Cornea

Serum Eyedrops Antagonize the Anti(lymph)angiogenic Effects of Bevacizumab In Vitro and In Vivo

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PURPOSE. The effect of autologous serum eyedrops on the corneal vasculature is undefined. Therefore, we analyzed the corneal vascular effects of serum eyedrops in comparison with and in combination with bevacizumab eyedrops.

METHODS. In vitro, blood and lymphatic endothelial cells were treated with serum eyedrops, bevacizumab eyedrops, or a combination of both, and cell proliferation was measured. In vivo, inflammatory corneal neovascularization was induced by suture placement. Subsequently, corneal blood and lymphatic vessel progression and regression were analyzed after treatment with serum or bevacizumab eyedrops or a combination of both. Hemangiogenesis and lymphangiogenesis were quantified in whole mounts using CD31 and lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1); inflammatory cell infiltration was analyzed using CD11b. Furthermore, corneal expression levels of interleukin 1β, tumor necrosis factor α, vascular endothelial growth factor (VEGF) A, VEGF-C, and VEGF-D were analyzed by real-time PCR.

RESULTS. In vitro, serum increased and bevacizumab decreased endothelial cell proliferation. In vivo, serum eyedrops had no significant effect on corneal vessel progression or regression. Bevacizumab eyedrops reduced blood and lymphatic vessel progression and promoted blood and lymphatic vessel regression. The combination of serum eyedrops and bevacizumab eyedrops attenuated the anti(lymph)angiogenic effects of bevacizumab. Inflammatory corneal cell counts were not significantly altered by serum or bevacizumab eyedrops. Serum eyedrops changed the proinflammatory and pro(lymph)angiogenic status of the cornea. Bevacizumab eyedrops significantly reduced proinflammatory and pro(lymph)angiogenic factor expression. Higher doses of bevacizumab did not restore its anti(lymph)angiogenic effects when used in combination with serum.

CONCLUSIONS. The counteracting effects of serum eyedrops and bevacizumab eyedrops on the corneal vasculature should be taken into account when combined therapeutic regimens are considered.

Keywords: autologous serum eyedrops, bevacizumab, corneal neovascularization, hemangiogenesis, lymphangiogenesis

Autologous serum eyedrops are widely used for the treatment of a variety of ocular surface disorders such as persistent corneal epithelial defects and severe forms of dry eye.1–3 One of the main reasons for the positive effects of autologous serum eyedrops on corneal wound healing is that these drops contain a plethora of epitheliotrophic and neurotrophic growth factors that promote accelerated epithelial wound closure, including epidermal growth factor, transforming growth factor β, fibroblast growth factor, nerve growth factor, and vascular endothelial growth factor (VEGF).3,4

Despite their well-defined wound healing profile, it is thus far unclear whether autologous serum eyedrops demonstrate positive effects in the treatment of ocular surface disorders in which there is both a lack of and an excess of certain growth factors (e.g., VEGF). In this context, the effect of serum eyedrops (which also contain VEGF) on corneal neovascularization has not been characterized to date. Pathologic ingrowth of blood and lymphatic vessels into the cornea not only reduces visual acuity directly but also is the main risk factor for immune-mediated graft rejection after corneal transplantation.5–7 In this regard, specifically lymphatic vessels have been shown to be responsible for higher rejection rates in vascularized corneas, mainly by serving as routes for antigen-presenting cell trafficking to secondary lymphoid organs and the initiation ofalloimmune responses.8,9 Recently, several studies10–12 demonstrated the beneficial effects of antiangiogenic therapy for the treatment of corneal neovascularization. We previously showed that bevacizumab (a recombinant, humanized, monoclonal antibody targeting VEGF-A that is approved by the U.S. Food and Drug Administration for the treatment of several metastatic carcinomas) inhibits not only corneal hemangiogenesis but also lymphangiogenesis.13

Several pathologic conditions of the ocular surface display nonhealing and relapsing epithelial defects coincident with corneal neovascularization. In these therapeutically challenging cases, autologous serum eyedrops are frequently used to provide the ocular surface with nutrients and growth factors and to promote wound closure.1,3,14 However, it is possible
that these supplied growth factors may worsen coexisting corneal neovascularization. Moreover, rational treatment of corneal neovascularization should require inhibition of elevated growth factor levels (e.g., VEGF) but not additional supplementation.

Therefore, we aimed to characterize the corneal vascular effects of serum eyedrops in comparison with and in combination with bevacizumab eyedrops. In vitro, we analyzed the effect of serum supplementation on blood and lymphatic endothelial cell proliferation. In vivo, we investigated whether serum eyedrops alter the progression or regression of pathologic corneal blood and lymphatic vessels. Furthermore, we analyzed inflammatory corneal cell infiltration and proinflammatory and pro(lymph)angiogenic cytokine expression after treatment with serum or bevacizumab eyedrops or a combination of both.

METHODS

Blood and Lymphatic Endothelial Cell Culture and Proliferation ELISA

Human blood and lymphatic microvascular endothelial cells (BECs and LECs, respectively; PromoCell GmbH, Heidelberg, Germany) were cultured in EGM2-MV medium containing 5% fetal calf serum (FCS). For cell proliferation analysis, a bromodeoxyuridine (BrdU)-based proliferation ELISA was used as previously described.15,16 Briefly, cells were seeded in a 96-well plate at a density of 3 to 4 × 10³ cells per well and cultured overnight before the medium was replaced with EGM2-MV medium containing 5% FCS and BrdU, along with allogenic human serum (10%), bevacizumab (50 ng/mL), or a combination of both. After 48 hours, cells were fixed and stained according to the manufacturer’s instructions. Colorimetric analysis was performed with an ELISA reader (Epoch Microplate Spectrophotometer; BioTek, Bad Friedrichshall, Germany). The mean extinction of the control wells was defined as 100%, and the extinction of all wells was then related to this value (cell proliferation ratio).

Animals and Anesthesia

All animal protocols were approved by the local animal care committee and were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All animals were 8-week-old female BALB/c mice (Charles River Laboratories, Sulzfeld, Germany). Mice were anesthetized with an intraperitoneal injection of a combination of ketamine hydrochloride (8 mg/kg) and xylazine hydrochloride (0.1 mL/kg).

Suture-Induced Inflammatory Corneal Hemangiogenesis and Lymphangiogenesis, Analysis of Vessel Progression, and Analysis of Vessel Regression

The mouse model of suture-induced corneal hemangiogenesis and lymphangiogenesis was described previously.17–19 Briefly, three 11-0 nylon sutures (Serag Wiessner, Nairi, Germany) were placed intrastromally into the right cornea, with two incursions each extending over 120° of the total corneal circumference. The outer point of suture placement was chosen near the limbus, and the inner suture point was chosen near the corneal center. Mice were randomly assigned to the various treatment groups, and corneal blood and lymphatic vessel progression, as well as corneal blood and lymphatic vessel regression, was analyzed. Each group comprised eight mice.

For the assessment of corneal blood and lymphatic vessel progression, treatment of mice started directly after suture placement and lasted 1 week. For the analysis of corneal blood and lymphatic vessel regression, sutures were left in place for 2 weeks, and mice received no treatment before the sutures were removed. After suture removal, mice were treated for 1 additional week. Mice received syngeneic serum eyedrops (three times daily), bevacizumab eyedrops (5 mg/mL three times daily), or a combination of both (serum eyedrops three times daily and bevacizumab eyedrops three times daily). Control mice received equal amounts of saline solution. Blood for the preparation of syngeneic serum was derived from healthy donor BALB/c mice and allowed to clot at room temperature. Serum was then separated by centrifugation, and serum eyedrops were applied undiluted. For the analysis of serum eyedrops combined with higher doses of bevacizumab, mice were treated with bevacizumab eyedrops (25 mg/mL six times daily) or a combination of syngeneic serum eyedrops (three times daily) and bevacizumab eyedrops (25 mg/mL six times daily). The volume of each eyedrop was 3 μL.

Preparation and Staining of Corneal Whole Mounts and Quantification of Corneal Hemangiogenesis and Lymphangiogenesis

Corneal whole mounts were prepared as described previously.15,20 Briefly, excised corneas were fixed in acetone for 20 minutes, blocked with 2% BSA in PBS for 2 hours, and stained overnight with a rabbit anti-mouse lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) antibody (AngioBio, Del Mar, CA) and FITC-conjugated rat anti-mouse CD31 antibody (Acris Antibodies, Herford, Germany) at 4°C. The next day, LYVE-1 was detected with a Cy3-conjugated secondary goat-anti-rabbit antibody (Dianova, Barcelona, Spain).

Stained whole mounts were analyzed with a fluorescence microscope (Olympus BX53; Olympus Corporation, Hamburg, Germany); photographs were obtained with a digital mono-chrome charge-coupled device camera (Olympus XM10; Olympus Corporation). Each whole mount photograph was automatically assembled from nine to twelve photographs taken at ×100 magnification. The hemvascularized and lymphvascularized areas detected and quantified with an algorithm established in the image analyzing program CellF (Soft Imaging System; Olympus Corporation, Hamburg, Germany) as previously described.21 For the analysis of blood and lymphatic vessel progression, the mean vascularized area of the control corneas was defined as 100%, and vascularized areas were then related to this value. For the analysis of blood and lymphatic vessel regression, the mean vascularized area of corneas excised 2 weeks after suture placement was defined as 100% (baseline vascularization after 2 weeks), and vascularized areas after 1 additional week of treatment were then related to this value.

Analysis of Corneal Immune Cell Infiltration

For the assessment of immune cell infiltration into the inflamed cornea, suture placement was performed, and mice were treated with syngeneic serum eyedrops (three times daily), bevacizumab eyedrops (5 mg/mL three times daily), or a combination of both (serum eyedrops three times daily and bevacizumab eyedrops three times daily). Control mice received equal amounts of saline solution (n = 5 per group and analyzed time point). After 2 days and 7 days of treatment, corneas were excised and fixed in acetone for 20 minutes, blocked with 2% BSA in PBS for 2 hours, and stained overnight.

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with FITC-conjugated rat anti-mouse CD11b antibody (Serotec, Raleigh, NC) at 4°C. Photographs of the central cornea of each whole mount were taken at ×200 magnification, with focus on the subepithelial corneal layer, and CD11b-positive cells were counted. The mean CD11b cell number per visual field of the control corneas was defined as 100%, and cell counts were related to this value (CD11b cell ratio).

**Analysis of Corneal Cytokine and Growth Factor Expression**

Corneal suture placement was performed, and mice were treated with syngeneic serum eyedrops (three times daily), bevacizumab eyedrops (5 mg/mL three times daily), or a combination of both (serum eyedrops three times daily and bevacizumab eyedrops three times daily). Control mice received equal amounts of saline solution (n = 5 per group per time point). After 2 days and 7 days of treatment, corneas were excised without the limbus and pooled for RNA isolation, cDNA synthesis, and real-time PCR analysis. The RNA was isolated by an RNeasy Micro Kit (Qiagen, Valencia, CA); cDNA was synthesized with random hexamers using reverse transcriptase (SuperScript III; Invitrogen, Carlsbad, CA), and primers (MWG Biotech, Ebersberg, Germany) were designed using Primer3 software (http://bioinfo.ut.ee/primer3/, in the public domain) and BLAST (Basic Local Alignment Search Tool; National Center for Biotechnology Information, Bethesda, MD) as described previously.15 The PCR reactions (25 μL) contained 20 to 40 ng of cDNA (depending on the analyzed gene), 0.4 μM of each forward and reverse primer, and master mix (SsoFast EvaGreen Supermix; Bio-Rad, Hercules, CA). Real-time PCR was performed under the following conditions: initial denaturation step of 95°C for 2 minutes, then 40 cycles of 95°C for 5 seconds and 60 to 63°C (depending on the analyzed gene) for 15 seconds, followed by an additional denaturation step of 95°C for 60 seconds and a subsequent melt curve analysis to check amplification specificity. All PCR products were analyzed by gel electrophoresis on a 2% agarose gel and were visualized by ethidium bromide staining. Primer sequences, product sizes, and respective annealing temperatures are summarized in the Table.

Real-time PCR results were analyzed by the comparative threshold cycle method with hypoxanthine guanine phosphoribosyl transferase (HPRT) as the endogenous reference gene for all reactions. The relative mRNA level in the saline-treated control group was used as the normalized control for all treatment groups. All assays were performed in triplicate, and a nontemplate control was included in all experiments to exclude DNA contamination.

**Statistical Analysis**

Statistical analyses were performed with Microsoft Excel 2010 (Microsoft Corp., Redmond, WA). Statistical significance was determined using the Student’s t-test. P < 0.05 was considered significant.

| Mouse Gene | Product Size, bp | Annealing Temperature, °C | Sequence (5’−3’)
|------------|-----------------|---------------------------|-----------------|
| HPRT       | 163             | 60–65                     | Forward: GTTGGATACAGGCCAGACTTTTG
|            |                 |                           | Reverse: AATCCCCGCTCATCTTTAGGC
| IL-1β      | 122             | 62.4                      | Forward: GTCCTCCTGTAATGGAAGAGCC
|            |                 |                           | Reverse: CAGAGCCACTGGCCTTTCC
| TNFα       | 176             | 63                        | Forward: CATGCGAATTCCTGGCCTTAC
|            |                 |                           | Reverse: CTGGTTGAGGTTGCTGATT
| VEGF-A     | 184             | 60                        | Forward: AGAAGGCGAACTGGCCTTTCC
|            |                 |                           | Reverse: CAGAGGCACCTGGCCTTAC
| VEGF-C     | 219             | 60                        | Forward: CATGCGAATTCCTGGCCTTTAC
|            |                 |                           | Reverse: CTGGTTGAGGTTGCTGATT
| VEGF-D     | 86              | 62.4                      | Forward: ATGGCGCTTTCCAAATAC
|            |                 |                           | Reverse: CAGAGGCACCTGGCCTTAC
RESULTS
Serum Addition Significantly Increases Blood and Lymphatic Endothelial Cell Proliferation In Vitro

The BECs and LECs were incubated with 10% additional serum, bevacizumab (50 ng/mL), or a combination of both; endothelial cell proliferation was assessed after 48 hours by ELISA. Serum addition significantly increased and bevacizumab significantly decreased endothelial cell proliferation (BEC serum mean of 155.3%, \( P < 0.01 \); bevacizumab mean of 86.4%, \( P < 0.05 \); LEC serum mean of 109.5%, \( P < 0.01 \); and bevacizumab mean of 67.0%, \( P < 0.001 \)). Combined treatment with serum and bevacizumab led to proliferation levels that ranged between those of the monotherapeutically treated groups (BEC serum and bevacizumab mean of 142.2%, \( P < 0.05 \); and LEC serum and bevacizumab mean of 94.1%, not significant [n.s.]) (Fig. 1).

Serum Eyedrops Antagonize the Inhibiting Effect of Bevacizumab Eyedrops on Blood and Lymphatic Vessel Progression In Vivo

Inflammatory corneal hemangiogenesis and lymphangiogenesis were induced by suture placement in BALB/c mice; corneas were then treated with serum eyedrops (three times daily), bevacizumab eyedrops (5 mg/mL three times daily), or a combination of both for 1 week. Top, Middle: Afterward, corneal whole mounts were analyzed using CD31 for blood (top green) and LYVE-1 for lymphatic vessels (middle red). Bottom: Quantification of blood (left) and lymphatic vascular progression (right). The mean vascularized area of the control corneas was defined as 100%, and vascularized areas were related to this value. Data are expressed as means ± SDs.

significant. Graphs were drawn using Prism™ version 4.03 (GraphPad Software, Inc., San Diego, CA).
Serum Eyedrops Antagonize the Hemangiogenesis and Lymphangioregressive Effect of Bevacizumab Eyedrops In Vivo

Pathologic blood and lymphatic vessels that grow into the cornea after suture placement have been shown to partially regress after removal of the sutures. Therefore, we analyzed the regression of corneal blood and lymphatic vessels 1 week after suture removal and treatment with serum eyedrops, bevacizumab eyedrops, or a combination of both. Regression rates were calculated by relating the hemvascularized and lymphvascularized areas of the treated corneas to the mean vascularized area of corneas that were excised on the day of suture removal. Vessel regression was not significantly altered by treatment with serum eyedrops, whereas treatment with bevacizumab eyedrops significantly promoted blood and lymphatic vessel regression compared with controls (blood vessel regression control mean of 70.2%, serum mean of 67.8%, n.s.; bevacizumab mean of 62.2%, \( P < 0.05 \); lymphatic vessel regression control mean of 77.4% and serum mean of 78.1%, n.s.; bevacizumab mean of 60.6%, \( P < 0.05 \)). The combination of serum eyedrops and bevacizumab eyedrops significantly antagonized the hemangioregressive and lymphangioregressive effects of bevacizumab (blood vessel regression serum and bevacizumab mean of 65.6%, n.s.; and lymphatic vessel regression serum and bevacizumab mean of 73.0%, n.s.) (Fig. 3).

Serum Eyedrops and Bevacizumab Eyedrops Do Not Significantly Alter the Quantity of Inflammatory Cell Infiltration In Vivo

We analyzed the contribution of inflammatory cells to the observed vascular changes in the cornea. Again, suture placement was performed, and CD11b-positive cells were quantified in corneal whole mounts after treatment with serum eyedrops, bevacizumab eyedrops, or a combination of both. We observed no significant differences in CD11b-positive cell counts after 2 days of treatment: compared with controls, serum-treated corneas showed similar quantities of CD11b-positive cells (mean of 103.1%, n.s.). Bevacizumab-treated corneas showed a slight but nonsignificant reduction in CD11b-positive cell counts (mean of 93.6%, n.s.), and the combination of serum and bevacizumab also led to comparable CD11b cell counts (mean of 105.6%, n.s.) (Fig. 4, top). Similarly, none of the treatment groups showed a significant alteration in the quantity of CD11b-positive cells after 7 days of treatment, although serum treatment appeared to slightly reduce cell counts (serum mean of 93.4%, n.s.; bevacizumab mean of 99.3%, n.s.; and serum and bevacizumab mean of 92.6%, n.s.) (Fig. 4, bottom).
Bevacizumab Eyedrops Have a More Significant Effect on Corneal Cytokine and Growth Factor Expression Than Serum Eyedrops In Vivo

Corneal expression levels of the proinflammatory and pro(lymph)angiogenic cytokines IL-1β, TNFα, VEGF-A, VEGF-C, and VEGF-D were analyzed by real-time PCR after suture placement and treatment with serum eyedrops, bevacizumab eyedrops, or a combination of both. After 2 days of treatment, only bevacizumab affected IL-1β expression (serum mean of 95.6%, n.s.; bevacizumab mean of 74.3%, P < 0.01; and serum and bevacizumab mean of 96.4%, n.s.), whereas after 7 days of treatment, all treatment groups had significantly reduced IL-1β expression (serum mean of 48.4%, P < 0.001; bevacizumab mean of 58.9%, P < 0.001; and serum and bevacizumab mean of 53.2%, P < 0.001) (Fig. 5, top left). After 2 days of treatment, TNFα expression was significantly impaired only by bevacizumab (serum mean of 106.1%, n.s.; bevacizumab mean of 68.4%, P < 0.05; and serum and bevacizumab mean of 93.0%, n.s.), while after 7 days of treatment, the combination of serum and bevacizumab also showed considerable inhibition of TNFα expression (serum mean of 107.5%, n.s.; bevacizumab mean of 86.6%, P < 0.01; and serum and bevacizumab mean of 84.3%, P < 0.01) (Fig. 5, top middle). VEGF-A expression was significantly inhibited by bevacizumab both after 2 days and after 7 days of treatment (bevacizumab after 2 days mean of 77.3%, P < 0.01; and bevacizumab after 7 days mean of 81.9%, P < 0.01). While exhibiting no effect after 2 days, serum treatment significantly upregulated VEGF-A expression after 7 days of treatment (serum after 2 days mean of 96.3%, n.s.; and serum after 7 days mean of 120.1%, P < 0.05). The combination of serum and bevacizumab showed no alteration in VEGF-A expression (serum and bevacizumab after 2 days mean of 100.7%, n.s.; and serum and bevacizumab after 7 days mean of 102.8%, n.s.) (Fig. 5, bottom left). VEGF-C expression was not significantly altered after 2 days of treatment with serum, bevacizumab, or a combination of both, although bevacizumab showed an apparent tendency to lower expression levels (serum mean of 90.5%, n.s.; bevacizumab mean of 82.8%, P = 0.06; and serum and bevacizumab mean of 95.4%, n.s.). After 7 days, serum had no effect, whereas bevacizumab and the combination of serum and bevacizumab inhibited VEGF-C expression (serum mean of 89.0%, n.s.; bevacizumab mean of 66.2%, P < 0.01; and serum and bevacizumab mean of 77.1%, P < 0.01) (Fig. 5, bottom middle). VEGF-D expression was reduced by bevacizumab both after 2 days and after 7 days.
bevacizumab after 2 days mean of 76.3%, \( P < 0.01; \) and bevacizumab after 7 days mean of 82.3%, \( P < 0.05 \). Serum and the combination of serum and bevacizumab did not modify VEGF-D expression after 2 days or after 7 days of treatment (serum after 2 days mean of 95.2%, n.s.; serum and bevacizumab after 2 days mean of 99.8%, n.s.; serum after 7 days mean of 104.7%, n.s.; and serum and bevacizumab after 7 days mean of 96.0%, n.s.) (Fig. 5, bottom right).

**Dose Increase of Bevacizumab Eyedrops Cannot Overcome the Antagonizing Effects of Serum Eyedrops**

To investigate whether higher doses of bevacizumab eyedrops can restore its anti(lymph)angiogenic effects when used in combination with serum eyedrops, we applied higher concentrations of bevacizumab eyedrops and increased the application frequency (high-dose bevacizumab of 25 mg/mL six times daily compared with 5 mg/mL three times daily). Higher doses of bevacizumab showed superior but nonsignificant antihemangiogenic effects, whereas the antilymphangiogenic effects remained comparable (blood vessel progression bevacizumab of 5 mg/mL three times daily mean of 80.2% versus bevacizumab of 25 mg/mL six times daily mean of 71.4%, n.s.; and lymphatic vessel progression bevacizumab of 5 mg/mL three times daily mean of 66.7% versus bevacizumab of 25 mg/mL six times daily mean of 67.8%, n.s.). The combination of serum eyedrops with higher doses of bevacizumab eyedrops still reduced the anti(lymph)angiogenic effects of bevacizumab on corneal blood and lymphatic vessel progression (blood vessel progression serum and bevacizumab of 25 mg/mL six times daily mean of 93.1%, n.s. versus control; and lymphatic vessel progression serum and bevacizumab of 25 mg/mL six times daily mean of 85.1%, n.s. versus control) (Fig. 6, top).

With regard to blood and lymphatic vessel regression, higher doses of bevacizumab showed slightly higher hemangioregressive effects (blood vessel regression bevacizumab of 5 mg/mL three times daily mean of 62.2% versus bevacizumab of 25 mg/mL six times daily mean of 57.4%, n.s.). Furthermore, the lymphangioregressive effects of higher doses of bevacizumab were higher compared with normal doses of bevacizumab (lymphatic vessel regression bevacizumab of 5 mg/mL three times daily mean of 60.6% versus bevacizumab of 25 mg/mL six times daily mean of 50.0%, n.s.). However, the combination of higher bevacizumab doses with serum eyedrops could not restore the hemangioregressive and lymphangioregressive effects of bevacizumab (blood vessel regression control mean of 70.2%; serum and bevacizumab of 25 mg/mL six times daily mean of 65.5%, n.s.; and lymphatic vessel regression control mean of 77.4%; serum and bevacizumab of 25 mg/mL six times daily mean of 73.9%, n.s.) (Fig. 6, bottom).

**DISCUSSION**

Autologous serum eyedrops have been shown to be an effective treatment option for several ocular surface disorders that generally display a lack of trophic factors.\(^1,3\) These eyedrops contain a large variety of nutrients and growth factors, which are responsible for their positive therapeutic effect.\(^2,4\) However, some of these epitheliotrophic and neurotrophic growth factors have also been shown to affect vascular cells and to promote corneal (lymph)angiogenesis.\(^23,24\) Therefore, it is possible that autologous serum eyedrops may aggravate corneal neovascularization.
The present study shows for the first time to date the following effects of serum eyedrops: (1) they promote BEC and LEC proliferation in vitro; (2) they cause no significant exacerbation of inflammatory corneal hemangiogenesis and lymphangiogenesis, with no alteration in vessel progression or regression in vivo; and (3) they seem to antagonize the anti(lymph)angiogenic effects of bevacizumab. These experimental findings may have important implications for the use of autologous serum eyedrops in patients having wound healing defects coincident with corneal neovascularization.

In vitro, serum addition significantly promoted BEC and LEC proliferation, which is not surprising because serum is the main culture medium supplement used to maintain growth and proliferation of various cell types. The proliferative effect of serum seems to be at least partially attributable to VEGF because addition of bevacizumab reduced serum-driven cell proliferation. However, this antagonism was incomplete, which may be due to additional (lymph)angioproliferative factors in serum (e.g., fibroblast growth factor) or overwhelming VEGF levels in serum.

In vivo, serum eyedrops alone did not alter the quantity of blood and lymphatic vessels developing after an inflammatory insult. Application of serum eyedrops to corneas with preexisting blood and lymphatic vessels did not attenuate spontaneous vessel regression, which is more comparable to the clinical setting, where patients often are seen with existing pathologic corneal vessels. Thus, these results suggest a safe vascular profile for serum eyedrops on neovascularized corneas; their use in patients with corneal wound healing deficiencies and corneal neovascularization will likely be unproblematic with respect to possible aggravation of corneal neovascularization.

The diverse findings in our in vivo experiments and our in vitro analysis indicate that in vivo several further mechanisms regulate corneal neovascularization and avascularity. In addition to the direct regulation of endothelial cell proliferation, various other steps are critical for the development of pathologic blood and lymphatic vessels in vivo. In this context, the cornea expresses different anti(lymph)angiogenic and decoy molecules, which are able to trap hemangiogenic and lymphangiogenic growth factors to a large extent, before these mechanisms are overwhelmed by the inflammatory upregulation of these growth factors. Furthermore, the indirect pathways that affect corneal hemangiogenesis and lymphangiogenesis (e.g., the regulation of immune cells that are able to secrete a plethora of hemangiogenic and lymphangiogenic growth factors) also have a crucial role in the development of blood and lymphatic vessels. Therefore, we investigated

**FIGURE 6.** A dose increase of bevacizumab eyedrops does not overcome the antagonizing effects of serum eyedrops on blood and lymphatic vessel progression and regression in vivo. Inflammatory corneal hemangiogenesis and lymphangiogenesis were induced by suture placement in BALB/c mice, and corneas were then treated with increased doses of bevacizumab eyedrops (25 mg/mL six times daily compared with 5 mg/mL three times daily) or a combination of serum eyedrops and increased doses of bevacizumab eyedrops. For the analysis of vessel progression, treatment started directly after suture placement; for the analysis of vessel regression, corneal sutures were removed after 2 weeks, and mice were then treated for 1 additional week. Afterward, blood and lymphatic vessels were quantified in corneal whole mounts. **Top:** Quantification of blood vascular (left) and lymphatic vascular progression (right). **Bottom:** Quantification of blood vascular (left) and lymphatic vascular regression (right). Data are expressed as means ± SDs.
whether serum eyedrops alter the quantity of CD11b-positive cells infiltrating the cornea after suture placement. No significant differences in CD11b cell counts in any of the treatment groups were observed. Thus, we conclude that serum and bevacizumab do not exhibit their vascular effects via the regulation of inflammatory cell infiltration. However, Nakao et al.28 have previously shown that bevacizumab is able to block leukocyte infiltration into corneas after IL-1β pellet implantation. This difference from our results may be due to the various assays used because corneal pellet implantation leads to a much lower inflammatory response than the suture-induced neovascularization assay. Therefore, our conclusions have assay-dependent limitations and apply to highly inflammatory conditions.

Serum eyedrops attenuated the anti(lymph)angiogenic effects of bevacizumab on corneal blood and lymphatic vessel progression, as well as regression. Furthermore, a dose increase of bevacizumab failed to restore its anti(lymph)angiogenic and (lymph)angioregressive effects. One obvious component in serum that can counteract bevacizumab is VEGF-A. According to the literature, murine serum contains 50 to 100 pg/mL of VEGF-A.50–52 Several studies have measured VEGF-A levels in the murine cornea in various inflammatory or infectious conditions; depending on the model used, the inflamed cornea contains VEGF-A concentrations that are also in the picogram range.33–36 Therefore, the bevacizumab-antagonizing effects of serum eyedrops could be at least partially attributable to an elevated level of VEGF-A. Nonetheless, our results cannot entirely rule out the contribution of other VEGF-independent mechanisms.

Analysis of corneal hemangiogenic and lymphangiogenic cytokine and growth factor expression revealed that the application of serum eyedrops led to several notable alterations. First, IL-1β expression was significantly downregulated via serum treatment, at least after 7 days. IL-1β has been shown to be an important mediator of corneal hemangiogenesis and lymphangiogenesis.29,37 The in vivo downregulation of IL-1β expression could be a further mechanism that causes a discrepancy between the in vivo and proliferative in vitro effects of serum. Second, serum treatment further upregulated VEGF-A expression after 7 days of treatment. We conclude that this may be a mechanism by which serum treatment counteracts the effects of bevacizumab in vivo. Indeed, the combination of serum and bevacizumab led to VEGF-A expression levels that were comparable to those of control corneas, whereas bevacizumab alone led to a significant downregulation of corneal VEGF-A expression. Third, VEGF-C and VEGF-D showed similar expression patterns: although not considerably affected by serum eyedrops alone, both growth factors were significantly downregulated by bevacizumab. However, the combination with serum neutralized the inhibitory effects of bevacizumab on VEGF-C and VEGF-D expression. This effect may also contribute to the observed vascular changes of the combined therapy.

The bevacizumab-antagonizing effects of serum eyedrops may have important consequences for the planning of combined treatment strategies in patients. Our experimental data indicate that bevacizumab is less effective if combined with serum eyedrops. Therefore, a possible adaptation of the therapeutic strategy could be that serum eyedrops are discontinued during a (lymph)angioregressive treatment interval, with bevacizumab then applied as short-term treatment. Afterward, treatment with serum eyedrops can be resumed. This strategy would also address the potential adverse effects of long-term treatment with bevacizumab because VEGF has also been shown to have epitheliotoxic and neurotrophic effects at the cornea.55,56

In conclusion, this study demonstrates that serum eyedrops alone do not alter the progression or regression of corneal blood and lymphatic vessels but seem to neutralize the anti(lymph)angiogenic effects of bevacizumab when used in combination. The opposing effects of serum eyedrops and bevacizumab eyedrops on the corneal vasculature should be taken into account when combined therapeutic regimens are considered such as in patients with corneal wound healing disorders coincident with corneal neovascularization.

Acknowledgments

The authors thank the following individuals at the Department of Ophthalmology, University of Cologne: Birgit Regenfuss for helpful discussions and proofreading of the article, Marie-Luise Dreisow for expertise with the whole mount preparations and stainings, and Frank Lacina for expert assistance at the animal facility.

Supported by the German Research Foundation Grants Sonderfor- schungsbereich SFB 643-B10 (CC), DFG Cu 47/4-1 (CC), DFG Cu 47/6-1 (CC), DFG HE 6743/2 (LMH); and the GEROK Programme, University of Cologne (DH, LMH).

Disclosure: D. Hos, None; K.R. Koch, None; F. Bucher, None; F. Bock, None; C. Cursiefen, None; L.M. Heindl, None

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