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Bcl-2 and the Regulation of Programmed Cell Death

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The terms programmed cell death (PCD)1 and apoptosis are often used interchangeably to describe a mechanism of cellular demise that is believed to play an important role in a wide variety of physiological situations, and that when dysregulated can contribute to the pathogenesis of many diseases ranging from cancer to AIDS. Recently, the bcl-2 gene has emerged as a critical regulator of PCD in a variety of physiological and pathological contexts.

Discovery of bcl-2 and Its Involvement in t(14;18) Translocations

Bcl-2 is the acronym for the B-cell lymphoma/leukemia-2 gene. As implied by its name, this gene was first discovered because of its involvement in B-cell malignancies, where chromosomal translocations activate the gene in the majority of follicular non-Hodgkin's B-cell lymphomas (Tsujimoto et al., 1985). In these translocations, the bcl-2 gene is moved from its normal chromosomal location at 18q21 into juxtaposition with powerful enhancer elements in the immunoglobulin heavy-chain (IgH) locus at 14q32, with the result being deregulation of the translocated bcl-2 gene and overproduction of bcl-2 mRNAs and their encoded proteins (reviewed by Nowell and Croce, 1987). Though originally thought to occur only in the setting of malignant lymphoma, use of polymerase chain reaction (PCR) methods capable of detecting t(14;18) chromosomes in as few as 1 in 106 cells suggests that this translocation commonly arises in rare cells in the setting of the benign follicular hypertrophy of lymph nodes and tonsils (33–54% of cases) that occurs during infections (Limpens et al., 1991; Aster et al., 1992). Fortunately, the presence of a t(14;18) in this circumstance appears to be insufficient by itself to lead to malignant lymphoma in the vast majority of cases, but nevertheless illustrates the malignant potential that perhaps exists in all of us.

Bcl-2 Blocks a Final Common Pathway for Programmed Cell Death

Vaux et al. (1988) were the first to report that bcl-2 can prolong cell survival. Using immature pre-B-cells that are dependent on the lymphokine Interleukin-3 (IL-3) for their growth and survival in culture, these investigators noticed that stable transfer of bcl-2 expression vectors permitted prolonged cell survival in the absence of IL-3, but without concomitant cell proliferation (Vaux et al., 1988). Since it was known that IL-3 and several other Colony Stimulating Factors help to maintain hemopoietic cell survival in vitro by preventing apoptosis (Tushinski et al., 1982; Loten and Sachs, 1989; Williams et al., 1990), these results obtained with IL-3-dependent hemopoietic cells suggested that Bcl-2 was capable of blocking PCD—a notion that was formally demonstrated by Hockenberg et al. (1990). Recent studies of bcl-2 function in cultured postmitotic neurons have further established a role for this gene in the suppression of PCD, in complete absence of cell proliferation. Specifically, microinjection of bcl-2 expression plasmids into Nerve Growth Factor (NGF)-dependent sympathetic neurons and into NGF-dependent or brain-derived neurotrophic factor (BDNF)-dependent central nervous system (CNS)-derived sensory neurons has been shown to markedly delay the rate of cell death that occurs upon removal of the neurotrophic factors from cultures (Garza et al., 1992; Allsopp et al., 1993). In contrast to the prolonged cell survival observed when elevations in the levels of Bcl-2 protein are achieved through gene transfer methods, antisense-mediated reductions in bcl-2 gene expression have been shown to accelerate the rate of cell death in the setting of growth factor withdrawal (Reed et al., 1990a,b), thus fulfilling another of Koch's postulates and confirming the importance of bcl-2 in the regulation of cell survival. Antisense experiments have additionally demonstrated that marked reductions in Bcl-2 protein levels, though rendering cells more prone to apoptosis, are by themselves often insufficient for causing cell death (Reed et al., 1990a,b; Kitada et al., 1993): a finding that is consistent with the observation that several types of cells do not contain detectable Bcl-2 protein in vivo (Hockenberg et al., 1991; LeBrun et al., 1993; Lu et al., 1993).

The ability of Bcl-2 protein over-production to prevent cell death without necessarily affecting cellular proliferation suggests that the bcl-2 gene defines a new category of oncogenes. If one thinks of the cancer problem as a simple balanced equation where:

\[
\text{Rate of Cell Accumulation} = \left[ \frac{\text{Rate of Cell Death}}{\text{Rate of Cell Proliferation}} \right]
\]

then, it becomes immediately obvious that one can arrive at a condition of excess cells by either increasing the rate at which cells divide or by decreasing the frequency with which they die. Since cell death is a normal homeostatic mecha-
Table I. BCL-2 and the Regulation of Cell Life and Death*

| Causes of cell death that Bcl-2 has been documented to block: |
|-------------------------------------------------------------|
| Chemotherapeutic drugs | Glutamate† |
| Gamma- and UV-radiation | Calcium |
| Heat shock | Glucose deprivation |
| Some viruses ( Sindbis, Baculovirus) | Growth Factor Deprivation |
| Free-radicals | Neurotrophic Factor Withdrawal |
| Lipid Peroxidation | Some cytotytic T-cells† |
| p53 | Azide |
| c-Myc | Ced 9 gene mutants in C-Elegans |
| Tumor Necrosis Factor | Hydrogen Peroxide |

| Documented causes of cell death that Bcl-2 does not block: |
|-----------------------------------------------------------|
| Complement-mediated lysis |
| Some cytotytic T-cells |
| Amyloid β peptide‡ |
| Tumor Necrosis Factor |
| Withdrawal of some lymphokines |
| Anti-Ig and TCR (positive and negative thymic selection)§ |
| Hydrogen Peroxide |
| Some types of neutrophic factor-dependent neurons (CNTF) |

* Some inducers of cell death appear on both lists, thus representing conflicts within the literature. Cell death induced by complement, cytotytic T-cells, amyloid β protein, TNF, and H2O2 often involves necrotic cell death.
† Unpublished observations (J. C. Reed).
§ A weak effect on negative selection has been seen in some transgenic lines.

nism that exists in essentially all tissues with capacity for self-renewal and which helps to ensure that the rate of new cell production in the body is offset by a commensurate rate of old cell death, it stands to reason that Bcl-2 and other genes that regulate the cell death side of the equation are at least as important as classical oncogenes which primarily exert their effect by accelerating the rate of cell proliferation.

In addition to delaying cell death in the setting of growth factor or neutrophic factor withdrawal, over-expression of bcl-2 can prevent or markedly reduce cell killing induced by a wide variety of stimuli (summarized in Table I). In all cases where examined, Bcl-2 appeared to block a relatively early event associated with apoptotic cell death in that none of the characteristic morphological changes such as cell shrinkage, chromatin condensation, and nuclear fragmentation occurred, and DNA degradation into oligonucleosomal length fragments was markedly reduced or prevented. Though few mechanistic details are known at this point, the data have suggested that bcl-2 blocks a final common pathway leading to apoptotic cell death. The participants in this pathway remain unknown, but the observation that bcl-2 blocks apoptosis induced by multiple chemotherapeutic drugs as well as γ-irradiation provides some clues that may help to provide at least a partial explanation (Miyashita and Reed, 1992, 1993; Lotem and Sachs, 1993; Fanidi et al., 1992; Collins et al., 1992; J. C. Reed, unpublished observations). For example, γ-irradiation and the wide variety of drugs to which Bcl-2 confers resistance have diverse mechanisms of action, but all induce DNA damage either directly or indirectly. The protection afforded by Bcl-2 does not involve reductions in drug-induced damage to DNA, increased rates of DNA repair, inhibition of drug-induced alterations in nucleotide pools, or changes in cell cycle kinetics (Walton et al., 1993; Fisher et al., 1993). Thus, Bcl-2 appears to act downstream of all of these events, perhaps preventing the presence of damaged DNA from being translated into a signal for activation of the genes involved in apoptosis, or blocking the action of the products of those genes once induced. One of the mediators of this pathway could be the tumor suppressor p53 which has been implicated in genomic surveillance, undergoes increases in its protein levels and activity in response to drug-induced DNA damage (Zhan et al., 1993), and which can induce apoptosis under a variety of conditions (reviewed by Sachs and Lotem, 1993). It is tempting to speculate therefore that a functional connection between p53 and Bcl-2 may exist. Indeed, preliminary indications are that the gene transfer-mediated over-production of Bcl-2 protein can partially suppress p53-induced apoptosis, at least in some hemopoietic cell lines (Wang et al., 1993). Clearly, however, p53 is not the whole story where Bcl-2 and the regulation of apoptosis are concerned, since recent investigations using p53 "knock-out" mice indicate that this tumor suppressor gene is required for induction of apoptosis by some stimuli such as γ-irradiation and etoposide (VP16) but not by photosensitizers and Ca2+-ionophores in thymocytes (Low et al., 1993; Clark et al., 1993), and yet it is known that Bcl-2 is very effective at blocking glucocorticoid- and Ca2+-induced cell death (Sentman et al., 1991; Strasser et al., 1991; Siegel et al., 1992; Miyashita and Reed, 1992, 1993).

The identity of other proteins that might help to regulate the induction of genes required for PCD in the absence of p53 are at present unknown, but p62-c-myc is a potential candidate given evidence that it can accelerate the rate of apoptosis in a variety of settings (Asken et al., 1991; Evan et al., 1992; Shi et al., 1992). Gene transfer experiments have shown that bcl-2 blocks apoptosis induced by c-myc (Bissonnette et al., 1992; Fanidi et al., 1992), suggesting an explanation for the aggressive nature of lymphomas and leukemias that contain chromosomal translocations involving both of these genes. Namely, since c-myc stimulates both mitogenesis and apoptosis, concomitant activation of bcl-2 can nullify the apoptotic influence of c-myc, thus unleashing the proliferative effects of c-myc and leading to a further selective growth advantage. Even in the absence of concomitant c-myc gene activation, however, bcl-2 expression appears to confer a worse prognosis on patients with at least some types of cancer, based on clinical correlation studies where the presence of either the Bcl-2 protein or bcl-2 gene rearrangements has been associated with poor responses to therapy in patients with non-Hodgkin's lymphomas, acute myelogenous leukaemia, and prostate cancer (Yunis et al., 1989; Campos et al., 1993; McDonnell et al., 1992).

Though centrally involved in the suppression of apoptosis, bcl-2 overexpression fails to protect cells in some circumstances from what appears to be apoptotic death (see Table I). Of course, it is possible that some of these routes to cellular demise that Bcl-2 fails to block are in fact more consistent with necrotic than apoptotic cell death, but this is not uniformly the case. Thus, either Bcl-2 alone is insufficient to prevent all forms of apoptotic cell death, or there exist Bcl-2-independent pathways for regulating PCD. Given that some tissues such as the liver lack bcl-2 expression altogether, and yet are subject to regulation by apoptosis (Oberhammer et al., 1992), Bcl-2-independent mechanisms clearly do exist.

The recent discovery of proteins that share sequence homology with Bcl-2 and that can repress its apoptosis-blocking
function however also suggests a possible explanation for why over-expression of bcl-2 is ineffective at preventing PCD in some types of cells and circumstances (Boise et al., 1993; Oltari et al., 1993).

**Structure of Bcl-2 and Related Proteins**

The amino-acid sequence of the 25–26 kD Bcl-2 protein, as predicted from cDNA cloning, contains no sequence motifs that might suggest a biochemical function for this regulator of PCD. The most noteworthy structural feature in the proteins from humans, mice, rats, and chickens is a stretch of 19 hydrophobic amino acids near the COOH terminus followed by just two charged residues that presumably serve to anchor the protein in membranes (Cazals-Hatem et al., 1992; Sato et al., 1993). Mutagenesis studies have confirmed that the hydrophobic COOH-terminal region alone posttranslational insertion into membranes such that the bulk of the Bcl-2 protein should be oriented towards the cytosol (Chen-Levy and Cleary, 1990), and have demonstrated the importance of membrane insertion for Bcl-2's function as a blocker of apoptosis (Hockenbery et al., 1990; Tanaka et al., 1993). A second shorter form of the Bcl-2 protein that lacks a hydrophobic tail can potentially be produced through alternative splicing (Tsujiimoto and Croce, 1986), but this protein (termed Bcl-2δ) has never been seen in vivo in any appreciable amounts.

Cleary et al. (1986) were the first to notice that Bcl-2 has weak homolog (22 % identity; 42 % similarity) with an open reading frame in the Epstein Barr Virus (EBV), termed BHRF-1. Like Bcl-2, the BHRF-1 protein has a hydrophobic tail, is associated with intracellular membranes, and can block programmed cell death caused by growth factor deprivation in human and murine hematolymphoid cells (Henderson et al., 1993; Takayama et al., 1993). The importance of these findings is in their demonstration that viruses can harbor Bcl-2 homologs which are capable of inhibiting PCD. When taken together with the observation that Bcl-2 can in some cases block or delay the apoptotic death of virus-infected cells and convert a lytic viral infection into a non-lytic persistent infection (Levine et al., 1993; Alimeni et al., 1992), the results suggest the possibility that virally encoded Bcl-2 homologs could perhaps contribute to viral latency or allow for persistent infections in the absence of cell lysis. Moreover, the recent discovery that the LMWS-HL protein of African Swine Fever virus has 26 % homology (47 % similarity) with Bcl-2 suggests that viral-regulators of the Bcl-2 pathway are not limited to EBV (Nielan et al., 1993). Of further interest with regards to viruses, EBV contains additional genes that can indirectly upregulate expression of the human bcl-2 gene in B-cells (Henderson et al., 1991; Finke et al., 1992), thus providing another example of a mechanism that potentially can be utilized by viruses to prevent host cell death.

Recently, cDNAs have been cloned for several novel human genes, revealing a family of Bcl-2-related proteins. One of these, termed Bcl-X, encodes a 241-amino acid protein with 74 % homology to Bcl-2 and that in preliminary gene transfer experiments functions essentially interchangeably with Bcl-2 to promote cell survival. An alternatively splice form of Bcl-X lacks a 63-amino acid region that is extremely well conserved among Bcl-2 homologs and inhibits Bcl-2 function (Boise et al., 1993). An additional Bcl-2 homolog called Bax was discovered through analysis of Bcl-2-associated proteins. A 21-kD (192 amino acids) isoform of Bax contains a transmembrane domain, has 21 % homology (43 % similarity) with Bcl-2, and appears to form heterodimers with the Bcl-2 protein an inactivate it (Oltari et al., 1993). The existence of Bcl-2/Bax heterodimers implies that Bcl-2 functions as a homodimer or oligomer, though biochemical evidence either supporting or opposing this idea is lacking at this time. Given that the Bcl-2-α and Bax-α proteins are 21 % homologous (43 % similarity when conservative replacements are permitted), it seems likely that much insight into Bcl-2 protein structure-function relations could be gained by creating chimeric Bcl-2/Bax proteins and thereby mapping the regions within Bcl-2 and Bax differentially responsible for suppression and acceleration of apoptosis, respectively. In contrast to Bax, the short form of Bcl-2 (Bcl-X-S) reportedly does not bind to the Bcl-2 protein and therefore presumably inhibits by competing either for unknown effector proteins that interact with Bcl-2 and lie downstream of it or for upstream activators of the Bcl-2 protein.

Additional members of the bcl-2 family termed mcl-1 and A1 were fortuitously identified through differential screening of cDNA libraries derived from myeloid leukemic and normal cells, respectively, induced to differentiate. Mcl-1 encodes a 37-kD protein that contains a stretch of 139 amino acids with 35 % homology (59 % similarity) with the corresponding region of human p26-Bcl-2 (Kozopas et al., 1993). A1 encodes a ~20-kD protein that has 40 % overall homology with Bcl-2 over a stretch of 80 amino acids in the carboxyl half of the A1 protein (Lin et al., 1993). Mcl-1 topographically resembles Bcl-2 with regards to having a hydrophobic domain near the COOH terminus but the corresponding segment in A1 contains a charged residue that makes it a poorer candidate for an integral membrane protein. The functions of Mcl-1 and A1 have not been reported to date. Whether other mammalian genes exist that share sequence and functional homology with Bcl-2 remains unknown, but considering that several tissues that are subject to regulation by PCD lack expression of bcl-2 and known related genes, it seems likely that additional members of the bcl-2 gene family will eventually be discovered. Furthermore, the finding that one of the members of the Bcl-2 protein family, Bax, can form heterodimers with Bcl-2 raises interesting possibilities with regards to potential combinatorial interactions among the various members of the Bcl-2 family.

**Intracellular Locations and Potential Mechanisms of Action of Bcl-2 Protein**

In the absence of any knowledge of the biochemical action of Bcl-2, considerable efforts have been expended on examining the subcellular location of this oncprotein in the hope that such information would provide some clues. The most recent observations based on use of confocal, laser scanning, and electron microscopy suggest that Bcl-2 resides in the nuclear envelope, parts of the endoplasmic reticulum (ER), and outer mitochondrial membrane but not in a variety of other intracellular membrane compartments including the plasma membrane (Monaghan et al., 1992; Jacobson et al., 1993; Krajewski et al., 1993). Subcellular fractionation studies have also provided convincing evidence of Bcl-2's as-
association with mitochondria (Hockenbery et al., 1990; Krajewski et al., 1993), a finding that is consistent with the punctate cytosolic immunostaining seen at the level of the conventional light-microscope in cells subjected to immuno-staining using Bcl-2-specific antibodies. Based on differential detergent extractions of outer and inner mitochondrial membranes, Hockenbery et al. (1990) concluded that Bcl-2 is an inner mitochondrial membrane protein, but the behavior of Bcl-2 in in vitro mitochondrial import assays is more consistent with an outer membrane protein (Krajewski et al., 1993). One potential explanation for the apparent discrepancy could lie in the observation that the pattern of immunostaining seen at the EM level suggests that Bcl-2 is present in a patchy, non-uniform distribution in the outer membrane, suggestive of the junctional complexes where inner and outer membranes abut.

The localization of Bcl-2 to mitochondria prompted an examination of the relevance of oxidative phosphorylation to Bcl-2 protein function. Using a human fibroblast cell line that had been depleted of its mitochondrial DNA by growth in media containing ethidium bromide, Jacobson et al. (1993) showed that no apparent requirement exists for oxidative phosphorylation for either induction of apoptosis or for Bcl-2 to function as a suppressor of cell death. Consistent with these data, no effect of Bcl-2 protein over-production was found on ATP levels or oxygen consumption in PC12 cells despite their resistance to induction of apoptosis by a wide variety of stimuli (Mah et al., 1993; Zhong et al., 1993). Similarly, Hennet et al. (1993) reported that transfection of bcl-2 expression constructs in L929 mouse fibrosarcoma cells did not affect oxygen consumption or the activity of the mitochondrial respiratory chain, but did appear to increase the mitochondrial membrane potential—at least as defined by crude measurements involving uptake of Rhodamine 123 into intact cells.

In contrast to the mitochondria, only scant efforts have been made thus far to evaluate the function of Bcl-2 in the nuclear envelope and ER compartments. Recent EM data suggest that Bcl-2 protein is not randomly distributed in the membranes of the nuclear envelope but rather is concentrated in patches highly reminiscent of nuclear pore complexes (NPCs) (Krajewski et al., 1993). If true, the finding raises the possibility of a role for Bcl-2 in some aspect of nuclear transport, NPC formation, or nuclear envelope assembly and maintenance. Double-labeling studies at the EM level are needed however to confirm the precise relation, if any, of Bcl-2 to NPCs. Still another possibility stems from the fact that the space between the inner and outer nuclear membranes, in its continuity with the lumen of the ER, is the major intracellular storage site for Ca2+. A variety of data have suggested that Ca2+ plays an important role in apoptosis, and several groups have characterized nucleus-associated Ca2+-dependent endonucleases that may be involved in the internucleosomal DNA digestion typical of apoptotic cells. In this regard, Bcl-2 has been shown to block apoptosis induced by Ca2+-ionophores in thymocytes, T-cell leukemia lines, and PC12 cells. Fluorometric measurements of cytosolic free Ca2+ concentrations in ionophore-treated PC12 cells have revealed that over-production of Bcl-2 does not prevent rises in intracellular Ca2+, suggesting that Bcl-2 blocks an apoptotic signal downstream of this event (Zhong et al., 1993). Baffy et al. (1993) also examined the calcium issue, focusing on the stores of Ca2+ sequestered in the ER and in mitochondria in IL-3-dependent cells deprived of lymphokine. Factor withdrawal led to a gradual loss of Ca2+ from the ER and a rise in the relative amounts of Ca2+ in the mitochondria. Gene transfer-mediated elevations in Bcl-2 protein prevented the alterations in Ca2+ and suppressed apoptotic cell death. These data suggest that Bcl-2 either directly or indirectly can influence Ca2+ partitioning, but the question now is whether those alterations in Ca2+ contribute functionally to the apoptotic process. In support of this possibility are the observations that: (a) glucocorticoid-treatment of T-cells has also been shown to result in a massive loss of Ca2+ from the ER prior to apoptotic cell death, suggesting that depletion of the ER pool of Ca2+ may be a common theme; (b) over-expression of an ER Ca2+- binding protein calbindin D28K in glucocorticoid-sensitive T-cells delays apoptosis induced by dexamethasone, forskolin and A23187, presumably by allowing the ER to better retain its Ca2+; and (c) poisoning the Ca2+-ATPase of the ER with thapsigargin induces apoptosis (Lam et al., 1993; Dowd et al., 1992). Thus, a connection between Bcl-2 and Ca2+ regulation makes an attractive hypothesis that fits nicely with the literature on Ca2+-dependent endonucleases and apoptosis, but that requires much further investigation.

An equally attractive hypothesis concerns the role of oxidative injury in the induction of cell death and the finding that Bcl-2 blocks the accumulation of lipid peroxides, as well as possibly other reactive oxygen species in at least some settings (Kane et al., 1993; Hockenbery et al., 1993). Though these findings in mammalian cells fall far short of establishing a direct cause and effect relation between Bcl-2 and control of cellular redox, a compelling argument can be made based on experiments performed in S. cerevisiae deficient in Mn-Superoxide Dismutase where expression of Bcl-2 was reported to restore growth in conditions requiring respiratory metabolism (Kane et al., 1993).

Clearly, many questions remain unanswered regarding the biochemical mechanism of action by which the Bcl-2 protein protects cells from apoptotic cell death and the functional significance of its association with mitochondria, nuclear envelope and ER. Given that several groups have recently cloned and are presently characterizing cDNAs that encode non-Bcl-2–related proteins that are capable of binding to the Bcl-2 protein (unpublished data and personal communications), perhaps the answers to these questions will not be far off. In this regard, the recent demonstration that Bcl-2 can interact with R-Ras in the yeast two-hybrid system and evidence that the two proteins co-immunoprecipitate in mammalian cells raises interesting possibilities (Fernandez-Sarabia and Bischoff, 1993). The effector domain of R-Ras for example is predicted to be capable of interacting with the Raf kinase, and we have observed in gene transfer experiments that Bcl-2 and Raf oncogenes can act synergistically to suppress apoptosis in some circumstances (manuscript submitted for publication). Thus, Bcl-2 may control a signal transduction pathway that, among other things, possibly could target signaling molecules to critical intracellular locations such as mitochondrial junctional and nuclear pore complexes. Regardless of the validity of these speculations, it seems likely that when the answers to the Bcl-2 mystery...
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