



Apoptosis in the Murine *rd1* Retinal Degeneration is Predominantly p53-Independent

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Purpose: To determine if p53 mediates apoptosis in photoreceptors of retinal degeneration, *rd1*, mice.

Methods: The *rd1/rd1* mice were interbred with p53 null mice to generate *p53^{-/-} rd1/rd1* and *p53^{+/+} rd1/rd1* mice. Rates of loss and incidence of apoptosis in rod photoreceptors were analyzed at appropriate ages (postnatal days 12, 14 and 16).

Results: The extent and kinetics of photoreceptor cell loss in *rd1* mice were nearly indistinguishable in the *p53^{+/+}* and p53 null mice.

Conclusions: Photoreceptor cell apoptosis in the *rd1* mouse model occurs by a predominantly p53-independent molecular pathway.

Several genes have recently been identified that either positively or negatively regulate apoptosis [1]. One such gene, p53, encoding the p53 tumor suppressor protein, mediates apoptosis in response to DNA damage and certain cell cycle perturbations. However, a variety of forms of p53-independent apoptosis have also been identified [2]. Importantly, nearly all normal developmental apoptosis must be p53-independent, since most p53 null mice develop essentially normally [3, 4]. Although the ability of p53 to regulate apoptosis in response to DNA damage or cell cycle perturbation as well as during development has been extensively examined, the role of p53 in post-mitotic cells, such as photoreceptors, has not been well addressed. If p53 does play a role in the regulation of programmed cell death in photoreceptors, then this could be a potential target for the treatment of retinal degeneration.

Inherited retinal degenerations lead to a progressive deterioration of vision resulting from the loss of function and viability of photoreceptors. Animal models of retinal degeneration in which the phenotype and genetic mutation correspond to the human disease have been widely studied. One such model, the *rd1* retinal degeneration mouse, exhibits a rapid retinal degeneration initiated by a recessive mutation of the gene encoding the rod β -subunit of the cyclic GMP phosphodiesterase [5, 6]. These mice develop fully differentiated photoreceptors during the second postnatal week, but then suffer nearly complete loss of the rod photoreceptors in the next week, followed by a slower loss of cone photoreceptors [7]. The photoreceptor death in this model exhibits the morphologic features and DNA fragmentation characteristic of apoptosis [8]. However, the regulation of retinal apoptosis in the *rd1* mouse model is still poorly understood.

To determine if the presence of p53 influences apoptosis in the rods of *rd1* mouse retinas, we interbred *rd1* mice with mice deficient for p53. We show that the rapid apoptotic loss of photoreceptors observed in this model is largely p53-independent.

METHODS

Experimental Animals—FVB/NJ (*rd1/rd1*) mice were obtained from the Jackson Laboratory, ME. TSG-p53 (p53-deficient) mice [3], obtained from GenPharm International, CA, are maintained in our laboratory in a C57Bl/6 X Balb/c genetic background. *rd1/rd1* and *p53^{-/-}* mice were interbred to generate *p53^{+/-} rd1/+* double heterozygous F1 offspring, and these were subsequently interbred to obtain mice with the following genotypes of interest: *p53^{+/+} rd1/rd1*, *p53^{-/-} rd1/rd1*, *p53^{+/-} +/+*, and *p53^{-/-} +/+*. Litters of mice were sacrificed at postnatal day 12, 14, or 16 (P12, P14, or P16; day of birth is defined as P0).

Identification of Genetic Mutations—Tail tissue was taken for DNA extraction and prepared as described [9]. Tail DNA was used to determine the presence of the p53 and *rd1* mutations by PCR-based strategies. The genetic status of p53, mutated or wild-type, was determined by using a three primer-based strategy previously described [10]. The *rd1* nonsense mutation within the β -PDE gene was identified by PCR amplification of the mutated region, followed by digestion with a restriction enzyme that distinguishes between the wild type and mutant allele, as described by Pittler and Baehr [6].

Detection of Apoptotic Cells—Eyes were enucleated with the optic nerve left intact to be used for orientation of anterior-posterior sections. Eyes were fixed overnight at room temperature in phosphate buffered 4% paraformaldehyde fixative and then embedded in Poly-Fin paraffin (Triangle Biomedical Sciences). Apoptotic cells were detected using the TUNEL (terminal deoxynucleotidyl transferase-mediated

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dUTP-biotin nick end labeling) procedure [11]. Incorporated biotin was detected using a streptavidin-horseradish peroxidase conjugate (DAKO) and visualized with a DAB (diaminobenzidine) chromagen (Sigma). Sections were counterstained with methyl green.

Quantitation of Apoptosis— Retinas were aligned along the maximal diameter of the 40x objective field (610 μ m length of retina) and numbers of TUNEL labeled nuclei in the outer nuclear layer (ONL) per high power field (hpf) were counted. Only those cells in which label was confined to the nucleus were counted. Both totally stained nuclei as well as those with punctate or peripheral staining were scored as TUNEL positive. The number of photoreceptor nuclei per row in the ONL were also counted, with a maximum index of 10 (the normal ONL thickness). Four hpfs were counted for each retina examined, two central hpfs (averaged to generate a single datapoint) and two peripheral hpfs (also averaged). The central hpf was aligned with the optic nerve at one edge of the field. The

peripheral hpf was aligned distal to the outer border of the central hpf and proximal to the tapering of the retina at the ciliary body. The second eye from each animal was not evaluated, unless the first eye was damaged due to handling and could not be assayed. Because the sample sizes were relatively small, data were summarized by plotting individual data points and medians, and compared using the Wilcoxon Rank Sum test, a nonparametric analogue of the two sample t-test that does not require the normality assumption.

RESULTS

Qualitative Analyses of Retinal Morphology— TUNEL assay results for $p53^{+/+} rd1/rd1$, $p53^{-/-} rd1/rd1$, and $p53^{+/+} +/+$ (wild-type) retinas at P12 and P16 are displayed in Figure 1. These micrographs illustrate qualitatively that at the onset of degeneration (P12), central retinal cross sections from all three genotypes appear virtually the same with regard to ONL thickness and morphology, although a slight increase in TUNEL label in $rd1$ retinas compared to wild-type is detectable. Only four days later, however, the $p53^{+/+} rd1/rd1$ and $p53^{-/-} rd1/rd1$ mice have lost nearly all rod photoreceptors. Moreover, the $p53^{-/-} rd1/rd1$ mice are nearly indistinguishable from the $p53^{+/+} rd1/rd1$ mice at P16. At all timepoints examined, $p53^{-/-} +/+$ retinas are indistinguishable from $p53^{+/+} +/+$ retinas (data not shown).

Quantitative Analyses of Photoreceptor Loss— The qualitative results clearly demonstrate that the retinal degeneration in $rd1$ mice is largely p53-independent. However, to determine whether p53 status might have a subtle effect on the kinetics of the process, the apoptotic index and ONL thickness were determined at P12, P14 and P16 for $rd1$ mice in the presence and absence of p53. We detected a slight decrease in TUNEL labeling in the central retina of $p53^{-/-} rd1/rd1$ mice at P14, and in the peripheral retina at P16, relative to the $p53^{+/+} rd1/rd1$ mice (Figure 2A,B). A slight statistically significant difference was also identified in the ONL thickness of the central retina at P16 (Figure 2C). However, no statistically significant differences were identified at the other timepoints for the parameters measured.

DISCUSSION

These results indicate that the retinal degeneration observed in $rd1$ mice is predominantly p53-independent (Fig. 1). While there may be a slight delay in the apoptosis observed in $p53^{-/-} rd1/rd1$ retinas, reflected in small differences in apoptotic cell indices at P14 and P16, this effect is subtle and does not affect the end result caused by the $rd1$ mutation, that is, complete loss of rod photoreceptors.

The variability in both TUNEL positive cells and ONL thickness, most apparent at P14, may indicate that the rate of photoreceptor loss varies somewhat between individuals. It may also suggest that at this critical midway point in the degeneration, small differences in age of the mice could lead

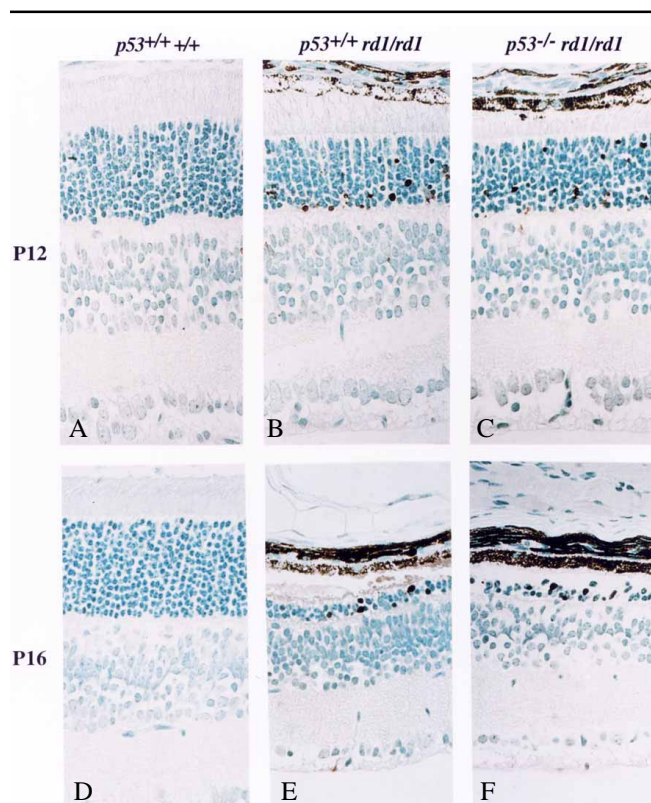


Figure 1. $p53^{+/+} rd1/rd1$, and $p53^{-/-} rd1/rd1$ mice at P12 and P16. Panels A-F compare the retinal morphology observed at P12 and P16 in normal mice and those of the $p53^{+/+} rd1/rd1$ and $p53^{-/-} rd1/rd1$ genotypes. Panels B and C illustrate the slight increases in TUNEL label in $rd1$ mutant mouse retinas, regardless of p53 status, as compared to normal mice (A) at this early stage of the degeneration. Panels E and F demonstrate the loss of photoreceptor nuclei in the outer nuclear layer (ONL) induced by the $rd1$ retinal degeneration only 4 days later in the presence or in the absence of the p53 gene, respectively. Panel D shows a normal retina at P16. The absence of the p53 gene does not protect the $rd1$ mutant retina from apoptosis. Retinas from $p53^{-/-} +/+$ and $p53^{+/+} +/+$ are indistinguishable at all ages (not shown). Paraffin sections were labeled by the TUNEL assay [11] and counterstained with methyl green (total magnification: x380).

to large differences in the extent of degeneration. The age of mice from different P14 litters could have differed by nearly as much as 24 hrs, since cages were checked for newborn pups once each day. Nevertheless, by P16, most of the photoreceptors in all mice have degenerated.

The timecourse of degeneration in this study, regardless of *p53* genotype, appears to be slightly accelerated relative to that reported in previous studies of the *rd1* phenotype [8, 12]. While the explanation for this difference in kinetics is not clear, one possibility is that the genetic background of the mice influences the rate of degeneration, since a different strain of mice carrying the *rd1* mutation was characterized in each study.

We have previously demonstrated that mice expressing HPV-E7 (which binds and inactivates the retinoblastoma gene product, pRb) in photoreceptor precursors undergo profound retinal apoptosis, but that when bred into a *p53*-deficient background, these mice develop retinal tumors [10]. However, examination of *E7/p53*^{-/-} retinas at early postnatal ages

revealed that apoptosis was slightly delayed, but not completely abrogated [13].

A variety of studies have demonstrated *p53*-independent apoptosis of neurons in response to withdrawal of neurotrophins [14, 15]. In addition, developmental apoptosis in the nervous system is generally *p53*-independent, since most *p53*-deficient mice develop normally [3]. Interestingly, a fraction of *p53*-deficient embryos exhibit exencephaly, but it is not clear whether a defect in apoptosis is responsible for the condition [4]. In contrast, cerebellar granule cells have been shown to exhibit *p53*-dependent apoptosis in response to gamma-irradiation [16]. Together, these studies and ours indicate that *p53* may be involved in neuronal apoptosis in some situations, including cell cycle perturbation or DNA damage, but is not generally involved in mediating developmental or other forms of apoptosis in neurons.

Why a particular cell type, neuronal or otherwise, exhibits *p53*-dependent or -independent apoptosis, or both, remains

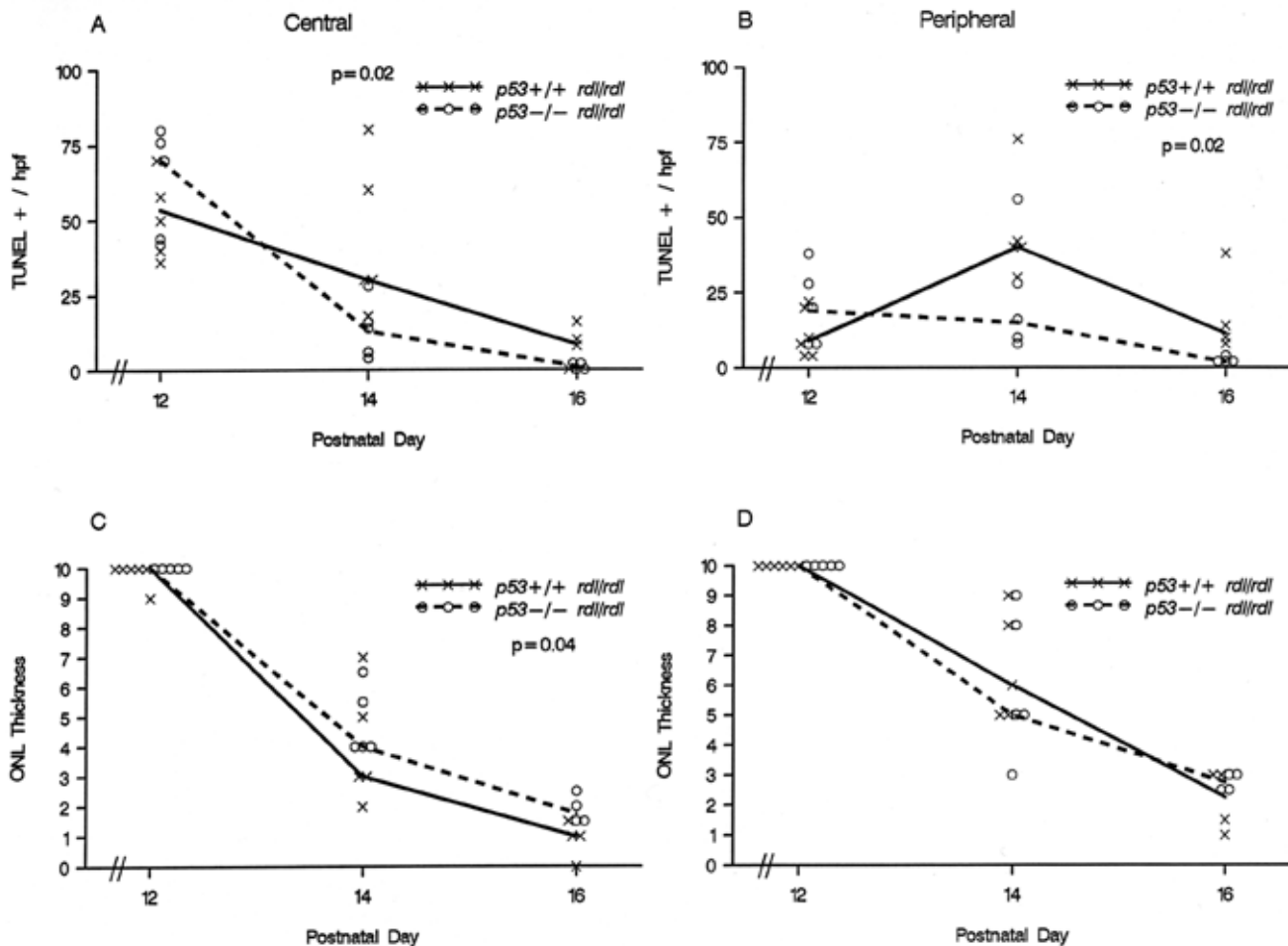


Figure 2. Time course of retinal degeneration in *p53*^{+/+}*rd1/rd1* and *p53*^{-/-}*rd1/rd1* mice. A minimum of 4 mice were examined for each genotype analyzed at each age (P12, P14, and P16). Each datapoint plotted in a given panel represents the average of counts from two high power fields from one eye of an individual animal. Both indices, TUNEL+ cells/hpf (A and B) and ONL thickness (C and D), indicate that the retinal degeneration occurring in the *p53*^{+/+}*rd1/rd1* and *p53*^{-/-}*rd1/rd1* mice follows a similar rate of rod cell destruction in the central and peripheral retina. Small significant differences were detected, however, and are indicated by the *p* values denoted in A, B, and C (Wilcoxon Rank Sum test within each time point). Solid or dashed lines connect the median values for each genotype.

poorly understood. There are clearly multiple molecular pathways regulating this process. In addition, a variety of factors, including cell type, stage of differentiation, cell-cell interactions, extracellular matrix interactions, availability of growth factors, the stimulus used to provoke the apoptosis, etc., can influence the cellular susceptibility to apoptosis. Understanding the mechanisms involved in the regulation of apoptosis of post-mitotic cells like the photoreceptors of *rdl* mice may lead to therapies directed at preventing these devastating degenerations.

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REFERENCES

- White E. Life, death, and the pursuit of apoptosis. *Genes Dev* 1996; 10:1-15.
- Liebermann DA, Hoffman B, Steinman RA. Molecular controls of growth arrest and apoptosis: p53-dependent and independent pathways. *Oncogene* 1995; 11:199-210.
- Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA Jr, Butel JS, Bradley A. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* 1992; 356:215-221.
- Sah VP, Attardi LD, Mulligan GJ, Williams BO, Bronson RT, Jacks T. A subset of p53-deficient embryos exhibit exencephaly. *Nat Genet* 1995; 10:175-180.
- Bowes C, Li T, Danciger M, Baxter LC, Applebury ML, Farber DB. Retinal degeneration in the *rd* mouse is caused by a defect in the beta subunit of rod cGMP-phosphodiesterase. *Nature* 1990; 347:677-680.
- Pittler SJ, Baehr W. Identification of a nonsense mutation in the rod photoreceptor cGMP phosphodiesterase beta-subunit gene of the *rd* mouse. *Proc Natl Acad Sci U S A* 1991; 88:8322-8326.
- Carter-Dawson LD, LaVail MM, Sidman RL. Differential effect of the *rd* mutation on rods and cones in the mouse retina. *Invest Ophthalmol Vis Sci* 1978; 17:489-498.
- Chang GQ, Hao Y, Wong F. Apoptosis: final common pathway of photoreceptor death in *rd*, *rds*, and rhodopsin mutant mice. *Neuron* 1993; 11:595-605.
- Hogan B, Beddington R, Costantini F, Lacy E. *Manipulating the mouse embryo: a laboratory manual*. 2nd ed. Cold Spring Harbor (NY): Spring Harbor Laboratory Press; 1994.
- Howes KA, Ransom N, Papermaster DS, Lasudry JG, Albert DM, Windle JJ. Apoptosis or retinoblastoma: alternative fates of photoreceptors expressing the HPV-16 E7 gene in the presence or absence of p53. *Genes Dev* 1994; 8:1300-1310.
- Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 1992; 119:493-501
- Portera-Cailliau C, Sung CH, Nathans J, Adler R. Apoptotic photoreceptor cell death in mouse models of retinitis pigmentosa. *Proc Natl Acad Sci U S A*. 1994; 91:974-978.
- Papermaster DS, Howes K, Ransom N, Windle JJ. Apoptosis of photoreceptors and lens fiber cells with cataract and multiple tumor formation in the eyes of transgenic mice lacking the p53 gene and expressing the HPV 16 E7 gene under the control of the IRBP promoter. In: Anderson RE, LaVail MM, Hollyfield JG, editors. *Degenerative Diseases of the Retina*. New York: Plenum Press; 1995. p. 39-49.
- Davies AM, Rosenthal A. Neurons from mouse embryos with a null mutation in the tumour suppressor gene p53 undergo normal cell death in the absence of neurotrophins. *Neurosci Lett* 1994; 182:112-114.
- Martinou I, Fernandez PA, Missotten M, White E, Allet B, Sadoul R, Martinou JC. Viral proteins E1B19K and p35 protect sympathetic neurons from cell death induced by NGF deprivation. *J Cell Biol* 1995; 128:201-208.
- Wood KA, Youle RJ. The role of free radicals and p53 in neuron apoptosis in vivo. *J Neurosci* 1995; 15:5851-5857.