The Journal of Neuroscience, October 1995, 15(10): 6364–6376

# Upregulation of Bax Protein Levels in Neurons following Cerebral Ischemia

**Stanislaw Krajewski,[1] Jürgen K. Mai,[2] Maryla Krajewska,[1] Marianna Sikorska,[3] Miroslaw J. Mossakowski,[4] and John C. Reed[4]**

[1]La Jolla Cancer Research Foundation, Oncogene and Tumor Suppressor Gene Program, La Jolla, California 92037, [2]University of Duesseldorf, Department of Neuroanatomy, Duesseldorf, D5000 Germany, [3]National Research Council of Canada, Institute Biological Sciences, Ottawa, Ontario, K1A OR6 Canada, and [4]Medical Research Centre of the Polish Academy of Sciences, Department of Neuropathology, Warsaw, 02-784 Poland

**The patterns of expression of the *bcl-2, bax,* and *bcl-X* genes were examined immunohistochemically in neurons of the adult rat brain before and after 10 min of global ischemia induced by transient cardiac arrest. High levels of the cell death promoting protein Bax and concomitant low levels of the apoptosis-blocking protein Bcl-2 were found in some populations of neurons that are particularly sensitive to cell death induced by transient global ischemia, such as the CA1 sector of the hippocampus and the Purkinje cells of the cerebellum. Moreover, within 0.5 to 3 hr after an ischemic episode, immunostaining for Bax was markedly increased within neurons with morphological features of degeneration in many regions of the brain. Use of a two-color staining method for simultaneous analysis of Bax protein and *in situ* detection of DNA-strand breaks revealed high levels of Bax immunoreactivity in many neurons undergoing apoptosis. Postischemic elevations in Bax protein levels in the hippocampus, cortex, and cerebellum were also demonstrated by immunoblotting. At early times after transient ischemia, regulation of Bcl-2 and Bcl-X protein levels varied among neuronal subpopulations, but from 3 hr on, those neurons with morphological evidence of degeneration uniformly contained reduced levels of Bcl-2 and particularly Bcl-X immunoreactivity. The findings suggest that differential expression of some members of the *bcl-2* gene family may play an important role in determining the relative sensitivity of neuronal subpopulations to ischemia and that postischemic alterations in the expression of *bax, bcl-2,* and *bcl-X* may contribute to the delayed neuronal cell death that occurs during the repurfusion phase after a transient ischemic episode.**

***[Key words: apoptosis, ischemia, neuronal cell death, Bcl-2, Bax, Bcl-X]***

Depending on the circumstances, the neuronal loss that occurs during and after ischemic events can probably occur through either of the two principal pathways for cell death, necrosis and apoptosis. Necrotic cell death clearly befalls neurons and glial cells within the core of infarcts, where insufficient oxygen tension caused by acute cessation of blood flow leads to arrest of aerobic metabolism, depletion of intracellular ATP, and loss of osmotic equilibrium across biological membranes, including those of the mitochondria, lysosomes, and plasma membrane. In contrast, neuronal loss in the brain tissue surrounding the core of the infarct (the so-called "penumbra") as well as the "delayed" neuronal cell death that occurs over a period of days in models of transient global ischemia may occur largely through other mechanisms that resemble apoptosis to some extent, with cell shrinkage, chromatin condensation, and nuclear pyknosis, among other morphological changes (Vogt and Vogt, 1925; Scholz, 1953; Araki et al., 1989; Kawai et al., 1992; Kreutzberg et al., 1992). In this regard, transient forebrain ischemia has been reported to result in oligonucleosomal DNA fragmentation within 12 hr in the gerbil hippocampus and cells with DNA strand breaks were detected *in situ* in the CA1 region (Sei et al., 1994), lending support to the hypothesis that at least some of the cell deaths that occur in ischemia-sensitive regions of the brain after transient ischemia involves apoptotic mechanisms. Furthermore, intraventricular administration of a nuclease inhibitor, aurintricarboxylic acid, has been shown to prevent some events associated with ischemia-induced neuronal cell death in the hippocampus (Roberts-Lewis et al., 1993). Similarly, in a model of transient global ischemia, systemic administration of protein synthesis inhibitors was reported to reduce the number of neurons lost in the hippocampus (Shigeno et al., 1990), consistent with the idea that at least some of the neuronal loss caused by transient ischemia involves an active cell death process similar to apoptosis as opposed to the passive cell demise typical of necrosis. Finally, evidence for intranucleosomal cleavage of genomic DNA in the ischemic penumbra has been obtained in an animal model of focal ischemia, and infusion into rats of the protein synthesis inhibitor cycloheximide reduced infarct sizes (Linnik et al., 1993).

Among the known regulators of apoptosis, the Bcl-2 protein stands out for its ability to suppress cell death induced by a wide variety of insults and stimuli, including some of the mediators suspected to be inducers of neuronal cell death associated with reperfusion injury. For example, gene transfer-mediated elevations in Bcl-2 protein levels in immortal neural cell lines have been shown to markedly inhibit cell death induced by L-glutamate, $Ca^{2+}$-ionophores, hypoglycemia, free radicals, and gluta-

April 20, 1995; revised June 1, 1995; accepted June 9, 1995.

We thank S. Januszewski and J. Przekop for expert technical assistance, Drs. D. E. Bredesen, W. A. Horne, and L. Fritz for critical review of the manuscript, S. Kitada for help with statistics, and C. Stephens for manuscript preparation. J.C.R. is a Scholar of the Leukemia Society of America.

Correspondence should be addressed to Dr. John C. Reed at the above address.

thione depletion (Zhong et al., 1993; Behl et al., 1994). In addition, a role for Bcl-2 in the control of neuronal susceptibility to ischemia-induced death has recently been suggested by studies of transgenic mice that overexpress *bcl-2* in the brain, in which infarct sizes caused by focal ischemia were reduced by ~50% compared to nontransgenic littermate controls (Martinou et al., 1994). Bcl-2 is an integral membrane protein associated primarily with the outer mitochondrial membrane, the nuclear envelope, and endoplasmic reticulum (Krajewski et al., 1993) that appears to block a distal event in what may represent a final common pathway for apoptotic cell death (reviewed by Reed, 1994). Though the biochemical mechanism by which the Bcl-2 protein prevents cell death remains enigmatic, evidence has been obtained suggesting an effect on regulation of intracellular $Ca^{2+}$ fluxes across membranes (Baffy et al., 1993; Lam et al., 1994) or control of an antioxidant pathway (Hockenbery et al., 1993; Kane et al., 1993), among other possibilities (reviewed by Reed, 1994).

Recently, a family of genes encoding homologs of Bcl-2 has been discovered. Some of these proteins, such as Bcl-X-L, function as blockers of cell death similar to Bcl-2 (Boise et al., 1993), whereas others, such as Bax, render cells more sensitive to apoptotic stimuli (Oltvai et al., 1993). Gene-targeting experiments in mice have recently demonstrated that loss of *bcl-X* expression is associated with massive death of immature postmitotic neurons in developing embryos, suggesting that this antiapoptotic member of the *bcl-2* gene family is important for mammalian neuronal survival at least during development (Motoyama et al., 1995). It has been shown that the Bcl-2 protein can physically interact with several of its homologous proteins, in the form of heterotypic dimers (Oltvai et al., 1993; Sato et al., 1994). One of the more critical interactions appears to be Bcl-2/Bax dimerization, since mutations in Bcl-2 that prevent its association with Bax also abrogate its function as a blocker of apoptosis (Yin et al., 1994). This and other findings have suggested that Bax actively promotes cell death, unless neutralized by binding of either Bcl-2 or Bcl-X-L (Sato et al., 1994; Yin et al., 1994).

Based on currently available data, the ratio of Bax to its antiapoptotic homologs such as Bcl-2 and Bcl-X-L appears to be a critical determinant of the relative sensitivity or resistance of cells to stimuli that trigger the physiological cell death pathway. We therefore examined the distributions of Bcl-2, Bax, and Bcl-X-L proteins by immunohistochemical methods in various subpopulations of neurons in the adult brains of rats, correlating the results with relative sensitivity to neuronal cell death in an animal model of transient global ischemia. In addition, we investigated whether changes in the expression of these genes occur during the repurfusion phase after an ischemic insult. The findings suggest a potentially important role for the ratios of Bcl-2, Bax, and Bcl-X proteins in determining the relative sensitivity of some subpopulations of neurons to ischemia-induced apoptosis and demonstrate dramatic increases in the levels of Bax protein in neurons that succumb to apoptosis following transient ischemic episodes.

## Materials and Methods

*Animals, surgical procedures, and tissue processing.* Normal brain tissue was obtained from nine adult female albino Wistar rats. Cardiac arrest was induced in deeply anaesthetized rats [1.5% Halothane in (2:1) nitrous oxide/oxygen] by compression of the heart vacular bundle against the sternum by use of a microsurgical hook, exactly as described in detail previously (Mossakowski et al., 1986; Majkowska, 1989; Kawai et al., 1992; Pluta et al., 1994). Complete cardiac arrest was generally achieved within 1.5 to 2 min. Intubated animals were resuscitated by external heart massage and controlled respiration after 10 min, of which 42 of 156 (27%) survived. Body temperature was maintained at 37°C by use of a heating pad and external radiation. During each experiment, the electrocardiogram (ECG) and electroencephalogram (EEG) were monitored, and only animals with complete EEG and ECG flattening during the 10 min after compression of the heart bundle were used. Sham operations were performed on 12 rats by introducing the microsurgical hook into the chest cavity for 10 min without vascular bundle compression. At various times after resuscitation or sham operations (0, 1–3 hr, 6–12 hr, 1 d, 3 d, 7 d, 14 d, 21 d), animals were deeply anesthetized with ether, and perfused by intracardiac puncture using ~200 ml of 4% neutral-buffered formalin (pH 7.4) ($n$ = 4–5 animals per time point). Tissues were then excised and postfixed for 24 hr in either 10% formalin or Bouin's solution (Sigma Chemicals, St. Louis, MO). Brain tissues were embedded in paraffin, sectioned (5 μm), and mounted on Superfrost-Plus glass slides (Fisher Scientific, Inc).

*Antibodies and immunohistochemical assays.* The production and characterization of anti-peptide antisera specific for the Bcl-2, Bax, and Bcl-X proteins have been described in detail previously (Krajewski et al., 1994a,b; Miyashita et al., 1994, available from PharMingen, Inc., San Diego, CA). Immunostaining of tissue sections was accomplished exactly as described, using a diaminobenzidine (DAB) colorimetric method with hematoxylin counterstaining, except that salt was eliminated from the preblocking and incubation solutions (Krajewski et al., 1994a,b). Antibodies were first preadsorbed in TMBT [0.1 M Tris, pH 7.6, 5% skim milk, 2% bovine serum albumin (BSA), 0.1% Triton X-100] containing 25 μg/ml ovalbumin (OVA) and either 0.01% normal goat serum (primary) or 0.05% normal rat serum (secondary). The specificity of all immunostaining results was confirmed by use of preimmune serum and by peptide competition experiments where antiserum was preincubated with 1–2 μg of either specific or irrelevant peptide per μl prior to immunostaining. The relative intensity of immunostaining was scored on an arbitrary five-point scale: 0, undetectable immunoreactivity; 1+, trace positive; 2+, weakly positive; 3+, medium-intensity staining; and 4+, strong immunostaining. Immunostained slides were independently scored by at least three, and in most cases four, investigators and the mean of the results were calculated. There was > 95% concordance among the independent reviewers.

In situ *detection of DNA cleavage.* DNA strand breaks were detected *in situ* by 3′ end labeling using a kit containing terminal deoxynucleotidyl transferase (TdT) and digoxigenin-11-dUTP according to the manufacturer's recommendations (ApopTag$^R$; Oncor, Inc.), with the exceptions that deparaffinized tissue sections were treated with 10 μg/ml proteinase K for 15 min and TdT reactions were performed for 1–3 hr. After incubation with HRPase-conjugated anti-digoxigenin antibody and color development with $H_2O_2$ and DAB, tissue sections were preblocked and immunostained for Bax exactly as described (Krajewski et al., 1994a; Miyashita et al., 1994), except that Vector-VIP was employed as a substrate for color detection.

*Immunoblot assays.* Animals were perfused with PBS containing 1 mM PMSF while under deep anesthesia. Immediately after sacrificing, brain tissues were dissected and homogenized in RIPA buffer containing protease inhibitors (Krajewski et al., 1994a). After sonication on ice and centrifugation (16,000 × $g$ for 5–10 min) to pellet debris, aliquots of protein samples (50 or 100 μg total protein) were analyzed by SDS-PAGE/immunoblotting (12% gels), using sequentially 0.1% (v/v) Bcl-2, 0.05% (v/v) Bax, and 0.03% (v/v) Bcl-X–specific preabsorbed antisera, followed each time by 0.15 μg/ml biotinylated goat anti-rabbit IgG and horseradish peroxidase (HRPase) avidin–biotin complex reagent (Elite; Vector Labs., Inc.) and then either Vector-SG substrate for 20 min, 3,3′-diaminobenzidine for 20 min, or 3-amino-9-ethyl-carbazol (AEC) for 15 min, respectively (Krajewski et al., 1994a,b). Blots were analyzed by scanning-laser densitometry (LKB Ultroscan; Bromma, Sweden) and the mean of three determinations was determined.

## Results

### *Basal patterns of Bcl-2, Bax, and Bcl-X protein production in the rat brain*

Some subpopulations of neurons are relatively more vulnerable to cell death induced after a transient ischemic episode, such as the pyramidal neurons in the CA1 sector of the hippocampus and Purkinje cells in the cerebellum (Vogt and Vogt, 1925;

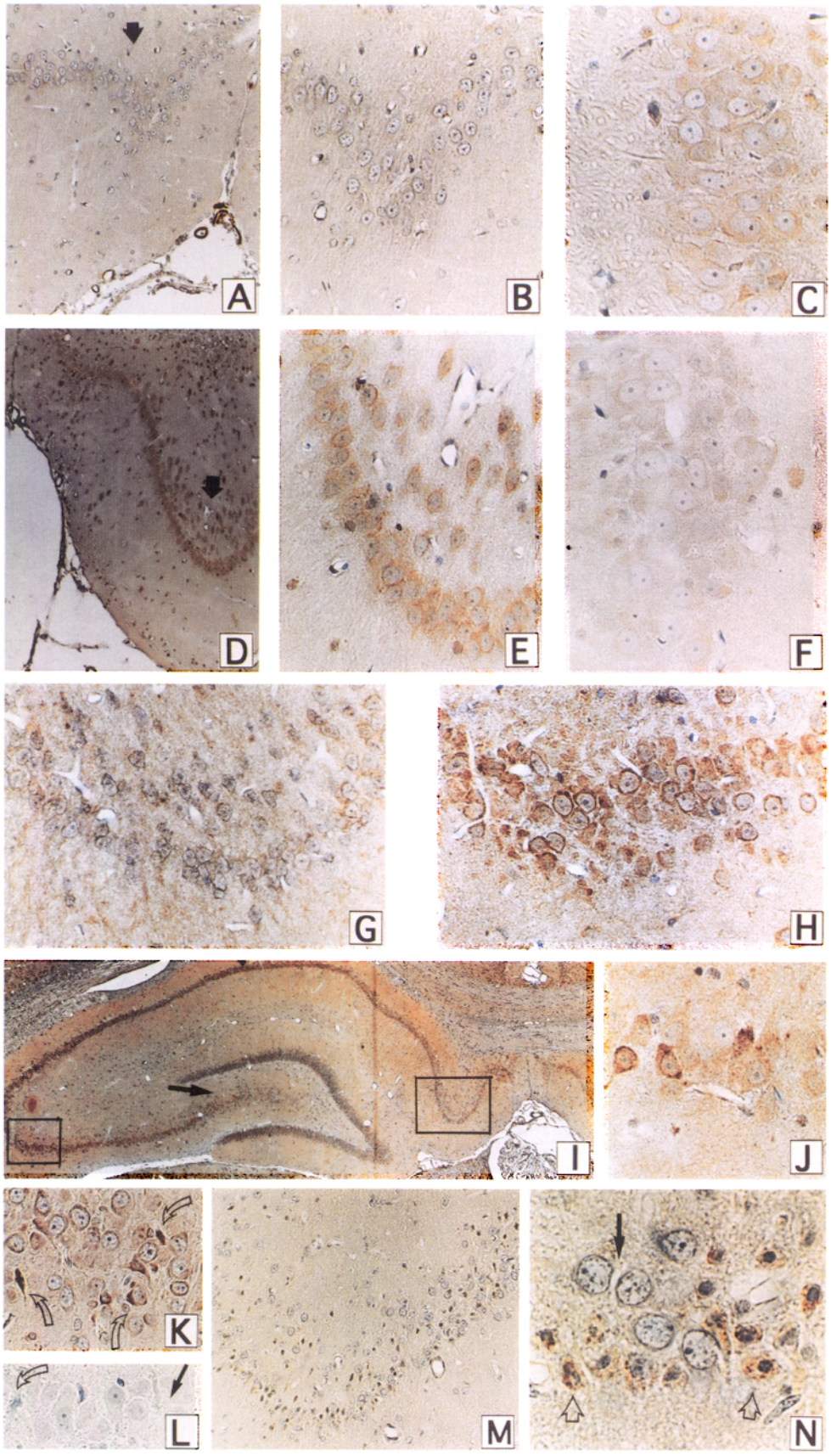



*Figure 1.* Immunohistochemical analysis of Bcl-2, Bax, and Bcl-X proteins in normal and postischemic hippocampus. Tissue sections from adult rat brains were immunostained using antisera specific for Bcl-2, Bax, or Bcl-X proteins, which were detected by a DAB method (*brown*). Counterstaining was with hematoxylin. Panels represent: *A* and *B*, pyramidal neurons in CA1 sector of hippocampus, stained for Bcl-2 at 150× and 400× original magnification, respectively. *Arrow in A* points to the region shown at higher power in B. In *C*, CA3 pyramidal neurons immunostained for Bcl-2 are shown at 400× magnification. Pairs of photomicrographs of CA1 and CA3 were taken from the same or adjacent immunostained tissue sections from the same animal but are presented separately at high power to show detail. In *D–F*, Bax immunostained hippocampal neurons in CA1 (100×; *arrow* points to area shown at higher magnification in panel *E*). Panels *E* and *F* represent CA1 (at 400×) and CA3 (at 400×), respectively, showing more intense Bax immunoreactivity in the smaller CA1 neurons than in the larger CA3 neurons. In *G–I*, Bcl-X immunostaining in CA1 at 400× (*G*) and CA3 at 400× (*H*), respectively. Panel *I* shows coronal section of hippocampus at 20×, (*boxed areas* represent the regions shown at higher power in *G* and *H*). *Arrow* indicates C4 and hilus region in which Bcl-X immunoreactivity is reduced relative to CA3. Panels *J–N* represent hippocampus after ischemic episode: *J*, Bcl-X immunostained CA3 sector neurons are shown at 0.5 hr after cardiac arrest (400×), demonstrating mixtures of Bcl-X–weak and Bcl-X–strong neurons, all with essentially normal morphology; *K*, Bax immunostaining in CA1/CA2 region at 0.5 hr (400×) (*open arrows* indicate degenerating neurons with Bax overexpression); *L*, Bax peptide competiton control for previoius panel in CA1 region using adjacent section (400×) (*solid arrow* indicates normal-appearing neurons; *open arrow* indicates neurons with early degenerative changes); *M,N*, Bax immunostaining in CA1 region at 7 d postcardiac arrest at 100× and 1000×, respectively. Note mixtures of strongly Bax-positive degenerating pyramidal neurons (*open arrows*) and normal-appearing neurons with lower Bax immunostaining (*dark arrows*) are seen.

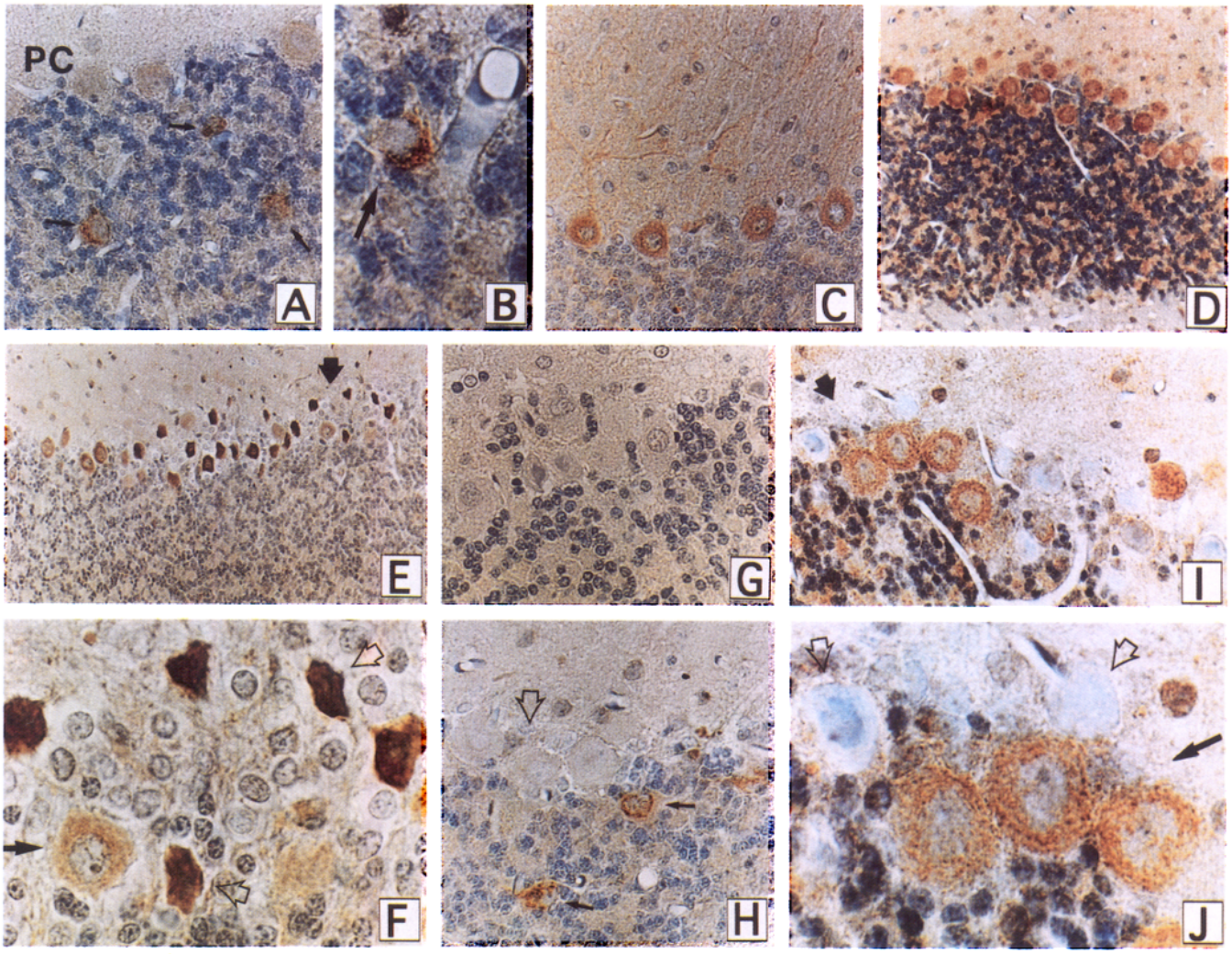



*Figure 2.* Bcl-2, Bcl-X, and Bax immunostaining in cerebellum before and after ischemia. *A* and *B*, Bcl-2 immunostaining in cerebellum of normal rat at 400× and 1000×, respectively. Note relatively low-intensity Bcl-2 immunostaining in Purkinje cells (*PC*) and high-level Bcl-2 in Golgi II neurons (*arrows*). *C* and *D*, Bax and Bcl-X, respectively, in normal Purkinje cells at 400× and 200× before ischemia. *E* and *F*, Bax immunostaining at 6 hr after cardiac arrest at 200× and 1000×, respectively (*arrow in E* indicates the region shown at higher magnification in *F*). *Thin arrow in F* indicates a normal-appearing Purkinje cell with relatively lower levels of Bax. *Thick open arrows* indicate ischemia-damaged neurons with marked upregulation of Bax. *G*, Bax peptide competition for cerebellum from same animal shown in *E* and *F* at 400×. *H*, Bcl-2 immunostaining in cerebellum at 0.5 hr after ischemia, showing negative Purkinje cells (*open arrow*) and nearby Golgi II cells that remain strongly Bcl-2 positive (*dark arrows*). *I* and *J*, Bcl-X in cerebellum at 0.5 hr after cardiac arrest at 400× and 1000×, respectively (*thick arrow* in *I* indicates the region shown at higher magnification in *J*). In *J*, Purkinje cells with early morphological features of degeneration and reduced Bcl-X immunostaining are shown (*dark arrow* indicates normal appearing Purkinje cells with high levels of Bcl-X immunostaining).

Scholz, 1953; Araki et al., 1989; Kawai et al., 1992; Kreutzberg et al., 1992), suggesting that intrinsic differences in the types and levels of the various gene products present in these cells influence their ultimate sensitivity to the biochemical mediators of neuronal cell death that are elaborated during the reperfusion phase after transient ischemia. Using anti-peptide antisera specific for Bcl-2, Bax, and Bcl-X, the normal patterns of production of these Bcl-2 family proteins were examined by immunohistochemical methods in the neurons of the adult rat brain, focusing particularly on comparisons of ischemia-prone and ischemia-resistant subpopulations of neurons. The immunostaining results for hippocampus, cerebellum, and cerebral cortex are presented in Figures 1–3, respectively. These studies demonstrated a generally good correlation between the patterns of Bcl-2 family protein production and the published literature regarding the relative resistance of some populations of neurons to cell death induced by ischemia, hypoglycemia, and status epilepticus.

With regards to Bcl-2, for example, immunoreactivity was present in the relatively ischemia-resistant pyramidal neurons of the CA3 sector of the hippocampus (2–3+) but not in the CA1 region (Fig. 1*A–C*). Figure 1*A* and *B* demonstrates low- and high-power views, respectively, of CA1 neurons, showing relatively little Bcl-2 immunoreactivity. In contrast, Bcl-2 immunostaining was considerably stronger in the CA3-sector pyramidal neurons, which were immunostained in the same experiment using tissue sections from the same animal. These CA3-sector hippocampal neurons are readily distinguished from their CA1 counterparts by their larger size (compare Fig. 1*B* and *C*).

Similarly, Bcl-2 protein was present at low levels in the Purkinje cells of the cerebellum (1–2+), a relatively sensitive pop-

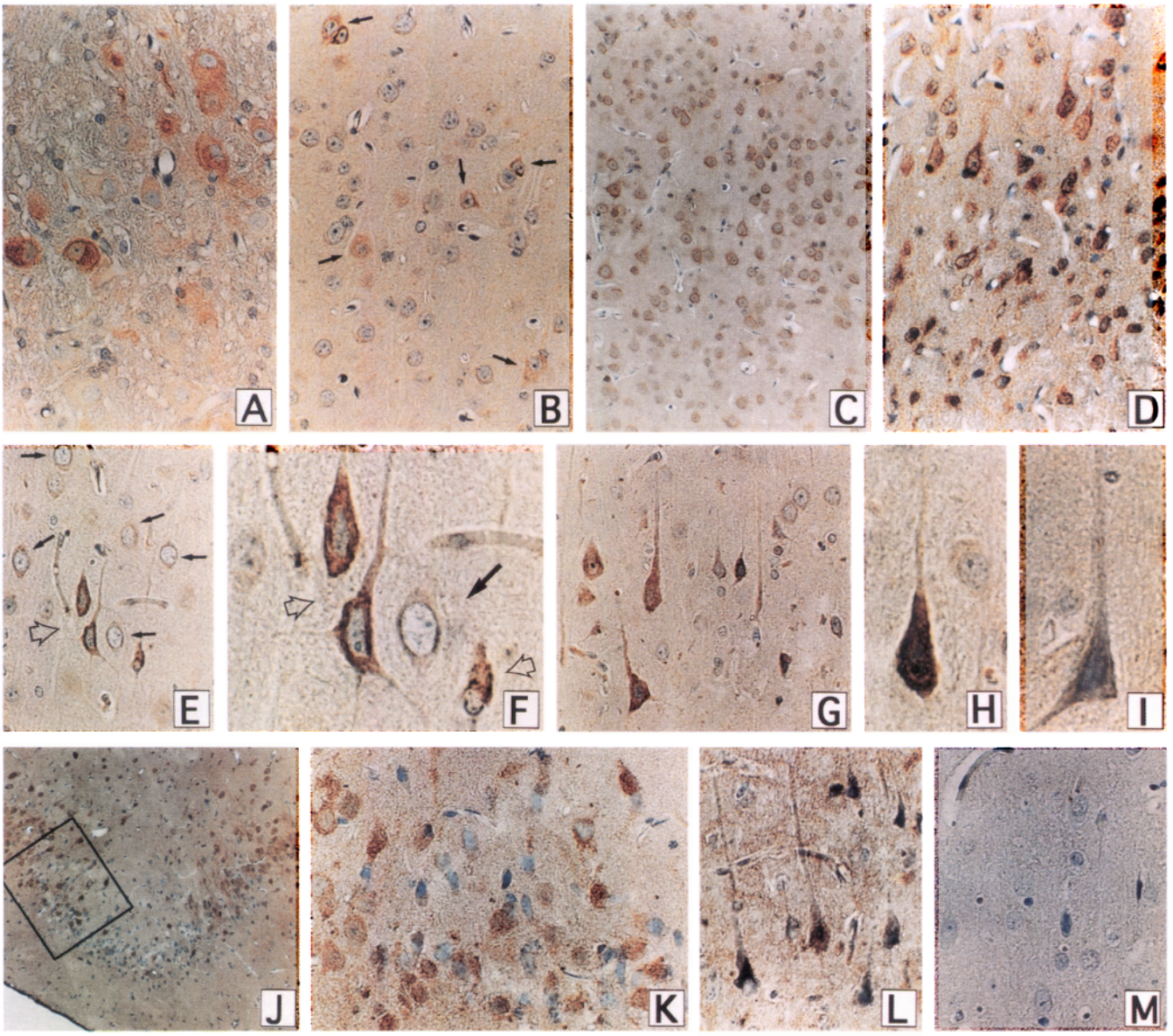
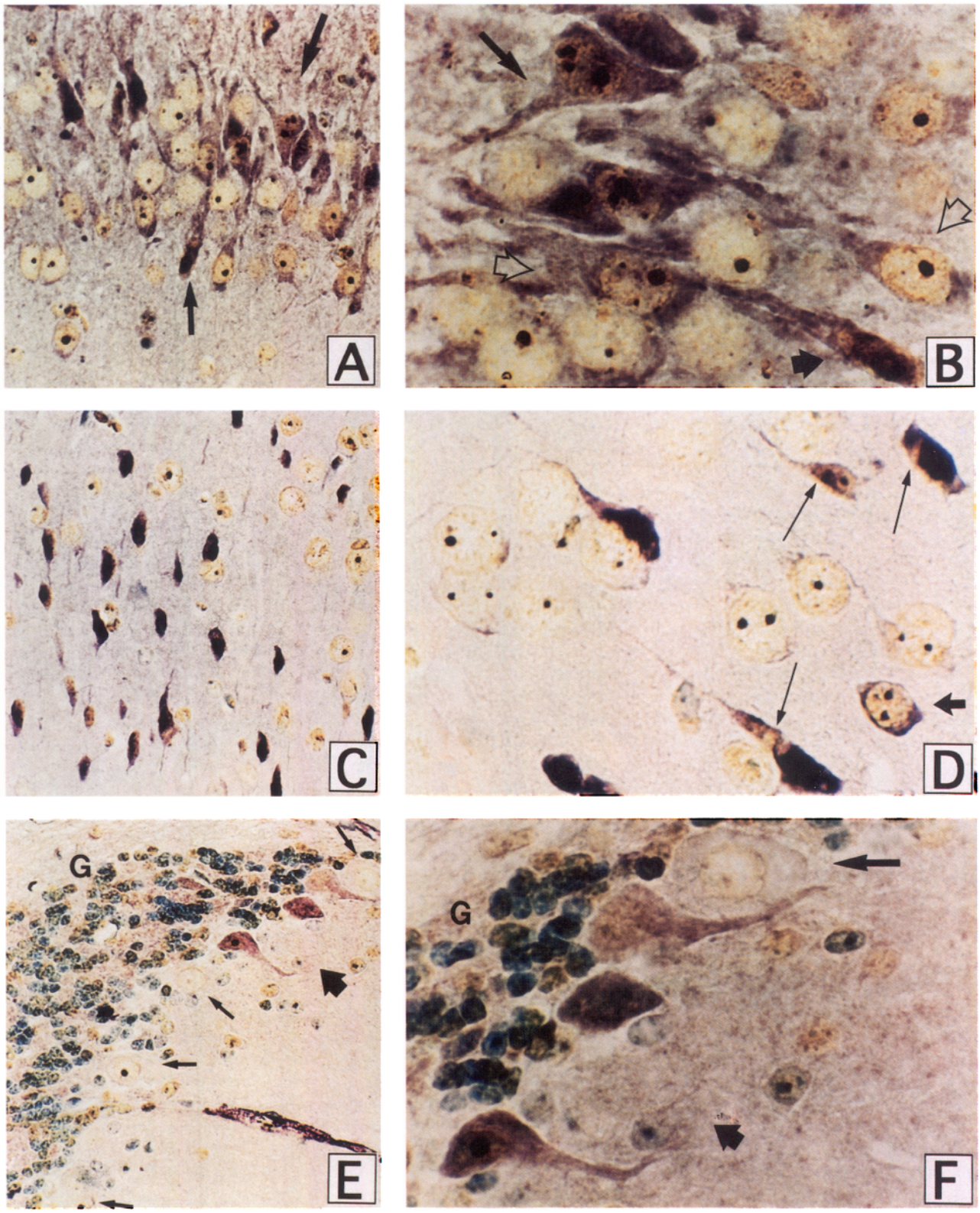



*Figure 3.* Bcl-2, Bax, and Bcl-X immunostaining in cerebral cortex and brainstem from rats before and after ischemia. *A* and *B*, Bcl-2 immunostaining in ventral cochlear nucleus of brainstem (400×) and frontal neocortex layers III–V (400×) of a sham-operated rat at 7 d, respectively. *Arrows in B* indicate scattered Bcl-2–positive neurons. *C* and *D*, Bax and Bcl-X immunostaining in frontal cortex of the same sham-operated animal at 250× and 400×, respectively, showing numerous Bax- and Bcl-X–positive cortical neurons. Postischemia cerebral cortex is shown in panels *E–M*. In *E* and *F*, Bax immunostaining of neurons in layer III of cortex is shown at 3 hr after ischemia at 400× and 1000×, respectively. *Thick, dark arrows* indicate normal-appearing cortical neurons with relatively lower levels of Bax immunostaining, whereas *thick, open arrows* denote ischemia-damaged neurons with intense Bax immunoreactivity. In *G* and *H*, Bax immunostaining of cerebral cortex is shown at 3 d after cardiac arrest, revealing persistent Bax elevations in degenerating neurons at 400× and 1000×, respectively. In *I*, Bcl-2 immunostaining of a degenerating cortical neurons is shown in an adjacent tissue section taken from the same animal shown in panels *G* and *H*, at 1000×. In *J* and *K*, Bcl-X immunostaining results are presented for primary olfactory cortex, at 100× and 400×, respectively. The *box in J* indicates region shown at higher power in panel *K*, which lies adjacent to a pale infarct in a animal at 3 hr after cardiac arrest. Note reduced Bcl-X immunostaining in the ischemia-damaged neurons compared to cells that retained normal morphology. Panels *L* and *M* show Bcl-X immunostaining in the absence or presence of competing Bcl-X peptide in cortex at 7 and 3 d, respectively, after cardiac arrest (400×).

→

*Figure 4.* Simultaneous detection of Bax and fragmented DNA in ischemia-damaged neurons. Bax was detected immunohistochemically (*red/purple*) and fragmented DNA was end labeled by a TdT-based method using DAB as the chromagen (*brown*). Counterstaining was with methylgreen in panels *E* and *F*. No counterstain was employed for others. Representative results are shown for *A* and *B* CA1 sector of hippocampus at 1 hr; *C*




and *D*, cerebral cortex at 3 hr; and *E* and *F*, cerebellum at 0.5 hr (400× and 1000×, respectively). *Dark arrows in A and B* indicate ischemia-damaged neurons with advanced degenerative changes that contain high levels of cytosolic Bax immunoreactivity and nuclear staining with DAB. *Open arrows in B* show neurons earlier in the degenerative process, with less advanced morphological alterations and less intense staining for Bax and DNA fragmentation. *Thin arrows in D* point to DAB-stained nuclei within severly damaged neurons that contain intense Bax immunoreactivity. *Thick arrow* indicates an ischemia-damaged neuron that is earlier in the degenerative process, with less shrunken size and more roundish shape. In *E* and *F*, *thin arrows* indicate normal nondamaged Purkinje cells, which were negative for DNA fragmentation and only weakly stained for Bax, compared to adjacent degenerating Purkinje cells (*thick arrow, E*). Note that some of the small neurons comprising the granular cell layer (*G*) and glial cells in the molecular layer of the cerebellum have DAB staining in the nucleus, but appear to be Bax negative. *A, C,* and *E* photomicrographs, 400× original magnifcation; *B, D,* and *F*, 1000×.

ulation of neurons, but was detected at considerably higher levels (3–4+ immunostaining) in the nearby Golgi II cells, which are relatively resistant to ischemia (Fig. 2*A,B*). In contrast, Bcl-2 immunoreactivity was present at high levels (2–3+) in the large neurons found in several brain stem nuclei (Fig. 3*A*). In this regard, the brainstem is one of the most ischemia-resistant regions of the brain, accounting for the preservation of many autonomic functions even after extensive loss of neuronal activity in the cerebrum following severe episodes of ischemia or hypoglycemia (1–3). Otherwise, Bcl-2 immunostaining was detected only in some subpopulations of neurons in the adult rodent brain (details to be published elsewhere). For instance, only occassional large neurons in the striatum, thalamus, and the layers III–VI of the cerebral cortex contained Bcl-2 immunostaining at significant levels (> 2+), thus suggesting that differential expression of *bcl-2* cannot explain the variable susceptibility of some subpopulations of neurons to transient ischemia in these regions of the brain. Figure 3*B* shows an example of Bcl-2 immunostaining in the frontal cerebral cortex, demonstrating that only occasional scattered neurons contain significant Bcl-2 immunoreactivity.

In contrast to Bcl-2, which was present in only some subpopulations of neurons, Bax immunoreactivity was found in most neurons at a ~2+ level of intensity. Figures 2*C* and 3*C*, for example, show fairly uniform 2–3+ Bax immunoreactivity in cerebellar Purkinje cells and cerebral cortical neurons, respectively. Interestingly, however, Bax immunostaining was usually stronger in the pyramidal neurons located in CA1 of the hippocampus compared to CA3 (Fig. 1*D–F*). Figure 1*D* and *E*, for example, shows lower and higher power views, respectively of CA1 neurons, demonstrating strong Bax immunoreactivity. In contrast, Bax immunostaining was much weaker in the large pyramidal neurons in the CA3 region, as revealed by photomicrograph in Figure 1*F*, which represents a tissue section derived from the same animal and immunostained simultaneously with the CA1 section shown in Figure 1*D* and *E*.

Like Bax, Bcl-X immunostaining was also present in many types of neurons, typically at an intensity of 3–4+ under the conditions with which anti-Bcl-X antibodies were employed here. Figures 2*D* and 3*D*, for example, show uniformly strong Bcl-X immunoreactivity in Purkinje cells in the cerebellum and in cerebral cortical neurons, respectively. Among the more notable exceptions to the high levels of Bcl-X expression seen in most CNS neurons, however, were the pyramidal neurons in the CA1 region of the hippocampus where Bcl-X immunoreactivity was lower (1–2+). Figure 1*I*, for example, shows Bcl-X immunostaining for a cross-section of the hippocampus at low power. When the CA1 and CA3 sector neurons from this tissue section were compared, the intensity of Bcl-X immunoreactivity was clearly stronger in the CA3 neurons then in the distal portions of the CA1 region. This difference in Bcl-X immunostaining was particularly obvious when the CA1 and CA3 regions were examined at higher power magnification, as shown in Figure 1*G* and *H*. Interestingly, Bcl-X immunoreactivity was also relatively lower in the distal portions of the CA4 sector and hilus of the hippocampus, which are ischemia-sensitive subregions of the brain (Kawai et al., 1992; Hossman, 1993) (Fig. 3*I*). However, Bcl-X immunoreactivity was relatively low and Bcl-2 immunostaining was absent from the granule cells of the dentate gyrus, an ischemia-resistant region, suggesting that the patterns of Bcl-2 and Bcl-X expression do not coincide with differential sensitivity to ischemia in all neuronal subpopulations.

At the subcellular level, immunostaining for Bcl-2, Bax, and Bcl-X was localized to the cell bodies, typically in a punctate pattern in the cytosol suggestive of association with organelles such as mitochondria and in what appeared to be perinuclear and, in some cases, nuclear membranes. Immunoreactivity was also seen in the neuropil but not in the long axons of the white matter.

### *Alterations in Bax, Bcl-X, and Bcl-2 immunostaining following transient global ischemia*

Ischemic brain damage due to sudden cardiac arrest represents a common cause of morbidity and mortality in developed countries where progress in resuscitation efforts are increasing (reviewed by Safar, 1986). We therefore employed a rat model of transient global ischemia to emulate this clinical situation. Cardiac arrest was induced in rats for 10 min as a means of achieving global ischemia in the brain, using the method originally described by Mossakaski et al. (1986) and subsequently employed by others (Majkawska et al., 1989; Kawai et al., 1992; Pluta et al., 1994). Animals were then resuscitated to restore perfusion to the brain and sacrificed at various times thereafter. Consistent with previous reports, two types of ischemic damage were seen: acute infarction and delayed neuronal death. Focal infarcts of variable size, characterized by pale areas of tissue with loss of nuclear basophilia and cytolysis of both neurons and glial cells, were seen at the earliest times examined within the cortical regions of occassional animals. The locations of these infarcts correspond to the arterial boundary zones where the network of arterioles and capillaries supplied by the anterior, middle, and posterior cerebral arteries become most distal. Immunostaining revealed a generalized decrease in intact proteins in this area for all antibodies tested, including Bcl-2, Bax, Bcl-X, and neuron-specific enolase (NSE) (not shown), and thus this type of damage will not be discussed further here.

In contrast to these pale infarcted areas, within 0.5 to 3 hr many cells in adjacent cortical regions (penumbra) had assumed a spindle-shape and somewhat shrunken size, with slightly condensed chromatin. Similar morphological changes were seen in the ischemia-sensitive regions of the brain, including many of the pyramidal neurons in the CA1 region of the hippocampus and many of the Purkinje cells in the cerebellum. Likewise, occassional large neurons scattered throughout the cortical hemispheres (particularly in layers III, V, and VI), ventral thalamus, and striatum also exhibited these early signs of degeneration. Over the next several hours and days, these morphological changes became more severe so that by day 3, most of these ischemia-damaged cells had assumed an extremely shrunken size and contained small pyknotic nuclei. Cells with these morphological features increased in frequency over the next hours to days, reaching maximum numbers between days 3 and 7 in the CA1 sector of the hippocampus and in the Purkinje cell layer of the cerebellum. Beginning on the third postischemic day and extending to day 14, neuronal loss was apparent in the regions most severely affected such as the hippocampal and cerebellar areas mentioned above and the penumbral cortical areas. Neurons with degenerative changes and neuronal loss were far less frequent or absent in sham-operated rats, and found in only scattered locations without any topographical predilection. The nature and kinetics of these postischemia changes and the neuronal cell loss thus were essentially the same as described previously in detail for this model (Mossakowski et al, 1986; Kawai et al., 1992). The somewhat more rapid kinetics of cell death seen in

this model compared to some others that employ four-vessel occlusion as opposed to cardiac arrest can probably be attributed to fewer possibilities for collateral circulation in our model.

As early as 0.5 to 3 hr after the ischemic episode, almost without exception neurons that developed early morphological features of degeneration contained markedly elevated levels of Bax immunoreactivity, compared to adjacent normal neurons. Figure 1*K*, for example, shows Bax immunostaining of hippocampal CA1 region neurons at 30 min after the ischemia. Smaller shrunken neurons with pyknotic nuclei and intense cytosolic Bax immunoreactivity can be seen admixed with normal-appearing pyramidal neurons that contain relatively lower intensity Bax immunoreactivity. Unlike the pyramidal neurons in the CA1, CA2, and CA4 areas of the hippocampus where Bax immunoreactivity selectively increased from 2–3+ to 4+ in intensity in ischemia-damaged neurons, increases in Bax immunostaining were not apparent in the more ischemia-resistant CA3 neurons and few of the neurons were lost from this region after the ischemic episode (not shown). Likewise, Bax immunostaining was strikingly elevated in the shrunken, pyknotic Purkinje cells of the cerebellum as early as 0.5 hr after global ischemia. Figures 2*E* and 2*F*, for example, show ischemia-damaged Purkinje cells with 4+ Bax immunostaining and mixed with normal-appearing Purkinje cells that contain considerably lower (2+) levels of Bax immunoreactivity. In contrast to the Purkinje cells, the cerebellar Golgi-II cells retained normal morphology and failed to express Bax at detectable levels. Peptide competition experiments (Figs. 1*L* and 2*G*) demonstrated that the Bax immunostaining was specific and did not reflect an artifact attributable for example to nonspecific adsorption of antibodies to dying neurons. In contrast, when equivalent amounts of irrelevant peptides were used, the patterns and intensity of Bax immunostaining were unaltered (not shown).

Similar changes were seen in ischemia-damaged neurons in several other regions of the brain, including scattered neurons in the cerebral cortex, ventral thalamus, striatum, and reticular thalamic nuclei. Figure 3*E* and *F*, for example, shows Bax immunostaining of cerebral cortical neurons in rats 3 hr after resuscitation. Note the striking increase in Bax immunoreactivity in the large cortical neurons in cortical layer III that have developed irregular shapes, somewhat smaller size, and more condensed chromatin, compared to adjacent neurons that retained normal morphological features and that contain comparatively less Bax immunoreactivity after ischemia. Similar findings were seen at later times in ischemia-damaged neurons, as demonstrated by the representative photomicrographs presented in Figure 3*G* and *H*. The increase in Bax immunostaining was specific in that immunostaining of adjacent tissue sections with antibodies for Bcl-2 revealed essentially no immunoreactivity in the ischemia-damaged neurons, thus precluding nonspecific adsorption of antisera to dying neurons as an explanation for the results (Fig. 3*I*).

In general, the increases in Bax immunoreactivity persisted surprisingly late into the degenerative process. Figure 1*M* and *N*, for example, shows small shrunken neurons with pyknotic nuclei containing medium to strong cytosolic Bax immunoreactivity in the CA1 sector of the hippocampus at 7 d postischemia. Note that the adjacent large neurons with normal morphology that survived the ischemic episode contained weaker Bax immunostaining.

Levels of Bcl-2 and Bcl-X were variably changed in ischemia-damaged neurons within the first few hours, exhibiting decreases, no change, or even a transient apparent increase in immunointensity, depending on the particular region examined. Typically from 3 hr on, however, neurons with more advanced degenerative changes consistently contained reduced immunostaining for Bcl-2 and Bcl-X compared to adjacent normal neurons. Similarly, NSE immunoreactivity was reduced in nearly all neurons with morphological signs of ischemic damage, but remained strong in healthy looking neurons at all time points examined after ischemia. Figure 3*J* and *K*, for example shows a borderline infarct in the cerebral cortex, where an area of cell lysis presumably presenting necrotic cell death is flanked by neurons that show the typical shrunken, spindle-shape, and hyperchromatic nuclei seen in other regions of the postischemic brain in this model. Immunostaining for Bcl-X demonstrated markedly reduced immunoreactivity in the small shrunken neurons as well as the infarcted tissue, compared to adjacent neurons that retained normal morphology. Similarly, reduced Bcl-X immunoreactivity was seen in the scattered ischemia-damaged neurons in the cerebral cortex and in the hippocampus, in regions not adjacent to borderline infarcts (Figs. 1*J* and 3*L*). Peptide competition experiments, such as the one shown in Figure 3*M*, demonstrated the specificity of these immunostaining results obtained for Bcl-X.

Though reductions in Bcl-X immunoreactivity were not obvious until > 3 hr in many ischemia-damaged neurons, in some neurons reductions in Bcl-X immunostaining were seen earlier and preceeded the development of gross morphological changes and nuclear pyknosis. For example, Bcl-X immunostaining was markedly reduced in cerebellar Purkinje cells that developed morphological signs of degeneration as early as 0.5 hr after ischemia (Fig. 3*I,J*). The nearby ischemia-resistant Golgi-II cells remained normal in appearance and continued to express *bcl-2* and *bcl-X,* thus serving as an internal control for immunostaining (Fig. 3*G–J*). Interestingly, loss of Bcl-X immunoreactivity was also seen in many CA3-sector hippocampal neurons in the absence of morphological changes. Figure 1*J*, for example, shows Bcl-X–negative pyramidal neurons admixed with strongly positive (4+) neurons at 0.5 hr after cardiac arrest; all of these cells retained essentially normal morphology and upregulation of Bax expression was rare in this region of the brain. Analysis of sections of the CA3 region of the hippocampus at later times (> 24 hr), however, revealed 3–4+ Bcl-X immunoreactivity in nearly all neurons, suggesting that the decrease in Bcl-X levels was transient in these cells (not shown). Since relatively little cell death occurred in the CA3 region after transient global ischemia, these findings suggest that transient postischemia reductions in Bcl-X are not necessarily associated with neuronal cell loss.

### *Correlation of Bax expression with* in situ *DNA cleavage*

One of the hallmarks of apoptosis is digestion of the genomic DNA by endogenous endonucleases (Wyllie et al., 1987), which can be detected *in situ* by labeling of free hydroxyl-groups on the 3′ end of DNA fragments using TdT (Wijsman et al., 1993). We therefore performed a two-color analysis to examine simultaneously the relative levels of Bax immunostaining (red/purple) and DNA cleavage (brown) in postischemic neurons. Figure 4 shows some typical results for neurons in the CA1 region of the hippocampus, cerebellum (Purkinje cells), and cerebral cortex at various times after resuscitation. In neurons with normal morphology, no staining or only weak nuclear staining was found indicating the presence of few if any DNA strand breaks; cytosolic Bax immunoreactivity was also relatively lower in these

normal cells compared to adjacent degenerating neurons. In contrast, ischemia-damaged neurons exhibited strong nuclear staining by the TdT-based DNA end-labeling procedure and invariably contained high levels of cytosolic Bax immunoreactivity, with the exception of granular cells of the cerebellum and dentate gyrus, which were Bax negative or which contained only low-levels (1+) of Bax but did occassionally stain positively with TdT (Fig. 4*E,Fp*; and data not shown). These elevations in Bax immunoreactivity appeared to be specific and not a reflection of a generalized increase in gene expression, since the levels of cytosolic Bcl- 2 and Bcl-X immunoreactivity were often reduced in ischemia-damaged neurons (not shown).

### *Immunoblot analysis of Bcl-2, Bcl-X, and Bax proteins in brain tissue before and after ischemia*

At least two forms of the Bcl-X protein can be produced through alternative splicing mechanisms that have opposing effects on cell life and death: Bcl-X-L, a cell death blocker, and Bcl-X-S a promoter of apoptosis (Boise et al., 1993). To determine which of these forms of the Bcl-X protein is produced in brain and whether ischemia induced production of the Bcl-X-S protein, protein-containing detergent lysates were prepared from hippocampus, cerebellum, or cerebral cortex before and after transient global ischemia and immunoblot assays were performed using an antiserum that can detect both forms of Bcl-X protein (Krajewski et al., 1994b). Only the ~28-kDa form of Bcl-X was detected; no 19-kDa Bcl-X-S protein was found in brain tissue samples either before or after transient ischemia (Fig. 5*A*). The failure to detect Bcl-X-S protein in normal brain is consistent with previous reports showing an absence of spliced mRNAs encoding this shorter form of Bcl-X that promotes cell death (Boise et al., 1993). Similarly, though other forms of the Bax and Bcl-2 proteins have been described that can result through alternative splicing (Tsujimoto and Croce, 1986; Oltvai et al., 1993), only the p21-Bax-α and p25-Bcl-2-α proteins were detected in pre- and postischemia brain tissue samples by immunoblotting (Fig. 5*A*).

The tissue samples obtained from rats after ischemia in the delay neuronal death model employed here are comprised of normal neurons admixed with degenerating cells. Thus, the contribution of ischemia damaged neurons to the protein samples is significantly diluted. Nevertheless, these immunoblotting experiments provided additional evidence that Bax protein levels become upregulated after transient ischemia. Figure 5*B*, for example, summarizes the results from three independent experiments where Bax protein levels were ascertained by immunoblotting and the data were quantified by scanning densitometry (all samples normalized for total protein content). As shown, Bax protein levels in the cerebral cortex rose by 2.14 ± 0.48-fold within 6 hr after the ischemic episode and remained persistently elevated for ~2 d, reaching a maximum increase of 2.28 ± 0.49-fold above baseline at 24 hr. Similarly, in the hippocampus, elevations in Bax protein (2.2 ± 0.5 above baseline) were observed at 6 hr after the ischemic episode but were less persistent, with a gradual decrease in Bax protein levels occurring over the next 2 d. Finally, in the cerebellum, increases in Bax protein levels were detected at 6 hr, became maximal at 24 hr (1.67 ± 0.35 above baseline), and then declined at 48 hr. In the cerebellum, however, the postischemia associated increases in Bax protein levels failed to reach statistical significance, unlike the cortex and hippocampus where the elevations in Bax protein levels were significant ($p < 0.05$ by paired $t$ test) for several of the time points examined (see Fig. 5*B* for details).

In contrast to Bax, no alterations in Bcl-X protein levels were detected by immunoblotting (Fig. 5*A*), thus serving as a control to verify loading of equal amounts of total protein derived from various samples of brain tissue. Only low levels of p25-Bcl-2 protein were detected (presumably reflecting the more selective expression of *bcl-2* in subpopulations of neurons in the adult brain and therefore the dilutional effects of Bcl-2-negative cells), making comparisons between pre- and postischemic tissues unreliable.

Recognizing that these tissue samples contain mixtures of ischemia-damaged and normal neurons, these semiquantitative immunoblot data are in general agreement with the immunohistochemical results. The decline in Bax protein levels detected at later times in the hippocampus by immunoblotting can probably be explained by the heterogenity of the cell populations as well as by the loss of ischemia-damaged neurons, which tends to occur more rapidly in the hippocampus than other regions of the brain.

## Discussion

Based on both animal models and clinical experience with humans, some subpopulations of neurons in the adult brain appear to be more susceptible to cell death induced by ischemia, hypoglycemia, status epilepticus, and other stimuli, with the pyramidal CA1 neurons of the hippocampus and the Purkinje cells of the cerebellum representing perhaps the most extreme examples of sensitive neurons (Vogt and Vogt, 1925; Scholz, 1953; Petito et al., 1987; Araki et al., 1989; Kawai et al., 1992; Kreutzberg et al., 1992). Though many possible explanations for this differential sensitivity among neurons may exist, the data reported here suggest that one potential contributor in at least some subregions of the brain could be the relative ratio of Bcl-2, Bax, and Bcl-X proteins. For example, in the hippocampus, levels of Bcl-2 protein were substantially lower in the ischemia-sensitive CA1 region than in the ischemia-resistant CA3 region. The intensity of Bcl-X immunoreactivity was also lower in the CA1 region than the other subregions of the hippocampus. In contrast, Bax immunoreactivity was stronger in CA1 than in CA3. Consistent with this differential expression of *bcl-2, bax,* and *bcl-X* in the hippocampus, neuronal cell loss after a global ischemic episode was more severe in the CA1 region than in CA3, a finding in keeping with previous reports (Vogt and Vogt, 1925; Scholz, 1953; Araki et al., 1989; Kawai et al., 1992; Kreutzberg et al., 1992). In addition, in the cerebellum, the ischemia-sensitive Purkinje cells contained only low (1+) levels of Bcl-2, whereas the resistant Golgi-II cells were strongly Bcl-2 positive. Conversely, Bax immunostaining was much stronger in Purkinje cells than Golgi II cells. Bcl-2 was also present at relatively high levels in most of the large neurons in the brain stem nuclei of adult rat brain, and the neuronal loss appeared to be rather minimal in this region of the brain after ischemia, consistent with previous reports (Vogt and Vogt, 1925; Scholz, 1953; Kreutzberg et al., 1992). Thus, in at least some of the areas of the brain that have been documented to exhibit clear differential sensitivity to ischemia-induced neuronal loss, a rough correlation exists between expression of *bcl-2* family genes and relative resistance.

One region where the expression of patterns of Bcl-2 family proteins did not correlate, however, was in the granule cells of the dentate gyrus, which represent a relatively ischemia-resistant population of neurons. These cells contained essentially no de-

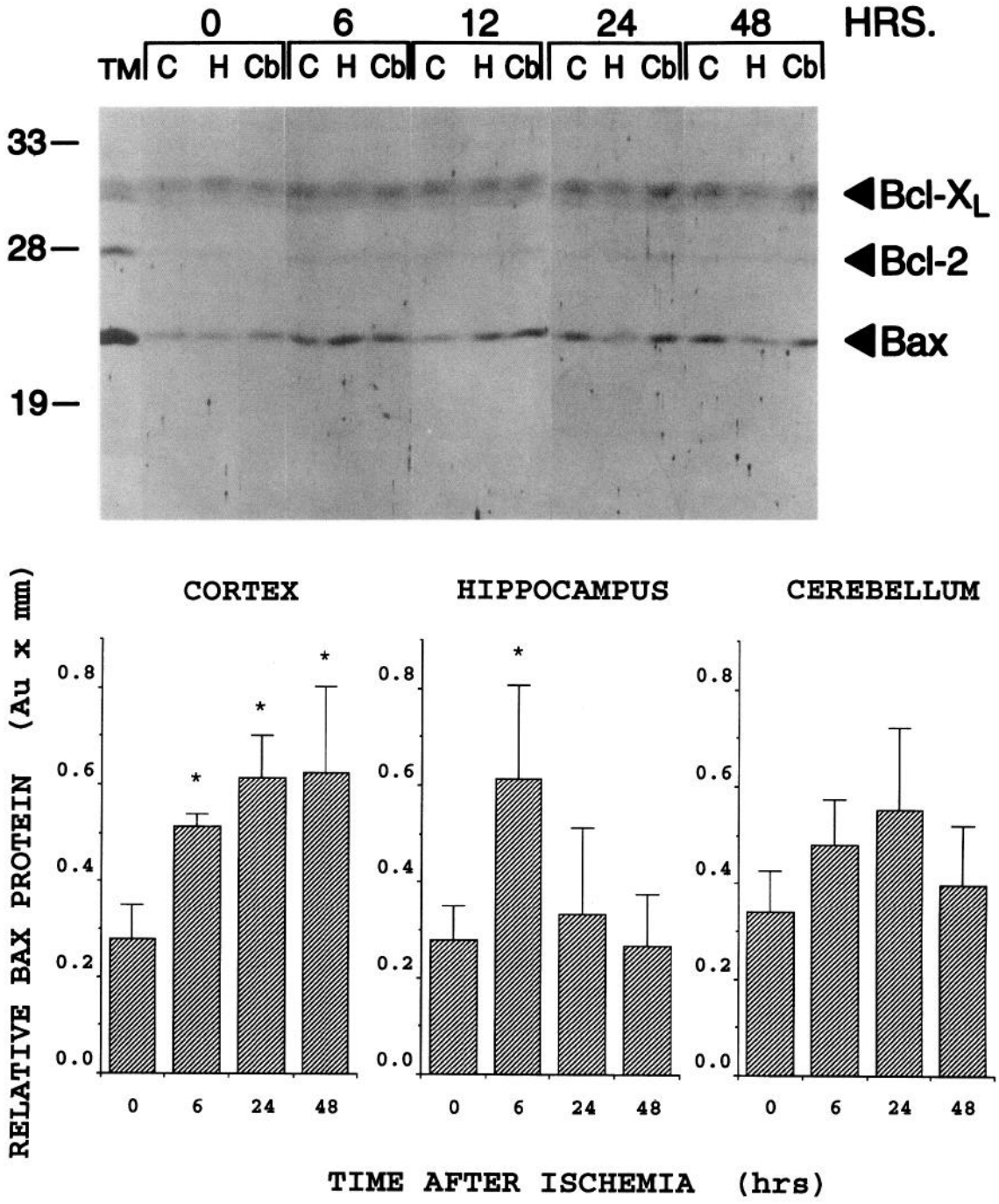



*Figure 5.* Immunoblot analysis of Bax, Bcl-2, and Bcl-X in ischemic brain tissues. In *A*, aliquots of protein samples (50 μg per lane) derived from cerebral cortex (*C*), hippocampus (*H*), or cerebellum (*Cb*) at various times before of after ischemia were subjected to SDS-PAGE/immunoblot analysis using sequentially anti-Bcl-2 antibody (Vector-SG substrate (*black*), anti-Bax (DAB, *brown*), and anti-Bcl-X (AEC, *red*). Normal thymus (*TM*) was included as a positive control. Molecular weight markers are in kilo-Daltons. The original three-color analysis can not be seen in the black-and-white reproduction presented here. In *B*, Bax immunoblot results from three independent experiments were quantified by scanning densitometry and the area under the tracings was determined [arbitrary absorbance units (AU) X mm]. Data represent mean ± standard deviation. Statistical significance (*) was determined by paired *t* test.

tectable Bcl-2 immunoreactivity, and the intensity of Bcl-X immunostaining was lower than in CA3 hippocampal region neurons, whereas Bax was lower in intensity than in CA1 neurons (but higher than CA3 neurons). Thus, the relative proportions of Bcl-2 family proteins probably cannot account entirely for the differential ischemia sensitivity of all neuronal populations. It should be noted, however, that Bcl-2, Bcl-X, and Bax are not the only known members of the Bcl-2 protein family expressed in brain. Though it remains unknown at present whether these other proteins are expressed in neurons, it is noteworthy that some of the more recently described pro-apoptotic Bcl-2 family proteins bind preferentially or exclusively to either Bcl-X or Bcl-2 (Chittenden et al., 1995; Yang et al., 1995). The actions therefore of other Bcl-2 family proteins conceivably may explain

observations such as the lack of apparent correlation between expression of Bcl-X and Bcl-2 and ischemia-resistance in the dentate gyrus and the marked ischemia-sensitivity of cerebellar Purkinje cells, despite their strong Bcl-X immunostaining. With regards to Purkinje cells, for example, it could be that co-expression of both Bcl-X and Bcl-2 is required for optimal protection, but these neurons contain very little Bcl-2 immunoreactivity.

In animal models of transient global ischemia, neuronal cell death typically occurs in a delayed fashion, requiring several days before much of the neuronal loss occurs (Vogt and Vogt, 1925; Scholz, 1953; Araki et al., 1989; Kawai et al., 1992; Kreutzberg et al., 1992). In the hours and days preceeding the neuronal cell loss described here in the adult rat brain after a 10 min period of cardiac arrest, the neurons almost uniformly underwent a series of morphological changes resembling apoptosis, with cell shrinkage, chromatin condensation, and nuclear compaction. The nuclei of most of these cells also appeared to contain increased amounts of fragmented DNA, based on *in situ* end labeling with TdT (Fig. 4 and data not shown). Bcl-2 family proteins are critical regulators of apoptosis that appear to control a relatively late step in what may represent a final common pathway for active cell suicide (reviewed by Reed, 1994). However, gene transfer-mediated elevations in Bcl-2 protein levels have also been reported to protect a neural cell line from necrotic cell death induced by depletion of intracellular glutathione by buthionine sulfoximine (BSO) (Kane et al., 1993), suggesting that in at least some circumstances the unknown biochemical events regulated by Bcl-2 and its homologs may be involved in both necrotic and apoptotic cell death. Thus, the correlations seen here between levels of Bcl-2 family proteins and susceptibility to ischemia do not necessarily add support to the hypothesis that some ischemic cell deaths occur through apoptosis as opposed to necrosis. Regardless of how these cell deaths are classified, however, our findings together with the recent results from *bcl-2* expressing transgenic mice (Martinou et al., 1994) suggest that the cell death pathway regulated by Bcl-2 and its homologs is often involved in neuronal cell death in the setting of ischemia.

Protein synthesis inhibitors such as cycloheximide and anisomycin have been shown to reduce the extent of ischemia-induced death of neurons in animal models of both global and focal ischemia (Linnik et al., 1993; Bredesen, 1994). This observation implies that the production of new proteins is required for at least some neuronal cell deaths that occur during ischemia. In this report, we show that one protein that increases during ischemia is p21-Bax. The Bax protein is a promoter of cell death whose pro-apoptotic function is directly opposed by Bcl-2 through formation of Bax/Bcl-2 heterodimers (Oltvai et al., 1993; Sato et al., 1994; Yin et al., 1994). The ratio of Bax to Bcl-2 and other homologs of Bcl-2 that can also antagonize Bax, such as Bcl-X-L therefore can be an important determinant of the relative vulnerability of cells to apoptotic stimuli. It is unlikely that the increases in Bax protein levels result from a concentration of proteins caused by volume contraction during neuronal shrinkage or reflect a generalized increase in gene expression, since immunostaining for Bcl-2, Bcl-X, and NSE was often diminished in cells with morphological evidence of degeneration. Also, it has been reported that a net decrease in protein synthesis occurs after ischemia in brain tissue (Hossman, 1993). For this reason, however, the reductions in Bcl-2 and Bcl-X immunoreactivity seen in some subpopulations of ischemia-damaged neurons, such as cerebellar Purkinje cells, could reflect a nonspecific decrease in protein synthesis. In fact, it is possible that rapid reductions in Bcl-2 and Bcl-X proteins reflect the postischemic activation of proteases, given that the half-life of the Bcl-2 protein is normally ~10 hr (Kitada et al., 1994). Moreover, rapid decreases in Bcl-X immunoreactivity (which were probably transient in nature) were seen in some CA3 sector hippocampal neurons, despite the normal appearance of these cells and the relative dirth of neuronal cell loss that occurs in this region of the brain after ischemia. Thus, reversible reductions in Bcl-X expression may not necessarily be associated with commitment to die, particularly in subtypes of neurons with a propensity to recover after transient hypoxia.

The finding of strikingly elevated levels of Bax in neurons that displayed morphological evidence of degeneration implies a potentially important role for Bax in the regulation of neuronal cell death in the global ischemia model employed here. Neurons with increased levels of Bax also generally contained fragmented DNA, based on two-color analysis of Bax immunostaining and DNA strand breaks by end labeling with TdT (Fig. 4), again suggesting the possibility of a functional connection between elevations in Bax and neuronal cell death caused by transient global ischemia. Moreover, elevations in Bax immunostaining were seen early after transient ischemia. It remains to be determined, however, whether a direct cause-and-effect relation can be attributed to elevations in Bax protein levels and cell death in neurons. Recent studies of *bcl-X* knock-out mice, for example, have demonstrated that loss of Bcl-$X_L$ protein, resulting presumably in a decrease in the Bcl-$X_L$: Bax ratio, is associated with massive neuronal cell death in the developing brain (Motoyama et al., 1995). On the other hand, investigations of *bcl-2* knock-out mice suggest that an elevated Bax/Bcl-2 ratio does not by itself trigger cell death in most tissues, including the brain, but does render many types of cells relatively more sensitive to induction of apoptosis by stimuli that can activate the physiological cell death pathway (Veis et al., 1993). Thus, while an elevation in Bax protein levels in ischemic neurons may not induce cell death per se, it presumably would render these cells more vulnerable to apoptotic stimuli and thus less likely to recover from transient ischemia. Also, increases in Bax are probably not required for all neuronal cell deaths that occur, since DNA fragmentation was detected *in situ* in some cerebellar granular cells that uniformly failed to express *bax*. In this regard, glutamate-induced death of cerebellar granular cells in culture was reported to be unaffected by inhibitors of RNA and protein synthesis, also consistent with an absence of a requirement for *bax* expression in at least some types of neurons (Dessi et al., 1993). Of course, this absence of a requirement for new gene expression may also be an indication that granule cell death occurs by necrosis rather than apoptosis, despite the presence of TdT-labeled DNA (Dessi et al., 1993).

One remarkable feature of the upregulation of Bax protein seen in degenerating neurons after ischemia was its persistence. Bax immunostaining increased early after transient ischemia and remained elevated for days in degenerating neurons. The stimuli that induce increases in Bax protein accumulation in ischemic neurons therefore appear to remain present long after the initial ischemic event and presumably occur during the reperfusion phase. The mechanisms responsible for these cell deaths that occur during reperfusion after transient ischemia are probably multifactoral but have been hypothesized to include release of excitotoxic neurotransmitters, production of oxygen free radi-

cals, disturbances in intracellular $Ca^{2+}$ regulation, alterations in gene expression, and other biochemical changes in neurons (reviewed by Choi and Rothman, 1990; Bredesen, 1994). Though the nature of the stimuli that account for upregulation of Bax remains unknown at present, one candidate is the tumor suppressor p53. Recently, we demonstrated that p53 binds directly to DNA containing typical 10-mer consensus sequences located in the bax gene promoter and strongly *trans*-activates reporter gene constructs containing the bax promoter (Miyashita and Reed, 1994). Furthermore, in p53 knock-out mice, basal levels of *bax* gene expression were demonstrably lower in a variety of CNS neurons than in normal littermate control animals that contained intact copies of the p53 gene (Miyashita et al., 1994). Interestingly, hypoxia has been reported to stimulate increases in p53 protein levels and p53 transcriptional activity in several established cell lines *in vitro* (Graeber et al., 1994). Furthermore, p53 expression has been shown to increase in ischemia-damaged regions of the brain after middle cerebral artery occlusion (Chopp et al., 1992; Li et al., 1994), and attenuation of p53 expression has been shown to protect against focal ischemic damage in transgenic mice (Crumrine et al., 1994). The glutamate analog kainic acid has been shown to induce elevations in p53 mRNA levels as detected by *in situ* hybridization in apoptosis-sensitive groups of neurons where ischemia-induced increases in Bax were seen in this study, such as the pyramidal neurons in CA1 of the hippocampus (Sakhi et al., 1994). By analogy to these data derived from focal ischemia models and kainic acid-treated animals therefore it is conceivable that postischemic increases in p53 account for the elevations in Bax protein levels seen in degenerating neurons after transient global ischemia. Further investigations, however, are required to establish whether direct functional relations exist between p53, Bax, and apoptotic cell death induced by ischemia and other insults in vulnerable populations of CNS neurons.

## References


Araki T, Kato H, Kogure K (1989) Selective neuronal vulnerability following transient cerebral ischemia in the gerbil: distribution and time course. Acta Neurol Scand 80:548–553.

Baffy G, Miyashita T, Williamson JR, Reed JC (1993) Apoptosis induced by withdrawal of Interleukin-3 (IL-3) from an IL-3-dependent hematopoietic cell line associated with repartitioning of intracellular calcium and is blocked by enforced Bcl-2 oncoprotein production. J Biol Chem 268:6511–6519.

Behl C, Hovey L III, Krajewski S, Schubert D, Reed JC (1993) Bcl-2 prevents killing of neuronal cell lines by L-glutamate but not by amyloid-beta peptide. BBRC 197:949–956.

Boise LH, Gonzalez-Garcia M, Postem, CE, Ding L, Lindsten T, Turka LA, Mao X, Nunez G, Thompson CB (1993) *bcl-x*, a *bcl-2*-related gene that functions as a dominant regulator of apoptotic cell death. Cell 74:597–608.

Bredesen DE (1994) Neuronal apoptosis: Genetic and biochemical modulation. In: Apoptosis II: the molecular basis of apoptosis in disease, pp 397–421. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Chittenden T, Harrington EA, O'Connor R, Flemington C, Lutz RJ, Evan GI, Guild BC (1995) Induction of apoptosis by the Bcl-2 homologue Bak. Nature 374:733–736.

Choi DW, Rothman SM (1990) The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. Annu Rev Neurosci 13:171–182.

Chopp M, Li Y, Zhang ZG, Freytag SO (1992) p53 expression in brain after middle cerebral artery occlusion in the rat. Biochem Biophys Res Commun 182:1201–1207.

Crumrine RC, Thomas AL, Morgan PF (1994) Attenuation of p53 expression protects against focal ischemic damage in transgenic mice. J Cereb Blood Flow Metab 14:887–891.

Dessi F, Charriaut-Marlangue C, Khrestchatisky M, Ben-Ari Y (1993) Glutamate-induced neuronal death is not a programmed cell death in cerebellar culture. J Neurochem 60:1953–1955.

Graeber TG, Peterson JF, Tsai M, Monica K, Fornace Jr, AJ, Giaccia AJ (1994) Hypoxia induces accumulation of p53 protein, but activation of a G1-phase checkpoint by low-oxygen conditions is independent of p53 status. Mol Cell Biol 14:6264–6277.

Hockenbery D, Oltvai Z, Yin X-M, Milliman C, Korsmeyer SJ (1993) Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell 75:241–251.

Hossmann K-A (1993) Disturbances of cerebral protein synthesis and ischemic cell death. Prog Brain Res 96:161–177.

Kane DJ, Sarafin TA, Auton S, Hahn H, Gralla FB, Valentine JC, Ord T, Bredesen DE (1993) Bcl-2 inhibition of neural cell death: decreased generation of reactive oxygen species. Science 262:1274–1276.

Kawai K, Nitecka L, Ruetzler CA, Nagashima G, Joó F, Mies G, Nowak TS Jr, Saito N, Lohr JM, Klatzo I (1992) Global cerebral ischemia associated with cardiac arrest in the rat: I. Dynamics of early neuronal changes. J Cereb Blood Flow Metab 12:238–249.

Kitada S, Takayama S, DeRiel K, Tanaka S, Reed JC (1994) Reversal of chemoresistance of lymphoma cells by antisense-mediated reduction of *bcl-2* gene expression. Antisense Res Dev 4:71–79.

Krajewski S, Tanaka S, Takayama S, Schibler MJ, Fenton W, Reed JC (1993) Investigations of the subcellular distribution of the *bcl-2* oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. Cancer Res 53:4701–4714.

Krajewski S, Krajewska M, Shabaik A, Miyashita T, Wang H-G, Reed JC (1994a) Immunohistochemical determination of *in vivo* distribution of *bax*, a dominant inhibitor of *bcl-2*. Am J Pathol 145:1323–1333.

Krajewski S, Krajewska M, Shabaik A, Wang H-G, Irie S, Fong L, Reed JC (1994b) Immunohistochemical analysis of *in vivo* patterns of Bcl-X expression. Cancer Res 54:5501–5507.

Kreutzberg GW, Klatzo I, Kleihues P (1992) Oskar and Cecile Vogt, Lenin's brain and the bumble-bees of the Black Forest. Brain Pathol 2:363–371.

Lam M, Dubyak G, Chen L, Nu;ěz G, Miesfeld RL, Distelhorst CW (1994) Evidence that Bcl-2 represses apoptosis by regulating endoplasmic reticulum-associated $Ca^{2+}$ fluxes. Proc Natl Acad Sci USA 91:6569–6573.

Li Y, Chopp M, Zhang ZG, Zaloga C, Niewenhuis L, Gautam S (1994) p53-immunoreactive protein and p53 mRNA expression after transient middle cerebral artery occlusion in rats. Stroke 25:849–856.

Linnik MD, Zobrist RH, Hatfield MD (1993) Evidence supporting a role for programmed cell death in focal cerebral ischemia in rats. Stroke 24:2002–2009.

Majkowska J (1989) Pathological characteristics of clinical death model in rats. Neuropatol Pol 27:85–96.

Martinou J-C, Dubois-Dauphin M, Staple JK, Rodriguez I, Frankowski H, Missotten M, Albertini P, Talabot D, Catsicas S, Pietra C, Huarte J (1994) Overexpression of *bcl-2* in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. Neuron 13:1017–1030.

Miyashita T, Reed JC (1994) Tumor suppressor p53 is a direct transcriptional activator of human *bax* gene. Cell 80:293–299.

Miyashita T, Krajewski S, Krajewska M, Wang HG, Lin HK, Hoffman B, Lieberman D, Reed JC (1994) Tumor suppressor p53 is a regulator of *bcl-2* and *bax* in gene expression *in vitro* and *in vivo*. Oncogene 9:1799–1805.

Mossakowski MJ, Hiligier W, Januszewski S (1986) Morphological abnormalities in the CNS of rats in experimental postresuscitation period. Neuropatol Pol 24:471–489.

Motoyama N, Wang F, Roth KA, Sawa H, Nakayama K-I, Nakayama K, Negishi I, Senju S, Zhang Q, Fujii S, Loh DY (1995) Massive cell death of immature hematopoietic cells and neurons in Bcl-X-deficient mice. Science 267:1506–1510.

Oltvai Z, Milliman C, Korsmeyer SJ (1993) Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. Cell 74:609–619.

Petito CK, Feldmann E, Pulsinelli WA, Plum F (1987) Delayed hippocampal damage in humans following cardiorespiratory arrest. Neurology 37:1281–1286.

Pluta R, Kida E, Lossinsky AS, Golabek AA, Mossakowski MJ, Wisniewski HM (1994) Complete cerebral ischemia with short term survival in rats induced by cardiac arrest. I. Extracellular accumulation

of Alzheimer's β-amyloid protein precursor in the brain. Brain Res 649:323–328.

Reed JC (1994) Bcl-2 and the regulation of programmed cell death. J Cell Biol 124:1–6.

Roberts-Lewis JM, Marcy VR, Zhao Y, Vaught JL, Siman R, Lewis ME (1993) Aurintricarboxylic acid protects hippocampal neurons from NMDA- and ischemia-induced toxicity *in vivo*. J Neurochem 61:378–381.

Safar P (1986) Cerebral resuscitation after cardiac arrest: a review. Circulation 74(Suppl IV):IV-138–ziv-153.

Sakhi S, Bruce A, Sun N, Tocco G, Baudry M, Schreiber SS (1994) p53 induction is associated with neuronal damage in the central nervous system. Proc Natl Acad Sci USA 91:7525–7529.

Sato T, Hanada M, Bodrug S, Irie S, Iwama N, Boise LH, Thompson CB, Golemis E, Fong L, Wang H-G, Reed JC (1994) Interactions among members of the *bcl-2* protein family analyzed with a yeast two-hybrid system. Proc Natl Acad Sci USA 91:9238–9242.

Scholz W (1953) Selective neuronal necrosis and its topistic patterns in hypoxemia and oligemia. J Neuropathol Exp Neurol 12:249–261.

Sei Y, Von Lubitz KJ, Basile AS, Borner MM, Lin RC, Skolnick P, Fossom LH (1994) Internucleosomal DNA fragmentation in gerbil hippocampus following forebrain ischemia. Neurosci Lett 171:179–182.

Shigeno T, Yamasaki Y, Kato G, Kusaka K, Mima T, Takakura K, Graham DI, Furukawa S (1990) Reduction of delayed neuronal death by inhibition of protein synthesis. Neurosci Lett 120:117–119.

Tsujimoto Y, Croce CM (1986) Analysis of the structure, transcripts, and protein products of *bcl-2*, the gene involved in human follicular lymphoma. Proc Natl Acad Sci USA 83:5214–5218.

Veis DJ, Sorenson CM, Shutter JR, Korsmeyer SJ (1993) Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell 75:229–240.

Vogt C, Vogt O (1925) Der Begriff der Pathoklise. J Psychol Neurol 31:245–257.

Wijsman JH, Jonker RR, Keijzer R, van de Velde CJH, Cornelisse CJ, van Dierendocnck JH (1993) A new method to detect apoptosis in paraffin sections: *in situ* end-labeling of fragmented DNA. J Histochem Cytochem 41:7–12.

Wyllie AH, Morris RG, Smith AL, Dunlop D (1984) Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. J Pathol 142:67–77.

Yang E, Zha J, Jockel J, Boise LH, Thompson CB, Korsmeyer SJ (1995) Bad: a heterodimeric partner for Bcl-XL and Bcl-2, displaces *bax* and promotes cell death. Cell 80:285–291.

Yin XM, Oltvai ZN, Korsmeyer SJ (1994) BH1 and BH2 domains of *bcl-2* are required for inhibition of apoptosis and heterodimerization with *bax*. Nature 369:321–333.

Zhong LT, Sarafian T, Kane DJ, Charles AC, Mah SP, Edwards RH, Bredesen DE (1993) *bcl-2* inhibits death of central neural cells induced by multiple agents. Proc Natl Acad Sci USA 90:4533–4537.