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The relationship between Bcl2, Bax and p53: consequences for cell cycle progression and cell death

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Each cell is under constant surveillance to maintain the integrity of its genome. Genomic lesions in a cell must be repaired before the onset of DNA replication and cell division. In the scenario that the genomic lesion is not repairable, the damaged cells are disposed in an orderly manner known as programmed cell death or apoptosis. Apoptosis and cell cycle progression are two intimately linked phenomena. Uncontrollable cell proliferation perturbs the cellular homeostasis and this can lead to malignancies, as well as organ dysfunction and developmental abnormalities. The biological pathway controlling cell fate is sequentially organized at the molecular level. Recent studies have made important contributions in advancing our knowledge of the mechanisms of cell cycle control and apoptosis regulation. A oncogene-derived protein, Bcl2, confers negative control in the pathway of cellular suicide machinery. A Bcl2-homologous protein, Bax, promotes cell death by competing with Bcl2. While Bax–Bax homodimers act as apoptosis inducers, Bcl2–Bax heterodimer formation evokes a survival signal for the cells. Both Bcl2 and Bax are transcriptional targets for the tumour suppressor protein, p53, which induces cell cycle arrest or apoptosis in response to DNA damage. In all, the coordinate performance of these molecules is crucial for controlling life and death of a cell.

Key words: apoptosis/Bax/Bcl2/cell cycle/p53

Introduction

Almost all tissues with a capacity for self-renewal undergo a continuous cycle of cell division and cell death. Eukaryotic cells replicate themselves by passing through the cell cycle, and the aged or damaged cells are removed through programmed cell death or apoptosis (Hartwell and Kastan, 1994; Basu and Haldar, 1996). The passage of cells from one stage of the cell cycle to another is under tight regulation of a number of negative and positive feedback loops. Negative controls may play an important role in preventing tumorigenesis and are exerted during development, differentiation, senescence and cell death. When the integrity of the genome is impaired, failure to arrest proliferation could cause the cells to evolve with highly unstable genomes. Thus programmed cell death is an important negative control regulatory event, and its disruption can lead to pathological states involving damaged cell accumulation, such as cancer, or to inappropriate cell loss, such as neurodegeneration (Hengartner, 1996; White, 1996; Yuan, 1997). The processes of cell loss and cell division must be homeostatically balanced not only in order to generate and maintain the complex dynamic architecture of tissues, but also to allow adaptation to changing circumstances. Therefore, there must be some system for linking these two antagonistic processes. It seems that the most plausible way in which this linkage might be established is through the direct coupling of the process of cell cycle progression and programmed cell death, perhaps through the use and control of shared molecular machinery.

An increasing number of genes that are involved in the evolutionary pathway for physiological cell death and its morphological counterpart, apoptosis, have been discovered. A number of these genes belong to the *bcl2* family. All of them are involved in the manipulation of cell survival outcomes after cytotoxic stress. The original family member, *bcl2* (B-cell leukaemia/lymphoma-2), was cloned from a patient with follicular lymphoma harbouring a t(14; 18) chromosomal translocation (Tsujimoto *et al*., 1984, 1985; Bakshi *et al*., 1985; Cleary *et al*., 1986). Deregulated Bcl2 expression confers interleukin-independent survival, allowing the life span of precancerous B cells to be extended, thus facilitating malignant transformation (Vaux *et al*., 1988; Hockenbery *et al*., 1990). Apart from its aetiological role in human follicular lymphoma, Bcl2 is also expressed in other neoplasms including prostate, breast and ovarian cancers (Haldar *et al*., 1994b, 1996; Lu *et al*., 1996; Marx *et al*., 1997). The family of Bcl2-related proteins constitutes one of the most biologically relevant classes of apoptosis regulators acting at the effector stage (Kroemer, 1997; Reed, 1997) of apoptosis, with some members functioning as suppressors of apoptosis and others as promoters of cell death. The ultimate vulnerability of cells to diverse apoptotic stimuli is determined by the relative ratio of various pro-apoptotic and anti-apoptotic members of the Bcl2 family (Oltvai *et al*., 1993; Yang and Korsmeyer, 1996). The Bcl2-interacting protein, Bax, is a pro-apoptotic member of the Bcl2 family, and its expression is induced by γ-radiation, chemotherapeutic drugs, and other forms of genotoxic stress (Kitada *et al*., 1996; Thomas *et al*., 1996).

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In addition to the Bcl2 family members, the tumour suppressor gene *p53* is required for checkpoint control during cell cycle progression, as well as induction of apoptosis (Meikrantz and Schlegel, 1995). During progression of the cell cycle, there are at least two checkpoints at which DNA damage is detected: one occurs at the G1–S transition and the other at the G2–M transition. The checkpoint controlling entry into S phase prevents cells from replicating damaged DNA and is mediated through the p53 protein, which induces either cell cycle arrest in G1 or apoptosis. Although the p53-mediated late G1 arrest is a well-known phenomenon (Merritt *et al.*, 1997), the implication of p53 in the regulation of G2 exit has also been reported (Guillouf *et al.*, 1995). More recently, a *p53*-related gene, called *p73*, has been mapped to chromosome 1p36, a region frequently deleted in neuroblastoma and other tumours (Kaghad *et al.*, 1997). The overexpression of p73 can activate the transcription of p53-responsive genes and can inhibit cell proliferation in a p53-like manner by inducing apoptosis (Jost *et al.*, 1997).

The Bcl2 family of apoptosis regulators

The growing Bcl2 family includes several representatives in mammals: Bcl-x (Boise *et al.*, 1993), Mcl-1 (Kozopas *et al.*, 1993), Bax (Oltvai *et al.*, 1993), A1 (Lin *et al.*, 1993), Bak (Chittenden *et al.*, 1995; Farrow *et al.*, 1995; Kiefer *et al.*, 1995), Bad (Yang *et al.*, 1995), Bik (Han *et al.*, 1996; Elongovan and Chinnadurai, 1997), Bcl-w (Gibson *et al.*, 1996), BID (Wang,K. *et al.*, 1996), bfl-1 (D'Sa-Eipper *et al.*, 1996) and Hrk (Inohara *et al.*, 1997) as well as the newly cloned Bok (Hsu *et al.*, 1997). In addition, the nematode homologue of Bcl2, ced-9, was identified in *Caenorhabditis elegans* (Hengartner and Horvitz, 1994). Furthermore, multiple homologues of Bcl2 have been found in viruses, e.g. BHRF1 in Epstein–Barr virus (Cleary *et al.*, 1986; Henderson *et al.*, 1993), E1B 19K in adenovirus (White *et al.*, 1992), as well as others (Neilan *et al.*, 1993). Such evolutionary conservation might reflect a pivotal role for this gene family in the cell death pathway. The members of the Bcl2 family, comprising both death antagonists and death agonists, differ in their structural features and in their expression patterns with regard to the tissue of expression and activators of expression. Most members of the Bcl2 family, except a few such as Bad, possess a carboxy terminal transmembrane region (Yang *et al.*, 1995), thereby influencing their subcellular distribution. One characteristic feature of this family of proteins is their propensity to form homodimers and heterodimers (Oltvai *et al.*, 1993). Most family members can dimerize with themselves and/or with other family members. The level of anti-apoptotic versus pro-apoptotic dimers is important in determining the resistance of a cell to apoptosis.

Expression of Bcl2 family members in tissues of the reproductive system

Breast

Several members of the Bcl2 family including the anti-apoptotic proteins Bcl2, Bcl-X and Mcl-1, and the pro-apoptotic protein Bax (Krajewski *et al.*, 1994), are expressed in normal mammary epithelium. Interestingly, the expression of Bcl2 in breast cancer cells is dependent on oestrogen, in contrast to Bax, whose expression is independent of oestrogen (Teixeira *et al.*, 1995). Immunohistochemical studies in primary adenocarcinoma of the breast establishes a strong correlation between oestrogen receptor (ER) positivity and Bcl2 immunostaining in primary adenocarcinoma of the breast (Leek *et al.*, 1994). Generally, the more aggressive types of breast tumours, which are hormone independent, are Bcl2 negative. Patients with ER-positive, Bcl2-positive tumours have a longer disease-free survival than patients with Bcl2-negative tumours. These observations have raised an interesting question regarding the favourable outcome with mammary carcinoma in the presence of a chemoresistant oncogene. A partial answer was given by recent studies demonstrating reduced Bax levels in tumours which had lost Bcl2 expression. The expression of Bax is not correlated with ER, p53, nor histological grade but is definitively correlated with Bcl2 (Krajewski *et al.*, 1995). It is noteworthy that an inverse relationship exists between p53 and Bcl2 in breast tumours (Leek *et al.*, 1994) and the ability of p53 to down-regulate Bcl2 was noted in a panel of breast cancer cell lines (Haldar *et al.*, 1994b). The normal epithelium from which breast carcinoma is developed expresses Bcl2. This is suggestive of its role in assisting cells to live longer and to accumulate genetic alteration leading to carcinoma.

Ovary

Studies on Bcl2 expression or p53 accumulation and the apoptosis phenomenon in ovarian carcinomas are also available in the literature (Henriksen *et al.*, 1995; Diebold *et al.*, 1996). Immunohistochemical studies with epithelial ovarian carcinomas indicate the presence of strong Bcl2 exression in tumours with low histological grade ($P = 0.004$), whereas p53 accumulation was associated with high grade tumours or advanced tumour stage (Diebold *et al.*, 1996). As observed in breast tumours (Haldar *et al.*, 1994b), the expression of Bcl2 was inversely related to the expression p53 in ovarian cancer (Henricksen *et al.*, 1995). Surprisingly, patients with Bcl2-positive ovarian carcinoma had a better outcome than p53-positive/BCL2-negative tumours. Clearly, apoptosis plays an important role in ovarian carcinoma, but apparently it is regulated in a different manner in neoplastic cells. Moreover, the cross-talk between Bcl2, p53 and Bax suggests that these apoptosis regulatory proteins can be important factors for modulating resistance to chemotherapy of ovarian cancer (Elipoulos *et al.*, 1995).

The isolation of a new pro-apoptotic gene called *bok* (*bcl2*-related ovarian killer) from a rat ovarian fusion cDNA library by a yeast two-hybrid system (Hsu *et al.*, 1997) indicates that there may be tissue-specific expression of apoptosis regulators. Northern blot analysis indicated that the expression of Bok was restricted to reproductive tissues including the ovary, testis and uterus. This tissue-specific expression of Bok suggests a potential role in regulating apoptosis in ovarian follicles as well as the monthly occurrence of apoptosis in uterine endometrial cells during menstruation.

Uterus

During early pregnancy, apoptosis is prevalent in both fetal and maternal tissues. A study by Lea *et al.* (1997) revealed the presence of Bcl2 protein in the stromal and glandular epithelial cells of decidual and placental tissue from women with a history of recurrent pregnancy loss as well as women undergoing sporadic miscarriage or surgical termination of pregnancy. Moreover, McLaren *et al.* (1997) reported the localization of both Bcl2 and Bax in human endometrium. Isolated peritoneal fluid macrophages in endometriosis also demonstrate the presence of these apoptosis regulatory proteins.

Prostate

While normal human prostatic secretory epithelial cells do not express Bcl2, immunohistochemical studies on neoplastic human prostate tissues demonstrated an elevated expression of this apoptosis inhibitory protein (Colombell *et al.*, 1993). A number of hormone-refractory prostatic adenocarcinomas were also found to express elevated levels of Bcl2 protein. Further immunohistochemical studies (Krajewska *et al.*, 1996) detected alterations in the expression of other anti-apoptotic as well as pro-apoptotic genes in the adenocarcinoma of the prostate. Out of 64 tumours evaluated, all of them expressed Bcl-X and its expression was found to be stronger in high grade (Gleason grade 8–10) tumours. Bcl2 expression was limited to 25% of adenocarcinomas and seemed to be more frequent in high grade tumours (grade 8–10; 41%) and nodal metastases (38%). The anti-apoptotic protein Mcl-1 was also typically expressed in higher grade tumors (81%). In contrast, the pro-apoptotic protein Bax was present in all types of prostate tumours regardless of the stage of progression.

In order to assess the incidence of intranuclear DNA fragmentation and the expression of the Bcl2 oncoprotein, the tumours obtained from newly diagnosed patients as well as hormone-treated prostatic carcinoma patients undergoing total prostatectomy were evaluated. There was no relationship between DNA fragmentation and Bcl2 positivity (Taniguchi *et al.*, 1996). Moreover, the results yielded from these studies also indicated that Bcl2-positive cells are potentially hormone resistant and that anti-androgen drugs might play a role in the induction of hormone-resistant cells.

Testis

Attempts to culture human seminoma (SE) cells *in vitro* have been unsuccessful because of massive degeneration of the tumour cells (Olie *et al.*, 1996). Investigations were therefore carried out to determine whether apoptosis was caused by disruption of the microenvironment of the seminoma cells by dissociation from tumour tissue. Indeed, these cells appear to rapidly enter the apoptotic pathway when they are deprived of their microenvironment surrounding the tumour tissue. Neither p53 nor Bcl2 were detectable in intact SE tissue or cell suspensions, although studies with testicular neoplasm clearly indicate the presence of apoptosis regulatory proteins Bcl2, Bax and p53. Metastatic testicular cancers are curable, whereas bladder cancers and most other solid tumours are not. The reason is attributed to the hypersensitivity of testicular tumours to chemotherapy due to the presence of functional p53 as well as a high Bax:Bcl2 ratio (Chresta *et al.*, 1996). The percentage of cells that underwent drug-triggered apoptosis was on average higher in the testicular tumour cell line than the bladder cell lines. Similar up-regulation of the pro-apoptotic Bax protein was found to be an early event in cisplatin-induced apoptosis in another human testicular germ-cell tumour cell line NT2 (Boersma *et al.*, 1997).

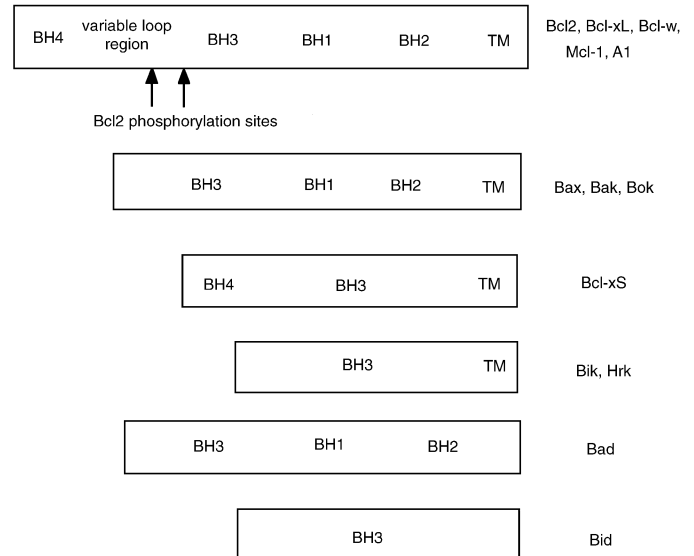

Figure 1. Structural organization of Bcl2-related apoptotic regulators. BH = Bcl2 homology domain; TM = transmembrane region.

Structure–function relationship of Bcl2 and Bax proteins

In the absence of a clear function of the Bcl2 protein, several laboratories have been engaged in determining the important structural domains in the protein and their roles in its anti-apoptotic function as well as its interaction with other proteins. Attempts have been made to identify novel proteins that interact with Bcl2 or enter into a multi-protein complex containing Bcl2. By sequence comparison of various Bcl2 proteins derived from different species, four structural domains have been characterized and termed as BH-1, BH-2, BH-3 and BH-4, where BH stands for Bcl2 homology domain (Oltvai *et al.*, 1993; Yin *et al.*, 1994; Hanada *et al.*, 1995; Zha,H. *et al.*, 1996) (Figure 1). Of interest is the absence of the BH4 domain in many of the pro-apoptotic Bcl2 family members including Bax, Bak, Bik and Bad. A unique role for this domain in regulating the function of the anti-apoptotic proteins such as Bcl2, Bcl-xL, Mcl1, A1 and Ced-9 cannot be unequivocally suggested because of its presence in the cell death-promoting protein Bcl-xS. This can be explained by two ways, one being no direct involvement of the BH4 domain in survival pathways. Alternatively, Bcl-xS protein may act as a dominant inhibitor of Bcl2 and its homologues by competing with protein domains that normally interact with BH4. Interestingly, another pro-apoptotic protein, Bik, contains only the BH3 domain, implying that this particular domain is responsible for promotion of apoptosis (Chittenden *et al.*, 1995). The regions

homologous to the BH3 domain are no doubt present in the anti-apoptotic proteins, but they are distinctly different in the sequence composition as well as the regulation of apoptosis. A relevant example is the conversion of the Bcl2 molecule from a cell death protector to death promoter by substituting its homologous BH3 domain with the corresponding domain of Bax (Hunter and Parslow, 1996). The recently cloned reproductive tissue-specific, pro-apoptotic Bok protein contains the BH1, BH2 and BH3 domains without BH4, similar to other pro-apoptotic family members.

Bcl2 proteins can form homodimers with themselves but the formation of these homodimers will be prevented if the *N*-terminal BH4 domain is deleted or if mutations in the downstream BH1 or BH2 regions are generated (Hanada *et al*., 1995). However, each of these mutant proteins is able to bind wild type Bcl2, resulting in mutant/wild type heterodimers which do not exert any effect in protecting mammalian cells from apoptosis (Borner *et al*., 1994). These observations suggest the importance of a correct Bcl2–Bcl2 homodimer formation for its anti-apoptotic function. It is apparent that a head-to-tail association is essential for Bcl2–Bcl2 homodimerization through a mutual interaction between the sequences located in the first 80 amino acid region including the BH4 domain and those located in the distal part of the protein where the BH1, BH2 and BH3 domains reside.

The first Bcl2-associated protein to be identified was Bax (Oltvai *et al*., 1993). As mentioned earlier, Bax is homologous to Bcl2 in sequence and co-immunoprecipitates with Bcl2 in cell extracts and *in vitro*. In functional assays, Bax can suppress the ability of Bcl2 to block apoptosis (Oltvai *et al*., 1993; Yin *et al*., 1994). Convincingly, excess Bax was always found to counter Bcl2 activity: accelerating apoptotic cell death, but only after a signal is received from an apoptotic trigger. In this context, it is interesting to know that the association between Bax and Bcl2 occurs in cells even before a death-inducing signal. When Bcl2 is in excess, either Bcl2–Bax heterodimers or Bcl2 homodimers predominate and the cells are protected. When Bax is in excess, Bax homodimers predominate and the cells are susceptible to apoptosis. The creation of Bax knockout mice further supported the role of Bax in accelerating apoptosis. The normal development of these mice accompanied by lymphoid hyperplasia was consistent with the pro-apoptotic role of Bax (Knudson *et al*., 1995). A number of biological systems indicate that, during development, cells vary in their inherent sensitivity or resistance to a given death stimulus. The ratio of Bcl2:Bax represents a cell-autonomous rheostat that predetermines the life or death response of a cell to an apoptotic stimulus.

The competitive dimerization between selective pairs of anti- and pro-apoptotic proteins is thought to determine the fate of a cell. The studies on pro-apoptotic proteins Bax and Bak involving deletion of different BH domains reveal that these proteins may function by forming dimers with both Bcl2 and Bcl-xL (Chittenden *et al*., 1995; Reed, 1997). Another pro-apoptotic member, Bok, preferentially binds with some of the anti-apoptotic proteins such as Mcl-1, BHRF1 or Bfl-1 but not with Bcl2, Bcl-xL or Bcl-W (Hsu *et al*., 1997). This preferential binding with Mcl-1 also explains the significance of overexpression of both Bok and Mcl-1 proteins in ovarian tissues. It is quite likely that selective pair formation of the pro- and anti-apoptotic partners can be an important factor for tissue-specific apoptosis regulation. Further elucidation of the dimeric partners for Bok in gonads and uterus will be very helpful for understanding the mechanism of apoptosis occurring in testicular germ cells and ovarian follicles.

Regulatory motifs for Bcl2–Bax interactions

The fact that the Bax protein lacks the BH4 domain suggests that the structural features by which Bcl2 interacts with Bax or interacts with itself are strikingly different. Substitution of certain single amino acids within the BH1 and BH2 domains completely abrogated the ability of Bcl2 to repress cell death. A particularly interesting finding was that those mutations which affected Bcl2 function also disrupted its heterodimerization with Bax, while still permitting Bcl2 homodimerization (Yin *et al*., 1994). These data build a strong case for the absolute necessity of Bcl2/Bax heterodimerization in favour of cell death suppression. However, the report by Zha,H. *et al*. (1996) indicating the requirement of the *N*-terminal region of Bcl2, the area of the BH4 domain, for its function, raises some logical concerns: (i) heterodimerization with Bax may not be sufficient for the ability of Bcl2 to inhibit on cell death; (ii) the Bcl2 protein must fulfil other functions as well. Mutant Bcl2 lacking the BH4 domain is not only unable to suppress apoptosis in mammalian cells but also fails to rescue yeast from Bax induced lethality (Sato *et al*., 1994; Hanada *et al*., 1995). On this basis, one provocative speculation would be that the BH4 domain of Bcl2 renders steric interference with the binding of other death effector proteins to Bax. The other explanation is that this domain is required for Bcl2–Bcl2 homodimerization and the interaction with other proteins that require the BH4 domain for association with Bcl2–Bax complex. In support of this hypothesis, it has been noted that the association of BAG-1 (which cooperates with Bcl2 in suppression of apoptosis) and Raf-1 with protein complexes containing Bcl2 involves the BH4 domain (Wang *et al*., 1994; Takayama *et al*., 1995). Thus the BH4 domain of Bcl2 could serve as an effector domain for linking other proteins to Bcl2 and the distal BH1 and BH2 domains could represent a dimerization domain that targets Bcl2 and its associated protein to Bax. Recent studies have shown that the BH1 and BH2 domains of Bax are expendible for both homodimerization and heterodimerization with Bcl2. In contrast, the BH3 domain of Bax is absolutely required for binding to both wild type Bax and Bcl2. This suggests that despite the striking homology between the amino acid sequences of the two proteins, Bcl2 and Bax differ extensively in three-dimensional structure. Unlike Bcl2–Bcl2 homodimerization, which involves a head to tail interaction, both Bcl2–Bax and Bax–Bax dimerization occurs through tail-to-tail interaction. Mutant Bax that has an altered BH3 domain can neither homodimerize nor promote cell death, indicating that Bax homodimerization is critical for a pro-apoptotic function.

Proposed mechanism of Bcl2 protein action

The mechanism by which Bcl2 prevents cell death remains enigmatic. The subcellular localization of Bcl2 may provide a clue to its function. Bcl2 has been found to be associated with mitochondria, the nuclear membrane, and smooth endoplasmic reticulum (Krajewski *et al*., 1993; Haldar *et al*., 1994a). These sites have in common an oxidation–reduction function that can result in oxygen free radical generation. Several stimuli that can promote apoptosis are linked to oxidative stress, including ionizing radiation, tumour necrosis factor and phorbol esters. Early studies attributed the anti-apoptosis function of Bcl2 to mitochondrial energy metabolism (Hockenbery *et al*., 1993). However, since enforced Bcl2 expression blocks apoptosis in a mutant fibroblast cell line lacking an intact respiratory chain, mitochondrial energy metabolism is not probably the key (Jacobson *et al*., 1993). Bcl2 may, however, be important in membrane lipid integrity by suppressing the generation of reactive oxygen species (Kane *et al*., 1993). Experimental evidence suggesting regulation of intracellular Ca^{2+} homeostasis by Bcl2 has also been reported (Baffy *et al*., 1993; Lam *et al*., 1994). It is possible that the effects of Bcl2 on lipid peroxidation and Ca^{2+} transfer are related since Ca^{2+} can influence the activity of some enzymes involved in lipid metabolism. Bcl2 has also been implicated in protein transport across biological membranes (Liu *et al*., 1996; Kluck *et al*., 1997; Yang *et al*., 1997). Recently, the three-dimensional structure of Bcl-xL, an anti-apoptotic homologue of Bcl2, revealed striking similarity to the pore-forming domains of diphtheria toxin and colicins (Muchmore *et al*., 1996). Subsequent studies have demonstrated that purified Bcl-xL and Bcl2 proteins are able to form ion channels on synthetic lipid membranes (Minn *et al*., 1997; Schendel *et al*., 1997). This finding has significant relevance as ion channels may regulate susceptibility to apoptosis. On the basis of genetic studies in the nematode *C.elegans*, it has been postulated that Bcl2 may control the activity of a family of cysteine proteases with homology to interleukin-1β converting enzyme (ICE) (Miura *et al*., 1993; Yuan *et al*., 1993). Recent experiments have demonstrated that overexpression of Bcl2 can prevent the proteolytic processing and activation of the ICE family homologue, CPP32/YAMA, in mammalian cells (Armstrong *et al*., 1996; Boulkia *et al*., 1996; Chinnaiyan *et al*., 1996). Additionally the association of Bcl2 with the kinase Raf-1 and possibly with the GTPase R-Ras, both of which are growth factor receptor signal transducers, could implicate a role in a signal transduction pathway that is exclusive for the intracellular membrane compartments where Bcl2 resides rather than the plasma membrane (Wang *et al*., 1994, 1995, 1996). Bcl2 has been shown to provide protection against cell death arising from a broad range of stimuli with numerous biological mechanisms of action in cells, suggesting that Bcl2 functions at a distal point in a pathway leading to cell death. It is true that these various stimuli transmit different upstream signals causing cells to die by apoptosis, but the final pathway utilized by them is identical. Currently it is not clear whether the effects of Bcl2 on redox state, Ca^{2+} compartmentalization, protein transport, ion channels, and protease activation, are directly responsible for controlling a downstream cell death program.

Balanced Bcl2 phosphorylation/dephosphorylation — the molecular determinant of cell survival?

Phosphorylation is an important mechanism for controlling the function of proteins involved in cellular signalling pathways. Since morphological changes typical of apoptosis were induced in mammalian cells following treatment with the protein phosphatase inhibitor okadaic acid (Boe *et al*., 1991), we investigated the effect of okadaic acid on the function of Bcl2 protein. We demonstrated that at high concentrations okadaic acid could induce phosphorylation of Bcl2 with simultaneous induction of apoptosis (Haldar *et al*., 1995). Moreover we showed that the effect of okadaic acid could be mimicked by microtubule targeting anti-neoplastic drugs, such as taxol, taxotere, vincristine etc. (Haldar *et al*., 1996, 1997, 1998). In the face of Bcl2 overexpression, a panel of human cancer cells could not be protected from taxol-induced apoptosis when there was simultaneous phosphorylation of Bcl2 (Haldar *et al*., 1995, 1996, 1997, 1998; Blagosklonny *et al*., 1996, 1997, Srivastava *et al*., 1998). A recent study (Chang *et al*., 1997) has identified a novel regulatory domain in Bcl2 and Bcl-xL. The three-dimensional structure of Bcl-xL revealed a 60 amino acid loop lacking a defined structure (Muchmore *et al*., 1996). Although this 60 amino acid loop is not conserved among all family members, structural modelling suggested that Bcl2 also contained this unstructured region. Compared with the full-length protein, loop deletion mutants of Bcl2 and Bcl-xL displayed an enhanced ability to inhibit apoptosis. Full length Bcl2 was unable to prevent anti-IgM-induced cell death of the immature B cell line, WEHI-231. In contrast, a Bcl2 deletion mutant lacking this loop region protected these cells from apoptotic cell death. Phosphorylation was found to be dependent on the presence of the intact loop domain. These results suggest that the loop domain in Bcl-xL and Bcl2 can suppress the anti-apoptotic function of these genes and may be a target for post-translational modification. The enhanced anti-apoptotic effect of deletion mutant (deletion of amino acid 51–85) of Bcl2 was also observed in transfected c-myc-transformed Rat1 fibroblasts (Uhlmann *et al*., 1996).

By co-immunoprecipitation and subsequent immunoblot analysis using antibodies against Bcl2 and Bax respectively, we have demonstrated that following exposure of prostate or breast cancer cells to the chemotherapeutic drug taxol, there is $>50\%$ reduction of Bax protein in the immunocomplex immunoprecipitated by Bcl2 antibody. Thus, the physical association of Bcl2 and Bax is disrupted to a certain extent by taxol-induced phosphorylation of Bcl2 (Haldar *et al*., 1996; Srivastava *et al*., 1998). These data are consistent with the previous report that a 50% reduction in the formation of Bcl2/Bax heterodimers can drive the cells toward apoptosis (Yang *et al*., 1995). Our working model, as proposed in Figure 2, suggests that Bcl2 might exist in a hypophosphorylated condition to maintain its anti-apoptotic property. Apparently, a microtubule-damaging drug, perhaps through activation of a

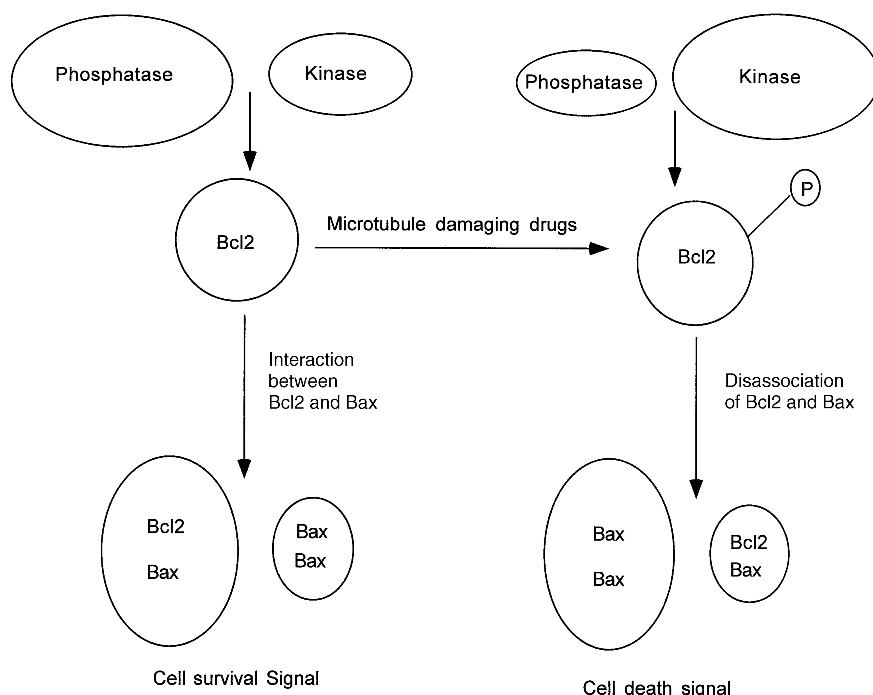

Figure 2. Working model for p53-independent apoptosis induced by microtubule-damaging drugs. The abundance of Bcl2-specific phosphatase/kinase or Bax–Bcl2/Bax–Bax dimers is indicted by the size of the oval.

kinase or cascade of kinases, can cause Bcl2 hyperphosphorylation and thus leads to its loss of function by dissociation of its dimeric partner Bax.

In this context, it is worth mentioning that the phosphorylation of another pro-apoptotic protein, BAD, by protein kinase Akt leads to its loss of function (Datta *et al.*, 1997; Peso *et al.*, 1997). Phosphorylated BAD has reduced ability to form heterodimers with its anti-apoptotic partner, Bcl-xL (Zha,J. *et al.*, 1996). Apparently, phosphorylation of Bcl2 family proteins (Bcl2, Bcl-xL, and BAD) interferes with their binding abilities to the respective partners, thus resulting in functional inactivation.

Induction of Bcl2 phosphorylation occurs at G2–M phase of the cell cycle

Anticancer drugs affecting either microtubule polymerization or depolymerization could evoke Bcl2 phosphorylation. On the contrary, drugs which damage DNA do not induce Bcl2 phosphorylation but induce apoptosis through a different, p53-dependent mechanism (Clarke *et al.*, 1993, 1994; Lowe *et al.*, 1993; Ziegler *et al.*, 1994; Merritt *et al.*, 1994, 1997). Phosphorylation of Bcl2 occurs at G2–M phase of the cell cycle. When B leukaemic cells were sorted into pools of cells in G0–G1–S and G2–M following treatment with vincristine, no significant Bcl2 phosphorylation was detected in the cells at G0–G1–S, whereas abundant phosphorylated Bcl2 was detected in the G2–M fraction of treated cells (Haldar *et al.*, 1997; 1998). Human chronic lymphocytic leukaemia (CLL) cells overexpress Bcl2, show increased survival, and are blocked at G0–G1 (Moore *et al.*, 1995). Treatment of these cells with microtubule-damaging drugs did not result in phosphorylation of Bcl2. A corollary of this finding is that drugs affecting microtubule integrity are effective in killing Bcl2-expressing tumours with high mitotic index. All these results suggest that Bcl2, through its phosphorylation, acts as guardian of microtubule integrity. Because microtubules play an important role in chromosome segregation, alteration in microtubules could cause genomic instability. Thus cells with altered microtubules should be eliminated through the induction of Bcl2 phosphorylation, probable loss of Bcl2 function, and apoptotic death. Recently, it has been shown that cells overexpressing the apoptosis-inhibitory protein Bcl-xL have an increased rate of spontaneous tetraploidization, suggesting that apoptosis may be important in eliminating cells failing to complete mitosis (Minn *et al.*, 1996). Not much information is available about the mammalian proteins that control the G2–M transition or those monitoring the formation of spindle or spindle poles. Several lines of evidence suggest that defects in the regulation of these processes could be important in human tumorigenesis. Our results suggest that post-translational modifications like phosphorylation at G2–M phase of the cell cycle could conceivably account for altered function of Bcl2, which in turn might determine the varied apoptotic thresholds of different cell types against antimicrotubule agents.

Potential role of Bcl2 in cell cycle events

That Bcl2 might be involved in controlling cell cycle events is reflected by its effect of delaying re-entry into the cell cycle after growth factor withdrawal (Marvel *et al.*, 1994). Interleukin-3 (IL-3)-dependent lymphoid cells will normally undergo rapid apoptosis following growth factor withdrawal and they die in the G0–G1 state of the cell cycle. The stable transfection of Bcl2 in these cells results in the prolonged survival under a non-cycling condition, halted in G0–G1

state. The re-addition of the growth factor (IL-3) to Bcl2-overexpressing cells enables them to re-enter into the cell cycle after some delay. The period of lag phase before entry into S phase is dependent on the length of the time of growth factor deprivation. The shorter the deprivation time, the lesser is the delaying effect of Bcl2 and vice-versa. These results are suggestive of the antagonistic function of Bcl2 for G0–G1 transition following a period of quiescence. Several models available in the literature indicate that cells accumulate in some specific phases of the cell cycle instead of committing suicide.

Another significant example of the indirect effect of Bcl2 on the cell cycle response was reported in the case of p53 induced apoptosis in murine erythroleukaemia cells. The constitutive overexpression of temperature-sensitive mutant p53 (p53-Val 135) in these cells can cause growth arrest at G1 phase and the elimination of cells by apoptosis. The co-transfection of Bcl2 not only abrogated the cell death, but also resulted in a different pattern of cell cycle arrest. Cells expressing both Bcl2 and p53 were found to be arrested throughout all phases of the cell cycle in contrast to the G1 phase arrest associated with p53-mediated apoptosis (Miyashita *et al*., 1994a,b). Although the above examples demonstrate some promise in the regulatory function of Bcl2 on p53-induced cell cycle arrest and apoptosis, little difference was noted in the cell cycle status when investigated with other apoptosis-inducing agents.

p53 and Bcl2 family proteins in relation to programmed cell death

Although Bcl2-related proteins have received most attention, other non-Bcl2-like proteins have also been identified as regulators of apoptosis. The best example is the tumour suppressor gene p53. It is now thought that the tumour suppression effect of p53 is related to induction of irreversible cell-suicide process or the imposition of reversible growth-arrest phenomenon (White, 1996). Although numerous studies have implicated deregulated expression of Bcl2 or loss of p53 function in human neoplasia, these changes have rarely overlapped. Bcl2 has been mainly studied in leukaemia and lymphoma, although it is clear now that its expression is not restricted to the haematopoietic system. p53 instead has been mainly studied in human solid tumours, where it appears to be the gene most frequently altered (Levine, 1997). Wild type p53 induces apoptosis. Numerous DNA-damaging agents that trigger programmed cell death also induce p53 expression. The mechanism of p53-mediated cell death is possibly associated with its function as transcriptional modulator and this may be mediated by activation or suppression of the transcription of other genes. p53 has been shown to induce cell cycle arrest at the G1–S transition. This action of p53 has been ascribed to its ability to induce the expression of a cellular gene p21^{WAF1} which encodes a 21 kDa inhibitor of cyclin-dependent kinases (El-Deiry *et al*., 1993). It has been demonstrated that both the expression of p53 and its transcriptional activity are elevated in cells exposed to radiation and DNA-damaging drugs. The specific functions of p53 in this process probably include the arrest of cycling cells before S phase to allow for repair of damaged DNA prior to DNA replication and the induction of apoptosis for cases where the DNA damage is too severe to be repaired properly. Loss of p53 could lead to the genomic instability common in tumour cells, by allowing damaged DNA to replicate and promoting cell survival so that the genomic alterations accumulate with time. Studies in *p53* knockout mice suggest that the effect of p53 on cell cycle arrest and apoptosis are two separable functions. It has been demonstrated that p53 is required for the induction of apoptosis *in vivo* by γ-radiation and other DNA-damaging drugs in thymocytes which are non-cycling cells in G0–G1 phase (Clarke *et al*., 1993; Lowe *et al*., 1993). Also in *p53* knockout mice, tumour formation was related to a reduced rate of cell death rather than an increased rate of cell proliferation (Symonds *et al*., 1994).

One of the mechanisms that plays a role in the dysregulation of Bcl2 expression in cancers is loss of the tumour suppressor p53. We have found an inverse correlation between the expression of the two proteins in several human breast cancer cell lines (Haldar *et al*., 1994b). This result suggested that loss of p53 function could substitute for increased Bcl2 activity in breast cancer cells and also that p53 could down-regulate Bcl2 expression. We found that overexpression of a mutant p53 in MCF-7 cells could down-regulate Bcl2 at both the protein and mRNA levels. In a p53-deficient murine leukaemia line, overexpression of wild type p53 also can decrease *bcl2* gene expression followed by apoptotic cell death (Miyashita *et al*., 1994a). Maintaining Bcl2 protein level high through gene transfer manipulations (Selvakumaran *et al*., 1994) could block p53-induced apoptosis. It has also been shown that treatment of human leukaemia line with γ-radiation, a known inducer of p53, results in a decreased level of Bcl2 mRNA (Zhan *et al*., 1994). A p53-negative response element has been mapped in the 5′ untranslated region of *bcl2* gene (Miyashita *et al*., 1994b). Thus loss of p53 in human tumours might account for the aberrant expression of Bcl2 protein in many types of cancer. Correspondingly, in these types of malignancies a major mode of resistance to antitumour treatments may be insensitivity to apoptosis induction due to elevated level of anti-apoptotic protein Bcl2.

In addition to down-regulation of Bcl2, restoration of p53 in the murine leukaemia cell M1 was associated with an increase in Bax mRNA and protein (Miyashita *et al*., 1994a, 1994b; Selvakumaran *et al*., 1994). Recent studies revealed that the pro-apoptotic *bax* gene is a direct transcriptional target of p53 (Miyashita and Reed, 1995). In reporter gene assay p53 has been shown to strongly transactivate *bax* gene promoter. Thus tumours with loss of p53 function are expected to contain relatively low levels of Bax protein. In these tumours, the ratio of Bcl2/Bax protein is markedly decreased as a result of the inverse effects of p53 on Bax and Bcl2. Under these circumstances, the malignant cells become more vulnerable to apoptotic stimuli, which is the key to successful chemotherapy or radiation therapy. However, the regulation of cell life and death *in vivo* is probably more complex and may be cell-type specific. In this context, it was recently reported that p53-mediated induction of new gene expression is not obligatory for triggering apoptosis in response to UV radiation

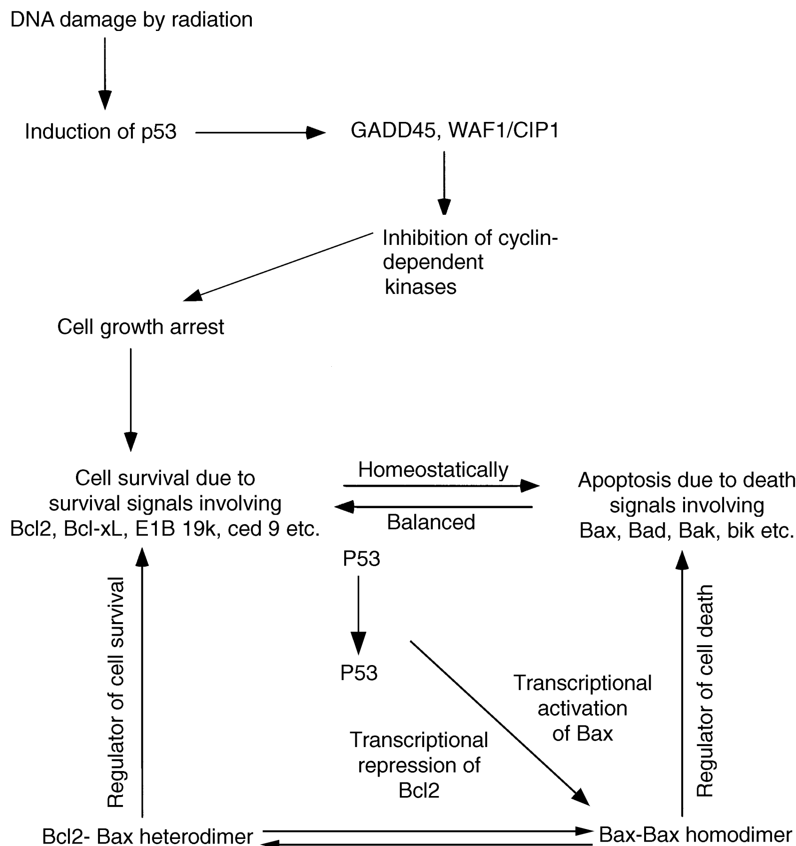

Figure 3. Control of cell life and death by Bcl2, Bax and p53.

in a pituitary tumour cell line (Caellas *et al.*, 1994). Irrespective of an obligatory requirement for stimulation of *bax* gene expression for the induction of p53-dependent apoptosis (McCurrach *et al.*, 1997) in all types of cells, it is logical to propose that p53-mediated elevation of Bax protein level would at least render the cells more susceptible to apoptosis. Studies with the *bcl2* knockout mice or with an antisense approach to reduce Bcl2 expression suggest that a low ratio of Bcl2:Bax is insufficient for triggering apoptosis but rather renders the cell more sensitive to various apoptotic stimuli. Interestingly, *bax*-deficient female mice displayed ovaries containing unusual atretic follicles with an excess of granulosa cells (Ratts *et al.*, 1995; Knudson *et al.*, 1995; Knudson and Korsmeyer, 1997), whereas the male counterparts were found to be infertile with no mature haploid spermatozoa. The same *bax*-deficient mice displayed hyperplasia in B and T lymphocytes. Thus it is apparent that the loss of Bax may result in hyperplasia or hypoplasia, depending on the cellular context. Another report by Perez *et al.* (1997) shows that oocytes isolated from *bax*-deficient, but not *p53*-deficient, null female mice confer complete resistance to doxorubicin. Doxorubicin, a well-known DNA-damaging, antitumour drug, can trigger apoptosis in unfertilized mouse oocytes but not in fertilized ones. Pretreatment of unfertilized oocytes with a specific inhibitor of caspases can reverse the apoptotic effect of doxorubicin. This observation is very important because female sterility, in many cases, is an inevitable consequence of chemotherapy.

p53 simply adjusts the sensitivity of the cell to respond to programmed cell death through transcriptional regulation of target genes. However, p53-mediated Bax induction may not be the only pathway by which p53 can induce cell death (Canman *et al.*, 1995). p53 is not essential for all aspects of apoptosis in murine development. Thus p53 appears to be a surveillance factor to induce apoptosis under specific circumstances, whereas apoptosis in normal development can proceed through a pathway independent of p53.

The activity of p53 as a transcription factor has been shown to be responsible for promotion of growth arrest by p53. In contrast, p53-induced apoptosis can be attributed to both transcriptionally dependent and independent mechanisms. When the Bax level is low, induction of apoptosis may require up-regulation of Bax expression by p53. When the Bax level is constitutively high, Bax induction to induce cell death may not be necessary, and in that scenario, an alternative transactivation-independent pathway for p53-dependent apoptosis might be revealed (Caelles *et al.*, 1994). Another possibility is that p53 promotes apoptosis through transcriptional repression of a survival factor, and this repression is normally prevented by anti-apoptotic members of the Bcl2 family.

Conclusion

With many regulators of the cell survival and apoptosis network being identified, it is now possible to define the

functional relationship between them in controlling the fate of the cell (Figure 3). Although life and death in a cellular environment will be controlled in a cell type-specific manner, the basic machinery for controlling cell viability might be universal. There is unceasing exchange of information between cell cycle progression and programmed cell death, the two important physiological modulators that ensure the maintenance of overall number of cells in an appropriate range. The susceptibility of cells to apoptosis is often dependent on their state of activation and position in the cell cycle. Phosphorylation of Bcl2 in cells with highest mitotic index implicates its functional regulation in a cell cycle-mediated manner. The most compelling evidence to date suggests that Bcl2 members may regulate apoptosis partly by modulating entry and progression through the cell cycle (Chao and Korsmeyer, 1998) by attenuating oxidative stress or by modulating the ICE-like cysteine protease cascade which is activated in most forms of apoptosis. Developmental cues directly or indirectly affect the function of Bcl2 family members. During development, coordination between cell growth and cell death is essential. Apoptosis during development is thought to occur through a p53-independent mechanism (Donehower *et al*., 1992). However, in damaged cells, p53 imposes growth arrest and death. Implementation of growth arrest is facilitated by the p53-mediated induction of the cell cycle inhibitor $p21^{WAF1}$. Also one cannot exclude the possibility of the functional redundancy between p53 and p73, a human p53-related protein that can induce apoptosis (Jost *et al*., 1997).

Stimulation of Bax gene expression by p53 promotes apoptosis as a result of a perturbed ratio of Bax to Bcl2. Overexpression of Bcl2 can block p53-dependent apoptosis. The level of Bcl2-like activity in the cell determines whether the cell will undergo apoptosis or growth arrest. Under these circumstances, cells with low Bcl2 activity will be destined to die whereas if there is enough Bcl2, to neutralize Bax activity, growth arrest will occur. The events upstream or immediately downstream of Bcl2/Bax heterodimerization are not yet mapped. However, the family of ICE-like cysteine proteases is likely to be involved. Bcl2 becomes phosphorylated at two serine residues, one (serine 70) lying in the loop region and another (serine 87) in close proximity to the loop region (Basu and Haldar, 1998). Phosphorylation of protein is a ubiquitous mechanism to regulate the function of the protein. A compelling correlation exists between phosphorylation of Bcl2 and loss of anti-apoptotic function in a variety of cancer cell lines. Despite the correlative nature of the data, this widespread phenomenon does stimulate future research addressing the mechanistic aspects of phosphorylation, such as identifying the kinase(s) and phosphatase(s) involved in Bcl2 activation/inactivation. It would be interesting to know how this post-translational modification interferes in its association with known partners such as Bax. The outcome of such investigations will have a tremendous impact on chemotherapeutic strategies of inducing apoptosis through reduced expression of anti-apoptosis oncogene *Bcl2*. However, translation of these new insights to clinical usefulness remains the ultimate and perhaps the most difficult task of the future.

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