Role of NF-κB-Mediated Interleukin-8 Expression in Intraocular Neovascularization

Ayako Yoshida, Shigeo Yoshida, Ahmad K. Kbalil, Tatsuro Ishibashi, and Hajime Inomata

Purpose. To investigate the role of interleukin (IL)-8 in intraocular neovascularization and the mechanism of its production.

Methods. Interleukin-8 was measured with enzyme-linked immunosorbent assays in vitreous and aqueous fluid obtained from patients with neovascular diseases. Localization of IL-8 was examined by immunohistochemistry. An in vitro angiogenesis assay was performed on collagen gels, by using bovine aortic endothelial cells to determine the effect of the vitreous fluid. In bovine retinal glial cells under hypoxia, NF-κB activation was evaluated by immunoblot analysis and by electrophoretic mobility shift assay, and IL-8 and vascular endothelial growth factor (VEGF) mRNA expression was determined by semiquantitative reverse transcription-polymerase chain reaction.

Results. The concentration of IL-8 in vitreous fluid of patients with retinal neovascularization was significantly higher than that of patients without neovascular disease. Interleukin-8 immunostaining was detected in vascular endothelial cells and glial cells in the retinas with neovascularization. Vitreous fluid with high concentrations of IL-8 induced tubular morphogenesis in endothelial cells, and this effect was inhibited to a similar extent by neutralizing antibodies to IL-8 or to VEGF. In glial cells, in vitro, hypoxia induced NF-κB activation and increased IL-8 and VEGF mRNA. Furthermore, pyrrolidine dithiocarbamate, a specific inhibitor of NF-κB activation, prevented the induction of the IL-8 gene, but not that of the VEGF gene.

Conclusions. These results suggest that IL-8 induced by hypoxia and mediated by NF-κB may contribute to the pathogenesis of intraocular neovascularization. (Invest Ophthalmol Vis Sci. 1998;39:1097-1106)

Intraocular neovascularization is a major cause of the loss of vision in patients with such diseases as diabetic retinopathy, retinal vein occlusion, and retinopathy of prematurity. Retinal ischemia often precedes the onset of such neovascularization, and the ischemic retina has been identified as a potential source of diffusible angiogenic factors. The amount of vascular endothelial growth factor (VEGF) in vitreous fluid (VF) and aqueous fluid (AF) is increased in persons with ischemic retinal diseases, and this agent is an important mediator of intraocular neovascularization. However, the stimulatory effect of VF on the growth of retinal vascular endothelial cells is not completely inhibited by antibodies to VEGF. Because angiogenesis depends on a balance between multiple angiogenic factors and angiogenic inhibitors, pathways other than those mediated by VEGF may be involved in intraocular neovascularization.

Interleukin (IL)-8, a chemotactic cytokine for T lymphocytes and neutrophils, induces an angiogenic response in vitro and in vivo. It also contributes to such angiogenesis-dependent disorders as rheumatoid arthritis, psoriasis, wound repair, malignant melanoma, bronchogenic carcinoma, and diabetic retinopathy.

The promoter of the IL-8 gene contains potential binding sites for the transcription factor NF-κB, which was originally identified as a heterodimeric complex of 50-kDa and 65-kDa (p65) subunits, thought to be central to the regulation of numerous inflammatory and proliferative response genes. Recent studies have shown that NF-κB regulates the initiation of angiogenesis in vitro. We have reported involvement of IL-8 through activation of NF-κB in tumor necrosis factor-α-induced angiogenesis. In addition to the IL-8 gene, genes regulated by this pleiotropic mediator, NF-κB, include those encoding tumor necrosis factor-α, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1, all of which may participate in neovascularization. Moreover, hypoxia induces expression of IL-8 in vascular endothelial cells through activation of NF-κB.

In this study we examined a possible pathologic role for NF-κB-mediated expression of IL-8 in intraocular neovascularization. We investigated the relation between the intraocular concentration of IL-8 and neovascularization in affected patients and characterized the NF-κB-mediated expression of IL-8 induced by hypoxia in bovine retinal glial cells.

Methods

Reagents

Rabbit polyclonal antibodies to p65 (anti-p65) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), rabbit poly-
clonal anti-human IL-8 from Sanbio (Uden, Netherlands), and rabbit polyclonal anti-gial fibrillary acidic protein and rabbit polyclonal anti–von Willebrand factor from Dakopatts (Glostrup, Denmark). Recombinant human IL-8, basic fibroblast growth factor (bFGF), and neutralizing antibodies to human IL-8 or to VEGF were obtained from R&D Systems (Minneapolis, MN), and recombinant human VEGF was obtained from Pepro Tech (Rocky Hill, NJ). [32P]deoxyadenosine triphosphate was obtained from Amersham (Little Chalfont, UK). NF-kB consensus sequences were synthesized with a DNA synthesizer (Applied Biosystems, Tokyo, Japan). The sequences of NF-kB consensus oligonucleotides of the IL-8 promoter region are 5'-GCAAATTCGTTGAGATTCCTCTGA-3' and 5'-GTCAGAGTCAATTCGATTTG-3'.

**Subjects**

Undiluted samples of AF (100 μl; n = 32), VF (0.5–0.8 ml; n = 82), and serum (n = 67) were obtained from patients undergoing intraocular surgery. Fourteen samples of AF, VF, and serum were simultaneously collected from 14 patients. Ten AF and serum samples were obtained from 10 patients, and 43 VF and serum samples were obtained from 43 patients. From 8 and 25 patients, only an AF or VF sample was available, respectively. The study protocol was approved by the Board of Clinical Research of Kyushu University and complied with the tenets of the Declaration of Helsinki. All patients gave their informed consent to participation. The primary diagnoses of neovascular disorders included proliferative diabetic retinopathy (PDR) (AF, n = 14; VF, n = 50; serum, n = 42) and ischemic retinal vein occlusion central retinal vein occlusion (CRVO) or hemispheric branch retinal vein occlusion (HBRVO) (AF, n = 4; VF, n = 8; serum, n = 6), and those of non-neovascular disorders included cataract (AF, n = 9; serum, n = 5), chronic open-angle glaucoma (AF, n = 3; serum, n = 2), proliferative vitreoretinopathy (VF, n = 5; serum, n = 2), macular hole (AF, n = 1; VF, n = 11; serum, n = 7), and preretal membrane (AF, n = 1; VF, n = 8; serum, n = 3).

Clinical data, including determinations of ocular neovascular activity, were obtained from the surgeon at the time of surgery by completion of standardized forms and were confirmed by standardized fundus photography whenever possible. Neovascularization was considered to be active if perfused, multiple preretal capillaries were apparent and to be quiescent if previously documented active proliferation had regressed fully or if only nonperfused, gliovascular tissues or fibrosis was present. The vitreous samples were collected by manual suction into a syringe through the air-flushed aspiration line of a vitrectome (Alcon, Tokyo, Japan), before opening the infusion line. Samples were immediately frozen and stored at −70°C.

Six eyes were also obtained and fixed immediately after enucleation with 4% paraformaldehyde. Three of them had PDR and were obtained by surgery and three eyes were from eye bank donors to be used as control subjects.

**Enzyme-Linked Immunosorbent Assay of Interleukin-8**

Measurements of IL-8 concentration were performed by enzyme-linked immunosorbent assay using a kit (TFB, Tokyo, Japan). Undiluted VF, AF, and serum were assayed in duplicate by adding 50 μl specimen per well. A standard curve was generated for each assay with predetermined amounts of human IL-8. The lower detection limit of the assay was 3 pg/ml.

**Immunohistochemistry of the Retina**

Three-millimeter sections of eyes were cut and after removal of paraffin, were rehydrated and incubated for 1 hour at room temperature with 1:13 rabbit polyclonal anti-human IL-8. Bound antibodies were detected by a conventional avidin-biotin–peroxidase protocol, with 3-amin-9-ethylcarbazole as substrate. For negative control, rabbit nonimmune IgG was used as the primary antibody. The specificity of the anti-IL-8 was demonstrated by preadsorption with IL-8, which abolished staining. To identify the cell types that express IL-8, we performed immunohistochemistry for glial fibrillary acidic protein and von Willebrand factor, which are markers for glial cells and endothelial cells, respectively.

**Cell Culture and Induction of Hypoxia**

Bovine retinal glial cells were isolated and cultured as previously described. For induction of hypoxia, bovine retinal glial cells were incubated in Anaerocult A mini (Merck–Clevenot, France) to produce hypoxic conditions as described. The Anaerocult A mini contains components that chemically bind oxygen quickly and completely, creating an oxygen-free milieu and an atmosphere of carbon dioxide. The culture dishes were immediately placed in the incubation bag, which was sealed and incubated at 37°C.

**Quantitative Analysis of Tube Formation by Bovine Aortic Endothelial Cells on Type I Collagen Gel**

The in vitro angiogenesis assay was performed as described previously. Bovine aortic endothelial cells were plated onto the surface of type I collagen gel in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum. When the cells achieved confluence, the medium was replaced with DMEM containing 1% fetal calf serum (in the absence or presence of recombinant human IL-8, VEGF, basic fibroblast growth factor, or VF) and incubated for an additional 72 hours. The cells were fixed and stained briefly in modified May–Gruenwald solution to visualize tubelike structures. Images of the cells were captured using a microscope coupled to a video camera, saved on disk as tagged image format files, and analyzed with the software package NIH Image 1.61 (National Institutes of Health, Bethesda, MD). The total length of tubelike structures per field was measured (magnification, ×200). Eight random fields were analyzed per dish, and the mean total length per field was calculated.

**Preparation of Nuclear and Cytosolic Extracts and Immunoblot Analysis**

Nuclear and cytosolic extracts of bovine retinal glial cells were prepared as described previously. These fractions were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 9% gels, and the separated proteins were transferred to nitrocellulose filters. The filters were then subjected to immunoblot analysis, with bound antibodies detected by the enhanced chemiluminescence protocol (Amersham).

**Electrophoretic Mobility Shift Assay**

Electrophoretic mobility shift assays were performed as described in a final volume of 20 μl containing nuclear extracts (4 μg protein), 10 mM HEPES (pH 7.9), 4% glycerol, 1 mM dithiothreitol, 1 mM EDTA, 0.1 μg bovine serum albumin, 1 μg...

Interleukin-8 in Intraocular Neovascularization 1099

Synthetic oligonucleotide primers based on the cDNA sequences of bovine IL-8, VEGF, and β-actin were prepared: IL-8, 5′-AGAATTCTGAGGCCAATGC-3′ and 5′-TCGAGCAACTTCGTCGTCGACGGGACCC-3′; VEGF, 5′-TGGTATGAGAAAAGTCTGTCAG-3′ and 5′-TCACCGCTTGGCCGTGACA-3′; and β-actin, 5′-ACCACTTGCGTATGTGACGGG-3′ and 5′-GCTTCTCCGTGATGTCACGC-3′. The VEGF primers are expected to yield amplification products of 186, 318, 390, and 441 bp, corresponding to VEGF121, VEGF165, VEGF189, and VEGF206, respectively.

Poly(dI-dC), and 2 μL 32P-labeled NF-κB target oligonucleotides, in the absence or presence of a 100-fold molar excess of nonradioactive competitor oligonucleotides. The reaction was initiated by the addition of the labeled oligonucleotides and was allowed to proceed for 30 minutes at 25°C, after which 2 μL 10X sample buffer (50% glycerol, 0.25% bromphenol blue, 0.25% xylene cyanol) was added, and the samples were subjected to electrophoresis on a 4% polyacrylamide gel (polyacrylamide-bisacrylamide ratio, 80:1) at 4°C in buffer (25 mM Tris base, 190 mM glycine, 1 mM EDTA [pH 8.5]). Gels were analyzed by autoradiography.

**Semiquantitative Reverse Transcription-Polymerase Chain Reaction**

Statistical Analysis

Data are expressed as means ± SEM and were compared between groups using the Wilcoxon rank sum test. A level of P < 0.01 was considered statistically significant.

**RESULTS**

Detection of Interleukin-8 in Ocular Fluid and Serum

With the use of an enzyme-linked immunosorbent assay, we assayed VF, AF, and serum from people with or without retinal neovascular disorders to determine IL-8 concentrations. There was no correlation between IL-8 concentrations and age or sex in any subgroup. For VF, IL-8 (>3 pg/ml) was detected in 30 (60%) of 50 samples from patients with PDR; 8 (100%) of 8 samples from patients with ischemic retinal vein occlusion (3 with CRVO and 5 with HBRVO); and 2 (8.3%) of 24 samples from patients with non-neovascular disorders (Table 1). Among patients with diabetes mellitus, IL-8 was detected in 22 (81%) of 27 VF samples from those with active proliferative retinopathy, but was detected in 8 (35%) of 23 samples from those with quiescent proliferative retinopathy. All eyes of patients with retinal vein occlusion exhibited active neovascularization. The concentration of IL-8 in VF was significantly increased in patients with PDR (51.8 ± 13 pg/ml; P < 0.0001) compared with those with retinal vein occlusion (227.1 ± 122.7 pg/ml; P < 0.0001), compared with concentrations in control subjects (0.9 ± 0.7 pg/ml) (Table 2; Fig. 1). The IL-8 concentration in VF from patients with active PDR (88.4 ± 21.8 pg/ml) was significantly higher than that in VF from patients with quiescent PDR (8.7 ± 5 pg/ml, P < 0.01). In AF, IL-8 was detected in 1 (7.1%) of 14 samples from patients with PDR, in 4 (100%) of 4 samples from patients with ischemic retinal vein occlusion (2 with CRVO and 2 with HBRVO), and in 1 (7.1%) of 14 samples from control subjects (Table 1). The IL-8 concentration was significantly increased in AF from patients with retinal vein occlusion (109.4 ± 192 pg/ml; P < 0.01), but not in that from patients with PDR (4.4 ± 4.4 pg/ml), compared with concentrations in control subjects (1.4 ± 1.4 pg/ml; Table 2). The IL-8 concentration in VF exceeded that in AF for simultaneously collected samples from each of 14 patients. Interleukin-8 was detected in serum from 11 (26%) of 42 patients with PDR, but only 3 of

<table>
<thead>
<tr>
<th>Group</th>
<th>Vitreous Fluid</th>
<th>Aqueous Fluid</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>All subjects</td>
<td>40/82 (49)</td>
<td>6/32 (19)</td>
<td>15/67 (22)</td>
</tr>
<tr>
<td>Control subjects</td>
<td>2/24 (8)</td>
<td>1/14 (7)</td>
<td>4/19 (21)</td>
</tr>
<tr>
<td>Proliferative diabetic retinopathy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All patients</td>
<td>30/50 (60)</td>
<td>1/14 (7)</td>
<td>11/42 (26)</td>
</tr>
<tr>
<td>Quiescent</td>
<td>8/23 (35)</td>
<td>4/4 (100)</td>
<td>0/6 (0)</td>
</tr>
<tr>
<td>Active</td>
<td>22/27 (81)</td>
<td>4/4 (100)</td>
<td>0/6 (0)</td>
</tr>
</tbody>
</table>

*Includes central and hemispheric branch retinal vein occlusion. All of these patients exhibited active retinal neovascularization.

**Table 1. Number (%) of People with more than 3 pg/ml Interleukin-8 in Ocular Fluid or Serum**
Table 2. Concentrations of IL-8 (pg/ml) in Ocular Fluid of Study Subjects

<table>
<thead>
<tr>
<th>Group</th>
<th>Vitreous fluid</th>
<th>Aqueous fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>P</td>
</tr>
<tr>
<td>Control subjects</td>
<td>0.9 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>Proliferative diabetic retinopathy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All patients</td>
<td>51.8 ± 13</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Quiescent</td>
<td>8.7 ± 3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Active</td>
<td>88.4 ± 21.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Retinal vein occlusion</td>
<td>227.1 ± 122.7</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*P values are relative to control subjects. Interleukin-8 was measured with enzyme-linked immunosorbent assays in 50 μl vitreous and aqueous fluid samples.*

these patients had increased amounts of IL-8 in VF or AF. Interleukin-8 concentrations in serum did not differ significantly among patients with PDR or retinal vein occlusion and control subjects. We observed no or a few neutrophils in AF and VF samples and found no correlation between IL-8 levels and the number of neutrophils (data not shown).

Expression of Interleukin-8 in the Retinal Glial Cells and Vascular Endothelial Cells of Patients with Active Retinal Neovascularization

The cellular origins of IL-8 in the retinas of patients with neovascularization were investigated by immunohistochemistry. Interleukin-8 expression surrounded the superficial vessels in the retina of patients with PDR (Fig. 2A). Interleukin-8-positive cells also stained for glial fibrillary acidic protein or von Willebrand factor (data not shown), suggesting that they were retinal glial cells and vascular endothelial cells, respectively. In contrast, IL-8 immunoreactivity was not detected in the retinas of control subjects (Fig. 2B). No specific immunoreactivity was detected with rabbit nonimmune IgG as the primary antibody (Fig. 2C).

Effect of Vitreous Fluid on Tubular Morphogenesis of Vascular Endothelial Cells

To investigate whether the increased concentrations of IL-8 in VF from patients with neovascular disorders contributes directly to retinal neovascularization, we used an in vitro model system to assay tubular morphogenesis in bovine aortic endothelial cells. Tubelike structures appeared on type I collagen gels in the presence of 50 ng/ml VEGF and 50 ng/ml basic fibroblast growth factor (Figs. 3C, 3D), but they were minimal in the absence of exogenous growth factors (Fig. 3A), as

![Figure 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933206/) Concentration of immunoreactive interleukin (IL)-8 in vitreous fluid from study participants. Vitreous fluid from patients undergoing intraocular surgery was assayed for IL-8 by enzyme-linked immunosorbent assay. Values of zero or below on the y-axis denote concentrations of less than 5 pg/ml. PDR, proliferative diabetic retinopathy.
FIGURE 2. Immunohistochemical localization of interleukin (IL)-8 in the retinas of a patient with proliferative diabetic retinopathy (A), in a control subject (B), and in negative control of serial section of the same retina stained with rabbit nonimmune IgG (C). (A) Interleukin-8 immunoreactivity was detected in endothelial cells (arrow) and glial cells (arrowhead) surrounding vessels in the retina of the patient with proliferative diabetic retinopathy. (B) No IL-8 expression was apparent in the retina of the control subjects. (C) No immunoreactivity was observed. Scale bar, 1 μm.

Previously described. Exogenous recombinant human IL-8 also induced tube formation in a dose-dependent manner (Figs. 3B, 4A). The extent of tube formation was greater in cells exposed to VF containing more than 20 pg/ml IL-8 than in cells exposed to VF containing less than 20 pg/ml IL-8 (Figs. 3E, 3G, 4B). The extent of tubular morphogenesis induced by VF was similar to that induced by the corresponding concentration of recombinant IL-8. To determine whether VF-induced formation of tubelike structures was attributable to IL-8 or VEGF, VF samples were preincubated with 10 μg/ml neutralizing anti-

FIGURE 3. Development of tubelike structures in bovine aortic endothelial cells. Cells were plated onto a collagen gel. After achieving confluence, vessel-like structures were analyzed 72 hours after incubation in Dulbecco's modified Eagle's medium containing 1% fetal calf serum (A) or after treatment with 1 ng/ml recombinant human interleukin (IL)-8 (B), 50 ng/ml vascular endothelial growth factor (C), 50 ng/ml basic fibroblast growth factor (D), or vitreous fluid (E, F, G). Samples of vitreous fluid containing 132 pg/ml endogenous IL-8 were pretreated for 6 hours with nonimmune IgG (E) or with neutralizing anti-IL-8 (F). Vitreous fluid with an IL-8 concentration of less than 3 pg/ml was pretreated with nonimmune IgG (G). The cells were fixed and stained briefly in modified May–Gruenwald solution to visualize tubelike structures. Scale bar, 20 μm.
IL-8 or 1 μg/ml anti-VEGF for 6 hours before addition to the endothelial cells. Treatment with anti-IL-8 or anti-VEGF significantly inhibited VF-induced tube formation by approximately 45% and 50%, respectively (Figs. 3F, 4B). Further increasing the antibody concentrations did not result in a greater inhibitory effect (data not shown).

Effects of Hypoxia on NF-κB Binding Activity and Interleukin-8 Gene Expression by Bovine Retinal Glial Cells

The mechanism responsible for the increase in IL-8 expression in retinal glial cells of patients with neovascular disorders was investigated with bovine retinal glial cells in vitro. We analyzed IL-8 and VEGF expression in bovine retinal glial cells by semiquantitative reverse transcription-PCR. Serially diluted cDNA amplified by PCR, the intensities of the resultant products were plotted against template amounts, and quantitative ranges in which the reactions proceeded exponentially were determined (data not shown). We chose 400-ng templates for IL-8 mRNA analysis and 100-ng templates for VEGF and β-actin mRNA analysis. Consistent with our clinical observation, the abundance of IL-8 and VEGF mRNA increased after exposure of the glial cells to hypoxia (Fig. 5). Of the various types of VEGF transcripts, those encoding VEGF121 and VEGF165 were detected. Sequence analysis confirmed that the respective PCR products corresponded to IL-8, VEGF121, and VEGF165 mRNA (data not shown). We then investigated the role of NF-κB in hypoxia-induced IL-8 expression in retinal glial cells. Subcellular fractionation and immunoblot analysis revealed that NF-κB was exclusively localized in cytosolic extracts of cells incubated in room air. However, incubation of cells under hypoxic conditions resulted in a time-dependent increase in the amount of NF-κB in nuclear extracts and a corresponding decrease in the amount in the cytosol, suggesting that hypoxia induces the translocation of NF-κB from the cytoplasm to the nucleus (Fig. 6A). Hypoxia also induced activation of NF-κB as measured by binding to oligonucleotides corresponding to the NF-κB consensus sites in the promoter region of the IL-8 gene (Fig. 6B); binding activity was increased 1 hour after the onset of hypoxia and remained increased at 3 and 6 hours.

Effect of Pyrrolidine Dithiocarbamate on the Hypoxia-induced Increases in NF-κB Binding Activity and IL-8 Gene Expression

To determine whether NF-κB activation is required for IL-8 gene expression in response to hypoxia, we investigated the effects of pyrrolidine dithiocarbamate (PDTC), a specific inhibitor of NF-κB activation. Pretreatment of bovine retinal glial cells with 100 μM PDTC for 1 hour prevented the hypoxia-induced activation of NF-κB (Fig. 7). Such pretreatment also abolished the hypoxia-induced increase in the amount of IL-8 mRNA, but not the increase in VEGF mRNA, in these cells (Fig. 5).
FIGURE 5. Effects of hypoxia and pyrrolidine dithiocarbamate (PDTC) on the amount of interleukin (IL-8) and vascular endothelial growth factor (VEGF) mRNA in bovine retinal glial cells. Cells were incubated in Dulbecco’s modified Eagle’s medium containing 1% fetal calf serum for 24 hours, exposed or not exposed to 100 μM PDTC for 1 hour, and subjected or not subjected to hypoxia for 3 hours, as indicated. Cells were lysed and mRNA was subjected to semiquantitative reverse transcription–polymerase chain reaction analysis with primers specific for IL-8 (upper panel), VEGF (middle panel), or β-actin (lower panel). Left-most lanes contain molecular size markers. The 186- and 318-bp VEGF products correspond to VEGF121 and VEGF165, respectively.

DISCUSSION

Various ischemic retinal diseases ultimately result in neovascularization of the retina and iris. Clinical evidence indicates that the proliferative response of the retina is initiated by the ischemia-induced release of angiogenic factors. Interleukin-8 is induced by hypoxia and is a potent angiogenic factor in vitro and in vivo. We have now shown that the IL-8 concentration in VF from patients with active retinal neovascularization was significantly higher than that in patients with quiescent neovascularization or without a neovascular disorder. Furthermore, VF containing a high concentration of endogenous IL-8 induced tubular morphogenesis in bovine aortic endothelial cells grown on a type I collagen gel, an effect that was significantly inhibited to a similar extent by neutralizing anti-IL-8 and anti-VEGF, suggesting that IL-8 contributes to retinal neovascularization.

Karakurum et al. showed that hypoxia increased IL-8 gene expression through activation of NF-κB in human vascular endothelial cells. We have previously shown that activated NF-κB and cytokine-induced neutrophil chemoattractant, a rat homolog of IL-8, are expressed in retinal vascular endothelial cells and glial cells in a rat model of retinal neovascularization, presumably caused by hypoxia. In the present study, we also detected IL-8 expression immunohistochemically in vascular endothelial cells and glial cells in the retinas of patients with neovascularization. Thus, retinal glial cells and endothelial cells are likely sources of IL-8 in the ocular fluid of patients with ischemic retinal diseases. Hypoxia induced NF-κB activation and IL-8 gene expression in bovine retinal glial cells, and both these effects were inhibited by PDTC. Thus, hypoxia-induced expression of IL-8, mediated by activation of NF-κB, in retinal glial cells and vascular endothelial cells probably plays a role in the pathogenesis of retinal neovascularization.

In most patients with detectable IL-8 in VF or AF, the serum concentration of IL-8 was normal. Therefore, even in the presence of breakdown of the blood-retina barrier or vitreous hemorrhage, IL-8 in VF or AF of these patients probably reflects only intraocular production. Moreover, the IL-8 concentrations in VF from patients with active retinal neovascularization were similar to those that induced the formation of tubelike structures by bovine aortic endothelial cells in the present study as well as to those that stimulate the proliferation of human umbilical vein endothelial cells in vitro, suggesting that IL-8...
gene. Where indicated, nonradioactive competitor oligonucleotides were present at 100-fold molar excess.

### Figure 7. Effects of pyrrolidine dithiocarbamate (PDTC) on hypoxia-induced activation of NF-κB in bovine retinal glial cells.

Cells were incubated for 24 hours in Dulbecco's modified Eagle's medium containing 1% fetal calf serum, were treated or not treated with 100 μM PDTC for 1 hour, and were subjected to hypoxia for 0 or 3 hours, as indicated. Nuclear extracts were analyzed by electrophoretic mobility shift assay with 32P-labeled oligonucleotides corresponding to the consensus of hypoxia-induced activation of NF-KB in bovine retinal glial cells. Where indicated, nonradioactive competitor oligonucleotides were present at 100-fold molar excess. Arrowheads indicate the position of the retarded DNA-protein complex.

<table>
<thead>
<tr>
<th>Competitor</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypoxia (h)</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>PDTC (μM)</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

NF-κB

### concentrations in the VF of such patients are sufficient to induce intraretinal neovascularization.

The predominant risk factor for the development of intraretinal neovascularization is the extent and duration of retinal ischemia (ischemic drive). In patients with PDR, retinal ischemia generally begins in the midperipheral retina, and retinopathy progresses with a relatively low ischemic drive. In contrast, the avascular region in patients with CRVO or HBRVO tends to appear rapidly and to spread extensively. In the present study, IL-8 concentrations in VF from patients with retinal vein occlusion were approximately three times those in VF from patients with active PDR. In addition, angiogenic factors diffuse from the posterior to the anterior segment of the eye as a result of a concentration gradient. The frequency of iris neovascularization that can progress to neovascular glaucoma, one of the most devastating complications of ischemic retinal disease, is 60% or 100% in CRVO, compared with 1.9% to 3.8% or 33% to 64% in PDR. In our study, IL-8 concentrations in AF were also significantly (P < 0.001) higher in patients with retinal vein occlusion than in those with PDR. The consistency of the clinical observations in connection with the IL-8 concentrations measured in the present study further implicates IL-8 in ischemia-associated retinal neovascular diseases such as PDR and retinal vein occlusion. The greater the ischemic drive in the retina, presumably the greater the extent of hypoxia experienced by retinal glial cells and the greater the amount of IL-8 produced.

Our immunohistochemical analysis showed that IL-8 was expressed in vascular endothelial cells and in glial cells whose processes surround the vessels in the retina of patients with retinal neovascularization. Such cells also produce VEGF in response to hypoxia and participate in retinal neovascularization. The production of IL-8 and VEGF by glial cells is consistent with the role of these cells as regulators of blood vessel structure and function. These observations suggest that IL-8 and VEGF released by glial cells and vascular endothelial cells contribute to retinal neovascularization in a paracrine or autocrine manner.

Our in vitro data suggested that hypoxia induced IL-8 production in retinal glial cells through activation of NF-κB. The mechanism by which hypoxia induces NF-κB activation is unknown. Schmidtje et al. have proposed that either incomplete oxidative metabolism or the presence of chemical oxidants in the cellular milieu may underlie the effect of hypoxia on NF-κB. Activation of NF-κB by hypoxia appears to occur by an antioxidant-sensitive pathway. In the present study, PDTC, an antioxidant that acts as a specific inhibitor of NF-κB activation, prevented the induction of IL-8 gene expression by hypoxia in bovine retinal glial cells.

Four types of alternatively spliced mRNA can be generated from the single VEGF gene, yielding protein products composed of 121, 165, 189, and 206 amino acids and designated VEGF121, VEGF165, VEGF189, and VEGF206, respectively. VEGF121 and VEGF165 are the predominant isoforms induced by hypoxia in endothelial cells. Hypoxia also increased the abundance of transcripts encoding these two secretory isoforms in bovine retinal glial cells, suggesting that the secreted proteins play an important role in retinal neovascularization. In contrast to IL-8, hypoxia-induced expression of VEGF was not inhibited by PDTC, suggesting the existence of different intracellular signaling pathways in hypoxia-induced retinal neovascularization. Hypoxia-inducible factor 1 has been implicated in the activation of VEGF gene transcription in hypoxic cells.

NF-κB is a pleiotropic regulator of many genes, including those encoding angiogenic factors or adhesion molecules. The promoter region of the gene encoding the VEGF receptor, Flk-1, also contains several potential binding sites for NF-κB. Therefore, in addition to IL-8 and VEGF, NF-κB may provide a target for therapeutic intervention in retinal neovascularization. Pyrrolidine dithiocarbamate has been shown to inhibit NF-κB activation and the angiogenic response in vitro. Moreover, NF-κB antisense oligonucleotides also inhibited angiogenesis in an in vitro model.

In conclusion, we showed that IL-8, through activation of NF-κB, participates in the pathogenesis of retinal neovascularization. It is possible that the use of the antibody against IL-8 and VEGF, the use of NF-κB antisense oligonucleotides, or the use of PDTC could play an important role in the therapy of neovascular diseases such as diabetic retinopathy and retinal vein occlusion.

### Acknowledgments

The authors thank Tetsushi Ejima, Yasuaki Hata, Takao Nakamura, Yuko Nishioka, Toyokazu Okada, Yukiko Sadamoto, and Shigeru Sugai for their assistance in the acquisition of patient specimens and the preparation of the manuscript.
References


ANNOUNCEMENT

Request for Proposals

The Glaucoma Research Foundation (GRF) is a national nonprofit organization dedicated to protecting the sight of persons with glaucoma, with the ultimate goal of finding a cure. We are soliciting grant requests for pilot projects. To be considered, all projects must meet one or more of the five GRF strategic research goals: (1) protect and restore optic nerve, (2) accurately monitor glaucoma’s progression, (3) find genes responsible for glaucomas, (4) understand intraocular pressure system and develop better treatments, and (5) determine risk factors for glaucoma damage.

For a copy of our grant guidelines and a grant request form, call (415) 986-3162, or write to the Glaucoma Research Foundation, 490 Post Street, Ste. 830, San Francisco, CA 94102. You can also complete a grant request form on our website at http://www.glaucoma.org/research.html. Request forms must be received and approved by Dec. 15, 1998.