that inhibits human vascular endothelial growth factor (VEGF)-A, has been considered for the treatment of corneal neovascularization (NV).^{2–12} Bevacizumab is approved by the US Food and Drug Administration for intravenous use for metastatic colorectal cancer or recurrent or metastatic nonsquamous non-small cell lung cancer, but is also used off-label intravitreally to treat VEGF-mediated diseases such as choroidal NV,¹³ central retinal vein occlusion,¹⁴ proliferative diabetic retinopathy,¹⁵ and neovascular glaucoma¹⁶ with encouraging results.

Bevacizumab which is a full-length immunoglobulin has a 12 nm long, Y-shaped configuration with a molecular weight of 149 kDa. Its three arms are rods approximately 3.5 nm in diameter. The healthy corneal epithelium is a stratified layer of cells connected by tight junctions that provide a barrier against compounds larger than 10 Å (1 nm).¹ Although it has been shown that engineered antibody fragments of 28 kDa and 67 kDa^{17,18} or single-chain antibodies¹⁹ can penetrate through intact corneas into the anterior chamber, topical administration of full-length immunoglobulins are typically considered ineffective because such molecules are too large to penetrate the intact cornea. However, the clinical effectiveness of topical bevacizumab in the treatment of corneal NV which has been shown before by our group¹² as well as other investigators^{2,3,7,10,20} indirectly implies that topical bevacizumab can go through the epithelial barrier in patients with ocular inflammation and corneal NV which may affect the integrity of the epithelial barrier. The purpose of this study was to examine corneal penetration of bevacizumab after topical application in a mouse model of corneal NV. Moreover, corneal penetration of bevacizumab injected subconjunctivally with an intact epithelium or administered topically with denuded corneal epithelium was evaluated.

METHODS

Animals and Anesthesia

Male 6- to 8-week-old BALB/c mice were used in all experiments. All animals were treated according to guidelines established by the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and the Public Health Policy on Humane Care and Use of Laboratory Animals (US Public Health Review), and all procedures were approved by the Institutional Animal Care and Use Committee. Anesthesia was administered intraperitoneally by ketamine/xylazine solution at a dose of 120 mg per kg body weight and 20 mg per kg body weight, respectively. Only the right eye of each animal was used for the experiments.

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Induction of Corneal NV

Corneal NV was induced by implantation of a micropellet containing basic fibroblast growth factor (b-FGF). Eighty nanogram b-FGF (gift from BRB Preclinical Repository, NCI-Frederick, Frederick, MD) micropellets were prepared as previously described by Kenyon et al.²¹ An initial half thickness linear incision was made at the center of the cornea using a disposable 30° microknife (Fine Science Tools, Inc., Foster City, CA). A lamellar pocket incision was then made parallel to the corneal plane using a Von Graefe knife (Fine Science Tools, Inc.) and advanced to the temporal limbus at the lateral canthal area. The micropellet was positioned into the pocket 1.0 mm apart from the limbal vascular arcade, and tetracycline ophthalmic ointment was applied to the eye after micropellet implantation. Because stimulation of robust hemangiogenesis with 80 ng b-FGF-impregnated micropellet peaks at 1 week after implantation,²² eyes were examined by slit lamp biomicroscopy on postoperative day 7. Those eyes which developed robust corneal NV were selected for the next phase of experiments.

Bevacizumab Administration

Bevacizumab 1% (10 mg/mL; Avastin; Genentech, Inc., South San Francisco, CA) was topically applied three times a day to the corneas of mice ($n = 28$). Animals were divided into two groups: the first group with intact corneas and without any obvious pathology or vascularization; the second group with corneal NV induced by a micropellet containing 80 ng b-FGF as explained earlier. Animals were euthanized at 1, 6, 12, and 24 hours, and 2, 4, and 7 days after the initiation of topical treatment for immunohistochemical analyses.

Because in normal corneas bevacizumab was barely detected beyond the very superficial layer of the epithelium even after 7 days of topical administration (see further details in the Results section), high frequency (every ½ hour for 8 hours; 16 times in total) topical bevacizumab 2.5% (maximum available concentration; 25 mg/mL) was tried in another group of mice ($n = 5$), and animals were euthanized 24 hours after the first drug application for immunohistochemical analyses. Additionally, topical bevacizumab 1% was tested in corneas with denuded epithelium. In this group ($n = 16$), corneas were scraped with a surgical scalpel blade (#15 BD Bard-Parker, Franklin Lakes, NJ) to remove the corneal epithelium demarcated with a 2-mm corneal trephine. One time topical bevacizumab 1% was applied to the corneal surface, and animals were euthanized at 1, 6, and 24 hours, and 2, 4,

7, 14, and 21 days for immunohistochemical analyses. To test corneal penetration of bevacizumab injected subconjunctivally, a single dose of 0.5 mg bevacizumab (0.02 mL of 25 mg/mL solution) was injected in the nasal side ($n = 16$), and animals were euthanized at 1, 6, and 24 hours, and 2, 4, 7, 14, and 21 days for further immunohistochemical studies.

Immunohistochemistry

In all experiments, immunohistochemistry staining was used to detect bevacizumab in corneal tissue. Mouse eyeballs were cryopreserved in optimal cutting temperature (OCT) embedding medium, and 8-μm cryosections were obtained. Sections were air-dried at room temperature for 30 minutes, and then fixed in acetone for 15 minutes on coated slides. Sections were then washed and blocked by 2% bovine serum albumin (BSA) at room temperature for 1 hour. For the detection of bevacizumab, donkey anti-human IgG antibody conjugated with fluorochrome Cy3 (1:500; Jackson ImmunoResearch, West Grove, PA) which can bind to the heavy and light chains of the humanized IgG, was added onto the tissue at room temperature for 2 hours. After a further washing step, the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) containing fluorescent mounting medium (VectaShield; Vector Laboratories, Burlingame, CA). The staining pattern of the tissue sections was observed by conventional fluorescence microscopy (model E800; Nikon, Melville, NY). Sections from eyes with intrastromal injection (as previously described by Stechschulte et al.²³) of bevacizumab were used as positive controls; sections from untreated eyes were also used as negative controls.

RESULTS

Immunoreactivity for bevacizumab was shown in the corneal stroma of eyes with intrastromal injection of bevacizumab, which were used as positive controls (Fig. 1A). Additionally, none of the untreated corneas which served as negative controls showed immunoreactivity for bevacizumab (Fig. 1B), confirming that the anti-IgG antibody used in this study recognized the bevacizumab molecule and that there was no nonspecific fluorescence of the corneal sections.

FIGURE 1. Immunohistochemistry staining for bevacizumab in corneal stroma of control eyes. Donkey anti-human IgG labeled with Cy3 was used for bevacizumab immunoreactivity detection. (A) Strong immunoreactivity for bevacizumab in a cornea with intrastromal injection of bevacizumab used as a positive control. *Arrow* shows site of injection. (B) Section from an untreated eye was used as a negative control showing no immunoreactivity for bevacizumab (magnification, $\times 200$).

FIGURE 2. Penetration of topical bevacizumab in cornea with intact epithelium. (A) One day, (B) 4 days, and (C) 7 days after topical application of bevacizumab 1%, in frequency of 3 times a day. (D) Twenty-four hours of high frequency (every $\frac{1}{2}$ hour for 8 hours; 16 times in total) application of topical bevacizumab 2.5%. Note that bevacizumab was detected merely on the very superficial layer of the epithelium (magnification, $\times 200$).

Penetration of Topical Bevacizumab in Cornea with Intact Epithelium

Tissue sections prepared at different time points after topical bevacizumab application were checked for their immunoreactivity against bevacizumab. In normal corneas with intact epithelium, bevacizumab was barely detected beyond the very superficial layer of the epithelium (Fig. 2). In fact, even after 7 days of topical administration of bevacizumab 1%, at a frequency of three times a day, bevacizumab did not show any appreciable penetration into the healthy corneal stroma (Fig. 2C). Moreover, high frequency (every $\frac{1}{2}$ hour for 8 hours; 16 times in total) application of topical bevacizumab 2.5% (maximum available concentration; 25 mg/mL) did not show any noticeable immunoreactivity for bevacizumab in the corneal stroma either; bevacizumab was detected merely on the very superficial layer of the epithelium (Fig. 2D).

Penetration of Topical Bevacizumab in Cornea with Neovascularization

Application of bevacizumab in mice with corneal NV showed variable detection in the corneal stroma. In this group, immunoreactivity for bevacizumab was noticeable in the stroma as early as 48 hours after initiation of topical bevacizumab, and staining became most intense on day 7 (Fig. 3). Also, bevacizumab localization was significantly more intense near the pellets. However, bevacizumab immunoreactivity was not present in all corneas, and staining intensity varied considerably among the corneas (Figs. 3A–C).

Penetration of Topical Bevacizumab in Cornea with Denuded Epithelium

Single application of topical bevacizumab in corneas with denuded epithelium showed significant staining within the whole corneal stroma as early as 1 hour which became more intense by 6 hours

FIGURE 3. Penetration of topical bevacizumab in cornea with neovascularization. (A–C) Seven days after initiation of topical bevacizumab in corneas with neovascularization. Note that immunoreactivity for bevacizumab was noticeable in stroma; however, staining intensity varied considerably among the corneas (magnification, $\times 200$).

FIGURE 4. Penetration of topical bevacizumab in cornea with denuded epithelium. (A) Six hours, (B) 24 hours, (C) 2 days, and (D) 14 days after single application of topical bevacizumab 1% in corneas with denuded epithelium. Note strong staining of corneal stroma which was seen at all time points up to 14 days (magnification, $\times 100$).

(Fig. 4A). Strong staining of corneal stroma was seen at all time points up to 14 days, as shown in Figures 4A–D. On day 21 (the last investigated time point), immunoreactivity for bevacizumab declined, but was still substantially detectable in central corneal stroma.

Corneal Penetration of Bevacizumab after Subconjunctival Injection

The corneal distribution of bevacizumab was investigated at different time points after subconjunctival injection. In low-

magnification views of whole globe sections at each time point, it was observed that by 1 hour of subconjunctival injection, staining for bevacizumab was readily detectable in many structures on the same side of the eye as the injection site (Fig. 5A); however, in 6 hours, immunohistochemical staining showed intracorneal diffusion of bevacizumab into the corneal periphery adjacent to the injection site (Fig. 5B). By 24 hours of injection, strong bevacizumab staining was detected in the whole cornea which remained almost unchanged in all layers

FIGURE 5. Corneal penetration of bevacizumab after subconjunctival injection. (A) One hour, (B) 6 hours, (C) 24 hours, and (D) 14 days after subconjunctival injection of a single dose of 0.5 mg bevacizumab (0.02 mL of 25 mg/mL solution). Arrow: site of subconjunctival injection. Note that intracorneal diffusion of bevacizumab started into the corneal periphery adjacent to the injection site and by 24 hours of injection, strong bevacizumab staining was detected in the whole cornea which remained almost unchanged up to 14 days (magnification, $\times 20$).

FIGURE 6. Corneal penetration of bevacizumab after subconjunctival injection. (A) One day, (B) 7 days, (C) 14 days, and (D) 21 days after subconjunctival injection of a single dose of 0.5 mg bevacizumab. Note that strong bevacizumab staining remained almost unchanged in all layers of stroma over the next time points up to 14 days; however, on day 21 bevacizumab staining started fading from the corneal periphery (magnification, $\times 100$).

of stroma over the next time points up to 14 days, as shown in Figures 5C, 5D, and 6A–C. On day 21 (the last investigated time point), the intensity of the fluorescence for bevacizumab decreased, but was still largely detectable in central corneal stroma. Immunohistochemical study of different corneal sections on day 21 showed that bevacizumab staining started fading from corneal periphery (Fig. 6D).

DISCUSSION

In the era of protein-based “biological” therapies, it is important for topically applied macromolecules (e.g., antibody) to gain access to the target cells. To be angiostatically effective, bevacizumab must penetrate the corneal epithelium to reach therapeutic targets within the corneal stroma where the vascular endothelial cells proliferate and form tubules. The corneal epithelial barrier almost completely excludes macromolecules ($r > 10 \text{ \AA}$).¹ In particular, the intercellular tight junctions of the most superficial epithelial cells provide a very effective barrier. Therefore, as we have also shown here, large full-length immunoglobulins including bevacizumab with a molecular weight of 149 kDa, likely have limited capacity to penetrate the normal corneas with intact epithelium. Our immunohistochemical studies in intact cornea with either 7 days of topical administration of bevacizumab 1%, in frequency of 3 times a day, or high frequency application (every $\frac{1}{2}$ hour for 8 hours; 16 times in total) of topical bevacizumab 2.5% did not show any appreciable penetration into the healthy corneal stroma. These results are in accordance with data derived from the study by Nomoto et al.²⁴ using enzyme-linked immunosorbent assay for bevacizumab which showed poor anterior chamber and intraocular tissue distribution of topically administered bevacizumab in rabbit eyes. On the other hand, quick penetration of topical bevacizumab in corneas with denuded epithelium merely underscores the significance of healthy corneal epithelium as a highly efficient barrier against large molecules such as bevacizumab. Thus, we demonstrate that bevacizumab can penetrate the neovascularized cornea after topical application, although, its penetration varies significantly among the

neovascularized corneas, reflective of the varying degrees of neovascularization/inflammation induced in these eyes. The assumption underlying this penetration is that in ocular surface diseases like corneal neovascularization, the integrity of epithelial tight junction diminishes and cornea becomes more porous so that it can allow penetration of macromolecules such as bevacizumab. Nonetheless, inflammation which is a common companion of neovascularization has a significant effect on the corneal epithelium; indeed, it is known that patients with ocular surface disease, such as dry eye disease for example, have an incompetent barrier function.²⁵ This means that large macromolecules that may have limited penetration into the intact eye may very well gain significant access through the epithelium of inflamed eyes; a phenomenon that has significant implications for other biological approaches in the treatment of ocular surface conditions. Clinical studies of effectiveness of topical bevacizumab in the treatment of corneal NV which has been shown before by our group¹² as well as other investigators^{2,3,7,10,20} indirectly support that topical bevacizumab can go through the epithelial barrier in patients with corneal NV. Although in these clinical studies, topical administration of bevacizumab resulted in a mild to moderate decrease in the severity of corneal NV; however, the clinical results varied drastically from case to case which are in accordance with the results of our current experiments.

Our study demonstrates that topically administered bevacizumab in eyes with denuded corneal epithelium or subconjunctivally injected bevacizumab in eyes with an intact cornea penetrates well and quickly into the corneal stroma. In both of these experiments, an intense staining for bevacizumab was present in the whole cornea up to 14 days after injection. By day 21 (the last investigated time point in our experiments), although immunoreactivity for bevacizumab declined, it was still largely detectable in central corneal stroma. Of interest, by day 21, bevacizumab staining started fading from corneal periphery. Heiduschka et al.²⁶ showed in their study that intravitreally injected radioactive labeled bevacizumab in cynomolgus monkeys, bevacizumab can be found in several retinal cell types 14 days after its injection. In a pharmacokinetic study by

Nomoto et al.,²⁴ it was shown that bevacizumab concentration in the retina/choroid after intravitreal injection was maintained for approximately 3 months. Given the long-lasting tissue presence of bevacizumab, it can be speculated that, due to its high molecular weight, diffusion beginning at the corneal periphery removes bevacizumab in a centripetal pattern which is slow and may require weeks to complete.

This study shows that subconjunctival injection is a more reliable administration route for full-length immunoglobulins such as bevacizumab in treatment of corneal NV and potentially other corneal pathologies. In an animal model of vascularized high-risk corneal transplantation to compare topical and subconjunctival bevacizumab treatments, we have also shown that while both topical and subconjunctival bevacizumab therapy can diminish corneal NV after high-risk transplantation, only the subconjunctival route is significantly effective in improving graft survival.²⁷

In summary, the data presented herein suggest that topically applied large proteins, such as full-length antibodies, have considerable capacity to penetrate into the cornea when the surface epithelial barrier function is not intact. This has potentially important implications for the topical application of biological therapies for a variety of ocular pathologies.

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