Deleterious Role of TNF-α in Retinal Ischemia–Reperfusion Injury

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PURPOSE. Tumor necrosis factor (TNF-α) is a mediator of neuronal cell death and survival in ischemia–reperfusion injury. This study was conducted to further elucidate the role of TNF-α and its receptor in an in vivo model of retinal ischemia–reperfusion injury by investigating its effects on retinal histopathology and function.

METHODS. Retinal ischemia–reperfusion injury was performed on p55 and p75 knockout (KO) mice and Sprague–Dawley rats using the high intraocular pressure method. The temporal expression of TNF-α was ascertained with immunohistochemical staining. Separate rats received intravitreal recombinant TNF-α or neutralizing antibody before or after ischemia. TUNEL labeling was performed to assess for cell death, and electroretinography was performed to assess function.

RESULTS. TNF-α expression peaked at 12 to 24 hours after ischemia–reperfusion injury. TUNEL staining was diminished after intravitreal TNF-α antibody. Both transgenic KOs demonstrated significantly less functional impairment. Rats receiving recombinant TNF-α 48 hours after ischemia showed exaggerated functional impairment. Animals treated with TNF-α antibody before ischemia displayed significant functional improvement.

CONCLUSIONS. TNF-α plays a largely deleterious role in ischemia–reperfusion injury in an in vivo model of retinal injury. Direct neutralization of this cytokine partially preserves retinal function. The diverse characteristics of TNF-α are attributed in part to the timing of its expression after injury. TNF-α receptor expression and function, along with combination treatments targeting death receptor–mediated apoptosis, should be further explored to develop neuroprotective therapeutic strategies for acute retinal ischemic disorders. (Invest Ophthalmol Vis Sci. 2008;49:3605–3610) DOI:10.1167/iovs.07-0817

Tumor necrosis factor (TNF-α) is an inflammatory mediator of neuronal death after ischemic injury in the brain and retina.1,2 TNF-α is a member of the death-inducing ligand (DIL) family; it triggers the extrinsic pathway of apoptosis and acts through its two primary receptors, TNFR1 (p55) and TNFR2 (p75).

Ischemia–reperfusion injury involves many signaling mechanisms resulting in necrotic and apoptotic cell death.3 Delayed neuronal cell death in the brain and retina secondary to transient ischemic injury occurs, in part, by apoptosis.4–6 TNF-α acts upstream of the caspases and participates in ischemic neuronal injury in the brain.1,7 Inhibition of TNF-α leads to protection in models of ischemia–reperfusion in the rat and mouse brain.8,9 and rat myocardium.10 The role of TNF-α in uveitis has been established, and its neutralization has been proven effective in preserving visual acuity and in decreasing apoptotic and inflammatory injury.11,12 Apoptotic damage to neurons secondary to TNF-α is also evident in spinal cord injury5 and in ischemic stroke.13,14,15 Studies have shown TNF-α and receptor upregulation in glaucomatous human retinas.16 Additionally, retinal ischemia induces TNF-α upregulation and apoptotic death in cultured retinal ganglion cells.2

Studies of TNF-α show a protective and a destructive role in cell death signaling. Opposing actions are thought to be directly related to which subtypes of the tumor necrosis factor receptors (TNFRs) are stimulated. TNFR1 mediates apoptotic cell death through the TNF receptor–associated death domain (TRADD),17 and the inhibition of TNFR2 has been shown, in in vitro studies, to exacerbate neuronal injury.18 In vivo models using TNFR1 knockout (KO) mice show a reduction in neurodegeneration, whereas TNFR2 KO animals show enhanced neurodegeneration.19 There is also evidence that TNF receptors activate the NFκB transcription factor,17,20 which has an antiapoptotic effect and may suppress apoptotic signals from TNF-α in a self-limiting fashion.21 The contrasting activity of these receptor subtypes leads us to believe that variability in their expression after ischemia–reperfusion injury may account for the potential destructive and protective actions of TNF-α.

In these experiments, we evaluated the role of TNF-α and its specific cellular expression in retinal ischemia–reperfusion injury. In addition, we investigated the effects of the inhibition or activation of TNF-α on retinal function.

MATERIALS AND METHODS

Retinal Ischemia

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA), weighing 150 to 175 g each, and C57BL wild-type, TNFRp55 knockout, and TNFRp75 knockout mice (Jackson Laboratory, Bar Harbor, ME) were subjected to high intraocular pressure (HIOP), as described previously.5,22,23 Briefly, after deep anesthesia with an intramuscular injection of ketamine (30–40 mg/kg) and xylazine (2.5 mg/kg) in rats or an intraperitoneal injection of ketamine and xylazine in mice, the anterior chamber of the right eye was cannulated with a 27-gauge needle in rats or a 30-gauge needle in mice under direct visualization, and the coronal puncture site was sealed with cyanoacrylate cement. The needle was

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then attached with silastic tubing and a manometer to an infusion of sterile 0.9% saline solution. Intraocular pressure (IOP) was raised to 120 mm Hg by raising the saline container to exceed systemic arterial blood pressure, thereby inducing retinal ischemia, for durations of 30, 45, or 60 minutes. Whitening of the iris and loss of the red reflex of the retina confirmed retinal ischemia. After completion of the target period of ischemia, the IOP was normalized and the needle was withdrawn from the eye. Reperfusion was confirmed by reappearance of the red reflex. Animals were kept normothermic at 36.7°C ± 0.5°C with a rectal probe and heating pad during the procedure. The untouched contralateral left eye of each animal served as a nonischemic control. One drop of ophthalmic solution (Ocuflox) was applied topically to the right eye before and after cannulation of the anterior chamber. The untouched contralateral left eye of each animal served as a nonischemic control. Animals were then humanely killed at various time points, and their eyes were enucleated for morphologic and immunohistochemical studies.

**Electroretinography**

The procedures used were those we have previously reported. Briefly, animals were anesthetized and their pupils dilated with tropicamide and cyclomydrl. Platinum electrodes were placed on the corneas, and the a-wave and b-wave were identified after presentation full-field stroboscopic flashes at the rate of 1.0/s at a distance of 15 cm. Electroretinograms (ERGs) were taken before the ischemic insult to determine the baseline and again before enucleation. ERGs of the right and left eyes were always taken together, with the left eye serving as the nonischemic control.

**Immunohistochemistry**

**TNF-α Labeling.** Eyes were enucleated at 0 (n = 3), 3 (n = 3), 6 (n = 3), 12 (n = 3), 24 (n = 3), 72 (n = 3), or 168 hours (n = 3) after 60 minutes of ischemia. This duration of ischemia was chosen because it causes severe injury and increases the expression of other cell death–related proteins. Eyes were then fixed in 4% paraformaldehyde (PFA) for 2 hours. After removing the anterior segment of the eye and the lens, the eyes were further fixed in 4% PFA for another 4 hours and then cryoprotected in 25% sucrose solution overnight. The eyecups were washed in ice-cold normal saline and frozen in OCT embedding medium over dry ice. Cryosections 12 μm-thick were prepared at −20°C, fixed in cold methanol for 10 minutes, rinsed in 1× phosphate-buffered saline (PBS) for 5 minutes, and incubated with 20% rabbit serum in PBS for 60 minutes at room temperature. Anti-TNF-α antibody (goat IgG; 1:50; R&D Systems, Minneapolis, MN) was diluted in 5% rabbit serum with 0.2% Triton X, and tissue cryosections were incubated with this primary antibody overnight at 4°C. Sections were then washed with 1× PBS three times and incubated with fluorescein-conjugated secondary antibody (anti-goat IgG; 1:200; AlexaFluor 488; Molecular Probes, Eugene, OR) at room temperature for 1 hour. Corresponding negative controls were performed by substitution of the primary antibody with 20% rabbit serum in PBS.

**TUNEL Staining.** TUNEL staining was performed using a cell detection kit (In Situ Cell Death Detection Kit; Roche Applied Science, Indianapolis, IN) according to the specifications and directions of the manufacturer. Briefly, 12 μm cryosections were incubated in methanol

![Figure 1](image1.png)  
**FIGURE 1.** TNF-1 immunohistochemistry. Cryosections of nonischemic rat retina and rat retina at 0, 3, 6, 12, 24, and 72 hours of reperfusion, after a 60-minute period of retinal ischemia. TNF-1 is present in the GCL and INL of ischemic retinas, with minimal expression in the nonischemic retina and at 72 hours of reperfusion. At the 24-hour time point, a representative negative control is provided in which the primary antibody was omitted (inset). Fluorescence microscopy; original magnification, ×20.

![Figure 2](image2.png)  
**FIGURE 2.** TUNEL immunohistochemistry. (A) Cryosections of nonischemic rat retina and of rat retina at 24 hours after 45 minutes of retinal ischemia. Retinas received intravitreal injection of denatured TNF-α antibody, vehicle (2 μL of 0.1% BSA), or TNF-α antibody (2 μL of 0.05 μg/μL) 1 hour before ischemia (n = 3). Fluorescence microscopy; original magnification, ×20. (B) TUNEL-positive cells are present in the ischemic retina, with minimal expression in the nonischemic retina. There were significantly fewer TUNEL-positive cells in the ischemic retina after TNF-α antibody injection compared with ischemic retinas treated with vehicle or denatured TNF-α antibody. Mean ± SEM are provided. TUNEL staining was compared among PBS vehicle, TNF-α antibody, and denatured TNF-α antibody groups using the Kruskal-Wallis test. *P < 0.05.
at −20°C for 10 minutes, washed in 1× PBS for 5 minutes, and incubated in a dUTP/TdT mixture (Roche Applied Science) at 37°C for 1 hour, followed by three rinses in 1× PBS and mounted with antifade. Corresponding negative (without terminal transferase) and positive (DNase I treated) control sections were also prepared. The sections were analyzed under fluorescence microscopy.

**Recombinant TNF-α and Inhibitor Experiments**

Rats received a single intravitreal injection of recombinant rat TNF-α or TNF-α neutralizing antibody 1 hour before ischemia. The TNF-α neutralizing antibody (goat anti-rat, 0.05 μg/μL; R&D Systems, Minneapolis, MN) was diluted in 1× PBS in accordance with the manufacturer’s specifications, and a 2 μL intravitreal injection was administered to the right eye using a 10-μL Hamilton syringe (Hamilton, Reno, NV) after recording of a baseline ERG. Control animals received 2-μL intravitreal injections of vehicle (1× PBS). A third group received denatured TNF-α blocking antibody. These rats were subjected to 45 minutes of retinal ischemia 1 hour after injection. Based on previous neuroprotection studies from our laboratory using ERG, this duration of ischemia is ideal for the detection of a protective effect.23

A separate group of rats received 5-μL intravitreal injections of recombinant TNF-α (recombinant rat TNF-α, 0.1 μg/μL; R&D Systems) diluted in 0.1% BSA. Control animals received 5-μL intravitreal injections of vehicle (0.1% BSA). These rats were subjected to 30 minutes of retinal ischemia and received the intravitreal injection after 2 or 48 hours of reperfusion. Thirty minutes of ischemia was chosen because it causes little injury.23 The purpose of these experiments was to demonstrate whether TNF-α itself leads to an exacerbation of damage, which may be masked by a longer duration of ischemia. In both groups, ERGs were obtained after 7 days of reperfusion and the animals were killed.

**FIGURE 3.** (A) Measurements of retinal layer thickness at 7 days after 60 minutes of ischemia (i). *P < 0.05 by ANOVA (n = 8 per group) and representative ERGs at baseline and at 7 days subsequent to ischemia. Histopathology demonstrates partial preservation of the retinal histoarchitecture (ii) in the ischemic p55−/− mice. Note preservation of the ERG a- and b-waves (iii) in the ischemic p55−/− animals compared with the ischemic wild-type animals. (B) Measurements of thicknesses of retinal layers 7 days after 60 minutes of ischemia (i). *P < 0.05 by ANOVA (n = 8 per group), and representative ERGs at baseline and 7 days after 60 minutes of ischemia. Histopathology demonstrates partial preservation of the retinal histoarchitecture (ii) in the ischemic p75−/− mice. Note preservation of the ERG a- and b-waves (iii) in the ischemic p75−/− animals compared with the ischemic wild-type animals.
Light Microscopy

Animals were anesthetized, and eyes were enucleated at the chosen time points after reperfusion and then fixed in paraformaldehyde and embedded in paraffin. Enucleated globes were then sectioned in the vertical meridian, and the inferior portion of the eye wall (retina, choroid, sclera). Sections 5 μm thick were stained with hematoxylin and eosin. The retinal histoarchitecture was evaluated as previously described by light microscopy.22–23 Measurements of the thickness of the retinal layers were carried out as follows: outer limiting membrane to inner limiting membrane (ILM); outer nuclear layer; outer plexiform layer; inner nuclear layer (INL); inner plexiform layer (IPL) to ILM. The mean value for these measurements, taken in four adjacent areas of the inferior retina within 1 mm of the optic nerve, was calculated.

RESULTS

TNF-α Expression and TUNEL

We examined the expression of TNF-α in the retina at 0, 3, 6, 12, 24, and 72 hours after 60 minutes of ischemia. The left eye, which received no surgical manipulation, served as the nonischemic control. TNF-α expression was detected as early as 3 hours after ischemia–reperfusion, peaked between 12 to 24 hours, and was dramatically decreased at 72 hours. Immunostaining for TNF-α was found primarily in the IPL-ILM and less in the INL (Fig. 1). Additionally, p55 and p75 KO mice showed TNF-α expression in the IPL-ILM 24 hours after 60 minutes of ischemia (not shown).

TUNEL-positive cells were prevalent in retinas from rats killed at 24 hours after 45 minutes of ischemia with or without an intravitreal vehicle injection 1 hour before ischemia compared with nonischemic controls. Interestingly, TUNEL staining was significantly diminished in eyes receiving intravitreal injections of TNF-α antibody 1 hour before ischemia compared with the vehicle-injected eyes (Fig. 2).

TNFRp55 and TNFRp75 KO Mice

Three groups of mice (n = 8 per group) were studied: TNFRp55 KO, TNFRp75 KO, and wild type. Seven days after 60 minutes of ischemia, both transgenic KO groups showed less functional impairment than did wild-type controls. Both KO groups had a similar degree of histologic and functional preservation as represented in the ERGs (Figs. 3A, 3B).

Effects of Intravitreal Recombinant TNF-α

In a separate set of experiments, rats that received intravitreal injections of recombinant TNF-α 48 hours after 30 minutes of retinal ischemia displayed exaggerated functional impairment based on decreased ERG a- and b-wave amplitudes 7 days after ischemia compared with vehicle-injected eyes (n = 5). The same injection given only 2 hours after ischemia did not show a statistically significant degree of functional attenuation (Fig. 4). Animals that received the injection 1 hour before 30 minutes of ischemia did not demonstrate any functional difference compared with controls (data not shown).

Effects of Intravitreal TNF-α Antibody

Rats that received intravitreal injections of TNF-α neutralizing antibody 1 hour before 45 minutes of ischemia (n = 9) displayed functional improvement, as evidenced by a statistically significant (P < 0.05) preservation of the ERG b-wave 7 days after ischemia, compared with the ischemic controls (Fig. 5).

FIGURE 4. (A, B) Measurements of ERG a-wave and b-wave amplitudes as a percentage of normal baseline 7 days after 30 minutes of ischemia and intravitreal injection of recombinant TNF-α (5 μL of 0.1 μg/mL) or vehicle (5 μL of 0.1% BSA) 2 or 48 hours after ischemia. *P < 0.05 by Student’s two-tailed t-test (n = 4–5 per group). (C) ERG subsequent to 30 minutes of ischemia and intravitreal injection of recombinant TNF-α (5 μL of 0.1 μg/mL) or vehicle (5 μL of 0.1% BSA) 2 hours after ischemia. (D) ERG 7 days after 30 minutes of ischemia and intravitreal injection of recombinant TNF-α (5 μL of 0.1 μg/mL) or vehicle (5 μL of 0.1% BSA) 48 hours after ischemia.

DISCUSSION

TNF-α has been implicated as a mediator of apoptotic neuronal damage after ischemia. However, no previously published data address intravitreal pharmacologic neutralization of TNF-α in the retina in an in vivo model of ischemia–reperfusion. TNF-α has been studied in a murine model of oxygen-induced retinopathy using intraperitoneal injections with a reduction in pathologic neovascularization.24 The present study shows the expression of TNF-α in the inner retinal layers in a time-dependent manner after ischemia–reperfusion injury. In vivo neutralization of TNF-α during retinal ischemia significantly preserved inner retinal function, as evidenced by ERG data. This might have been caused largely by a decrease in TNF-α-mediated apoptotic signaling, as suggested by the decreased TUNEL staining observed in eyes treated with TNF-α-neutralizing antibody. Apoptotic neuronal cell death in the HIOP model of retinal ischemia has been well established.5,6 TNF-α has been shown to act...
through the caspase-mediated pathway, but a recent study suggests that TNF-α acts through a caspase-independent pathway and that greater protection is achieved with neutralization of reactive oxygen species after TNF-α stimulation.25 It is unclear whether a caspase-independent-mediated component to cell death is present in our in vivo model, which examines delayed retinal cell death.

TNF-α plays a significant role in functional and morphologic retinal damage after ischemia–reperfusion injury acting through its receptors, TNFR1 and TNFR2.19,26 There are conflicting data in in vitro and in vivo systems of cerebral ischemia concerning whether TNF-α acts to promote or to inhibit neuronal survival.19,27,28 The neurodegenerative and neuroprotective effects of TNF-α in retinal ischemia have been suggested to be a function of the opposing roles of TNF receptors 1 and 2, respectively.19 Our data show that the inhibition of TNF-α during and immediately after ischemia–reperfusion injury preserves retinal function. However, the administration of recombinant TNF-α 48 hours after ischemia–reperfusion injury appears to exacerbate functional retinal damage compared with injection 2 hours after reperfusion. This suggests that TNF-α may play a protective or a destructive role with respect to time after ischemia–reperfusion injury. Whether this effect results from differential upregulation or downregulation of TNFR1 or TNFR 2 or from a caspase-independent mechanism is unclear.

Activation of NF-κB by TNF-α may also account for the opposing effects of TNF-α. A recent study has shown that TNFR 1 and TNFR 2 induce the NFκB pathway, but with distinguishable kinetics, and that the TNFR2-induced persistent NF-κB activity is essential for neuronal survival.20 In our study, we found that the absence of either TNFR 1 or TNFR 2 resulted in the preservation of retinal function after ischemic injury. The administration of recombinant TNF-α 48 hours after reperfusion may disproportionately stimulate one receptor because of upregulation or increased receptor sensitivity. To further elucidate the mechanism behind the contrasting role of TNF, TNF-α activity should be studied with attention directed toward the timing and specific location of activity.

The partial preservation of retinal function after TNF-α inhibition leads us to consider other mediators in the pathway of apoptosis in ischemia–reperfusion injury. Caspases and caspase-independent mediators should be investigated with TNF-α to further elucidate the role of TNF-α and any synergistic effects in ameliorating ischemia–reperfusion injury. Combination treatment may further preserve retinal function subsequent to ischemia–reperfusion injury.

References

Figure 5. (A) Measurements of ERG a- and b-wave amplitudes as a percentage of normal baseline 7 days after 45 minutes of ischemia and intravitreal injection of TNF-α-blocking antibody. *P < 0.05 by Student’s two-tailed t test (n = 8–9 per group). (B) Representative ERGs at baseline and 7 days after ischemia. Note the preservation of the ERG a- and b-waves in the ischemic TNF-α antibody-injected animals compared with the ischemic vehicle-injected animals.


