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Review

BCL-2 family members in the development and degenerative pathologies of the nervous system

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Abstract

Neuronal death is an essential feature in the normal development of the nervous system and in neurodegenerative states of the adult or ageing brain. Bcl-2 is the prototype of a growing family of proteins which control cell death. Many of these proteins are expressed in the nervous system during development and in the adult. Numerous observations have suggested that this family of proteins plays a central role in the control of naturally occurring and pathological neuronal death. In this review, I will discuss the possible mechanisms of action of these proteins as well as their potential use in treating neurodegenerative diseases.

Keywords: Bcl-x; Bax; neurodegeneration; apoptosis; neuronal death; mitochondria

Abbreviations: BH, Bcl-2 homology domain; Bax, Bcl-2 associated protein x; CF, carboxy fluorescein; CNTF, ciliary neurotrophic factor; BDNF, brain derived neurotrophic factor; NGF, nerve growth factor; NT-3, neurotrophin-3; IGF-1, insulin like growth factor; NOCD, naturally occurring cell death, HSV, herpes simplex virus; CCP, cyanide M-chlorophenyl hydrazone; AD, Alzheimer's disease; PD, Parkinson's disease; ALS, amyotrophic lateral sclerosis; SOD1, superoxide dismutase 1; SMA, spinal muscular atrophy; NAIP, neuron-anti-apoptosis protein; SMN, spinal motor neuron; IAP, inhibitor of apoptosis

Introduction

For decades, cell death has been recognised by embryologists as an outstanding feature of normal neuronal development (Oppenheim, 1991). Today, it is widely accepted that neurotrophins, electrical activity, hormones or cell contacts stimulate neuronal survival throughout critical periods of the development. One essential role of these survival factors is to block an active cell death program which is initiated by default (Hamburger and Oppenheim, 1982; Raff *et al*, 1993). Today the term 'programmed cell death' refers to this chain of molecular events which underlies active cell death. As in the embryo, massive cell death also occurs in the adult and ageing nervous system in acute and chronic pathologies referred to as neurodegenerative diseases.

The demonstration that overexpression of the proto-oncogene Bcl-2 protects neurons from naturally occurring cell death and from a variety of pathological insults has suggested that the control of neuronal death by proteins of the Bcl-2 family is common to development and diseases (Sadoul *et al*, 1994). These findings have shown that mimicking the effect of Bcl-2 may allow to block neuronal cell death by agents that do not prevent the initial damage nor facilitate its repair. This opens new avenues for the treatment of neurodegenerative diseases for which the causative agents as well as the mechanisms of cell destruction are largely unknown.

The present review summarises results demonstrating the role of Bcl-2 family members in controlling neuronal death and discusses the role that Bcl-2-like proteins may have during development and in acute and chronic diseases.

The Bcl-2 family

bcl-2 was identified at the chromosomal breakpoint of t(14;18) an alteration that occurs in almost all (over 95%) of human follicular B lymphomas (Tsujimoto *et al*, 1984). Bcl-2 favours malignancy, not by acting on proliferation *per se*, but by extending cell survival. Indeed, Bcl-2 extends cell survival by blocking cell death following a variety of signals. (Hockenbery *et al*, 1990). Bcl-2 is the prototype of a growing family of cytosolic proteins (Figure 1) which includes antiapoptotic members such as Bcl-2, which inhibit cell death when overexpressed (Bcl-xL Mcl-1, Bfl-1, Bcl-w and A1), and proapoptotic proteins which are sufficient to induce cell death when overexpressed (Bax, Bak, Bok, Bad, Bik/NBK, Bid and Hrk) (Kroemer, 1997; Rinkenberger and Korsmeyer, 1997). Viruses have also been shown to encode for proteins belonging to the Bcl-2 family (Rao and White, 1997).

The homologies between Bcl-2 family members are clustered in discrete regions referred to as the Bcl-2 homology domains (BH1–BH4). Bik, Bid and Hrk each contain a BH3 domain but no BH1 or BH2. The BH4 domain is only found in antiapoptotic proteins with the exception of an alternatively spliced form of *bcl-x* called Bcl-xS, which may induce cell death. Some members of the Bcl-2 family contain a carboxy terminal transmembrane domain, which may anchor these cytosolic proteins into intracellular membranes.

Bax was first characterised as the Bcl-2 associated protein x, since it forms heterodimers with Bcl-2 (Oltvai *et al*, 1993). Since then, homo- and heterodimer formation has been shown to be a common theme between almost all members of the Bcl-2 family (Farrow and Brown, 1996). For

example, Bax may not only dimerise with Bcl-2 but also with Bcl-xL, Mcl-1 and A1, as well as with itself. Bcl-2 and Bcl-xL may in turn also form homodimers.

Interaction between Bcl-2 family members are regulated at least in part, by the BH1 and BH2 domains (Yin *et al*, 1994). Mutations in these regions of Bcl-2 which disrupt its ability to form heterodimers with Bax correlate with a loss in its ability to inhibit cell death. BH3 binding domains of proapoptotic proteins like Bax, Bak, Hrk or Bik are necessary and sometimes sufficient for binding to Bcl-2 or Bcl-xL and for inducing cell death (Diaz *et al*, 1997; Han *et al*, 1996; Ink *et al*, 1997; Inohara *et al*, 1997). One interpretation of these results, has been put forward by Korsmeyer and collaborators who proposed a model in which Bcl-2 or Bcl-xL heterodimerises with Bax to suppress cell death (Merry and Korsmeyer, 1997). However mutations in Bcl-xL have been described which disrupt heterodimerisation with Bax and Bak, but have only weak effects on the antiapoptotic activity of the protein (Cheng *et al*, 1996) . This suggests that besides blocking proapoptotic proteins, Bcl-x and perhaps Bcl-2, may act independently to suppress cell death (Knudson and Korsmeyer, 1997).

Molecular mechanisms by which Bcl-2 related proteins may regulate cell death

In most cases Bcl-2 family members have been shown to control the activation of caspases, a family of aspartate

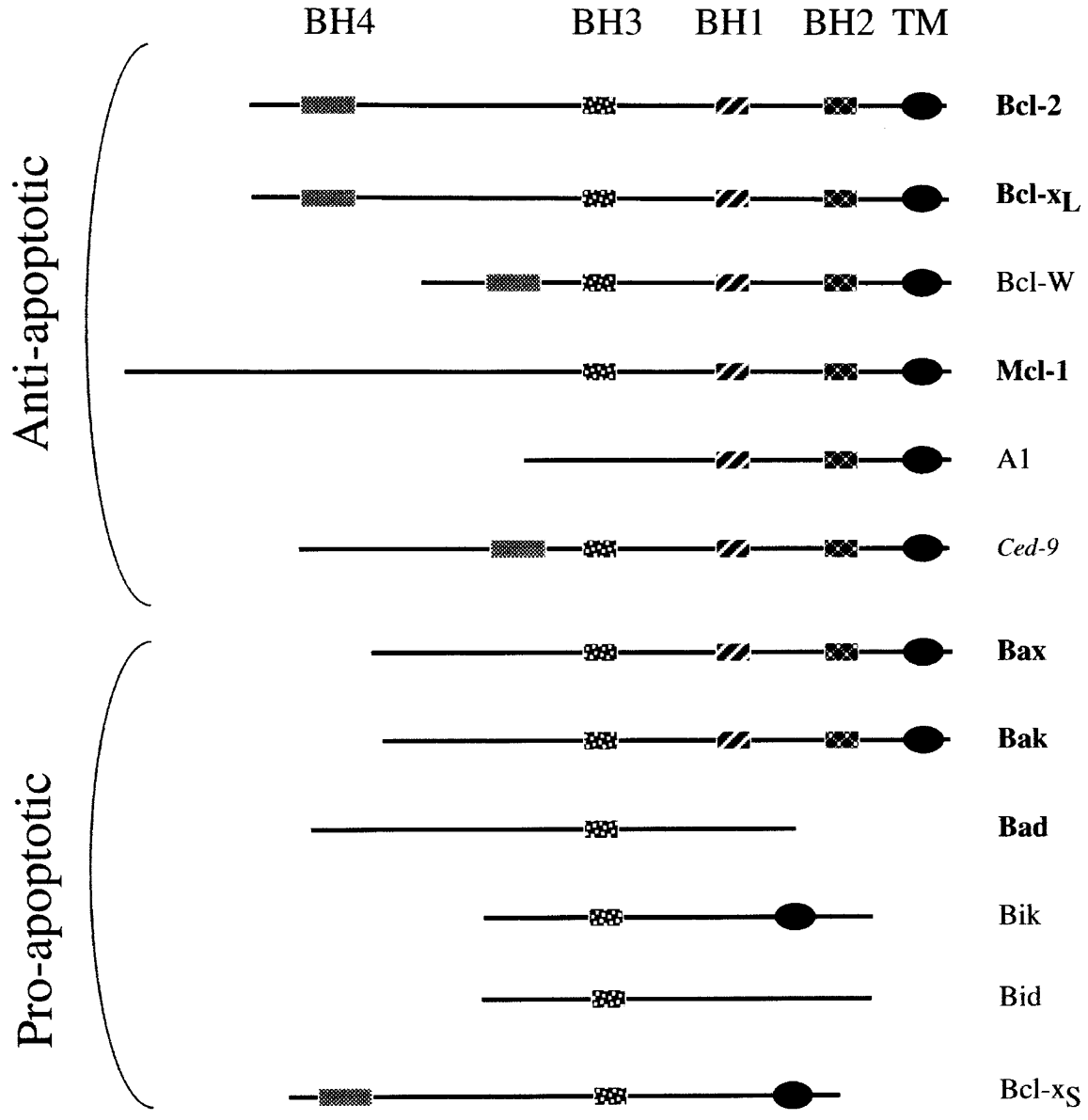

Figure 1 Bcl-2 family members. Only mamalian proteins are shown with the exception of Ced-9 which is a *C. elegans* protein. In bold are proteins with a proven expression in the nervous system. Viral proteins of the Bcl-2 family have been omitted. BH domains are shown as BH1-BH4. TM: putative transmembrane domain

directed cysteine proteases central to the execution of programmed cell death (Salvesen *et al*, 1997; Frielander and Yuan, this volume). Several laboratories have shown that following a cell death stimulus, cytochrome c is released from the mitochondria into the cytosol where it indirectly activates caspase 3/CPP32, a critical step in cells undergoing apoptosis (Figure 2A) (Kluck *et al*, 1997b; Zou *et al*, 1997). Antiapoptotic Bcl-2 related proteins block the release of cytochrome c into the cytosol, as shown from experiments using cells overexpressing Bcl-2 or Bcl-xL or purified mitochondria (Kluck *et al*, 1997a; Yang *et al*, 1997). On the other hand Bax can directly induce release of cytochrome c from isolated mitochondria (Jürgensmeier *et al*, 1998).

A link between cytochrome c and caspase 3 activation has been recently described: cytochrome c binds an adaptor protein, Apaf-1, which in turn can bind to pro-caspase 9. caspase 3 activation begins when caspase 9 binds Apaf-1 in a reaction triggered by cytochrome c and dATP. caspase 3 activation occurs through the cleavage of pro-caspase 3 by caspase 9 (Figure 2A). Interestingly, Apaf-1 shows significant sequence homologies to the *C elegans* protein Ced-4 and to the terminal part of the caspase Ced-3 which are both required for cell death to occur in the worm (Zou *et al*, 1997).

The mechanism by which Bcl-2 related proteins control the release of cytochrome c into the cytosol may be related to their capacity to form channels in artificial membranes. It is the structural similarity between Bcl-xL and the pore forming bacterial toxins diphtheria toxin and colicins (Muchmore *et al*, 1996) which has prompted several laboratories, including ourselves, to examine the capacity of Bcl-2 family members to form pores in membranes (Antonsson *et al*, 1997; Minn *et al*, 1997; Schendel *et al*, 1997; Schlesinger *et al*, 1997). Bcl-xL structure consists of two long hydrophobic α helices arranged in an anti-parallel fashion, surrounded by five amphipatic helices. BH-1, BH-2 and BH-3 are in close proximity forming a hydrophobic cleft that may represent the binding site for other BCL-2 like proteins (Sattler *et al*, 1997).

The potential channel activities of Bcl-2 family proteins were tested using recombinant soluble Bax, Bcl-xL and Bcl-2, lacking their carboxy terminal transmembrane domain. Two groups (Schendel *et al*, 1997; Schlesinger *et al*, 1997) have demonstrated that Bcl-2 can form discrete channels at pH 7 with mild cation selectivity, a property reminiscent of that described by Minn *et al* (1997) for Bcl-xL. In contrast Bax exhibits an anionic selectivity. Noteworthy is the fact that the structure of the α5 and α6 helices which potentially form the channel varies among Bcl-2 family members, suggesting that these differences determine ion selectivity and/or conductance. The positively charged residues of Bax and negatively charged residues of Bcl-2 may reflect their selectivities for anions and cations respectively.

The channel activities of both Bcl-xL and Bcl-2 are pH sensitive being greatly enhanced at pH 4. In our hands, only Bax but not Bcl-2, was capable of forming detectable channels in lipid bilayers at physiological pH. Consistent with all other studies, we found that both Bax and Bcl-2 pore-forming activities as measured by Carboxyfluorescein (CF) efflux from liposomes, were greatly enhanced at lower pH with Bax having a broader pH optimum. Using liposomes, we showed that at pH 7, Bcl-2 can antagonise Bax-induced leakage of the negatively charged CF (Antonsson *et al*, 1997).

Overexpressed Bax colocalises with Bcl-xL and Bcl-2 at the mitochondria, although Bcl-2 is also detected in nuclear and reticular membranes (Rinkenberger and Korsmeyer, 1997). One may therefore hypothesize that following a death signal, Bax forms an anionic channel at the mitochondrial membrane leading to the opening of an unidentified pore through which cytochrome c may be released (Jürgensmeier *et al*, 1998) (Figure 2A). One cannot exclude however, that Bax itself forms a pore large enough to allow cytochrome c to flow through. Bax channel activity would also result in the loss of mitochondrial membrane potential which represents an early feature of apoptosis and occurs in cells overexpressing Bax (Xiang *et al*, 1996). Based on our results using liposomes, one may hypothesize that Bcl-2 or Bcl-xL inhibits cell death following the same death signal, by directly interacting with Bax and counteracting its channel activity and thereby inhibiting cytochrome c release and depolarisation (Figure 2Ba). Another hypothesis is that Bcl-2 or Bcl-xL form a cationic channel which allows to restore the ionic balance compromised by Bax (Figure 2Bb).

We now have evidence that classical channel blockers can mimic the effect of Bcl-2 to block the release of CF from liposomes induced by Bax (D. Church and R. Sadoul unpublished observations). The final demonstration that Bax channel activity is essential to the cell death process, will await the demonstration that compounds which selectively block Bax channel activity can also block cell death. On the other hand, the development of Bcl-2 channel agonists may also allow to mimic the effect of Bcl-2 and thereby block active cell death.

Channel formation by Bcl-2 family members is only one of the models which may account for their role in cell death. A number of interactions observed between Bcl-2 family members and other proteins like ced 4, calcineurin, Raf-1, rRas, Bag-1 and p53-binding protein 2, which may also participate in cell death regulation, has led to numerous speculations regarding the role of Bcl-2 like proteins in the control of cell death. These mechanisms have been extensively described in a number of recent reviews and will therefore not be developed herein (Kroemer, 1997; Reed, 1997; Rinkenberger and Korsmeyer, 1997).

Bcl-2 family proteins in the nervous system

Until now, only Bcl-2, Bcl-xL, Mcl-1, Bax, Bak and Bad have been shown to be expressed by cells of the central and/or peripheral nervous system (Kitada *et al*, 1998; Krajewski *et al*, 1995a; Krajewski *et al*, 1996). Bcl-2 is widely expressed during nervous system development whereas in the adult, its expression is low in the central nervous system but stays high in the peripheral nervous system (Merry *et al*, 1994). In contrast Bcl-xL is highly expressed in both developing and adult nervous system (Frankowski *et al*, 1995; Gonzalez-

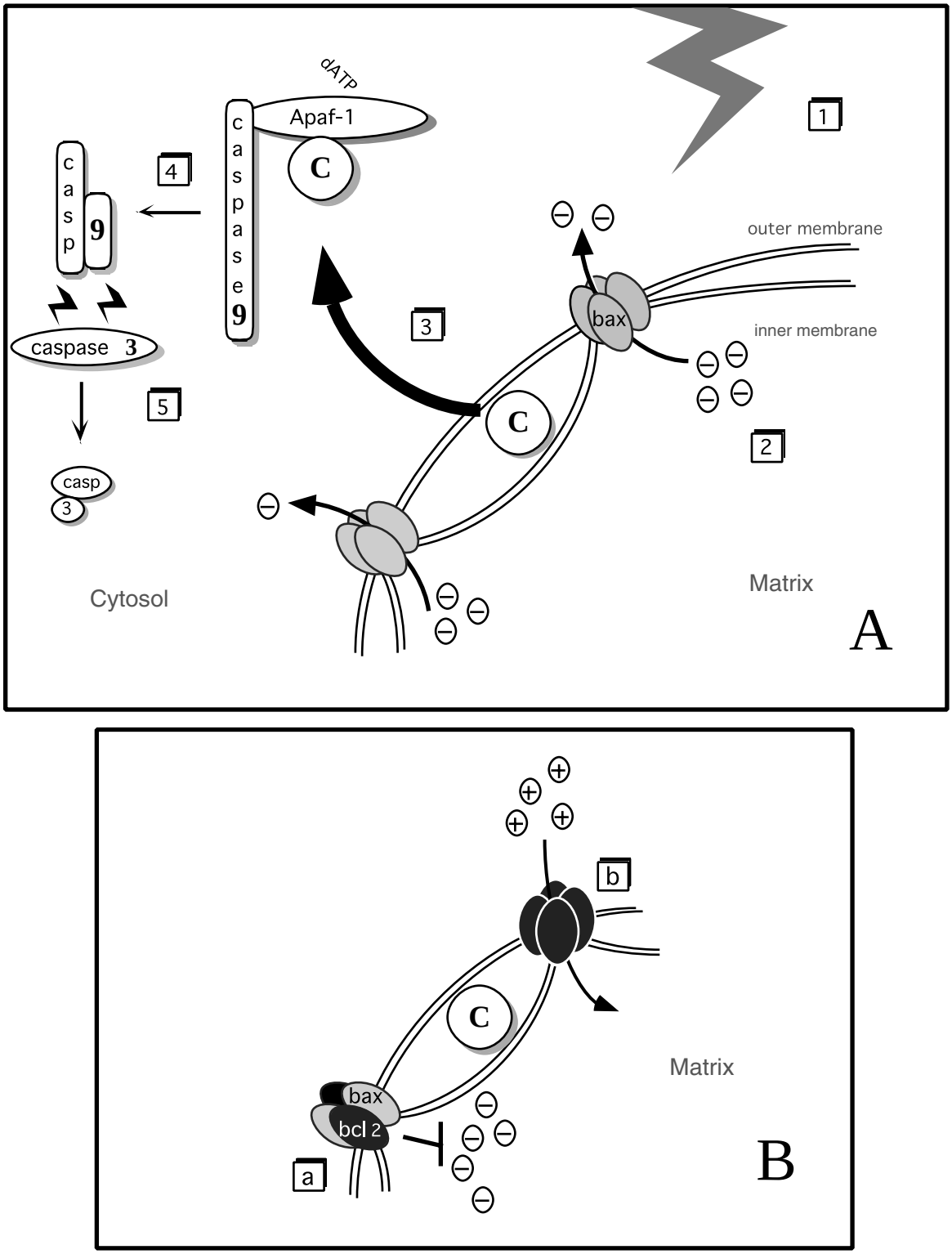

Figure 2 Hypothetical model explaining how Bax and Bcl-2 may control cell death: (**A**) Following an apoptotic stimulus (1), mitochondrial Bax channels open to allow anion (−) efflux (2). This efflux results in a drop of mitochondrial membrane potential and release of cytochrome c (C) into the cytosol (3). Cytochrome c then interacts to Apaf-1, which together with dATP binds and activates procaspase 9 (4). Caspase 9 in turn activates procaspase 3 (5). (**B**) possible mechanisms explaining inhibition by Bcl-2 of Bax-induced cytochrome c leakage. (a) Bcl-2 associates with Bax to form a hybrid channel non permeable to anions (−) and thereby blocks anionic efflux. (b) Bcl-2 itself forms a channel allowing influx of cations (+) to counteract Bax induced anionic efflux

Garcia *et al*, 1995). Bax is also highly expressed in the developing nervous system but its expression is strongly reduced after the period of naturally occurring cell death (Vekrellis *et al*, 1997).

Bcl-2 family proteins regulate neuronal apoptosis *in vitro*

The demonstration that Bcl-2 overexpression through microinjection of an expression vector into sympathetic neurons is able to protect from death induced by removal of nerve growth factor (NGF), provided the first indication that Bcl-2 family members play a central role in neuronal death and survival (Garcia *et al*, 1992). Using the same neurons, we demonstrated that Bcl-xL overexpression also blocks NGF-deprivation-induced neuronal death (Martinou *et al*, 1995). Bcl-2 can also protect against neurotrophin deprivation in chick sympathetic neurons that depend on one or more neurotrophins (brain derived neurotrophic factor (BDNF), NGF, or neurotrophin-3 (NT-3)). In contrast, Bcl-2 does not rescue ciliary neurons from Ciliary Neurotrophic Factor (CNTF) deprivation, suggesting that Bcl-2 dependent and independent cell death pathways may be used by neurons or that CNTF removal initiates the death program downstream of Bcl-2 (Allsopp *et al*, 1993).

Overexpression of Bax or Bak is sufficient to induce death of sympathetic neurons cultured in presence of NGF (Farrow *et al*, 1995; Martinou *et al*, 1998; Vekrellis *et al*, 1997). In this case, Bax-induced neuronal death can be blocked by inhibitors of caspases, which are also known to block neuronal death induced by NGF deprivation. These results suggest that neurotrophins regulate Bax activity which in turn controls the activation of caspases and thereby neuronal death. Regulation of Bcl-2 like proteins by growth factors was recently demonstrated through Bad which controls the equilibrium between Bax and Bcl-2/Bcl-xL (Datta *et al*, 1997; del Peso *et al*, 1997). Indeed Bad interacts with Bcl-2 and Bcl-xL and thereby inactivates their antiapoptotic properties (Yang *et al*, 1995). Upon phosphorylation Bad is sequestered by 14-3-3 proteins and is no longer available to interact with Bcl-2 or Bcl-xL which can counteract the proapoptotic effect of Bax. Bad has been demonstrated to be phosphorylated by AKT/PKB, a protein kinase controlled by growth factors such as IL-3, Insulin like growth factor (IGF-1) and NGF, through the activation of the phosphatidylinositide-3′-OH kinase. Thus, according to the current model, survival factors like NGF may promote cell survival by activating AKT phosphorylation of Bad, thereby allowing Bcl-2 or Bcl-xL to fulfil their antiapoptic function.

Bcl-2 family proteins regulate neuronal survival during development

During nervous system development, many neuronal cell populations including motoneurons, undergo a period of naturally occurring cell death (NOCD). The extent of neuronal death varies from region to region, but in most neuronal populations accounts for 25–70% of the initial number. The timing of NOCD occurs when neurons are receiving or making synapses, suggesting a relationship between neuronal death and the establishment of connectivity. The exact role of NOCD is still unclear, but may serve to match the number of neurons to the size of their target territory and to the number of their afferent projections. It may also allow the elimination of neurons having made aberrant connections. Neurotrophins may be involved in the mechanisms driving the selection of neurons since they are thought to be secreted in limiting amounts by the target cells. Selection would occur through competition for the survival factors.

In order to explore the role of Bcl-2 family members in regulating neuronal death *in vivo*, Martinou *et al* (1994) and Farlie *et al*, (1995) have generated transgenic mice containing *bcl-2* under the control of the neurone specific enolase promoter which drives the protein's expression in neurons. In these mice, Bcl-2 expression begins before the onset of NOCD. Quantitative analysis of defined neuronal populations from the central and peripheral nervous system, demonstrated that Bcl-2 overexpressing mice display 30–40% more neurons than wild animals. For example, a vast majority of the retinal ganglion cells found in the retina of neonatal Bcl-2 overexpressors are maintained until adulthood, whereas only 40% survive in wild-type animals (Bonfanti *et al*, 1996). These findings demonstrate that during development some neurons use death pathways which can be blocked by Bcl-2.

Bcl-2 overexpressing motoneurons of young animals are protected from axotomy of the facial- and sciatic nerve (Dubois-Dauphin *et al*, 1994; Farlie *et al*, 1995). Facial motoneurons overexpressing Bcl-2 were shown to conserve functional electrophysiological properties similar to those of unlesioned motoneurons despite a reduction of the size of their soma (Alberi *et al*, 1996). Similarly, all ganglion cells of transgenic animals survive transection of the optic nerve, an axotomy which induces degeneration of 50% of ganglion cells in normal animals. In these experimental paradigms, the massive neuronal death following nerve section is only seen in very young animals around the time of NOCD, and can be rescued by neurotrophins (Lewin and Barde, 1996; Weibel *et al*, 1995).

The findings related to Bcl-2 trangenic mice show that Bcl-2 overexpression can protect neurons *in situ* from death induced by removal of trophic support and suggest that members of the Bcl-2 family may control neuronal selection during development.

Studies of mice deficient for one or more proteins of the Bcl-2 family made it possible to test their relative contribution during development. Deckwerth *et al*, (1996) have established that sympathetic neurons isolated from *bax* knock out (−/−) mice are independent of NGF for their survival, a finding which correlates well with the protection of the same neurons by Bcl-2 ectopic expression. As in the case of Bcl-2 overexpressing mice, facial nuclei from $bax^{-/-}$ mice contain more neurons, which may correspond to neurons which have not undergone NOCD. Here again, motoneurons of the facial nucleus of $bax^{-/-}$ mice are fully protected against axotomy, demonstrating that Bax is necessary for neuronal death after neurotrophic factor deprivation and during development.

In contrast, Bcl-x is required for cell survival early in development, since a massive apoptotic neuronal loss is observed in postmitotic neurons of the brain, spinal cord and dorsal root ganglia of homozygous mutant *bcl-x* $^{-/-}$ mouse embryos (Motoyama *et al*, 1995). In these mutants, cell death occurs in differentiated neurons prior to establishment of synaptic connections, and therefore is unlikely to represent an exacerbation of the target-dependent cell death described above.

Shindler *et al* (1997) demonstrated using mice deficient in both *bcl-x* and *bax* (*bcl-x*$^{-/-}$/*bax*$^{-/-}$) that Bax deficiency can prevent the increased apoptosis of *bcl-x*$^{-/-}$ mutants. These results suggest that Bax acts as a dominant death effector protein which regulates cell death in the early developing nervous system, an activity tightly regulated through interactions with Bcl-xL. The remaining apoptosis observed in *bax*$^{-/-}$ and *bax*$^{-/-}$/*bcl-x*$^{-/-}$ animals suggests a role for other members of the Bcl-2 family or for death pathways independent of the Bcl-2 family in developmental neuronal death.

At birth, *bcl-2* KO animals have the same number of facial motoneurons, sensory and sympathetic neurons as their wild-type counterparts (Michaelidis *et al*, 1996). Furthermore, axotomy-induced degeneration of facial motoneurons can still be prevented by BDNF or CNTF. However, a progressive degeneration of motor, sensory and sympathetic neurons from *bcl-2*$^{-/-}$ animals occurs after the period of NOCD. Therefore Bcl-2 does not appear to be a permissive factor for neurotrophic factors but seems crucial for the maintenance of survival in selective neurons.

The neuronal phenotypes observed in mice deficient in *bcl-2*, *bcl-x* or *bax* are consistent with the hypothesis that the balance between pro- and antiapoptotic proteins of the Bcl-2 family regulate the neuronal fate during development, even though the identity of those regulating neuronal selection stays unclear. They also suggest that the model put forward by Korsmeyer, which states that Bcl-2 or Bcl-xL suppress the death program by blocking Bax deleterious activity, is particularly relevant for neurons.

Bcl-2 overexpression protects neurons from ischaemia

The availability of the Bcl-2 overexpressing mice has allowed to test whether such a balance between pro- and antiapoptotic proteins may also regulate neuronal survival following pathological insults in the adult brain. The first paradigm tested was the resistance to ischaemia following permanent occlusion of the middle cerebral artery, an experimental correlate of human stroke (Martinou *et al*, 1994). Bcl-2 efficiently protects neurons from ischaemic damage, since one week after the operation the estimated volume of the infarct was 40% less in Bcl-2 overexpressors than in wild-type animals. Using herpes simplex virus (HSV) capable of overexpressing Bcl-2 in intact brain, two groups have shown that Bcl-2 protects neurons from ischaemia in the striatum and cortex (Lawrence *et al*, 1997; Linnik *et al*, 1995). Bcl-2 could still protect striatal neurons even when the HSV vector was delivered 1.5 h after focal ischaemia (Lawrence *et al*, 1997). These data demonstrate the potential use of up regulating Bcl-2 expression through gene therapy or of mimicking Bcl-2 effect in order to limit the infarct damage following human stroke.

The mechanism by which Bcl-2 overexpression may protect neurons from ischaemia is still unclear. Indeed neuronal death induced by ischaemia is due to both necrosis and apoptosis as demonstrated recently by several laboratories (Choi, 1996; Li *et al*, 1997). Necrosis appears to be mediated by hypoxia and over-activation of ionotropic glutamate receptors, especially N-methyl-D-aspartate receptors, with subsequent excessive Ca^{2+} influx. Bcl-2 overexpression protects cultured neurons against glutamate excitotoxicity (Jia *et al*, 1996; Lawrence *et al*, 1997) but cerebellar neurons isolated from *Bax*$^{-/-}$ mice are not protected against glutamate (Miller *et al*, 1997) suggesting that, in this case, Bcl-2 protective role is independent of Bax.

A rapid and prolonged entry of calcium through glutamate receptors may lead to an increased Ca^{2+} load in the mitochondria. Up to a certain point this accumulation may be beneficial to the cell, since it decreases the intra-cytosolic concentration of Ca^{2+} which is a potent activator of several enzymes contributing to the active swelling and lysis typical of necrosis. However, in excess mitochondrial calcium can become deleterious since it induces an abrupt loss in mitochondrial polarisation and respiratory impairment (White and Reynolds, 1996; White and Reynolds, 1997). This impairment induces a number of harmful consequences such as ATP depletion, imbalance of ionic homeostasis or free radical generation which all drive necrotic cell damage (Nicotera *et al*, 1996). Murphy *et al* (1996) have shown that Bcl-2 potentiates the maximal uptake capacity of neural cell mitochondria and that this potentiation correlates with increased resistance of mitochondria to Ca^{2+} induced respiratory damage. Bcl-2 expression also reduces Ca^{2+} efflux from the endoplasmic reticulum to the cytosol in thapsigargin treated cells (Lam *et al*, 1994) and modulates sustained increases in intranuclear Ca^{2+} (Marin *et al*, 1996). These effects of Bcl-2 on intracellular Ca^{2+} homeostasis may represent mechanisms by which the protein's overexpression protects neurons from the deleterious effect of Ca^{2+}.

Bcl-2 and Bcl-xL have been shown to retard necrosis in non neuronal cells induced by oxygen depletion and respiratory chain inhibitors such as KCN and antimycin A (Shimizu *et al*, 1996). Loss of mitochondrial polarisation renders neurons more susceptible to low glutamate concentrations which induce typical signs of necrosis if the glutamate is applied together with cyanide m-chlorophenyl hydrazone (CCP), an uncoupler of mitochondria (Nicotera *et al*, 1996). The capability of Bcl-2 to block the loss of mitochondrial membrane potential in compromised neurons may also serve to protect cells against mild calcium entry.

Beside necrosis, global and focal ischaemia also induce DNA fragmentation and morphological signs of apoptosis (Choi, 1996; Li *et al*, 1997). This ischaemic-apoptotic cell death may be a remote result of damage through necrosis,

resulting from initiation of cytokine cascades, mild oxidative stress, synaptic or trophic deprivation induced by a loss of cellular or synaptic partners. It may also be a direct effect of mild glutamate toxicity, since Nicotera and colleagues have shown in neuronal cultures that short duration or low concentrations of glutamate may induce typical signs of apoptosis in some neurons (Ankarcrona *et al*, 1995; Bonfoco *et al*, 1995). Bax protein expression was shown to be induced after global ischaemia in neurons showing signs of apoptosis (Chen *et al*, 1996a; Krajewski *et al*, 1995b). One may therefore hypothesise that Bcl-2 overexpression blocks Bax activity and as a consequence ischaemic apoptosis. This model could be tested by comparing infarct size in $bax^{-/-}$ animals with that of Bcl-2 overexpressors. This demonstration is particularly important in order to validate the therapeutical approach based on the development of Bax channel blockers to limit neuronal death in stroke.

Interestingly Bcl-xL and Bcl-2 protein expression is upregulated in neurons in regions of the hippocampus which are the least affected by degeneration following global ischaemia suggesting, a role of antiapoptotic genes as inducible neuroprotective agents (Chen *et al*, 1997; Krajewski *et al*, 1995b).

A potential role for blocking neuronal death by mimicking Bcl-2 function to treat other acute pathologies of the nervous system

Beside ischaemia, overexpression of Bcl-2 in normal photoreceptors was also shown to decrease the damaging effects of constant light exposure (Chen *et al*, 1996b). Other types of experimental acute stresses can reproduce pathologies of the nervous system observed in human. None of these stresses have yet been tested in mice which overexpress Bcl-2, but the detection of typical apoptotic cells in several regions affected by these pathologies suggests that blocking apoptosis through Bcl-2 could be a way to limit neuronal damage.

Typical features of apoptosis have been detected in the hippocampus of animals injected with kainate, an activator of non NMDA receptors. This model is often used as a correlate to status epilepticus in humans and suggests that *in vivo*, active cell death may be a consequence of epileptic seizures. Bcl-2 protein expression is upregulated only in the CA1 region of the hippocampus which contains neurons surviving the kainate induced seizures. (Graham *et al*, 1996). This contrasts the Bcl-2 protein upregulation following global ischaemia, which occurs only in neurons from the dentate and CA3 regions of the hippocampus which are most resistant to this type of ischaemia (Chen *et al*, 1997; Krajewski *et al*, 1995b). Interestingly *bcl-2* mRNA expression is upregulated in both CA1 and CA3 following kainate injection or global ischaemia suggesting, a role for translational blockage or for protein degradation in the regulation of Bcl-2 protein expression.

Neuronal death which occurs after experimental brain injury in the rat has features of both apoptosis and necrosis (Rink *et al*, 1995). In this system apoptosis may account for the delayed neuronal death which occurs up to 72 h following the trauma. *bcl-2* mRNA and protein expression is also upregulated following traumatic brain injury in the rat in the peritrauma cortex and hippocampus. Here again, neurons which express Bcl-2 survive preferentially following the trauma (Clark *et al*, 1997).

Recently, two independent groups have carefully described the death features of cells dying after spinal cord injury (Crowe *et al*, 1997; Liu *et al*, 1997). Typical signs of necrosis occur following spinal cord contusion in rats and monkeys. Apoptotic neurons and glial cells were also detected from 6 h to 3 weeks following injury. Apoptotic cells were observed adjacent to myelin sheets and were demonstrated to be oligodendrocytes. This apoptotic death of oligodendrocytes may be a result of the degeneration of the fibre tracts following the injury. Therefore both secondary degeneration at the site of SCI and the chronic demyelination of tracts away from the injury may be due in part to apoptosis. The long time course of the secondary injury processes suggests that intervention for blocking programmed cell death may be of great value in limiting the damage to neurons and myelin forming cells. This last example of secondary apoptosis following trauma in the central nervous system shows that delayed cell death is not restricted to neurons but affects also glial cells which play a vital role in maintaining a fully functional nervous system.

A role for Bcl-2 family proteins in chronic neurodegeneration??

Up till now it has been difficult to ascertain a role for Bcl-2 like proteins in slowly-progressing neurodegenerative diseases which are characterised by a degeneration of specific populations of neurons. However, a beneficial role of antiapoptotic Bcl-2 members in these pathologies may be suggested in view of the increasing evidence showing that defects in mitochondrial energy production and in Ca^{2+} buffering may underlie neuronal death in Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) (Beal, 1996).

Parkinson's and Alzheimer's diseases

Several studies have shown that PD is associated with reduced mitochondrial complex I activity in platelets, muscle and substantia nigra. Mitochondria from PD patients have been transferred into rho^o recipient neuroblastoma cells (mitochondrial-DNA-free) to create cybrids. These PD cybrids were shown to have increased oxygen radical production and susceptibility to 1-methyl-4-phenyl pyridinium-induced programmed cell death (MPP+) (Sheehan *et al*, 1997b; Swerdlow *et al*, 1996). MPP+ is the reactive metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) which produces a syndrome, both in man and in animals, that closely mimics idiopathic PD. This involves production of MPP+ which is taken up by dopaminergic neurons, inhibition of the mitochondrial complex I, and free radical generation which contributes to the destruction of the dopaminergic neurons. Dopaminergic neurons from trans-

genic mice overexpressing Bcl-2 are protected from MPTP toxicity (Offen *et al*, 1998). Hockenbery *et al* (1993) have shown that Bcl-2 protects cells by an unknown mechanism, from damaging effects of reactive oxygen species such as lipid peroxydation. Therefore, Bcl-2 protection against MPP+ cytotoxicity may be in part related to its antioxidant properties.

Mitochondria from AD have also been used to create AD cybrids. Here as well, these AD cybrids display an increase in free radical production. Both AD- and PD-cybrids show impairment of Ca^{2+} buffering as revealed by a slower recovery from elevation in cytosolic calcium induced by the inositol-1,4,5-triphosphate (InsP3) agonist carbachol (Sheehan *et al*, 1997a,b) compared to control cybrids. Since the mitochondria is today recognised as one of the major regulator of cytosolic Ca^{2+}, one may hypothesize that PD and AD mitochondria are impaired in their Ca^{2+} buffering function. Alterations in calcium homeostasis and ROS generation might lead to increased susceptibility to cell death under circumstances not ordinarily toxic. In view of the increased maximum uptake capacity of mitochondria conferred by Bcl-2 (Murphy *et al*, 1996), it will be interesting to test whether overexpression of Bcl-2 in PD or AD cybrids is capable of restoring normal calcium homeostasis.

Interestingly, βAmyloid which is the major component of amyloid plaques seen in AD brains, may have a direct role on the Bax/Bcl-2 equilibrium inside the cell. Indeed βAmyloid incubated with cultured human neurons down regulates Bcl-2 and upregulates Bax-expression by these neurons (Paradis *et al*, 1996). This peptide is a hallmark of AD but the question of its direct toxicity remains controversial. The findings of Paradis may suggest a mechanism by which accumulation of βAmyloid may render neurons more vulnerable to age-dependent stress and thereby contribute to the disease.

Motoneuron diseases

The observation that mutations in Cu/Zn superoxide dismutase 1 (SOD1) accounts for 20% of the inherited forms of ALS suggested that a perturbation in free radical homeostasis might play a role in the development of the disease (Rosen *et al*, 1993). Overexpression of SOD1 mutations associated with ALS in transgenic mice causes motoneuron degeneration resembling that seen in this human pathology, despite normal or increased SOD1 activity (Ripps *et al*, 1995; Wong *et al*, 1995) . Recently, Kostic and colleagues have reported the effect of neuronal Bcl-2 overexpression on this pathology by crossing SOD1 transgenic animals with Bcl-2 overexpressors (Kostic *et al*, 1997). The overexpression of human Bcl-2 delayed the onset of the disease by 19%, but had no effect on the duration of the pathology. Interestingly, overexpression of Bcl-2 attenuated the motor neuronal loss seen in the SOD1 ovexpressor suggesting that neuronal death does not account for all clinical signs of the disease. In these SOD1/Bcl-2 overexpressors one potential site of Bcl-2 activity may be the mitochondria which are affected in ALS: an early abnormality in the transgenic ALS mice is a mitochondrial swelling (Wong *et al*, 1995). Also, studies of muscle biopsies of ALS patients have shown that nerve terminals have significant increases in calcium (Siklos *et al*, 1996). This suggests a use for Bcl-2 overexpression as a therapeutical tool to protect mitochondria from damage induced by calcium overloads as discussed above.

Three candidate genes have been implicated in spinal muscular atrophy (SMA), another motoneuron disease which affects the anterior horn cells of the spinal cord. From these three genes, two may encode proteins directly controlling the cell death programme: the neuronal-anti-apoptosis protein (NAIP) and the spinal motor neuron protein (SMN).

NAIP gene encodes a protein which belongs to a family of viral and cellular proteins called IAP (inhibitor of apoptosis) most of which have anti-death activities (Clem and Ducket, 1997). IAP proteins may block apoptosis through different mechanisms since some are central components of the TNF receptor pathway, others bind to the *drosophila* Reaper while others inhibit directly caspases (Clem and Ducket, 1997; Devereaux *et al*, 1997; Vucic *et al*, 1997). NAIP overexpression has been shown to inhibit apoptosis induced by a variety of signals through an unknown mechanism (Liston *et al*, 1996).

The SMN gene is deleted or interrupted in almost all SMA patients. Mice with homozygous SMN disruption display massive cell death during early embryonic development, indicating that the SMN gene product is necessary for cellular survival and function (Schrank *et al*, 1997). Recently, Tsujimoto and colleagues have demonstrated that SMN can directly interact with Bcl-2. Furthermore, they showed that although SMN had only weak antiapoptotic activity, coexpression of SMN with Bcl-2 confers a synergistic preventive effect against Bax induced or Fas-mediated toxicity. However, SMN carrying a missense mutation found in one SMA patient had no synergistic effect with Bcl-2 (Iwahashi *et al*, 1997). These results are important since they are the first to indicate that genetic defects in regulation of Bcl-2 family members could underlie some type of neurodegenerative diseases.

Conclusion

The demonstration that neuronal death can be blocked by manipulation of the cell death program, regardless of the cell death signal, has raised enormous hopes for the treatment of neurodegenerative diseases in which the cell death signals are of unknown origin or have already occurred. These hopes have been reinforced with the recent findings that Bcl-2 family members may form channels in intracellular membranes, since it is now possible that pharmacological modulation of these channels may give new opportunities to block cell death. Furthermore this approach may also turn out to be usefull in helping regeneration of injured neurons as Bcl-2 was recently found to play a role in regulating neurite outgrowth (see Chen and Tonegawa, this volume). Bax is well suited for this type of manipulation since blocking its channel activity is likely to be a readily achievable goal. Bax blocking compounds will allow to test if Bax channel activity is an early and central step in neuronal apoptosis. If this is the case, these compounds may prove to be powerful drugs to block the neuronal loss characteristic of neurodegenerative diseases. However, the potential use of antiapoptotic drugs mimicking

Bcl-2 for treating chronic neurodegenerative diseases could be hampered by their possible side effects which may be foreseen when given over long periods of time. Indeed prolonged use of general suppressors of apoptosis may lead to hyperplasia and possibly contribute to tumour formation in other cell types and trigger uncontrolled inflammatory responses. In contrast, antiapoptotic drugs given over a short period of time may be of tremendous interest to treat acute pathologies of the central nervous system. These pathologies involve apoptosis especially during delayed cell death which participates in the damage due to ischaemia, brain and spinal cord injury and perhaps epileptic seizures. Here the timing of delayed death should give a reasonable time frame for the treatment of these pathologies with cell death blockers. Nevertheless it should be kept in mind that neuronal death in the pathological brain may be the end result of a process involving loss of connections, impaired axonal transport or impaired metabolism. Therefore, even though they raise enormous hopes, the success of antiapoptotic therapies will depend on the ability of neurons not only to survive but also to recover from these different types of damages. At least we may soon have the opportunity to give neurons a chance to do so!

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