Bcl-2 and Bcl-x<sub>L</sub> in Peroxide-Resistant A549 and U87MG Cells

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Overexpression of the bcl-2 and the related bcl-x<sub>L</sub> protooncogene proteins enhance cell survival by inhibiting apoptosis induced by many agents including oxidants. Whether these proteins contribute to survival in oxidant-resistant cells is not known. The current study assessed the expression of bcl-2 and bcl-x<sub>L</sub> proteins in human glioblastoma U87MG cells and human lung adenocarcinoma A549 cells selected for resistance to 0, 50, 100, 200, and 400 μM H<sub>2</sub>O<sub>2</sub> by exposure to this oxidant one time each passage for 9 months. When examined 7 to 32 days after cessation of peroxide exposure (times when peroxide resistance was maintained), bcl-2 protein levels were significantly increased in most peroxide-resistant U87MG cells. However, the increase was not dose dependent and was not accompanied by an increase in mRNA levels. A549 cells did not express significant levels of bcl-2 or bcl-x<sub>L</sub> proteins, although bcl-2 mRNA was detectable. A549 cells expressed large amounts of bcl-x<sub>L</sub> and immunohistochemistry demonstrated extensive localization of this protein around the nucleus. However, expression of this protein was not altered in peroxide-resistant lines nor was bcl-2 protein increased to a measurable level. U87MG cells also expressed bcl-x<sub>L</sub> but it was not altered in peroxide-resistant cells. Although the increased bcl-2 protein in peroxide-resistant U87MG cells may contribute to their oxidant tolerance, the lack of a dose–response relationship, the failure to induce bcl-x<sub>L</sub> protein, and the absence of any bcl-2 or bcl-x<sub>L</sub> protein induction in peroxide-resistant A549 cells suggest these genes are not primary factors in oxidant resistance.

Key Words: bcl-2; bcl-x<sub>L</sub>; hydrogen peroxide; oxidant toxicity; A549 cells; U87MG cells.

The mechanism(s) by which cells defend themselves against an oxidative stress has been studied in cell lines that become stably resistant to H<sub>2</sub>O<sub>2</sub>-induced toxicity following stepwise exposure to increasing concentrations of H<sub>2</sub>O<sub>2</sub>. Initial work in a H<sub>2</sub>O<sub>2</sub>-resistant Chinese hamster fibroblast cell line revealed increases in a number of antioxidant systems and supported the hypothesis that catalase activity was a major determinant of resistance to exogenous bolus peroxide doses (Spitz et al., 1992). Recently, using a protocol similar to that used with the hamster cell lines, H<sub>2</sub>O<sub>2</sub>-resistant human glioblastoma cells derived from the U87MG parental line demonstrated increases in catalase activity, but inconsistent or no changes in glutathione peroxidase, Mn-SOD, and Cu/Zn-SOD activities (Spitz et al., 1996). The changes in catalase activity, while significant, did not appear to be in proportion to the extent of resistance. Thus, the mechanism by which these cell lines are resistant to H<sub>2</sub>O<sub>2</sub> toxicity may involve other as yet unidentified factors.

Oxidants, including reactive oxygen species (ROS) and free radicals, can modulate various cellular processes leading to alterations in growth and differentiation and possibly death (Burdon, 1995). The cytotoxic effects of ROS are well established in terms of necrosis. However, ROS may also trigger apoptotic cell death in a variety of tissues and cell lines (Korsmeyer et al., 1995; Payne et al., 1995; Safarian and Bredesen, 1994). The mechanisms involved in controlling oxidant-induced apoptosis are unknown but one possible factor is the protooncogene bcl-2. The protein product of the bcl-2 gene appears to inhibit apoptosis, as well as necrosis, and is induced by a variety of stimuli including oxidants, radiation, hyperthermia, chemotherapy agents, glucocorticoids, and growth factor withdrawal (Miyashita and Reed, 1993; Walton et al., 1993; Zhong et al., 1993).

Bcl-2 was discovered by analysis of B-cell lymphomas which revealed that the t(14;18) chromosomal translocation of bcl-2 is found in more than two-thirds of this cancer. This translocation leads to transcriptional deregulation and overproduction of the hydrophobic 26-kDa bcl-2 protein. The protein product of bcl-2 is localized primarily in the nuclear envelope, outer mitochondrial membrane, and endoplasmic reticulum (Krajewski et al., 1993). The protein product of the related bcl-x<sub>L</sub> gene appears to function similarly (Boise et al., 1993) and to have a similar localization (Gonzalez-Garcia et al., 1994).

A link between free radicals or oxidants, apoptosis, and the protooncogene bcl-2 is supported by several studies indicating that the protein encoded by this gene suppresses the apoptosis and toxicity induced by various oxidative processes (Hockenbery et al., 1990; Kane et al., 1992; Miyashita and Reed, 1993; Park and Hockenbery, 1996; Veis et al., 1993; Walton et al., 1993; Zhong et al., 1993). In addition, various antioxidants...
appear capable of substituting for bcl-2 expression in preventing apoptosis (Hockenbery et al., 1993). However, bcl-2’s function, and thus the mechanism by which it prevents apoptosis and protects cells from free radical-mediated injury, is not known. Bcl-2 is clearly not a classical antioxidant, but seems to decrease the net cellular generation of ROS in a neural cell line (Kane et al., 1992; Wiedau-Pazos et al., 1996). It has been suggested that mitochondria are not the source of these ROS since overexpression of bcl-2 does not appear to affect function of this organelle (Hockenbery et al., 1993) and bcl-2 blocks apoptosis in human fibroblast cells lacking a functional respiratory chain (Jacobson et al., 1993). However, mitochondrial bcl-2 protein may still be an important factor in apoptosis as suggested by recent work showing the ability of bcl-2 to prevent the release of cytochrome c (Kluc et al., 1997; Yang et al., 1997) and to control the mitochondrial permeability transition (Petit et al., 1996).

While the overexpression of the bcl proteins can prevent oxidative injury, the converse study as to whether bcl expression is altered in oxidant resistant cells has not been reported. The present study was designed to examine the expression of both bcl-2 and bcl-xL proteins in a human glioblastoma cell line and a human lung adenocarcinoma cell line made tolerant to increasing amounts of H2O2. The results show that bcl-2 protein levels were increased in peroxide tolerant U87MG cells, but not in A549 cells which largely lack this protein. Bcl-xL protein levels were not altered in either cell line.

MATERIALS AND METHODS

Materials. Bcl-2 standard protein was a lysate of S49 cells stably transfected with a human bcl-2 complementary DNA (Caron-Leslie et al., 1994). The lysate was a gift from Dr. Rosemary B. Evans (National Institute of Environmental Health Sciences). Bcl-xL standard protein was a lysate of KMS6 leukemia cells stably transfected with human bcl-xL. These cells were provided by Dr. Gabriel Nuñez (University of Michigan). Monoclonal bcl-2 antibodies and polyclonal bcl-xL antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Oligonucleotides for RT-PCR were obtained from Genosys (The Woodlands, TX). KpnI, reverse transcriptase, and Taq polymerase were obtained from Promega (Madison, WI). Sense, antisense, and nonsense 18-mer phosphorothioate oligonucleotides to the translation start site of bcl-xL (Boise et al., 1993) were obtained from Oligos Etc. Inc. (Wilsonville, OR).

U87MG and A549 cell maintenance and H2O2 adaptation protocol. The U87MG human glioblastoma cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in BME medium ( Gibco, Grand Island, NY) supplemented with 10% bovine calf serum (Hyclone, Logan, UT). A549 cells, originally obtained from ATCC, were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (Atlanta Biologicals, Northcross, GA) and penicillin (50 units/ml)/streptomycin (50 mg/ml). Cells were incubated in a humidified atmosphere of 95% air:5% CO2 at 37°C. The medium was changed every second day and cells were subcultured every 3 to 4 days when they reached 80 to 90% confluency.

H2O2-resistant variants were derived by culturing U87MG and A549 cells in complete medium containing progressively increasing concentrations of H2O2. U87MG and A549 cells cultured for the same length of time were used as controls. Initially, exponentially growing monolayers were trypsinized and 2.5 x 10^5 cells were plated into complete medium and incubated 24 h at 37°C. Then, H2O2 was directly added to the flasks to achieve final concentrations of 50 or 100 μM. Peroxide-treated cell lines received H2O2 (50 or 100 μM) once weekly and were passaged weekly for approximately 20 passages. At this time, both peroxide-exposed cell lines were capable of forming monolayers when treated with H2O2, something control cells could not do. To generate cells resistant to even higher H2O2 concentrations, 2.5 x 10^6 of 100 μM adapted cells were transferred into a flask and treated weekly with 200 μM H2O2. Within four passages these cells grew into confluent monolayers when treated with H2O2. At this point, 2.5 x 10^6 of the 200 μM adapted cells were treated weekly with 400 μM H2O2 for four additional passages. The final result of this procedure was cell lines tolerant to 50, 100, 200, or 400 μM H2O2. All H2O2-resistant cell lines were then maintained by weekly treatment with appropriate concentrations of H2O2. The last peroxide exposure was a minimum of 7 days prior to analyses to expand the populations sufficiently for experimentation. The resistant phenotype was confirmed using bolus doses of peroxide for 1 h at 37°C and clonogenic survival assays (Spitz et al., 1988, 1989).

Western blot assay. Cells were lysed with 1 ml RIPA buffer (10 mM sodium phosphate, 150 mM NaCl (pH 7.4), 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml of PMSF, 0.3% NP-40, and 1 mM sodium orthovanadate) by aspirating slowly four to five times with a 26-gauge 1/2-in. needle. The lysed cells were centrifuged at 400g for 10 min and supernatants were run on 15% reducing SDS polyacrylamide gels (buffer composition: 20% glycerol, 4% β-mercaptoethanol, 4% SDS, 0.2 M Tris–HCl, pH 6.8, 0.02% bromophenol blue). Protein content was determined by the method of Lowry et al., (1951). Proteins were transferred on PVDF membranes and blocked overnight. The membrane was then incubated with either anti-bcl-2 or anti-bcl-xL antibodies (1:150 dilution) for 4 h. After membrane washing, horseradish peroxidase-conjugated secondary antibodies were used (1:2000 dilution; Sigma Chemical Co., St. Louis, MO). Blots were detected using a chromogen mixture with 0.1% H2O2 in methanol. Bcl-xL antibodies (1:150 dilution) for 4 h. After membrane washing, horseradish peroxidase was quenched with 0.1% H2O2 in methanol. Bcl-xL antibodies were obtained from Dr. Gabriel Nufiez (University of Michigan). Monoclonal bcl-2 antibodies (Sigma Chemical Co., St. Louis, MO) were then added and incubated with gentle rocking at 4°C overnight. The precipitate was collected by centrifugation at 200g for 5 min at 4°C. The pellet was suspended in 40 μl electrophoresis sample buffer. The sample was then heated at 100°C for 2 min and subjected to Western blotting as described above.

Immunoprecipitation. Bcl-2 or bcl-xL protein was immunoprecipitated by taking the cell lysate prepared for Western blotting and incubating with 1 μg of either bcl-2 or bcl-xL antibody, respectively, for 1 h at 4°C. Twenty microliters of agarose conjugate (Protein A, Sigma Chemical Co., St. Louis, MO) was then added and incubated with gentle rocking at 4°C overnight. The precipitate was collected by centrifugation at 200 g for 5 min at 4°C. The pellet was then washed three times with 1 ml RIPA buffer. After the final wash the supernatant was removed and the pellet was suspended in 40 μl electroblotting sample buffer. The sample was then heated at 100°C for 2 min and subjected to Western blotting as described above.

Immunohistochemistry. Methanol-fixed A549 cells were incubated with anti-bcl-xL-polyclonal antibody at a dilution of 1:50 in PBS (pH 7.4) containing 1% BSA. Biotinylated rabbit IgG (1:226 dilution in PBS containing 1% BSA) was used to detect anti-bcl-xL binding. A549 cells incubated without primary antibody were used as controls. Cells were then incubated for 30 min with an avidin-biotinylated horseradish peroxidase complex. Endogenous horseradish peroxidase was quenched with 0.1% H2O2 in methanol. Bcl-xL was detected with diaminobenzidine. Slides were rinsed three times with PBS between all incubations and counterstained with hematoxylin.

RT-PCR. Competitive RT-PCR was performed as described by Gilliland et al. (1990) and modified by Vanden Heuvel et al. (1994). RNA was reverse transcribed in a final volume of 20 μl containing 25 mM Tris–HCl (pH 8.3, 25°C), 50 mM (NH4)2SO4, 0.3% β-mercaptoethanol, 0.1 mg/ml bovine serum albumin, 5 mM MgCl2, 1 mM of each deoxynucleotide triphosphate, 1 unit RNase inhibitor, 2.5 units MMLV reverse transcriptase, 2.5 mM oligo(dT)15, and 0.1 μg each of internal standard RNA (rRNA) and sample RNA. For
reverse transcription, samples were incubated for 15 min at 42°C followed by 5 min at 95°C to inactivate reverse transcriptase. Immediately following, amplification was performed by adding 30 µl of 85°C PCR mix to bring the final volume to 50 µl. The final MgCl₂ concentration was 4 mM and contained 2.5 units of Taq polymerase and 6 pmol of forward and reverse primers. PCR primer reactions were performed using 30 cycles (30s at 95°C, 30 s at annealing temperature 53°C, and 30 s at 72°C). Following amplification, each 50-µl reaction was incubated with 1 unit KpnI restriction enzyme (37°C, 1 h), followed by heat inactivation to improve the separation and visualization of the internal standard cDNA vs target cDNA. PCR products were analyzed by 3% agarose gel electrophoresis and ethidium bromide staining. Densitometry was performed on gel photographs using an LKB Gel Scan II laser densitometer. The ratio of the volume of rRNA to target RNA was plotted against the amount of rRNA added to each reaction, and quantitation of sample RNA was performed as described previously (Gonzalez-Garcia et al., 1995). All PCR reactions were completed using a Perkin Elmer Cetus GeneAmp PCR system (Norwalk, CT). Primer sequences were (Bcl-2) 5' - GCC AAC CAC ATC CAA TAA - 3' and reverse, 5' - CCA CTC GTA GCC CCT CTG - 3'.

**Antisense.** Sense, nonsense, and antisense 18-mer phosphorothioate oligonucleotides to the translation start site of bcl-xL (Bouse et al., 1993) were incubated at a concentration of either 10 or 20 µM for 3 days with A549 cells. On day 4, 10 or 20 µM of the same oligomer was added. Cells were analyzed for bcl-xL protein expression after 3 and 7 days.

**Statistics.** Data are expressed as means ± SE. Statistical comparisons were performed using Wilcoxon's two-sample test. A p value of less than 0.05 was considered significant.

**RESULTS**

Both A549 and U87MG cells exhibited resistance to bolus doses of H₂O₂ following the selection protocol (Table 1). The resistance was greatest in cells selected to be tolerant of the highest chronic doses, and U87MG cells were relatively more resistant than A549 cells. Although appearing to decrease with time, the resistance to H₂O₂ in U87MG cells remained eight-fold above controls 32 days following removal from the chronic exposure protocol (Table 2). The lower surviving fraction in this particular study compared to Table 1 was due to the use of a higher bolus peroxide dose.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Control</th>
<th>50 µM</th>
<th>100 µM</th>
<th>200 µM</th>
<th>400 µM</th>
</tr>
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<tr>
<td>U87MG</td>
<td>0.06 ± 0.01</td>
<td>0.22 ± 0.03</td>
<td>0.49 ± 0.03</td>
<td>1.08 ± 0.08</td>
<td>0.99 ± 0.07</td>
</tr>
<tr>
<td>A549</td>
<td>0.09 ± 0.02</td>
<td>0.24 ± 0.01</td>
<td>0.20 ± 0.01</td>
<td>0.32 ± 0.01</td>
<td>0.58 ± 0.04</td>
</tr>
</tbody>
</table>

*Data are expressed as the mean surviving fraction ± SE of one dish treated 7 days after the last peroxide exposure and plated into four or five dishes in a clonal survival assay after exposure to 2 pmol/cell H₂O₂ for 1 h at 37°C. The surviving fraction is normalized to untreated control plating efficiency for each cell line.*

are shown in Fig. 1. There was a significant increase in bcl-2 expression in peroxide-tolerant cells compared to control cells (Fig. 1, Table 3). The darkest bcl-2 band was seen in cells tolerant to 100 µM H₂O₂ and the weakest in control cells. Cells tolerant to 50 µM H₂O₂ had less bcl-2 than higher doses, although there was no obvious dose–response relationship. The results of density integration of these blots are presented in Table 3. This analysis showed significant increases in bcl-2 mRNA levels in all 8 of the 12 treatment groups. There was no significant time-dependent change in bcl-2 protein postperoxide within any resistance level.

In contrast to the findings in U87MG cells, there was no detectable expression of bcl-2 protein in a control A549 cell lysate using peroxidase/diazobenzidine detection (Fig. 2A). Immunoprecipitation of 1.67 mg A549 protein did yield a faint band (Fig. 2B), demonstrating the presence of a small amount of bcl-2 protein. This was in contrast to the major band found following immunoprecipitation of 135 µg U87MG lysate protein (Fig. 2C).

Although bcl-2 protein levels were increased in most peroxide-resistant U87MG cells, bcl-2 mRNA content was not altered (Fig. 3). Interestingly, despite expression of very low levels of bcl-2 protein in A549 cells, RT-PCR analysis revealed a significant content of bcl-2 mRNA (Fig. 3, lanes 7–11). Based on this finding, analyses for any increased bcl-2 expression in H₂O₂-tolerant A549 cells were performed. Bcl-2 protein levels in A549 cells resistant to 50, 100, 200, or 400

**TABLE 1**

Effect of Acute Peroxide Exposure on H₂O₂-Resistant Cells

<table>
<thead>
<tr>
<th>Peroxide resistance level</th>
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<tbody>
<tr>
<td>7</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>100 µM</td>
</tr>
</tbody>
</table>

*Data are expressed as the mean surviving fraction × 10⁻² of two or three culture plates assayed in a single experiment using a clonal survival assay after exposure to 4 pmol/cell H₂O₂ for 1 h at 37°C.*
μM H₂O₂ lysed one passage (7 days) following the last peroxide treatment did not increase to measurable levels using peroxidase diazobenzidine detection (data not shown).

Bcl-xL is a 29- to 30-kDa member of the bcl-2 family with similar activity to bcl-2 (Boise et al., 1993). In contrast to bcl-2, it is expressed in a greater variety of tissues and often persists beyond the embryonic period (Gonzalez-Garcia et al., 1995). Immunoblot analyses of control A549 cells revealed a large level of expression of this protein. The identity of this protein as bcl-xL was confirmed by comparison to lysate from K562 cells transfected to overexpress this protein as well as by immunoprecipitation (Fig. 4). Interestingly, basal bcl-xL expression in A549 cells was greater than that in the transfected K562 cell line (Fig. 5A). Analyses of peroxide-tolerant A549 cells did not reveal any changes in bcl-xL expression 7 days after the last peroxide exposure (Fig. 5A). This protein was also not altered in peroxide tolerant U87MG cells 7 (Fig. 5B), 18, or 32 days (data not shown) after the last peroxide exposure.

The large basal expression of bcl-xL in A549 cells made it of interest to determine the localization of this protein. Immunohistochemistry revealed extensive localization of bcl-xL protein around the nucleus (Fig. 6). Mitochondrial localization was not visible due to the resolution of this experiment, but is likely based other published work (Krajewski et al., 1993; Gonzalez-Garcia et al., 1994).

Previous work indicated that antisense oligonucleotides to bcl-xL in WEHI-231 lymphoma cells were able to partially block upregulation of this protein (Wang et al., 1995). Following a similar protocol, no change in basal bcl-xL expression could be achieved in A549 cells (data not shown). This could be due to the long half-life of this protein and its mRNA making basal expression more difficult to decrease than up-regulation or may be related to differences between A549 and WEHI-231 cells.

**DISCUSSION**

Induction of antioxidant enzyme systems has been considered a major mechanism of tolerance to oxidative injury. This concept has received strong support from work on various models including animals resistant to pulmonary oxygen toxicity (Briehl and Baker, 1996; Frank, 1991). More recent studies have demonstrated that other mechanisms, including cytokines such as interleukin 1 (Lee et al., 1994) and keratinocyte growth factor (Panos et al., 1995), may also be important for protecting lung tissue from hyperoxia. This raises the possibility that unrecognized factors play a role in cellular resistance to oxidative injury.

Examination of antioxidant enzyme activities in peroxide resistant U87MG cells revealed only moderate changes except for catalase activity that was increased four- to sixfold (Spitz et al., 1995). Follow-up experiments confirmed a major mechanism of tolerance to oxidative injury. This was also shown in the WEHI-231 lymphoma cell line.

**TABLE 3**

<table>
<thead>
<tr>
<th>Days after Peroxide</th>
<th>H₂O₂ dose (μM)</th>
<th>Control</th>
<th>50</th>
<th>100</th>
<th>200</th>
<th>400</th>
</tr>
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<tbody>
<tr>
<td>7</td>
<td></td>
<td>1.00 ± 0.11</td>
<td>1.19 ± 0.11</td>
<td>1.42 ± 0.09*</td>
<td>1.03 ± 0.07</td>
<td>1.16 ± 0.12</td>
</tr>
<tr>
<td>18</td>
<td></td>
<td>1.00 ± 0.11</td>
<td>1.14 ± 0.06*</td>
<td>1.33 ± 0.08*</td>
<td>1.10 ± 0.13</td>
<td>1.24 ± 0.08*</td>
</tr>
<tr>
<td>32</td>
<td></td>
<td>1.00 ± 0.18</td>
<td>1.26 ± 0.07*</td>
<td>1.58 ± 0.16*</td>
<td>1.28 ± 0.10*</td>
<td>1.38 ± 0.08*</td>
</tr>
</tbody>
</table>

*Data are expressed as the mean ratio to control (no peroxide) ± SE of integrated density values from immunoblots. Each sample was run five separate times for Day 7, and four times for Days 18 and 32.

* Significantly different from control; Wilcoxon’s two-sample test (p < 0.05).
Bcl IN PEROXIDE-RESISTANT CELLS

al., 1996). Since this increase did not correlate with the extent of peroxide resistance, additional factors were considered. Bcl-2 is an obvious gene to examine for a role in resistance to oxidant-induced injury since it has been linked to protection from free radical-mediated necrosis and apoptosis by a number of studies (Hockenbery et al., 1993; Kane et al., 1992; Korshmeier et al., 1995). Furthermore, bcl-2 prevents the loss of antioxidant enzyme activity, particularly catalase, during dexamethasone-induced apoptosis in a lymphoid cell line (Baker et al., 1996).

The results of the current study showed increases in bcl-2 protein, but not bcl-xL, another bcl family member with anti-apoptotic activity, in U87MG cells selected for resistance to H2O2. Cooperation between bcl-2 and bcl-xL in cells expressing both proteins has not been reported, but it is possible that most bcl activity in U87MG cells is provided by bcl-2 and that adaptive changes induced by chronic H2O2 exposure may have increased bcl-2 expression to a maximum effective level. The absence of a dose–response relationship between the level of peroxide resistance and the level of bcl-2 indicates that any protective effect is unlikely to be direct, but does not preclude this gene from contributing to the observed resistance. These results are similar to findings in multiple myeloma cells exposed acutely to 0.5 mM H2O2 (Tu et al., 1996) in that bcl-2 protein was increased and bcl-xL protein was unaffected, but differed in that no dose–response relationship was seen nor were bcl-2 mRNA levels increased. Comparisons between acute and chronic H2O2 exposure studies using different cell lines may be, however, of limited value.

Bcl-2 is reportedly expressed at low levels in alveolar epithelial cells (Lu et al., 1996). This was evident in A549 cells, a type 2 alveolar epithelial cell line, that did not express significant amounts of bcl-2 protein despite containing bcl-2 mRNA. However, A549 cells did express a large amount of bcl-xL. This expression may already be maximal, thereby explaining the failure to induce either bcl-2 or bcl-xL in H2O2-tolerant A549 cells. Of interest is the fact that A549 cells are constitutively relatively resistant to damage by radiation (Mortyn et al., 1984) as well as other toxicants (Perry et al., 1995). This resistance may be related to the very high basal expression of bcl-xL or the relatively high levels of glutathione found in these cells.

The extensive localization of the bcl-xL protein to the nuclear envelope in A549 cells is similar to that reported previously in murine FL5.12 cells that overexpress this protein (Gonzalez-Garcia et al., 1994). The similarity between a non-transfected (but transformed) human cell line and a transfected murine line suggests that, in addition to mitochondria, this is a normal site for this protein. It is interesting that the bcl proteins localize in membranes with electron transport capabilities; mitochondria, endoplasmic reticulum, and nuclear membranes (Krajewski et al., 1993; Gonzalez-Garcia et al., 1994) and bcl-proteins are protective against oxidant injury. This is suggestive for a role in some aspect of electron transport or redox regulation of cell function.

The function of the bcl proteins remains unknown. Although the protection provided against oxidant-induced cell death by overexpression of bcl-2 has resulted in this protein being referred to as an antioxidant, it appears that the bcl-2 protein lacks any direct antioxidant activity (Lee and Shacter, 1997; Marshall et al., 1995). It has even been suggested that bcl-2 might be a prooxidant based on the induction of gene products associated with oxidant resistance in superoxide dismutase-deficient Escherichia coli transfected to overexpress bcl-2 (Steinman, 1995), although alternate explanations seem possible.

Recent work with bcl-xL has shown structural similarity to the membrane channel formed by diphtheria toxin which facilitates passage of a protein fragment (London, 1992) and is supportive of a regulatory role for ions or small molecules (Minn et al., 1997). A potential candidate is cytochrome c (Kluck et al., 1997; Yang et al., 1997) which is reportedly

![FIG. 3. RT-PCR analysis for bcl-2 mRNA. RT-PCR products from U87MG and A549 cells 7 days after the last peroxide exposure were separated by agarose gel electrophoresis and detected with ethidium bromide staining as described under Materials and Methods. Lane 1, U87MG control; lane 2, U87MG 50 μM; lane 3, U87MG 100 μM; lane 4, U87MG 100 μM; lane 5, U87MG 200 μM; lane 6, U87MG 400 μM; lane 7, A549 control; lane 8, A549 50 μM; lane 9, A549 100 μM; lane 10, A549 200 μM; lane 11, A549 400 μM.](https://academic.oup.com/toxsci/article-abstract/42/2/109/1685742/figure-3)
necessary for the initiation of apoptosis. The electron transport ability of this protein provides further evidence that the bcl proteins may protect cells from oxidant-induced death through an ability to affect cellular redox regulation, perhaps by affecting glutathione transport (Bojes et al., 1997).

Expression of high levels of bcl-2 does not protect Burkitt's lymphoma cells from hydrogen peroxide-induced killing (Lee and Shacter, 1997) suggesting that this protein has no role in resistance to acute peroxide toxicity under conditions where cell death is primarily necrotic in nature. Increased bcl-2 expression was recently shown in response to heat shock in the human cell lines U937, HL-60, and CCRF-CEM (Polla et al., 1996). These increases may have played a role in the resistance of heat-shocked cells to H$_2$O$_2$-induced mitochondrial damage. Together with our findings of increased bcl-2 expression in peroxide-tolerant U87MG cells, these data suggest that induction of bcl-2 may be a relatively common response to stress, at least in cells that have basal levels of expression. The lack of induction of bcl-x$_L$ indicates it does not respond to stress like bcl-2.
In summary, the expression of bcl-2, but not bcl-xL, was enhanced in U87MG cell lines tolerant to H_2O_2. This increased expression was not dose related and was not accompanied by a change in mRNA levels, but was persistent for at least 1 month following cessation of peroxide exposure. Peroxideresistant A549 cells did not exhibit any increase in the very low basal levels of bcl-2 expression, nor were the high bcl-xL protein levels altered. The failure to induce bcl-2 protein, or additional bcl-xL protein in A549 cells, may be related to the very high basal levels of bcl-xL found in this cell line. Bcl-xL protein was found at the same subcellular sites previously reported in transfected cell lines suggesting that this localization is normal. Although implicated in oxidant resistance by numerous studies in overexpressing cell lines, a direct role for bcl family proteins in resistance to peroxide toxicity was not supported by the current study in nontransfected oxidant-resistant human cell lines, although a contributory role remains possible.

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