Simultaneous Increase in Multiple Proinflammatory Cytokines in the Aqueous Humor in Neovascular Glaucoma With and Without Intravitreal Bevacizumab Injection

Saori Ohira, Toshihiro Inoue, Kohei Shobayashi, Keiichiro Iwao, Mikiko Fukushima, and Hidenobu Tanihara

Department of Ophthalmology, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan

Correspondence: Toshihiro Inoue, Department of Ophthalmology, Faculty of Life Sciences, Kumamoto University, 1-1-1 Honjo, Chuo-ku, Kumamoto, Japan; noel@da2.so-net.ne.jp.
Submitted: October 23, 2014
Accepted: April 10, 2015

DOI:10.1167/iovs.14-15918

PURPOSE. To investigate aqueous humor proinflammatory cytokine levels of patients with neovascular glaucoma (NVG), and to analyze the effects of background factors in the expression of these molecules.

METHODS. This cross-sectional study enrolled 137 participants who were grouped into (1) primary open-angle glaucoma (POAG; n = 36) patients; (2) NVG patients (NVG; n = 33); and (3) cataract surgery patients as a comparative group (CG; n = 68). Aquous humor samples were collected from the anterior chamber at the start of surgery, deposited in Cryo Tubes, registered, and stored at −80°C until processing. Multiplex microparticle-based immunodetection was performed by using xMAP and the Human Cytokine/Chemokine Panel I. Bevacizumab was injected into the vitreous cavity 1 to 2 days before surgery in 22 NVG patients (IVB group), whereas 11 NVG patients received no antivascular endothelial growth factor (VEGF) therapy 3 months preoperatively (N group). The Wilcoxon rank sum test or Fisher’s exact test for two variables and the Tukey-Kramer honestly significant difference test for multiple variables were used to compare the cytokine levels.

RESULTS. The NVG patients had higher levels of interleukin (IL)-6, IL-8, monocyte chemotactic protein (MCP)-1, tumor necrosis factor-α (TNF-α), and platelet-derived growth factor (PDGF)-AA compared to both the CG and POAG groups. The levels of IL-6, IL-8, MCP-1, and PDGF-AB/BB were higher in the IVB group than the N group, whereas the VEGF level was significantly lower in the IVB group (P < 0.01).

CONCLUSIONS. Intravitreal bevacizumab injection decreased VEGF levels, but not those of the other cytokines.
Keywords: neovascular glaucoma, MCP-1, interleukin, VEGF, bevacizumab

Neovascular glaucoma (NVG) normally develops after ischemic intraocular disease, including proliferative diabetic retinopathy and central retinal vein occlusion. During NVG development, the ocular fluid vascular endothelial growth factor (VEGF) level increases, stimulating neovascularization in the iris and trabecular meshwork, thereby elevating intraocular pressure (IOP). Compared to primary open-angle glaucoma (POAG), NVG cases are often resistant to surgical treatment, including filtering surgeries, probably because of the relatively young age, history of intraocular surgeries, and chronic ocular microinflammation. Although previous clinical reports have suggested that anti-VEGF therapy effectively reduces IOP by suppression of neovascularization in the iris and iridocorneal angle at an early stage of NVG, the effect appears to be temporary. In addition, anti-VEGF therapy reduces vascular permeability and diminishes the inflammatory reaction, whereas newly formed vessels are still present in the iris (particularly in the stroma) and iridocorneal angle. In addition, increased permeability exposes the tissues to redundant cytokines, which can initiate and accelerate proinflammatory reactions. The multiplex microparticle-based immunodetection system is useful for simultaneously assessing cytokine levels in small clinical samples. In addition to VEGF, other bioactive molecules, including interleukin (IL)-6, transforming growth factor (TGF)-β1 and β2, and erythropoietin, are also elevated in the aqueous humor in NVG and might play significant roles in NVG pathophysiology. To our knowledge, however, the levels of multiple cytokines/growth factors in the aqueous humor in NVG and their related background factors have not been investigated in the peer-reviewed literature.

We have previously reported that phacoemulsification is a possible risk factor for the poor surgical outcome of mitomycin-C trabeculectomy in POAG and exfoliation glaucoma. Applying recently developed techniques to determine low levels of cytokines and growth factors, we have found that an elevated aqueous proinflammatory cytokine level after phacoemulsification is a possible reason for the poor outcome. The abundant cytokines and growth factors in the aqueous humor may contribute to aberrant wound-healing activity in
glaucomatous eyes after filtration surgeries, and thereby decrease the surgical success rate.

Since the aqueous humor supplies nutrients to avascular tissues in the anterior ocular segments and drains through the outflow pathway, including the trabecular meshwork and Schlemm’s canal, the biologically active factors in aqueous humor are thought to play important roles in the pathophysiology of glaucoma.\textsuperscript{4,31–33} In particular, VEGF plays a major role in glaucoma development and progression in NVG. To the best of our knowledge, this is the first report of elevated IL-8, monocyte chemotactic protein (MCP)-1, tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), and platelet-derived growth factor (PDGF)-AA levels in the aqueous humor in NVG. Therefore, in addition to VEGF, these cytokines and growth factors might modulate multiple pathologic aspects of this disease. Here, we report a simultaneous increase in proinflammatory cytokines in aqueous humor samples obtained from eyes with NVG and analyze the effect of intravitreal injection of bevacizumab (IVB), a major anti-VEGF therapy.

**PATIENTS AND METHODS**

**Clinical Study**

**Patients and Sample Collection.** This cross-sectional study was approved by the Institutional Review Board of Kumamoto University (reference no. Senshin-1319). All procedures conformed to the Declaration of Helsinki, and informed consent was obtained from each participating patient. Patients who had undergone trabeculectomy for POAG or NVG were recruited. In contrast, patients with systemic diseases other than hypertension and hyperlipidemia, ocular diseases other than cataracts, a history of ocular surgeries, or an IOP > 21 mm Hg were excluded. Intraocular pressure was determined by using a noncontact tonometer. None of the patients were using topical or systemic anti-inflammatory drugs, including corticosteroids. When both eyes of a patient met the inclusion criteria, only the eye treated first was included in the analysis. In all of the participants, the anterior eye segment was examined by glaucoma specialists using a slit-lamp biomicroscope and all of the changes were recorded. Through dilated pupils, the optic disc was evaluated with a stereo fundus lens to make a diagnosis of NVG. None of the participants had LASER treatments within 6 months before beginning the study. Preoperative aqueous humor was obtained at the start of the phacoemulsification surgery before any incisional procedures, as described previously.\textsuperscript{28} Briefly, aqueous humor was obtained gently at the start of surgery from the anterior chamber, through limbal paracentesis by using a syringe with a 30-gauge needle (Nipro, Osaka, Japan) attached. Approximately 70 to 100 \(\mu\)L (to avoid excessive hypotony) was collected in CryoTubes (Thermo Fisher Scientific, Waltham, MA, USA), registered, and stored at \(-80^\circ\text{C}\) until processing.

**Intravitreal Injection of Bevacizumab.** Two days before trabeculectomy, IVB was conducted in surgeon-selected NVG cases, as described previously.\textsuperscript{34} Briefly, following topical anesthesia using lidocaine (AstraZeneca, Osaka, Japan) and disinfection using povidone-iodine (Nitten, Nagoya, Japan) 0.05\% bevacizumab (Avastin; Genentech, San Francisco, CA, USA) at 25 mg/mL was injected into the vitreous cavity by a 25-gauge needle (Nipro, Osaka, Japan) attached. Approximately 70 to 100 \(\mu\)L levofloxacin (Santen, Osaka, Japan) and xylazine hydrochloride (Celactal, 10 mg/kg body weight; Bayer Medical, Leverkusen, Germany), IVB was

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cataract</th>
<th>POAG</th>
<th>NVG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients, (n)</td>
<td>68</td>
<td>36</td>
<td>33</td>
</tr>
<tr>
<td>Male/female</td>
<td>31/37</td>
<td>26/10</td>
<td>22/11</td>
</tr>
<tr>
<td>Age, y</td>
<td>61–90</td>
<td>46–88</td>
<td>26–85</td>
</tr>
<tr>
<td>Preoperative IOP, mm Hg</td>
<td>Mean ± SD</td>
<td>11.9 ± 2.9</td>
<td>26.8 ± 7.9</td>
</tr>
<tr>
<td>Range</td>
<td>6.5–21.0</td>
<td>11.0–46.0</td>
<td>20.0–67.7</td>
</tr>
<tr>
<td>Glaucoma eye drops</td>
<td>No. mean ± SD</td>
<td>0 ± 0.6</td>
<td>3.4 ± 0.7†</td>
</tr>
<tr>
<td>Range</td>
<td>0 ± 1.4</td>
<td>2–5</td>
<td></td>
</tr>
<tr>
<td>β-blocker, n (%)</td>
<td>0</td>
<td>33 (91.7)</td>
<td>33 (100.0)</td>
</tr>
<tr>
<td>PG, n (%)</td>
<td>0</td>
<td>34 (94.4)</td>
<td>33 (100.0)</td>
</tr>
<tr>
<td>CAI, n (%)</td>
<td>0</td>
<td>33 (91.7)</td>
<td>32 (97.0)</td>
</tr>
<tr>
<td>Others, n (%)</td>
<td>0</td>
<td>5 (13.9)</td>
<td>19 (57.6)</td>
</tr>
</tbody>
</table>

Duration of glaucoma therapy, mo

| No. mean ± SD | 0 | 140.8 ± 222.8 | 10.4 ± 14.2† |
| Range         | 0 | 1.3–1303.5 | 0.2–61.5 |
| History of phacoemulsification, n (%) | 68 (100) | 9 (25.0) | 19 (57.6)† |
| History of vitrectomy, n (%) | 0 (0) | 0 (0) | 15 (39.4)† |

PG, prostaglandin analog; CAI, carbonic anhydrase inhibitor; SD, standard deviation.

* \(P < 0.01\) compared to the POAG group.

† \(P < 0.05\) compared to the POAG group.

immunoassays, xMAP, and Human Cytokine/Chemokine Panel I (Luminex, Austin, TX, USA), as previously described.\textsuperscript{29} Briefly, a 25-\(\mu\)L aliquot of aqueous humor sample was transferred to a plate, and some of each aliquot was placed into one of the capture microsphere multiplexes. After incubation at 4 \(^\circ\text{C}\) for 18 hours, multiplexed cocktails of biotinylated reporter antibodies were mixed and then incubated at room temperature for 1 hour. Multiplexes were developed by using an excess of streptavidin–phycocerythrin solution (Merck Millipore, Billerica, MA, USA). The solution was mixed with each multiplex and was then incubated at room temperature for 30 minutes. After filtration was used to reduce the volumes of the multiplexed reactions, and then the volumes were increased by dilution with a matrix buffer (Merck Millipore). A Luminex 200 instrument (Hitachi Solutions, Tokyo, Japan) was used for the analysis, and data were interpreted with proprietary data-analysis software (DNASIS Plex version 2.5; Hitachi Software Engineering, Tokyo, Japan).

**Animal Experiment**

Experiments were conducted according to the guidelines of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and approved by the Animal Use Committee of Kumamoto University. Five male Dutch rabbits (1.2–1.8 kg, 12–14 weeks old) were used. They were maintained in a temperature-, humidity-, and light-controlled room at 22 \(^\circ\text{C}\) \(±\) 2 \(^\circ\text{C}\), 50% to 70% relative humidity, and a 12-hour light/dark cycle. Food and water were provided ad libitum. Under general anesthesia via an intramuscular injection of ketamine hydrochloride (Ketalar, 25 mg/kg body weight; Daiichi Sankyo, Tokyo, Japan), 10 mg/kg body weight; Bayer Medical, Leverkusen, Germany), IVB was
administered in the right eye by using the same method as in the clinical study. The controls (left eyes) received single injections of 0.05 mL balanced salt solution. Aqueous samples were collected 2 days after injection from both eyes and stored as described previously.29

**Enzyme-Linked Immunosorbent Assay (ELISA)**
Interleukin 6 (CSB-E06903Rb; Cusabio, Wuhan, China), IL-8 (CSB-E06905Rb; Cusabio), and MCP-1 (SEA087Rb; USCN Life Science, Wuhan, China) concentrations in the rabbit aqueous humor were determined by using an ELISA kit, according to the manufacturer’s protocol. Briefly, 100 μL aqueous samples or standards was added to each well in microtiter plates precoated with an antibody specific to each cytokine and incubated for 2 hours at 37°C. Then, 100 μL biotin antibody was added to each well and incubated for 1 hour at 37°C before 0.1 mL streptavidin–horseradish peroxidase enzyme solution was added to each well. After adding 90 μL tetramethylbenzidine substrate to each well, the solution was incubated for 20 minutes at 37°C and protected from light. Then, 50 μL Stop Solution was added to each well, and the optical density of each well was immediately determined by using a microplate reader (Thermo Fisher Scientific) set to 450 nm. We averaged duplicate readings for each standard and sample, and subtracted the average zero standard optical density. A standard curve was created on a log-log graph by plotting the mean optical density for each standard on the y-axis against the concentration on the x-axis using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

**Cell Culture and Hypoxic Treatment**
Human iris pigment epithelial cells were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). Cells were cultured and expanded by using the manufacturer’s protocol. At passage 3 or 4, the iris pigment epithelial cells were seeded into poly-L-lysine-coated culture dishes (IWAKI, Tokyo, Japan) at a seeding density of 5000 cells/cm² and allowed to reach 80% confluence. Subsequently, the medium was changed to serum-free medium (Wako, Osaka, Japan), and the cells were cultured under normoxic or hypoxic conditions for 48 hours by using a BIONIX-1 hypoxic culture kit (Sugiyamagen, Tokyo, Japan). Next, conditioned medium fractions were collected from the normoxic and hypoxic cells,
centrifuged at 13,000g at 4°C for 10 minutes, and stored at −80°C before use. ELISA was performed as described above. To assess the effects of the neutralization of VEGF cells were immediately treated with 0.25 mg/mL bevacizumab before starting the hypoxic treatment.

Statistical Analysis

Data were analyzed by using the JMP V8 statistical software package (SAS Institute, Cary, NC, USA). In the clinical study, the Wilcoxon rank sum test or Fisher’s exact test for two variables and the Tukey-Kramer honestly significant difference test for multiple variables were performed to compare the cytokine and growth factor levels between the subgroups. In the animal experiment, a paired two-tailed t-test was performed to compare cytokine values. In the cell experiment, Dunnett’s test was used to compare the cytokine values in which the control was the normoxic condition. A value of \( P < 0.05 \) was taken to indicate statistical significance. Graphs were created with GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA).

RESULTS

Clinical Study: Patient Characteristics and Ophthalmologic Data

Aqueous humor samples were obtained from 137 participants who included (1) POAG patients \(( n = 36)\); (2) patients with NVG \(( n = 33)\); and (3) cataract surgery patients (nonglaucoma) who comprised the comparative group \(( n = 68)\). The patient characteristics are summarized in Table 1. Mean ages of both glaucoma groups were lower and mean IOPs were significantly higher than those of the cataract group.

Clinical Study: Biochemical Data

Figure 1 shows the cytokine and growth factor levels. There were no significant differences in the cytokine and growth factor levels between the cataract (nonglaucomatous) and POAG cases. In contrast, the NVG cases displayed significantly higher levels of IL-6, IL-8, MCP-1, TNF-α, and PDGF-AA (all \( P < 0.001 \)) than both the cataract and POAG cases. When cases with IVB were excluded, the VEGF level was also higher in the NVG cases than in the cataract and POAG cases (both \( P < 0.001 \)).

We assigned the NVG cases to two groups, as based on the history of IVB 2 days before the trabeculectomy, and compared the cytokine levels. Table 2 presents the characteristics of the patients of these two groups. In the 22 NVG cases with IVB (IVB group), the aqueous VEGF level was significantly lower, whereas the level of the other cytokines was higher, than in the 11 NVG cases without IVB (N group) (Fig. 2). The IVB group included more cases with rubeosis iridis and received a shorter glaucoma therapy than the N group. We subdivided the NVG cases by the presence of rubeosis iridis, a history of cataract surgery, and a history of vitrectomy, and found that only the presence of rubeosis iridis was significantly associated with the aqueous cytokine levels. There was a statistically significant difference between the NVG cases with and without rubeosis iridis in the IL-6 \(( P = 0.036)\), IL-8 \(( P < 0.001)\), MCP-1 \(( P = 0.011)\), and VEGF levels \(( P = 0.033)\) (Fig. 3).

Animal Experiment: Effects of IVB Injection on Aqueous Cytokine Levels

To investigate the effect of IVB on aqueous cytokine levels, we compared the levels between bevacizumab-injected eyes and the contralateral untreated eyes. The aqueous IL-6 and IL-8 levels were not different between the two groups. In contrast, the aqueous MCP-1 level was significantly higher in the bevacizumab-injected eyes than in the untreated eyes (Table 3).

Hyopxic Treatment

Figure 4 shows the cytokine concentrations produced by cultured iris pigment epithelial cells. In the hypoxic condition, IL-8 and MCP-1 concentrations were increased compared to the normoxic condition \(( P = 0.0095 \) and 0.0495, respectively); anti-VEGF treatment did not inhibit the increase in those cytokines. In comparison, the concentration of IL-6 did not differ between the conditions.

DISCUSSION

Elevated levels of these cytokines and growth factors may be explained in part by increased local production in the anterior ocular segments, because ischemic tissues produce more angiogenic factors. Chalam et al. report a strong VEGF immunoreaction in the nonpigmented epithelial cells of the ciliary processes and in the retina in eyes with NVG, whereas minimal VEGF immunostaining is observed in the control eyes. Furthermore, the mRNA levels of inflammatory cytokines, including IL-2, IL-6, and TNF-α, are significantly increased in the NVG group compared to the POAG controls. Our results suggest that iris pigment epithelial cells were induced to secrete proinflammatory cytokines such as IL-8 and MCP-1 in the hypoxic condition (Fig. 4). Therefore, a source of elevated cytokines in NVG may be uveitic tissues in the anterior segments, and the inflammatory process may contribute to NVG development. Another possible cause of elevated levels of multiple cytokines/growth factors may be an impaired blood–aqueous barrier, because ocular neovascular vessels are more perme-
**FIGURE 2.** Comparison of the levels (pg/mL) of analytes in the eyes with and without IVB. Data points ($n = 22$ and $11$ for with and without IVB, respectively) represent individual samples. *Error bars* represent medians ± interquartile range. **$P < 0.01$.**

**FIGURE 3.** Comparison of the levels (pg/mL) of analytes in the eyes of NVG cases with and without rubeosis iridis. Data points ($n = 20$ and $13$ for with and without rubeosis iridis, respectively) represent individual samples. *Error bars* represent medians ± interquartile range. **$P < 0.01$.**
able to blood proteins than normal vessels. In regard to this point, our results that NVG cases with rubeosis iridis presented higher aqueous levels of IL-6, IL-8, and MCP-1 would be expected; redundant neovascular vessels in the iris are considered to release large amounts of biologically active factors. However, the effects of rubeosis iridis on aqueous cytokine levels were inconclusive in the present study, because more cases with rubeosis iridis were included in the IVB group. Intravitreal injection of bevacizumab is reported to reduce aqueous VEGF concentrations in NVG. Zhou et al. have demonstrated that the elevated level of erythropoietin in NVG is unchanged approximately 1 week after IVB. By contrast, Matsuyama et al. have found, when determining the serum levels of VEGF and pigment epithelium-derived factor before and after IVB (1 day, 7 days, and 1 month), that the levels of both factors are decreased until 1 month after injection in patients with proliferative diabetic retinopathy. Furthermore, Forooghian et al. report elevated levels of aqueous IL-8 and TGF-β2 after IVB (mean of 10 days) in patients with proliferative diabetic retinopathy undergoing pars plana vitrectomy in a prospective study. Similarly, Jeon and Lee report elevated levels of aqueous IL-6 and IL-8 at 1 and 7 days after IVB in patients with proliferative diabetic retinopathy. Therefore, the direct effects of IVB on multiple biologically active factors in the aqueous humor in NVG remain unclear. Because compensatory reactions of other angiogenic and inflammatory factors have been reported after VEGF inhibition, the levels of some cytokines/growth factors may be elevated in response to anti-VEGF therapy.

A further explanation for the IVB-induced elevation of proinflammatory cytokines may be that the procedure itself could induce an inflammatory reaction, including the recruitment of proinflammatory cells that produce proinflammatory cytokines. Interestingly, in this study, in healthy rabbits the IVB procedure itself elevated aqueous MCP-1, but not IL-6 and IL-8. Since NVG eyes might be more sensitive to invasive procedures than healthy eyes, it remains possible that the IVB procedure induces other inflammatory reactions in NVG. Taken together, IVB alone is unlikely to decrease proinflammatory cytokine levels. Further prospective studies are required to clarify the effects of IVB on aqueous cytokine/growth factor levels in NVG.

Aqueous cytokine levels are related to several clinical features in ocular ischemic diseases. For example, increased IL-8, PDGF-AA, TNF-α, and VEGF levels in the aqueous humor at the onset of retinal vein occlusion are associated with the subsequent development of retinal ischemia. In eyes with diabetic retinopathy, the IL-1β, IL-6, IL-8, MCP-1, and interferon-γ-induced protein-10 levels in the aqueous humor increase with greater retinopathy severity. Following vitrectomy, the level of aqueous IL-8 is associated with the occurrence of recurrent vitreous hemorrhage. Although it is not fully understood whether the elevated cytokine/growth factor levels are the cause or result of the clinical features, monitoring these cytokines may provide clinically useful biomarkers in ocular ischemic diseases, including NVG.

In a previous study, we have found that the history of phacoemulsification is associated with high levels of IL-8 and MCP-1 in open-angle glaucoma. Since the differences in cytokine levels between the POAG and NVG cases were prominent, the effect of past intraocular surgery may have been masked by the effect of NVG severity. Therefore, the history of intraocular surgery may affect aqueous cytokine levels in both POAG and NVG, although the effects of other factors may be more prominent in NVG. Similarly, glaucoma eye drops could affect the aqueous cytokine levels, although this could be relatively limited in NVG.

In conclusion, the levels of multiple cytokines in NVG cases were higher than in cataract and POAG cases. Intravitreal bevacizumab injection decreased the VEGF level, but not that of other cytokines.

### Acknowledgments

Supported in part by Japan Society for the Promotion of Science KAKENHI Grants 23390403, 23659814, and 25791994. The sponsor or funding organization had no role in the design or conduct of this research.

Disclosure: S. Ohira, None; T. Inoue, None; K. Shobayashi, None; K. Iwao, None; M. Fukushima, None; H. Tanihara, None

### References


4. Tripathi RC, Li J, Tripathi BJ, Chalam KV, Adamis AP. Increased level of vascular endothelial growth factor in aqueous humor...


