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BCL-2 Family Proteins: Regulators of Cell Death Involved in the Pathogenesis of Cancer and Resistance to Therapy

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Abstract The *BCL-2* gene was first discovered because of its involvement in the t(14;18) chromosomal translocations commonly found in lymphomas, which result in deregulation of *BCL-2* gene expression and cause inappropriately high levels of Bcl-2 protein production. Expression of the *BCL-2* gene can also become altered in human cancers through other mechanisms, including loss of the p53 tumor suppressor which normally functions as a repressor of *BCL-2* gene expression in some tissues. Bcl-2 is a blocker of programmed cell death and apoptosis that contributes to neoplastic cell expansion by preventing cell turnover caused by physiological cell death mechanisms, as opposed to accelerating rates of cell division. Overproduction of the Bcl-2 protein also prevents cell death induced by nearly all cytotoxic anticancer drugs and radiation, thus contributing to treatment failures in patients with some types of cancer. Several homologs of Bcl-2 have recently been discovered, some of which function as inhibitors of cell death and others as promoters of apoptosis that oppose the actions of the Bcl-2 protein. Many of these Bcl-2 family proteins can interact through formation of homo- and heterotypic dimers. In addition, several nonhomologous proteins have been identified that bind to Bcl-2 and that can modulate apoptosis. These protein–protein interactions may eventual serve as targets for pharmacologically manipulating the physiological cell death pathway for treatment of cancer and several other diseases.

Key words: *BCL-2* gene, Bcl-2 protein, homologs, homo- and heterotypic dimers, cancer

Cell death is a physiological process that plays a critical role in the regulation of tissue homeostasis by ensuring that the rate at which new cells are produced in the body through cell division is offset by a commensurate rate of cell loss. The amount of cell death that occurs constantly within cell renewing tissues such as bone marrow, gut, and skin is enormous. In fact, some estimates suggest that in the course of a typical year, each of us will lose through cell death and have replenished through cell division a mass of cells equivalent to our entire body weight. Although largely overlooked until recently, it is now becoming increasing appreciated that disturbances in the physiological cell death process that prevent or delay normal cell turnover can be just as important to the pathogenesis of cancer as abnormalities in the regulation of the cell cycle. Perhaps of even greater clinical importance is the recent realization that defects in the cell death pathway are important not only for the origins of cancer, but also because they may markedly influence our ability to treat it. Since nearly all chemotherapeutic drugs, as well as radiation, ultimately tap into endogenous physiological pathways for cell death in order to ultimately kill cancer cells, the loss of genes required for cell death or the over-activation of genes that block it can render tumor cells relatively more resistant to the cytotoxic effects of a broad spectrum of anticancer drugs.

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DISCOVERY OF *BCL-2* IN HUMAN LYMPHOMAS AND DELINEATION OF A FAMILY OF HOMOLOGOUS GENES

Like cell division, which is controlled through a complex interplay of cell cycle stimulators and repressors, the physiological cell death pathway is precisely regulated under normal circum-

stances by a delicate balance of genes whose encoded proteins either induce or inhibit cell death. The first realization that defects in the cell death pathway could contribute to the development of human neoplasia came from the discovery of a gene involved frequently in non-Hodgkin's lymphomas, called *BCL-2* for "B-cell lymphoma-2" [Tsujimoto and Croce, 1986; Vaux et al., 1988]. In ~90% of low-grade follicular non-Hodgkin's lymphomas, as well as ~30% of more aggressive B-cell lymphomas, chromosomal translocations move the *BCL-2* gene from its normal location on chromosome 18 into juxtaposition with the immunoglobulin (Ig) heavy-chain gene locus on chromosome 14, probably as the result of errors in the normal DNA recombination mechanisms that cut and splice together the V, D, and J gene segments to create functional Ig genes during B-cell differentiation in the bone marrow [Tsujimoto et al., 1988]. The resulting t(14;18) translocations place the *BCL-2* gene under the influence of powerful transcriptional enhancers associated with the Ig locus, thus dysregulating the expression of *BCL-2* primarily at the transcriptional level. Because the protein encoded by the *BCL-2* gene blocks programmed cell death, B cells containing a t(14;18) translocation enjoy a selective survival advantage relative to their normal counterparts, and begin to clonally expand without necessarily experiencing an increase in their doubling times. When one considers that that average life span of B cells is only 5–7 days, it becomes immediately obvious how a genetic alteration that prevents cell death can impact on the homeostatic mechanisms that control the number of these cells in the body. This mechanism for clonal expansion, which is based on a selective survival advantage as opposed to an increased rate of cell division, probably explains the low growth fraction of most follicular lymphomas, which are generally regarded as low-grade malignancies in which the accumulation of malignant B cells in the body occurs slowly over time but nevertheless ultimately leads to patient demise.

The protein encoded by the *BCL-2* gene is unique, and has no significant amino-acid homology with other proteins whose biochemical mechanism of action is known. Comparisons of the sequences of the human, mouse, rat, and chicken homologs of Bcl-2 have suggested a four-domain structure where a ~40-amino acid conserved N-terminal domain is followed by a nonconserved region of variable length that is often rich in prolines and thus unlikely to fold into higher-order structures such as α-helicies or β-sheets. This is then followed by another well-conserved region of ~100-amino acids length and then a stretch of hydrophobic aminoacids at the C-terminus that has been shown to constitute a transmembrane domain [Cazals-Hatem et al., 1992]. Thus, Bcl-2 is an integral membrane protein. The intracellular membranes into which the Bcl-2 protein post-translationally inserts include predominantly the outer mitochondrial membrane, nuclear envelope, and parts of the endoplasmic reticulum [Krajewski et al., 1993]. Although mutagenesis studies suggest that the transmembrane (TM) domain of Bcl-2 is essential for optimal function in some types of cells, in some circumstances C-terminal truncation mutants of Bcl-2 that lack a membrane anchore are equally effective as the wild-type Bcl-2 proteins at blocking cell death [Tanaka et al., 1993; Borner et al., 1994]. Such TM-deficient versions of Bcl-2, however, probably still retain the ability to interact with other membrane-associated proteins.

Since the discovery of *BCL-2*, several homologs of this gene and its encoded protein have been identified. Interestingly, several of these Bcl-2-related proteins can physically interact with each other in the form of homo- and heterotypic dimers or oligomers (the actual stoichiometry is unknown at present). Furthermore, some of these homologs function as blockers of cell death, whereas others are promoters of apoptosis. At present, six mammalian homologs of Bcl-2 have been reported, including Bax, Bcl-X, Mcl-1, A1, Bad, and Bak [Oltvai et al., 1993; Boise et al., 1993; Kozopas et al., 1993; Lin et al., 1993; Yang et al., 1995; Chittenden et al., 1995; Kiefer et al., 1995; Farrow et al., 1995]. Some of these proteins have additional forms that arise through alternative splicing, the most interesting of which are the long and short forms of Bcl-X. Bcl-X-L (47% identical to Bcl-2 at the amino acid level) and Bcl-X-S (missing a well-conserved 63-amino acid region) have opposing functions, with Bcl-X-L acting as a cell death blocker and Bcl-X-S as an antagonist of Bcl-2 and Bcl-X-L that promotes apoptosis [Boise et al., 1993]. In addition to Bcl-X-S, the Bax, Bad, and Bak proteins function as promoters of cell death. Conversely, the Mcl-1 and A1 proteins appear to be suppressors of cell death, like Bcl-2 and Bcl-X-L. Finally, several homologs of Bcl-2 have been discovered in viruses, including the

E1b–19-kD protein of adenovirus and the BHRF-1 protein of Epstein-Barr virus (EBV)—both of which function as suppressors of cell death [Chiou et al., 1994; Takayama et al., 1994]. The sparce economy of viral genomes implies that intense evolutionary pressures must have selected for retention of these viral homologs of Bcl-2 and suggests that Bcl-2 represents a critical point for regulating the physiological cell death pathway.

When expressed in yeast (*Saccharomyces cerevisiae*), the Bax protein confers a lethal phenotype, suggesting that it promotes cell death through a mechanism that may be evolutionarily conserved [Sato et al., 1994, 1995]. Co-expression in yeast of fusion proteins that represent Bcl-2, Bcl-X-L, or Mcl-1 without their TM domains neutralizes Bax-mediated cytotoxicity, thus a TM domain is not absolutely required for any of these Bcl-2 family proteins in this system. Conversely, a variety of deletion mutants of Bcl-2 and Mcl-1 and the Bcl-X-S protein do not suppress Bax lethality in yeast [Sato et al., 1994, 1995; Hanada et al., 1995; Bodrug et al., 1995]. In particular, Bcl-2 deletion mutants missing any of 3 domains that are generally well conserved among members of the Bcl-2 protein family, BD(A), BD(B), and BD(C), are unable to rescue yeast from the lethal effects of Bax [Hanada et al., 1995]. Investigations of equivalent deletion mutants of Bcl-2 in mammalian cells have produced similar results, showing inability to protect cells from apoptotic stimuli [Borner et al., 1994]. Interestingly, co-immunoprecipitation experiments have demonstrated that some of these Bcl-2 deletion mutants, such as those lacking BD(B) and BD(C), have lost the ability to heterodimerize with Bax [Yin et al., 1994]. Thus, binding to Bax appears to be one important feature of Bcl-2 function, presumably preventing the formation of Bax/Bax homodimers. However, in vitro binding studies indicate that some Bcl-2 deletion mutants, such as those lacking BD(A), still bind to Bax and appear to do so with roughly the same efficiency as the wild-type Bcl-2 protein, although quantitive measurement of affinities have not been performed [Hanada et al., 1995]. Consequently, it appears that while binding to Bax may be important for Bcl-2 function, it is not the only requirement. What those other requirements are remains to be determined but they may include binding to other proteins by Bcl-2 or masking sites on Bax so that other proteins cannot bind to or post-translationally modify Bax.

Although Bax appears to directly promote cell death through still poorly understood mechanisms, another class of Bcl-2 homologs, which include Bcl-X-S and Bad, indirectly induce apoptosis by binding to Bcl-2 and Bcl-X-L and preventing them from heterodimerizing with Bax (Fig. 1). The complexity of these interactions among Bcl-2 family proteins has undoubtedly evolved to provide multiple opportunities for fine-tuning the relative sensitivity or resistance of cells to apoptotic stimuli through differential regulation of the expression of various *BCL-2* family genes [Sato et al., 1994; Yang et al., 1995].

Bcl-2 AND CHEMORESISTANCE

Using gene transfer methods to overexpress *BCL-2* in leukemic and solid tumor cell lines that normally contain low levels of Bcl-2 protein, as well as antisense approaches to reduce the levels of Bcl-2 protein in t(14;18)-containing lymphoma cell lines that contain high levels of this protein, we have shown that levels of Bcl-2 protein correlate with relative resistance to a wide spectrum of chemotherapeutic drugs as well as γ-irradiation [Miyashita and Reed, 1992, 1993; Hanada et al., 1993; Kitada et al., 1994]. Included among the drugs that Bcl-2 has been experimentally shown to render cells more resistant to are: dexamethasone, cytosine arabinoside (Ara-C), methotrexate, cyclophosphamide, Adriamycin, daunomycin, 5-fluorouracil, 2-chlorodeoxyadenosine, fludarabine, taxol, etoposide (VP-16), camptothecin, nitrogen mustards, mitoxantrone, and cisplatin. The extent to which Bcl-2 provides protection from the cytotoxic effects of these drugs varies, depending on the particular drug and the cell line, but can be as much as four or more logs (×10,000) or as little as half a log (×5). Given that so-called "high-dose" aggressive chemotherapy typically involves a mere doubling of the concentration of drugs delivered to patients, even a fivefold increase in relative resistance however could be enormously significant in clinical terms.

The observation that Bcl-2 provides protection against such a wide variety of drugs which have markedly diverse mechanism of action suggests that they all use the same final common pathway for ultimately inducing cell death and that Bcl-2 is a regulator of this pathway. Indeed, several studies have provided evidence that che-

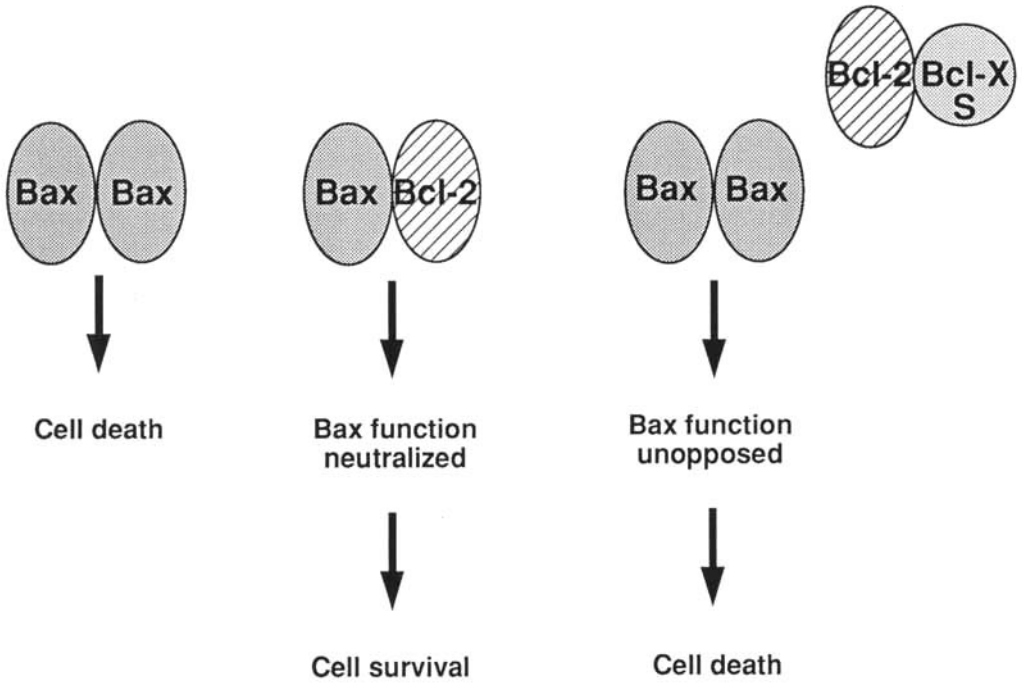

Fig. 1. Model for Bcl-2 family protein interactions. Based on evidence available to date, a model can be envisioned in which Bax promotes apoptosis, probably through formation of homodimers. Bax-mediated cell death is opposed when Bcl-2, Bcl-X-L, Mcl-1, or possibly other homologs of Bcl-2 that have antideath activity (e.g., A1) heterodimerize with Bax, thus neutralizing its function. A second class of cell death promoters, which include Bcl-X-S and Bad, indirectly induce apoptosis by binding to Bcl-2, Bcl-X-L, and probably other anti-apoptotic members of the Bcl-2 protein family, thus sequestering them and preventing them from heterodimerizing with Bax. This leaves Bax homodimers unopposed. A recently described member of the Bcl-2 protein family, Bak, may function equivalent to Bax (not shown).

motherapeutic drugs, as well as γ-radiation, when administered in vitro to tumor cell lines induce cell death through mechanisms consistent with apoptosis as opposed to necrosis. It stands to reason, therefore, that genes such as *BCL-2*, which block the apoptotic pathway, could also block cell killing induced by anticancer drugs.

The mechanism by which Bcl-2 confers resistance to anticancer drugs is distinct from other previously recognized forms of chemoresistance. Traditionally, pharmacologists have thought of the chemoresistance problem in cancer in terms of four issues: (1) delivery of drug to the target such as occurs when the *mdr-1* gene product, P-glycoprotein, is overproduced in the plasma membrane of cancer cells and pumps drugs out of the cell or when a drug is metabolized to an inactive product; (2) modification of the drug target, an example of which is amplification of the gene for dihydrofolate reductase which often occurs following exposure to methotrexate; (3) increased rates of repair of damage to DNA or other structures; and (4) diminished rates of drug-induced damage to DNA or other macromolecules, as can occur for some drugs when glutathione levels are elevated in tumors. Bcl-2, in contrast, appears not to interfere with the ability of drugs to enter cells, bind to their appropriate targets, and induce damage. Indeed, Bcl-2 does not protect cancer cells from drug-induced cell cycle arrest but does prolong their survival during this period so that proliferation can resume upon withdrawal of the drug, as typically occurs between cycles of chemotherapy. Rates of DNA repair are also not affected by Bcl-2. Thus, the drugs induce cell cycle arrest and damage to DNA, but this damage somehow is not translated effectively into signals for cell death. As such, Bcl-2 defines a new category of chemoresistance gene; namely, those that regulate downstream events in the normal physiological pathway for programmed cell death and that convert anticancer drugs from cytotoxic to merely cytostatic. It remains to be determined within the clinical context of patients to what extent Bcl-2 controls treatment outcomes, but at least some clinical correlative studies have suggested a connection between Bcl-2 and either poor response to therapy, shorter disease-free

survival, or shorter overall survival in some groups of patients with large cell non-Hodgkin's lymphomas, myeloid leukemias, and adenocarcinomas of the prostate [reviewed in Reed, 1994, 1995].

MECHANISMS OF *BCL-2* GENE DYSREGULATION IN HUMAN CANCERS

Although the *BCL-2* gene was first discovered because of its involvement in t(14;18) translocations found frequently in non-Hodgkin's lymphomas, high levels and aberrant patterns of *BCL-2* gene expression have been reported in a wide variety of human cancers, including ~90% of colorectal, ~60% of gastric, ~30–60% of prostate, ~20% of non-small cell lung cancers, ~30% of neuroblastomas, and variable percentages of melanomas, renal cell, and thyroid cancers as well as acute and chronic lymphocytic and nonlymphocytic leukemias [reviewed in Reed, 1994, 1995]. In essentially all these nonlymphomatous cancers, no evidence for structural alterations of the *BCL-2* gene has been found and instead alterations in trans-acting factors that control *BCL-2* gene expression are suspected to be at flaut.

One of the mechanisms that may play a role in dysregulation of *BCL-2* expression in cancers is loss of the tumor suppressor p53. Loss of p53 function occurs in about one-half of all human cancers. This DNA-binding protein can both induce cell cycle arrest and promote apoptosis, and functions at least in part as a transcriptional regulator. In experiments where p53 function was conditionally restored to a p53-deficient murine leukemia line, p53 was shown to induce marked decreases in *bcl-2* gene expression followed by apoptotic cell death [Miyashita et al., 1994a]. When Bcl-2 protein levels were maintained at high levels through gene transfer manipulations, p53-induced apoptosis was partially blocked but cell cycle arrest occurred normally [Selvakumaran et al., 1994]. γ-Radiation, a known inducer of p53, has also been shown to downregulate *BCL-2* mRNA levels in a human leukemia line [Zhan et al., 1994]. Thus, p53 either directly or indirectly appears to be able to suppress *BCL-2* gene expression, leading to the speculation that p53 loss in human tumors may contribute to the high levels and abnormal patterns of Bcl-2 protein production observed in many types of cancer. Indeed, using reporter gene assays, we have mapped a p53-negative response element (NRE) to the 5′ untranslated region of the *BCL-2* gene [Miyashita et al., 1994b]. However, our analysis of p53 knock-out mice suggests that loss of p53 may be sufficient to result in elevated levels of Bcl-2 protein production in only some tissues, suggesting that either other p53-independent mechanisms for repression of *BCL-2* gene expression exist or that critical transactivators of *BCL-2* are missing from some types of cells [Miyashita et al., 1994a].

DYSREGULATION OF EXPRESSION OF OTHER *BCL-2* FAMILY GENES IN CANCER

In addition to inhibition of *BCL-2* gene expression, the tumor suppressor p53 can also induce increases in *BAX* gene expression [Miyashita et al., 1994a]. These effects of p53 on *BCL-2* and *BAX* gene expression can result in a marked decrease in the ratio of Bcl-2 to Bax protein, and thus render cells more vulnerable to apoptotic stimuli (Fig. 2). The *BAX* gene promoter contains four 10 bp motifs with homology the consensus p53-binding sites and is strongly transactivated by p53 in reporter gene assays [Miyashita and Reed, 1995]. Thus, *BAX* represents the first pro-apoptotic gene to be identified which is a direct transcriptional target of p53. Clearly, however, p53 represents only one of the inputs into the *BAX* gene promoter and other undelineated factors may modulate the effects of p53 on this gene. In this regard, radiation has been shown to induce expression of genes associated with genotoxic stress and cell cycle arrest in a p53-dependent fashion in many types of tumor cell lines, but triggers elevations in *BAX* mRNA and apoptosis only in a subset of cancer lines in vitro [Zhan et al., 1994]. The mechanisms that prevent p53 from transactivating the *BAX* gene in many tumor lines remains to be determined, but once delineated could potentially provide insights into strategies for improving tumor responses to radiotherapy and DNA-damaging chemotherapeutic drugs.

A prediction of the observations that p53 can bind to and transactivate the *BAX* gene is that tumors with loss of p53 function will contain relatively lower levels of Bax protein. Indeed, we have observated that Bax protein levels are markedly reduced in about one-third of advanced breast cancers [Krajewski et al., 1995] but do not correlate with p53-immunostaining results. We have also found striking decreases in *BAX* expression in drug-resistant ovarian cancers and leukemias (submitted). In some of these

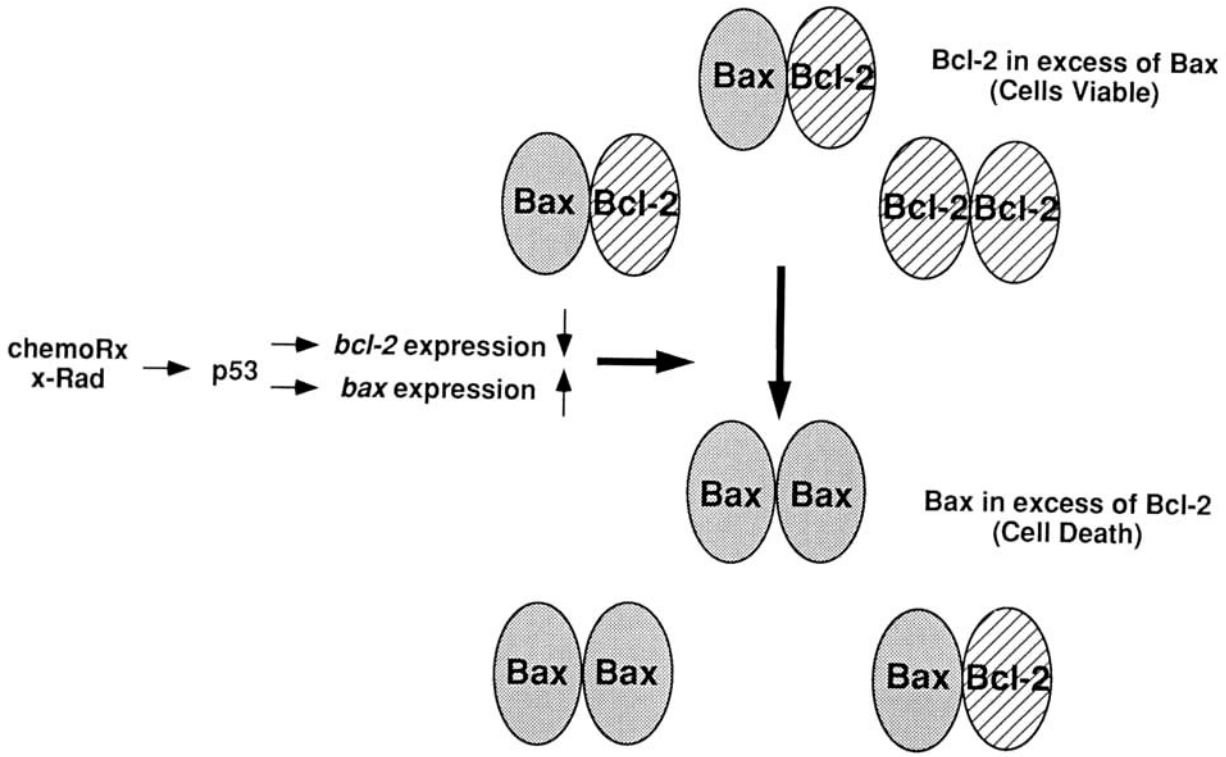

Fig. 2. Model for regulation of chemosensitivity by tumor suppressor p53. Radiation or DNA-damaging chemotherapeutic drugs are known to induce increases in p53 protein and p53 transcriptional activity. These elevations in p53 then directly upregulate *BAX* gene transcription and also downregulate *BCL-2* gene expression, at least in some types of cells and tissues where the effects of p53 are dominant. As a consequence, the ratio of Bcl-2 to Bax protein declines, resulting in an excess of Bax protein and the promotion of apoptotic cell death.

cases, *BCL-2* expression was not significantly altered relative to less aggressive, drug-sensitive tumors. These findings suggest therefore that *BCL-2* and *BAX* can be independently regulated and that either increases in Bcl-2 or decreases in Bax protein levels can be associated with drug-resistant phenotypes in human cancers. It will be of interest in future studies to determine whether assessment of *BAX* expression, alone or in combination with *BCL-2* or p53, is of prognostic significance for some subgroups of cancer patients. In this regard, reduced Bax-immunostaining was associated with poor responses to combination chemotherapy and shorter overall patient survival in a small study of women with metastatic breast cancer [Krajewski et al., 1995]. Since loss of *BAX* expression was strongly associated with loss of Bcl-2 immunopositivity in these breast cancers, the results suggest that reductions in Bcl-2 levels were tolerated because of diminished Bax protein and provide a potential explanation for the previously reported paradoxical association of Bcl-2-immunostaining with good prognosis in breast cancer patients [Silvestrini et al., 1994]. Finally, we have observed prominent increases in Bcl-X-L protein production (rather than elevations in Bcl-2 or decreases in Bax), in association with drug-resistant phenotypes in some leukemias and solid tumors, implying that alterations in the expression Bcl-X-L may also be relevant to mechanisms of drug-resistance in some types of cancer. Though these studies are just beginning, the preliminary observations suggest that dysregulation of the expression several members of the *BCL-2* gene family is likely to contribute directly or indirectly to drug-resistance in cancers.

POSSIBLE MECHANISMS OF Bcl-2 PROTEIN ACTION

At present, a biochemical understanding of how Bcl-2 and its homologs control cell life and death remains elusive. Indirect evidence has been obtained in support of an effect of Bcl-2 on regulation of antioxidant pathways in cells, particularly protecting against lipid peroxidation [Kane et al., 1993; Hockenbery et al., 1993].

Experimental evidence suggesting regulation of intracellular Ca^{2+} homeostasis has also been obtained. In a hemopoietic cell where apoptosis is induced by lymphokine deprivation and in glucocorticoid-treated lymphoid cells, massive loss of Ca^{2+} from the ER occurs as a relatively early event prior to apoptosis. Enforced production of high levels of Bcl-2 protein in these cells delays apoptosis and loss of ER Ca^{2+} stores [Baffy et al., 1993; Lam et al., 1994]. In the hematopoietic cell model, Bcl-2 was also found to influence mitochondrial pools of Ca^{2+}, preventing accumulation of Ca^{2+} in this organelle, which can serve as a sink for Ca^{2+} under conditions of high cytosolic Ca^{2+} concentrations [Baffy et al., 1993]. Bcl-2 has also been reported to prevent the transient decrease in cytosolic free Ca^{2+} that occurs rapidly after withdrawal of growth factors from hemopoietic cells and fibroblasts [Magnelli et al., 1994]. Furthermore, Bcl-2 was reported to delay the efflux of Ca^{2+} from the ER in cells treated with thapsigargin, a specific inhibitor of that organelle's Ca^{2+}-ATPase. Although no direct link between Bcl-2 and Ca^{2+}-channels or other Ca^{2+}-regulating proteins has been found, it is of interest that two recently described Bcl-2-binding proteins, Nip-2 and Nip-3, contain sequences that resemble Ca^{2+}-binding sites or that have homology to calbindin-D [Boyd et al., 1994], an ER protein that has been shown to delay apoptosis when over-expressed in glucocorticoid-sensitive lymphoid cells [Dowd et al., 1992]. Of course, it is possible that effects of Bcl-2 on lipid peroxidation and Ca^{2+} transport are related, since Ca^{2+} can influence the activity of some enzymes involved in lipid metabolism and oxidative damage to membranes can compromise Ca^{2+} compartmentalization.

It has also been suggested that Bcl-2 may participate in protein transport across biological membranes [reviewed in Reed, 1994]. For example, immunoelectromicroscopic studies indicate that Bcl-2 is located in discrete patches distributed nonuniformly in the outer mitochondrial membrane and nuclear envelope, not unlike proteins targeted to the mitochondrial junctional complexes (MJCs) and nuclear pore complexes (NPCs), where the inner and outer membranes of these DNA-containing organelles come into contact and where transport of peptides, RNA and probably some ions occurs [Krajewski et al., 1993; deJong et al., 1994]. In this regard, nuclear accumulation of p53 and some cyclin-dependent kinases has been reported to be antagonized by gene transfer-mediated elevations in Bcl-2 protein [Ryan et al., 1994; Meikrantz et al., 1994], but several other groups have failed to find effects of Bcl-2 on translocation of temperature-sensitive versions of p53 from cytosol to nucleus. Studies using enucleated cells as well as a cell-free system involving apoptotic cytosolic extracts from *Xenopus* eggs have also provided convincing support for the idea that apoptosis is largely a cytoplasmically regulated process with the nucleus serving as a mere passive substrate for degradation, at least in circumstances in which the induction of cell death does not require new gene expression [Jacobson et al., 1994; Newmeyer et al., 1994]. These observations however do not exclude an important role for Bcl-2 in regulation of protein transport in mitochondria.

A link between Bcl-2 and regulation of proteases has also been suggested both by genetic studies in the nematode *C. elegans* and gene transfer studies in mammalian cells where cell death induced by *ced-3*, a cysteine protease, and its homologs was shown to be inhibitable by either Bcl-2 or its equivalent in the worm ced-9 [Yuan et al., 1993; Miura et al., 1994]. The discovery of a mammalian Bcl-2-binding protein BAG-1 that contains a ubiquitin-like domain has also raised the possibility of a direct connection between Bcl-2 and proteases. BAG-1 has anti-cell death activity in transfection studies and cooperates with Bcl-2 in the suppression of apoptosis, providing enhanced protection from apoptotic stimuli beyond that conferred by either Bcl-2 or BAG-1 alone [Takayama et al., 1995]. One interesting revelation to come from studies of BAG-1 is that some cell death stimuli previously thought to function through a Bcl-2-independent mechanism, such as apoptosis induced by Fas or cytolytic T cells, were inhibited by the combination of Bcl-2 and BAG-1. These findings thus suggest that in the absence of adequate levels of appropriate partner proteins, elevations in Bcl-2 protein levels can be insufficient to render some types of cells resistant to some cell death stimuli. Other mechanisms, such as high levels of Bax, production of dominant inhibitors of Bcl-2 (Bcl-X-S; BAD), or possibly post-translational modifications such as phosphorylation, could conceivably also account for the failure of Bcl-2 to protect against apoptosis in some circumstances [Haldar et al., 1995], thus begging the question of whether pathways for apoptotic cell death truly exist that do not

involve Bcl-2 or one of its homologs at some level.

Finally, an additional potential mechanism of action for the Bcl-2 protein has been raised by the recent finding that Bcl-2 can physically associate with signal transducing proteins, including the GTPase R-Ras and the serine/threonine-kinase Raf-1 [Fernandez-Sarbia and Bischoff, 1993; Wang et al., 1994]. In gene transfer experiments, a constitutively activated version of Raf-1 kinase was shown to synergize with Bcl-2 in preventing apoptosis induced by lymphokine withdrawal from a factor-dependent hemopoietic cell line, yet did not induce phosphorylation of the Bcl-2 protein [Wang et al., 1994]. Conversely, in the same cell model, activated versions of R-Ras accelerated apoptosis through a mechanism that was completely suppressible by co-expression of Bcl-2 [Wang et al., 1995]. These findings thus suggest the possibility of a signal transduction system that is centered presumably around the membranes where Bcl-2 resides, including the outer mitochondrial membrane, nuclear envelope and ER, and that may be uniquely involved in regulating cell death pathways, as opposed to the traditional roles for Ras and Raf-1 family proteins at the plasma membrane where they participate in signal transduction pathways linked to mitogenesis. Consistent with this idea are observations derived from use of a cell-free system for apoptosis where it was shown that mitochondria are required for apoptotic-like degradation of nuclei in cytosolic extracts prepared from *Xenopus* eggs [Newmeyer et al., 1994], implying that some kind of cell death "signal" was originating from the mitochondria. Experiments with respiratory chain inhibitors and free-radical scavengers suggested that reactive oxygen species were not significantly involved, whereas phosphotyrosine and Zn^{2+}, a known inhibitor of some protein tyrosine phosphatases, were effective at preventing apoptotic distruction of nuclei. Precisely how Bcl-2 might participate in the regulation of a hypothetical signal transduction system centered around mitochondrial and other intracellular membranes remains to be established. In this regard, it remains unproven that the interaction of Bcl-2 with Raf-1, R-Ras, or any of the other recently identified Bcl-2-interacting proteins such as BAG-1, Nip-1, Nip-2, and Nip-3 is essential for the function of Bcl-2 as an inhibitor of cell death. Only when the domains involved in these protein-protein interactions have been precisely mapped and appropriate mutagenesis studies performed, will the relative importance of these interactions be revealed. Information of this type represents an essential first step towards the ultimate goal of identifying novel pharmaceuticals that may one day improve our ability to treat cancer and many other diseases that involve dysregulation of the physiological cell death pathway.

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