Intraocular Concentrations of Growth Factors and Cytokines in Retinal Vein Occlusion and the Effect of Therapy with Bevacizumab

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PURPOSE. To investigate concentrations of growth factors and inflammatory cytokines in eyes with central (CRVO) and branch (BRVO) retinal vein occlusion before and during therapy with bevacizumab and to identify associations with disease activity.

METHODS. In a prospective clinical trial, 13 eyes of patients with CRVO (n = 5) or BRVO (n = 8) were included. Bevacizumab was administered intravitreally at baseline and months 1 and 2. Retreatments were given at monthly visits if OCT showed edema or when vision loss occurred. Aqueous humor samples were taken each time injections were performed. Follow-up was 15 months. Samples from patients with cataract served as the control. Multiplex bead assays were used for measurement of 28 growth factors and cytokines.

RESULTS. During therapy with bevacizumab, VEGF levels were reduced to below detection in the first 2 months. Whenever criteria for retreatment were met, VEGF was measurable again. The decrease in VEGF was associated with a decrease in central retinal thickness (CRT) and improvement in visual acuity (VA). Significanty increased concentrations of VEGF, IL-6, IL-8, IP-10, MCP-1, and PDGF-AA were observed in aqueous humor samples of patients with CRVO compared with the control samples.

CONCLUSIONS. VEGF levels were significantly elevated in patients with CRVO compared with control subjects. Intravitreal injections of bevacizumab resulted in a substantial decrease of VEGF under physiologic levels and remained low under the loading dose of three consecutive monthly retreatments. Macular edema was related to VEGF levels in the aqueous humor.

1

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Submitted for publication June 30, 2008; revised September 4, 2008; accepted January 16, 2009.

Disclosure: M. Funk, None; K. Kriechbaum, None; F. Prager, None; T. Benesch, None; M. Georgopoulos, None; G.J. Zlabinger, None; U. Schmidt-Erfurth, None

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METHODS

The study was conducted prospectively in the Department of Ophthalmology at the Medical University of Vienna. The protocol was approved by the Ethics Committee of the Medical University of Vienna, registered at the European clinical database (EUDRACT-2005-003288-21) and adhered to the tenets of the Declaration of Helsinki. Informed consent was obtained from all patients before study inclusion.

Eligibility Criteria and Diagnostic Procedures

Inclusion criteria were clinically significant macular edema (CSME) involving the fovea; visual acuity (VA) better than 0.4 or no response to previous focal laser coagulation; central retinal thickness of at least 250 μm due to intraretinal; or subretinal edema, as measured by standard OCT imaging.

Exclusion criteria included active neovascular disease defined as neovascularization on the disc or elsewhere and rubeosis iridis; severe
ischemic retinopathy with >10 disc areas of nonperfusion detected by fluorescein angiography (FA); previous vitrectomy; laser coagulation within the past 3 months; previous participation in any studies using investigational drugs within the 3 months preceding day 0 including intravitreal triamcinolone, intraocular surgery (including cataract surgery) in the study eye within 2 months preceding day 0, glaucoma in the study eye, diabetes mellitus, use of immunosuppressive drugs, malignant tumors of any location.

Best corrected visual acuity (BCVA) was measured by using the Early Treatment Diabetic Retinopathy Study (ETDRS) charts at a 2-m distance. Slit lamp examination and measurement of intraocular pressure were performed. Ophthalmoscopy and fundus photography were performed in mydriasis. Central retinal thickness (CRT) was measured by optical coherence tomography (Stratus OCT; Carl Zeiss Meditec AG, Jena, Germany). Progression of avascular areas and the incidence of neovascular complications were examined by FA (Retina Angiograph II; Heidelberg Engineering, Heidelberg, Germany). All diagnostic procedures followed a standardized protocol and were performed at each monthly visit. FA was indicated at baseline and at months 3 and 12.

### Treatment with Intravitreal Bevacizumab

All patients with RVO were given three initial intravitreal injections of 1.25 mg bevacizumab (Avastin, Genentech and Hoffmann La Roche, Basel, Switzerland) in intervals of four weeks (visit 0, 1, and 2). Starting from visit 3, patients received retreatment at monthly control visits only if OCT showed evidence of intraretinal or subretinal fluid, the amount of fluid had increased, or a vision loss of at least five ETDRS letters was documented since the previous visit.

Aqueous humor samples were taken each time an intravitreal injection was performed. Before surgery, patients received topical lidocaine 4% and betaxolodona in the study eye. The lids and pericocular skin were cleaned with betaxolodona solution, the eye was draped in a sterile fashion, and a sterile lid speculum was inserted. A mean volume of 0.1 mL of aqueous humor was collected by anterior chamber limbal paracentesis with a 27-gauge needle attached to an insulin syringe. The intravitreal injection of bevacizumab was then administered through the pars plana, 3.5 mm from the limbus. Antibiotic ointment was given after surgery for 4 days. Immediately after collection, aqueous humor samples were transferred to sterile plastic tubes and stored at −80°C until analysis.

### Control Group

Reference samples were obtained from patients with cataract. Exclusion criteria were any type of retinal disease, glaucoma, previous vitrectomy, laser coagulation, diabetes mellitus, use of immunosuppressive drugs, malignant tumors, and participation in any study of investigational drugs within the 3 months preceding inclusion. Aqueous humor samples were obtained immediately before routine cataract surgery by limbal paracentesis. Samples were immediately frozen and stored at −80°C.

### Measurement of Cytokines and Growth Factors

Samples were analyzed using the suspension array technology (xMAP; Luminex Corp. Austin, TX). Capture bead kits (Beadlyte; Upstate Biotechnology, Lake Placid, NY) were used for the detection of interleukin (IL)-1α, -1β, -2, -3, -4, -5, -6, -7, -8, -10,-12(p40), -12(p70), -13, and IL-15; interferon (IFN)-γ inducible protein (IP)-10; ctaxin; interferon (IFN)-γ; granulocyte/macrophage colony-stimulating factor (GM-CSF); monocyte chemoattractant protein 1 (MCP-1); macrophage inflammatory protein 1α (MIP-1α); RANTES; tumor necrosis factor (TNF)-α; epidermal growth factor (EGF); fibroblast growth factor (FGF)-2; Flt-3 ligand; platelet-derived growth factor (PDGF)-AA and -AB/BB; and VEGF. Aqueous humor samples (30 μL) were used undiluted and incubated overnight. Kits were used according to the manufacturers’ instructions.

### Statistical Analysis

Standard descriptive statistics were used to summarize the variables studied. Statistical analysis was performed using a general linear model with fixed-factors patient group, eye, sex, age, duration of disease and VEGF, CRT, or VA, respectively, and random factor patient number. P < 0.05 was considered significant. Statistical calculations were performed with commercial software (SAS ver. 8; SAS Institute Inc., Cary, NC).

### Results

Thirteen eyes of patients with CSME involving the fovea due to CRVO (n = 5) and BRVO (n = 8) were enrolled. The BRVO group consisted of six eyes with major BRVO (occlusion of one of the major branch retinal veins) and two eyes with macular BRVO (occlusion of one of the macular venules). There was no hemiretinal vein occlusion included. Thirteen eyes of patients undergoing cataract surgery served as the control. Mean age was 58.2 ± 11.5 years in CRVO, 62.5 ± 7.9 years in BRVO, and 66.2 ± 5.3 years in control patients. The mean duration of the disease at baseline was 10.4 ± 15.5 months in BRVO and 16.3 ± 21.7 months in CRVO. The mean CRT at baseline was 542.4 ± 192.7 μm in BRVO and 572 ± 156.3 μm in CRVO. The mean VA at baseline was 0.48 ± 0.25 logMAR in BRVO and 0.97 ± 0.55 logMAR in CRVO.

Follow-up time was up to 15 months (mean 11 months). One patient was excluded at month 3 and one at month 6, for reasons unrelated to the underlying disease. None of the patients had undergone treatment with intravitreal triamcinolone before study inclusion.
Concentrations of Cytokines at Baseline Presentation

At baseline, the median level of VEGF in control patients was 40.4 pg/mL (interquartile range [IQR], 25.5–41.5). VEGF levels in BRVO patients were not statistically significant elevated (median, 106.9 pg/mL; IQR, 78–154; \( P = 0.57 \)). Highly elevated levels were observed in patients with CRVO (median, 351.8 pg/mL; IQR, 128–1957.8) and the difference was statistically significant (\( P = 0.04 \); Fig. 1).

A significant negative association was observed between VEGF levels and the duration of RVO before onset of treatment (\( P = 0.033 \)). The longer the duration of disease, the lower the levels of VEGF found in the aqueous humor at the beginning of treatment.

Concentrations of the entire series of cytokines differed widely, from very high levels of MCP-1 (median, 649.9 pg/mL) or IP-10 (median, 346.6 pg/mL) to very low levels near to or below detection limits (GM-CSF; IFN-\( \gamma \); IL-1\( \beta \); -2, -3, -4, -5, -7, -10, -12p40, -12p70, -13, and -15; eotaxin; MIP-1\( \alpha \); RANTES; EGF; FGF-2; Flt-3-ligand; PDGF-AB/BB; and TNF-\( \alpha \)). These findings were consistent in patients and control subjects.

Statistical analysis revealed significantly increased concentrations of IL-6, -8, and -10; MCP-1; and PDGF-AA in CRVO compared with control patients (\( P < 0.05 \)) and a trend was observed for IL-1-\( \alpha \) (\( P < 0.1 \)). In BRVO patients IL-1-\( \alpha \) was significantly elevated (\( P < 0.05 \); Table 1, Fig. 1).

Changes in Cytokine Levels during Treatment

All patients received three consecutive initial injections. Due to signs of recurrent disease activity, 10 patients were also treated at month 3. During the following months, retreatment was necessary in one to four patients, except for month 6, when seven patients received retreatment. In total, a mean of 5.9 \pm 2.2 injections per patient was necessary.

Under therapy with intravitreal bevacizumab, VEGF levels were reduced from a median value of 117 pg/mL to values below the detection limit during the first 2 months (\( P = 0.062 \)). The first three consecutive injections were accompanied by constantly low VEGF levels in most patients (Fig. 2). Exceptions were three patients with CRVO. The course of VEGF levels in the loading phase of the first 2 months is shown in Figure 2. After the initial therapy, VEGF levels were measurable again after discontinuation whenever criteria for retreatment were met. Noteworthy, VEGF concentrations did not return to below detection after retreatment of reoccurrence.
The treatment of a variety of retinal diseases, the effect on VEGF in RVO. Although anti-VEGF therapy is routinely used in the cases were excluded in our series. This finding supports the treated patients. Afterward, IL-1 remained low below detection at month 3 in 10 consecutively (OCT, VA, and VEGF concentrations. Besides VEGF, IL-1α also showed a similarly pronounced decrease during initial treatment of three consecutive injections. Median levels decreased from 46.15 pg/mL (IQR, 8.58–40.9) at baseline to 9.39 pg/mL (IQR, 9.39–44.53) after the first treatment and below detection after the second treatment (P = 0.03, Fig. 2). IL-1α levels remained low below detection at month 3 in 10 consecutively treated patients. Afterward, IL-1α was measurable again whenever retreatment was necessary. Subgroup analysis revealed no significant difference between CRVO and BRVO eyes in changes of IL-1α under treatment. The other cytokines and growth factors were not significantly altered by treatment with bevacizumab. Analysis of possible relationships of all cytokines and growth factors with changes in VEGF levels revealed significant associations with changes in levels of PDGF-AA (P < 0.0001), MCP-1 (P = 0.006), IL-8 (P = 0.027), and IL-15 (P = 0.027).

**DISCUSSION**

Our results provide evidence of a disturbed balance of cytokines and growth factors in eyes with RVO compared with control eyes without vascular disease. Intraocular VEGF levels were significantly increased in the aqueous humor of our patients with CRVO, and as a trend in patients with BRVO. We also demonstrated measurable levels of VEGF in the control group providing evidence for a physiological expression of this angiogenic factor in the human eye.

In contrast to previous studies in which elevated levels of VEGF in patients with RVO were observed,1,13,14 ischemic cases were excluded in our series. This finding supports the importance of VEGF in pathogenesis after vascular dysfunction in RVO. Although anti-VEGF therapy is routinely used in the treatment of a variety of retinal diseases, the effect on VEGF concentrations in the human eye in vivo has not been examined so far. We evaluated for the first time the influence of antiangiogenic therapy on VEGF and other intraocular cytokines and growth factors. After initial intravitreal application of bevacizumab, VEGF levels rapidly decreased to values below detection and physiologic values. This effect of anti-VEGF treatment was measurable 4 weeks after injection. During consecutive monthly treatments in the first 2 months, VEGF levels remained continuously low. After discontinuation of treatment whenever there were clinical signs of disease activity and retreatment was necessary, VEGF levels were measurable again and did not return to values below detection limits as had been seen during the first 2 months. These observations could reflect underlying molecular mechanisms of rebound phenomena, that have frequently been observed during anti-VEGF therapy. VEGF levels were also significantly associated with clinically measurable features such as VA and CRT, which may therefore be considered as an index for biological disease conditions. The evidence of a direct correlation of intraocular VEGF and retinal thickness under therapy also highlights the value of OCT parameter for guidance of VEGF inhibitory therapy and retreatment regimen.

In addition, baseline VEGF levels correlated negatively with the duration of the disease. Lower levels were found with a long history of disease, suggesting that VEGF levels decrease during the natural course of RVO. A clear benefit of early therapeutic intervention has been observed clinically and may be explained by these findings.

None of the patients showed signs of conversion into a nonperfusion type with ischemia-related complications under therapy, although VEGF levels were extremely low. These findings suggest that the angiogenic factor is not relevant for recanalization or vessel survival. Obviously there is a different pathway in RVO than in other ischemic diseases of the retina such as ROP, where selective stimulation of VEGF receptor 1 prevents oxygen-induced retinal vascular degeneration.13 Also no severe systemic adverse effects such as arterial thromboembolic events have been observed in our patients.

In addition to VEGF, we analyzed a broad spectrum of pro- and anti-inflammatory cytokines and growth factors. So far, the analysis of aqueous humor of patients with RVO focused on VEGF and IL-6.3,14 Using a multiplexed assay in our study, the microarray assay (xMAP; Lumex Corp.) allowed a simultaneous measurement of 28 representative cytokines and growth factors in a small sample of only 30 μL. Several of these factors

**FIGURE 3.** VEGF levels (picograms/milliliter; median and IQR) in the initial loading phase, when all patients were retreated according to a fixed regimen. From month 3, only eyes with disease recurrence or persistence were retreated, based on a flexible regimen during the maintenance phase.
were near to or below detection limits in both patients and the control group. Treatment with bevacizumab did not have an influence on these low concentrations. Whether this finding is due to a limited sensitivity of the used method or whether these factors do not have a role in physiology and disease of the eye, requires further investigation.

Several other cytokines besides VEGF appear to play a role in the vascular dysfunction and pathologic course of RVO. Concentrations of IL-1α, -6, and -8; IP-10; MCP-1; and PDGF-AA were significantly increased in eyes of patients with RVO compared with concentrations in control subjects. Subgroup analysis revealed that some parameters were slightly elevated in BRVO, either as a trend or with statistical significance, but were more intensively increased in CRVO eyes. Differences in the extension of area in BRVO and CRVO could be an explanation for this observation, which has to be clarified in further studies with larger sample sizes.

IL-6 levels in RVO have been investigated previously. The elevated levels of IL-6 in CRVO and BRVO in our study confirm findings of Noma et al.,1,4 who have also shown a correlation of IL-6 levels with the severity of macular edema in RVO.

Very little is known about the role of the other significantly increased cytokines and growth factors in the physiology of the eye as well as under pathologic conditions in retinal diseases. IL-1α was significantly elevated in eyes of our RVO patients and was the only factor apart from VEGF that was substantially decreased after intraocular treatment with bevacizumab. IL-1α is a proinflammatory cytokine involved in beneficial and destructive responses to injury.16 Observations of an upregulation of IL-1α during reperfusion after induced retinal ischemia have been made previously in rats.17 Our results revealed an upregulation of IL-1α in retinal vascular disease in humans as well and a close connection to antiangiogenic treatment. Levels of IP-10, IL-8, MCP-1, and PDGF-AA were also elevated before treatment and IL-8, MCP-1, and PDGF-AA significantly associated with changes in VEGF during treatment. An upregulation of these inflammatory factors after retinal ischemia has been restricted to observations in animal models so far.18 IL-8 is important in the regulation of the acute inflammatory response.19 MCP-1 is a potent chemotactic cytokine and has a critical role in monocyte recruitment to the vessel wall after vascular injury.20 The increased levels of an eosinophil chemotactic factor like MCP-1 support the idea that eosinophils play a role in tissue and vascular remodelling after RVO. Eosinophils have been identified as an important source of VEGF21 and several fibrogenic cytokines and modulators of
remodelling such as TGF-α and -β.\textsuperscript{22} Their importance in tissue fibrosis has been established in recent studies in animal models and humans.\textsuperscript{23,24} Overexpression of PDGF has also been linked to different types of fibrotic disorders and malignancies. It is mainly believed to be an important mitogen for connective tissue, especially for fibroblasts that serve in wound healing. IP-10 exerts antiangiogenic properties by inhibition of proliferation and induction of apoptosis in ECs\textsuperscript{25} and inhibits VEGF-induced endothelial motility,\textsuperscript{26} but augmented IP-10 expression has also been linked to proinflammatory functions of VEGF.\textsuperscript{27} Several other cytokines and growth factors, that have well-known roles in inflammation and angiogenesis, such as IL-10 and -12; EGF; FGF-2; MIP-1α; and RANTES, did not appear to have a significant role in RVO in our study.

**Figure 5.** Patient presenting with BRVO. Intravitreal injections at baseline, month 1, 2, 6, 9, 12, and 15. BL, baseline; M, month; VA, visual acuity (logMAR); CRT, central retinal thickness (micrometers). VEGF levels are given in picograms/milliliter.
Limitations of the Study
We are aware of some limitations that apply to our study: Undoubtedly, because of the small sample size significant differences in cytokine levels may have been missed, and on the contrary, observed significances represent tendencies and need to be confirmed in further studies. However, the concept of the study was to give first evidence on which of the wide variety of the growth factors and immunologic cytokines is detectable at all and which of them seems to be of particular interest to be followed any further.

CONCLUSIONS
In conclusion, we demonstrated differences of cytokine and growth factor profiles in the eyes of patients with RVO compared with control subjects. Our results provide information on which of them may be of particular importance in RVO and should be investigated in further studies. In addition, our study analyzed for the first time the influence of anti-VEGF therapy on VEGF and other cytokines and provides evidence of an inhibition of intraocular VEGF in human eyes after antiangiogenic therapy. Initial monthly treatment reduced VEGF levels to undetectable values and below physiologic levels. The changes of intraocular VEGF levels were also associated with disease activity.

Acknowledgments
The authors thank Margarethe Merio for excellent technical work and Renate Amon for assistance in sample collection.

References