Identification of the \textit{bcl-2} Family of Genes in the Rat Retina

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\textbf{Purpose.} Retinal ganglion cells die by apoptosis after axotomy, and this process may reflect altered expression of cell-death genes. Several of these genes, including \textit{bcl-2}, \textit{bcl-x}, and \textit{bax}, share homology at the amino acid level in the BH1 and BH2 domains, through which they also interact. To understand their role in the neuronal response to axotomy, the authors studied their expression in the adult rat retina and after optic nerve crush.

\textbf{Methods.} An initial survey was conducted with reverse transcription-polymerase chain reaction (RT-PCR), using oligonucleotides against identified members of this family and against the conserved BH1 and BH2 domains. Retinal \textit{bd-x} expression at the messenger RNA (mRNA) and protein level was studied by RT-PCR, Northern blotting, RNase protection analysis, in situ hybridization, Western blotting, and immunofluorescence staining. The effect of retinal ganglion cell axotomy on the steady-state level of \textit{bd-x} mRNA was investigated.

\textbf{Results.} RT-PCR results indicated that rat retinal cells predominantly express the long form of \textit{bd-x}. Both clonal analysis and quantitative measurements using RNase protection assays demonstrated that \textit{bd-x} message was at least 16 times more abundant than that of \textit{bcl-2}. In situ hybridization and indirect immunofluorescence demonstrated that nearly all neuronal cells of the retina express \textit{bd-x}. Northern and RNase protection analyses showed a moderate decrease in \textit{bd-x} message shortly after optic nerve crush.

\textbf{Conclusions.} These findings suggest that the antideath gene \textit{bd-x} is the predominant member of the \textit{bd-2} family in the adult retina, and that its level decreases after optic nerve crush. Changes in \textit{bd-x} expression may correlate with increased retinal ganglion cell apoptosis after axotomy. Invest Ophthalmol Vis Sci. 1997; 38:2545–2553.

Neuronal apoptosis typically involves transcriptional regulation of cell-death genes. One of the most studied group of genes is the \textit{bcl-2} gene family, the members of which include \textit{bcl-x}, \textit{bax}, \textit{bad}, \textit{bak}, and \textit{bik}. Many of these genes share nucleotide and amino acid homology at two or more sites. Two initially described conserved homology domains are named BH1 and BH2 (for Bcl-2 homology 1 and 2 domains), with interactions between gene family members occurring at these sites. Control of cell death appears to be regulated by these interactions and by constitutive activities of the various family members (e.g., blockade of lipid membrane peroxidation by Bcl-2). Subsequently, the BH3 and BH4 homology domains were described; the former is also a site for interaction between members of this extended family. Although \textit{bcl-2} is expressed in a variety of cell types, especially stem cells and epithelia, \textit{bcl-x} is expressed in a more restricted subset of cells. Similarly, \textit{bcl-2} is widely expressed in the developing nervous system and adult peripheral nervous system, but adult central neurons preferentially express \textit{bcl-x}.

In the retina, retinal ganglion cells respond to axotomy by undergoing apoptosis, probably triggered by loss of retrograde transport of brain-derived neurotrophic factor or other neurotrophins. Overexpression of \textit{bcl-2} in transgenic animals prevents retinal ganglion cell death after optic nerve injury, as well as death of sympathetic and other neuronal cell types after deprivation of nerve growth factor and...
other neurotrophins.\textsuperscript{28-30} Although these and similar experiments demonstrate the capacity for \textit{bcl-2} overexpression to rescue dying neurons, the role of physiological expression of \textit{bcl-2} and similar genes in neurons susceptible to neurotrophic deprivation injury is not well understood. To determine the constitutive cell-death genes in the retina, especially the retinal ganglion cell, we studied the expression of \textit{bcl-2} family members in normal and axotomized retinas using degenerate polymerase chain reaction (PCR) and RNA analysis.

**MATERIALS AND METHODS**

**Optic Nerve Crush**

Animal procedures were in accordance with institutional, state, ARVO, and federal guidelines. All surgery was performed on the right eye of adult Long-Evans rats. Animals were anesthetized with ketamine–xylazine and a limited lateral canthotomy was performed. The conjunctiva was incised at the limbus and the subtenon's space was bluntly dissected posteriorly. The muscle cone was then entered and the optic nerve exposed. The optic nerve was crushed with blunt forceps for 5 seconds 3 mm posterior to the globe under direct visualization. The ocular fundus was observed ophthalmoscopically, and animals with evidence of central retinal artery occlusion were not used in subsequent procedures. The skin was closed with absorbable sutures, topical bacitracin was applied, and the animal was returned to the cage. After recovering from anesthesia, animals were observed to feed and drink normally. Typically, 6 to 10 animals were operated on at each sitting.

**RNA Isolation**

Rats were killed with carbon dioxide and the eyes removed. For studies of axotomized retinas, animals were killed 24 and 96 hours after optic nerve crush, and the contralateral eyes and eyes from unoperated animals were used as controls. Retinas were dissected in sterile Hank's salt solution and flash-frozen on dry ice. Total RNA was then isolated using the guanidinium thiocyanate–phenol–chloroform method, treated with RNase-free DNase I, phenol-chloroform extracted, and precipitated with ethanol. This DNA-free RNA was used for reverse transcription–PCR (RT-PCR), Northern blotting, and RNase protection assays.

**Degenerate PCR**

Degenerate primers specific to the most conserved portions of the BH1 and BH2 domains were designed with the aid of MacVector version 4.5 (Kodak Scientific Imaging, New Haven, CT) and a local primer analysis program. The forward primer was 5'-AAC-TGGGGKMSATYGTGGC-3', the reverse primer 5'-CCCAKCKCCGTTMTCTTGGATC-3'. These primers were designed to maximize amplification of sequences similar to the antiapoptosis genes \textit{bcl-2} and \textit{bcl-x}\textsubscript{L} and were synthesized by Operon Technologies (Alameda, CA).

Total retinal RNA (10 \textmu g) was treated with RNase-free DNase and reverse-transcribed in a 50-\textmu l reaction with MMLV-reverse transcriptase (Gibco, Gaithersburg, MD) at 37°C. Approximately 1 ng ssDNA was amplified in a PCR reaction containing 1 nmol dNTP (Boehringer-Manheim, Indianapolis, IN), 2.5 U AmpliTaq (Perkin-Elmer, Norwalk, CT), and 50 pmol of each degenerate primer in 50 mM KCl, 10 mM Tris (pH 8.3), and 1.5 mM MgCl\textsubscript{2} to a total volume of 50 \textmu l. Polymerase chain reaction amplification was performed on a Perkin-Elmer 480 thermal cycler (Perkin-Elmer) at 95°C for 5 minutes, 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 55°C for 30 seconds, and 72°C for 60 seconds, followed by 72°C for 5 minutes.

**Cloning of PCR Products**

Reaction products were electrophoretically separated on 1.5% low-melting-point agarose gels. The gel slice containing the predicted 160-base pair (bp) band was excised with a new razor blade, digested with \beta-agarase (New England Biolabs, Beverly, MA), and ethanol precipitated. The PCR amplimer, containing a single overhanging A at each 3' end, was ligated into the pT7Blue TA-cloning vector (Novagen, Madison, WI) and used to transform supercompetent cells. The presence of inserts was detected with blue-white selections. Standard procedures were used for overnight cultures and plasmid preparations.

**Clone Analysis**

To facilitate rapid analysis of clones, the presence of an insert was detected by PCR, using primers for the T7 and M13-40 flanking sites of the vector. To screen for clones containing \textit{bcl-2} or \textit{bcl-x}\textsubscript{L} inserts, the following internal primers were designed using portions of the nucleotide sequence where there was minimal homology: \textit{bcl-x}\textsubscript{L}: (forward) 5'-AACCTGGGGTGATGTGGTCT-3' and (reverse) 5'-GATTCAAGGGTCTAGGTTGCTGTC-3'; \textit{bcl-2}: (forward) 5'-TTGACTTCTGGGATGGTATGTC-3' and (reverse) 5'-GATTCAAGGGTCTAGGTTGCTGTC-3'. These were used in PCR performed directly after insert detection, using standard methods. Manual dyeideoxy sequencing was performed using the Sequenase 2.0 kit (Amersham, Arlington Heights, IL).

**Cloning of Rat \textit{bcl-x}\textsubscript{L}**

Rat retinal complementary DNA (cDNA) was amplified using \textit{bcl-x} primers and conditions as described previously.\textsuperscript{7} These primers amplify a 764-bp cDNA
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from the messenger RNA for bcl-xL and an approximately 600-bp cDNA from the alternately spliced bcl-xL. Reverse transcription-PCR using rat retinal cDNA as a template yielded only bcl-xL in our experiments. For cloning, the 764-bp cDNA was gel purified, incubated with Klenow fragment in the absence of dNTP to remove overhanging 3' ends, and filled in with Klenow and dNTP. This blunt-ended amplimer was coprecipitated with appropriate amounts of total yeast tRNA. After precipitation, the samples were air dried and then resuspended in 20 µl hybridization buffer (80% formamide, 40 mM PIPES, 1 mM ethylenediaminetetraacetic acid, 200 mM sodium acetate, pH 6.8) and heated to 95°C for 5 minutes in a Perkin Elmer 9600 Thermocycler followed by rapid cooling to 50°C, where the samples were incubated for 16 to 18 hours. After hybridization, the samples were rapidly diluted with 180 µl buffer containing 10 mM Tris (pH 7.4), 5 mM ethylenediaminetetraacetic acid, and 200 mM sodium acetate. The unhybridized probe in each reaction was digested using 20 units of RNase ONE (Promega, Madison, WI) for 1 hour at 22°C. Protected probe was then precipitated and analyzed by denaturing gel electrophoresis followed by autoradiography. Quantification of protected molecules was made by cutting them out of a dried gel and counting them in a Beckman LS6500 liquid scintillation counter (Fullerton, CA).

In Situ Hybridization

In situ hybridization was carried out using a whole-mount procedure designed for vertebrate embryos and later modified for blocks of rat retina as previously described. Briefly, weaned rats were killed by carbon dioxide inhalation and the eyes enucleated. An incision was made at the midline of the globes, which were then placed in 4% paraformaldehyde in 100 mM phosphate buffer (pH 7.2) for 1 hour at 22°C. Eyes were then placed in 0.4% paraformaldehyde and stored at 4°C for as long as 6 months. After fixation, the eyes were placed in 0.9% NaCl and the retinas dissected from the surrounding tissue, then further cut into small blocks (about 1 mm). These small blocks were used for the whole-mount hybridization protocol as described. Antisense RNA probes were synthesized using the pXEK plasmid and digoxigenin-labeled uridine triphosphate (Boehringer-Mannheim, Indianapolis, IN). Tissue preparation and hybridization conditions were identical to those previously described, except that samples were hybridized with 500 ng/ml antisense pXEK for 24 to 36 hours at 60°C. Control probes containing sense pXEK sequence and sequence specific for a gene expressed in photoreceptor cells (rod opsin) were also generated to monitor nonspecific hybridization using these conditions. After hybridization, unbound probe was removed by washing in 50% formamide at 60°C followed by incubation with RNase A, as described by Li et al. Hybrid-
Amplification of the BH1–BH2 Region From Retinal cDNA

RESULTS

Amplification of the BH1–BH2 Region From Retinal cDNA

Rat retinal cDNA was amplified by PCR using degenerate primers specific to BH1 and BH2 homology domains shared by antideath bcl-2 family members. Agarose gel electrophoresis of the reaction product demonstrated a single 160-bp band, matching the length predicted by the distance between the two homology domains plus the length of the primers.

To identify which members of the bcl-2 family might be present in the amplimers contained within the 160-bp band, amplified DNA was extracted from low-melting-point agarose and ligated into a TA-cloning vector, and clones of transformed bacteria were isolated using ampicillin resistance and blue-white selection. White colonies were used for an initial screen by amplifying the insert within the vector’s flanking sites. Of the 152 clones initially analyzed, 79 had inserts of the appropriate size. Clones with inserts were further analyzed by manual sequencing, PCR amplification using internal primers specific to bcl-2 or bcl-xL sequences, or both methods.

Analysis of all 79 of these clones revealed that 63 contained bcl-xL sequence and 3 contained bcl-2 sequence. The other 13 clones contained sequences lacking complete BH1 and BH2 domains, presumably resulting from mispriming of the degenerate primers at nucleotide sequences weakly homologous to BH1 and BH2. The identification of 21 of the 63 bcl-xL clones and all 3 of the bcl-2 clones was confirmed by direct sequencing. The analysis of the other clones was performed by PCR amplification using bcl-xL- and bcl-2-specific internal primers, generating a 141-bp amplimer with bcl-xL primers and a 116-bp product with bcl-2 primers. No clones were identified that contained complete BH1 and BH2 domains, suggesting that expression of other bcl-2 family members is relatively low in the retina. PCR of rat retinal cDNA using primers outside the BH1–BH2 area amplified bcl-xL, but not bcl-xS.

Relative Expression of Retinal bcl-2 and bcl-xL

Given that the vast majority of identified clones contained bcl-xL sequence and relatively few contained bcl-2, we used RNase protection assays and Northern blotting to establish the relative abundance of mRNA for bcl-xL and bcl-2 in the retina. Total retinal RNA was incubated with 32P-labeled 160-bp probes corresponding to the BH1–BH2 regions of rat bcl-xL and bcl-2. After RNase digestion, protected fragments were electrophoresed and autoradiograms prepared (Fig. 1). The bcl-xL probe protected 2 fragments of mRNA of 160 and 140 nt. This reflects a polymorphism of the bcl-xL gene at nucleotide 546 of the coding sequence, where there is a C→T transition in a degenerate isoleucine codon. This polymorphism is exactly 20 nucleotides from the end of the BH2 region, resulting in the two different protected fragments when derived from RNA pooled from different outbred animals. No such polymorphism was evident for the bcl-2 probe, which protected a full-length 160 nt mRNA fragment. There was minimal bcl-2 mRNA expression, compared with bcl-xL. Direct counting of the labeled gel slices demonstrated that bcl-xL mRNA was 16 times more abundant than bcl-2 in the retina, which is similar to the 21-fold greater frequency of bcl-xL clones than bcl-2 clones found in the clonal analysis. Similar findings were seen when Northern blots of total retinal RNA were probed in parallel for bcl-xL and bcl-2 (data not shown).

Localization of bcl-xL in the Retina

Retinas were prepared for in situ hybridization using a digoxigenin-labeled RNA probe corresponding to the 423 nt of the 3’ coding region of rat bcl-xL. Nonisotopic detection revealed expression of bcl-xL in all cell

Immunofluorescence Labeling

Rat eyes were harvested and fixed as described for in situ hybridization. For immunofluorescence, the entire eye was equilibrated in 30% sucrose and embedded in Histo Prep (Fisher Scientific, Pittsburgh, PA). Cryosections of 5 to 7 μm were cut for immunolabeling. Immunolabeling was carried out as described with the following modifications. Sections were blocked in 100 mM phosphate buffer (pH 7.2) containing 4% bovine serum albumin for 2 hours at 22°C. A polyclonal antibody against Bcl-x, recognizing both the long and short protein products of rat and human bcl-x (Santa Cruz Biotechnology, Santa Cruz, CA), was diluted 1:100 in phosphate buffer and incubated with the sections overnight at 4°C. For detection, the sections were then incubated with goat antirabbit IgG conjugated to Texas Red (Jackson ImmunoResearch, West Grove, PA) for 2 hours at 22°C in the dark. Indirect immunofluorescence was monitored and photographed using a Zeiss Axiophot Photomicroscope (Thornwood, NY).
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FIGURE 1. RNase protection assays demonstrating predominantly bcl-xL expression in the retina. Increasing amounts of total retinal RNA or yeast tRNA were incubated with a32P-labeled 210 nt RNA probe, which contained 160 nt of sequence specific to the BH1-BH2 region of bcl-xL or bcl-2. A polymorphism in bcl-xL resulted in digestion to both 140 and 160 nt protected fragments. (A) Lanes 1 to 5 correspond to bcl-xL probe, lanes 6 to 10 to bcl-2 probe. Lane order is as follows: 1, 6: native probe; 2, 7: 10 µg yeast tRNA; 3, 8: 5 µg retinal total RNA; 4, 9: 10 µg retinal total RNA; 5, 10: 25 µg retinal total RNA. Two-hour exposure. (B) Same RNase protection assays as in A but a 6-hour exposure to demonstrate faint bcl-2 protected fragment.

FIGURE 2. In situ hybridization of rat retina using a digoxigenin-labeled RNA probe for the 3'-most 423 nt of bcl-xL. Hybridized probe is detected as a dark reaction product after incubation with substrate for alkaline phosphatase. There is moderate staining of all nuclear layers (A), as well as focal staining adjacent to the outer limiting membrane of the photoreceptors (B) and in the ganglion cell layer (C). No staining was detected in retina incubated with a sense probe (D). Retinas probed with a positive control opsin probe stained in the inner segments and outer nuclear layer (E). OS: outer segments; IS: inner segments; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar = 40 µm in A, D, and E and 20 µm in B and C.

Prepared from eyes 1 and 4 days after optic nerve crush and amplified by PCR using degenerate primers specific to the BH1 and BH2 regions. This resulted in the previously detected 160-bp amplimer. Unexpectedly, the intensity of this band was observed to decrease over time. This finding was confirmed in three different experiments using separate groups of experimental animals.

Previous experiments had demonstrated that the predominant bcl-2 family member in the retina was bcl-xL. To see whether the species decreasing in the degenerate PCR amplifications corresponded to decreases in retinal bcl-xL after axotomy, we studied the expression of the latter with Northern analysis and RNase protection assays. Blots of total RNA from axo-

layers of the retina, including the ganglion cell layer (Fig. 2). The cytoplasm and nuclei of many of the cells labeled in the ganglion cell layer were large, which is a structure consistent with ganglion cells. However, the possibility that some displaced amacrine cells were labeled could not be excluded. Hybridization with control probes demonstrated no background staining.

The Bcl-x protein product was localized using a polyclonal primary antibody followed by an immunofluorescent secondary antibody. The specificity of the antibody was confirmed by its ability to detect a single band of approximately 29 kDa on Western blots, consistent with rat Bcl-x (data not shown). Immunofluorescence studies revealed immunoreactive cells in the photoreceptor inner segments, the outer plexiform layer, the inner plexiform layer, and especially the retinal ganglion cell layer (Fig. 3). Again, many of the labeled cells in the retinal ganglion cell layer had a structure suggestive of retinal ganglion cells.

bcl-xL Expression Decreases After Retinal Ganglion Cell Axotomy

To understand the expression of bcl-2 family genes after retinal ganglion cell axotomy, retinal cDNA was

FIGURE 2. In situ hybridization of rat retina using a digoxigenin-labeled RNA probe for the 3'-most 423 nt of bcl-xL. Hybridized probe is detected as a dark reaction product after incubation with substrate for alkaline phosphatase. There is moderate staining of all nuclear layers (A), as well as focal staining adjacent to the outer limiting membrane of the photoreceptors (B) and in the ganglion cell layer (C). No staining was detected in retina incubated with a sense probe (D). Retinas probed with a positive control opsin probe stained in the inner segments and outer nuclear layer (E). OS: outer segments; IS: inner segments; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar = 40 µm in A, D, and E and 20 µm in B and C.
FIGURE 3. Immunodetection of Bcl-x in the rat retina. A polyclonal antibody against Bcl-x was followed by a Texas Red secondary antibody. (A) There is strong staining in the photoreceptor inner segments, the outer plexiform layer, the inner plexiform layer, and the ganglion cell layer. Several large cells in the ganglion cell layer (B, C) are focally immunoreactive for Bcl-x, suggestive of ganglion cells. Scale bar = 20 μm in all panels.

Tomized and control retinas were probed with a 32P-labeled full-length rat bcl-xL probe, and relative expression was estimated by densitometry of multiple exposures of the autoradiograms. This demonstrated a decrease of approximately 30% at 4 days after optic nerve crush (Fig. 4). Similarly, total RNA from axotomized and control retinas incubated with a 32P-labeled RNA probe for bcl-xl, digested with RNase, and electrophoretically separated demonstrated a decline in the intensity of the protected fragment, corresponding to a moderate decrease at 1 day and 32% at 4 days, compared with the contralateral control (see Fig. 4).

DISCUSSION

The translocation of a locus on chromosome 18 adjacent to the immunoglobulin heavy-chain promoter on chromosome 14 in human follicular B-cell lymphomas is associated with inhibition of cell death. Subsequent studies demonstrated that overexpression of the gene derived from the breakpoint, B-cell lymphoma/leukemia 2 gene, or bcl-2, prevented cell death in a variety of paradigms, including glucocorticoid and radiation treatment of immature thymocytes, cytokine deprivation of hematopoietic cells, and neurotrophin deprivation of culture neurons. Boise et al. probed a chicken lymphoid library with a murine bcl-2 probe and identified a related gene bcl-xL, which could be differentially spliced into two forms. The long form, or bcl-xL, prevented apoptosis resulting from IL-3 deprivation of an IL-3-dependent prolymphocytic cell line; the short form, bcl-xS, inhibited the ability of bcl-2 to block apoptosis induced by growth-factor withdrawal. Subsequently, several other genes sharing homology to bcl-2 have been identified through various approaches, including bax, bad, bak, and bik. Some of these genes (bcl-2 and bcl-xL) appear to inhibit cell death; others (bcl-xL, bax, bad, bak) induce it. Interactions between members of this family may serve as a mechanism for regulating relative levels of cell death, with binding occurring at bcl-2-related homology domains BH1, BH2, and BH3.

Although bcl-2 and bcl-xL are expressed predominantly in populations of proliferating cells in adult animals, bcl-xS is frequently expressed in postmitotic...
cells. In the central nervous system, embryonic neurons express both bcl-2 and bcl-x<sub>L</sub>,<sup>16,17,39</sup> but adult neurons predominantly express bcl-x<sub>L</sub>.<sup>17</sup> The importance of bcl-x for development of the central nervous system was supported by studies of transgenic mice homozygous for disrupted bcl-x genes.<sup>40</sup> These mice have widespread apoptosis of central neurons during development, dying at embryonic day 13, in contrast to transgenic mice with disrupted bcl-2 genes, which have apparently normal central nervous systems<sup>31,42</sup> and develop loss of facial motor nucleus, dorsal root ganglion, and sympathetic ganglion neurons only after the period of developmental cell death.<sup>43</sup>

Our data demonstrate that bcl-x<sub>L</sub> is the predominant bcl-2 family member expressed in the retina of adult rats. This supports the concept that the neural retina, a central nervous system structure, has a pattern of bcl-2 family member expression similar to that of other central neurons. The degenerate PCR approach used in these studies makes unlikely (but does not exclude) the possibility that another molecule sharing the BH1 and BH2 homology domains is the predominant member of the bcl-2 family in the retina. Genes coding for molecules lacking these domains would not have been detected by our methodology (e.g., that encoded by the death-inducing molecule bik, which lacks BH1 and BH2 and interacts with the protein products of bcl-2 and bcl-x through its BH3 domain).<sup>11</sup> Significant expression of mRNA for the death-inducing molecule bcl-x<sub>L</sub>, the protein product of which lacks BH1 and BH2 domains, was excluded by the absence of the appropriate size species when primers to the full-length bcl-x<sub>L</sub> were used. Furthermore, genes such as bax, which we have amplified by specific RT-PCR from rat retina (Levin and Spieldoch, unpublished data), might not have been detected by the primers used in this study because of their degenerate design.

In addition, we found that bcl-x<sub>L</sub> expression is modulated in response to retinal ganglion cell axotomy, decreasing after optic nerve crush. This suggests that bcl-x<sub>L</sub> downregulation may be part of the pathway by which retinal ganglion cells die by apoptosis after axotomy<sup>18–21</sup> and is similar to the findings of Krajewski et al,<sup>44</sup> who found decreased expression of bcl-x<sub>L</sub> after ischemic neuronal injury. The decrease in retinal ganglion cell bcl-x<sub>L</sub> is unlikely to be caused simply by neuronal loss, because levels decrease at 24 hours after axotomy, a time when ganglion cell loss is minimal, and there is a significant decrease in levels of bcl-x<sub>L</sub> mRNA at 4 days after axotomy, a time when there is relatively little ganglion cell loss.<sup>18</sup> More significantly, decreases in bcl-x<sub>L</sub> levels are unlikely to reflect neuronal loss: we have demonstrated that another axotomy-induced gene, ceruloplasmin, increases in expression within retinal ganglion cells after optic nerve crush (Levin and Geszvain, unpublished data).

We detected minimal bcl-2 expression in the normal adult retina and observed no overt change 1 to 4 days after optic nerve transection. Chen et al<sup>45</sup> demonstrated increased retinal Bcl-2 expression 30 days after optic nerve transection in the adult rat, apparently in Müller cells, but did not look earlier. Our results suggest that neuronal expression of bcl-2 in the retina is minimal, but we cannot exclude a small change in expression early after ganglion cell axotomy that was undetected by our methodology. The role of bcl-2 in response to neuronal injury in other nervous system areas is unclear because both increases and decreases in bcl-2 levels have been described after neuronal insults in a variety of paradigms, including ischemia and excitotoxicity.<sup>44,46–50</sup>

Although the level of bcl-x<sub>L</sub> mRNA is several times that of bcl-2 in the adult retina, the latter retains capacity for inhibiting apoptosis of retinal ganglion cells. For example, retinal ganglion cells from mice overexpressing bcl-2 under the control of the neuron-specific enolase promoter are relatively resistant to axotomy-induced apoptosis.<sup>26,27,51</sup> These mice appear to undergo less developmental cell death of the retinal ganglion cell layer and facial nucleus,<sup>27,52</sup> consistent with a role for bcl-2 in preventing neuronal apoptosis induced by growth-factor deprivation. However, the relative preservation of central neuronal numbers in bcl-2-deficient mice<sup>43</sup> indicates that other mechanisms can substitute for bcl-2 expression in preventing apoptosis. A likely candidate is bcl-x<sub>L</sub>, bcl-x<sub>L</sub> can better prevent apoptosis induced by IL-3 deprivation than bcl-2,<sup>52</sup> the protein product of which may inhibit the death-inducing molecules Bax and Bak by binding by the BH1, BH2, and BH3 domains,<sup>12,53</sup> but has anti-death activity independent of Bax or Bak interactions.<sup>54</sup> The mechanism by which the Bcl-x<sub>L</sub> protein product might modulate retinal neuronal survival is controversial and may include interactions with Bax, Bad, and Bak through BH1 and BH2 domains,<sup>2,53</sup> blockage of apoptosis induced by free radicals,<sup>55</sup> or some other process.<sup>56</sup> However, Bcl-x<sub>L</sub>, which lacks the BH1 and BH2 domains, induces cell death independent of heterodimerization with other bcl-2 family members, suggesting a direct toxic effect.<sup>57</sup> The recent solution of the Bcl-x<sub>L</sub> crystal structure<sup>58</sup> may help suggest structural similarities to other proteins associated with cell death and lead to a definitive role for Bcl-x<sub>L</sub> and other family members in preventing neuronal cell death.

**Key Words**

axotomy, bcl-2, bcl-x, optic nerve, retinal ganglion cell

**References**


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