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

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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). Aqueous humor samples were collected from the anterior chamber at the start of surgery, deposited in CryoTubes, 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 via 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 usage before filtration surgery reduces surgical complications, although its effect on long-term surgical results is limited. Histopathologic analysis indicates that 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
Aqueous Humor Cytokines in Neovascular Glaucoma

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, or 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, and was then incubated at room temperature for 1 hour. Multiplexed cocktails of biotinylated reporter antibodies were mixed and then incubated at room temperature for 18 hours, multiplexed cocktails of biotinylated reporter antibodies were mixed and then incubated at room temperature for 18 hours. Multiplex 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 Animal Experiment 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°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) and xylazine hydrochloride (Celaclat, 10 mg/kg body weight; Bayer Medical, Leverkusen, Germany), IVB was administered using a tuberculin syringe with a 30-gauge needle through the conjunctiva. The aqueous humor samples obtained from eyes with NVG and analyze the simultaneous increase in proinflammatory cytokines in aqueous humor are thought to play important roles in the pathophysiology of glaucoma. In particular, VEGF, monocyte chemotactic protein (MCP)-1, tumor necrosis factor-α (TNF-α), 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.

Clinical Study

Intravitreal Injection of Bevacizumab. Two days before trabeculectomy, IVB was conducted in surgeon-selected NVG cases, as described previously. Briefly, following topical anesthesia using lidocaine (AstraZeneca, Osaka, Japan) and disinfec-

TABLE 1. Patient Characteristics

<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>11.9 ± 2.9</td>
<td>26.8 ± 7.9</td>
<td>37.3 ± 10.7†</td>
</tr>
<tr>
<td>Glaucoma eye drops</td>
<td>0.2–61.5</td>
<td>11.0–60.0</td>
<td>20.0–67.7</td>
</tr>
<tr>
<td>Duration of glaucoma therapy, mo</td>
<td>0.2–61.5</td>
<td>1.3–1303.5</td>
<td>0.2–61.5</td>
</tr>
<tr>
<td>History of phacoemulsification, n (%)</td>
<td>68 (100)</td>
<td>9 (25.0)</td>
<td>19 (57.6)†</td>
</tr>
<tr>
<td>History of vitrectomy, n (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>15 (39.4)†</td>
</tr>
</tbody>
</table>

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.
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 x-axis against the concentration on the y-axis by 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-I-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.

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**Figure 1.** Comparison of the levels (pg/mL) of analytes in the eyes of controls (cataractous [CAT]), with open-angle glaucoma (OAG), and with NVG. Data points (n = 68, 36, and 33 for CAT, OAG, and NVG, respectively) represent individual samples. Error bars represent medians ± interquartile range. **P < 0.01.
centrifuged at 13,000 g 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-$\alpha$, 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 $P < 0.001$).

**Hypoxic 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.35 Chalam et al.36 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-$\alpha$, are significantly increased in the NVG group compared to the POAG controls.37 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.
Matsuyama et al. have found, when determining the serum is unchanged approximately 1 week after IVB. By contrast, demonstrated that the elevated level of erythropoietin in NVG aqueous VEGF concentrations in NVG. Zhou et al. have more cases with rubeosis iridis were included in the IVB group. cytokine levels were inconclusive in the present study, because factors. However, the effects of rubeosis iridis on aqueous levels of IL-6, IL-8, and MCP-1 would be considered to release large amounts of biologically active factors. 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. reported 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.

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