Role of MCP-1 and MIP-1α in retinal neovascularization during postischemic inflammation in a mouse model of retinal neovascularization

Shigeo Yoshida,[*] Ayako Yoshida,[*] Tatsuro Ishibashi,[*] Susan G. Elner,[†] and Victor M. Elner,[†]

[*]*Department of Ophthalmology, Kyushu University Graduate School of Medicine, Fukuoka, Japan; and* [†]*Department of Ophthalmology & Visual Sciences, W.K. Kellogg Eye Center, University of Michigan, Ann Arbor*

Abstract: Macrophages are important participants in neovascularization. This study was designed to examine the role of the monocyte/macrophage chemotactic proteins, monocyte chemotactic protein-1 (MCP-1), and macrophage inflammatory protein-1α (MIP-1α) in a mouse model of oxygen-induced ischemic retinopathy and to determine whether the morphology and distribution of macrophages/microglia are concomitantly altered. The MCP-1, MIP-1α mRNA levels increased at 3 h after ischemia. MCP-1, MIP-1α, and vascular endothelial growth factor protein levels were also increased markedly and were maximal on days 1, 0.5, and 1, respectively, after ischemia. In situ hybridization showed that MCP-1 and MIP-1α were localized in the hypoxic inner retina. Immunostaining demonstrated that the macrophages/microglia in the retina had morphological changes with enlarged processes, and some were closely associated with neovascular tufts at postnatal day 17. Coadministration of the neutralizing antibodies against MCP-1 and MIP-1α inhibited retinal neovascularization by 30%. Our data suggest that MCP-1 and MIP-1α are involved in the induction of retinal neovascularization and play a role in the inflammation induced by the ischemic retinopathy, possibly by modulating or attracting macrophages/microglia. *J. Leukoc. Biol.* 73: 137–144; 2003.

Key Words: *cytokines · macrophages · retinal ischemia · angiogenesis*

INTRODUCTION

Intraocular neovascularization is a major cause of decreased vision in patients with diseases such as proliferative diabetic retinopathy (PDR), retinal vein occlusion, and retinopathy of prematurity [1]. Although these diseases differ in many aspects, it is generally believed that the tissue ischemia common to all of them initiates a series of events that leads to the compensatory angiogenesis. Cellular inflammation is initiated at the blood-microvascular endothelial-cell interface, and leukocytic infiltration has been observed after focal ischemia in central nervous system (CNS) lesions [2].

Among the inflammatory cells, macrophages carry out a wide variety of biologic functions including participation in neovascularization [3]. Macrophages are important among the key angiogenic effector cells that produce a number of growth stimulators and inhibitors, proteolytic enzymes, and cytokines capable of modulating new vessel formation. Polverini et al. [4] have demonstrated that conditioned media derived from activated macrophages can induce angiogenesis. In addition, tumor-associated macrophage infiltration is correlated with tumor angiogenesis in individuals with invasive breast cancer [5]. Macrophage infiltration also occurs in a number of retinal and choroidal angiogenic diseases, such as PDR and age-related macular degeneration [6]. However, it is not completely clear how macrophages/microglia are involved in retinal angiogenesis.

Chemokines, a family of structurally related cytokines involved in the activation and directed migration of immune cells, may be pathophysiologically important mediators of inflammation [7]. Two well-studied CC chemokines are monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α). MCP-1 mediates the recruitment of monocytes [8] and can also induce corneal neovascularization [9]. A strong induction of MCP-1 expression has been reported following diverse CNS insults, including ischemic injury models [10, 11]. MCP-1 is also increased in the vitreous of patients with PDR [12].

MIP-1α, similar to MCP-1, has been shown to mediate the recruitment of monocytes in several inflammatory diseases [13]. A recent study has documented the importance of this cytokine in mediating leukocytic infiltration and neovascularization using a dermal wound pouch model [14]. We have also demonstrated that MIP-1α is induced during inflammatory neovascularization in the mouse cornea [15].

The angiogenic potential of cells and substances has been determined in several angiogenic model systems, such as the in vivo model of ischemia-induced retinal neovascularization [16,

Correspondence: Shigeo Yoshida, M.D., Ph.D., Department of Ophthalmology, Kyushu University Graduate School of Medicine, 3-1-1 Maidashi, Higashi-ku, Fukuoka, 812-8582, Japan. E-mail: usyosi@yahoo.com

Received March 10, 2002; revised July 28, 2002; accepted July 29, 2002; doi: 10.1189/jlb.0302117.

17]. Using this model, we previously demonstrated that the transcription nuclear factor-κB plays a significant role in regulating the expression of many genes, including chemokines [18, 19]. However, it still remains unclear what roles the chemotactic mediators play in ischemia-associated retinal neovascularization.

Thus, we have investigated whether the monocyte/macrophage-attracting proteins MCP-1 and MIP-1α can be induced in a mouse model of oxygen-induced ischemic retinopathy and whether the morphology and distribution of macrophages/microglia are concomitantly altered. Additionally, we examined the possible involvement of these chemokines in retinal neovascularization.

MATERIALS AND METHODS

Murine model of oxygen-induced ischemic retinopathy

All experimental procedures concerning animals were performed according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

A reproducible model of ischemia-induced retinal neovascularization has been described in detail previously [19]. Briefly, litters of 7-day-old [postnatal day 7 (P7)] C57BL/6 pups with their mothers were exposed to 75 ± 2% oxygen for 5 days and then returned to room air at age P12. The intraocular injections were performed at P12 as described below. Pups of the same age that had been kept in room air were used as controls. Mice were killed by an overdose of intraperitoneal (i.p.) sodium pentobarbital.

Northern blot analysis

The cDNA for mouse MCP-1, MIP-1α, vascular endothelial growth factor (VEGF), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was constructed by reverse transcriptase-polymerase chain reaction (RT-PCR). The primers were: for MCP-1, 5′-ATCCCAATGAGTAGGCTGGAGAGC-3′ and 5′-CAGAAGTGCTTGAGGTGGTTGTG-3′; for MIP-1α, 5′-ACCTGCTCAACATCATGAAGG-3′ and 5′-AGATGGAGCTATGCAGGTGG-3′; for VEGF, 5′-TGTAACGATGAAGCCCTGGAG-3′ and 5′-TCACCGCCTTGGCTTGTCACA-3′; and for GAPDH, 5′-CCCACTAACATCAAATGGGG-3′ and 5′-ATCCACAGTCTTCTGGGTGG-3′. These sequences were derived from published sequences [20–23].

The PCR conditions were 10 min at 94°C, followed by 35 cycles at 72°C for 20 s, 94°C for 30 s, and 60°C for 30 s, with a final extension step at 72°C for 5 min. The resulting PCR fragments were confirmed by direct dideoxynucleotide chain termination [24].

Retinas were removed at selected periods after ischemia, and total RNA was extracted as described previously [25]. Eight retinas were pooled for each time point, and experiments were repeated at least three times. The RNAs were fractionated on a 1% agarose gel containing 2.2 M formaldehyde, transferred onto a nylon membrane (Hybond N+, Amersham, Buckinghamshire, UK), and then cross-linked using UV radiation at 0.25 J/cm^2 with FLUO-LINK (Viler Lourmat, Marne-La-Vallée, Cedex, France). The membrane was hybridized to ^{32}P-labeled DNA probes in a Hybrisol (Oncor, Inc., Gaithersburg, MD) at 42°C for 24 h, washed twice at room temperature in 2x saline sodium citrate (SSC; 1xSSC=0.15 M NaCl-0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate (SDS), once in 1x SSC and 0.1% SDS, and finally in 0.2x SSC and 0.1% SDS. The mRNA levels were quantified by densitometry using a Scion Image Beta 3b (Scion Corp., Frederick, MD), and signal intensities of various transcripts were normalized by GAPDH mRNA level.

Real-time quantitative (q)RT-PCR

Real-time qRT-PCR was performed on the purified RNAs from retinas from selected times. Total RNA was extracted from dissected mouse retinas, treated with DNase, diluted by 20-fold in water, and subjected to reverse transcription using standard procedures. PCRs were performed with the Roche LightCycler system (Roche Molecular Biochemicals, Mannheim, Germany) with a SYBR-Green Master kit (Roche Molecular Biochemicals). The primer pairs for MCP-1 and MIP-1α were the same as used in the Northern blot analysis described above.

The optimal cycle programs were determined for each gene in preliminary PCR runs to obtain a single, specific PCR product as verified by melting curve analysis followed by gel electrophoresis. The cDNA quantities were calculated with the LightCycler analysis software as described previously [26, 27]. MCP-1 and MIP-1α were normalized to hypoxanthine guanine phosphoribosyl transferase (HPRT; 5′-CTACGAATCTCCGACCACCACTAC-3′ and 5′-GGCTTATCATCTTTCAACACGCAG -3′) cDNA fragments.

Enzyme-linked immunosorbent assay (ELISA)

Cytokine levels were measured using ELISA kits for MCP-1 (Biosource International, Camarillo, CA), MIP-1α, and VEGF (R&D Systems, Minneapolis, MN), as described previously [28].

Retinas were removed from mice at 0, 0.5, 1, 2, 3, and 5 days after ischemia. Each test sample was made up of four whole retinas. The retinas were individually immersed in 500 μl lysis buffer containing 20 mM imidazole HCl, 10 mM KCl, 1 mM $MgCl_2$, 10 mM EGTA, 1% Triton, 10 mM NaF, 1 mM Na molybdate, 1 mM EDTA (pH 6.8), supplemented with a protease inhibitor cocktail (Roche Biomedical Systems, Indianapolis, IN), and were stored at -80°C until use. When used, the samples were thawed, homogenized in Polytron homogenizer (Kinematica AG, Lucerne, Switzerland), sonicated for 30 s, and clarified by centrifuging at 150 g for 10 min. The clarified retinal lysates were then assayed using ELISA.

The total protein was determined with a commercial assay (Coomassie plus protein assay reagent kit; Pierce, Rockford, IL) The sensitivities of the assays for MCP-1, MIP-1α, and VEGF were 9.0, 1.5, and 3.0 pg/ml, respectively.

Generation of riboprobes

The MCP-1 and MIP-1α cDNAs used for Northern blot analysis were subcloned into TOPO-2 (Invitrogen, San Diego, CA) according to the manufacturer's protocol. Their nucleotides were sequenced, and the inserts were identified as murine MCP-1 and MIP-1α. Clones with both orientations were selected so that the same RNA polymerase (SP6) could be used to generate antisense and sense riboprobes. The templates were linearized with EcoRV, and in vitro transcription was performed using digoxigenin (DIG)-labeled uridine triphosphate (Roche Biomedical Systems) according to the manufacturer's protocol.

In situ hybridization

Mice retinas obtained 12 h after ischemia were fixed in 4% paraformaldehyde for 2 h and embedded in paraffin. In situ hybridization was performed as described in detail previously [15]. Briefly, retinas were rehydrated and treated with proteinase K, followed by refixing in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min. The retinas were then acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl buffer (pH 8.0) for 10 min, dehydrated, and dried. Hybridization was performed with fresh hybridization buffer [600 mM NaCl, 10 mM Tris-HCl (pH 7.6), 5 mM EDTA (pH 8.0), 1xDenhardt's solution, 50% formamide, 17 mg/ml yeast tRNA, and 10% weight/vol dextran] plus a sense or antisense DIG-labeled RNA probe for 12–16 h at 60°C. After hybridization, the retinas were washed to remove nonspecifically bound RNA probe, and immunologic detection was performed by anti-DIG Fab fragments conjugated to alkaline phosphatase as described in the system protocol (Roche Biomedical Systems).

Immunohistochemistry of the retina

The eyes were enucleated, and the retinas were removed. They were fixed in absolute ethanol at 4°C for 10 min, rehydrated in PBS for 15 min, and blocked in normal serum for 30 min. The retinas were then incubated for 90 min at 37°C with rat monoclonal anti-F4/80 antibody (1:10 dilution; Biosource International). After three washes with PBS, specimens were incubated with fluorescein-conjugated goat anti-rat immunoglobulin G (IgG; Cappel, Durham, NC) at room temperature for 3 h. Following three washes with PBS, specimens were flat-mounted on microscope slides and examined under a Leica fluorescence microscope (Wetzlar, Germany).

Other eyes were fixed with 4% paraformaldehyde in PBS and embedded in paraffin. Thin sections (3 μm) were cut, and after removal of the paraffin, they were rehydrated, blocked, and incubated for 1 h at room temperature with anti-F4/80 antibody. Bound antibody was detected by a conventional avidin-biotin-peroxidase protocol with 3-amino-9-ethylcarbazole as the substrate. For negative controls, rat nonimmune IgG was used as the primary antibody.

Intravitreal injections

Mice were deeply anesthetized by i.p. injections of sodium pentobarbital. The lid fissure was opened, and the eyes were proptosed. Intravitreal injections were performed on P12 and P14 by delivering 0.5 μl (100 ng) anti-MCP-1 antibody and anti-MIP-1α antibody (R & D Systems) to the left eye and control preimmune antibody to the right eye with a 32-gauge needle on a Hamilton syringe, 200 μm posterior to the limbus. The eyes were then repositioned, and the lids were approximated over the cornea. For simultaneous administration of the anti-MCP and anti-MIP-1α antibody, premix of both antibodies was generated, and then 0.5 μl (100 ng each) was injected daily. Repeated injections were performed through a previously noninjected area.

Quantification of neovascularization

The eyes of P17 mice were enucleated, fixed with 4% paraformaldehyde in PBS, and embedded in paraffin. Serial 3-μm-thick axial sections of the retina were obtained starting at the optic nerve head. After staining with hematoxylin and eosin, 10 intact sections of equal length, each 30 μm apart, were evaluated for a span of 300 μm. All retinal vascular cell nuclei anterior to the internal-limiting membrane were counted in each section under a fully masked protocol. Averaging of all 10 counted sections yielded the mean number of neovascular cell nuclei per 3-μm section per eye. No vascular cell nuclei anterior to the internal-limiting membrane were observed in normoxic control animals.

Statistical analysis

Data were analyzed by one-way ANOVA followed by Bonferroni *t*-test. Results are reported as the means ± SEM.

RESULTS

Induction of MCP-1 and MIP-1α in the retinas of mice with oxygen-induced ischemic retinopathy

MCP-1 and MIP-1α have recently been reported to play a critical role in the pathogenesis of postischemic inflammation in several ischemic injury models [2]. Therefore, we determined the mRNA levels of these inflammatory cytokines using Northern blot analysis in mice with ischemic retinopathy (**Fig. 1A**). As it has already been reported that in the mouse model of ischemic retinopathy, hyperoxia led to a significant down-regulation of VEGF, and the subsequent hypoxia induced an up-regulation of VEGF level [29–31], the level of VEGF mRNA was determined as a positive control.

The expression of the mRNAs of MCP-1 and MIP-1α was very low or undetectable in the retinas of the normal controls and in P12 mice killed just 5 days after hyperoxia. A dramatic increase in MCP-1 mRNA expression was observed 12 h after the onset of hypoxia. The profile of MIP-1α mRNA expression was similar to that of MCP-1 mRNA, except that a slight increase of MIP-1α mRNA was detected in the retinas of mice 5 days (P12) after hyperoxia.

In contrast to MCP-1 and MIP-1α, a steady level of VEGF mRNA expression was found in the retinas of control, normal mice. Hyperoxia resulted in a twofold decrease in the level of VEGF mRNA, and the subsequent hypoxia led to a significant up-regulation of VEGF expression by approximately fourfold compared with that in the control retinas.

We also performed quantitative RT-PCR to examine the level of chemokine gene expression at an earlier time point (Fig. 1B). MCP-1 and MIP-1α mRNAs were slightly increased in the retinas of mice 5 days (P12) after hyperoxia, and those

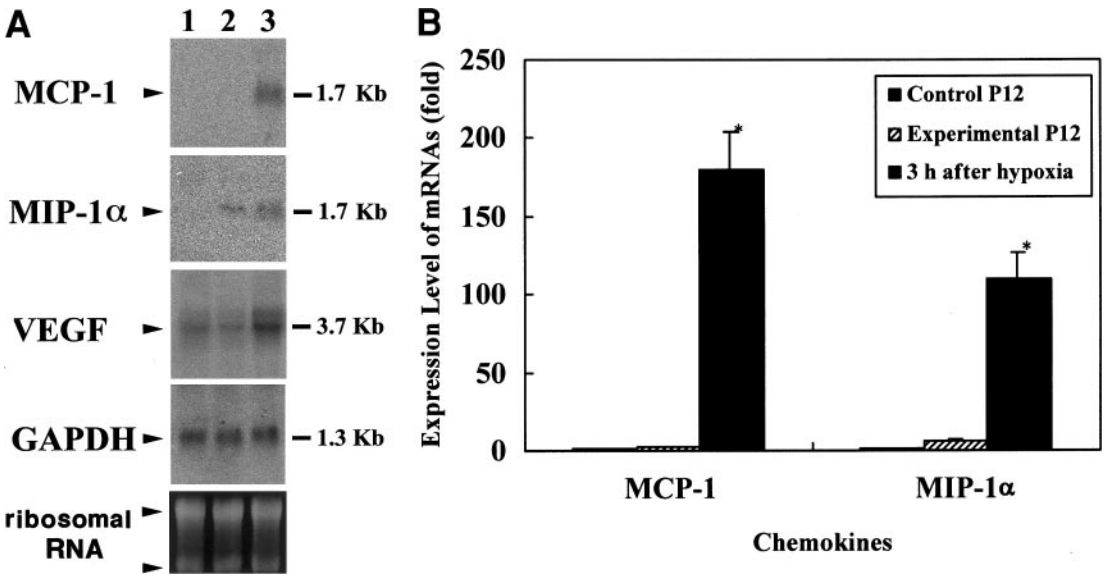

Fig. 1. (A) Northern blot determination of mRNA expression of MCP-1, MIP-1α, and VEGF in mice with ischemic retinopathy. Representative blots of three independent experiments are shown. Lane 1, Control retina (P12); lane 2, retina after 5 days exposure to hyperoxia (P12); lane 3, retina 12 h after ischemia (P12.5). For control, the same blot was stripped and reprobed with GAPDH, and 18 S and 28 S ribosomal RNA were used for equal loading of RNA. Each lane contains 10 μg total RNA. (B) Real-time, quantitative RT-PCR determining the mRNA levels of MCP-1 and MIP-1α at control P12, experimental P12, and 3 h after hypoxia. All data were standardized by HPRT and divided by control value at P12. The bars show the mean ± SEM of four independent experiments per time point. *, Statistically significant differences ($P<0.01$) compared with normal subjects.

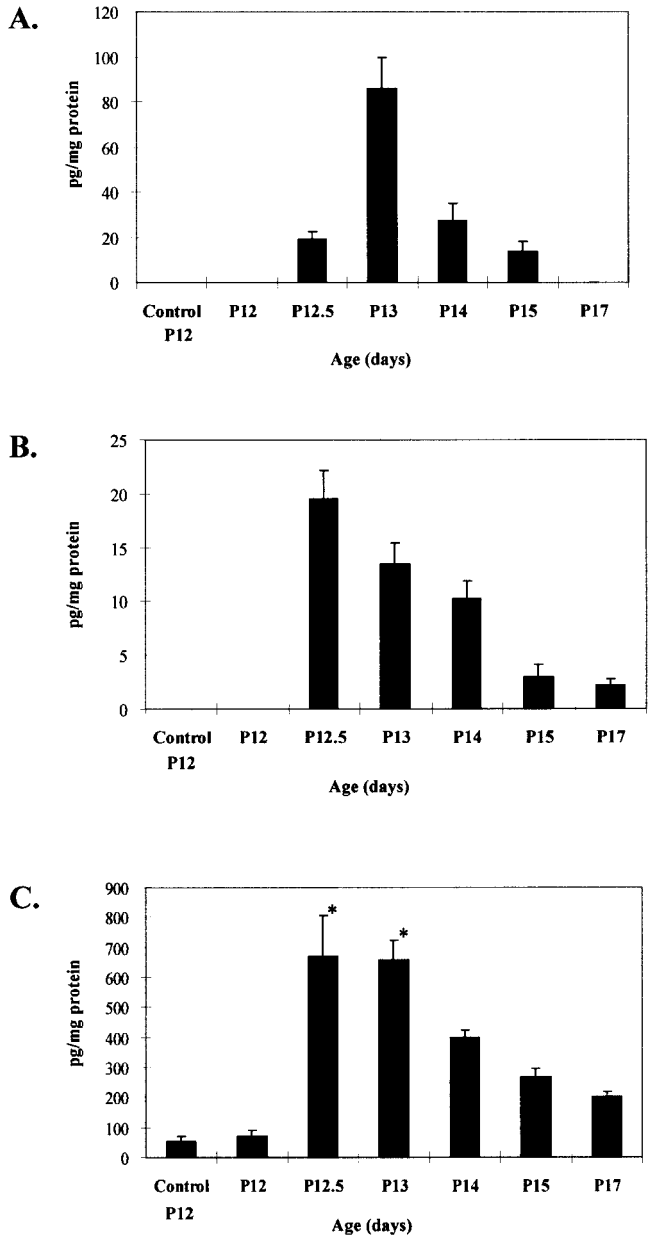

Fig. 2. Kinetics of changes in protein levels of MCP-1 (A), MIP-1α (B), and VEGF (C) in mice with ischemic retinopathy. Four retinas were measured at the indicated times. Retinal lysates were prepared and were individually assayed by ELISA. The bars show the mean ± SEM of four independent experiments per time point. *, Statistically significant differences ($P<0.01$) compared with normal subjects.

levels were dramatically enhanced by 180- to 100-fold, respectively, as early as 3 h after ischemia.

ELISA was performed to determine the protein levels of MCP-1, MIP-1α, and VEGF. At 12 h after ischemia, the MCP-1 protein level was markedly increased, peaked at 1 day after ischemia (P13; 86.2 pg/mg total protein), and then decreased rapidly to return to undetectable levels by P17 (**Fig. 2A**). The MIP-1α protein levels were also markedly increased at 12 h after ischemia (P12.5; 19.5 pg/mg total protein) and then decreased more slowly than MCP-1 (Fig. 2B). The VEGF protein levels similarly increased markedly after ischemia (670.8 pg/mg total protein), reached a plateau between 12 h and 1 day after ischemia, and showed a relatively gradual decline compared with MCP-1 and MIP-1α (Fig. 2C).

The protein levels of MCP-1 and MIP-1α in the control retinas and retinas of mice killed just after 5 days of hyperoxia were below the level of detection (Fig. 2, A and B). Thus, we could not apply any statistics to evaluate the significance of the changes. In contrast, 50–70 pg/mg total protein of VEGF was observed in the normal and hyperoxia-exposed retina (Fig. 2C).

Localization of MCP-1 and MIP-1α in the retinas by in situ hybridization

To determine the cellular source of MCP-1 and MIP-1α, serial sections of retinal tissues obtained ~12 h after ischemia were subjected to in situ hybridization with antisense or sense riboprobes. In ischemic P12.5 retinas, MCP-1 was present predominately in the hypoxic inner retina and most strongly, in the retinal ganglion cell layer and in the cells located in the inner nuclear layer (**Fig. 3B**), and cells located at both borders of the inner nuclear layer showed distinct signals (Fig. 3B). In situ hybridization with an antisense probe specific for MIP-1α showed a staining pattern similar to that seen with MCP-1 (Fig. 3E). Only a weak signal was seen in the negative controls hybridized with sense probes (Fig. 3, C and F). The expression of MCP-1 and MIP-1α with antisense riboprobes was very weak, if any, in control P12.5 normal retinas (Fig. 3, A and D).

Morphology and location of F4/80-reactive macrophage/microglia in normal and ischemic retinas

These findings strongly suggest that MCP-1 and MIP-1α are likely to be involved in the pathogenesis of ischemia-induced retinopathy. We examined the morphology of macrophages/

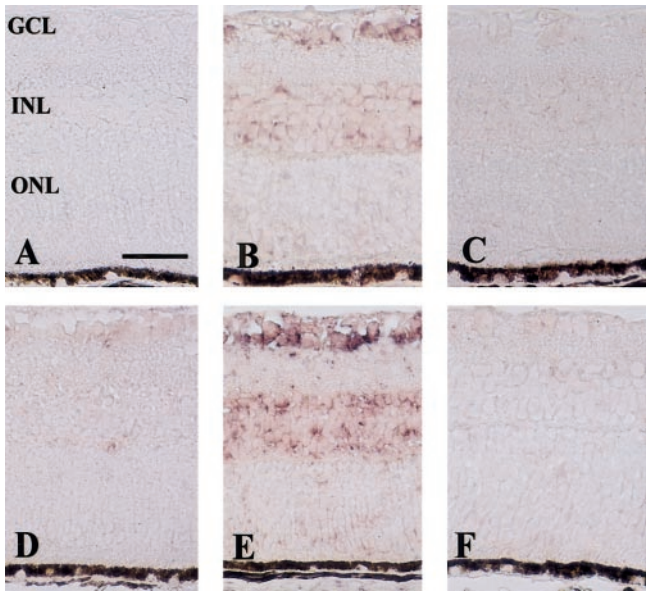

Fig. 3. In situ hybridization for MCP-1 and MIP-1α in the retinas of mice with ischemic retinopathy. Hybridization was performed with antisense (A, B, D, E) or sense (C, F) probes specific for MCP-1 (A–C) and MIP-1α (D–F). A, D, Control normal retina (P12); B, C, E, and F, retina 12 h after ischemia (P12.5). GCL, Ganglion cell layer; INL, inner-nuclear layer; ONL, outer-nuclear layer. Original bar = 50 μm.

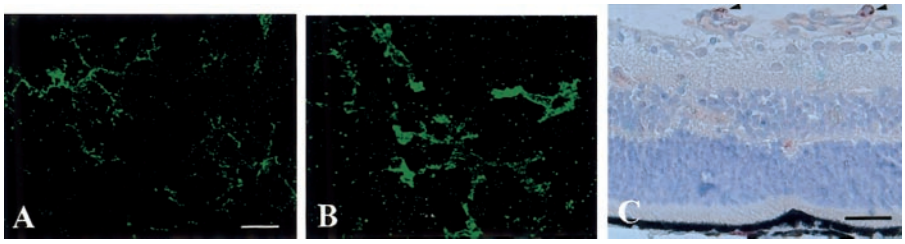

Fig. 4. Retinal macrophage/microglia visualized using F4/80 immunohistochemistry in mice with ischemic retinopathy. (A, B) Microglial changes in the whole mount retina during retinal ischemia. In control P16 mice, retinal microglia display long, fine processes typical of resting microglia (A). In P16 mice retina, the processes of microglia are thicker, and the distended processes are more evident (B). (C) Retinal sections showing macrophages/microglia associated with neovascular sprouts (arrow) in experimental P17 animals. Original bars = 10 μm (A) and 50 μm (C).

microglia immunohistochemically to determine if they underwent specific morphological changes following ischemic retinopathy. Whole mounts of experimental and control mice retinas were prepared, fixed in 4% formaldehyde, and stained with F4/80 antibody. The whole mounts of the control retina showed extensively ramified macrophages/microglia with slender cell bodies (**Fig. 4A**). In contrast, microglial cells changed from the resting, ramified morphology to a more distended shape from approximately day 4 after ischemia (P16; Fig. 4B). To localize these F4/80+ cells, paraffin-embedded sections were also prepared from P17 eyes. Cells positive to F4/80 were found immediately adjacent to the neovascular tufts (Fig. 4C). Nonimmune serum controls showed no staining (data not shown).

Inhibition of retinal neovascularization by anti-MCP-1 and anti-MIP-1α antibodies

To determine whether MCP-1 and MIP-1α were directly involved in retinal neovascularization in this model, we next examined whether neutralization of those chemokines would attenuate the angiogenic response. The neovascularization was assessed histologically by counting the number of endothelial cell nuclei vitreal to the inner-limiting membrane. The retinas of the control, antibody-treated eyes from the hypoxic mice contained multiple neovascular tufts extending into the vitreous, whereas retinas from control eyes of normoxic mice treated with or without control antibody did not contain endothelial cells at this location (**Fig. 5**).

Intravitreous injections of anti-MCP-1 or anti-MIP-1α antibodies alone significantly affected the ischemia-induced retinal neovascularization, although the degree of neovascularization following each antibody was lower than in the control mice (**Fig. 6**). However, when injected together, they inhibited the neovascularization by ~30% (Fig. 6).

DISCUSSION

Our results demonstrated a dramatic increase of MCP-1 and MIP-1α mRNA and proteins by Northern blot, qRT-PCR, and ELISA, respectively, in response to ischemia in retinas of mice (Figs. 1 and 2). In ischemic retinopathies, the inner retina, which is supplied by retinal vessels, is hypoxic, whereas the outer retina, which is supplied by the choroidal vessels, is not [30]. In situ hybridization for MCP-1 and MIP-1α showed a prominent increase of positive cells located in the inner retina 12 h after the ischemia (P12.5) in comparison to the lower level of staining in the nonischemic retinas of control mice (Fig. 3). Together, these results clearly demonstrated that in vivo ischemia enhances the expression of those leukocytic chemoattractants, and this ischemic, proinflammatory activation may constitute a key event in initiating postischemic inflammation. Additionally, simultaneous injection of anti-MCP-1 and MIP-1α antibodies depressed the inflammatory neovascularization (Figs. 5 and 6), indicating these molecules are indeed involved in ischemia-induced retinal neovascularization. Re-

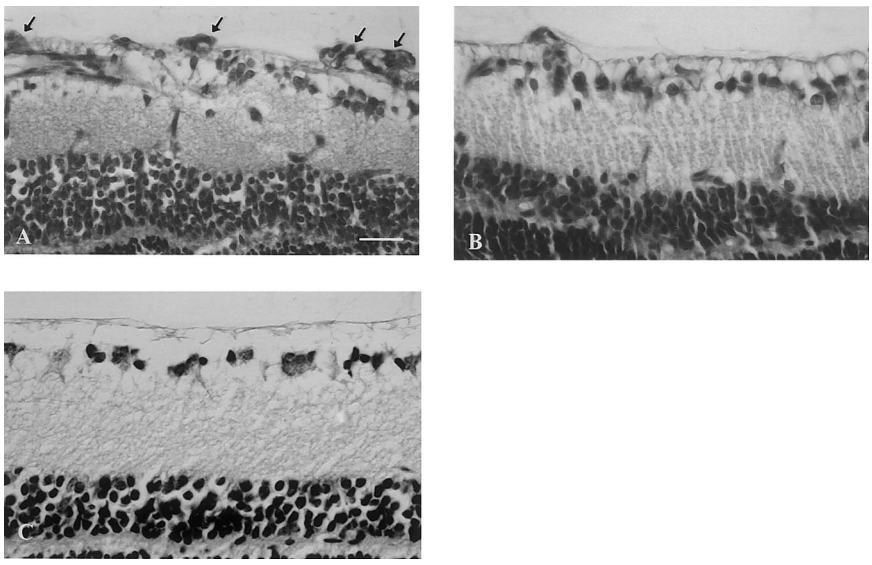

Fig. 5. Retinal sagittal sections: the effect of antibodies against MCP-1 and MIP-1α on oxygen-induced retinopathy. Animals were subjected to 7 days of normoxia, followed by 5 days of hyperoxia, and then by 5 days of normoxia. (A) Retinas of animals that received only control antibody. Extensive preretinal neovascular loops are apparent (arrows). (B) Retinas of animals that received antibodies against MCP-1 and MIP-1α. A significant reduction in preretinal neovascular loops is apparent compared with control antibody-treated eye. (C) Retina of normal control animals exposed only to room air. No preretinal neovascularization is seen. Original bar = 50 μm.

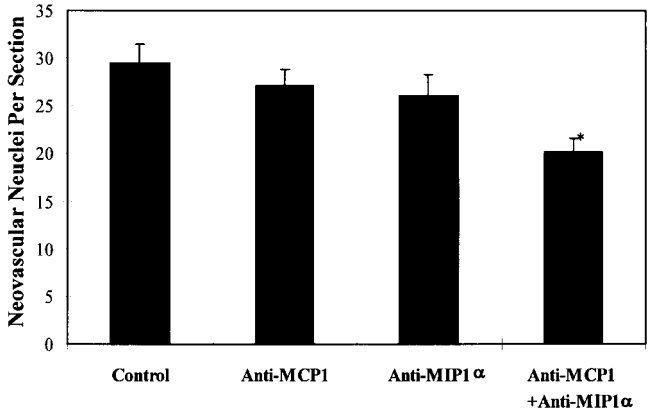

Fig. 6. Results of quantification of cell nuclei occurring on the vitreous side of the internal-limiting membrane in sections from whole eyes of hyperoxia-treated mouse pups. Antibodies to MCP-1 and MIP-1α alone did not significantly inhibit ischemia-induced retinal neovascularization. In combination, these antibodies reduced significantly the degree of oxygen-induced neovascularization. *, $P < 0.05$ versus control antibody. Data are shown as mean ± SEM.

cently, a direct effect of MCP-1 on angiogenesis, which was not related to its monocyte/macrophage recruitment, has been suggested [32, 33]. This indicates that MCP-1 may have two independent effects in this model: enhancement of the chemotaxis of monocytic cells and angiogeneic activity.

The inner retinal layer where ganglion cells and astrocytes are located was the most prominent cellular site of MCP-1 and MIP-1α gene expression in the hypoxic inner retina (Fig. 3, B and D). This suggests that MCP-1 and MIP-1α expressed in this region attract resident macrophages/microglia, vitreous macrophages, and/or circulating monocytes through the blood: retinal barrier toward the superficial layer of the retina where neovascularization occurs. In support of this, macrophages were observed in the vicinity of neovascular tufts at P17 (Fig. 4C) [34]. This observation further demonstrated the involvement of MCP-1 and MIP-1α in the activation and/or recruitment of macrophages/microglia by the ischemic retina. However, the extent to which MCP-1 and MIP-1α can coordinate and/or differentially regulate the activation and recruitment of macrophages/microglia still remains unknown. Further studies are necessary to determine how the number and pattern of distribution of the macrophages/microglia are altered by these chemokines in the retinas of mice with ischemic retinopathy.

Only activated macrophages/microglia and granulocytes and stimulated resident macrophages/microglia and granulocytes induced angiogenesis after their implantation into the corneas in several species [35]. Among the environmental stimuli, macrophage/microglia gene expression following ischemia is becoming increasingly well-characterized to have angiogenic potential [36]. Macrophages/microglia in the ischemic retina exhibited thicker and more distended processes compared with those in normal, control retinas (Fig. 4B), and such "hypoxia-activated" macrophages have the potential to produce an array of angiogenic cytokines and growth factors such as tumor necrosis factor-α (TNF-α) and VEGF, which can contribute to the progression of neovascularization. TNF-α is an angiogenic molecule produced by hypoxic macrophages [37, 38] and is a likely candidate for angiogenic regulation [39]. It has been reported that TNF-α level is up-regulated in patients with PDR [40, 41], and we have observed that the molecule is also expressed in the macrophages/microglia in this model (S. Yoshida and A. Yoshida, unpublished observation). TNF-α released by activated macrophages may trigger retinal neovascularization through induction of interleukin-8, VEGF, or basic fibroblast growth factor in an autocrine or paracrine manner [25, 42].

Another chemotactic factor of monocytes assayed in this study was VEGF [43], which is a major mediator of retinal ischemia-associated neovascularization [44]. Similar to MIP-1α, the expression of VEGF increased dramatically as early as 12 h after ischemia and declined relatively gradually toward the prehypoxic level by P17 (Fig. 2C). VEGF might thus have a dual effect on neovascularization in this model, enhancement of the chemotaxis of monocytic cells, and angiogenesis activity.

A steady level of VEGF mRNA and protein was expressed in the normal retina (Figs. 1 and 2). This is consistent with the fact that VEGF is constitutively expressed in the adult retina and is consistent with the role proposed for it in normal retinal homeostasis and/or function [45, 46]. It has also been reported that VEGF receptors are found outside the vasculature in mouse neural retina [47], and abnormal expression of VEGF may constitute a previously unknown risk factor for motor-neuron degeneration [48]. These observations indicate that manipulation of the VEGF pathway to inhibit pathologic neovascularization would result in unexpected disturbances of normal homeostasis in the retina and thus should be approached carefully [47]. In contrast, it is important to mention that MCP-1 and MIP-1α are supposed to be nonfunctional in normal retinas, in keeping with the very low levels of MCP-1 and MIP-1α in the normal control retinas (Figs. 1 and 2). This raises the possibility that these chemokines might be an attractive, therapeutic target to regulate "disease-specific" pathways in ischemia-induced retinopathy.

To the best of our knowledge, our study provides the first evidence for the involvement of MCP-1 and MIP-1α and the potential role of macrophage/microglia in the mouse model of ischemic retinopathy. The results suggest that the postischemic inflammation contributes to the pathogenesis of ischemia-induced retinal neovascularization, possibly by modulating or attracting inflammatory cells to the ischemic area. Thus, modulating the expression of MCP-1 and MIP-1α or the cognate receptors and downstream signaling steps that activate quiescent macrophages/microglia as well as inhibiting microglial secretion of angiogenic factors by ischemia could be a potential, novel, therapeutic strategy for inhibiting ischemia-associated retinal neovascularization.

ACKNOWLEDGMENTS

This work was supported in part by grants from Sumitomo Life Social Welfare Services Foundation (S. Y.), Japan National Society for the Prevention of Blindness (A. Y.), Japan Eye Bank Association (A. Y.), and NIH grants EY09441 (V. M. E.) and EY7003.

REFERENCES

1. Yoshida, A., Yoshida, S., Ishibashi, T., Inomata, H. (1999) Intraocular neovascularization. *Histol. Histopathol.* **14,** 1287–1294.
2. Kim, J. S. (1996) Cytokines and adhesion molecules in stroke and related diseases. *J. Neurol. Sci.* **137,** 69–78.
3. Sunderkotter, C., Steinbrink, K., Goebeler, M., Bhardwaj, R., Sorg, C. (1994) Macrophages and angiogenesis. *J. Leukoc. Biol.* **55,** 410–422.
4. Polverini, P. J., Cotran, P. S., Gimbrone Jr., M. A., Unanue, E. R. (1977) Activated macrophages induce vascular proliferation. *Nature* **269,** 804–806.
5. Lewis, J. S., Landers, R. J., Underwood, J. C., Harris, A. L., Lewis, C. E. (2000) Expression of vascular endothelial growth factor by macrophages is up-regulated in poorly vascularized areas of breast carcinomas. *J. Pathol.* **192,** 150–158.
6. Elner, S. G., Strieter, R. M., Elner, V. M., Rollins, B. J., Del Monte, M. A., Kunkel, S. L. (1991) Monocyte chemotactic protein gene expression by cytokine-treated human retinal pigment epithelial cells. *Lab. Invest.* **64,** 819–825.
7. Kunkel, S. L. (1999) Through the looking glass: the diverse in vivo activities of chemokines. *J. Clin. Invest.* **104,** 1333–1334.
8. Strieter, R. M., Standiford, T. J., Huffnagle, G. B., Colletti, L. M., Lukacs, N. W., Kunkel, S. L. (1996) The good, the bad, and the ugly. The role of chemokines in models of human disease. *J. Immunol.* **156,** 3583–3586.
9. Goede, V., Brogelli, L., Ziche, M., Augustin, H. G. (1999) Induction of inflammatory angiogenesis by monocyte chemoattractant protein-1. *Int. J. Cancer* **82,** 765–770.
10. Che, X., Ye, W., Panga, L., Wu, D. C., Yang, G. Y. (2001) Monocyte chemoattractant protein-1 expressed in neurons and astrocytes during focal ischemia in mice. *Brain Res.* **902,** 171–177.
11. Yamagami, S., Tamura, M., Hayashi, M., Endo, N., Tanabe, H., Katsuura, Y., Komoriya, K. (1999) Differential production of MCP-1 and cytokine-induced neutrophil chemoattractant in the ischemic brain after transient focal ischemia in rats. *J. Leukoc. Biol.* **65,** 744–749.
12. Elner, S. G., Elner, V. M., Jaffe, G. J., Stuart, A., Kunkel, S. L., Strieter, R. M. (1995) Cytokines in proliferative diabetic retinopathy and proliferative vitreoretinopathy. *Curr. Eye Res.* **14,** 1045–1053.
13. Lukacs, N. W., Strieter, R. M., Elner, V. M., Evanoff, H. L., Burdick, M., Kunkel, S. L. (1994) Intercellular adhesion molecule-1 mediates the expression of monocyte-derived MIP-1 alpha during monocyte-endothelial cell interactions. *Blood* **83,** 1174–1178.
14. DiPietro, L. A., Burdick, M., Low, Q. E., Kunkel, S. L., Strieter, R. M. (1998) MIP-1alpha as a critical macrophage chemoattractant in murine wound repair. *J. Clin. Invest.* **101,** 1693–1698.
15. Ogawa, S., Yoshida, S., Ono, M., Onoue, H., Ito, Y., Ishibashi, T., Inomata, H., Kuwano, M. (1999) Induction of macrophage inflammatory protein-1alpha and vascular endothelial growth factor during inflammatory neovascularization in the mouse cornea. *Angiogenesis* **3,** 327–334.
16. Campochiaro, P. A. (2000) Retinal and choroidal neovascularization. *J. Cell. Physiol.* **184,** 301–310.
17. Smith, L. E., Wesolowski, E., McLellan, A., Kostyk, S. K., D'Amato, R., Sullivan, R., D'Amore, P. A. (1994) Oxygen-induced retinopathy in the mouse. *Invest. Ophthalmol. Vis. Sci.* **35,** 101–111.
18. Yoshida, A., Yoshida, S., Hata, Y., Khalil, A. K., Ishibashi, T., Inomata, H. (1998) The role of NF-kappaB in retinal neovascularization in the rat. Possible involvement of cytokine-induced neutrophil chemoattractant (CINC), a member of the interleukin-8 family. *J. Histochem. Cytochem.* **46,** 429–436.
19. Yoshida, A., Yoshida, S., Ishibashi, T., Kuwano, M., Inomata, H. (1999) Suppression of retinal neovascularization by the NF-kappaB inhibitor pyrrolidine dithiocarbamate in mice. *Invest. Ophthalmol. Vis. Sci.* **40,** 1624–1629.
20. Rollins, B. J., Morrison, E. D., Stiles, C. D. (1988) Cloning and expression of JE, a gene inducible by platelet-derived growth factor and whose product has cytokine-like properties. *Proc. Natl. Acad. Sci. USA* **85,** 3738–3742.
21. Davatelis, G., Tekamp-Olson, P., Wolpe, S. D., Hermsen, K., Luedke, C., Gallegos, C., Coit, D., Merryweather, J., Cerami, A. (1988) Cloning and characterization of a cDNA for murine macrophage inflammatory protein (MIP), a novel monokine with inflammatory and chemokinetic properties. *J. Exp. Med.* **167,** 1939–1944.
22. Claffey, K. P., Wilkison, W. O., Spiegelman, B. M. (1992) Vascular endothelial growth factor. Regulation by cell differentiation and activated second messenger pathways. *J. Biol. Chem.* **267,** 16317–16322.
23. Sabath, D. E., Broome, H. E., Prystowsky, M. B. (1990) Glyceraldehyde-3-phosphate dehydrogenase mRNA is a major interleukin 2- induced transcript in a cloned T-helper lymphocyte. *Gene* **91,** 185–191.
24. Yoshida, A., Yoshida, S., Khalil, A. K., Ishibashi, T., Inomata, H. (1998) Role of NF-kappaB-mediated interleukin-8 expression in intraocular neovascularization. *Invest. Ophthalmol. Vis. Sci.* **39,** 1097–1106.
25. Yoshida, S., Ono, M., Shono, T., Izumi, H., Ishibashi, T., Suzuki, H., Kuwano, M. (1997) Involvement of interleukin-8, vascular endothelial growth factor, and basic fibroblast growth factor in tumor necrosis factor alpha-dependent angiogenesis. *Mol. Cell. Biol.* **17,** 4015–4023.
26. Morrison, T. B., Weis, J. J., Wittwer, C. T. (1998) Quantification of low-copy transcripts by continuous SYBR green I monitoring during amplification. *Biotechniques* **24,** 954–958, 960, 962.
27. Simpson, D. A., Feeney, S., Boyle, C., Stitt, A. W. (2000) Retinal VEGF mRNA measured by SYBR green I fluorescence: a versatile approach to quantitative PCR. *Mol. Vis.* **6,** 178–183.
28. Yoshida, A., Elner, S. G., Bian, Z. M., Kunkel, S. L., Lukacs, N. W., Elner, V. M. (2001) Thrombin regulates chemokine induction during human retinal pigment epithelial cell/monocyte interaction. *Am. J. Pathol.* **159,** 1171–1180.
29. Alon, T., Hemo, I., Itin, A., Pe'er, J., Stone, J., Keshet, E. (1995) Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. *Nat. Med.* **1,** 1024–1028.
30. Ozaki, H., Yu, A. Y., Della, N., Ozaki, K., Luna, J. D., Yamada, H., Hackett, S. F., Okamoto, N., Zack, D. J., Semenza, G. L., Campochiaro, P. A. (1999) Hypoxia inducible factor-1alpha is increased in ischemic retina: temporal and spatial correlation with VEGF expression. *Invest. Ophthalmol. Vis. Sci.* **40,** 182–189.
31. Pierce, E. A., Avery, R. L., Foley, E. D., Aiello, L. P., Smith, L. E. (1995) Vascular endothelial growth factor/vascular permeability factor expression in a mouse model of retinal neovascularization. *Proc. Natl. Acad. Sci. USA* **92,** 905–909.
32. Salcedo, R., Ponce, M. L., Young, H. A., Wasserman, K., Ward, J. M., Kleinman, H. K., Oppenheim, J. J., Murphy, W. J. (2000) Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. *Blood* **96,** 34–40.
33. Low, Q. E., Drugea, I. A., Duffner, L. A., Quinn, D. G., Cook, D. N., Rollins, B. J., Kovacs, E. J., DiPietro, L. A. (2001) Wound healing in MIP-1alpha(−/−) and MCP-1(−/−) mice. *Am. J. Pathol.* **159,** 457–463.
34. Naug, H. L., Browning, J., Gole, G. A., Gobe, G. (2000) Vitreal macrophages express vascular endothelial growth factor in oxygen-induced retinopathy. *Clin. Experiment. Ophthalmol.* **28,** 48–52.
35. Sholley, M. M., Gimbrone Jr., M. A., Cotran, R. S. (1978) The effects of leukocyte depletion on corneal neovascularization. *Lab. Invest.* **38,** 32–40.
36. Crowther, M., Brown, N. J., Bishop, E. T., Lewis, C. E. (2001) Microenvironmental influence on macrophage regulation of angiogenesis in wounds and malignant tumors. *J. Leukoc. Biol.* **70,** 478–490.
37. Yun, J. K., McCormick, T. S., Villabona, C., Judware, R. R., Espinosa, M. B., Lapetina, E. G. (1997) Inflammatory mediators are perpetuated in macrophages resistant to apoptosis induced by hypoxia. *Proc. Natl. Acad. Sci. USA* **94,** 13903–13908.
38. Scannell, G., Waxman, K., Kaml, G. J., Ioli, G., Gatanaga, T., Yamamoto, R., Granger, G. A. (1993) Hypoxia induces a human macrophage cell line to release tumor necrosis factor-alpha and its soluble receptors in vitro. *J. Surg. Res.* **54,** 281–285.
39. Fahey III, T. J., Sherry, B., Tracey, K. J., van Deventer, S., Jones II, W. G., Minei, J. P., Morgello, S., Shires, G. T., Cerami, A. (1990) Cytokine production in a model of wound healing: the appearance of MIP-1, MIP-2, cachectin/TNF and IL-1. *Cytokine* **2,** 92–99.
40. Limb, G. A., Hollifield, R. D., Webster, L., Charteris, D. G., Chignell, A. H. (2001) Soluble TNF receptors in vitreoretinal proliferative disease. *Invest. Ophthalmol. Vis. Sci.* **42,** 1586–1591.
41. Spranger, J., Meyer-Schwickerath, R., Klein, M., Schatz, H., Pfeiffer, A. (1995) TNF-alpha level in the vitreous body. Increase in neovascular eye diseases and proliferative diabetic retinopathy. *Med. Klin.* **90,** 134–137.
42. Elner, V. M., Strieter, R. M., Pavilack, M. A., Elner, S. G., Remick, D. G., Danforth, J. M., Kunkel, S. L. (1991) Human corneal interleukin-8. IL-1 and TNF-induced gene expression and secretion. *Am. J. Pathol.* **139,** 977–988.
43. Clauss, M., Gerlach, M., Gerlach, H., Brett, J., Wang, F., Familletti, P. C., Pan, Y. C., Olander, J. V., Connolly, D. T., Stern, D. (1990) Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration. *J. Exp. Med.* **172,** 1535–1545.
44. Aiello, L. P., Pierce, E. A., Foley, E. D., Takagi, H., Chen, H., Riddle, L., Ferrara, N., King, G. L., Smith, L. E. (1995) Suppression of retinal neovascularization in vivo by inhibition of vascular endothelial growth

factor (VEGF) using soluble VEGF-receptor chimeric proteins. *Proc. Natl. Acad. Sci. USA* **92,** 10457–10461.
45. Gerhardinger, C., Brown, L. F., Roy, S., Mizutani, M., Zucker, C. L., Lorenzi, M. (1998) Expression of vascular endothelial growth factor in the human retina and in nonproliferative diabetic retinopathy. *Am. J. Pathol.* **152,** 1453–1462.
46. Kim, I., Ryan, A. M., Rohan, R., Amano, S., Agular, S., Miller, J. W., Adamis, A. P. (1999) Constitutive expression of VEGF, VEGFR-1, and VEGFR-2 in normal eyes. *Invest. Ophthalmol. Vis. Sci.* **40,** 2115–2121.
47. Robinson, G. S., Ju, M., Shih, S. C., Xu, X., McMahon, G., Caldwell, R. B., Smith, L. E. (2001) Nonvascular role for VEGF: VEGFR-1, 2 activity is critical for neural retinal development. *FASEB J.* **15,** 1215–1217.
48. Oosthuyse, B., Moons, L., Storkebaum, E., Beck, H., Nuyens, D., Brusselmans, K., Van Dorpe, J., Hellings, P., Gorselink, M., Heymans, S., Theilmeier, G., Dewerchin, M., Laudenbach, V., Vermylen, P., Raat, H., Acker, T., Vleminckx, V., Van Den Bosch, L., Cashman, N., Fujisawa, H., Drost, M. R., Sciot, R., Bruyninckx, F., Hicklin, D. J., Ince, C., Gressens, P., Lupu, F., Plate, K. H., Robberecht, W., Herbert, J. M., Collen, D., Carmeliet, P. (2001) Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration. *Nat. Genet.* **28,** 131–138.