Apoptosis and Caspasases after Ischemia–Reperfusion Injury in Rat Retina

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PURPOSE. Extensive cell loss in the retinal ganglion cell layer (RGCL) and the inner nuclear layer (INL) was noted in a rat model of retinal ischemia–reperfusion injury by transient elevated intraocular pressure (IOP). The possible involvement of apoptosis and caspases was examined in this model of neuronal loss.

METHODS. Transient elevated IOP was induced in albino Lewis rats through the insertion of a needle into the anterior chamber connected to a saline column. Elevated IOP at 110 mm Hg was maintained for 60 minutes. Groups of animals were euthanatized at various times after reperfusion, and their retinas were evaluated by morphologic, agarose gel electrophoresis of DNA, in situ terminal deoxynucleotidyl transferase-mediated biotin-deoxyuridine triphosphate nick-end labeling (TUNEL), and morphometry. YVAD.CMK, a tetrapeptide inhibitor of caspases, was used to examine the involvement of caspases.

RESULTS. A marked ladder pattern in retinal DNA gel analysis, typical of internucleosomal DNA fragmentation and characteristic of apoptosis, was present 12 and 18 hours after reperfusion. Labeling of nuclei in the RGCL and the inner nuclear layer (INL) by TUNEL was noted between 8 and 18 hours after reperfusion. Histologic and ultrastructural features typical of apoptosis were also observed in the inner retina after ischemia. YVAD.CMK administered during the ischemic period inhibited apoptotic fragmentation of retinal DNA and ameliorated the tissue damage. When administered intravitreally 0, 2, or 4 hours after reperfusion, YVAD.CMK was also effective in preserving the inner retina but had no significant effect when administered 6 or 8 hours after reperfusion. The inner retina showed transient elevated immunoreactivity of caspases II and III 4 and 8 hours after reperfusion.

CONCLUSIONS. Retinal ischemia–reperfusion after transient elevated IOP induced apoptosis of cells in the retinal ganglion cell layer and the INL. Caspases may have a pivotal role in the early events of the apoptotic pathway(s). Rescue by using anti-apoptotic agents after ischemia–reperfusion is feasible. (Invest Ophthalmol Vis Sci. 1999;40:967-975)

Apoptosis, a highly regulated and energy-dependent form of cell death, has characteristic histologic and morphologic features showing scattered involvement of individual cells in an asynchronous pattern, a minimum or an absence of inflammatory reaction around the dying cells, intact cytoplasmic membrane, perinuclear chromatin condensation with subsequent nuclear disintegration, and apoptotic cell bodies.1,2 Biochemically, it is characterized by internucleosomal double-stranded DNA cleavage, producing DNA fragments that are multiples of 180 bp to 200 bp in length and therefore show a typical ladder pattern in DNA gel electrophoresis.1,2 In contrast, the random breakdown of DNA in necrosis shows a smear in gel electrophoresis.1,2 The double-stranded DNA nicks in apoptosis can be demonstrated histochemically in situ with the terminal deoxynucleotidyl transferase (TdT)-mediated biotin-deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) technique in paraffin sections.3 Using these criteria and methods, we noted apoptosis in the retinas of the retinal dystrophic Royal College of Surgeons (RCS) rat, and in rat retinas after light injury or N-methyl-D-aspartate-induced excitotoxicity.6 Others have also noted its involvement in retinal degeneration in the RCS rat,7 rd1-9 and rds mice,9 and transgenic mice carrying mutations in the rhodopsin gene.9 Clinically, apoptosis has been shown to occur in retinas with glaucoma,10 traumatic detachment,11 macular degeneration,12 retinoblastoma,13 or retinitis pigmentosa.14 Therefore, apoptosis seems to be the final common pathway of retinal neuronal degeneration.

Retinal degeneration after ischemia–reperfusion injury by transient elevation of intraocular pressure (IOP) in rats shows extensive loss of neuronal elements of the retinal ganglion cell layer (RGCL) and the inner nuclear layer (INL).15 These features closely resemble those after retinal ischemic insult and acute glaucoma.16 Certain ultrastructural features typical of apoptosis have been noted among the dying cells in a similar animal model.16 Therefore, it seems possible that apoptosis...
may have an important role in ischemia-reperfusion insult to the retina.

Yuan and Horvitz\textsuperscript{19} reported the requirement of \textit{ced-3} and \textit{ced-4} genes for apoptotic cell death in the nematode \textit{Caenorhabditis elegans}. Subsequent sequence analysis of the gene products by Miiuru et al.\textsuperscript{20} found a highly conserved noncysteine-rich region of the \textit{CED-3} protein homologous to the sequence of the human interleukin-1B-converting enzyme (ICE), a cysteine protease, and suggested that ICE may have a central role in apoptotic cell death in mammalian cells. The exact human genes corresponding to \textit{ced-3} in \textit{C. elegans} remains undefined, but various studies have shown that ICE-like enzymes or caspases may play a pivotal role in apoptosis in mammalian cells.\textsuperscript{21,22} Whether caspases play similar significant roles in retinal degeneration is not clear.

In the present study, we showed that the loss of inner retinal elements after retinal ischemia-reperfusion insult with transient elevated IOP involved apoptosis. In addition, we used immunohistochemistry and a caspase inhibitor, the tetrapeptide YVAD.CMK,\textsuperscript{23} to probe the possible involvement of caspases in apoptosis of retinal neurons after ischemia-reperfusion insult. We also explored the timing of the window for effective treatment with YVAD.CMK after the insult.

**Materials and Methods**

**Induction of Transient Elevated Intraocular Pressure**

Transient elevated IOP was induced with an established method\textsuperscript{18} in adult male albino Lewis rats (Harlan, Indianapolis, IN), 45 to 50 days old. Briefly, the animals were anesthetized with intraperitoneal injections of 400 mg/kg chloral hydrate (Sigma, St. Louis, MO). After topical application of 0.5% proparacaine hydrochloride, the anterior chamber was cannulated with a 26-gauge needle connected to a normal saline container (Sigma, St. Louis, MO). Intraocular pressure was raised to 110 mm Hg by elevating the saline container. Body temperature of the animals was maintained at 37.5°C with a probe and heating pad during the experimental period. The infusion needle was removed from the anterior chamber 60 minutes later. Reperfusion of the retinal vasculature was confirmed by fundus examination. The animals were then euthanatized at various times, and their eyes were enucleated for morphologic, morphometric, and immunohistochemical studies; DNA agarose gel electrophoresis analysis; or TUNEL assay. All procedures involving animals were in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research.

**Agarose Gel Electrophoresis of Retinal DNA**

The eyes enucleated for DNA agarose gel electrophoresis were bisected at the ora serrata, and the lenses were removed. The retinas were dissected from the underlying tissues, transferred to liquid nitrogen, and kept frozen in −70°C until analysis with a previously described procedure.\textsuperscript{3} Briefly, the frozen retinas were thawed and vortexed in a buffer containing 50 mM EDTA, 0.5% sodium dodecyl sulfate (SDS), and 20 mM Tris-HCl (pH 8.0). RNase A was added to a final concentration of 50 μg/ml. After incubation for 30 minutes at 42°C, proteinase K was added to a final concentration of 400 μg/ml. The samples were then incubated at 55°C until clear lysates were produced. The lysates were extracted with phenol/chloroform/isooamyl alcohol (25:24:1) and chloroform/isooamyl alcohol (24:1), respectively. DNA was precipitated with 3 M sodium acetate and ice-cold ethanol and analyzed for internucleosomal cleavage by electrophoresis using a 2% agarose gel (10 μg DNA per lane). DNA in the gel was visualized by UV light after ethidium bromide staining and photographed (MP-4 system; Polaroid, Cambridge, MA).

**Morphologic Study**

Eyes enucleated for morphologic and immunohistochemical studies were fixed in 10% buffered formaldehyde. The anterior segment was removed, and the posterior segment was prepared for paraffin sectioning. Morphologic evaluations were performed on 5-μm sections stained with hematoxylin and eosin.

Eyes were used for electron microscopy 18 hours after reperfusion. The enucleated eyes were fixed in 1% glutaraldehyde and 4% paraformaldehyde in phosphate buffer for 1 hour. The anterior segment was removed, and the whole eyecup was divided into four strips, one each from the nasal, inferior, temporal, and superior quadrants. Samples were postfixed by 1% osmium tetroxide, dehydrated with graded alcohol and propylene oxide, embedded in epoxy resin, sectioned, and stained with Mallory's azure II-methylene blue. Ultrathin sections containing representative areas were obtained for electron microscopy.

**In Situ TUNEL**

In situ TUNEL was performed according to the procedure described by Gavioli et al.\textsuperscript{5} except that the proteinase K digestion step was omitted. The 5-μm paraffin sections obtained from the preceding section were used. The tissue sections were deparaffinized, rehydrated, and incubated in methanol containing 3% H₂O₂ to block the endogenous peroxidase. They were washed in distilled water, and TdT buffer (30 mM Trizma base [pH 7.2], 140 mM sodium cacodylate, 1 mM cobalt chloride). The sections were incubated with TdT buffer containing TdT and dUTP for 60 minutes, and then with TB buffer (300 mM sodium chloride, 30 mM sodium citrate) to terminate the reaction. They were washed with double-distilled water, incubated with peroxidase-conjugated streptavidin, and developed with 3,3-diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO).

**Caspase Inhibitor Study**

The effects of YVAD.CMK, a tetrapeptidyl caspase inhibitor, on apoptotic and tissue responses were evaluated. Vehicle (0.1 M phosphate-buffered saline [PBS]; pH 7.2), or 100 μM YVAD.CMK\textsuperscript{25,26} (Bachem, Philadelphia, PA) in PBS was introduced into the anterior chamber during the induction of elevated IOP through the needle and Silastic tubing, as previously described.\textsuperscript{23} Animals were killed 12 hours after reperfusion for DNA analysis by electrophoresis, when the characteristic ladder pattern was apparent, or at 7 days after reperfusion for morphometric evaluation, when the tissue responses were established. A second series of experiments was conducted to determine the timing of the window for the effectiveness of the caspase inhibitor YVAD.CMK. Groups of animals received 2 μl of 500 μM YVAD.CMK intravitreally at 0, 2, 4, 6, or 8 hours
after the initial reperfusion of retinas previously exposed to 60 minutes of ischemia and were euthanatized 7 days after reperfusion, when the tissue responses had stabilized. The effectiveness of the treatment was evaluated morphometrically with inner retinal thickness (IRT) measurements.\(^{15}\)

Morphometry

Tissue response after retinal ischemia-reperfusion injury was evaluated using a previously published method: measurement of the IRT.\(^{15}\) Tissue sections were prepared as described in the morphologic study with electron microscopy. The IRT was assessed by measuring the thickness between the internal limiting membrane and the interface of the outer plexiform layer and outer nuclear layer (ONL) on the projected image of a stained 1-μm section onto a digitizing pad coupled with a customized image-processing work station. Two readings were obtained for each tissue sample. The average readings of the four quadrants of the same eye represented the mean of the whole eye. Analysis of variance and Student's t-test were used for statistical comparisons.

Immunohistochemistry of Caspases

Paraffin sections obtained from the morphologic study were deparaffinized, rehydrated, and incubated in 10 mM sodium citrate buffer pH 6.0 at 90°C for 40 minutes. Endogenous peroxidases were blocked with incubation in 3% H\(_2\)O\(_2\) for 4 minutes. The sections were permeabilized in 0.3% Triton X-100 for 1 hour. Anti-caspase II (anti-ICh1; 1:100) or anti-caspase III (anti-CPP32; 1:100; Transduction Laboratory, Lexington, KY) was added to the sections separately and incubated at 4°C overnight. Immunoreactivity was detected using the standard ABC complex (StreptABC Complex; Dako, Carpinteria, CA) and DAB.

RESULTS

Agarose gel electrophoresis of DNA samples from retinas at 0 to 48 hours of reperfusion after 60 minutes of ischemia by elevated IOP showed a recognizable ladder pattern at 12 and 18 hours after reperfusion (Fig. 1; lanes 5 and 6, respectively).

Histopathologically, at 4 hours, the retina showed occasionally condensed nuclei in the RGCL, markedly edematous inner plexiform layer (IPL) and condensed nuclei with various degrees of cytoplasmic vacuolation in the INL, especially in the inner part of the INL (Fig. 2B). The outer retina was unremarkable. At 8 hours (Fig. 2C), condensed nuclei scattering in the RGCL and the INL and various degrees of cytoplasmic vacuolation in the INL persisted. At 12 (Fig. 2D) and 18 (Fig. 2E) hours, scattered condensed nuclei remained in the RGCL and the INL, whereas vacuolation in the INL subsided. After 48 hours (Fig. 2F), only occasionally condensed nuclei were still seen in the RGCL. The IPL was markedly atrophic. Thinning of the INL was noted. The ONL remained unremarkable throughout the observation period.

Figure 3 depicts TUNEL of a normal retina (A) and retinas at 4 (B), 8 (C), 12 (D), 18 (E), or 48 (F) hours of reperfusion after 60 minutes of ischemia. At 8 hours of reperfusion (Fig. 3C) there were obvious TUNEL-positive nuclei scattered in the RGCL and the INL (especially the inner part of the INL), some with a ringlike staining pattern. More evenly stained nuclei in the RGCL and the INL (especially the inner part of the INL) were noted at 12 (Fig. 3D) and 18 hours (Fig. 3E) of reperfusion, but no labeled nuclei in the RGCL and INL were noted at 48 hours of reperfusion (Fig. 3F). Positively labeled nuclei were seen only occasionally in the ONL (not shown).

Ultrastructural studies of the sections obtained 18 hours after reperfusion showed cells with typical features of apoptotic cells. Perinuclear condensation of the nuclear chromatin and apoptotic bodies in the RGCL were noted (Fig. 4A). Scattered condensed cells were also present in the INL (Fig. 4B). The cytoplasm and the nuclear chromatin were condensed, and the formation of apoptotic bodies was noted.

Figure 5 shows the gel analysis of retinal DNA from animals at 12 hours of reperfusion after 60 minutes of ischemia, with or without the caspase inhibitor YVAD.CMK treatment. Without YVAD.CMK, there were typical ladder patterns in retinal DNA gel analysis (lanes 2 and 3) characteristic of apoptotic cell death. This ladder pattern was inhibited by treatment with 100 μM YVAD.CMK (lanes 4 and 5), similar to results in our previous report with MK801, which also inhibited the formation of the ladder pattern in retinal DNA analysis after ischemia.\(^{26}\) Figure 6 shows the retinas at 7 days of reperfusion, with or without YVAD.CMK treatment. The YVAD.CMK-treated retina (Fig. 6B) shows significantly better preserved inner retina than does the vehicle-treated control (Fig. 6A). This ameliorative effect was confirmed by measuring the IRT (Fig. 7) 7 days after the insult. YVAD.CMK treatment totally abolished the loss of IRT (Fig. 7; \(P < 0.001\)). In addition, YVAD.CMK administered intravitreally from 0 to 4 hours of reperfusion also abolished the loss of IRT when measured 7 days after the insult, whereas YVAD.CMK administered at 6 or 8 hours of reperfusion showed no rescuing effect (Fig. 8).

Immunohistochemical detection of caspases II (ICHI) and III (CPP32) showed similar changes; and therefore, only the results with anti-caspase III are shown, because the changes were more apparent. Normal rat retina showed minimum diffused background staining in all layers of the retina (Fig. 9A). Four hours after reperfusion, scattered nuclei with positive

![Figure 1. Agarose gel electrophoresis of retinal DNA from animals at various times of reperfusion after 60 minutes of ischemia. Lane 1: DNA markers, multiples of 126 bp; lanes 2, 3, 4, 5, and 7: 0, 4, 8, 12, 18, and 48 hours of reperfusion, respectively (10 μg DNA per lane).](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933211/ on 10/06/2018)
staining in the RGCL and the INL (especially the inner part of the INL) were noted (Fig. 9B). At 8 hours, immunoreactivity in the RGCL subsided, whereas that of the INL (inner part of the INL) persisted (Fig. 9C). At 12 hours, the staining of the retina (Fig. 9D) was similar to the normal (Fig. 9A).

**DISCUSSION**

In this study, we examined the possible involvement of apoptosis in the loss of retinal elements after ischemia-reperfusion insult. In addition to histologic and morphologic evidence, we noted the presence of internucleosomal DNA fragmentation, which elicited the typical ladder pattern in agarose gel electrophoresis of DNA, and TUNEL-positive nuclei in the RGCL and the INL but only occasionally in the outer retina. We were able to demonstrate that internucleosomal fragmentation of retinal DNA after the insult was inhibited by the caspase inhibitor, YVAD.CMK, resulting in tissue preservation. We also noted that YVAD.CMK was effective in ameliorating the neuronal loss when administered up to 4 hours after reperfusion but not afterward. Immunohistochemistry of caspases II and III showed an early transient increase of immunoreactivity in the inner retina. This evidence suggested a pivotal role of apoptosis in retinal ischemia-reperfusion injury; an important role of caspases in the early events of apoptosis of retinal elements after ischemia-reperfusion; that modulation of tissue response after ischemia-reperfusion insult with apoptotic modulators is feasible; and that there exists a time window of effective treatment against ischemia-reperfusion injury after the insult with apoptotic modulators.

In the central nervous system, neuronal death induced by hypoxia-ischemia had been presumed to be through necrosis. However, recent re-examination of the available data has suggested that apoptosis may make a major contribution. Evidence of ischemia-induced apoptosis has been noted in transient global cerebral ischemia, in which the CA1 hippocampal neurons died at 48 to 72 hours after the insult, and in focal cerebral ischemia. The involvement of apoptotic regulatory genes such as bcl-2 and p53 in cerebral ischemic injury has been demon-
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Strated using transgenic mice. In this report, we provide evidence that apoptosis also played a pivotal role in ischemia-reperfusion-induced neuronal loss in the retina, also part of the central nervous system.

Earlier ultrastructural studies by Büchi in a similar model showed typical apoptotic features. However, in his study, inflammatory reaction was noted, and the involvement of apoptosis was suggested to be variable. Biochemical evidence of internucleosomal fragmentation was absent. In addition, in Büchi's settings, the tissue damage was close to the maximum, with significant loss of ONL. In contrast, under our experimental conditions, only the occasional disappearance of photoreceptors and no inflammatory reaction were noted. Although our histologic and ultrastructural studies also suggested variations in the morphology of the degenerating cells (not shown), distinctive necrosis was not noted. Therefore, under our mild conditions, apoptosis may play a more prominent role than in severe conditions in which necrosis may be more prominent. The diverse ultrastructural features noted in Büchi's and our studies may also be variants of apoptotic cell death. In a recent report, Portera-Cailliau et al. also observed variations of ultrastructural changes in the excitotoxic death of central neurons. They proposed that there is a morphologic continuum in apoptosis-necrosis. However, more detail studies are needed to provide further evidence of this possibility.

Nett-Fiordalisi et al. and Henkart in inhibition studies have suggested that the role of caspases in apoptosis may be cell- and stimulus-specific. In this study, we noted the inhibition of internucleosomal DNA fragmentation by YVAD.CMK, suggesting the arrest of the apoptotic process after ischemia-reperfusion insult. By evaluating the tissue response 7 days after reperfusion, we also noted the efficacy of YVAD.CMK in ameliorating the tissue damage. In addition, we demonstrated an early effective window for treatment with YVAD.CMK. Therefore, it is likely that YVAD.CMK works through the block of the apoptotic pathway(s), probably by inhibiting the caspases, and that caspases may play a pivotal role in the early events of apoptosis after ischemia-reperfusion insults. Because YVAD.CMK has a broad spectrum of action against caspases, the specific caspase involved remains to be examined.

That caspases may play key roles in the early events of apoptosis of inner retinal neurons after ischemia-reperfusion insult is further supported by our observation on the immunohistochemical detection of caspases II (ICH1) and III (CPP32). An early and transient increase in immunoreactivity was noted in the inner retina after ischemia-reperfusion damage. This observation is consistent with our preceding hypothesis that there is an early involvement of caspases in the apoptotic pathway(s). Whether those cells that showed increased immu-

Figure 3. TUNEL of the inner retinas at various times after reperfusion. (A) Normal retina; (B) 4 hours; (C) 8 hours; note ringlike positive labeling pattern of nuclei scattered in the RGCL and the INL (especially the inner part of the INL); (D) 12 and (E) 18 hours; note more evenly labeled nuclei scattered in the RGCL and INL (especially the inner part of the INL); (F) 48 hours. Scale bar, 10 μm.
Figure 4. Electron micrographs of degenerating cells in the RGCL and the INL. (A) RGCL: note perinuclear condensation of nuclear chromatin and intracytoplasmic apoptotic body (arrow); (B) INL: note shrunken, dense cells (arrows) and formation of apoptotic bodies (arrowhead). Scale bar, (A) 2.5 μm; (B) 1 μm.

noreactivity go through apoptosis later is not known, but our results regarding the timing of the window for treatment with YVAD.CMK support the thesis that caspases may be involved in the early events of apoptosis of those neurons. Therefore, taking all the evidence together, it is likely that caspases may have an important role in the early events of apoptotic death of inner retinal neurons after ischemia–reperfusion insult. However, the exact regulatory role of caspases in the apoptotic pathway(s) is not clear. Poly-adenylribose polymerase39 and P21-activated kinases40 have been suggested to be the substrates for these proteases and may play important roles in the apoptotic pathways. The downstream events also remain to be examined.

It has been suggested that apoptotic modulators may be used for ameliorating cell death in degenerative diseases. This report presents results of a few in vivo studies showing the feasibility of this hypothesis in retinal degeneration. In addition, our window-of-treatment experiment showed that postinjury treatment with apoptotic modulators was effective. Whether there are other more effective modulators controlling later steps of the apoptotic pathway(s) affording a longer lasting window for effective treatment remains to be explored. We have noted the effectiveness of other inhibitors such as aurintricarboxylic acid,41 an endonuclease inhibitor, and 3-amino-benzamide,42 a poly-adenylribose polymerase inhibitor, in ameliorating the loss of retinal elements after ischemia–reperfusion insult. Therefore, it seems likely that agents with a longer lasting window for effective treatment may be found.

It is possible that these apoptotic changes may be caused by the elevated hydrostatic pressure on the retina. However, Siliprandi et al.43 showed that it is more likely that the injury is caused by ischemia–reperfusion insult. In addition, we noted similar apoptotic responses after intravitreal injection of the excitotoxin, N-methyl-D-aspartate,5 which is believed to play an important role in retinal ischemia–reperfusion injury.26 Nonetheless, the contribution of pressure-induced damage is not defined in this model.

Figure 5. Effect of YVAD.CMK on internucleosomal fragmentation of retinal DNA collected 12 hours after reperfusion. Lane 1: DNA markers, multiples of 126 bp; lanes 2 and 3: ischemia-reperfused retinas; note the characteristic ladder pattern of internucleosomal fragmentation of DNA; lanes 4 and 5: ischemia-reperfused retinas with YVAD.CMK treatment. The ladder pattern was inhibited compared with that in lanes 2 and 3 (10 μg DNA per lane; each lane represents a sample from 1 retina).
In summary, our observations that moderate ischemia-reperfusion insult induced apoptotic cell death and that blocking the apoptotic pathway(s) protected against retinal neuronal loss suggest a new approach to therapy for the loss of retinal elements after injury, namely the use of agents that block the apoptotic pathway(s). In addition, the application of the caspase inhibitor YVAD.CMK even 4 hours after reperfusion was as effective as at 0 hours, which suggests that there is a reasonable window for effective treatment in using these antiapoptotic agents. It is possible that other agents, such as
Acknowledgments

The authors thank Jun Fu and Suhui Li for their technical assistance and Ann Wong for her editorial assistance.

References


