BCL-2 Gene Family and the Regulation of Programmed Cell Death

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I hope he doesn’t mind me saying this, but Dr. Stan Korsmeyer has achieved an enormous amount in a relatively short scientific career. You can take that whichever way you want to. Good, now I have larded myself in it.

Stan Korsmeyer did his undergraduate degree in the University of Illinois and then did an M.D. at the same university and graduated in ’76. Then, after doing an internship in UCSF Medical School, he came to the NIH where he was a clinical associate working in collaboration with Phil Leder and Tom Waldmann.

There are some interesting parallels with Suzanne’s career at this stage. Stan worked quite a lot on the structure of the immunoglobulin genes, their rearrangements in normal B cell development, and also studied aberrant rearrangements in leukemias.

He set up his own lab in, I believe it was, ’83 at NIH and then moved to Washington University School of Medicine at St Louis where he became an associate professor in about 1986. The first pivotal contribution in relation to this prize, was his isolation, at the same time as others, of the bcl-2 oncogene at the breakpoint of a translocation between chromosomes 14 and 18 in B cell follicular lymphoma.

Very shortly after Suzanne and colleagues published their seminal study showing that bcl-2 promotes and extends B cell survival, Stan showed essentially the same thing in transgenic mice where he overexpressed the bcl-2 oncogene in B cells and was able to show that B cells had extended survival and went on to develop lymphomas. So, a wonderful convergence of in vitro findings in cell culture from Suzanne’s lab and Stan’s finding in mice.

Stan has continued over the years to make some very important contributions to our understanding of bcl-2 function and also of bcl-2 relatives, as you have already heard from Suzanne. One of the first things he showed was that bcl-2 may have a function in mitochondria, and this notion received a great deal of support from findings published last year by several groups.

Using a very elegant set of biochemical approaches, he was able to show that bcl-2 is a member of a larger family and that these family members can interact with each other. As you have heard, some of them act to promote and some to inhibit apoptosis. So, we have a very interesting network of proteins here.

By knocking out different members of these families, Stan was also able to show that these genes really do play vital roles in cell survival in whole animals, obviously, a very important thing to do. He continues to work on the function of the proteins, and it is remarkable to think that, while doing this, he also was able to show the important roles of Hox-11 and Mll both in normal development and in leukemias.

Since 1992 Stan has been the Chief of the Division of Molecular Oncology at Washington University School of Medicine, St. Louis and since 1993 has been an investigator of the Howard Hughes Medical Institute. In recognition of his pioneering work, Stan has already received a number of awards including the Ciba-Drew Award in 1996, the GHA Clowes Award, American Association for Cancer Research in 1997 and the Bristol-Myers Squibb Award for distinguished achievements in cancer research, 1997. He was elected to the National Academy of Sciences in 1997.

So, we look forward very much to hearing Stan’s talk on the bcl-2 family and the regulation of cell death.

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Abstract

The BCL-2 gene was identified at the chromosomal breakpoint of t(14;18)-bearing human follicular B cell lymphomas. BCL-2 proved to block programmed cell death rather than promote proliferation. Transgenic mice that overexpress Bcl-2 in the B cell lineage demonstrate extended cell survival and progress to high-grade lymphomas. Thus, BCL-2 initiated a new category of oncogenes, regulators of cell death. Bcl-2-deficient mice demonstrate fulminant apoptosis of lymphocytes, profound renal cell death and loss of melanocytes. BCL-2 protein duels with its counteracting twin, a partner known as BAX. When BAX is in excess, cells execute a death command; but, when BCL-2 dominates, the program is inhibited and cells survive. Bax-deficient mice display cellular hyperplasia, confirming its role as a pro-apoptotic molecule. An expanded family of BCL-2-related proteins shares homology clustered within four conserved regions termed BCL-2 homology 1 through 4 (BH1–4). These novel domains control the ability of these proteins to dimerize and function. An amphipathic α helix, BH3, is of particular importance for the proapoptotic family members. BID and BAD represent an evolving set of proapoptotic molecules, which bear sequence homology only at BH3. They appear to reside more proximal in the pathway serving as death ligands. BAD connects upstream signal transduction paths with the BCL-2 family, modulating this checkpoint for apoptosis. In the presence of survival factor interleukin-3, cells phosphorylate BAD on two serine residues. This inactivated BAD is held by the 14-3-3 protein, freeing BCL-XL and BCL-2 to promote survival. Activation of BAX results in the initiation of apoptosis. Downstream events in this program include mitochondrial dysfunction, as well as Caspase activation. The pro- and antiapoptotic BCL-2 family members represent central regulators in an evolutionarily conserved pathway of cell death. Aberrations in the BCL-2 family result in disordered homeostasis, a pathogenic event in diseases, including cancer.

Introduction

Programmed cell death plays an indispensable role in the development and maintenance of homeostasis within all multicellular organ-
isms. Genetic and molecular analysis from nematodes to humans has indicated that the pathway of cellular suicide is highly conserved. Although the capacity to carry out apoptosis seems to be inherent in all cells, the susceptibility to apoptosis varies markedly and is influenced by external and cell-autonomous events. Considerable progress has been made in identifying the molecules that regulate the apoptotic pathway at each level. Of note, both positive and negative regulators, often encoded within the same family of proteins, characterize the extracellular, cell surface, and intracellular steps.

The t(14;18) Breakpoint of Human Follicular Lymphoma Provides the BCL-2 Gene. The t(14;18):(q32;q21) constitutes the most common chromosomal translocation in human lymphoid malignancies, being present in 85% of follicular and 20% of diffuse B-cell lymphomas. As a disease, follicular lymphoma provided many clues concerning the ultimate function of the BCL-2 molecule. Follicular lymphoma often presents as a low-grade malignancy composed of small resting IgM/IgD B cells. Over time, conversion to an aggressive high-grade lymphoma with a diffuse large-cell architecture frequently occurs in these patients. The location of the immunoglobulin heavy chain locus at 14q32 and the B-cell phenotype of this lymphoma provided the rationale for cloning the chromosomal breakpoint. Aberrant immunoglobulin heavy chain rearrangements in t(14;18) lymphomas proved to be the chromosomal breakpoint and delivered a candidate oncogene, BCL-2, at 18q21 (1–3). BCL-2 is normally located on chromosome segment 18q21.3 in a telomere to centromere orientation. A molecular consequence of the translocation is the movement of the BCL-2 gene to the der (14) chromosome (Fig. 1). This places BCL-2 in the same transcriptional orientation as the immunoglobulin heavy chain locus, giving rise to chimeric RNAs. However, translocation does not interrupt the protein-encoding region so that normal and translocated alleles produce the same-sized, 25-kDa protein. t(14;18)-bearing B cells have inappropriately elevated levels of the BCL-2-immunoglobulin fusion RNA and BCL-2 protein (4, 5).

BCL-2-immunoglobulin Transgenic Mice Progress from Follicular Hyperplasia to High-Grade Lymphoma. A stringent test of a gene’s oncogenic capacity is to place it into the germline of mice to observe its effects during the development of an entire organism. Transgenic mice bearing a BCL-2-immunoglobulin minigene initially displayed a polyclonal follicular lymphoproliferation that selectively expanded a small resting IgM/IgD B-cell population (Refs. 6, 7; Fig. 1). Cell cycle analysis confirmed that ~97% of the expanded B cells reside...
in G1/G0. These recirculating B cells accumulate because of an extended survival rather than increased proliferation. Despite a 4-fold increment in resting B cells, BCL-2-immunoglobulin transgenic mice are initially quite healthy. However, over time, these transgenics progress from indolent follicular lymphomas to diffuse large-cell immunoblastic lymphoma. A long latency period and progression from polyclonal hyperplasia to monoclonal high-grade malignancy is an indictment of secondary genetic abnormalities.

Approximately half of the high-grade tumors possess a second genetic alteration that confers an inherent survival advantage in their wild-type form. These high-grade tumors had also lost the bcl-2 transgene and retained a c-myc translocation involving an immunoglobulin heavy-chain locus (8). These tumors may have complemented an inherent survival advantage (bcl-2) with a gene that promotes proliferation (myc). When bcl-2 transgenic mice were mated with myc transgenic mice, rapidly emerging undifferentiated hematopoietic leukemia occurred, providing further testimony for the hypothesis that deregulation of bcl-2 is sufficient to confer the effect. Category II represents the classic tumor suppressor genes that, in their wild-type form, inhibit growth and proliferation (18). The first oncogenes discovered, category I, promote cell growth and proliferation. Most of these can be classified as transcription factors or molecules involved in signal transduction. In general, these genes contribute to cancer after an alteration resulting in a gain of function. They usually display an autosomal dominant mechanism, in which a single altered allele is sufficient to confer the effect.

BCL-2 Blocks Apoptosis Initiating a New Category of Oncogenes: Regulators of PCD. BCL-2 blocks cell death after a wide variety of stimuli. BCL-2 conferred a death-sparing effect to hematopoietic cell lines after growth factor withdrawal, including the IL-3-dependent early hematopoietic cell lines FDCP1, FL5.12, and 32D (14-16). A detailed examination of the interference of BCL-2 with cell death revealed it does not alter the dose response to limiting concentrations of IL-3 (16). Instead, BCL-2 blocked the plasma membrane blebbing, volume contraction, nuclear condensation, and endonucleolytic cleavage of DNA, (15) termed apoptosis by Wyllie (17). By preventing the apoptotic demise of activated lymphocytes, BCL-2 enables the acquisition of additional genetic aberrations and the emergence of monoclonal neoplasms. By repressing normal apoptotic pathways, BCL-2 allows cells that sustain DNA damage to avoid a suicide response.

Oncogenic events had concentrated on mechanisms of increased growth and proliferation. However, increased cell number, which violates normal homeostasis, could arise from either increased proliferation or decreased death. BCL-2 provided the first example of oncogenesis mediated by decreased cell death. Table 1 depicts two classes of oncogenes that regulate growth and proliferation (18). The first oncogenes discovered, category I, promote cell growth and proliferation. Most of these can be classified as transcription factors or molecules involved in signal transduction. In general, these genes contribute to cancer after an alteration resulting in a gain of function. They usually display an autosomal dominant mechanism, in which a single altered allele is sufficient to confer the effect. Category II represents the classic tumor suppressor genes that, in their wild-type form, inhibit growth and proliferation (18). A unique aspect of two experimentally proven members, Rb and p53, is their frequent contribution to unchecked growth by loss of function. The effects of deregulated BCL-2 argue that it does not qualify as either a category I or II oncogene. Instead, BCL-2 seems to be the cardinal member of a new category of oncogenes: regulators of PCD (Table 1).

**BAX, a Proapoptotic Member and Expansion of the Family.** The first proapoptotic homologue, BAX, was identified by coin-
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Table 2  Bcl-2 −/−

<table>
<thead>
<tr>
<th>T cell apoptosis</th>
<th>Immune deficiency</th>
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<tr>
<td>Renal mesangial apoptosis</td>
<td>Polycystic kidney</td>
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<tr>
<td>Melanocyte death</td>
<td>Hypopigmentation</td>
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<td>Primordial follicles</td>
<td>Infertility</td>
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Table 3  Bax −/−

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<tr>
<th>Lymphoid hyperplasia</th>
<th>Sterility</th>
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<tr>
<td>Spermatogonia hyperplasia</td>
<td>NGF independence; protection from stroke</td>
</tr>
<tr>
<td>Primordial follicles</td>
<td>Oocyte chemoresistance; no menopause</td>
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<tr>
<td>Acclerated onset of tumors in the presence of an oncogene</td>
<td>Bax as a tumor suppressor</td>
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* NGF, nerve growth factor.

The family has further expanded to include the death antagonists BCL-2, BCL-XL (21), MCL-1 (22), and A1 (23), as well as the proapoptotic molecules BAX, BCL-XS (21), and BAK (24–26). Conserved domains BH1, BH2, and BH3 participate in the formation of various dimer pairs, as well as the regulation of cell death (Refs. (27 and 28); Fig. 3).

Mutational analysis of BCL-2 and BCL-XL identified key residues within BH1 and BH2 domains required for both heterodimerization with BAX and repression of cell death (29). However, other BCL-XL mutants lost heterodimerization with BAX, but still retained some death-repressor activity, suggesting that these two functions were separable (30). An additional domain, BH3, had also been noted in BCL-2 proteins and proved essential for proapoptotic function (31, 32, 33). The multidimensional nuclear magnetic resonance and X-ray crystallographic structure of a BCL-XL monomer indicated that BH1–4 domains corresponded to α-helices 1–7. The α-helices of BH1–3 are closely juxtaposed to form a hydrophobic pocket (34). Nuclear magnetic resonance analysis of a BAK peptide/BCL-XL interaction and a detailed mutational analysis of the BH3 amphipathic α2-helix of BAX indicates that BH3 forms critical interactions with BCL-2/BCL-XL (35). Moreover, the hydrophobic face of this amphipathic helix is critical for its death-effector function (Ref. (33); Fig. 4).

Loss of Function Models Reveal Critical Roles for the BCL-2 Family in Homeostasis. The normal developmental roles of BCL-2 family members have been addressed by gene disruption. These loss of function models have proven most instructive in revealing the cell type specificity and singular roles for the BCL-2 members. Newborn Bcl-2 −/− knockout mice are viable, but the majority die at a few weeks of age (Ref. (36); Table 2). They develop polycystic kidney disease with marked dilation of proximal and distal tubules and collecting ducts, resulting in renal failure (36–38). In the normal fetal kidney, BCL-2 maintains cell survival during inductive interactions between epithelium and mesenchyme. BCL-2 −/− kidneys contain many fewer nephrons and greatly increased apoptosis within metanephric blastemas of metanephroi at embryonic day 12 (39). Bcl-2 −/− mice turn gray at 5–6 weeks of age, at the time of the second hair follicle cycle. The hypopigmentation of Bcl-2 −/− mice reflects decreased melanocyte survival. The lymphoid organs, thymus, and spleen are initially normal in Bcl-2 −/− mice. Thymocyte development is normal, and B and T cells undergo selection successfully. However, at 4–8 weeks of age, the lymphoid organs undergo massive cell death and involution, showing a failure to maintain homeostasis in both the B- and T-cell populations in the absence of BCL-2.

Bax-deficient mice represent the first knockout of a death-promoting family member (Ref. (40); Table 3). Bax −/− mice appear healthy, indicating that BAX is not essential for development of a viable organism. However, male Bax −/− mice are infertile, and Bax −/− seminiferous tubules are markedly disorganised.
BCL-2 FAMILY REGULATES APOPTOSIS

Normal Tissue

(In) ↓ Homeostasis

(Out) ↓

Diseases of Disordered Cell Death

Neurodegenerative

Immunoedeficiency

Infertility

Proliferation ↓ Cancer Autoimmunity

Death ↓

Fig. 5. Schematic representation of normal tissue homeostasis with balanced input and output reactions. Primary aberrations in the cell death pathway that result in diminished death can manifest as cancer or autoimmunity, whereas increased death can result in neurodegenerative disease, immunodeficiency, or infertility.

Fig. 6. Dual impact of IL-3-induced phosphorylation of BAD on two serine sites by distinct kinase pathways. The phosphorylated BAD is sequestered by 14-3-3 in the cytosol as the inactive form, which releases BCL-X\textsubscript{L} to promote survival. Only nonphosphorylated BAD can heterodimerize with membrane-bound BCL-X\textsubscript{L}, which seems to be the active form that inhibits BCL-X\textsubscript{L}.

Proapoptotic BAX as a Tumor Suppressor. Prolonged cell survival with resistance to apoptosis can be a primary oncogenic event. This predicted that proapoptotic genes in the death pathway might contribute to oncogenesis through loss of function mutations (Table 1). Indeed, a principal contribution from the loss of p53 function is the elimination of a death pathway (43, 44). In select settings, stimuli that induce p53 result in the expression of Bax (45). Bax-deficient mice indicate that approximately half of certain p53-dependent cell deaths require BAX. Elimination of BAX blocks approximately half of chemotherapy-induced cell death (46). A transgenic model, TGT121 expresses a truncated T antigen that inhibits Rb but leaves p53 intact, resulting in cell proliferation with counterbalancing cell death. The elimination of Bax in this model markedly decreased the apoptosis. Moreover, the TGT121 transgenic mice displayed a markedly accelerated progression to malignancy on a Bax-deficient background (Ref. (47); Table 3). These experimental models argue that Bax can also be considered a tumor suppressor.

Recently, mutations in \textit{BAX} were noted in ~20% of human hematopoietic malignancies studied (48). Approximately half were frameshifts confined to a single mononucleotide (G\textsubscript{1} and G\textsubscript{2}) tract (nt 114–121) that results in a stop codon and the loss of BAX protein. The others represent point mutations within conserved BH1 and BH3 domains that alter dimerization and eliminate proapoptotic activity. Rampino \textit{et al.} (49) described the presence of frameshift mutations in the identical (G\textsubscript{1}) tract of BAX in about 50% of human colon adenocarcinomas with the microsatellite mutator phenotype. Mutations of the proapoptotic BAX molecule within human malignancies support the role of BAX as a tumor suppressor.

BH3 Only Molecules as Death Ligands That Connect the BCL-2 Checkpoint to Signal Transduction. To extend the cell death pathway, BCL-2 and BAX were used as bait in interactive cloning strategies. This provided a series of interacting molecules that bear sequence homology to BCL-2 principally within only the BH3 domain. These now include BIK (50, 51), BID (52), BAD (53, 54), BIM (55), HRK (56), and BLK (Ref. (57); Fig. 3). Mutagenesis of...
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Two of these molecules, BID and BAD, lack a COOH-terminal hydrophobic segment (Fig. 3). These molecules can translocate between cytosol and classic membrane based family partners such as BCL-2 or BCL-X<sub>L</sub>. Moreover, mutants of BID and BAD that fail to bind their partner proteins were functionally inert. Thus, BID and BAD can be envisioned as “death ligands” that bind to downstream membrane-based BCL-2 full members, “receptors.” These characteristics make BID and BAD candidates for an upstream link between the BCL-2 checkpoint and proximal signal transduction.

**Extracellular Survival Factors Reset the BCL-2 Checkpoint by Phosphorylation of BAD.** Support for the above thesis comes from the recognition that BAD is rapidly phosphorylated on two serine residues in response to a survival factor, IL-3 (58). These serines represent canonical 14-3-3 binding sites. Phosphorylated BAD seems to be the inactive form incapable of binding BCL-X<sub>L</sub> and sequestered in the cytosol bound to 14-3-3 (Fig. 6). This would release BCL-X<sub>L</sub> at membrane sites to promote cell survival. Consistent with this model, substitution of the serine phosphorylation sites eliminated binding to 14-3-3 and further enhanced the death-promoting activity of BAD. Despite the similarity of the phosphorylation motifs surrounding Ser112 and Ser136 of BAD, distinct BAD kinases seem responsible for the differential phosphorylation of each site after engagement of a single receptor (58, 59). This illustrates the complexity of the posttranslational modifications that will regulate this pathway.

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**Fig. 7.** Schematic model of BAX activation cell death. A death signal activates BAX, resulting in the translocation of a BAX monomer in the cytosol to homodimerized, integral mitochondrial membrane BAX. Downstream effects include the activation of Caspases and a program of mitochondrial dysfunction.

**Fig. 8.** Schematic model of mammalian cell death pathway. A major checkpoint in the common portion of the pathway is the ratio of proapoptotic (BAX) to antiapoptotic (BCL-2) members.
BAX Activation Induces Mitochondrial Dysfunction and Apoptosis. Although the ratio of BCL-2:BAX determines the response to an apoptotic signal, a question remained as to whether BAX itself could initiate death and, if so, how. Transient transfection and inducible expression systems for BAX or BAK proved sufficient to induce apoptosis without an additional stimulus (60, 61). BAX-induced death activated caspases which cleaved endogenous substrates in the nucleus (PARP) and cytosol (D4-GDI; Fig. 7). However, although caspase inhibitors successfully blocked protease activity and could prevent a FAS-induced death, it did not block BAX-induced death. The localization of BCL-2 (15), and subsequently other family members, to the mitochondria prompted an assessment of mitochondrial function. Although the cleavage of substrates and the final degradation of DNA was prevented, mitochondrial dysfunction still occurred. BAX-induced alterations in mitochondrial membrane potential, production of reactive oxygen species, cytoplasmic vacuolation, and plasma membrane permeability apparently occurred despite the inhibition of measurable caspase activity (60).

BAX has been shown to have both cytosolic and membrane-associated locations. Death signals such as IL-3 withdrawal, staurosporine, dexamethasone, and γ-irradiation induce the translocation of BAX protein from cytosol to mitochondrial membrane (62, 63). Although BAX forms both homo- and heterodimers, questions remained concerning its native conformation in vivo and which moiety was functionally active. Physiological death stimuli result in the translocation of monomeric BAX from the cytosol to the mitochondria, where it can be cross-linked as a BAX homodimer (62–64). In contrast, cells protected by BCL-2 demonstrate a block in this process in that BAX does not redistribute or homodimerize in response to a death signal. Moreover, enforced dimerization of FKBP-BAX by the bivalent ligand FK1012 resulted in its translocation to mitochondria and induced apoptosis (63). Enforced dimerization of BAX overrode the protection of BCL-X(L) and IL-3 to kill cells.

The ability of BAX dimers to kill cells is consistent with genetic evidence that BAX can function independently of BCL-2 (65). Moreover, a point mutant of BAX that fails to form homodimers or heterodimers in classic binding assays still inserted into mitochondria and induce apoptotic DNA fragmentation and cytochrome c release (66, 67). This may prove to regulate an electrochemical potential. Evidence that BAX can function independently of BCL-2 (65) suggests that BAX is a death-inducing protein that can function independently of other BH3-only proteins.

References
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