Safety Profile of Topical VEGF Neutralization at the Cornea

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PURPOSE. Bevacizumab eyedrops inhibit corneal neovascularization. The purpose of this study was to analyze the safety profile of VEGF-A neutralization at the ocular surface.

METHODS. Bevacizumab eyedrops (5 mg/mL) and an antimurine VEGF-A antibody (250 μg/mL) were applied to normal murine corneas five times a day for 7 and 14 days. Subsequently, corneas were analyzed for morphologic changes by light and electron microscopy. In a mouse model of corneal epithelial abrasion, the effects of topically applied anti-VEGF antibodies on epithelial wound healing were analyzed: the treatment group received bevacizumab (5 mg/mL) or the antimurine VEGF-A antibody (250 μg/mL) as eyedrops, and the control group received an equal volume of saline solution. After 12, 18, and 24 hours, corneas were photographed in vivo with and without fluorescein staining for morphometry. Afterwards the mice were killed, and eyes were removed for histology, immunohistochemistry with Ki67/DAPI, and electron microscopy. The effect of midterm anti-VEGF therapy on corneal nerve density was assessed by staining corneas treated with an FITC-conjugated anti-neurofilament antibody and morphometric analysis.

RESULTS. Murine corneas treated with two different types of anti-VEGF antibody eyedrops did not show obvious corneal morphologic changes at the light and electron microscopic levels. Furthermore, anti-VEGF antibody eyedrops had no significant impact on the wound healing process after corneal epithelial injury or on normal murine corneal nerve fiber density.

CONCLUSIONS. Topical neutralization of VEGF-A at the corneal surface does not have significant side effects on normal corneal epithelial wound healing, normal corneal integrity, or normal nerve fiber density. Therefore, anti-VEGF eyedrops seem to be a relatively safe option to treat corneal neovascularization.


The healthy human cornea is devoid of blood and lymphatic vessels and actively maintains its avascularity. This "angiogenic privilege" of the cornea is essential for corneal transparency and good vision. Therefore, in all higher animals that depend on good vision, the cornea is normally avascular. Corneal angiogenic privilege also contributes to the remarkable success of corneal transplantsations performed in avascular, so-called low-risk recipient beds. Conversely, corneal neovascularization both before and after keratoplasty significantly increases the risk for corneal graft rejection.

Until recently, no specific antiangiogenic treatment options against corneal neovascularization had been available. The mainstay of antiangiogenic therapy was anti-inflammatory and only partly antiangiogenic steroids that often were not able to prevent or stop corneal neovascularization. Because of the rapid progress in angiogenesis research, novel specific antiangiogenic drugs are now available for use in oncology and against neovascular diseases at the posterior pole of the eye. Bevacizumab (Avastin; Genentech, South San Francisco, CA), a humanized anti-VEGF antibody, is the first US Food and Drug Administration (FDA)-approved antiangiogenic drug on the market. Initially approved for systemic treatment of colorectal cancers, bevacizumab now is widely used off label to treat age-related maculopathy, proliferative retinopathy, and neovascular glaucoma.

Recent studies demonstrate that bevacizumab eyedrops inhibit inflammatory corneal neovascularization in animal models. This effect can be achieved by topical and systemic application of bevacizumab. Therefore, it is reasonable to use bevacizumab eyedrops to treat patients with progressive corneal neovascularization who do not respond to conventional steroid therapy. Reports have been published of successful clinical experiences with bevacizumab applied as eyedrops and subconjunctivally in patients with progressive corneal neovascularization. It is important to know whether bevacizumab eyedrops cause morphologic side effects in the cornea or affect corneal epithelial wound healing. Corneal epithelium contains VEGF and expresses receptors for VEGF (e.g., soluble VEGF receptor 1 and VEGF receptor 3). In addition, VEGF is an important neurotrophic growth factor. Several side effects of VEGF-A neutralization at the ocular surface are possible. Although no toxic side effects of intravitreal bevacizumab have been reported at the posterior pole of the eye, we set out to investigate whether anti-VEGF antibodies such as bevacizumab eyelids have adverse effects on normal corneal morphology, epithelial wound healing, or corneal nerve fiber density in vivo. This is of clinical relevance because corneal neovascularization often occurs in the presence of epithelial defects. Given the low affinity of bevacizumab to murine VEGF, we also used a specific high-affinity antimurine VEGF-A antibody.

METHODS

Animals

For the wound healing and eyelid application assay, female C57BL/6 mice (6–8 weeks of age) were used. The local animal care committee, in compliance with the ARVO Statement for the Use of
Animals in Ophthalmic and Vision Research, approved all animal protocols.

Wound Healing Assay

Before surgery, each animal was deeply anesthetized with an intramuscular injection of 8 mg/kg ketamine (Ketanest S; Gödecke AG, Berlin, Germany) and 0.1 mL/kg xylazine (Rompun; Bayer, Leverkusen, Germany). The central 1.8 mm² of corneal epithelium of the right eye was scraped off in a standardized fashion (using 1.5-mm diameter trephine [1.8-mm² area]), and the treatment group received bevacizumab (5 mg/mL; every 2 hours) or a blocking antimurine VEGF-A antibody (250 μg/mL; R&D Systems, Wiesbaden, Germany) as eyedrops (n = 5). The control group received an equal volume of saline solution (n = 5) as eyedrops. After 12, 18, and 24 hours, digital images of the corneas were taken with a camera (F-View II; Olympus GmbH, Hamburg, Germany) in vivo with and without fluorescein staining to localize and measure the epithelial defect (corneal erosion). Afterward, the mice were killed and eyes were removed. The deep epithelialized area was measured with an image analysis program (Cell®F; Olympus GmbH, Mainz, Germany).

To analyze the effect of bevacizumab on healthy corneas and to exclude that neutralization of VEGF-A at the ocular surface causes new epithelial defects as a consequence of, for example, neurotrophic keratopathy, mice with normal corneas received bevacizumab (5 mg/mL) or the antimurine VEGF-A antibody (250 μg/mL; R&D Systems) as eyedrops 5 times per day for 7 and 14 days (n = 5). The control group received an equal volume of saline solution (n = 5) as eyedrops.

Nerve Fiber Density Assay

C57BL/6 mice received bevacizumab (5 mg/mL) four times daily, in accordance with off-label treatments and the literature23 or a blocking antimurine VEGF-A antibody (250 μg/mL; R&D Systems) as eyedrops for 2 or 4 weeks. Control mice received an equal volume of saline solution. Mice were killed after 2 or 4 weeks.

Corneas were excised and radially incised four or five times, and the epithelium was scraped off. Prepared corneas were fixed for 5 hours in paraformaldehyde and afterward digested with a Triton X-100/Tween 20/PBS mix (1 mL Triton X-100 + 1 mL Tween 20 + 1 mL PBS). Corneas were incubated overnight at 4°C. The next day the specimens were rinsed three times with PBS and blocked with 2% BSA (in PBS) for

Figure 1. No morphologic differences were observed in the central cornea between eyes treated with anti–VEGF antibody eyedrops and controls. (a) Paraffin sections of control and anti–VEGF antibody (bevacizumab)-treated murine eyes after 7 and 14 days (hematoxylin and eosin, 200×). No differences were observed in the shape or morphology of the epithelial cells. EP, epithelium; S, stroma. (b) Ultrastructural analysis of the corneal epithelium of bevacinumab (anti–VEGF antibody)-treated and control eyes. Normal corneal epithelium with basal keratinocytes (BK) and flattened keratinocytes at the surface. Bevacizumab-treated eyes displayed equal structures of the epithelial layer. SK, surface keratinocytes; S, stroma.
Corneas were stained for 48 hours at 4°C with a rabbit anti–mouse neurofilament antibody (1:500; BioTrend, Cologne, Germany). After a further washing step with PBS on day 3, the neurofilament was detected with a goat anti–rabbit Alexa 488 antibody (1:2000 in PBS; Invitrogen, Karlsruhe, Germany; incubated at room temperature for 4 hours). Corneas were rinsed again in PBS and then covered on a slide with fluorescent mounting medium (DakoCytomation, Hamburg, Germany).

Stained wholemounts were analyzed with a fluorescence microscope (BX51; Olympus GmbH), and digital pictures were taken with a 12-bit monochrome CCD camera (F-View II; Olympus GmbH). The central area of each wholemount was photographed (100× magnification), and the nerve fibers in this area were measured manually with an image analyzing program (Cell^F; Olympus GmbH).

**Histology**

**Light and Electron Microscopy.** For light microscopy, eyes were enucleated and preserved in paraffin. Microsections of the eyes were deparaffinized by graded alcohol up to aqua destillata. Nuclei were stained by hematoxylin for 20 minutes. After washing in water, sections were corroded for differentiation by 1% HCl-alcohol for 5 seconds, washed three times in water, washed in ammonia water for 30 seconds, and washed again three times in water. Afterward the sections were stained blue in water for 30 minutes. Connective tissue was stained with eosin solution for 4 minutes, followed by a washing step in water. The specimens were dehydrated with graded alcohol up to xylol, and the slides were covered with resin.

**Epithelial Cell Counting.** To analyze the number of epithelial cells in the central cornea, digital images of hematoxylin and eosin-stained sections of the central cornea were taken. An area measuring 400 μm in length was outlined, and cell nuclei were counted by particle analysis with an image analysis program (Cell^F; Olympus GmbH).

**Electron Microscopy.** For transmission electron microscopy, eyes were bisected and fixed with 2% buffered osmium tetroxide, dehydrated in graded alcohol concentrations, and embedded in epoxy resin (Epon 812; Fluka, Buchs, Germany) according to standard protocols, as previously described.25

**Immunohistochemistry.** For cryosection staining, murine eyes were cryopreserved in OCT embedding medium, and 5- to 7-μm cryosections were obtained. Sections were dried (15 minutes, 37°C), fixed in acetone for 15 minutes on coated slides (Superfrost; Erie Scientific, Portsmouth, NH), and again given three 5-minute washes in PBS. Each section was circumscribed with a DakoCytomation fat-based pen and again given three 5-minute washes in PBS. Unspecific antigens were blocked with 2% BSA in PBS at room temperature in a moist chamber. After blocking, slides were incubated with a rabbit anti–mouse Ki67 antibody (1:100 in 2% BSA in PBS; Abcam, Cambridge, UK) overnight at 4°C. Excess antibody was washed off with five 5-minute washes in PBS. The primary antibody was detected by a Cy3 goat-anti–rabbit antibody (1:500 in 2% BSA in PBS; Dianova, Hamburg, Germany).

**Figure 2.** No significant differences were observed in the number of epithelial cells in the central cornea of anti–VEGF-treated and control eyes. A 400-μm long area in hematoxylin and eosin-stained paraffin sections of the central cornea was outlined, and cell nuclei were counted by particle analysis (n = 5, control versus bevacizumab [P = 0.152]; control versus murine VEGF antibody [P = 0.5]).

**Figure 3.** Healthy corneas treated with anti–VEGF antibody (bevacizumab) eyedrops or control showed no new epithelial defects after 7 and 14 days. In vivo images of murine eyes after 7- and 14-day treatment with bevacizumab eyedrops or control solution.
in PBS incubated for 45 minutes in a moist chamber in the dark. Afterward, slides were given five 5-minute washes at room temperature in the dark. For staining of the nuclei, 20 μL DAPI in 50 mL PBS for 10 minutes was used, and slides were given three 5-minute washes in PBS. Slides were covered with fluorescent mounting medium (Dako) and stored at 4°C.

**FIGURE 4.** Anti-VEGF eyedrops do not impair the nerve density of normal murine corneas after midterm application. (a) Representative image of the central area of a normal murine cornea stained with a neurofilament antibody (FITC-labeled). Original magnification, 100×. (b) Representative image measuring the nerve fiber length in the corneal center by retracing the fibers with the coarse (red lines). NF, nerve fiber. (c, d) Nerve density of control was given as 100%. (c) After 2 weeks, no significant differences were observed between bevacizumab-treated and control corneas (P = 0.694; n = 10). (d) After 4 weeks, still no significant differences were observed between bevacizumab-treated and control corneas (P = 0.698; n = 10).

**FIGURE 5.** Similar epithelial wound healing in anti–VEGF-treated and control mice. (a) Mice treated with bevacizumab eyedrops. (b) Mice treated with an antimurine VEGF-A antibody as eyedrops. At 0 hours (immediately after corneal abrasion), 12 hours, 18 hours, and 24 hours, in vivo images of the epithelial defect were taken after staining with fluorescein. Deepithelialized areas were measured with an image analysis program (CellˆF, Olympus GmbH). (a) No significant differences in size were observed in the deep epithelialized area, as follows: 12 hours: P > 0.73; control, 0.8706 ± 0.067 mm²; bevacizumab, 0.8980 ± 0.1754 mm²; 18 hours: P > 0.84; control, 0.2303 ± 0.179 mm²; bevacizumab, 0.1790 ± 0.088 mm²; 24 hours: P > 0.99; control, 0.004 ± 0.004 mm²; bevacizumab, 0.081 ± 0.169 mm²; n = 5. (b) For the antimurine VEGF antibody, similar results were obtained, as follows: 12 hours: P > 0.99; control, 0.5397 ± 0.042 mm²; antimurine VEGF antibody, 0.5391 ± 0.053 mm²; 18 hours: P > 0.96; control, 0.1977 ± 0.049 mm²; antimurine VEGF antibody, 0.1948 ± 0.059 mm²; 24 hours: P > 0.96; control, 0.0282 ± 0.024 mm²; antimurine VEGF antibody, 0.0059 ± 0.004 mm²; n = 5.
Statistical Analysis

Statistical analysis for the wound healing assay was performed with the Mann-Whitney U test, and for the epithelial cell count and the nerve density assay an unpaired t-test was used. For analysis, statistical programs (InStat and Prism; GraphPad Software, Inc., San Diego, CA) were used.

RESULTS

Effects of Anti–VEGF Antibody Eyedrops on Normal Murine Corneas In Vivo

To analyze whether anti–VEGF antibody eyedrops have any effect on healthy corneas, mice with healthy corneas received bevacizumab (5 mg/mL)/antimurine VEGF-A antibody (250 μg/mL) or saline solution as eyedrops for 1 or 2 weeks. Afterward the eyes were collected for histologic analysis.

First, we analyzed the morphology of corneal epithelial cells of the central cornea in histologic specimens and performed ultrastructural analyses of the corneal epithelium by electron microscopy after 7 and 14 days’ treatment (only for bevacizumab eyedrops). In each group at all time points, the epithelium was five to six layers thick, and basal keratinocytes were well shaped and not cornified. Keratinocytes became flattened near the surface. There was no obvious difference between the control group and the bevacizumab-treated group on the light and electron microscopic levels (Fig. 1).

Second, we analyzed the amount of epithelial cells in the central cornea. Therefore, we took digital images of this area and analyzed them with an image analysis program (Cell F; Olympus GmbH). An area of 400 μm in length was outlined, and cell nuclei were counted by particle analysis. Again, no significant differences were detectable between treatment groups (antimurine VEGF antibody; bevacizumab) and the control group (Fig. 2).

Next we sought to determine (e.g., by inducing neurotrophic keratopathy in vivo) whether anti–VEGF antibody (bevacizumab) eyedrops can cause corneal epithelial defects. Therefore, the corneas of treated and control eyes were analyzed by slit lamp examination and by photography after 7 and 14 days’ treatment. No epithelial defects were observed (Fig. 3).

According to the recently demonstrated neuroprotective effect of VEGF-A,20 we also wanted to analyze whether mid-term application of anti-VEGF antibody eyedrops can lead to neurodegeneration in the healthy corneas of mice. Therefore, mice received bevacizumab (5 mg/mL) or saline solution as eyedrops for 2 and 4 weeks. Afterward, we collected the corneas and analyzed nerve density by staining the specimens with a neurofilament antibody and by measuring the total nerve fiber length in the center of each cornea. We observed no significant differences between the bevacizumab-treated group and the control group after both time periods (2 weeks: \( P > 0.7, \text{NS} \); 4 weeks: \( P > 0.7, \text{NS} \); Figs. 4c, 4d). Additionally, specifically blocking with an antimurine VEGF-A antibody (R&D Systems; Figs. 4e, 4f) was performed. With this high-affinity antibody, no effect on the innervation of the intact cornea was detectable (2 weeks: \( P = 0.1465, n = 8 \); 4 weeks: \( P = 0.9521, n = 9 \); Figs. 4e, 4f).

Effects of Anti-VEGF Eyedrops on Corneal Epithelial Wound Healing

To analyze whether VEGF neutralization by bevacizumab has an effect on wound healing of the corneal epithelium, we scraped off the central 1.8 mm² of the corneal epithelium and treated eyes with bevacizumab as eyedrops (5 mg/mL) or saline solution as control. After 12, 18, and 24 hours, we stained the corneas in vivo with fluorescein and took digital images (Figs. 5, 6). The deep epithelialized area was measured with an image analysis program. No significant differences in the size of the deep epithelialized area at any time point were observed between both groups (12 hours: \( P > 0.73 \); control, 0.8706 ± 0.067 mm²; bevacizumab, 0.8980 ± 0.1754 mm²; 18 hours: \( P > 0.84 \); control, 0.2303 ± 0.179 mm²; bevacizumab, 0.1790 ± 0.088 mm²; 24 hours: \( P > 0.99 \); control, 0.004 ± 0.004 mm²; be-
vacizumab, 0.081 ± 0.169 mm²; n = 5; Fig. 5a). We also could not find any significant differences when we used the blocking antimurine VEGF-A antibody (250 μg/mL [see Ref. 23]; R&D Systems; 12 hours: P > 0.99; control, 0.5397 ± 0.042 mm²; antimurine VEGF-A antibody, 0.5391 ± 0.053 mm²; 18 hours: P > 0.96; control, 0.1977 ± 0.049 mm²; antimurine VEGF-A antibody, 0.1948 ± 0.039 mm²; 24 hours: P > 0.96; control, 0.0282 ± 0.024 mm²; antimurine VEGF-A antibody, 0.0059 ± 0.004 mm²; n = 5; Fig. 5b). Subsequently, we collected the eyes and performed immunohistochemistry with Ki67 as a proliferation marker and routine histologic staining. We observed no obvious differences in the amounts of proliferating cells or in the shape and morphology of the corneal epithelial cells (Fig. 7).

**DISCUSSION**

VEGF-A is a key modulator not only of corneal angiogenesis but also of corneal lymphangiogenesis. Given that corneal neovascularization impairs corneal graft survival and reduces corneal transparency and vision, pharmacologic strategies to specifically target corneal VEGF-A are needed. The ultimate goal is to have specific antiangiogenic agents applied as eyedrops. Topical application of antiangiogenic drugs reduces the risk for systemic side effects.

Bevacizumab is the first FDA-approved antiangiogenic drug widely used off label in ophthalmology. Although bevacizumab applied topically at the cornea potentely inhibits neovascularization in human patients and in rodent models, not much is known about the safety profile of anti-VEGF eyedrops in vivo. Therefore, we performed in vivo experiments to analyze whether VEGF-A neutralization at the ocular surface causes side effects.

Our studies show, for the first time, that VEGF-A neutralization at the ocular surface with bevacizumab eyedrops or blocking antimurine VEGF-A antibody does not affect normal corneal anatomy and integrity (at least not short term), does not cause new epithelial defects (at least not short term), does not significantly impair the nerve density of a resting, normal murine cornea, and does not significantly affect corneal epithelial wound healing in vivo.
Taken together, these experimental animal in vivo data suggest topical application of anti-VEGF antibody to be relatively safe. This is consistent with the clinical experience of our\(^8\) and other groups,\(^12,15\) of the safety data acquired for retina and choroid,\(^29,30\) and of several ocular cell types on the in vitro level.\(^31,32\) In contrast, a recent clinical study\(^15\) demonstrates new epithelial defects under topical bevacizumab therapy. That discrepancy may be related to different degrees of VEGF-A neutralization because of different affinities to VEGF-A, different dependencies on VEGF-A of ocular surface in mice and humans, different concentrations of drugs used here and in the clinical setting, and different effects of VEGF-A neutralization in normal and diseased tissue and corneas.

To verify our findings and because of the low affinity of bevacizumab to murine VEGF-A,\(^29,30\) we also used an antimumire VEGF-A specific blocking antibody. Using this high-affinity antibody to murine VEGF-A, we were able to replicate the findings that epithelial wound healing, corneal integrity, and normal corneal innervation are not significantly affected by the blockade of VEGF-A in an otherwise healthy cornea. That finding is in contrast to the general wound healing–inhibiting effect of antiangiogenic drugs in vascularized tissues.\(^33,54\)

The FDA demonstrated that most serious, and sometimes fatal, toxicities—in addition to, for example, gastrointestinal perforation, hemorrhage, and arterial thromboembolic events—resulting from bevacizumab plus FOLFOrX (5-fluorouracil, leucovorin, oxaliplatin) administration are complications of wound healing.\(^55\) Accordingly, negative effects of topical VEGF-A neutralization at the cornea on the wound healing of corneal abrasion were to be expected. Our ultrastructural and histologic analysis, however, indicated that the neutralization of VEGF-A at the ocular surface does not affect corneal integrity or epithelial wound healing. Recently, it was shown that bevacizumab eye drops impair murine corneal nerve fiber regeneration.\(^36\) That we did not observe a significant effect of bevacizumab eye drops/antimurine VEGF-A antibody on normal resting nerve fibers might have indicated that a higher level of VEGF-A is present during corneal wound healing\(^57\) and that, therefore, more VEGF-A might be needed during nerve fiber repair. Santos et al.\(^38\) demonstrated that in vitro wounding of endothelial cell monolayers leads to internalization of VEGF and VEGF-R2 to the nucleus, which is essential for monolayer recovery. Additionally, it was shown that VEGF-R1 blockade impedes VEGF and VEGF-R2 activation and internalization, blocking endothelial monolayer recovery.\(^38\) Although there is no evidence yet for VEGF-R2 expression on corneal epithelium, it has been shown that VEGF-A, VEGF-R1, and VEGF-R3 are expressed in the corneal epithelium.\(^2,19\) Therefore, a similar mechanism could be involved in the wound healing of corneal epithelial cell layers. Nevertheless, we demonstrated here that there is no impact on speed or morphology of normal murine epithelium wound healing under anti-VEGF treatment.

Not only is VEGF-A an angiogenic growth factor, it is also chemotactic and neuroprotective.\(^40\) Reduction in VEGF-A levels leads to a murine form of amytrophic lateral sclerosis.\(^39\) VEGF has a neuroprotective effect on retinal ganglion cells.\(^40\) Given that the normal corneal epithelium is one of the most densely innervated tissues of the body\(^41\) and that both VEGF-A as its receptor VEGF-R1 and VEGF-R3 are normally present in the corneal epithelium, blocking of VEGF-A at the ocular surface could lead, for example, to neurotrophic keratopathy.\(^19,2\)

Neurotrophic keratopathy is a degenerative disease of the cornea caused by reduced corneal innervation.\(^42\) Loss or reduction of corneal innervation leads to a reduced aqueous phase of the tear film and due to reduced supply with neurotransmitters/trophic factors also to reduced epithelial healing capacity (impaired mitosis and migration). The combined presence of tear film deficiency and impaired epithelial healing capacity predispose to persistent epithelial defects. We observed no new epithelial defects as indications for neurotrophic keratopathy after 2-week application of anti-VEGF as eye drops. In addition, we observed no difference in corneal nerve density of corneas treated with anti-VEGF antibodies compared with control after 2 or 4 weeks. Therefore, our data suggest that bevacizumab does not cause significant neurotrophic keratopathy, at least not during short-term use in healthy corneas.

In conclusion, topical anti-VEGF antibodies such as bevacizumab eye drops do not seem to affect normal murine corneal structure, epithelial wound healing, or nerve fiber density. They should be relatively safe for use in patients with corneal neovascularization. Nevertheless, careful observation of the side effects of anti-VEGF antibodies, such as bevacizumab eye drops, are necessary, as are controlled trials.

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