Necrosis and Apoptosis after Retinal Ischemia: Involvement of NMDA-Mediated Excitotoxicity and p53

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Purpose. Accumulated evidence has shown that apoptosis and necrosis contribute to neuronal death after ischemia. The present study was performed to study the temporal and spatial patterns of neuronal necrosis and apoptosis after ischemia in retina and to outline mechanisms underlying necrosis and apoptosis.

Methods. Retinal ischemia was induced by increasing intraocular pressure to a range of 160 mm Hg to 180 mm Hg for 90 minutes in adult rats. The patterns of neuronal cell death were determined using light and electron microscopy and were visualized by Tdt-dUTP nick-end labeling (TUNEL). The mRNA expression profile of p53 was examined using reverse transcription-polymerase chain reaction (RT-PCR) and in situ hybridization histochemistry. Immunohistochemistry was performed using anti-p53, anti-microtubule associated protein-2, and anti- glial fibrillary acidic protein antibodies.

Results. Within 4 hours after ischemia, neurons in the inner nuclear cell layer (INL) and ganglion cell layer (GCL) underwent marked necrosis, made apparent by swelling of the cell body and mitochondria, early fenestration of the plasma membrane, and irregularly scattered condensation of nuclear chromatin. After 3 days, the INL and GCL neurons showed further degeneration through apoptosis marked by cell body shrinkage, aggregation, and condensation of nuclear chromatin. Apoptotic neurons were also observed sparsely in the outer nuclear cell layer. Intravitreal injections of MK-801 prevented early neuronal degeneration after ischemia. Of note, mRNA and protein levels of p53, the tumor suppressor gene known to induce apoptosis, were increased in the retinal areas undergoing apoptosis 1 to 3 days after ischemic injury.


Glutamate, an excitatory neurotransmitter, becomes toxic through overactivation of receptors sensitive to N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate.1,2 Intra-cellular accumulation of Ca2+ is known to cause glutamate-mediated neuronal degeneration.3 Glutamate neurotoxicity is marked by swelling of the cell body and mitochondria, early fenestration of the plasma membrane, and irregularly scattered condensation of nuclear chromatin. After 3 days, the INL and GCL neurons showed further degeneration through apoptosis marked by cell body shrinkage, aggregation, and condensation of nuclear chromatin. Apoptotic neurons were also observed sparsely in the outer nuclear cell layer. Intravitreal injections of MK-801 prevented early neuronal degeneration after ischemia. Of note, mRNA and protein levels of p53, the tumor suppressor gene known to induce apoptosis, were increased in the retinal areas undergoing apoptosis 1 to 3 days after ischemic injury.

ischemic necrosis and apoptosis appeared in the retina. The present study was performed to investigate temporal and spatial degeneration patterns of retinal neurons exposed to ischemia. In addition, the expression profiles of p53 mRNA and protein and NMDA-mediated excitotoxicity were also analyzed to correlate with cell death patterns.34

**MATERIALS AND METHODS**

**Induction of Retinal Ischemia**

Retinal ischemia was induced as previously described with minor modifications.35 In brief, adult male Sprague-Dawley rats (180–220 g) were anesthetized intraperitoneally with 400 mg/kg chloral hydrate. A 30-gauge needle connected to a pneumatic pressure device was inserted into the anterior chamber to increase the intraocular pressure (IOP) to a range of 160 mm Hg to 180 mm Hg for 90 minutes. Retinal ischemia was monitored externally by absence of red reflex of the fundus, hardness of the eyeball, and the immediate return of the red fundus reflex after removal of the needle from the anterior chamber. In experiments to examine NMDA-dependent retinal degeneration after ischemia, 10 μl 5 mM MK-801, a selective NMDA antagonist, or vehicle was injected into the eye through a 10-μl Hamilton syringe inserted into the vitreous chamber 15 minutes before ischemic injury.

Animals were euthanatized 4 hours or 1, 3, 7, or 14 days after reperfusion. Eyes were removed, immersed in 10% formalin for hematoxylin–eosin staining (n = 10 for condition), or fixed and processed for electron microscope study after removing the cornea and lens (n = 6 for condition). An additional group of eyes (n = 8–10 for each) were removed and immediately processed to isolate total RNA for reverse transcription–polymerase chain reaction (RT-PCR). For experiments with in situ hybridization histochemistry, eyes (n = 6 for condition) were quickly frozen on dry ice after the superior limbus was marked. All animals were used in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Quantitative Analysis of Retinal Lesion**

Enucleated eyes were fixed in 2% paraformaldehyde plus 2% glutaraldehyde in phosphate-buffered saline (PBS) and postfixed in 1% osmium tetroxide. Blocks of retina were embedded in epoxy resin, cut at 1-μm thickness, and stained with 1% toluidine blue. The ischemic injury was manifested by the decrease in retinal thickness. To determine neuronal survivorship in the retinal layers, the number of viable neurons was counted in 100 X 25-μm squares (five squares randomly chosen in each rat; three rats per experimental condition) underlying GCL or INL in retinal sections stained with toluidine blue. The ischemic injury was manifested by the decrease in retinal thickness.

**Statistical analysis** between groups was performed using analysis of variance and the Student-Newman–Keuls test.

**Transmission Electron Microscopy**

Transmission electron microscopy was performed as described.36 In brief, retinal tissues were fixed in Karnovsky’s fixative solution (2% paraformaldehyde, 2% glutaraldehyde, 2 mM calcium chloride, and 100 mM cacodylate buffer [pH 7.4]). The tissues were washed with cacodylate buffer, postfixed in 1% osmium tetroxide, and dehydrated in a series of graded ethanol. The tissues were then embedded in Araldite resin (Taab Laboratory Equipment, Alder Mastro, UK). Ultrathin sections were mounted on grids, stained with uranyl acetate and lead citrate, and examined with an electron microscope (model 902A; Carl Zeiss, Oberkochen, Germany).

**Reverse Transcriptase–Polymerase Chain Reaction**

The retina was removed from each eye immediately, 4 hours, 1 day, and 3 days after elevation of IOP to 160 mm Hg to 180 mm Hg for 90 minutes. Total RNA was isolated by a modified guanidine isothiocyanate-phenol method (Ultraspec-III, Biotecx Laboratories, Houston, TX). To prepare single-stranded cDNA, 5 μg total RNA was incubated in a reaction mixture containing oligo(dT)12–18, 1 mM each deoxycytidine triphosphate, deoxyguanosine triphosphate, and deoxytymidine triphosphate; 1 U/μl Rnasin; and 0.6 U/μl AMV reverse transcriptase at 42°C for 90 minutes. The PCR reaction was performed using 1/100 of synthesized single-stranded cDNA as a template and specific sets of oligonucleotide primers for each gene. The annealing temperature was adjusted to 58°C for p53 and 59°C for actin. The sequences of primer sets for each gene and the size of products are as follows: p53, forward primer 5'-GCCATCTACAAGAAGTCATGCAGC-3' and reverse primer 5'-GATGATGATGAAAGTAGGTCGGG-3' (expected size, 362 bp); actin, forward primer 5'-GAGATATGCGCAGCGCTCGTGCAAGC-3' and reverse primer 5'-AGCCAGTCCAGACGAGATGGCACTG-3' (expected size, 538 bp). After polymerization, the reaction mixture was subjected to electrophoresis on a 2% agarose gel.

**In Situ Hybridization**

In situ hybridization experiments using [35S] labeled cRNA probes were performed as described previously.35

**Probe Synthesis**

The p53 cDNA was prepared by an RT-PCR reaction of total RNA extracted from retinal tissues using the primers described. The sequence analysis of amplified cDNA verified a 362-bp product corresponding to part of exons 5 and 6 of rat p53. The PCR product was inserted in the EcoRV site of pGEM-5zf (+). The antisense probes for p53 were labeled with [35S]Juridine triphosphate (UTP), using T7 polymerase.

**Tissue Section and Prehybridization**

Whole eyes were horizontally sectioned on a cryostat at −23°C, 10-μm-thick sections were thaw-mounted onto slides pretreated with gelatin, and sections were stored at −80°C. Sections were dried at room temperature, fixed in 3% paraformaldehyde in 0.1 M PBS (pH 7.4) for 5 minutes at room temperature, and then acetylated for 10 minutes in 0.1 M triethanolamine (pH 8.0) with 0.25% acetic anhydride. The sections were then dehydrated through a series of graded ethanol (70%, 80%, 95%, and 100%) and air dried.

**Hybridization and Autoradiography**

Sections were then hybridized with [35S]UTP-labeled cRNA probes in a reaction buffer containing 40% deionized formamide, 2X SSC, 1 mg/ml tRNA, 1 mg/ml sperm DNA, 10% dextran sulfate, 10 mM dithiothreitol, and 1X Denhardt’s solution at 50°C for 4 hours. During the hybridization, sections were covered with parafilm. Immediately after the hybridiza-
Figure 1. Temporal pattern of degeneration caused by retinal ischemia. The retina was exposed to ischemic injury by increasing the IOP to a range of 160 mm Hg to 180 mm Hg for 90 minutes. (A) Bright-field photomicrographs of retinal sections stained with hematoxylin-eosin (a, b) or TUNEL (c, d) 1 day after a sham operation (a, c) or retinal ischemia (b, d). Note that the ischemic injury produced dark, shrunken, condensed neurons (arrows). (B) Bright-field photomicrographs of retinal sections immunolabeled with anti-MAP-2 (a, b, arrows indicate MAP-2-positive neurons) or anti-GFAP (c, d, arrows indicate GFAP-positive glia) 3 days after a sham operation (a, c) or retinal ischemia (b, d). (C) Quantitative analysis of retinal damage after ischemia. The number of viable neurons in a 100 x 25 μm square overlying the GCL or the INL was analyzed after staining with toluidine blue (mean ± SEM; n = 15 fields randomly chosen from three rats per condition), scaled to the mean number of viable neurons in the corresponding retinal area after a sham operation (set at 100). *Significant difference from relevant control (CTRL) at P < 0.05, using analysis of variance and the Student-Newman-Keuls test. IPL, inner plexiform layer; OPL, outer plexiform layer; ONL, outer nuclear layer. Bar, (A, B) 50 μm.
INL

FIGURE 2. Occurrence of early neuronal necrosis and latent apoptosis after 90 minutes of retina ischemia. Electron photomicrographs of retinal sections prepared 4 hours (left), 1 day (middle), and 3 days (right) after a 90-minute ischemic injury. Note that neurons in the INL and the GCL 4 hours after ischemia showed necrotic morphology marked by swelling of the cell body and mitochondria (mi), disintegration of the plasma membrane (pm), and scattering of nuclear chromatin with relatively conserved nuclear membrane (nm). Within 1 day after ischemia, some neurons in the inner nuclear layer (INL) and outer nuclear layer (ONL) underwent apoptosis (*) with cell body shrinkage, aggregation, and condensation of nuclear chromatin. The INL showed no further degeneration 3 days later, whereas a few neurons in the ONL showed apoptosis-like degeneration. Bar, 5 μm.

Immunohistochemistry and TdT-dUTP Nick-End Labeling

The retina was fixed in 2% paraformaldehyde for 2 hours, washed in PBS, dehydrated in alcohol, and embedded in paraffin. The paraffin-embedded retina was sectioned on a microtome at room temperature at a thickness of 5 μm, deparaffinized in xylene for 10 minutes, and rehydrated in a graded alcohol series. For immunocytochemistry using antibodies specific for microtubule associated protein-2 (anti-MAP-2) or glial fibrillary acidic protein (anti-GFAP), 40 μm-thick vibratome sections were prepared. The retinal sections were incubated in 2% H₂O₂ for 5 minutes and then in 20% normal horse serum containing 0.05% Triton X-100 for 1 hour. The sections were reacted with anti-p53 at room temperature for 2 hours or anti-MAP-2 or anti-GFAP at 4°C for 10 to 12 hours. The sections were then incubated in biotinylated goat anti-mouse IgG for 10 minutes and ABC reagents (Vectorstain; Vector, Burlingame, CA) for 10 minutes and then reacted with 3-amin-9-ethylcarbazole and 0.01% H₂O₂ (HRP/AEC; Laboratory Vision, Fremont, CA). The immunolabeled sections were counter-stained with hematoxylin (for p53) or observed under a microscope with a differential interference contrast filter (MAP-2 and GFAP).

For TdT-dUTP nick-end labeling (TUNEL) staining, the paraffin-embedded sections were treated with 20 μg/ml proteinase K and incubated in 3% H₂O₂ for 5 minutes. As previously reported, DNA was end-labeled at 3' using biotinylated dUTP and terminal deoxyuridyltransferase in a reaction buffer containing 30 mM Tris, 140 mM sodium cacodylate, and 1 mM cobalt chloride at 37°C for 2 hours. The signal was visualized with an ABC staining kit (TACS apoptotic DNA laddering kit, Trevigen, Gaithersburg, MD) using diaminobenzidine as a substrate.

RESULTS

Temporal Degeneration of Retinal Neurons after Retinal Ischemia

Elevation of the intraocular pressure to 160 mm Hg to 180 mm Hg for 40 to 100 minutes resulted in widespread neuronal...
Figure 3. The neuroprotective effect of MK-801 against retinal ischemia. Rats underwent 90 minutes of ischemia 15 minutes after intravitreal injections of 10 μl saline (CTRL) or 5 mM MK-801 (MK). MK-801-treated rats also received additional injections of 5 mM MK-801 24 and 48 hours after ischemia (MK(multi)). Neuronal death in the GCL and INL was analyzed 4, 24, and 72 hours later by counting viable neurons in 100 X 25-μm square (mean ± SEM; n = 15 fields randomly chosen from three rats per condition), as described in Figure 1. *Significant difference from relevant control in viable neurons (P < 0.05; analysis of variance and Student-Newman-Keuls test).

Degeneration throughout the retina over the 1 to 3 days after ischemia. For the present study, the 90-minute retinal ischemia paradigm was chosen to produce reproducible moderate neuronal injury manifested by a decrease in retinal thickness. Neurons in the GCL and INL showed pyknotic nuclei and DNA fragmentation revealed by the TUNEL stain within 1 day after ischemia (Fig. 1A). This neuronal injury was confirmed by a marked decrease in MAP-2 immunoreactivity throughout the cell bodies and processes in the INL, inner plexiform layer, and GCL 3 days later, whereas glial cells immunoreactive to GFAP were not decreased in number, but rather increased in intensity (Fig. 1B). Neurons in the INL and GCL underwent rapid degeneration by 30% to 40% within 4 hours after retinal ischemia (Fig. 1C). The number of degenerating neurons was further increased in the retinal layers. Three days after ischemia, surviving neurons in the GCL and INL were approximately 40% in the sham-operation control (Fig. 1C). The retinal thickness gradually decreased over 7 days after ischemia. At this point, the INL and GCL neurons were intermingled but did not show further degeneration.

**Necrosis and Apoptosis of Retinal Neurons after Ischemia**

Electron microscopy showed that most of the degenerating neurons in the INL and GCL 4 hours after retinal ischemia accompanied swelling of the cell body and mitochondria, a marked collapse of the plasma membrane with preservation of the nuclear membrane, and irregular scattering of nuclear chromatin (Fig. 2). All these effects are typically seen in the course of necrosis. The necrotic neurons were rarely detectable 1 day after ischemia. Instead, neurons in the INL and GCL showed apoptosis-like morphologic changes characterized by aggregation and condensation of nuclear chromatin and cell body shrinkage 1 day later. Neurons in the outer nuclear layer also underwent apoptotic degeneration gradually over 1 to 7 days after ischemia, although it was sparsely distributed.

**Effect of MK-801 against Ischemic Retinal Degeneration**

Experiments were performed to study the possibility that the NMDA receptor antagonist MK-801 would protect against the necrosis component of neuronal death in ischemia. The intravitreal injections of MK-801 substantially attenuated the necrosis of neurons appearing in the GCL and INL 4 hours after retinal ischemia (Fig. 3). The neuroprotective effect of MK-801 lasted over the next 1 to 3 days, but neurons in the GCL and INL continued to undergo extensive degeneration over time. Repeated injections of MK-801 every 24 hours after ischemia did not show additional protection. This implies that the delayed neuronal apoptosis in retina occurs in an NMDA-independent fashion.
Upregulation of p53 mRNA and Protein

The expression pattern of mRNA encoding p53 or actin was analyzed at 4 hours, and 1, 3, and 7 days after retinal ischemia. RT-PCR experiments showed that levels of actin mRNA in the retina remained unchanged at any time tested after ischemia. Retinal ischemia did not change the mRNA expression of p53 over the next 4 hours. The levels of p53 mRNA were significantly increased 1 day after ischemia (Fig. 4). Thereafter, the ischemia-induced expression of p53 mRNA seemed to decline but was still evident 3 days later. The temporal induction of p53 mRNA expression after ischemia was confirmed by in situ hybridization histochemistry using [35S]-labeled antisense cRNA probes (Figs. 4B, 4C, 4D, 4E, 4F). p53 mRNA expression in the retina returned to the control level within 7 days after ischemia. In emulsion-dipped sections after hybridization with p53 cRNA probe, an increase in the p53 mRNA hybridization signal was observed exclusively in neurons overlaying the GCL and INL 1 day after ischemia (Fig. 5B). Immunoreactivity to p53 also increased in the GCL and INL neurons 1 day after ischemia (Fig. 5D), which suggests that retinal ischemia increases levels of p53 protein and mRNA.

DISCUSSION

The present study shows that retinal neurons undergo two morphologically distinct patterns of degeneration after ischemic injuries. One evolves rapidly in the INL and GCL within 4 hours, marked by prominent necrosis and prevented by blocking NMDA receptors. The second phase of neuronal death was observed in all retinal layers over the next 1 to 3 days. The slowly evolving death was characterized by shrinkage of the cell body, aggregated condensation of nuclear chromatin, and expression of p53 primarily in the GCL and INL.

Excitotoxicity has been well documented as a primary cause of neuronal death in ischemia in the retina and the brain.23 Glutamate antagonists protect retinal neurons against ischemic insult in vivo and in vitro.30,32 Neurons treated with excitotoxins show the typical morphology of necrosis accompanied by cell body swelling, early disruption of plasma membrane, and irregularly scattered condensation of nuclear chromatin.6 Excitotoxicity was observed in the course of the rapidly evolving necrosis of the GCL and INL neurons after retinal ischemia. In addition, the administration of MK-801 substantially prevented the necrotic degeneration of retinal neurons induced by ischemia. The present findings support the possibility that retinal ischemia produces necrosis of the GCL and INL neurons in part through the activation of NMDA receptors.

Although necrosis is a predominant form of neuronal death in ischemia, accumulating evidence supports apoptosis as an additional pattern of death. For example, internucleosomal DNA fragmentation, chromatin condensation, and cycloheximide-sensitive death have been observed in degenerating areas after ischemic insults in brain and spinal cord.30 Mixed patterns of necrosis and apoptosis have been shown in GCL and INL neurons after retinal ischemia.33 We also found that retinal ischemia produced slowly evolving neuronal apoptosis subsequent to the rapid onset of necrosis in the INL and GCL neurons. In addition, apoptotic neurons were sparsely and continually observed in the outer nuclear layer at late time points after ischemia.

Even though the two different types of death, apoptosis and necrosis, both contribute to ischemic injury, the underlying mechanisms remain to be delineated. Recently, neurons...
deprived of oxygen and glucose have been shown to undergo slowly evolving and cycloheximide-sensitive apoptosis when excitotoxic necrosis is blocked, indicating that ischemic apoptosis may occur through nonexcitotoxic mechanisms. This is supported by the present results showing that the intravitreal injections of MK-801 attenuate the early neuronal necrosis without interfering with the slowly evolving neuronal death in retina after ischemia. Parallel occurrence of excitotoxicity and apoptosis has been observed in cortical areas after focal cerebral ischemia. Expression of c-Jun, Bax, and cyclooxygenase-2 is increased in degenerating neurons after brain ischemia, supporting the present results showing that the intravitreal injections of MK-801 attenuate the early neuronal necrosis and increase in p53 immunoreactivity after ischemia. Bar, 50 μm.

The p53 tumor suppressor gene is upregulated in neurons vulnerable to brain ischemia or adrenalectomy. Although these molecular and cellular events are thought to play a role in the process of ischemic neuronal degeneration in the retina, further study is needed to determine whether they are specifically required for execution of apoptosis, not excitotoxic necrosis, after retinal ischemia.

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FIGURE 5. Concurrent induction of p53 mRNA and protein after retinal ischemia. Retinal sections prepared 1 day after a sham operation (A, B) or 90 minutes of ischemia (C, D) were hybridized with a [35S]-labeled antisense cRNA probe for p53 (A, C) or immunolabeled with an anti-p53 antibody (B, D). (A, C) Dark-field photomicrographs of the emulsion-dipped sections showing ischemia-induced increase in p53 mRNA hybridization signal in retina (arrows). (B, D) Bright-field photomicrographs of retinal sections showing ischemia-induced increase in p53 immunoreactivity (arrows). Bar, 50 μm.

days after retinal ischemia. Increase in p53 expression does not necessarily mean causality in the delayed neuronal apoptosis, but the expression pattern in the apoptosis zone probably contributes to the progress of neuronal apoptosis in retinal ischemia. In support of this, neuronal loss in ischemia in the retina and brain was reduced in transgenic mice deficient in p53. Neuronal death dependent on p53 in the hippocampus after seizure. These findings suggest that p53 plays a dynamic role in the process of apoptotic neuronal degeneration after brain injuries such as ischemia or seizure.

The overexpression of p53 has been shown to downregulate Bcl-2 expression and to upregulate Bax expression in vitro. The p53-dependent increase in Bax protein was also observed in the degenerating cortical and hippocampal neurons. The transgenic expression of Bcl-2 reduces the size of ischemic brain lesion in mice and rats. It is conceivable that the Bcl-2 family is coupled to downstream events in the p53-mediated apoptosis pathway after ischemia.

The notion that necrosis is the sole pattern of ischemic neuronal death has been challenged by a flood of evidence showing apoptosis after ischemic insults. Our findings suggest that retinal ischemia causes the fulminant neuronal necrosis of the GCL and INL neurons within several hours, in part through the activation of NMDA receptors. In contrast, apoptosis appears as the latent component of neuronal death in the same retinal areas. Cooperative strategy to counteract apoptosis (e.g., modulation of p53 expression) and excitotoxic necrosis (e.g., block of NMDA neurotoxicity) should be contemplated for efficient protection of neurons against ischemia.

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References


