The Role of the p53 Protein in the Selective Vulnerability of the Inner Retina to Transient Ischemia

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Purpose. To determine whether the p53 protein plays a role in the selective vulnerability of the inner retina to transient ischemia.

Methods. Transient retinal ischemia was induced using a high intraocular pressure (HIOP) model in the Sprague-Dawley rat for 60 minutes. Histopathologic outcome was determined 7 days after ischemia. In addition, analysis for evidence for apoptosis (TdT-dUTP terminal nick-end label [TUNEL] staining) and p53 protein expression (immunohistochemistry) was performed at several points during the reperfusion period. In a separate set of experiments, wild-type mice and two groups of transgenic mice, one homozygous and the other heterozygous for the *p53* null gene, were also subjected to HIOP for 60 minutes, and histopathology was performed 7 days later.

Results. At 7 days subsequent to 60 minutes of ischemia in the rat, there was marked thinning of the inner retinal layers. There were scattered TUNEL-positive cells within the inner retina, peaking at 24 to 48 hours and persisting for at least 7 days. p53 immunochemistry demonstrated elevated protein levels within the inner retina; this finding peaked at 24 to 48 hours but was no longer present at 4 days after ischemia. TUNEL staining of the inner retina of the mouse was most prominent 24 hours subsequent to ischemia but persisted at 48 hours. Seven days subsequent to 60 minutes of ischemia in the wild-type and transgenic mice, histopathologic evaluation demonstrated preservation of the retinal histoarchitecture in the heterozygous group compared with the wild-type or homozygous animals.

Conclusions. These data further support the hypothesis that the delayed cell death that occurs after transient retinal ischemia is, in part, apoptotic. In addition, they suggest a role for the p53 protein in the selective vulnerability of the inner retina to transient ischemia. p53 protein may be a target for future therapeutic agents in the treatment of disorders of the retina where ischemia plays a pathogenetic role. (*Invest Ophthalmol Vis Sci.* 1998;39:2132-2139)

Recent insights into the mechanisms of cell death have shed light on the pathophysiology of a number of retinal degenerative disorders.[1] Cell death can occur by two mechanisms, necrosis and apoptosis.[2] Necrosis is characterized by cell swelling, with early loss of plasma membrane integrity, nuclear swelling with major changes of the organelles, and prominent inflammatory infiltrates. It has been commonly associated with acute pathologic states such as ischemia, trauma, and radiation injury. In contrast, in apoptosis the nucleus and cytoplasm shrink and fragment with rapid phagocytosis by neighboring cells or macrophages. Apoptosis is a part of normal development of virtually all organs, and in the nervous system it leads to the orderly death of more than 50% of embryonic neurons. More recently, it has been recognized that many adult cells retain the capacity to undergo apoptosis and that pathways leading to apoptosis can be activated by a wide array of stimuli, including radiation, growth factor withdrawal, treatment with calcium ionophores, and chemotherapeutic agents.[3,4]

Recent studies, both in vitro[5] and in vivo,[6] suggest that delayed neuronal death that occurs as a consequence of transient cerebral ischemia may in part occur by apoptosis. Similarly, we have recently shown that the delayed cell death that occurs in a model of transient retinal ischemia is in part apoptotic.[7] Moreover, this ischemic retinal damage may be ameliorated by the administration of aurintricarboxylic acid, an endonuclease inhibitor that inhibits apoptosis.[7,8] These observations suggest that the use of anti-apoptotic agents could potentially play a role in the treatment of ischemia-reperfusion injury in the retina.

The molecular events regulating apoptosis are complex and involve genes that are both pro-apoptotic and anti-apoptotic.[9] The nuclear phosphoprotein p53 is a key determinant in this process in many cell types, acting to promote apoptosis.[10] p53 is a DNA-binding transcription factor originally recognized as a tumor suppressor, and mutations in this gene are found in approximately half of all human tumors.[11] p53 is involved in

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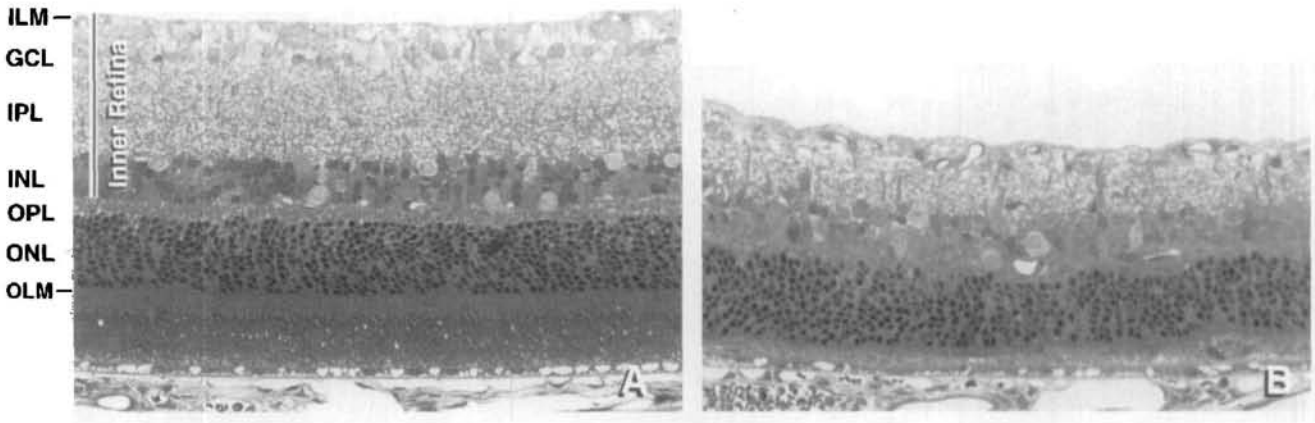

FIGURE 1. Light microscopy of rat retina in nonischemic, control (**A**) and ischemic (**B**) eyes. The control retina demonstrates a normal histoarchitectural pattern, whereas in the ischemic retina there is marked atrophy of the inner retina with a decrease both in its thickness and in the number of cells in the inner nuclear and ganglion cell layers. Mild disorganization of the nuclei in the outer nuclear layer and of the photoreceptor inner and outer segments is also seen. Epon-embedded, 1-μm-thick, toluidine blue; magnification, '100. *ILM*, inner limiting membrane; *GCL*, ganglion cell layer; *IPL*, inner plexiform layer; *INL*, inner nuclear layer; *OPL*, outer plexiform layer; *ONL*, outer nuclear layer; *OLM*, outer limiting membrane.

DNA damage and repair, in which it halts the cell cycle and thereby provides time needed to repair damaged DNA before cell division.[12,13] Increases in p53 expression may accompany apoptosis in cultured cells or during tumor regression, suggesting additional functions of this gene.[14] Hypoxia also stimulates p53 levels and activates the p53 protein; this may represent one mechanism by which p53 regulates tumor growth.[15] *p53* expression increases after neuronal injury in the central nervous system (but not the peripheral nervous system),[16] and transgenic mice deficient in p53 are resistant to excitotoxic[17] and focal ischemic lesions in the brain.[18] The role of the *p53* gene in retinal pathology, however, is unknown. This study therefore sought to determine whether the *p53* gene product is involved in cell death after transient retinal ischemia and whether transgenic mice deficient in *p53* are protected against transient ischemic retinal cell death.

METHODS

Animals

Male Sprague–Dawley rats weighing 150 to 250 g were obtained from Charles River Farms (Wilmington, MA). Transgenic C57 mice were obtained from Taconic Farms (Germantown, NY) at 5 to 6 weeks of age. The mice were transgenic for the null allele construct of the *p53* gene. The homozygous transgenic mice had two null *p53* genes and were therefore devoid of the p53 protein. The heterozygous mice had one copy of the wild-type gene and one copy of the null gene construct. Wild-type mice had two normal copies of the gene. Each animal's genotype was determined by the supplier before shipment. All procedures involving the animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Retinal Ischemia

The rats were anesthetized with an intraperitoneal injection of ketamine (30 mg/kg) and xylazine (2.5 mg/kg). For the studies involving mice, ketamine (100 mg/kg), and xylazine (15 mg/kg) were given intraperitoneally. The anterior chamber of the right eye was cannulated with a 27-gauge needle attached to a normal saline infusion and a manometer via a three-way stopcock. The corneal puncture site was sealed with cyanoacrylate cement. Intraocular pressure was raised to 150 mm Hg for 60 minutes. Retinal ischemia was confirmed by whitening of the iris and loss of the red reflex. After 60 minutes of ischemia, the needle was withdrawn and the intraocular pressure normalized. One drop of gentamicin ophthalmic solution and atropine 1% ophthalmic solution were applied topically to the right eye before and after cannulation of the anterior chamber.

Light Microscopy

The right (experimental) and left (untouched control) globes were enucleated 1 week after ischemia and fixed in Trump's

TABLE 1. Measurements of Thickness of Retinal Layers and INL Cell Count at 7 Days Reperfusion after 60 Minutes of Ischemia in the Rat

	OLM-ILM	ONL	OPL	INL	IPL-ILM	INL Cell Count
Control	181.0 ± 16.1*	43.0 ± 2.9	9.3 ± 1.2	40.0 ± 1.7†	78.0 ± 1.5*	119.8 ± 4.7‡
Ischemia	118.3 ± 13.1	45.3 ± 1.9	7.3 ± 1.5	21.7 ± 2.6	38.9 ± 6.4	67.3 ± 5.8

OLM, outer limiting membrane; ILM, inner limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. Values are mean ± SEM; $n = 4$ for all.

* $P < 0.05$ compared with ischemia.

† $P < 0.01$ compared with ischemia.

‡ $P < 0.001$ compared with ischemia.

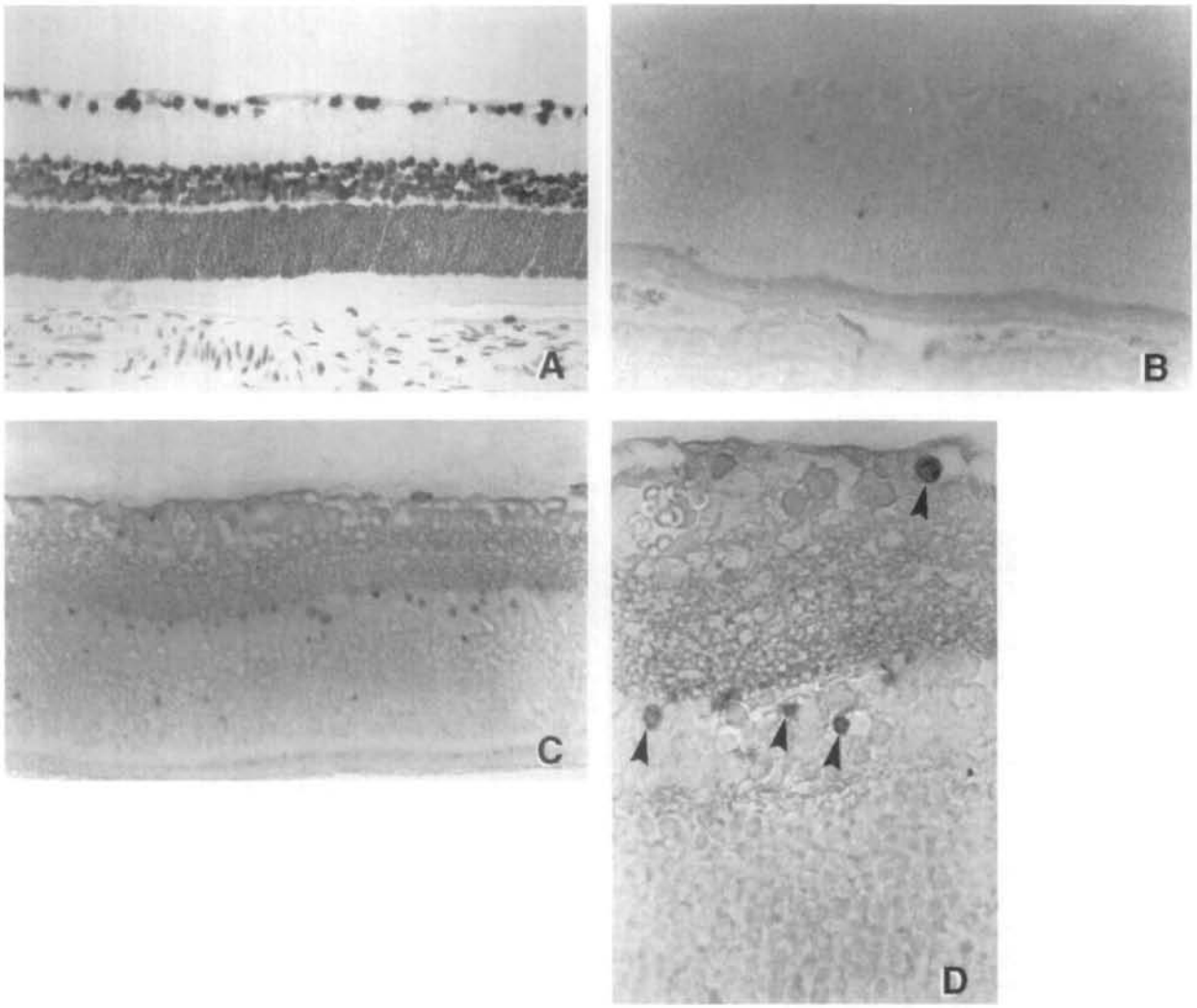

FIGURE 2. TUNEL-stain-treated rat retina viewed by light microscopy. (**A**) A normal retina treated with DNase before the TUNEL reaction serves as the positive control. The expected uniform red-brown positive reaction product is seen in all cell nuclei. (**B**) In the nonischemic control retina, no TUNEL-positive cells are present. (**C, D**) Sections of retina 24 hours subsequent to a 60-minute period of ischemia demonstrate TUNEL-positive cells scattered within the ganglion cell and inner nuclear layers (*arrowheads*; **D**). Paraffin-embedded. Magnification, (**A, B, C**) ×100; (**D**) ×300. $n = 5$ per time point.

fixative.[19] The globes were sectioned in the vertical meridian and the inferior portion of the eye wall (retina, choroid, and sclera) embedded in epoxy-resin. Sections (1 μm-thick) were stained with 1% toluidine blue. The retinal histoarchitecture was evaluated by light microscopy. The thickness of the retinal layers was measured as follows: outer limiting membrane to inner limiting membrane; outer nuclear layer; outer plexiform layer; inner nuclear layer (INL); and inner plexiform layer to inner limiting membrane. Averages for these measurements taken in four adjacent areas within 1 mm of the optic nerve were calculated. Additionally, manual cell counts of the INL were performed over a length of 200 μm in the inferior peripapillary region. Selection of the same topographic region of the retina for all these measurements is important to protect against possible regional anatomic variation. The measurements were performed in a blinded fashion.

TdT-dUTP Terminal Nick-End Label Staining

This technique was based on the method described by Gavrielli et al.[20] but with several modifications. Paraffin sections were prepared, paraffin was removed in a xylene bath, and sections were then rehydrated in decreasing ethanol concentrations. The sections were rinsed in diethyl pyrocarbonate-treated double distilled water and treated with proteinase K (20 μg/ml) for 15 minutes at room temperature in a humidified chamber. Sections were then incubated in a biotinylated deoxyuridine triphosphate/terminal deoxynucleotidyl transferase mixture at 37°C for 1 hour in a humidified chamber followed by rinses in SSC buffer. The sections were then visualized with 3′3-diaminobenzidine HCl using a horseradish peroxidase-labeled ABC kit (Vector Laboratories, Burlingame, CA). Corresponding negative and positive control sections were also prepared. These studies were performed in the rat and in the mouse.

p53 Immunohistochemistry

The enucleated globes were fixed with 4% paraformaldehyde in phosphate-buffered sulfate (PBS) at 4°C for 30 minutes and incubated overnight in 25% sucrose in PBS at 4°C. Frozen sections 10-μm-thick were cut. The sections were

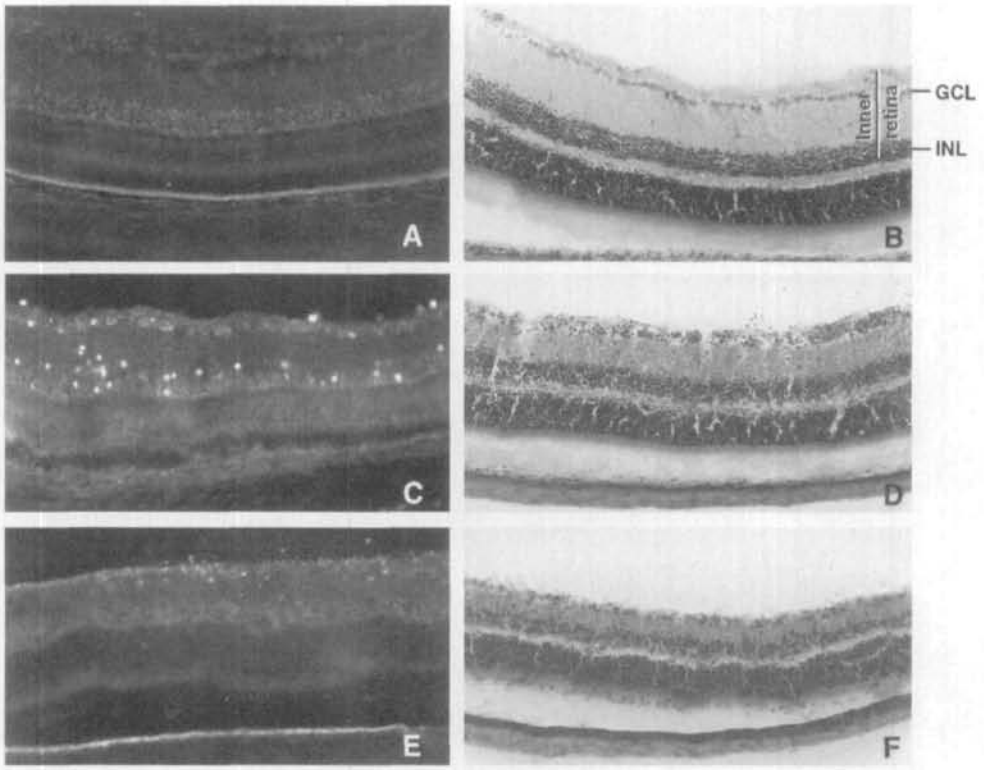

FIGURE 3. p53 immunohistochemistry and corresponding hematoxylin-eosin–stained cryosections of nonischemic control rat retina (**A**, **B**) and of rat retina at 24 hours' (**C**, **D**) and 48 hours' (**E**, **F**) reperfusion subsequent to a 60-minute period of ischemia. No p53 protein immunoreactivity is present in the control retina (**A**). In the ischemic retinas (**C**, **E**), significant immunofluorescence is present in the ganglion cell layer (GCL) and inner nuclear layer (INL) of the inner retina, indicating marked p53-immunoreactive protein expression in these cells (**E**). (**A**, **C**, **E**), p53 rabbit polyclonal antibody; (**B**, **D**, and **F**), hematoxylin-eosin; magnification, ×50. n = per time point.

preincubated in PBS containing 10% normal rabbit serum (Vector) for 45 minutes They were then incubated with 1:30 p53 rabbit polyclonal antibody (FL-393, Santa Cruz Biotechnology, Santa Cruz, CA) in PBS for 1 hour in a humidified chamber at room temperature. After rinsing, sections were incubated with 1:6 FITC-conjugated anti-rabbit IgG (Vector) for 30 minutes in a dark humidified chamber at room temperature. Corresponding control sections and hematoxylin-eosin–stained sections were also prepared. The slides were examined using a fluorescent microscope using a fluorescein filter (BX50; Olympus, Tokyo, Japan). These studies were only performed in the rat.

Transmission Electron Microscopy

Sections of the retina (1-μm-thick) stained with uranyl acetate and lead citrate were examined for the ultrastructural features of apoptosis. These studies were performed in the mouse.

Statistical Analysis

Data were analyzed with ANOVA. Significance between groups was assigned at a level of <5% probability ($P < 0.05$). Type 1 error was controlled using the Bonferroni correction for multiple comparisons.

RESULTS

Ischemia in the Rat

Ischemia produced by this technique resulted in the typical histopathologic features expected subsequent to acute retinal ischemia.[21] In the ischemic eyes, the thickness of the entire retina was reduced compared with the untouched control retina. Specifically, there was a 35% reduction in the thickness of the ischemic retina (outer limiting membrane– inner limiting membrane) compared with the untouched controls (Fig. 1, Table 1). This resulted predominantly from marked thinning of the inner retinal layers, which also disclosed a reduction in the number of cells in the inner nuclear and ganglion cell layers. The number of cells in the inner nuclear layer was reduced by 44% in the ischemic retina compared with the untouched control (Table 1). Mild disorganization of the cells in the outer nuclear layer and of the photoreceptor inner and outer segments were also noted. The histologic features of this model have been previously reported and are consistent with the present data.[7]

TdT-dUTP Terminal Nick-End Label Staining in the Rat

We have previously demonstrated in the rat that the cell death that occurs in this model has the characteristic morphologic

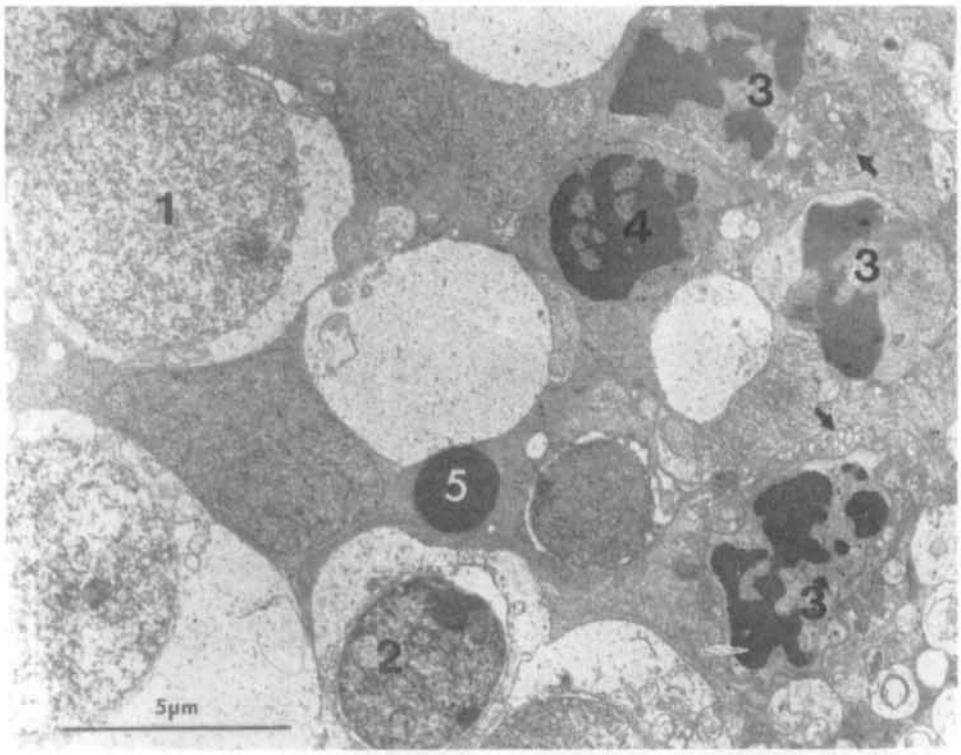

FIGURE 4. Transmission electron micrograph of cells in the inner nuclear layer of a retina of a wild-type mouse at 12 hours' reperfusion after a 60-minute period of ischemia. The inner nuclear neurons show progressive stages of apoptosis and are labeled as follows: No. 1 is a normal cell with dispersed chromatin and a single nucleolus; No. 2 shows mild clumping of chromatin along the nuclear border and an increased electron density of the nucleoplasm; No. 3s show chromatin clumping throughout the nucleus and mild vacuolation of the cytoplasm (*arrows*); No. 4 demonstrates nuclear condensation around very dark chromatin, with the cytoplasm becoming more dense and reduced in volume, perhaps due to blebbing. Finally, one dark mass of chromatin (No. 5) appears to be phagocytosed by a cell adjacent to a large empty space where the healthy neuron may have resided. Scale bar, 5 μm.

(ultrastructural) and biochemical (DNA ladder) changes consistent with apoptosis.[7] These changes were seen at 12 and 24 hours after ischemia. To determine which retinal cell layers were undergoing apoptosis, TdT-dUTP terminal nick-end label (TUNEL) staining was performed at different times after 60 minutes of transient ischemia in the rat. No TUNEL-positive cells were seen in the control retinas. However, subsequent to ischemia TUNEL-positive cells were seen as early as 6 hours in the ganglion cell layer (GCL), and INL and was most prominent at 24 to 48 hours (Fig. 2). At 7 days there continued to be TUNEL-positive cells in the GCL and INL, and a few scattered TUNEL-positive cells were seen in the photoreceptor layer as well.

p53 Expression in the Rat

Because the *p53* oncogene has been identified as playing an important role in pathologies in which apoptosis is believed to occur, its expression was examined in the retina after ischemia. Transient retinal ischemia was induced in the rat for 60 minutes, and p53 immunohistochemistry was performed at different times during the reperfusion periods. No p53 protein immunoreactivity was noted in control retinas. However, after ischemia, p53 immunoreactivity progressively increased and peaked at 24 to 48 hours. It decreased thereafter and was absent by the seventh day. p53-immunoreactive protein expression was localized to cells within the inner retina (Fig. 3); these are the cells that appear most sensitive to ischemia and in which cell death occurs in a delayed manner ("maturation phenomenon").[22]

Ischemic Damage in Mice with p53 Expression Deficiency

Because p53 expression in the retina increased subsequent to ischemia, its role in cellular responses was examined in mice deficient in expression of the tumor suppressor gene. Transient retinal ischemia was induced for 60 minutes in three groups of mice: wild type and heterozygous and homozygous for the *p53* null mutation. In the wild-type animals, transient retinal ischemia resulted in cell death in the inner retinal layers. Ultrastructural analysis performed after 12 hours of reperfusion showed changes that conformed to previous descriptions of apoptosis[7,23] (Fig. 4). These ultrastructural features of apoptosis were also consistent with our previous transmission electron microscopic demonstration of apoptosis in the rat.[7] TUNEL staining of the inner retina was most prominent at 24 hours subsequent to reperfusion but persisted at 48 hours (Fig. 5). Light microscopy in the wild-type mice performed at 7 days after reperfusion also demonstrated cell death in the inner layers. In contrast, there was significant resistance to ischemia in the heterozygotes with preservation of the inner layer com-

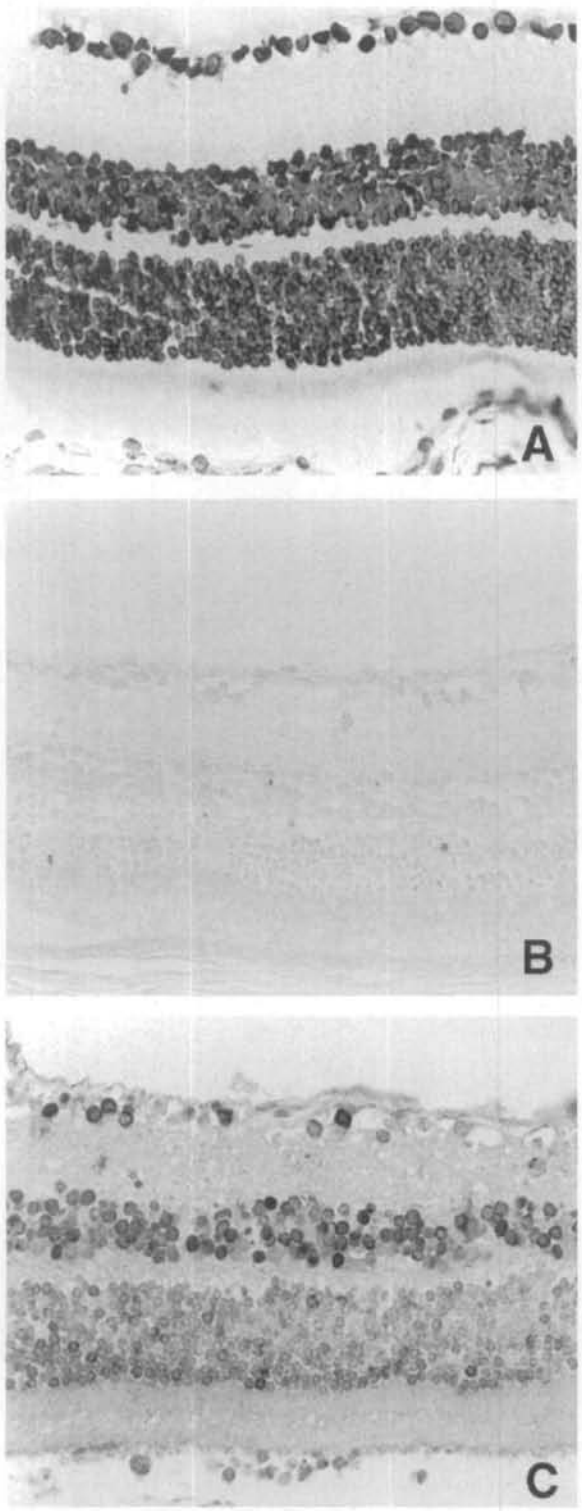

FIGURE 5. TUNEL-stain-treated mouse retina viewed by light microscopy. (A) A normal retina treated with DNase before the TUNEL reaction serves as the positive control, demonstrating the red-brown reaction product in all cell nuclei. (B) In the nonischemic control retina no TUNEL-positive cells are seen. (C) Sections of retina 24 hours subsequent to a 60-minute period of ischemia show strong TUNEL-positive staining within the ganglion cell and inner nuclear layers and only mild staining within the outer nuclear layer. Magnification (paraffin-embedded sections), ×100. $n = 4$ per time point.

pared with the ischemic wild-type retinas (Fig. 6, Table 2). Interestingly, the ischemic retinas of the homozygotes did not differ significantly from those of the ischemic retinas of wild-type mice.

DISCUSSION

We have previously shown that transient retinal ischemia results in apoptotic cell death of the inner retina, which may explain the delayed cell death that occurs after ischemia/reperfusion. In addition to the delayed cell death that occurs after ischemia/reperfusion, some populations of neuronal cells in the retina appear to be more susceptible to ischemia-induced cell damage. Inner layer neurons show a significantly increased susceptibility to ischemia compared with outer layer neurons.[22,24,25] This selective vulnerability cannot be attributed to changes in blood flow to the retina. In the experimental model used in some of these studies, choroidal blood flow is diminished to the same degree or greater than retinal blood flow.[26] Furthermore, this selective susceptibility has been found in a variety of models of transient retinal ischemia, such as the suture ligation model, eliminating a direct pressure phenomenon as the cause.[25,27] These differences have been attributed to differential response to neurotransmitter,[25] free radical[22,28] or rate of protein synthesis.[24] Because the p53 protein plays a key role in apoptotic cell death in other organ systems, we hypothesized that increased expression of this protein may be involved in ischemia-induced apoptosis in the retina as well. In this study, we also found an increase in p53 expression after ischemia in the inner retina—that same portion of the retina that morphologically appears to be most sensitive to ischemia. Furthermore, we found that mice heterozygous for the null mutation of the *p53* gene are resistant to retinal ischemia. These observations suggest that activation of p53 may participate in the delayed apoptotic death seen in the inner retina after ischemic insults and, also, that it may explain the selective vulnerability of the inner retina as well.

Although the retinas of mice heterozygous for the *p53* null mutation were protected against ischemia-mediated cell death, the retinas of homozygotes were not similarly protected. This finding is consistent with those of Crumrine et al.[18] who noted a similar difference between the *p53* heterozygotes and homozygotes after cerebral ischemia. These findings are also consistent with those of Li et al.[29] in a model of excitotoxic injury to the inner retina. However, in a study of excitotoxic injury to the brain there was a dose-response relationship between the degree of injury and expression of p53.[17] The explanation for the lack of protection in homozygotes remains unclear. p53 participates in a large number of regulatory events in cells,[30] and it may be that a certain minimal level of the protein is necessary for the cell to handle metabolic stresses. However, our observations suggest that in the retina, and in other organ systems,[14,31-33] excessive levels of the protein may lead to apoptosis.

Whether a cell decides to enter p53-mediated cell cycle arrest or apoptosis is multifactorial. Conditions that lead to DNA damage preferentially result in p53-mediated apoptosis, thereby eliminating cells with unstable genomes. Thus, p53 may have several opposing roles after ischemia, some of which may be protective, and others may be detrimental. It is therefore possible that the ability of p53 to confer protection is dose

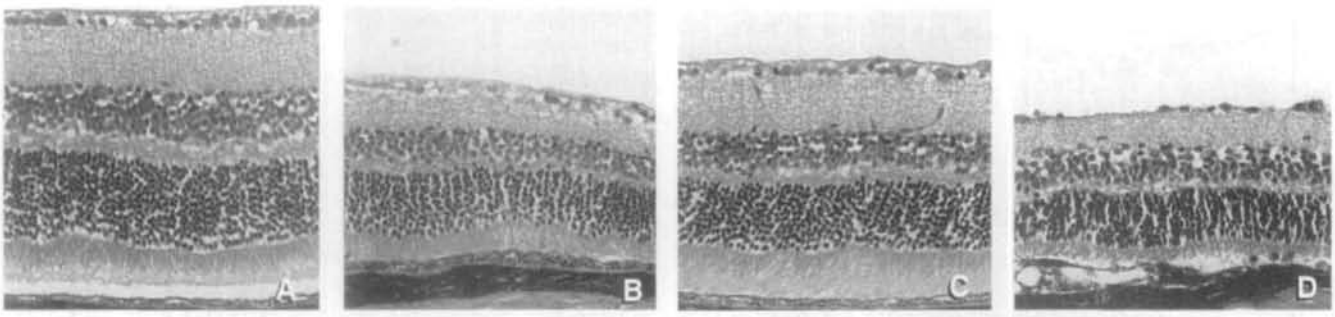

Figure 6. Light microscopy of the retinas of wild-type mice and of mice heterozygous and homozygous for the *p53* null mutation 7 days subsequent to a 60-minute period of ischemia. There is inner retinal atrophy and dropout of cells in the inner nuclear and ganglion cell layers of the ischemic wild-type retina (**B**) compared with the control (**A**). In contrast, the ischemic heterozygote (**C**) shows less ischemic retinal atrophy than the ischemic wild-type (**B**) and the ischemic homozygote (**D**) retinas. Epon-embedded sections, 1 μm-thick, toluidine blue. Magnification, ×100.

Table 2. Measurements of Thickness of Retinal Layers and INL Cell Count at 7 Days Reperfusion after 60 Minutes of Ischemia in the Mouse

	OLM-ILM	ONL	OPL	INL	IPL-ILM	INL Cell Count
Control (n = 12)	176.2 ± 7.8	63.8 ± 3.6	11.5 ± 0.7	37.6 ± 1.5	63.8 ± 4.0	195.0 ± 9.1
Wild-type (n = 4)	108.4 ± 13.2*	41.8 ± 4.8†	7.0 ± 0.9†	24.2 ± 4.1*	38.0 ± 5.8†	130.0 ± 8.9*
Heterozygote (n = 4)	155.5 ± 10.6	49.5 ± 5.3	9.0 ± 0.9	35.3 ± 2.7	62.2 ± 2.8	193.0 ± 20.4
Homozygote (n = 4)	116.8 ± 15.4†	35.0 ± 4.6	7.8 ± 1.2	29.8 ± 3.2	45.3 ± 9.8	131.8 ± 12.3†

OLM, outer limiting membrane; ILM, inner limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. Values are mean ± SEM.

* $P < 0.01$ compared with control.

† $P < 0.05$ compared with control.

dependent and that too little or too much results in more severe damage. This may explain why the heterozygotes were protected from ischemic damage, whereas the homozygotes were not. The *p53*-knockout mice used in this study express a global attenuation of p53 expression in all tissues and are susceptible to tumor formation.[34] It is therefore possible that the resistance noted in the heterozygotes was related to non-neuronal factors, but this is unlikely because increased expression was noted primarily in the inner layer of the retina that consists primarily of neural cell types. Furthermore, others have demonstrated that expression of p53 in pure neuronal culture results in apoptotic cell death of hippocampal and cortical neurons.[14,35] p53 expression was not measured in the mouse. It is possible that, although p53 levels in the heterozygotes are intermediate compared with the wild type mice or homozygotes,[34] it may not be the case that the levels of p53 expressed in response to ischemia in heterozygote animals are also intermediate. This question needs to be addressed in future studies.

In conclusion, the results of this study further support the hypothesis that ischemia-reperfusion injury in the retina results, at least in part, in apoptotic cell death of the inner retina. The p53 protein may be the target for future therapeutic agents in the treatment of disorders of the retina in which ischemia plays a pathogenetic role.

Acknowledgments

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