Interleukin-6 in Retinal Ischemia Reperfusion Injury in Rats

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Purpose. To study the role of interleukin (IL)-6 after retinal ischemia-reperfusion (I/R) injury in rats.

Methods. Intraocular pressure of adult male Lewis albino rats was raised to create retinal ischemia for 1 hour. Retinal reperfusion was reestablished, and the animals were killed at various time points after the injury. Their eyes were enucleated and processed for immunohistochemistry to detect IL-6 and ED-1 (a marker of microglial/phagocytic cells), enzyme-linked immunosorbent assay (ELISA) of IL-6 protein, and semiquantitative real-time RT-PCR for IL-6 mRNA. The neuroprotective effect of IL-6 was evaluated by giving intravitreal injections of 150 or 300 ng rat recombinant IL-6 to eyes immediately after I/R injury and counting cresyl violet–stained retinal ganglion cell layer cells (RGCLCs) and fluorochrome-labeled retinal ganglion cells (RGCs) on flat preparations of retinas at 7 days.

Results. IL-6–positive cells appeared after I/R injury in the inner plexiform layer (IPL) and the inner nuclear layer (INL). Their numbers were significantly higher 18 hours after the injury, and most of these cells were also ED-1 positive. ELISA showed noticeable increases in endogenous retinal IL-6 protein levels 8 hours after I/R injury. Semiquantitative real-time RT-PCR showed significant increases in endogenous retinal IL-6 mRNA levels between 2 and 18 hours. Exogenously added IL-6 prevented between 50% and 70% of RGC loss after I/R injury.

Conclusions. IL-6 is upregulated after retinal I/R injury, and its expression by microglia/phagocytic cells may protect RGC and RGCLCs from cell death. Exogenously added IL-6 protects the inner retina after I/R injury. (Invest Ophthalmol Vis Sci. 2003;44:4006–4011) DOI:10.1167/iovs.03-0040

Interleukin (IL)-6 is a multifunctional cytokine that belongs to the GPH-10 family of cytokines. It is expressed in the brain in neurons,1,2 microglia,1,3–5 and glial cells1 after middle cerebral artery occlusion (MCAO) or intrastriatal injection of quinolinic acid. IL-6 protects cerebral neurons from ischemia7 and N-methyl-D-aspartate (NMDA)–induced excitotoxicity8 in vivo, enhances retinal ganglion cell (RGC)9 and dorsal root ganglion cell10 survival in vitro, and protects cortical cell cultures from NMDA-induced excitotoxicity.11 However, it has been recently reported that IL-6 knockout (−/−) mice have an increased number of RGCs after optic nerve crush injury or glutamate-induced excitotoxicity,12 when compared with their wild-type counterparts, suggesting a neurodegenerative role for IL-6. In addition, increased plasma levels of IL-6 in patients who had had a stroke were associated with a poorer neurologic outcome.13 Therefore, the role of IL-6 in neuronal injury may be complicated.

Ischemia reperfusion (I/R) injury to the retina causes degeneration of inner retinal elements, including the RGCs,14–16 and leads to visual deficiency. Understanding the pathophysiology of I/R injury to the retina is important for development of therapeutic strategies for protecting these neurons. It has been reported that multiple cytokines such as IL-1β,17 TNF-α, IFN-γ, TGF-β, and IL-6 are upregulated18 and that IL-1β mediates the degeneration of inner retinal elements after I/R injury.19 However, the roles of IL-6 and other cytokines in retinal I/R injury are not clear.

In this study, we examined the possible involvement of IL-6 by localizing it with immunohistochemistry, profiling the temporal changes of its protein and mRNA with enzyme-linked immunosorbent assay (ELISA) and semiquantitative real-time RT-PCR, and evaluating the effect of exogenous IL-6 after I/R injury.

Materials and Methods

Induction of Retinal I/R Injury in Rats

All animals used in this study adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Adult male Lewis albino rats (45–50 days old) were kept under controlled light–dark conditions with food and water available ad libitum. A previously described procedure was used to induce retinal I/R in the rats.20 Briefly, animals were anesthetized with ketamine-xylazine (10 mg/kg and 4 mg/kg, respectively; Fort Dodge Animal Health, Fort Dodge, IA) intramuscularly, and retinal ischemia was induced by elevating the intraocular pressure to 110 mm Hg by cannulating the anterior chamber of the eye with a tube connected to an elevated reservoir containing saline. Ischemia was confirmed by ophthalmoscopic examination by noting the blanching of retinal arteries and loss of the red reflex. One hour later, the cannulating needles were removed to allow for reperfusion of the retinal vasculature. Animals were killed at various times with a pentobarbital overdose, and their eyes were enucleated.

Immunohistochemistry of IL-6 and ED-1

Normal eyes (n = 3) and eyes at 18 (n = 3) or 48 (n = 3) hours after reperfusion were obtained and fixed in 10% formalin in 0.1 M phosphate-buffered saline (PBS; pH 7.4) at 4°C overnight, processed, embedded in paraffin, and sectioned. Only sections containing the whole retina with visible portions of the optic nerve head were used for the studies. Standard protocol for immunohistochemistry of IL-6 using the
The homogenates were centrifuged for 10 minutes at 17,000 rpm. The supernatant was collected and homogenized for 30 seconds in 0.1 M PBS (pH 7.4) to prepare cell lysates (R&D Systems, Minneapolis, MN). Normal retinas and retinas at 0, 4, 8, and 100 hours after reperfusion were used for cell lysate preparation. The lysates were centrifuged at 20,000 rpm for 15 minutes. The supernatants were collected and used for ELISA for IL-6 according to the manufacturer’s instructions and reported as picograms per milligram protein. The IL-6 content was calculated according to the manufacturer’s instructions and reported as picograms per milligram protein.

Fluorescent double immunolabeling of IL-6 and ED-1 was performed according to a standard protocol, with a monoclonal mouse anti-rat ED-1 antibody (1:100; Serotec, Raleigh, NC) and a polyclonal rabbit anti-rat IL-6 antibody (1:100; Research Diagnostics, Inc.) as primary antibodies, and an anti-mouse antibody conjugated to tetra-methylrhodamine isothiocyanate (TRITC; Dako) and an anti-rabbit antibody conjugated to FITC (Dako) as secondary antibodies on sections of eyes at 18 hours after I/R injury. Colocalization of IL-6 and ED-1 was visualized with a fluorescence confocal microscope (model 510; Carl Zeiss Meditec, Thornwood, NY).

ELISA for IL-6

Standard protocol for IL-6 ELISA was followed to measure the levels of immunoreactive IL-6 in retinas after I/R injury, according to the manufacturer’s instructions. The homogenates were collected and homogenized for 30 seconds in 0.1 M PBS (pH 7.4). R&D Systems, Minneapolis, MN). Normal retinas and retinas at 0, 4, 8, 12, 18, 24, and 72 hours after I/R injury (n = 4 for each time point) were collected and homogenized for 50 seconds in 0.1 M PBS (pH 7.4). The homogenates were centrifuged for 10 minutes at 17,000 rpm. The supernatant was used for each determination. The optical density of each sample (in triplicate) was determined with a microplate spectrophotometer (Benchmark Plus; Bio-Rad, Hercules, CA). IL-6 content was calculated according to the manufacturer’s instructions and reported as picograms per milligram protein. The assay was repeated, and the averages of the two runs were reported.

Relative Quantitative Real-Time RT-PCR of IL-6 mRNA

Retinal samples were obtained at 0 (n = 13), 2 (n = 4), 4 (n = 12), 8 (n = 12), 12 (n = 5), 18 (n = 5), 24 (n = 5), and 48 (n = 5) hours after reperfusion and homogenized with extraction RNA reagent (TRizol; Invitrogen-Gibco, Carlsbad, CA). Total RNA was isolated by a standard guanidinium thiocyanate-phenol-chloroform extraction protocol, and DNA contamination was eliminated with a DNase I kit (Ambion, Austin, TX). One microgram of RNA was reverse transcribed with commercial reverse transcriptase (Promega, Madison, WI) under the following conditions: 22°C for 5 minutes; 42°C for 60 minutes; and 95°C for 5 minutes.

Primers for real-time PCR were designed on computer (Primer Express software; Applied Biosystems, Foster City, CA) and synthesized by Operon (Alameda, CA): TCAACTCTCAGGGGCCCCCTCAG (sense) and AAGGCAATCTGGCTGGAAATCT (antisense) for IL-6 and TAAAGGGAACCTGCGGAAAC (sense) and CAGGATCGGCCCCCTGAACT (anti-sense) for the housekeeping gene L-32. Semi-quantitative real-time PCR was performed with a Light Cycler (Gene Amp 5700; Applied Biosystems). The reaction was initiated using 12.5 μL of gelfoam soaked in 2% aqueous gelfoam. An incision was made in the conjunctiva close to the superior orbital rim, to expose the optic nerve (ON), which was subsequently transected approximately 1 mm from the posterior pole without damaging the retinal blood supply. A piece of gelfoam soaked in 2% aqueous fluorescein was placed on the stump of the transected ON. Rats were killed 2 days later (7 days after I/R injury) by an overdose of pentobarbital. Retinas were dissected, fixed in 4% paraformaldehyde, and flattened onto glass slides. Cell counting was performed as previously described under a fluorescence microscope (Nikon, Melville, NY) equipped with a UV filter set (365/397 nm). RGC densities were determined by counting RGC-like cells in 16 distinct areas of 1.36 × 10^7 mm² each (four areas per retinal quadrant at 0.5 [central retina] and 4 mm [peripheral retina] from the optic nerve head). The averages of the four quadrants were recorded, and each flat preparation was counted by two individuals independently with the samples masked.

Statistical Analysis of Cell Counts with Fluorochrome Labeling or Cresyl Violet Staining

Cell densities for each retina are described in the text and shown in scatterplot format as the mean ± SD, representing the results in four to seven retinas. Statistical analyses were performed by the Student-Newman-Keuls method (ANOVA). Differences in cell numbers were considered significant at P < 0.05.

RESULTS

IL-6 Immunoreactivity in Rat Retinas after I/R Injury

Figure 1 illustrates the presence of IL-6-positive cells in retinas at 18 hours after I/R injury. These IL-6 immunoreactive cells were pleiomorphic and were localized to the IPL and INL. Counting of the IL-6-positive cells showed very few (2.2 ± 1.5 cells) in normal retinas, significant elevations in the number of cells (41.3 ± 9.1, n = 3; P < 0.001 versus normal, Tukey test). For some retinas, the effect on RGCL cell (RGCLc) loss was evaluated by using a previously described procedure for flattened whole retinas, cresyl violet staining, and cell counting. Briefly, RGCLc densities were determined by counting RGC-like cells in 16 distinct areas of 1.36 × 10^7 mm² each (four areas per retinal quadrant at 0.5 [central retina] and 4 mm [peripheral retina] from the optic nerve head). The averages of the four quadrants were recorded, and each flat preparation was counted by two individuals independently with the samples masked.

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test) at 18 hours, and a return to normal (13.0 ± 3.5) at 48 hours (Fig. 2). Double immunostaining of retinas at 18 hours showed colocalization of IL-6 and ED-1, a marker for microglial/phagocytic cells, in most of the ED-1–positive cells (Fig. 3).

**Endogenous IL-6 Protein and mRNA Levels**

Retinal IL-6 protein was significantly elevated 8 hours after I/R injury (40 ± 27 pg/mg, n = 4; P < 0.050 versus normal, Dunnett method; Fig. 4A). There were no detectable levels of IL-6 mRNA in the normal nonischemic retinas; (Ct > 40 for all normal samples; n = 6; data not shown). Therefore, relative multiples of changes in IL-6 gene expression using L-32 as a reference were calculated in comparison with levels at hour 0 (n = 13) after I/R injury, rather than in normal retinas. IL-6 mRNA showed a rapid increase as early as 2 hours (270 ± 241-fold, n = 4; P < 0.05 vs. hour 0, Dunn method) and remained elevated 12 hours (349 ± 388-fold, n = 6; P < 0.05 versus 0 hours, Dunn method) after I/R injury (Fig. 4B). Gene expression of IL-6 approached levels similar to 0 hour levels by 48 hours. Efficiency analysis demonstrated that IL-6 and L-32 primers had comparable efficiencies (data not shown).

**Effect of Exogenous IL-6 after I/R Injury**

Intravitreal injection of 150 or 300 ng recombinant IL-6 protected cells of the inner retina after I/R injury, as indicated by the elevated number of fluorochrome-labeled RGCs in central areas of 150-ng IL-6 treated retinas (1806 ± 222 RGCs/mm², n = 7; P < 0.050 versus vehicle; Student-Newman-Keuls method) and 300 ng IL-6–treated retinas (1739 ± 159 RGCs/mm², n = 6; P < 0.050 versus vehicle; Student-Newman-Keuls method) when compared with the corresponding areas of vehicle-treated retinas (1400 ± 246 RGCs/mm², n = 7). There were also significantly higher numbers of fluorochrome labeled RGCs in peripheral areas of 150-ng IL-6 treated retinas (1521 ± 167 RGCs/mm², n = 7; P < 0.050 versus vehicle; Student-Newman-Keuls method) and 300 ng IL-6 treated retinas (1454 ± 171 RGCs/mm², n = 6; P < 0.050 versus vehicle; Student-Newman-Keuls method) when compared with the cor-

**FIGURE 2.** Quantitation of IL-6–positive cells in the inner retina. Note a significant increase in the number of IL-6–positive cells in the IPL and INL of retinas 18 hours after I/R injury (n = 3, *P < 0.001 versus normal. Tukey test). Data are expressed as the mean ± SEM of results in three retinas.

**FIGURE 3.** Double immunolabeling of IL-6 and ED-1. Note colocalization of IL-6 (A) and ED-1 (B) in cells (arrows) in the IPL and the INL at 18 hours after I/R injury.

**FIGURE 4.** IL-6 levels after I/R injury. (A) IL-6 protein levels were significantly elevated 8 hours after I/R injury (40 ± 27 pg/mg protein, n = 4; *P < 0.050 versus normal; Dunnett method). Data are expressed as the mean ± SEM of results in four retinas measured twice, each time in triplicate. (B) Relative IL-6 mRNA expression. Semiquantitative real-time RT-PCR showed significantly elevated levels (*P < 0.001 versus 0 hour, Tukey test) of IL-6 mRNA at 2 (n = 4), 4 (n = 12), 8 (n = 12), 12 (n = 5), and 18 (n = 5) hours when compared with 0-hour (n = 13) samples. L-32 was used as a housekeeping gene. Data are expressed as the mean ± SEM of results in 4 to 12 retinas.
Significantly higher numbers of fluorochrome-labeled RGCs in central areas of 150-ng IL-6–treated retinas (1866 ± 222 RGCs/mm², n = 7; P < 0.050 versus vehicle; Student-Newman-Keuls method) and 300-ng IL-6–treated retinas (1759 ± 159 RGCs/mm², n = 6; P < 0.050 versus vehicle; Student-Newman-Keuls method) when compared with the corresponding areas of vehicle-treated retinas (1400 ± 246 RGCs/mm², n = 7). Also note the significantly higher number of fluorochrome-labeled RGCs in peripheral areas of 150-ng IL-6–treated retinas (1521 ± 167 RGCs/mm², n = 7; P < 0.050 versus vehicle; Student-Newman-Keuls method) and 300-ng IL-6–treated retinas (1454 ± 171 RGCs/mm², n = 6; P < 0.050 versus vehicle) when compared with the corresponding areas of vehicle-treated retinas (1235 ± 99 RGCs/mm², n = 7). Each data point represents the number of RGCs/mm² per retina evaluated. The error bars represent the mean ± SD.

**DISCUSSION**

In this study, we demonstrated the presence of IL-6–containing ED-1–positive cells in the rat retina after retinal I/R injury. ELISA and relative quantitative real-time RT-PCR showed an upregulation of endogenous IL-6 protein and its mRNA, respectively, after I/R injury. In addition, exogenous IL-6 introduced immediately after I/R injury protected the RGCs. These results are consistent with the hypothesis that endogenous IL-6 is upregulated in the inner retina after neuronal injury as a self-defensive mechanism for RGCs, including RGCs after I/R injury, and that microglial/phagocytic cells play important roles in this self-defensive–protective action.

Elevated IL-6 levels have been demonstrated in neurons of the substantia nigra and entopeduncular nucleus and microglial cells of the ischemic core and penumbra after MCAO. Expression of IL-6 in neurons and microglia in the gerbil forebrain that sustained direct ischemic injury during MCAO has also been reported. In addition, glial cells expressing IL-6 in striatal tissue that underwent neuronal degeneration as a direct result of stereotaxic injection of quinolinic acid were also noted. Hence, neurons, microglia/phagocytic cells, and other glial cells in neuronal tissue may express IL-6 after injury but the cell types that express IL-6 may vary. In contrast to these earlier studies showing a wide distribution of IL-6 in neuronal tissue after injury, we found little expression of IL-6 in neurons of the retina, and most IL-6 was localized in microglial/phagocytic cells that appeared after I/R injury. The reason for these differences is not clear. One of the possible explanations is that the studies that noted IL-6 exclusively in neurons focused on tissue response to I/R injury in areas of the brain that did not have an interruption of blood flow and that the response resulted from transsynaptic degeneration. In addition, our study is consistent with other studies that demonstrated microglial activation in many tissues during neurodegeneration and that microglia/phagocytic cells synthesize and secrete IL-6 on activation. Further studies are under way to examine the signaling pathways regulating the generation of IL-6 by these phagocytic cells in rat retinas after I/R injury.
It has been reported that optic nerve ligation–induced I/R injury stimulates endogenous IL-6 gene expression in the retina at 6 and 12 hours.18 The locations or cell types containing IL-6 were not examined. We noted IL-6 mRNA elevation as early as 2 hours, suggesting that one of the earliest responses by the retina to I/R injury is gene expression of neuroprotective cytokines such as IL-6. This was similar to other studies in the brain showing IL-6 mRNA expression as early as 3 hours after MCAO11–13 and stereotaxic injection of quinolinic acid.6 Our study also suggests that IL-6 mRNA is translated into its protein by demonstrating a peak in IL-6 immunoreactivity at 8 hours with our ELISA and at 18 hours with our morphometric analysis of IL-6–positive cells. These findings correlate with the data in Suzuki et al.,7 which demonstrated a peak in IL-6 protein within the first 24 hours after MCAO. Hence, early expression of IL-6 mRNA and subsequent synthesis and secretion of its protein may be a universal response to I/R injury in neural tissues.

In this study, we used two widely used techniques to assess the effect of IL-6 on retinal ganglion cell layer neurons: cresyl violet staining and RGCLC counting and fluorochrome labeling and RGC counting. Our study showed large differences between the number of RGCLCs observed in the cresyl violet assay and the RGCs counted in the fluorochrome labeling assay, with the former showing much higher numbers. This is not unexpected, because quantification through cresyl violet staining probably overestimates the number of RGCs, that displaced amacrine cells constitute a substantial population of cells in the GCL.27 In contrast, our fluorochrome labeling may underestimate the total number of RGCs, because axotomy may affect retrograde transport of fluorescent gold label to the RGC bodies. Nonetheless, the number of fluorochrome-labeled RGCs/mm² in the normal adult rat retina that we reported herein is between 200 and 500 RGCs/mm² less than what has been reported in other studies.28,50–58 In addition, consistent with the report of Gelrich and Gelrich27 our cresyl violet assay gave a good indication of changes in RGC number in the retina.27

Our observation that IL-6 is neuroprotective of the inner retina is consistent with other studies. IL-6 deficiency exacerbated 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)–induced toxicity in dopaminergic neurons,9/aggravated degeneration of hippocampal neurons after hippocampal injection of kainic acid,61 and induced higher rates of apoptosis31 and decreased survival20 of cortical neurons in the frontoparietal cortex subjected to focal cryo-induced injury. In earlier studies, site-specific administration of IL-6 has also been shown to be neuroprotective after permanent MCAO in rats7 and NMDA-induced excitotoxicity of rat striatal cholinergic neurons.8 However, these findings are in contrast to the IL-6 knockout (−/−) mouse study by Fisher et al.,12 suggesting that IL-6 may exacerbate retinal injury. The reason for the discrepancy between these former findings and those of Fisher et al. remains unclear. Although, there is little information on the neuroprotective mechanism of IL-6 in the retina, it is widely believed that IL-6 exerts its activity through its receptor (IL-6R) and its mRNA and that exogenous IL-6 protected neurons in the RGCL from I/R injury. These findings are consistent with our hypothesis that I/R injury induces phagocytic/microglial cells to protect RGCs against I/R injury through expression of IL-6. Although this hypothesis deviates from other studies that demonstrated that microglia aggravate cerebral ischemia through IL-1β and TNF-α expression,17–20 it is possible that there are heterogeneous populations of microglial/phagocytic cells in the retina and that they may synthesize and secrete different spectra of bioactive molecules after I/R injury. Therefore, it is important to characterize further these populations of phagocytic/microglial cells on the basis of the cytokines that they secrete, because future neuroprotective strategies may incorporate mechanisms to upregulate protective populations of microglial/phagocytic cells and/or downregulate neurodegenerative populations in response to neuronal injury.

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