Bevacizumab as a Potent Inhibitor of Inflammatory Corneal Angiogenesis and Lymphangiogenesis

Felix Bock,1 Jasmine Onderka,1 Tina Dietrich,1 Björn Bachmann,1 Friedrich E. Kruse,1 Matthias Pascbke,2 Grit Zahn,2 and Claus Cursiefen1

PURPOSE. To analyze whether bevacizumab can inhibit inflammatory angiogenesis and lymphangiogenesis in the cornea. Bevacizumab (Avastin; Roche, Welwyn Garden City, UK) is a recombinant, humanized, monoclonal antibody against VEGF-A that has been approved by the U.S. Food and Drug Administration for the treatment of colon carcinomas.

METHODS. The mouse model of suture-induced corneal neovascularization was used to assess the antihemangiogenic and antilymphangiogenic effect of bevacizumab by systemic and topical application. Corneal flaps were stained with LYVE-1 as a specific lymphatic vascular endothelial marker and CD31 as a pan-endothelial marker, and blood and lymph vascularized areas were analyzed morphometrically. The inhibitory effect of bevacizumab on lymphatic endothelial cells (LECs) was analyzed with a colorimetric (BrdU) proliferation ELISA. The binding ability of bevacizumab to murine VEGF-A was analyzed by Western blot, ELISA, and surface plasmon resonance.

RESULTS. The systemic and topical applications of bevacizumab significantly inhibited the outgrowth of blood (P < 0.006 and P < 0.0001, respectively) and lymphatic (P < 0.002 and P < 0.0001, respectively) vessels. Inhibition of the proliferation of LECs was also significant (P < 0.0001). Western blot analysis, ELISA, and the surface plasmon resonance assay showed that bevacizumab binds murine VEGF-A.

CONCLUSIONS. Topical or systemic application of bevacizumab inhibits both inflammation-induced angiogenesis and lymphangiogenesis in the cornea. This finding suggests an important role of VEGF-A in corneal lymphangiogenesis. Bevacizumab may be useful in preventing immune rejections after penetrating keratoplasty or tumor metastasis via lymphatic vessels. (Invest Ophthalmol Vis Sci. 2007;48:2545–2552) DOI: 10.1167/iovs.06-0570

A normal, healthy cornea is devoid of both blood and lymphatic vessels, but inflammatory conditions such as chemical burns or herpes infection can lead to a breakdown of this "angiogenic privilege." Consequently, in severe corneal inflammation, blood and lymphatic vessels grow in parallel into the cornea, thereby reducing visual acuity and increasing the risk of graft rejection after subsequent penetrating keratoplasty. But, lymphangiogenesis not only plays an important role in mediating immune reactions after (corneal) transplantation but also facilitates tumor metastasis.

Many endogenous and exogenous antiangiogenic factors are known already. In contrast, to inhibit lymphangiogenesis, so far there are only a few experimental approaches (e.g., the use of a VEGFR1R2-Trap or of a blocking anti-VEGFR3-antibody) but there is no U.S. Food and Drug Administration (FDA)-approved substance available yet.

Bevacizumab (rhUAB VEGF) is a recombinant, humanized, monoclonal antibody that binds to VEGF-A and prevents VEGF-A from ligating to its receptor. The antibody was engineered by assembling VEGF-A-binding residues from the murine-neutralizing antibody into a framework of a human immunoglobulin. Bevacizumab was FDA-approved in 2004 as a first-line treatment for patients with metastatic colorectal cancer. In addition, it is now widely used off-label for treatment of neovascular forms of age-related maculopathy.

Recently several reports have indicated that VEGF-A not only mediates hemangiogenesis but also lymphangiogenesis. Therefore, we tested the hypothesis, that blocking VEGF-A using bevacizumab can inhibit the outgrowth of both blood and lymphatic vessels in the cornea. So far, no FDA-approved antiangiogenic agent for use against corneal neovascularization is available, and in addition it is unclear whether bevacizumab inhibits lymphangiogenesis. This possibility has therapeutic implications beyond ophthalmology.

METHODS

Western Blot Analysis

The Western blot analysis was performed as described previously. Before SDS-PAGE, the samples were denatured at 95°C for 10 minutes. A sample volume of recombinant murine VEGF_A40 (RELIA Tech GmbH, Braunschweig, Germany) and recombinant human VEGF_A105 (Chemicon International Inc., Hampshire, UK) served as the control (2 μg each). For detection, 10 μg bevacizumab was added as the primary antibody and an alkaline phosphate-conjugated, Fab-specific goat anti-human IgG (Sigma-Aldrich-Fluka, Diesenhofen, Germany) as the secondary antibody.

Bevacizumab-Binding Analysis with Murine and Human VEGF-A

Solid-Phase Binding Assay. The binding activity of bevacizumab to human and murine VEGF-A was determined in a solid-phase binding assay by using soluble antibody and coated VEGF-A. Binding of antibodies was then detected by specific secondary antibody in an enzyme-linked immunosorbent assay. Culture plates (Nunc-immunoMaxisorp; Nalge Nunc Europe, Ltd. Neeeijse, Belgium) were coated overnight at 4°C with human or murine VEGF-A (5 μg/mL; Chemicon International, Inc.) in 15 mM Na2CO3 and 35 mM NaHCO3 (pH 9.6). All subsequent washing and binding was performed in TPBS (PBS with 0.05% Tween 80) with 1 mg/mL BSA. The plates were blocked with 3% BSA in PBS 0.1% Tween 80 for 1 hour at room temperature. A serial dilution of bevacizumab (Roche, Welwyn Garden

From the 1Department of Ophthalmology, University of Erlangen-Nürnberg, Erlangen, Germany; and 2Jerini AG, Berlin, Germany.

Supported by the Interdisciplinary Center for Clinical Research (IZKF) Erlangen (A9) and ELAN Funds Erlangen.

Submitted for publication May 26, 2006; revised November 17, 2006, and January 24, 2007; accepted April 2, 2007.

Disclosure: F. Bock, None; J. Onderka, None; T. Dietrich, None; B. Bachmann, None; F.E. Kruse, None; M. Pascbke, Jerini AG (E); G. Zahn, Jerini AG (E); C. Cursiefen, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Claus Cursiefen, Department of Ophthalmology, University of Erlangen-Nürnberg, Schwabachanlage 6, 91054 Erlangen, Germany; claus.cursiefen@augen.imed.uni-erlangen.de.

Copyright © Association for Research in Vision and Ophthalmology
City, UK) or human isotype control IgG1k (5 μg/mL–0.05 ng/mL; Southern Biotech, Birmingham, AL) were added to the VEGF-A-coated plates and incubated for 1 hour at room temperature. The detection antibody (anti-human-Fc-HRP [horseradish peroxidase] antibody conjugate, Sigma-Aldrich-Fluka) was then applied for 1 hour at room temperature. The detection of HRP was performed with HRP substrate (Chemicon International, Inc.) and stored at 4°C in the dark.

**Surface Plasmon Resonance Assay.** For qualitative binding analysis surface plasmon resonance measurement was performed (Biacore X, Biacore Life Sciences, Freiburg, Germany). Carboxymethylated dextran biosensor chip (CM5; Biacore AB) were activated with EDC (1-ethyl-3-(3-dimethylaminopropyl) and NHS (N-hydroxysuccinimide) according to the supplier’s instructions. Human isotype control IgG1k (Southern Biotech) was immobilized in Fc1 and bevacizumab (Roche) in Fc2. Different concentrations (2–25 nM) of human and murine VEGF-A (both Chemicon International Inc.) were injected in HBS-EP buffer at a flow rate of 5 mL/min, and the association and dissociation behavior were compared. Association and dissociation constants were not determined.

**Animals**

For the suture-induced inflammatory corneal neovascularization assay, female BALB/c mice (age range, 6–8 weeks) were used. The local animal care committee approved all animal protocols, in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Suture-Induced Inflammatory Corneal Neovascularization Assay**

Before surgery, each animal was deeply anesthetized with an intramuscular injection of ketamine (8 mg/kg) and xylazine (0.1 mL/kg). Three 11-0 nylon sutures (Serag Wiessner, Naila, Germany) were placed intrastromally with two stromal incisions extending over 120° of corneal circumference each. The outer point of suture placement was chosen near the limbus, and the inner suture point was chosen near the corneal center equidistant from the limbus, to obtain standardized angiogenic responses. Sutures were left in place for the duration of the experiment. The systemic treatment group received bevacizumab intraperitoneally on the day of surgery and 3 days later (5 mg/kg in saline solution, according to the manufacturer’s instruction). Control mice received an equal volume and concentration of a human IgG1k/isotype control (Southern Biotech) or an equal volume of saline solution. After 7 days, the mice were killed. In the long-term treatment group, the mice received 5 mg/kg bevacizumab in saline solution on the day of surgery, and on days 3, 6, 9, and 12. Control mice received an equal volume of saline solution. After 14 days, the mice were killed. For topical treatment, the central 2 mm of the corneal epithelium was scraped off before suturing, and the mice received bevacizumab (5 mg/mL; four times daily; according to off-label treatments and the literature) in eye drops for 5 days. Control mice received an equal volume of saline solution. The mice were killed after 5 days.

**Corneal Wholemounts and Morphologic Determination of Hem- and Lymphangiogenesis**

The corneas were excised, rinsed in PBS, and fixed in acetone for 30 minutes, as described previously. After three additional washing steps in PBS and blocking with 2% BSA in PBS for 2 hours, the corneas were stained overnight at 4°C with rabbit anti-mouse LYVE-1 (1:500; a kind gift of David G. Jackson, Oxford University, Oxford, UK), as described previously. On day 2, the tissue was washed, blocked, and stained with FITC-conjugated rat anti-CD31 (Acros Antibodies GmbH, Hildenhausen, Germany) antibody overnight at 4°C. After a last washing and blocking step on day 3, LYVE-1 was detected with a Cy3-conjugated secondary antibody (rabbit anti-mouse; 1:100; Dianova, Hamburg, Germany). Isotype control was assured with an FITC-conjugated normal rat IgG for CD31-FITC and with a normal rabbit IgG (both from Santa Cruz Biotechnology, Santa Cruz, CA) for LYVE-1.

For cryosection staining, vascularized murine eyes were cryopreserved in OCT embedding medium and 5- to 7-μm cryosections were obtained. Sections were dried (15 minutes, 37°C) and fixed in acetone on slides for 15 minutes (Superfrost; Fisher Scientific, Pittsburgh, PA). After the slides were rinsed with PBS (three times for 5 minutes each on a shaker) and 1-h incubation in 2% BSA (bovine serum albumin) at room temperature, the LYVE-1 antibody (1:500) was incubated overnight at 4°C. On the second day, we rinsed the slides with PBS (five times for 5 minutes each on a shaker), blocked them in 2% BSA (1 hour), and incubated them with a podoplanin antibody (monoclonal syring-hamster-anti-mouse antibody, 1:200; Acris Antibodies GmbH) overnight at 4°C in the dark. On the third day, after the slides were rinsed with PBS (five times for 5 minutes), secondary antibodies were incubated for 45 minutes at room temperature in the dark (goat-anti-syring hamster FITC antibody 1:100; rabbit anti-mouse; 1:500; Dianova). All dilutions were 2% BSA in PBS and all incubations were performed in a humid chamber. After a final rinsing step (five times for 5 minutes in PBS), the sections were covered with fluorescent mounting medium (Dako, Carpinteria, CA) and stored at 4°C in the dark.

**Functional and Statistical Analysis**

Double stained wholemounts and cryosections were analyzed with a fluorescence microscope (BX51; Olympus Optical Co., Hamburg, Germany), and digital images were taken with a 12-bit monochrome CCD camera (F-View II; Soft Imaging System, Münster, Germany). Each wholemount image was assembled from nine images taken at 100x magnification. The areas covered with blood or lymphatic vessels were detected with an algorithm established in image-analysis program (analySIS B; Soft Imaging System). Before analysis, gray-scale images of the wholemount photographs were modified by several filters. Blood and lymphatic vessels were detected with a threshold setting that included the bright vessels and excluding the dark background. Quantitative analysis was performed using rectangles of a standardized size (1.11 mm²) aligned along the limbus. Blood or lymphatic vessels in each rectangle were measured and correlated with the rectangle area (vessel ratio). Statistical analysis was performed (InStat 3, ver. 3.06) and graphs were drawn (Prism 4, ver. 4.03; both GraphPad Software Inc, San Diego, CA).

**Lymphatic Endothelial Cell Proliferation ELISA**

Human lymphatic microvascular endothelial cells (HLMLECs; Cambrex BioScience, Walkersville, MD) were cultured in EGM2-MV medium (Cambrex BioScience) according to the manufacturer’s instructions. For the ELISA, cells were seeded in a 96-well plate in the medium at a density of 2 × 10⁴ cells/well and left overnight to attach. To analyze, whether bevacizumab inhibits LEC proliferation (bevacizumab experiment), medium was replaced with serum-, bFGF- and VEGF-A-free EGM2-MV medium (minimal medium). Bevacizumab (0.05 μg/mL) and BrDU (0.1 μL/mL; Cell Proliferation ELISA for BrdU; Roche) were added. As the control, equal volumes of saline solution or IgG1k/isotype control were added instead of bevacizumab. After 3 hours, the medium was supplemented with 0.0025 ± 0.002 μg/mL (customer data) human VEGF-A (EGM2-MV supplement; Cambrex BioScience). Cells were fixed and stained after 3 days according to the manufacturer’s instructions (Cell Proliferation ELISA for BrdU; Roche). Colorimetric analysis was performed with an ELISA plate reader (Spectra; SLT Labinstruments Deutschland GmbH, Crailsheim, Germany).

To analyze directly whether VEGF-A induces proliferation of LECs (VEGF-A experiment), we added 0.05 μg/mL VEGF-A (Chemicon International, Inc.) to the minimal medium. Saline solution served as the control. Again, cells were fixed and stained after 3 days according to the manufacturer’s instructions (Cell Proliferation ELISA for BrdU;
Roche). Colorimetric analysis was again performed with the ELISA plate reader (Spectra; SLT Labinstruments Deutschland GmbH).

RESULTS

Bevacizumab Binding to Murine and Human VEGF-A

We performed three different experimental approaches using Western blot analysis, ELISA, and BIAcore analysis to assess whether bevacizumab binds to murine VEGF-A. For Western blot analysis, recombinant murine VEGF-A164, a 24-kDa protein consisting of 164 amino acids residues, and the 19.1-kDa homologous human VEGF165, which consists of 165 amino acid residues, were used. In ELISA and the BIAcore analysis, recombinant human and the homologous murine VEGF-A were used as well. Our Western blot analysis showed that bevacizumab bound murine and human VEGF-A (Fig. 1), when VEGF-A was immobilized on a membrane.

To confirm these results, we performed two different experimental approaches for analysis of the binding of bevacizumab to murine VEGF-A. The first assay, an ELISA, analyzed the interaction of soluble bevacizumab with VEGF-A immobilized on a microtiter plate surface. In the second assay, the surface plasmon resonance technology (BIAcore) was used for the analysis of interaction of soluble VEGF-A with bevacizumab immobilized on the sensor chip surface. Both assays allow a qualitative characterization of the interaction between bevacizumab and human or murine VEGF-A.

In the ELISA binding assay, bevacizumab was able to bind to both human and murine VEGF-A; however, an ~1000-fold higher bevacizumab concentration was necessary to reach a similar level of binding to murine VEGF-A compared with binding to the human protein (Fig. 2). There was no significant binding of isotype control IgG1k to murine VEGF-A at this concentration.

During surface plasmon resonance experiments using BIAcore, different concentrations of human and murine VEGF-A were injected, to analyze their binding to immobilized bevacizumab. As shown in Figure 3, a more than more than 10-fold higher concentration of murine VEGF-A than of human VEGF-A was necessary to reach similar resonance units (representing the interaction). The qualitative comparison of the interaction profiles showed significantly different dissociation behavior. Thus, bevacizumab bound the human VEGF-A very strongly. No significant dissociation was detectable during the time course of the experiment. In contrast, the interaction of the murine VEGF-A with bevacizumab was prone to a more rapid dissociation.

Effect of Systemic Application of Bevacizumab on Hem- and Lymphangiogenesis

We first investigated the effect of systemically applied bevacizumab on the outgrowth of blood and lymphatic vessels in a suture-induced corneal neovascularization assay (5 mg/kg injected intraperitoneally14). The treatment group (n = 9) showed a significant decrease in angiogenesis (P < 0.01) and lymphangiogenesis (P < 0.001) compared with the control (Fig. 4). We tested the specificity of LYVE-1 in the murine cornea by double staining cryosections of vascularized murine corneas with podoplanin and LYVE-1 (Fig. 5).

Effect of Topical Application of Bevacizumab on Hem- and Lymphangiogenesis

Next, we determined whether the topical application of bevacizumab also significantly affects corneal neovascularization.
Therefore, we modified the corneal neovascularization assay by scraping off the central 2 mm of corneal epithelium before suture placement. By application of 5 mg/mL bevacizumab as eye drops (0.25 mL/drop) five times daily the outgrowths of blood ($P < 0.0001$) and lymphatic ($P < 0.0001$; $n = 8$) vessels again was inhibited significantly (Fig. 6).

**FIGURE 3.** Binding analysis of murine and human VEGF-A to bevacizumab with surface plasmon resonance (BIAcore assay). VEGF-A bound to bevacizumab immobilized on the chip surface using surface plasmon resonance.

**FIGURE 4.** Systemic application of bevacizumab significantly decreased lymph- and hemangiogenesis in the cornea in comparison to the control. (a–l) Representative segments of corneal wholemounts. *Arrows*: limbus, (a, d, g) LYVE-1$^+$ lymphatic vessels; (b, e, h) CD31$^+$ blood vessels; (c, f, i) merger of (a) and (d), (b) and (e), and (g) and (h), respectively. Inhibition of (j) hemangiogenesis ($P < 0.01$) and (k) lymphangiogenesis ($P < 0.001$; $n = 9$) after treatment with bevacizumab over 1 week in a suture-induced neovascularization assay. Vessel ratio: area covered by blood/lymphatic vessels (%) in relation to the control (set to 100%). Original magnification, $\times 40$. 
Long-Term Application of Bevacizumab

To assess the ability of bevacizumab to inhibit hem- and lymphangiogenesis over an extended period, we treated the mice intraperitoneally five times over 2 weeks with 5 mg/kg bevacizumab per injection after suture placement. Hem- and lymphangiogenesis were inhibited significantly. Therefore, prolonged administration of bevacizumab leads to stable reduction of hemangiogenesis ($P < 0.0001$) and lymphangiogenesis ($P < 0.003$; Fig. 7).

IgG-Isotype Control Backup

Given that the unspecific part of an antibody sometimes can have related effects as the specific binding domain of an antibody, we compared bevacizumab with its isotype control (human IgG1k). Bevacizumab inhibited hem- and lymphangiogenesis significantly in comparison to the isotype control ($P < 0.0003$; $P < 0.003$; Fig. 8). The outgrowth of blood and lymphatic vessels was decreased by 24% and 31%, respectively. Using saline solution as control we obtained a decrease in hemangiogenesis by 23% and of lymphangiogenesis by 40%.

**Figure 5.** Cryosections of vascularized, murine corneas were double-stained with podoplanin (green) and LYVE-1 (red). Both markers colocalize on lymphatic vessels in the stroma. LV, lymphatic vessels. Original magnification: ×1000.

**Figure 6.** Topical application of bevacizumab significantly decreased lymph- and hemangiogenesis in the cornea in comparison with the control. Inhibition of (a) hemangiogenesis ($P < 0.0001$) and (b) lymphangiogenesis ($P < 0.0001$; $n = 8$) after topical treatment with bevacizumab over 5 days in a suture-induced neovascularization assay. Vessel ratio is as in Figure 4.

**Figure 7.** Inhibition of hem- and lymphangiogenesis in the cornea by long-term application of bevacizumab. Inhibition of (a) hemangiogenesis ($P < 0.0001$) and (b) lymphangiogenesis ($P < 0.003$; $n = 8$) after treatment with bevacizumab over 2 weeks in a suture-induced neovascularization assay. Vessel ratio is as in Figure 4.
due to the bevacizumab treatment (Fig. 4). The results of these two control approaches were not significantly different (blood vessels: $P > 0.7$; lymphatic vessels: $P > 0.5$).

**Inhibition of Lymphatic Endothelial Cell Proliferation In Vitro**

Subsequently, we wanted to know whether the novel antilymphangiogenic effect of bevacizumab is due to a direct effect of VEGF-A on LECs. Therefore, we analyzed the effect of neutralizing VEGF-A on the LECs in an in vitro proliferation assay. We supplied the LEC culture with 50 ng/mL bevacizumab and human VEGF-A. As a control for bevacizumab, we used saline solution and an IgG1k-isotype control at equal volumes. The statistical analysis showed that the proliferation of the LECs was significantly inhibited by bevacizumab treatment (saline control: $P < 0.0003$; control IgG: $P < 0.0001$; Figs. 9a, 9b). To ensure that VEGF-A can induce the growth of lymphatic endothelial cells in vitro, we added 50 ng/mL human recombinant VEGF-A to the LEC culture. The proliferation was increased significantly under the influence of VEGF-A ($P < 0.0001$; Fig. 9c).

**DISCUSSION**

The results of the experiments allow the following three conclusions to be drawn:

**FIGURE 8.** Inhibition of (a) hemangiogenesis ($P < 0.003$) and (b) lymphangiogenesis ($P < 0.0003$; $n = 8$) after treatment with bevacizumab over 1 week in a suture-induced neovascularization assay in comparison with a human IgG1k-isotype control. Vessel ratio is as in Figure 4.

**FIGURE 9.** Bevacizumab significantly inhibited proliferation of lymphatic endothelial cells (LEC) in vitro. Bevacizumab (0.05 μg/mL) significantly inhibited LEC proliferation in comparison to (a) saline solution (NaCl; $P < 0.0003$; $n = 60$) and (b) a human IgG1k-isotype control ($P < 0.0001$; $n = 60$). (c) VEGF-A significantly promoted cell proliferation in comparison to the control ($P < 0.0001$; $n = 60$); proliferation was measured by a cell proliferation ELISA with BrdU.
1. Bevacizumab is the first FDA-approved antiangiogenic drug that is shown to inhibit lymphangiogenesis in addition to hemangiogenesis.

2. Both topical and systemic application of bevacizumab inhibits inflammatory hem- and lymphangiogenesis in the cornea.

3. The in vivo findings together with the observation that bevacizumab in vitro inhibits proliferation of lymphatic endothelial cells support the concept of an important role of VEGF-A in lymphangiogenesis. The LEC in vitro data furthermore suggest an VEGFR3-independent pathway of lymphangiogenesis induction by VEGF-A, possibly through VEGFR2 expressed on LECs.

A plethora of antiangiogenic drugs is currently in clinical trials for use especially in oncology and ophthalmology. Numerous compounds have entered phase-II/III testing for the treatment of neovascular forms of age-related maculopathy or diabetic retinopathy. None of these compounds has been tested yet for its antilymphangiogenic effects. Therefore, bevacizumab is the first FDA-approved antiangiogenic drug that has been shown to inhibit the outgrowth of lymphatic vessels as well. According to our results and the expression of VEGF-A during inflammatory hem- and lymphangiogenesis in the cornea, bevacizumab could be used to improve graft survival after penetrating keratoplasty, by reducing the ingrowths of both blood and lymphatic vessels and thereby interrupting the so called “immune reflex arc.” In addition, several studies have recently shown that tumor-induced lymphangiogenesis is an important risk factor for tumor metastasis. Antilymphangiogenic treatment could reduce the incidence of metastasis in animal experiments. Therefore, the antilymphangiogenic effect of bevacizumab should be useful for treating cancer patients with other than metastatic colorectal cancer.

Similarly, plenty of clinical indications exist for antihem- and antilymphangiogenic treatment of the cornea (i.e., to stop vision-threatening corneal neovascularization or to improve graft survival after keratoplasty by inhibiting postoperative hem- and lymphangiogenesis). Yet, so far there are no FDA-approved antiangiogenic agents available for use at the cornea. Bevacizumab, as mentioned, is the first FDA-approved antiangiogenic drug which shows topical antihemangiogenic and surprisingly also antilymphangiogenic effects against corneal neovascularization. The topical application allows easier use of bevacizumab in ophthalmology. Clinical trials will have to determine the efficacy, optimal dosage, and safety of topical application of bevacizumab and its potential effects on corneal epithelium. In our study, we did not observe toxic effects on corneal epithelium when bevacizumab was used in normal eyes (data not shown).

Finally, our findings also shed light on the role of VEGF-A in lymphangiogenesis. Blocking VEGF-A by bevacizumab inhibited not only the ingrowths of lymphatic vessels into the cornea in vivo but also significantly impaired proliferation of lymphatic endothelial cells in vitro. Although we cannot completely rule out an indirect effect on lymphangiogenesis via primary inhibition of hemangiogenesis, our in vivo and in vitro data suggest a primary effect on lymphangiogenesis. In vitro, bevacizumab directly inhibited the growth of LECs. In vivo, a primary effect of bevacizumab on the outgrowth of lymphatic vessels in the cornea is supported by recent articles analyzing the time course of lymphangiogenesis. Under inflammatory conditions in the cornea, lymphangiogenesis can occur in parallel with or even precede hemangiogenesis.

Although, bevacizumab was previously described as binding specifically to human VEGF-A, we have clearly shown its binding abilities to murine VEGF-A in three independent molecular biological assays (Western blot analysis, ELISA, and BIacore assay). In the current study, we made an interesting observation: The molecular biological assays demonstrated that bevacizumab bound to murine VEGF-A with a lower affinity in comparison to human VEGF and dissociated faster. Nevertheless, when bevacizumab was used at a high concentration (5 mg/kg) in the mouse model, it displayed a significant inhibitory effect on lymphangiogenesis. Therefore, an initial removal of some or all the VEGF-A molecules in the inflamed corneal tissue seems to be sufficient to impair hemangiogenesis and lymphangiogenesis permanently. This finding is in line with previous experiments demonstrating that a brief blockade of VEGF-A (using a cytokine trap) permanently inhibits inflammatory lymphangiogenesis.

In addition, VEGF-A added to LECs in vitro increased proliferation of lymphatic endothelial cells. VEGF-A was previously thought to act as a specific blood angiogenic factor through activation of VEGFR1 and -2. Recent studies demonstrate, that VEGF-A is also able to induce lymphangiogenesis in animal models. In 2004 we reported, that VEGF-A activated inflammatory macrophages release VEGF-C and -D, which indirectly leads to lymphangiogenesis. Our current findings support the growing body of evidence suggesting that VEGF-A can directly induce lymphangiogenesis through a VEGF-C/D/VEGFR3-independent pathway (potentially through LECs expressing VEGFR2).

In summary, bevacizumab is the first FDA-approved antiangiogenic agent that also significantly inhibits lymphangiogenesis. Applied topically or systemically, it effectively inhibits corneal hem- and lymphangiogenesis, thus opening new avenues for topical treatment of corneal neovascularization.

Acknowledgments

The authors thank Elke Lütjen-Drecoll, Erlangen, for general help, Carmen Runnelt, Erlangen, for technical support; and Martin Baier, Erlangen, for preparation of the bevacizumab eye drops.

References


