Expression of Bcl-2 Protects against Photoreceptor Degeneration in *retinal degeneration slow (rds)* Mice

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The *retinal degeneration slow* or *rds* gene encodes rds/peripherin, an integral membrane glycoprotein in the outer segments of rod and cone photoreceptors. Mice homozygous for a null mutation in *rds* fail to develop outer segments and undergo subsequent degeneration of photoreceptors by the apoptotic pathway. Mutations in the human *RDS* gene are responsible for several forms of inherited blindness including autosomal-dominant retinitis pigmentosa and macular degeneration. Here, we examined the effects of ectopic Bcl-2 expression in transgenic photoreceptors on the rate of retinal degeneration in *rds* mutant mice. We observed an approximately twofold preserva-

tion of photoreceptors compared with nontransgenic *rds* mutant mice at 3 months. Immunoblot analysis showed similar levels of Bcl-2 in 2-, 3-, and 4-week-old transgenic mice. Expression of Bcl-2 in the *rds* mouse did not lead to outer segment formation and did not induce cell death. These results suggest that Bcl-2 expression may be an effective therapeutic strategy in humans with mutations in *RDS* or other genes that affect the integrity of photoreceptor outer segments.

Key words: *rds* mice; *rds/peripherin*; *retinal degeneration*; *retinitis pigmentosa*; *Bcl-2*; *transgene*; *apoptosis*

The mammalian retina is a useful system for studying neuronal degeneration. Mutations in multiple genes have been shown to cause blindness because of photoreceptor cell death (for review, see Molday, 1998; Travis, 1998). An example is the *retinal degeneration slow* or *rds* mouse. Photoreceptors in mice homozygous for a null mutation in the *rds* gene fail to develop outer segments, the site of photon capture and of the reactions of visual transduction (Jansen and Sanyal, 1984). Photoreceptor cell death starts between the second and third postnatal week and is completed at ~1 year (Sanyal et al., 1980; Nir et al., 1990). A light response can be elicited in *rds* mice although at a greatly reduced sensitivity (Reuter and Sanyal, 1984). The residual photoactivity is mediated by opsin that is localized in the inner segment and connecting cilium plasma membranes (Nir and Papermaster, 1986).

In humans, over 50 mutations in the *RDS* gene have been implicated in dominant forms of retinitis pigmentosa (RP) and macular degeneration (for review, see Keen and Inglehearn, 1996; Shastri, 1997). *RDS* encodes rds/peripherin, a structural glycoprotein in the rims of outer segment disks (Connell et al., 1991; Travis et al., 1991; Kedzierski et al., 1999). In previous studies, we demonstrated complete rescue of the *rds* phenotype in transgenic mice that express normal rds/peripherin (Travis et al., 1992). Subsequently, we showed that the *rds* rescue transgene integrated

into chromosome X in line 113 mice. Hemizygous females from line 113 showed mosaic expression of the transgene and partial rescue of the *rds* phenotype, whereas males of this line were completely rescued (Kedzierski et al., 1998).

The events that lead from primary mutation to the onset of cell death are not known for any form of retinal degeneration. In every system studied, however, photoreceptors have been shown to die by the apoptotic pathway (Chang et al., 1993; Lolley et al., 1994; Portera-Cailliau et al., 1994; Tso et al., 1994; Cook et al., 1995; Abler et al., 1996; Molthagen et al., 1996). An important step in apoptosis is the release of cytochrome c from the intermembrane space of mitochondria (Krippner et al., 1996; Li et al., 1997). Members of the Bcl-2 protein family exert antiapoptotic effects by blocking the release of cytochrome c from mitochondria (Kluck et al., 1997; Yang et al., 1997). Bcl-2 is not expressed in mature photoreceptors, although the related protein Bcl-X_L is present in these cells (Levin et al., 1997).

Prevention of photoreceptor death by ectopic expression of Bcl-2 was attempted in several mouse models of inherited retinal degeneration. These studies showed either no effect (Joseph and Li, 1996) or limited and short-lived effects (Chen et al., 1996; Tsang et al., 1997). In the current study we examined the effects of Bcl-2 expression on photoreceptor death in *rds* mutant mice that undergo a relatively slow degeneration, similar to many forms of retinal degenerations in humans. We show that expression of Bcl-2 causes a significant and long-term suppression of photoreceptor cell death in homozygous *rds* mutants.

MATERIALS AND METHODS

Transgenic mice. Mice carrying a transgene (line B) containing the complete human Bcl-2-coding region downstream of a mouse rhodopsin promoter (Chen et al., 1996) were crossed onto an *rds*^{−/−} homozygous mutant background on the C57BL/6 strain. The resulting F1 mice were crossed with *rds* line 113-transgenic mice, also on an *rds*^{−/−} background,

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to yield F2 progeny of the desired genotypes. Mice were maintained under a 12 hr dark/light cycle at 40 lux illumination. Tail-cut DNA from mice was analyzed for the presence of the *rd*s transgene by PCR with the following primers: 5'-CCTGGAGTTGCGCTGT and 5'-GTCTTTTTCATGAAGCACC from the mouse rhodopsin promoter and the *rd*s-coding region, respectively (Travis et al., 1989). Mice were analyzed for the presence of the Bcl-2 transgene by PCR with the following primers: 5'-CCTGGAGTTGCGCTGT and 5'-CCTGTTCTCCCAGCGT from the mouse rhodopsin promoter and the human Bcl-2-coding region, respectively.

Microscopy. Mice were killed by cervical dislocation; the eyes were enucleated and placed in 4% formaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer for fixation. After 30 min, the eyes were bisected along the vertical meridian. The two hemispheres were fixed for an additional 3 hr. The tissue was then treated in 1% OsO₄, embedded in araldite. One micrometer sections were prepared for analysis of the outer nuclear layer (ONL) thickness by light microscopy. Thin sections from the same regions of the retina were used for ultrastructural analysis.

Measurement of photoreceptor survival. Light microscopy was used for quantitative assessments of photoreceptor survival. Sections of 1 μ m thickness were cut along the superior-inferior meridian. Each section included the full retina from optic nerve head to ora serrata. The number of photoreceptor nuclei within the ONL was counted along the posterior-periphery axis at a distance of 150–1500 μ m from the optic nerve head in the superior and inferior hemispheres. Measurements were performed at 150 μ m intervals using an eyepiece graticule. Data are expressed as the number of photoreceptor nuclei in a 50 μ m retinal length.

Immunoblot analysis of human Bcl-2. Dissected mice retinas were homogenized on ice in 10 mM Tris-HCl, pH 7.5, 1% SDS, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN) and cleared by a 2 min centrifugation in a microfuge at 4°C. The extracts were mixed with 2 $\times$ Laemmli buffer and incubated at 65°C for 2 min. Samples were resolved by 10% PAGE in 10% 0.75 mm gels and electrotransferred to Immobilon P membranes (Millipore, Bedford, MA) in a transfer buffer containing 25 mM Tris-HCl, 190 mM glycine, 15% methanol, and 0.05% SDS. Blots were preblocked for 1 hr by treating with 1% nonfat dry milk in PBS. The blots were incubated for 2 hr with a mouse monoclonal antibody against human Bcl-2 (Sigma, St. Louis, MO) diluted 1:1000 with PBS containing 1% dry milk. After being washed with PBS containing 0.05% Tween 20 for 1 hr, blots were incubated for an additional hour with goat anti-mouse IgG-horseradish peroxidase conjugate (Bio-Rad, Richmond, CA) diluted 1:10,000 with PBS containing 1% dry milk. Blots were detected with the LumiGLO chemiluminescent substrate (Kirkegaard & Perry, Gaithersburg, MD).

RESULTS

Photoreceptor survival in 3-month-old retinas

We studied photoreceptor survival by light microscopy in 3-month-old mice of the following genotypes: (1) nontransgenic *rd*s^{-/-} homozygotes (*rd*s), (2) Bcl-2-transgenic, *rd*s^{-/-} homozygotes (*rd*s:*bcl*), (3) *rd*s rescue transgenic, *rd*s^{-/-} homozygotes (*rd*s:*113*), and (4) Bcl-2 and *rd*s rescue double-transgenic, *rd*s^{-/-} homozygotes (*rd*s:*113*+*bcl*). Representative retinal sections from males of these four genotypes are shown in Figure 1. Nontransgenic *rd*s mice showed significant retinal degeneration at 3 months, with reductions in ONL thickness to two to three rows of nuclei (Fig. 1A). Expression of Bcl-2 in *rd*s:*bcl* mice resulted in a partial rescue of retinal degeneration, with retention of five to six rows (Fig. 1B). Expression of normal *rd*s/peripherin in male *rd*s:*113* mice prevented retinal degeneration, with retention of eight to nine rows of nuclei (Fig. 1C). Expression of both *rd*s/peripherin and Bcl-2 in *rd*s:*113*+*bcl* mice also resulted in full protection from retinal degeneration, with retention of eight to nine rows of nuclei (Fig. 1D).

We performed detailed morphometric analysis to define accurately the extent of photoreceptor survival in mice of these four genotypes. Data were collected separately from male and female sibs of seven litters. Figure 2 shows the results of this analysis.

Figure 1. Light micrographs of retinal sections from 3-month-old male mice of different genotypes. The densely stained photoreceptor nuclei layer is indicated in each panel by a white vertical bar. *A*, Homozygous *rd*s retina (*rd*s). Up to three rows of photoreceptor nuclei are present. *B*, Homozygous *rd*s retina expressing the Bcl-2 transgene (*rd*s:*bcl*). Up to six rows of nuclei are present. *C*, Homozygous *rd*s retina expressing the normal *rd*s/peripherin transgene (*rd*s:*113*). Up to eight rows of nuclei are present. *D*, Homozygous *rd*s retina expressing the normal *rd*s/peripherin plus Bcl-2 transgenes (*rd*s:*113*+*bcl*). Up to eight rows of nuclei are present. *ris*, Rod inner segment layer; *ros*, rod outer segment layer. Magnification, 400 $\times$.

Figure 2. Quantitation of the number of photoreceptor nuclei. Analysis was performed on 3-month-old male and female retinas of the four genotypes: *rd*s, *rd*s:*bcl*, *rd*s:*113*, and *rd*s:*113*+*bcl*. Data are presented as means $\pm$ SEM ($n = 6$). Males: ^a $p < 0.001$, *rd*s:*bcl* versus *rd*s and *rd*s:*113*. Females: ^b $p < 0.001$, *rd*s:*bcl* versus *rd*s; $p < 0.05$, *rd*s:*bcl* versus *rd*s:*113*.

Photoreceptor survival was increased by $\sim 100\%$ in both male and female *rd*s:*bcl* mice compared with nontransgenic *rd*s mice. However, the effect of Bcl-2 expression on the *rd*s phenotype was significantly less than the complete rescue observed in *rd*s:*113* males (Kedzierski et al., 1998). Mosaic expression of *rd*s/peripherin in *rd*s:*113* females caused partial rescue of the *rd*s phenotype, as described (Kedzierski et al., 1998). Coexpression of Bcl-2 with *rd*s/peripherin in *rd*s:*113*+*bcl* females conferred no additional rescue effect.

Figure 3. Immunoblot analysis showing the levels of Bcl-2 in retinas from 2-, 3-, and 4-week-old mice on an *rd*^{−/−} background. TG denotes Bcl-2 transgenic, and nTG denotes nontransgenic. Note the nearly adult levels of Bcl-2 expression in 2-week-old retinas. w, Week.

Photoreceptor survival in 1-month-old retinas

Approximately one-third of photoreceptors are lost during the first postnatal month in *rd* retinas. We sought to determine whether the preponderance of photoreceptors lost in *rd:bcl* mice occurred during this accelerated cell-death period. Accordingly, we compared the number of photoreceptor nuclei present at 35–38 d in *rd* and *rd:bcl* mice. Morphometry was conducted on retinas from *rd* males and females, five of each, and *rd:bcl* males and females, four of each. The *rd* retinas had an average of 50.8 ± 4.7 photoreceptor nuclei ($n = 5$) per 50 μm , whereas the *rd:bcl* retinas had 66.6 ± 0.53 nuclei ($n = 4$) per 50 μm , representing a 31% increase. Thus, the protective effect of Bcl-2 was present during the early phase of cell death. A comparison of these values with the number of nuclei remaining at 3 months (23 ± 1.7 for *rd* and 47 ± 2.3 for *rd:bcl*) indicates that photoreceptors continue to die in *rd:bcl* mice during the ensuing 2 months, although at a slower rate than in *rd* mice.

Levels of transgenic Bcl-2 expression

To confirm the levels and stability of Bcl-2 expression, we performed immunoblot analysis on retinal homogenates from transgenic and nontransgenic mice at 2, 3, and 4 weeks of age. These intervals bracket the period of accelerated photoreceptor degeneration in *rd* mice. No difference was observed in the levels of Bcl-2 in transgenic mice at these different ages (Fig. 3). Bcl-2 immunoreactivity was undetectable in the nontransgenic littermates.

Ultrastructural analysis

We examined the effects of Bcl-2 expression on the cellular organization of photoreceptors by electron microscopy. In *rd:bcl* retinas, photoreceptors completely lacked outer segments (Fig. 4A), similar to those observed in nontransgenic *rd* mice (Jansen and Sanyal, 1984; Nir and Papermaster, 1986). Thus, the protective effect of Bcl-2 expression on photoreceptor death was not mediated by a correction of the outer segment phenotype in *rd* mice. The antiapoptotic effect of Bcl-2 is manifested in the increased number of photoreceptor nuclei. In the *rd:113+bcl* male retina, the expression of the Bcl-2 transgene had no adverse effect on the ultrastructure of outer segments (Fig. 4B).

DISCUSSION

In the present study, we examined the effect of Bcl-2 expression on photoreceptor degeneration in *rd* mutant mice. At 3 months,

Figure 4. Electron micrographs of 3-month-old mice. *A*, Homozygous *rd* retina expressing Bcl-2 (*rd:bcl*). Note the absence of outer segments and the abnormal position of photoreceptor rod inner segments (RIS) against the retinal pigment epithelium (RPE). The effect of Bcl-2 on photoreceptor survival is evident by the presence of a significant number of photoreceptor nuclei (N). *B*, Homozygous *rd* retina expressing both normal *rd*/peripherin and Bcl-2 transgenes (*rd:113+bcl*). Note the normal morphology of intact rod outer segments (ROS) adjacent to the RPE. The expression of Bcl-2 did not adversely affect photoreceptor organization. Magnification: *A*, 3200 $\times$; *B*, 2900 $\times$.

mice expressing the Bcl-2 transgene retained approximately twice the number of photoreceptors as did nontransgenic *rd* mice. This is in contrast to previous studies on mice with null mutations in the genes for the β - and γ -subunits of cGMP phosphodiesterase (PDE) and dominant mutations K269E and S334ter in the gene for rhodopsin. Expression of Bcl-2 in these mice resulted in minor and transient increased photoreceptor survival (Chen et al., 1996; Joseph and Li, 1996; Tsang et al., 1997).

In the *rd* mouse, a mutation in the β -subunit of PDE results in accumulation of cGMP with severe metabolic consequences, leading to early photoreceptor death. Apoptosis starts at postnatal day 7 (P7), and by P20 almost all rods die (Carter-Dawson et al., 1978; Farber et al., 1988; Portera-Cailliau et al., 1994). In the *Pdeg*^{tm1}/*Pdeg*^{tm1} mouse with a mutation in the γ -subunit of PDE, almost all cells die between P14 and P21 (Tsang et al., 1996). In the opsin S334ter mutant, as in the *rd* mouse, the mutation leads to the onset of apoptosis at P8 and completion by P21 (Chen et al., 1996). In contrast to these mutants, cell death in the *rd* retina starts at P14–P17 and progresses slowly for a full year (Sanyal et al., 1980; Nir et al., 1990). The increased effect of Bcl-2 expression on the preservation of photoreceptors in *rd* mice may be caused by the slower pace of apoptosis in this mutation. In assessing the significance of the present data it should be noted that the rate of cell death in *rd* mice more closely resembles the rate observed in human RP and other forms of progressive neuronal degeneration.

A key feature of the *rd* phenotype is absent outer segments (Jansen and Sanyal, 1984; Nir and Papermaster, 1986), because of loss of the structural protein *rd*/peripherin (Travis et al., 1989, 1991; Connell et al., 1991). Not surprisingly, Bcl-2 expression did not reverse the *rd* phenotype of absent outer segments (Fig. 4) because expression of this antiapoptotic protein does not correct the inherent genetic mutations leading to the structural defect. The protective effect of Bcl-2 on photoreceptor survival must be by an alternative mechanism. Because Bcl-2 was shown to protect against diverse cytotoxic insults (Adams and Cory, 1998), it is not

certain what type of stress triggers apoptosis in the *rd*s retina. One possibility is that the absence of outer segments in *rd*s mice may predispose photoreceptors to oxygen toxicity. The loss of outer segments might be accompanied by the loss of the cGMP-gated cation channels and a dramatic reduction in cation influx. This may “unload” the Na⁺/K⁺ ATPase pumps, resulting in significantly reduced oxygen consumption by inner segment mitochondria and a much flatter pO₂ gradient across the distal retina. Also, with the loss of outer segments, the photoreceptor cell bodies are physically closer to the oxygen-rich choriocapillaris. These two effects may conspire to produce toxic levels of oxygen in the microenvironment of *rd*s photoreceptor cell bodies. The association between apoptotic cell death and the presence of reactive oxygen species is well established. Bcl-2 was shown to prevent neuronal cell death by decreasing the net generation of oxygen species (Kane et al., 1993). Overexpression of Bcl-2 suppressed lipid peroxidation completely (Hockenbery et al., 1993). Also, in a study of Bcl-2-deficient mice, enhanced oxidative stress and susceptibility to oxidants were found (Hochman et al., 1998). Thus, Bcl-2 may be particularly effective in preventing cell death attributable to oxidative damage.

Although expression of Bcl-2 in *rd*s:*bcl* mice resulted in a near doubling of surviving photoreceptors compared with that in non-transgenic *rd*s mutants, only ~60% of the photoreceptors were protected from degeneration compared with fully rescued *rd*s:*113* male controls. This persistent cell loss was probably not caused by direct Bcl-2 toxicity, because we observed similar numbers of photoreceptors in *rd*s:*113* and *rd*s:*113*+*bcl* mice. In this study we used a low-expressing Bcl-2-transgenic line (line B), which did not cause photoreceptor degeneration on an otherwise wild-type genetic background (Chen et al., 1996). Higher levels of Bcl-2, or additional members of the Bcl family (besides Bcl-X_L), may be required for complete protection. Alternatively, it may be impossible fully to prevent photoreceptor degeneration attributable to mutations in a critical gene by expression of proteins that inhibit apoptosis.

We showed in a previous study that female mice hemizygous for the 113 transgene displayed a mosaic pattern of normal *rd*s/peripherin expression, both by *in situ* hybridization and outer segment ultrastructure, because of random inactivation of chromosome X (Kedzierski et al., 1998). However, retinal degeneration in these mice was uniform, with no mosaic pattern of cell loss. This observation indicates a non-cell-autonomous component of photoreceptor degeneration in *rd*s mice (Kedzierski et al., 1998). Before the current study, we anticipated that Bcl-2 expression in *rd*s:*113*+*bcl* hemizygous females may preferentially protect photoreceptors that express the *rd*s rescue transgene, because these cells are entirely normal in the context of transgenic male or homozygous female retinas. This was not observed, however. Expression of Bcl-2 in *rd*s:*113*+*bcl* hemizygous females resulted in no additional protection against photoreceptor degeneration compared with that in *rd*s:*113* hemizygous females (Fig. 2). The degree of rescue in *rd*s:*113* hemizygous females was higher here than in our previous study (Kedzierski et al., 1998). This may be attributable to strain effects on the time of X-chromosome inactivation, which would affect the coarseness of the mosaic.

In summary, Bcl-2 had the greatest protective effect on unrescued *rd*s photoreceptors. Because *rd*s photoreceptors fail to form outer segments (Jansen and Sanyal, 1984; Nir and Papermaster, 1986), we suggest that the major activity of Bcl-2 is to protect against oxygen-mediated toxicity. Shortening of outer segments is an ultrastructural feature of multiple inherited retinal degenera-

tions, including several that affect humans (Travis, 1998). The results of this study suggest that somatic gene therapy involving the delivery of Bcl-2 to diseased photoreceptors may slow the progression in some forms of human inherited blindness.

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