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Localization of Bax and Bcl-2 proteins, regulators of programmed cell death, in the human central nervous system

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Abstract Bax and Bcl-2 proteins are identified as regulating molecules for programmed cell death. In the central nervous system, programmed cell death or apoptosis is considered to be an important phenomenon that is related to neuron vulnerability to a variety of toxic effects, including ischaemic insult. In this study, localization of Bax and Bcl-2 proteins was investigated in the human central nervous system using autopsy cases without any neurological disorder. Results were compared with findings in the rat. Most neurons in human cerebral cortex, basal ganglia and brain stem were positive for both Bax and Bcl-2 proteins, whereas Purkinje cells in cerebellum and neurons in hippocampal CA1, CA2 and CA3 regions were positive for Bax but negative or weakly positive for Bcl-2. Glial cells examined in all sections were negative for both proteins. Choroid plexus, ependymal cells and arachnoid villi showed positive reactivity for both proteins. A possible relationship between the localization of Bax or Bcl-2 proteins and the cell vulnerability in central nervous system is discussed.

Key words Bax · Bcl-2 · Apoptosis · Central nervous system · Human

Introduction

Bax and its related protein, Bcl-2, are essential for the regulation of apoptotic cell death, an active cell-deletion process operating during embryonic development, tissue remodelling and tumour regression [16, 22]. Bax, which forms heterodimers with Bcl-2, has extensive amino acid sequence homology with Bcl-2 protein [18]. Overexpression of Bax is known to promote programmed or apoptotic cell death and conversely Bcl-2 inhibits this type of cell death and promotes cell survival [12, 21]. The anti-apoptotic activity of Bcl-2 is achieved by suppressing the apoptosis-inducing function of Bax by forming heterodimer with Bax protein [7, 18, 23]. Thus, Bax and Bcl-2 proteins play opposing parts in the physiological control of cell survival and death [5].

Immunohistochemical localization of Bax and Bcl-2 proteins has been investigated in various tissues of mice [14]. Bax expression is more widespread than expression of Bcl-2, and it is sometimes, but not always, localized to tissues characterized by high apoptotic death rates [14]. It is speculated that the ratio of Bax to Bcl-2 plays a critical part in regulating the relative sensitivity of some tissues to apoptotic cell death. In central nervous system, Bcl-2 protein is abundant in the developing rat brain [3, 4], and a higher level of bcl-2 mRNA was detected in late prenatal development than in postnatal and adult rat brain [3]. Strong Bcl-2 immunoreactivity occurs in the neocortex and hippocampus of the developing rat during the 1st postnatal week. Bcl-2 immunoreactivity then rapidly decreases with ageing. Very low levels of Bcl-2 protein, hardly detectable by immunohistochemistry, are recognized in rats during adult life [4]. In contrast, intense immunoreactivity of Bax protein has been detected in some neurons of mouse adult central nervous system [14]. Bax is suggested to contribute to the vulnerability of some neurons to a variety of toxic effects, including hypoxia, hypoglycaemia and ischaemic insult [6, 10].

In this study, we used immunohistochemistry to determine in vivo localization of Bax and Bcl-2 proteins in the human central nervous system, using polyclonal antibodies specific for Bax and Bcl-2 proteins. The study was performed in neural and related tissues from human autopsy cases without neurological disorder, and the results were compared with those obtained in the rat central nervous system.

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Materials and methods

Five adult autopsy cases without neurological disorder were used in this study. Specimens obtained at autopsy were fixed in formalin as soon as possible and immersed for 7 days, and then embed-

ded in paraffin. Fisher 344 rats 8 weeks of age were also used for tissue preparation. Animals were anaesthetized with pentobarbital, and perfused transcardially with saline and then with 10% neutral buffered formalin. Brains were then immersed in 10% neutral buffered formalin for 24 h and processed for paraffin embedding.

Sections (5 μm) were cut for conventional haematoxylin and eosin (H&E) staining, and for immunohistochemistry.

Sections for immunohistochemistry were deparaffinized and then hydrated by transferring them through the following solutions: xylene bath twice for 5 min, and then 96% ethanol twice, 90% ethanol, 80% ethanol, and distilled water, for 3 min each.

Anti-Bax antibody (P-19) and anti-Bcl-2 antibody (N-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). Anti-Bax antibody is an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to amino acids 43-61 mapping within an amino terminal domain of Bax protein of mouse origin. Anti-Bax antibody reacts with Bax protein of mouse, rat and human origin and is non-cross-reactive with Bcl-2 and Bcl-x proteins (data from manufacturer). Anti-Bcl-2 antibody is an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to amino acids 4–23 mapping at the amino terminus of Bcl-2 protein of human origin. Anti-Bcl-2 antibody reacts with Bcl-2 protein of mouse, rat and human origin and is non cross-reactive with Bax and Bcl-x proteins (data from manufacturer). The deparaffinized sections were heated and boiled for 1 min by microwaving in 10 mM citrate buffer, pH 6.0. To diminish nonspecific staining, each section was treated with methanol containing 3% hydrogen peroxide for 3 min. Anti-Bax and anti-Bcl-2 antibodies used at dilutions of 1:2000 and 1:1000, respectively, in 0.05 M Tris-buffered saline, pH 7.6 (TBS) were added to the slides and incubated overnight in 4°C. Expression of Bax and Bcl-2 proteins was shown by the labelled streptavidin biotin (LSAB) method using the LSAB kit (Dako, Carpinteria, Calif.) containing blocking reagent, biotinylated link antibody and peroxidase-labelled streptavidin reagents. The peroxidase-binding sites were detected by staining with 3,3′-diaminobenzidine in TBS. Finally, counterstaining was performed with Mayer's haematoxylin. Slides were photographed with a light microscope (Olympus model AH2) equipped with a 35-mm camera (Olympus model C-35A4-4) with Ektar 100 film (Eastman Kodak, Rochester, N.Y.)

Results

The results are summarized in Table 1.

In the human central nervous system, positive Bax immunostaining is seen in various neurons. In particular, neurons in the cerebral cortex (Fig. 1A), hippocampus (Fig. 1B), brain stem and cerebellar cortex (Fig. 1C) show strongly positive immunoreactivity. Axonal fibres are also positive for Bax immunostaining (Fig. 1A). Glial cells in all locations, however, demonstrate completely negative immunostaining for Bax. No Bax immunostaining is seen in the granular layer of the cerebellar cortex (Fig. 1C). The choroid plexus (Fig. 1D), ependymal cells in all ventricles, and the arachnoid villi in the superior sagittal sinus show positive Bax immunostaining. In the choroid plexus, Bax immunoreactivity is seen in epithelial cells but not in the central connective tissue core. In the same manner, the immunostaining is present in the arachnoid cells of arachnoid villi but not in the surrounding connective tissues. The same pattern of the expression of Bax immunoreactivity is recognized in the rat central nervous system (Table 1).

Positive Bcl-2 immunostaining is also seen in various neurons (Fig. 1E), except for hippocampal neurons and Purkinje cells. Neurons in hippocampal CA1, CA2 and CA3 regions show only a weak level of immunoreactivity for Bcl-2 (Fig. 1F) and no immunostaining is seen in Purkinje cells (Fig. 1G). Glia cells in all the location of human central nervous system demonstrate completely negative immunostaining for Bcl-2 in the same manner as Bax. Golgi type II cells in the granular layer of the cerebellar cortex are positive for Bcl-2 although most granular cells show negative reactivity for Bcl-2 (Fig. 1G). Choroid plexus (Fig. 1H) and ependymal cells in all the ventricles, and arachnoid villi in the superior

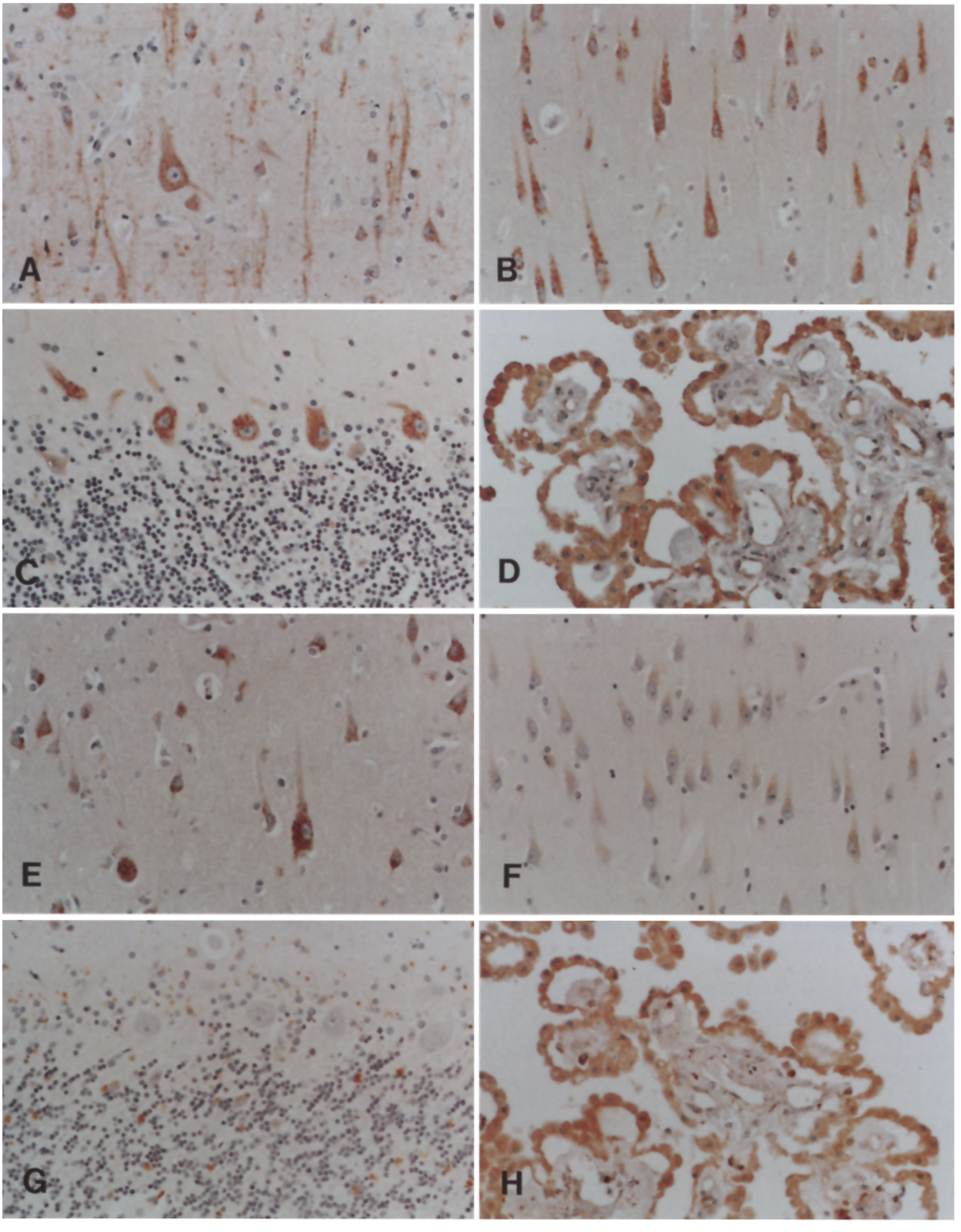
Table 1 Expression of Bax and Bcl-2 proteins in rat and human central nervous system (– negative, ± weakly positive, + positive, ++ strongly positive)

	Bax		Bcl-2	
	Human	Rat	Human	Rat
Cerebral cortex				
Neuron	++	+	+	±
Glia	–	–	–	–
Basal nuclei				
Neuron	+	+	+	±
Glia	–	–	–	–
Hippocampus				
Neuron[a]	++	+	±	–
Glia	–	–	–	–
Brain stem				
Neuron	++	+	++	+
Glia	–	–	–	–
Cerebellum				
Purkinje cell	++	++	–	±
Granular cell	–	–	+[b]	–
Cerebellar nuclei	++	+	++	+
Glia	–	–	–	–
Choroid plexus	++	++	+	+
Ependymal cell	+	+	+	+
Arachnoid villi	+	+	+	+

[a] These include neurons in hippocampal CA1, CA2 and CA3 regions

[b] Golgi type II cells in granular layer of cerebellar cortex are positive for Bcl-2 although most granular cells are negative for Bcl-2

Fig. 1A–H Immunohistochemical detection of Bax and Bcl-2 proteins in human central nervous system from autopsy cases. (Immunostaining for **A–D** Bax, **E–H** Bcl-2; ×150.) **A** Neurons of cerebral cortex in frontal lobe show strongly positive immunostaining in both perikarya and axonal fibres. **B** Hippocampal pyramidal neurons show strongly positive immunostaining in perikarya and apical dendrites. **C** In cerebellar cortex, Purkinje cells express intense immunostaining for Bax in their perinuclear cytoplasm, whereas no staining is recognized in the granular layer. **D** In choroid plexus, the Bax immunoreactivity is seen only in epithelial cells, and not in the central core comprised of connective tissues. **E** Neurons of cerebral cortex in frontal lobe show positive immunostaining in perikarya but not in axonal fibres. **F** Hippocampal pyramidal neurons show weakly positive immunostaining in perikarya. **G** No immunostaining is seen in Purkinje cells. Golgi type II cells in granular layer of cerebellar cortex are positive for Bcl-2 although most granular cells showed negative reactivity for Bcl-2. **H** In the same manner as Bax, the Bcl-2 immunoreactivity is seen in only epithelial cells of choroid plexus but not in the central core. Macrophages in the connective tissue of the choroid plexus are stained as immunoreactive single cells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sagittal sinus show positive Bcl-2 immunostaining. Macrophages in the connective tissue of the choroid plexus are stained as immunoreactive single cells (Fig. 1H). Almost the same pattern of the expression of Bcl-2 immunoreactivity is recognized in rat central nervous system, although the immunostaining intensity of Bcl-2 in rat is not so intense as that in human (Table 1).

Discussion

Neurons in central nervous system are vulnerable to cell death induced by hypoxia, hypoglycaemia, ischaemia and a variety of other insults. Recent studies have revealed that the delayed neuronal death in the hippocampal CA1 region of rodents induced by transient forebrain ischaemia was characterized by nuclear DNA fragmentation, an important phenomenon in apoptotic cell death [9, 11, 13, 15, 17]. In the central nervous system, various disease situations such as neurodegenerative disorders, toxic neuropathy and ischaemic neuronal change are thought to have some possible relationship with apoptosis in their pathogenesis. Information on the distribution of apoptosis-regulating proteins, Bax and Bcl-2, in central nervous system seems to be important in the understanding of the pathogenesis of above disease situations.

The following two patterns of Bax and Bcl-2 expression in neurons were found in this study. One was recognized as a localization pattern of both Bax and Bcl-2 in the cytoplasm of the neurons. However, Bax was recognized in both perikarya and axon, and Bcl-2 only in perikarya, suggesting that Bax was predominant. Most human neurons in the central nervous system, such as those in the cerebral cortex, basal ganglia, and brain stem, belonged to this group. The other pattern was recognized in cerebellar Purkinje cells, which expressed intense immunostaining for Bax in their perinuclear cytoplasm. Bcl-2 was absent from the cells. The neurons in hippocampus also belonged to this pattern because of their strong expression of Bax but low level of immunoreactivity for Bcl-2. It is known that Purkinje cells and hippocampal neurons are extremely vulnerable to ischaemic insult and we suggest that the high ratio of Bax to Bcl-2 plays an important role in vulnerability of these cells to ischaemia.

Interestingly, Golgi type II cells, (several percent of all cells in the granular layer of the cerebellar cortex) were positive for Bcl-2 although most granular cells showed negative reactivity. A different vulnerability of the various cell types in the cerebellar cortex to ischaemic insults has been described [1, 2]. Even within the granular layer, the differing vulnerability to transient ischaemia has been demonstrated [8]. Golgi type II cells, for example, are more resistant to transient ischaemia than the other cells in the granular layer and have been observed as surviving cells in lobular atrophy following transient ischaemia [19]. The expression of Bcl-2 in Golgi type II cells may contribute to the resistance to ischaemic insult.

The epithelial cells of choroid plexus, ependymal cells and arachnoid cells of arachnoid villi were positive for both Bax and Bcl-2. These cells are usually resistant to ischaemia, and the potential relationship between vulnerability to cell death and expression of both proteins is unclear. In the immunohistochemical analysis of Bax and Bcl-2 in mouse tissues, epithelial cells in other organs showed various patterns of expression for both proteins [14]. For example, reciprocal localization of Bax and Bcl-2 is recognized in salivary glands. They express Bax in the serous cells but not the mucinous cells and conversely Bcl-2 in mucinous cells but not serous cells. In contrast, the outer layer of germinal epithelial cells within developing ovarian follicles and breast epithelium contain both Bax and Bcl-2, as detected in the epithelial cells of the choroid plexus, ependymal cells and the arachnoid cells in the present study.

Glial cells in all locations of the human central nervous system were completely negative for both proteins. Glial cells usually survive and then proliferate even when cerebral infarction occurs and many neurons have disappeared. Glial cells appear to have little relationship with apoptotic cell death.

The fixation of neural tissue in experimental rat model for Bax or Bcl-2 immunohistochemical analysis is usually performed using cardiac perfusion with buffered formalin [13] or paraformaldehyde [4, 20]. In this study the human central nervous system specimens obtained at autopsy were fixed in nonbuffered formalin (immersion for 7 days) and then embedded in paraffin. Before the immunohistochemical procedure, the deparaffinized sections were heated and boiled by microwaving in citrate buffer. The results reveal that the preservation of antigenicity of the human Bax and Bcl-2 proteins was good enough for immunohistochemical analysis.

Tthe localization of Bax and Bcl-2 proteins in the human central nervous system has been demonstrated, and the distribution pattern of the proteins is found to be almost the same as in the rat. Specimens obtained from autopsy cases can be examined for Bax and Bcl-2 immunohistochemical analysis using the microwave heating and boiling technique. We suggest that the high ratio of Bax to Bcl-2 plays an important part in making Purkinje cells and hippocampal neurons vulnerable to ischaemic insults. Recent studies have revealed dynamic changes in Bax and Bcl-2 expression during or immediately after ischaemic or toxic insults in hippocampus in certain animal models [6, 10]. It is important to understand the localization of Bax and Bcl-2 and the dynamic change in the Bax and Bcl-2 expression in some disease states both in the human central nervous system and in animal models, in order to clarify how apoptotic cell death is involved in their pathogenesis.

References

1. Brierley JB, Cooper JE (1962) Cerebral complications of hypotensive anaesthesia in a healthy adult. J Neurol Neurosurg Psychiatry 25:24–30

2. Brierley JB, Graham DI (1984) Hypoxia and vascular disorders of the central nervous system. In: Adams JH, Corsellis JAN, Duchen LW (eds) Greenfield's neuropathology, 4th edn. Wiley, New York, pp 125–207
3. Castren E, Ohga Y, Brezaghi MP, Tzimagiorgis G, Thoenen H, Lindholm D (1994) bcl-2 Messenger RNA is located in neurons of the developing and adult rat brain. Neuroscience 61:165–177
4. Ferrer I, Tortosa A, Condom E, Blanco R, Macaya A, Planas A (1994) Increased expression of bcl-2 immunoreactivity in the developing central cortex of the rat. Neurosci Lett 179:13–16
5. Gillardon F, Wickert H, Zimmermann M (1994) Differential expression of bcl-2 and bax mRNA in axotomized dorsal root ganglia of young and adult rats. Eur J Neurosci 6:1641–1644
6. Gillardon F, Wickert H, Zimmermann M (1995) Up-regulation of bax and down-regulation of bcl-2 is associated with kainate-induced apoptosis in mouse brain. Neurosci Lett 192:85–88
7. Guillouf C, Grana X, Selvakumaran M, De Luca A, Giordano A, Hoffman B, Liebermann DA (1995) Dissection of the genetic programs of p53-mediated G1 growth arrest and apoptosis: blocking p53-induced apoptosis unmasks G1 arrest. Blood 85:2691–2698
8. Hara A, Yoshimi N, Hirose Y, Ino N, Tanaka T, Mori H (1995) DNA fragmentation in granular cells of human cerebellum following global ischemia. Brain Res 697:247–250
9. Hara A, Yoshimi N, Mori H, Iwai T, Sakai N, Yamada H, Niwa M (1995) Hypothermic prevention of nuclear DNA fragmentation in gerbil hippocampus following transient forebrain ischemia. Neurol Res 17:461–464
10. Hara A, Iwai T, Niwa M, Uematsu T, Yoshimi N, Tanaka T, Mori H (1996) Immunohistochemical detection of Bax and Bcl-2 protein in gerbil hippocampus following transient forebrain ischemia. Brain Res 711:249–253
11. Heron A, Pollard H, Dessi F, Moreau J, Lasbennes F, Ben-Ari Y, Charriaut-Marlangue C (1993) Regional variability in DNA fragmentation after global ischemic evidenced by combined histological and gel electrophoresis observations in the rat brain. J Neurochem 61:1973–1976
12. Hockenbery DM, Zutter M, Hickey W, Nahm M, Korsmeyer SJ (1991) Bcl-2 protein is topographically restricted in tissues characterized by apoptotic cell death. Proc Natl Acad Sci USA 88:6961–6965
13. Iwai T, Hara A, Niwa M, Nozaki M, Uematsu T, Sakai N, Yamada H (1995) Temporal profile of nuclear DNA fragmentation in situ in gerbil hippocampus following transient forebrain ischemia. Brain Res 671:305–308
14. Krajewski S, Krajewska M, Shabaik A, Miyashita T, Wang HG, Reed JC (1994) Immunohistochemical determination of in vivo distribution of Bax, a dominant inhibitor of Bcl-2. Am J Pathol 145:1323–1336
15. MacManus JP, Buchan AM, Hill IE, Rasquinha I, Preston E (1993) Global ischemia can cause DNA fragmentation indicative of apoptosis in rat brain. Neurosci Lett 164:89–92
16. McConkey DJ, Orrenius S, Jondal M (1990) Cellular signalling in programmed cell death (apoptosis). Immunol Today 11:120–121
17. Nitatori T, Sato N, Waguri S, Karasawa Y, Araki H, Shibanai K, Kominami E, Uchiyama Y (1995) Delayed neuronal death in the CA1 pyramidal cell layer of the gerbil hippocampus following transient ischemia is apoptosis. J Neurosci 15:1001–1011
18. Oltvai ZN, Millman CL, Korsmeyer SL (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, bax that accelerates programmed cell death. Cell 74:609–619
19. Scholz W (1953) Selective neuronal necrosis and its topistic patterns in hypoxemia and oligemia. J Neuropathol Exp Neurol 12:249–261
20. Shimazaki K, Ishida A, Kawai N (1994) Increase in bcl-2 oncoprotein and the tolerance to ischemia-induced neuronal death in the gerbil hippocampus. Neurosci Res 20:95–99
21. Vaux DL, Cory S, Adams JM (1988) Bcl-2 promotes the survival of hematopoietic cells and cooperates with c-myc to immortalize pre-B cells. Nature 335:440–442
22. 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 (Lond) 142:67–77
23. 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 369:321–323