Increased Interleukin-6 in Aqueous Humor of Neovascular Glaucoma

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PURPOSE. To demonstrate the involvement of proinflammatory cytokines in intraocular neovascularization by detecting the presence of interleukin (IL)-6, IL-2, and tumor necrosis factor (TNF)-α in aqueous humor and serum of patients with neovascular glaucoma (NVG) secondary to central retinal vein occlusion (CRVO).

METHODS. According to the grade of iris neovascularization (NVI), patients with CRVO were divided into three groups: CRVO without NVI, CRVO with NVI, and CRVO with regressed NVI. Healthy patients with cataract were enrolled as control subjects. Enzyme-linked immunosorbent assay was used to quantitate the concentrations of the cytokines IL-6, IL-2, and TNF-α in aqueous humor and serum from patients with NVG and control subjects.

RESULTS. In serum, the levels of IL-6, IL-2, and TNF-α did not differ among groups. In aqueous humor, only IL-6 showed significant change among groups. IL-6 levels in aqueous humor of group 2, CRVO with NVI (1532.0 ± 221.1 pg/ml; P < 0.001), and group 3, CRVO with regressed NVI (234 ± 154.6 pg/ml; P < 0.001), were significantly higher. There was no significant difference in IL-6 levels between the control group (26.4 ± 21.8 pg/ml) and group 1 (15.6 ± 0.9 pg/ml).

CONCLUSIONS. The inflammatory cytokine IL-6 in aqueous humor increased spatially and temporally correlated with the grade of NVI in patients of NVG secondary to CRVO. The aqueous IL-6 increased in NVI and decreased after vessels regressed. It is possible that the significantly higher level of IL-6 was due to intraocular synthesis because of the minimal change in serum. The increased level of IL-6 may have a putative role along with other angiogenic factors in angiogenesis of NVG as a possible predictor of NVI. (Invest Ophthalmol Vis Sci. 1999;40:2627–2632)

Pathologic intraocular neovascularization is a complication of most blinding eye diseases. Such disorders include retinopathy of prematurity (ROP), diabetic retinopathy, age-related macular degeneration, and neovascular glaucoma (NVG). NVG is defined as rubeosis iridis, or iris neovascularization (NVI), with secondary angle-closure glaucoma. It is a serious sequela of many ischemic eye problems. Clinical studies imply that central retinal vein occlusion (CRVO), diabetic retinopathy, and sickle cell retinopathy are leading causes of the development of NVG.1,2 In 1948, Michaelson3 proposed that “there exists in the retina a factor or factors affecting the budding of new vessels.” Ashton4 and others later expand the model to suggest that hypoxia of the retina was the primary stimulus of retinal production of angiogenic factors. Almost 50 years later, mounting evidence has prompted many to speculate that vascular endothelial growth factor (VEGF) is the sole “vasoformative factor” proposed by Michaelson.3 Current reports support that VEGF and its receptors serve an important role in various steps of intraocular angiogenesis by virtue of its ability to be induced by hypoxia and its diffusible nature. However, angiogenesis is a multistep process in which many growth factors and cytokines are involved and have essential roles.5 Besides VEGF, other molecules that have been demonstrated to be correlated with the development of NVG include basic fibroblast growth factor,6 platelet-derived growth factor, insulin-like growth factor-1,7,8 and interferon-α.9,10 But except for VEGF, none of these molecules has had its bioactivities inhibited to show it to be causal in the production of NVG.

Hjelmeland et al.1,2 established an experimental model of NVI and ectropion uveae in the cat by retinal vein cauterization. It was demonstrated histologically that inflammatory cells were present in the iris stroma, iris epithelium, and on the surface of fibrovascular membrane during the development of NVI. Histologically, NVI is not only the new vessel formation on iris but also fibrotic scarring, which is characteristic of inflammatory fibrosis.1,2 Furthermore, Shabo et al.13 have demonstrated that NVG can be induced by an inflammatory response in the anterior chamber of sensitized monkey. Schulze14 also noted the inflammatory changes in aqueous humor before the development of NVI. Clinical findings show that NVG develops in nonischemic inflammatory eye diseases such as Vogt–Koyanagi–Harada disease,15 endophthalmitis,14,16 chronic uveitis,17 sympathetic ophthalmia,16 syphilitic retinitis,18 and Fuchs’ heterochromic iridocyclitis.19 Based on previous reports, we suggested that NVI implied not only formation of new vessels on iris but also a process of complex mechanisms involving ischemic, immunologic, and
inflammatory responses. The proinflammatory cytokines, such as interleukin (IL)-6, IL-2, and tumor necrosis factor (TNF)-α, may play a role in mediating the inflammation associated with these various ocular diseases.

IL-6, IL-2, and TNF-α share many common characteristics with VEGF and are also reported to be hypoxia induced. Intravitreal injection of IL-6, IL-2, or TNF-α caused ocular inflammation in experimental animals. Increased IL-6 in aqueous humor was noted in intraocular inflammation such as uveitis and endophthalmitis, cataract extraction, and Vogt–Koyanagi–Harada disease. IL-6 and TNF-α gene expression markedly increases in rat retina after transient ischemia. Furthermore, Cohen et al. showed that proinflammatory cytokines regulate VEGF expression. In previous studies, increased levels of VEGF have been reported in samples of vitreous and aqueous humor in patients with NVI. In this study, we measured the concentrations of IL-6, IL-2, and TNF-α in aqueous humor and serum samples from patients with NVG secondary to CRVO.

**Methods**

**Study Subjects: Patients with CRVO**

Twenty-four patients with CRVO, 20 men and 4 women, were included in the group with eye disease after they provided informed consents according to a protocol approved by the Institutional Review Board of the Veterans General Hospital-Taipei. This study was performed according to the tenets of the Declaration of Helsinki for research involving human subjects. The gender, age, vision, intraocular pressure, age of CRVO onset, duration of CRVO, and grade of NVI were recorded. NVI was graded according to the grading system of Teich and Walsh. Eyes of patients in the eye disease group were checked by slit lamp microscopy, and the grades of NVI were determined by fluorescein angiography. The patients were divided into three groups according to the disease process and iris conditions: group 1, CRVO without NVI (n = 5); group 2, CRVO with NVI (n = 10); and group 3, CRVO with regressed NVI after laser treatment (n = 9). Patients who had ever undergone ocular or systemic steroid treatment were excluded. Intraocular pressure in groups 2 and 3 was controlled by β-adrenergic antagonists, carbonic anhydrase inhibitors, and/or hypotensive agents. Laser treatments had been performed in the patients of group 3 at least 6 months before the study began. Six healthy patients with cataract, who were sex and age matched with the disease groups, were included as a control group. The basic data of the groups are listed in Table 1.

**Collection of Aqueous Humor and Serum**

Approximately 0.1 ml aqueous humor and serum samples were obtained with documented permission from patients in these four groups when they underwent cataract surgery. Aqueous humor was collected through anterior chamber paracentesis before cataract surgery began. The blood samples were centrifuged at 1000g for 20 minutes, and serum was then removed. Aqueous and serum samples were stored at −70°C in liquid nitrogen until they were assayed.

**Grading of NVI**

We adopted the grading system of NVI that was proposed by Teich and Walsh: grade 0, no neovascularization of iris observed; grade 1, fine surface neovascularization of the pupillary zone of iris involving less than two quadrants; grade 2, surface neovascularization of the pupillary zone of iris involving more than two quadrants; grade 3, in addition to neovascularization of pupillary zone, neovascularization of the ciliary zone of the iris, and/or ectropion uveae involving one to three quadrants; and grade 4, neovascularization of the ciliary zone of the iris and/or ectropion uveae involving more than three quadrants. Patients in group 2 had either grade 3 or grade 4 NVI and those in group 3 had grade 1 or grade 2. No NVI was found in group 1 and the control group.

**Enzyme-Linked Immunosorbent Assay**

IL-6, IL-2, and TNF-α in aqueous humor and serum samples were quantitated with enzyme-linked immunosorbent assay (ELISA) kits (Boehringer, Mannheim, Germany). The assays were processed according to the manufacturer’s instruction. Briefly, the assays were based on the quantitative sandwich enzyme immunoassay principle, using two monoclonal antibodies from mouse, directed against two different epitopes of cytokines. Both antibodies recognize epitopes that are essential for receptor binding. These enabled the specific determination of biologically active cytokines in this assay system. During the first incubation step, cytokines in standard and samples were simultaneously bound by the biotin-labeled antibody and peroxidase-conjugated detection antibody forming a complex that bound by biotin-labeled antibody to the streptavidin-coated surface of the microtiter plate. Subsequent to the washing step, the peroxidase bound in the complex was developed by tetramethylbenzidine as a substrate and concentr-
tions determined photometrically. The developed color was proportional to the concentration of cytokine. Standards of defined concentrations were run in each assay, allowing the construction of a calibration curve by plotting absorption versus concentration. The cytokine concentrations of samples were then calculated from this calibration curve.

The measuring range of this test system has been shown to be between 5 and 1000 pg/ml, and a value of more than 10 pg/ml is usually obtained. If the measurement was more than 1000 pg/ml, the sample was diluted 10-fold and checked again.

Analysis of Data

Data are presented as mean ± SEM. Student’s paired t-test (two-tailed) was used to analyze the data of photometric counts. \( P < 0.05 \) was accepted as statistically significant.

RESULTS

IL-6 in Aqueous Humor

We measured the levels of IL-6, IL-2, and TNF-\( \alpha \) in samples of aqueous humor and serum from patients with NVG and healthy patients with cataract. Figure 1 shows the results of IL-6 measurement in aqueous humor in the four groups. IL-6 levels in aqueous humor of the control group ranged from 21.2 to 63.1 pg/ml (mean, 26.4 ± 21.8 pg/ml). In group 1, IL-6 levels of aqueous humor averaged 15.6 ± 0.9 pg/ml, (range, 15–17.5 pg/ml). The difference between these two groups was not significant (\( P > 0.4 \)).

In group 2, all IL-6 levels were higher than 1000 pg/ml, which was the upper limit of detection of this assay. In the patients with CRVO with NVI, the IL-6 levels, 1532 ± 221.1 pg/ml, were dramatically and significantly greater than in the other three groups (\( P < 0.001 \)). Group 2 had a 98-fold increase of IL-6 in aqueous humor over group 1.

In group 3, patients with CRVO with regressed NVI, IL-6 levels of aqueous humor decreased to 234.3 ± 154.6 pg/ml (range, 16.3–790.1 pg/ml). This level of IL-6 was significantly lower than in patients in group 2 (\( P < 0.05 \)). Unlike the trend in the variation of IL-6 levels in aqueous humor that correlated with the grade of NVI, the iris vessels had regressed.

IL-6 in Serum

Figure 2 shows the results of measuring IL-6 in the sera of these four groups. The IL-6 concentration in every sample of the four groups was close, ranging from 12.6 to 27.0 pg/ml. No significant difference in serum IL-6 levels was noted among the groups (\( P > 0.4 \)). In the control group, no significant difference was found between the IL-6 levels in aqueous humor and serum (\( P > 0.5 \)).

In groups 2 and 3, IL-6 levels in serum did not increase. There were significantly higher levels of IL-6 in aqueous humor of these two groups than in their respective serum samples. (\( P < 0.001 \)). Unlike the trend in the variation of IL-6 levels in aqueous humor that correlated with the grade of NVI, serum IL-6 levels remained the same during NVI formation and regres-
Therefore, the IL-6 found in the aqueous samples of groups 2 and 3 were probably produced intraocularly.

**IL-2 and TNF-α in Aqueous Humor and Serum**

IL-2 levels in the four groups ranged from 49.0 to 51.0 pg/ml in aqueous humor and 49.5 to 58.0 pg/ml in serum. Both were not significantly different (P > 0.1) among the four groups. TNF-α levels in the four groups ranged from 13.0 to 15.0 pg/ml in aqueous humor and 13.0 to 22.0 pg/ml in serum, and neither was significantly different among the four groups (P > 0.1). Therefore, levels of IL-2 and TNF-α in aqueous humor and in serum during CRVO with NVI were unchanged.

**DISCUSSION**

The pathologic characteristics of ischemic ocular tissues share similar features with inflammatory response, including production of proinflammatory cytokines such as IL-6.23 We have shown that the IL-6 levels in aqueous humor increased dramatically in patients who had CRVO with NVI and was in parallel with the change of grade of NVI. The mean concentration of IL-6 in aqueous humor of patients with NVI was significantly higher than that of control subjects and patients who had CRVO without NVI. After laser treatment, the NVI regressed grossly, and the IL-6 levels in aqueous humor were significantly lower than those of patients with NVI. IL-6 levels in serum did not change in any patient in spite of the condition of the iris. It appears that increased IL-6 in aqueous humor may originate intraocularly, rather than in systemic circulation. This finding is compatible with the conclusion of Hoekzema et al.35 in demonstrating the significance of intraocular IL-6 in endotoxin-induced uveitis in rat. However, because the blood-ocular barrier is destroyed in NVG patients, we cannot exclude the possibility that increased IL-6 in aqueous humor was serum-derived and accumulated in the eye because of decreased clearance.

Normally, cells do not synthesize and secrete IL-6 unless the cells are stimulated by other cytokines or by some physiological event. It has been shown that a wide range of ocular tissues can produce IL-6 in vitro and in vivo, such as cultures of cornea epithelial, stromal, and endothelial cells36; iris and ciliary body explants37; cytokine-stimulated human pigment epithelial cells 38,39; ischemic retina22; and hypoxia-induced or cytokine-stimulated vascular endothelial cells and vascular smooth muscle cells.40 Inflammatory cells, especially mast cells, are known to be able to stimulate IL-6 secretion from leukocytes and human vascular endothelial cells in ischemic and inflammatory conditions.41,42 Therefore, there is a strong possibility that IL-6 in aqueous humor of eyes with NVI comes from intraocular sources, such as ocular and inflammatory cells.

IL-6 is a multifunctional cytokine that acts on a wide range of cells. Several reports have demonstrated its major role as a mediator of inflammatory and immune responses.43 The pleiotropic effects of IL-6 include the stimulation and secretion of immunoglobulin, induction of neuronal differentiation, and activation of acute-phase protein synthesized by liver cells. Yan et al.44 suggested that IL-6 contributes to neovascularization in pulmonary vessels as a result of prolonged hypoxic exposure. IL-6 expression has been identified during angiogenesis in
wound healing and tumor growth.\textsuperscript{44,45} It has been reported that the in vivo expression of IL-6 accompanies vascularization in female reproductive tissues.\textsuperscript{46} Motro et al.\textsuperscript{46} found that maximal IL-6 mRNA levels coincided with the period of formation of a capillary network, and no expression was detected once angiogenesis had been completed. Holzinger et al.\textsuperscript{47} indicated that IL-6 was a potent mediator in inducing the proliferation of human umbilical vein endothelial cells in culture. Moreover, it has been suggested that IL-6 may be involved in the progression of vascular tumors induced by Polyomavirus.\textsuperscript{48} and Kaposi's sarcoma\textsuperscript{49} and may stimulate the migration of vascular endothelial cells in culture.\textsuperscript{50} At least 15 intraocular angiogenic growth factors are known\textsuperscript{51} and the most extensively studied of these molecules include basic fibroblast growth factor, VEGF, transforming growth factor-β, TNF-α, growth hormone, insulin-like growth factor-1, IL-8, integrins, and the angiopoietins.\textsuperscript{52} That IL-6 involvement in angiogenesis is not limited to hypoxia conditions but also occurs in ovulation, wound healing, and tumor formation suggests that IL-6 should also be considered an angiogenic factor. Previous evidence\textsuperscript{44–46} and our own findings indicate that the level of IL-6 in the eye is highly correlated with the initiation and formation of new vessels.

The role IL-6 plays in NVI formation and intraocular angiogenesis is not clear. In different experiments of human vascular endothelial cells in vitro, IL-6 stimulated,\textsuperscript{53} inhibited,\textsuperscript{50,53} or had no effect on endothelial cell proliferation.\textsuperscript{53} In proliferative diabetic retinopathy, IL-6 was detected in the vitreous, and its levels correlated with disease activity.\textsuperscript{54} Intravitreal injection of IL-6 in animal experiments of uveitis did not lead to intraocular neovascularization. In all these studies, however, the animals were usually killed before NVI was allowed to form.\textsuperscript{12,24,25} In some circumstances, IL-6 acts in an autocrine fashion in vascular endothelial cells stimulated with inflammatory cytokines\textsuperscript{56–57} and, consequently, induces endothelial cells growth.\textsuperscript{47,58} A recent study indicates that there is a causal relationship between IL-6 and VEGF in neovascularization. Even though VEGF alone is sufficient to produce NVI and NVG in nonhuman primates by intravitreal injection of VEGF,\textsuperscript{59,60} it does not rule out the possibility that other growth factors may be involved in the intraocular angiogenic process. Cohen et al.\textsuperscript{53} suggested that IL-6 may induce angiogenesis indirectly by stimulating VEGF expression comparable with the documented induction of VEGF mRNA by hypoxia. The increase of IL-6 as an inflammatory cytokine in the aqueous humor of NVG secondary to ischemic retinal disease seems to complement the relationship between VEGF and NVI in ocular inflammatory diseases.

However, from our data it is not possible to conclude whether the increased IL-6 caused NVI or whether IL-6 was produced because of NVI. It may be that the increased level of IL-6 in patients with NVG is an epiphenomenon and that IL-6 is an unrelated bystander, is a cytokine that is synthesized to inhibit NVI, or is secondary to the higher intraocular pressure. Furthermore, because antiglaucoma medication was used in patients of groups 2 and 3 who have higher IL-6 levels in aqueous humor, drug effects cannot be ruled out as a confounding variable factor. So far, there has been no report indicating that the antiglaucoma drugs used in this study increase the IL-6 level in aqueous humor. Steroid is the only drug used in treating NVG that has been reported to alter the IL-6 level. We excluded the group of patients treated with steroids.

In our study, we describe the spatial and temporal correspondence of IL-6 in aqueous humor with the rubecosis iridis or NVG secondary to CRVO. IL-6 levels in aqueous humor correlated with clinically graded NVI. Although questions remain in exploring the relationships between intraocular pathologic neovascularization and ischemia and proinflammatory cytokines, we suggest that intraocularly produced IL-6 may have a role in the formation of NVG. In clinical studies, IL-6 levels in body fluids were documented as indicators and predictors of disease processes such as intraoperative splanchic ischemia,\textsuperscript{61} unstable angina,\textsuperscript{62} and ovarian hyperstimulation syndrome.\textsuperscript{63} It is possible that IL-6 can be successfully used as a predictor of NVG. Also, normalization of IL-6 activity in the eye may be a useful treatment strategy for regulating the extent of NVI in NVG.

References


