Overexpression of bcl-x<sub>L</sub> Protects Astrocytes from Glucose Deprivation and Is Associated with Higher Glutathione, Ferritin, and Iron Levels

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Background: The possibility of altering outcome from ischemia-like injury by overexpressing the anti-cell death gene bcl-x<sub>L</sub> was studied. Cells are known to die by different pathways including apoptosis, or programmed cell death, and necrosis. The bcl-x<sub>L</sub> gene is a member of a family of apoptosis regulating genes and often displays the death-inhibiting properties of the prototype of this family, bcl-2. It is of special interest to study bcl-x<sub>L</sub> for possible brain protection, because, unlike bcl-2, it is important for normal brain development.

Methods: Overexpression of bcl-x<sub>L</sub> was achieved in primary astrocyte cultures using a retroviral vector. Cultures of astrocytes overexpressing bcl-x<sub>L</sub> or a control gene were injured by hydrogen peroxide, glucose deprivation, or combined oxygen and glucose deprivation. Outcome was assessed morphologically and by release of lactate dehydrogenase. We assessed antioxidant effects by measuring glutathione using monochlorobimane, ferritin by immunoblotting, the level of iron spectrophotometrically, and superoxide using iodonitrotetrazolium violet and dihydrothreidium.

Results: Protection by bcl-x<sub>L</sub> was found against glucose deprivation and hydrogen peroxide exposure but not combined oxygen and glucose deprivation. Higher levels of superoxide were found, without increased levels of lipid peroxidation. Overexpression of bcl-x<sub>L</sub> was associated with elevated glutathione levels, elevated ferritin levels, and increased amounts of iron. The increased glutathione contributed to the protection from glucose deprivation.

Conclusions: Overexpression of bcl-x<sub>L</sub> protects astrocytes from oxidative injury with the same spectrum of protection seen previously for bcl-2. The increased antioxidant defense observed should be beneficial against both apoptotic and necrotic cell death. The effects on levels of ferritin and iron are novel and identify a new area of interest for this gene family. Whether this relates to the effects of these genes on mitochondrial function remains to be elucidated. (Key words: Antioxidant; bcl-2; free radical; hydrogen peroxide; hypoglycemia; ischemia; mouse; primary culture; retrovirus.)

The gene bcl-x is a member of the evolutionarily conserved bcl-2 gene family. Two principal bcl-x splice variants have been reported, bcl-x<sub>S</sub> and bcl-x<sub>L</sub>, referring to long and short forms. Only bcl-x<sub>L</sub> prevents apoptosis, a type of cell death that involves a cascade of closely regulated intracellular events leading to cell suicide. Many studies have demonstrated wide-ranging protective effects of bcl-2 and bcl-x<sub>L</sub> against both apoptotic and necrotic cell death. These genes are therefore of great interest for protection from ischemic brain injury, in which both necrotic and apoptotic cell death occurs. The original distinction between apoptotic and necrotic cell death was a morphologic one. Necrotic cell death was initially considered cell-independent; however, recent studies suggest greater overlap between apoptosis and necrosis. Mitochondrial dysfunction, cellular adenine triphosphate levels, calcium loading, and reactive oxygen species all influence both types of cell death. Furthermore, many of the same stimuli induce either apoptotic- or necrotic-appearing cell death depending on the severity of the insult. This concept also applies in cerebral ischemia. Delayed cell death, seen in the hippocampus after global ischemia, is often apoptotic. During focal ischemia, cells dying in the penumbra, that area bordering the core that retains a reduced level...
of perfusion, show signs of apoptosis; more of the cells in the center of the infarct have features of necrosis.

Similar but not identical anti-death effects are possessed by bcl-x\textsubscript{L} and bcl-2.\textsuperscript{9,10} Unlike bcl-2, bcl-x\textsubscript{L} plays a critical role in brain development.\textsuperscript{11} Survival of bcl-2 knockout mice into postnatal life is without major central nervous system (CNS) deficits.\textsuperscript{12} Knockout of bcl-x\textsubscript{L} produces embryonic death and marked CNS deficits.\textsuperscript{11} The gene bcl-x\textsubscript{L} is important in neuronal survival \textit{in vitro},\textsuperscript{13} and higher expression in mature brain suggests a role in CNS cell survival \textit{in vivo}.\textsuperscript{14,15} Members of the bcl-2 family have been shown to protect from cerebral ischemia \textit{in vivo}\textsuperscript{16,17} and from related insults \textit{in vitro}.\textsuperscript{18,19} Overexpression also prevented neuronal death from trophic-factor deprivation or oxidative stress induced necrosis.\textsuperscript{2,16,18}

Both genes protect in animal models of stroke.\textsuperscript{15–17} Overexpression of bcl-2 is associated with higher superoxide levels\textsuperscript{20} but decreased generation of oxidized cell constituents.\textsuperscript{16,19,21} There is evidence that bcl-2 family members affect ischemic cell death. The ratio of bcl-2 family cell death repressors (bcl-2, bcl-x\textsubscript{L}) to cell-death promoters (bax) decreases in areas that die after cerebral ischemia.\textsuperscript{8,22–25} The prevention of necrotic neuronal death by bcl-2\textsuperscript{22,24} suggests that bcl-2 affects a physiologic step common to both apoptotic and necrotic death pathways, perhaps improving antioxidant defense or improving mitochondrial function.\textsuperscript{21,25} This also applies to bcl-x\textsubscript{L}.\textsuperscript{26}

Our previous studies showed that overexpression of bcl-2 reduced necrotic oxidative death of primary mouse astrocytes.\textsuperscript{19} Astrocytes were studied because of their importance in antioxidant defense.\textsuperscript{27–30} Astrocytes have higher glutathione (GSH) levels than neurons\textsuperscript{27,28} and protect neurons from peroxide toxicity \textit{in vitro}.\textsuperscript{29} Oxidative stress is implicated in CNS injury, including stroke, head trauma, Alzheimer’s disease, and amyotrophic lateral sclerosis.\textsuperscript{5} We have now tested the protective ability of bcl-x\textsubscript{L} and focused on antioxidant defenses, characterizing changes GSH, iron, and ferritin. A preliminary report has appeared.\textsuperscript{31}

**Materials and Methods**

All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise specified.

**Astrocyte Cultures**

Primary astrocyte cultures were prepared from postnatal (days 1–3) Swiss Webster mice (Simonsen, Gilroy, CA) as previously described.\textsuperscript{32} All procedures were carried out according to a protocol approved by the Stanford University animal care and use committee, in keeping with the National Institutes of Health guide. In brief, neocortices were dissected free of meninges, treated with 0.09% trypsin, triturated and plated as a single-cell suspension in Falcon Primaria 24-well plates (Becton Dickinson, Lincoln, IL) at a density of two hemispheres per multiwell, in Eagle’s Minimal Essential Medium (Gibco, Grand Island, NY), supplemented with 10% equine serum (HyClone, Logan, UT), 10% fetal bovine serum (HyClone), 21 mM (final concentration) glucose and 10 ng/ml epidermal growth factor. The cultures were infected with retroviral vectors encoding bcl-x\textsubscript{L}, bcl-2, or lacZ, the \textit{Escherichia coli} gene encoding β-galactosidase and selected with G418 as previously described.\textsuperscript{19} Cells were used for experiments between days 21 and 45 \textit{in vitro}. Experiments were performed at least three times on cultures from a minimum of three different dissections.

**Retroviruses**

The mouse bcl-x\textsubscript{L} gene was cloned by reverse-transcriptase polymerase chain reaction from Swiss Webster mouse RNA, and both strands were sequenced for verification. The bcl-x\textsubscript{L} sequence was then inserted into the LXSN retroviral backbone.\textsuperscript{33} Retroviruses to express human bcl-2 or \textit{E. coli} β-galactosidase in the MPZen vector\textsuperscript{34} were provided by Dr. David Vaux. We have previously found that after antibiotic selection, more than 95% of the astrocytes express the gene of interest.\textsuperscript{19}

**Immunobistochemistry**

Immunostaining of cultures for ferritin was accomplished after fixing in methanol:acetone (1:1) at −20°C for 20 min. The cells were air-dried, rinsed in phosphate-buffered saline (PBS), and incubated in a polyclonal antiferritin antibody (Boehringer Mannheim, Indianapolis, IN) diluted 1:100 in PBS containing 5% fetal calf serum and 0.09% sodium azide for 2 h at room temperature. The cells were rinsed in PBS and incubated in a horseradish peroxidase-linked antirabbit antibody (Amersham, Arlington Heights, IL) diluted 1:100, for 1 h. After rinsing in PBS, antibody binding was visualized by exposing to diaminobenzidine and urea H2O2 for 5 min. No staining was observed if the primary antibody was omitted. Counterstaining was with Gill no. 3 hematoxylin. Astrocytes expressing β-galactosidase were stained with X-gal (Molecular Probes, Eugene, OR).
Immunoblotting

Expression of ferritin or bcl-xL was estimated by immunoblotting. Equal amounts of protein as determined by the bicinchoninic acid method (Pierce, Rockford, IL), 30 μg per condition, were separated on a 12.5% polyacrylamide gel and electrotransferred onto Immobilon polyvinylidene fluoride membrane (Millipore Corp., Bedford, MA). Gels and membranes were stained with 0.15% Coomassie blue and Ponceau S red solutions respectively, to ensure equal loading and transfer of protein. Membranes were blocked with 5% nonfat dry milk in water with 0.1% Tween 20 and 0.02% sodium azide overnight, incubated for 2 h with 1:1000 polyclonal rabbit antihuman ferritin antibody (Boerhringer Mannheim) or 1:1000 polyclonal rabbit anti–bcl-xL antibody (PharminGen, San Diego, CA), washed with 1% bovine serum albumin in PBS and incubated with 1:2000 horseradish peroxidase linked antirabbit antibody (Amersham), 1% bovine serum albumin in PBS, for 1 h. Immunoreactive bands were visualized with the enhanced chemiluminescence detection system (Amersham). After visualizing bcl-xL the blot was striped in distilled H₂O for 10 min, 0.2% NaOH for 5 min, and H₂O for 5 min; blocked overnight in nonfat milk; and reprobed using 1:1,000 goat polyclonal anti-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in 1% bovine serum albumin. The membrane was washed and then incubated with 1:3,000 horseradish peroxidase linked antigoat antibody (Santa Cruz Biotechnology) for 1 h followed by enhanced chemiluminescence detection. Densitometric quantitation was performed using a Gel Doc 1000 densitometer (BioRad Laboratories, Hercules, CA) with Molecular Analyst software.

Staining for Iron

The method of Hill and Switzer³⁵ was used with modifications.³⁶ Cells were fixed, stained in Perls’ solution, and counterstained with Gill no. 3 hematoxylin.

Fig. 1. Astrocyte cultures transfected with a retrovirus encoding bcl-xL express higher levels of bcl-xL than do control astrocytes transfected with a retrovirus encoding β-galactosidase or mock-transfected cells. Cultures were transfected with the indicated retroviral vectors or sham washed, subjected to selection with G418 to eliminate untransfected cells, and allowed to grow to confluence. Sham-transfected cells were not exposed to G418. Cell extracts were prepared from each culture and 30 μg protein was loaded in each lane. (A) The result of probing with anti-bcl-x antibody shows an immunopositive band just under 30 kDa. (B) The result of stripping the same membrane and then probing for actin (approximate weight 42 kDa) to verify that the lanes had equal amounts of protein loaded.

Fig. 2. Overexpression of bcl-xL morphologically protects astrocytes from H₂O₂ injury. Astrocytes overexpressing bcl-xL (A and B) or β-galactosidase (C and D) or mock-infected (E and F) were exposed to 400 μM H₂O₂ for 6 h, then stained with Trypan blue and photographed with phase contrast (A, C, and E) or bright field (B, D, and F) optics. Astrocytes infected with bcl-xL were resistant to injury (A and B), showing few Trypan blue–stained cells; β-galactosidase–expressing (C and D) and mock-infected astrocytes (E and F) were nearly all dead, as demonstrated by Trypan blue staining.
Iron Measurements

Total cellular iron content in the cultures was measured about 25 days after infection with the retroviral vectors. The cells were washed three times with 0.9% NaCl, then harvested in 1% sodium dodecyl sulfate, 50 mM Tris (pH 7.5). The protein content was determined using bicinchoninic acid, and 300 μg total protein in 1 ml was assayed for each condition, in triplicate. The colorimetric reaction was initiated by addition of 0.5 ml 0.6 N HCl, 2.25% KMnO₄ for 2 h at 60°C, followed by 0.1 ml reagent B (ferrozine 3.25 μM, neocuproine 6.54 μM, L-ascorbic acid 1 M, and ammonium acetate 2.54 M). Absorbance at 562 nm was measured after 30 min at room temperature. Iron standards were prepared using iron (II) ethylenediammonium sulfate (Alfa ASAR, Ward Hill, MA). The level of iron was calculated from a standard curve and expressed in nanomoles per milligram protein.

Injury paradigms were carried out as previously described.

Injury Paradigms

Exposure to Hydrogen Peroxide. Thirty percent H₂O₂ was diluted in balanced salt solution (BSS₅.₅) at pH 7.4, containing (in mM) glucose 5.5, NaCl 116, CaCl₂ 1.8, MgSO₄ 0.8, KCl 5.4, NaH₂PO₄ 1, NaHCO₃ 14.7, and HEPES 10, and phenol red 10 mg/l to a final concentration of 400 μM.

Glucose Deprivation. Cultures were deprived of all substrate by replacing the culture medium with BSS₀, identical to BSS₅.₅ but lacking glucose.

Oxygen Glucose Deprivation. Cultures were transferred to an anaerobic chamber (Forma Scientific, Marietta, OH) with an atmosphere of 5% CO₂, 10% H₂, and 85% N₂. The culture medium was replaced with warmed, deoxygenated BSS₀. Oxygen tension was < 0.2%, monitored with an oxygen electrode (Microelectrodes, Bedford, NH). Oxygen glucose deprivation (OGD) was ended by adding glucose to a final concentration of 5.5 mM and returning the cultures to the normoxic incubator. The medium was sampled at the end of OGD and 24 h later to measure lactate dehydrogenase (LDH).

Assessment of Injury. Astrocyte injury was evaluated morphologically by phase-contrast light microscopy and was quantitated by measuring the activity of LDH released into the culture medium. LDH release is appropriate in assessing these injuries, as they appear necrotic in nature, lacking evidence of nuclear fragmentation or DNA laddering (data not shown). Total LDH release corresponding to complete astrocyte death was determined at the end of each experiment following freezing at −70°C and rapid thawing.

Table 1. bcl-xL Overexpression Has Little Effect on Combined Oxygen–Glucose Deprivation Injury

<table>
<thead>
<tr>
<th>Cells</th>
<th>Percent LDH Release, 6-h OGD</th>
<th>% Reperfusion 24 h</th>
<th>Percent LDH Release, 8-h OGD</th>
<th>% Reperfusion 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>bcl-xL</td>
<td>18.8 ± 2.4*</td>
<td>50.4 ± 4.7</td>
<td>33.9 ± 6.5</td>
<td>58.9 ± 5.0</td>
</tr>
<tr>
<td>β-galactosidase</td>
<td>33.3 ± 6.5</td>
<td>49.9 ± 5.7</td>
<td>37.6 ± 8.0</td>
<td>62.9 ± 6.3</td>
</tr>
</tbody>
</table>

Astrocytes were subjected to oxygen–glucose deprivation for 6 or 8 h and then oxygen and glucose were returned to the medium. Lactate dehydrogenase release was assessed acutely at the end of the period of deprivation and after 24 h reoxygenation and refeeding.

OGD = oxygen–glucose deprivation; LDH = lactate dehydrogenase.

* Significant difference from β-galactosidase expressing control (P < 0.05; n = 18–22).

Fig. 3. Astrocytes that overexpress bcl-xL are significantly more resistant to H₂O₂ injury. LDH release was determined 4 and 24 h after addition of 400 μM H₂O₂. # = significant difference from β-galactosidase–expressing and mock-infected control (P < 0.05, n = 16).
GSH Measurements
Reduced GSH was measured before and after glucose deprivation (GD) for 7 h as described previously using a modification of the method of Fernandez-Checa and Kaplowitz. The concentration of GSH was calculated from standard curves and expressed as nanomoles per milligram protein. Preincubation with the GSH synthesis inhibitor \( \text{l-buthionine sulfoximine} \) (BSO; 100 \( \mu \text{M} \)) was for 12 h in growth medium. GSH was then measured immediately, or the astrocytes were subjected to GD in BSS0 without added BSO. LDH release was measured after 28 h.

Assay for Lipid Peroxidation
Loss of cis-parinaric fluorescence, as an indicator of lipid peroxidation, has been shown to correlate with the generation of thiobarbituric acid reactive substances. The cis-parinaric assay was performed on astrocyte suspensions as previously described.

Assays for Superoxide
We assayed the superoxide anion in two ways. (1) The colorimetric assay using iodonitrotetrazolium violet, described by Bagchi et al. was performed as previously described. (2) Dihydroethidium (Molecular Probes) was also used to determine relative superoxide levels according to the method of Bindokas et al. The probe is oxidized by superoxide to ethidium. Dihydroethidium was prepared as a 3.2 mM stock in dimethylsulfoxide stored under \( \text{N}_2 \) at \(-20^\circ\text{C} \). The cells were washed with BSS5.5 twice. The dihydroethidium (6 \( \mu \text{M} \)) was added in the dark. The fluorescence of ethidium was measured at 510 nm excitation, 590 nm emission.

Statistical Analysis
Differences between groups underwent analysis of variance followed by the Student-Newman-Keuls test for multiple comparisons, or \( t \) test if there were only two conditions, using Sigmastat (SPSS, Chicago, IL). Values given are mean \( \pm \) SEM, with the number of cultures tested indicated in the figures.

Results
Transfection of primary cultured astrocytes with the mouse \( \text{bcl-x}\text{L} \) gene using a retroviral vector resulted in markedly higher levels of expression of this protein (fig. 1) compared with the level seen in astrocytes expressing...
bcl-xL PROTECTION, HIGHER FERRITIN, AND GLUTATHIONE

Table 2. Glutathione Levels

<table>
<thead>
<tr>
<th>Cells</th>
<th>Growth Conditions</th>
<th>7-h Glucose Deprivation</th>
<th>l-buthionine Pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>bcl-xL</td>
<td>20.1 ± 1.0*</td>
<td>15.3 ± 0.7*</td>
<td>4.3 ± 0.8</td>
</tr>
<tr>
<td>β-galactosidase</td>
<td>17.2 ± 0.8</td>
<td>11.7 ± 1.3</td>
<td>3.9 ± 0.5</td>
</tr>
</tbody>
</table>

Levels of glutathione in astrocytes that overexpress β-galactosidase or bcl-xL were measured before (growth conditions) and after 7-h glucose deprivation. Glutathione values were standardized to protein. Data are pooled from experiments conducted in three different dissections. The level of glutathione in each group decreased after 7-h glucose deprivation but was still higher in bcl-xL-overexpressing astrocytes than in β-galactosidase-overexpressing controls. The levels of glutathione were also measured after preincubation with 100 µM l-buthionine sulfoximine for 12 h in growth medium. The level of glutathione was significantly decreased after preincubation with l-buthionine. *P < 0.05.

β-galactosidase or mock-infected cells. Astrocytes overexpressing bcl-xL were significantly protected from H2O2 (400 µM) injury (fig. 2) as assessed morphologically and by LDH release (fig. 3). Four hours after addition of 400 µM H2O2, astrocytes overexpressing bcl-xL suffered only half the cell death seen in β-galactosidase-overexpressing controls, and protection was stable at 24 h. Because we have found the half-life of peroxide in astrocyte cultures to be about 15 min, with the release of LDH occurring primarily in the first 5 h,37 the protection seen with bcl-xL appears to be long-lasting. In contrast, if exposed to OGD, bcl-xL-overexpressing astrocytes were less injured than β-galactosidase-overexpressing controls only acutely at the end of a mild insult, 6 h of OGD, but this protection was lost after 24 h of reoxygenation and refeeding (table 1). Similarly, if exposed to a more severe insult, 8 h OGD, no protection was seen immediately at the end of the period of OGD or 24 h later.

Protection from GD alone was significant. After 24 h deprivation bcl-xL astrocytes suffered 22 ± 6% death compared with 81 ± 6% in β-galactosidase-overexpressing controls (P < 0.01; fig. 4A). Injury in bcl-xL overexpressing cells was still less even at 30 h. The level of GSH was significantly higher in the astrocytes that overexpress bcl-xL compared with mock-infected or β-galactosidase-overexpressing controls (P < 0.01; table 2) under normal growth conditions. After GD for 7 h, the level of GSH decreased in each group but remained higher in bcl-xL than β-galactosidase-overexpressing or mock-infected controls (table 2). To determine the importance of this elevation of GSH to protection by bcl-xL we reduced the levels of GSH in all groups so that they were equal prior to starting the injury paradigm. Preincubation with BSO (100 µM) for 12 h in growth medium resulted in equivalent, reduced GSH levels in bcl-xL-overexpressing astrocytes and controls (table 2). Astrocyte cultures preincubated with or without BSO for 12 h in growth medium were then deprived of glucose. Although the degree of protection was reduced compared with the extent of protection without BSO pretreatment, the bcl-xL-overexpressing cells still suffered significantly less injury than controls (fig. 4B).

The level of superoxide in the bcl-xL-overexpressing cells was higher than in controls under normal growth conditions, whether determined by reaction with iodonitrotetrazolium violet (fig. 5) or hydroethidium (data not shown). After GD for 7 h, the levels of superoxide were decreased to about 40% of the level seen in growth medium, but the level in bcl-xL-overexpressing cells was still higher than in β-galactosidase expressing (1.6 times) or mock-infected control (two times) astrocytes (fig. 5). Levels of lipid peroxidation were assessed with cis-parinaric acid and found to be similar in all three groups of astrocytes under normal growth conditions (data not shown).

bcl-xL-overexpressing astrocytes (fig. 6A) showed greater staining for iron than β-galactosidase-expressing cells (fig. 6B) or mock-infected cells (fig. 6C). Perl’s reagent revealed a granular distribution of iron staining.

Anesthesiology, V 91, No 4, Oct 1999

Fig. 5. The levels of superoxide are higher in bcl-xL-expressing than β-galactosidase-expressing astrocytes. Superoxide was significantly higher in bcl-xL-overexpressing cells than mock-infected controls or β-galactosidase-expressing controls. The levels of superoxide decreased about 40% after 7 h glucose deprivation in each group but were still higher in bcl-xL-expressing cells. Superoxide values were standardized against the amount of protein. # = significant difference from mock-infected control and β-galactosidase-expressing control (P < 0.05, n = 12).
Colorimetric determination of iron content showed significantly higher levels in \( bcl-x_L \) and \( bcl-2 \) infected astrocytes compared with \( \beta \)-galactosidase expressing (twofold) and mock-infected controls (fourfold; table 3).

In situ immunohistochemistry showed greater staining for ferritin in \( bcl-x_L \)-overexpressing astrocytes (fig. 6D) than in \( \beta \)-galactosidase-expressing (fig. 6E) or mock-infected controls (fig. 6F). Semiquantitative assessment of ferritin by immunoblot showed two bands: the heavy (H-) subunit (about 22 kDa) and the light (L-) subunit (about 19 kDa). Increased amounts of both H- and L-subunits were seen in both \( bcl-2 \)- and \( bcl-x_L \)-overex-

### Table 3. Total Iron Levels in Astrocytes under Normal Growth Conditions

<table>
<thead>
<tr>
<th>Cells</th>
<th>Protein (mu/mg)</th>
</tr>
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<tbody>
<tr>
<td>( bcl-x_L )</td>
<td>9.70 ± 0.82*</td>
</tr>
<tr>
<td>( bcl-2 )</td>
<td>9.41 ± 1.17*</td>
</tr>
<tr>
<td>( \beta )-galactosidase</td>
<td>4.82 ± 0.38</td>
</tr>
<tr>
<td>Mock-infected control</td>
<td>2.62 ± 0.60</td>
</tr>
</tbody>
</table>

Total iron levels were measured colorimetrically. Iron was higher in \( bcl-x_L \)- and \( bcl-2 \)-infected astrocytes than \( \beta \)-galactosidase-expressing or mock-infected controls (n = 8–10). Data are pooled from determinations conducted on cells from three different dissections.

* Significant difference from \( \beta \)-galactosidase-expressing and mock-infected control (\( P < 0.05; \ n = 9 \)).
bcl-\(x_L\) PROTECTION, HIGHER FERRITIN, AND GLUTATHIONE

Discussion

There are similarities between bcl-\(x_L\) and bcl-2 in structure, intracellular localization, and effect.\(^{26,44,45}\) However, bcl-\(x_L\) and bcl-2 also differ.\(^{9–11}\) The former has been suggested to be the predominant protective protein in gerbil hippocampus.\(^{18}\) We investigated the ability of bcl-\(x_L\) to protect primary astrocytes in culture from ischemia-like injury. It provided robust protection of primary astrocytes from \(H_2O_2\) exposure and GD but was relatively ineffective against combined OGD. This protection is associated with increases in at least two aspects of cellular antioxidant defense: increased glutathione levels and increased ferritin levels. The increased levels of GSH were found to contribute to the extent of protection from glucose deprivation, because pretreatment with BSO reduced the extent of protection about 50%. This is consistent with prior observations that hypoglycemia represents an oxidative stress,\(^{38}\) which is worsened by reduction of GSH levels and ameliorated by artificially elevating GSH levels.\(^{38}\) OGD is not worsened by reducing GSH levels.\(^{19}\) GSH is an important hydrophilic-free radical scavenger that also participates in enzymatic free-radical scavenging. Although several reports document increased GSH levels in neural cells and fibroblasts overexpressing bcl-2,\(^{21,46}\) there are fewer data on the effects of bcl-\(x_L\) on GSH levels. Two recent studies in lymphocytes report that bcl-\(x_L\) can preserve GSH levels and intracellular thiols and block induction of apoptosis.\(^{47,48}\)

There is no evidence that either bcl-2 or bcl-\(x_L\) acts directly as an antioxidant. The mechanism by which they influence levels of reactive oxygen intermediates and endogenous cellular antioxidants is not yet understood. Superoxide is primarily a byproduct of mitochondrial function, and bcl-2 and bcl-\(x_L\) affect mitochondrial function under stress conditions.\(^{26,49}\) The possibility that

Fig. 7. Ferritin protein levels were estimated in bcl-\(x_L\)-overexpressing, bcl-2-overexpressing, \(\beta\)-galactosidase-expressing, and mock-infected controls by immunoblotting. Quantitation by scanning densitometry and averaging of three experiments is shown (A and B). *\(P < 0.05\) compared to control or \(\beta\)-galactosidase. The two immunopositive bands of ferritin, the heavy (H) subunit (approximately 22 kDa) and the light (L) subunit (approximately 19 kDa) are indicated by arrows in an individual western blot (C). Thirty micrograms total protein was loaded per condition. The immunopositive bands for ferritin were greater in bcl-\(x_L\) and bcl-2 overexpressing cultures than in controls. The ferritin L-subunit band was barely detected in controls.
bcl-2 has a prooxidant effect that induces a cellular antioxidant response was first suggested by Steinman and is consistent with our prior observations on bcl-2 overexpression in astrocytes. We report here that bcl-xL increases superoxide levels yet is associated with improved antioxidant defense—higher levels of ferritin and GSH. Both of these antioxidants are induced by oxidative stress, so the observed increases if bcl-2 or bcl-xL is overexpressed imply that there may be some level of oxidative stimulus leading to up-regulation under normal growth conditions. That the potential oxidative stress is compensated for is suggested by the inability to detect increased lipid peroxidation. Although no assay is completely specific, we used two different assays to confirm a change in superoxide. The dihydroethidium assay has been shown by Bindokas et al. not to be affected by many other common oxidants including -OH, NO, ONOO, H2O2, hypochlorite, or singlet O2. The increased level of ferritin, which binds Fe3+, observed with bcl-xL may prevent increased hydroxyl radical production despite the higher superoxide levels. That the cells are not suffering greater oxidative stress is suggested by the observed lack of difference in the extent of lipid peroxidation. This finding is reminiscent of the results of Hockenbery et al., who found no lipid peroxidation in bcl-2-overexpressing cells in the absence of exogenous stress. Although the precise mechanism of action of bcl-2-related proteins is still not known, recent studies have focused on the role of these proteins in mitochondrial function, their ability to associate in protein complexes, and their ability to form pores in membranes. Several reports suggest that the relative levels of expression of the pro- and antiapoptotic members of the bcl-2 gene family may play an important role in determining the sensitivity of the brain to ischemia. Many of these genes can homodimerize and heterodimerize with each other. The relative levels of expression of these proteins may determine whether a cell undergoes apoptosis or not. Postischemic alterations in expression, relatively increased levels of bax in cells that go on to die, and increased levels of bcl-2 or bcl-xL in cells that survive have been reported in models of both global and focal ischemia. We report here the novel observation that overexpression of bcl-xL and bcl-2 is associated with elevated ferritin levels. Brain ferritin is composed of H- and L-subunits. The ferritin observed in our primary cultured astrocytes was predominantly the H-subunit. With bcl-xL or bcl-2 overexpression the amount of ferritin in the cells increased. In addition to higher levels of the H-subunit, significant expression of the L-subunit was found. The roles suggested for H-subunit rich ferritin are as a stress or early-response protein allowing rapid iron binding, as an antioxidant, and in cell proliferation. In contrast, L-subunit-rich ferritin, such as is found in liver, is thought to be primarily a storage protein. Both subunits show largely post-transcriptional control. However, after iron administration, both subunits show transcriptional induction, with the L-subunit having a greater response. The observed accumulation of the L subunit may reflect the higher levels of iron seen in bcl-xL-overexpressing astrocytes. The H-subunit has ferritinase activity and has been found to be upregulated in oligodendrocytes after hypoxia, through what is thought to be an oxidative stress-induction mechanism. It is possible that the increased levels of superoxide found in astrocytes overexpressing bcl-xL or bcl-2 induce ferritin H, which allows the cells to capture more iron and subsequently leads to induction of ferritin L. Further studies are required to determine the validity of this hypothesis.

Iron has been detected by histochemical staining in many cell types of the CNS, including neurons, microglia, oligodendroglia, and astrocytes. A large proportion of the soluble brain iron was shown to be present in ferritin. Iron can catalyze oxidant-induced cell injury, and iron can be released from ferritin by acid and reduction. Ferrous iron in the presence of oxygen can catalyze the production of active oxygen species. Recent findings indicate that an increase in ferritin can compensate for increased iron levels and prevent accumulation of protein carbonyls, evidence of protein oxidation. By sequestering iron, ferritin may prevent iron-catalyzed lipid peroxidation injury. Although excess iron can be deleterious, a certain level of iron is essential for cell growth and viability. Iron depletion was found to induce apoptosis in human leukemic cells. Ferritin may both provide the iron required to sustain oxidative metabolism and protect the cell from oxidant mediated injury.

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bcl-xL PROTECTION, HIGHER FERRITIN, AND GLUTATHIONE

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