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Insights from Transgenic Mice regarding the Role of bcl-2 in Normal and Neoplastic Lymphoid Cells

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Insights from transgenic mice regarding the role of *bcl*-2 in normal and neoplastic lymphoid cells

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SUMMARY

The *bcl*-2 gene was first discovered by molecular analysis of the 14;18 chromosome translocation which is the hallmark of most cases of human follicular lymphoma. To date, it is unique among proto-oncogenes because, rather than promoting cell proliferation, it fosters cell survival. This review summarizes the impact of constitutive *bcl*-2 expression on the development and function of lymphocytes as well as their malignant transformation. Expression of a *bcl*-2 transgene in the B lymphoid compartment profoundly perturbed homeostasis and, depending on the genetic background, predisposed to a severe autoimmune disease resembling human systemic lupus erythematosus. T lymphoid cells from *bcl*-2 transgenic mice were remarkably resistant to diverse cytotoxic agents. Nevertheless, T lymphoid homeostasis was unaffected and tolerance to self was maintained. Expression of high levels of Bcl-2 facilitated the development of B lymphoid tumours but at relatively low frequency and with long latency. Co-expression of *myc* and *bcl*-2, on the other hand, promoted the rapid onset of novel tumours which appeared to derive from a lympho-myeloid stem or progenitor cell. Introduction of the *bcl*-2 transgene into scid mice facilitated the survival and differentiation of pro-B but not pro-T cells, suggesting that a function necessary to supplement or complement the action of Bcl-2 is expressed later in the T than the B lineage. Crosses of the *bcl*-2 transgenic mice with $p53^{-/-}$ mice have addressed whether loss of p53 function and gain of *bcl*-2 function are synergistic for lymphoid cell survival.

1. INTRODUCTION

The 14;18 chromosome translocation typical of human follicular lymphoma (Yunis *et al.* 1987) results from a reciprocal recombination event involving the *bcl*-2 gene and the immunoglobulin heavy chain locus (Tsujimoto *et al.* 1984; Bakhshi *et al.* 1985; Cleary *et al.* 1986). Unlike most other proto-oncogenes, *bcl*-2 does not play a role in cellular proliferation. Instead, it fosters cell survival. This function was first revealed by infection of IL-3-dependent mouse cell lines with a retrovirus engineered to express human Bcl-2 protein (Vaux *et al.* 1988). Such cells normally perish when the growth factor is withdrawn, because they undergo apoptosis (Williams *et al.* 1990), a suicidal process characterized by shrinkage of the cytoplasm, membrane blebbing, chromatin condensation and DNA fragmentation (Wyllie *et al.* 1980). Cells infected with the *bcl*-2 retrovirus did not die after removal of IL-3; although they ceased proliferating, they remained viable for at least two weeks (Vaux *et al.* 1988). Similar effects were subsequently observed for certain cell lines dependent on other growth factors (Nuñez *et al.* 1990).

The *bcl*-2 gene appears to be the mammalian homologue of *ced*-9, which determines cell survival during development of the nematode *Caenorhabditis elegans* (Hengartner *et al.* 1992). Until recently, *bcl*-2 was the only known mammalian cell survival gene. However, an homologous gene has now been identified and shown to encode a protein, Bcl-x_L, which also fosters cell survival (Boise *et al.* 1993). The 26 kDa cytoplasmic Bcl-2 protein has a hydrophobic carboxy terminus and is associated with the nuclear envelope, endoplasmic reticulum and mitochondrion (Chen-Levy *et al.* 1989; Monaghan *et al.* 1992; Lithgow *et al.* 1994), where it resides in the outer (Lithgow *et al.* 1994), not the inner membrane as claimed earlier (Hockenbery *et al.* 1990). Bcl-x_L apparently has a similar distribution. Bcl-2 appears to function as a heterodimer with another homologous protein, Bax (Oltvai *et al.* 1993). High levels of Bax inhibit the survival function of Bcl-2. A similar property has been ascribed to Bcl-x_S, which is produced by alternative splicing of *bcl*-x transcripts (Boise *et al.* 1993). The biochemical basis for the ability of Bcl-2 and its homologues to regulate cell survival is unknown but its protective function is well conserved, because Bcl-2 preserves mammalian neurons (Garcia *et al.* 1992) and can even spare the cells of *C. elegans* fated to die during ontogeny (Vaux *et al.* 1992; Hengartner & Horvitz 1994).

Mice expressing a *bcl*-2 transgene have been developed by several laboratories to investigate the role of *bcl*-2 in lymphoid development and lymphomagenesis. The transgenes we constructed (Strasser *et al.* 1990*b*)

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express human *bcl*-2 cDNA (Cleary *et al.* 1986) under the control of the intronic enhancer from the immunoglobulin heavy chain locus (Eμ). As expected, most Eμ-*bcl*-2 lines expressed the trangene exclusively in the B lymphoid compartment. However, a few expressed it in both B and T cells and one line expressed the transgene in only the T lymphoid compartment. This paper reviews insights derived from analysis of the Eμ-*bcl*-2 mice and the progeny of crosses with other mutant mice.

2. BCL-2 AND B LYMPHOID DEVELOPMENT

Expression of the Eμ-*bcl*-2 transgene rendered B and T cells remarkably robust, enhancing their longevity *in vitro* in the absence of growth factors and enabling them to survive exposure to diverse cytotoxic agents *in vitro* and *in vivo*, including γ-radiation and corticosteroids (Sentman *et al.* 1991; Strasser *et al.* 1991*a*,*b*; Siegel *et al.* 1992). T lymphoid development appeared normal (see below) but B lymphoid homeostasis was profoundly perturbed. The mice displayed a polyclonal excess of mature, phenotypically normal B cells in all lymphoid organs (McDonnell *et al.* 1989; Strasser *et al.* 1991*b*). Most of these cells were non-cycling but responded to mitogens and growth factors. The imbalance was not confined to B cells, as the number of pre-B cells and Ig-secreting cells was also elevated and serum Ig levels were abnormally high (Strasser *et al.* 1991*b*). Immunization provoked a greatly amplified and prolonged immune response. All of these properties are consistent with an increased lifespan for *bcl*-2-expressing B lymphoid cells.

Our *bcl*-2 lines were initially maintained on a mixed genetic background equivalent to (C57BL/6 × SJL)F2. The lines having B lymphoid expression of the transgene proved to be highly prone to develop a fatal autoimmune disease which resembled human systemic lupus erythematosus, being characterized by immune complex glomerulonephritis and high levels of anti-nuclear antibodies (Strasser *et al.* 1991*b*). This autoimmune disease may reflect pathological accumulation of autoreactive antibodies due to the longevity of cells with anti-self reactivity. However, further studies have revealed that the disease has a strong genetic component. Serial crosses of the *bcl*-2 transgene on to a C57BL/6 or BALB/c background seem to have eliminated the kidney disease (Strasser *et al.* 1993; A. W. Harrris and M. L. Bath, unpublished results). Thus the onset of the autoimmune disease apparently requires the presence of alleles from the SJL background.

The inference from these results for normal B cell differentiation is that cells with useful antigenic specificities may be triggered to express Bcl-2 to ensure their survival. Conversely, down-regulation of Bcl-2 may allow the demise of cells incapable of interacting with antigen. Consistent with this hypothesis, both B cells undergoing positive selection in germinal centres and circulating B cells express high levels of Bcl-2 protein (Liu *et al.* 1991) and expression of the *bcl*-2 transgene partially inhibits deletion of autoreactive B cells in immunoglobulin transgenic mice (Hartley *et al.* 1993).

3. BCL-2 AND T LYMPHOID DEVELOPMENT

Most T lymphocytes which develop in the thymus are doomed to die (Egerton *et al.* 1990), having failed the stringent selection criteria which ensure that only cells bearing useful antigen receptors can mature and emigrate to the periphery. To survive, immature thymocytes must express antigen receptors capable of binding to molecules of the major histocompatibility complex (MHC) on thymic epithelial cells (positive selection). However, those with receptors which bind with high affinity to MHC molecules complexed to self-antigens are censored (negative selection). The self-reactive thymocytes are believed to die by apoptosis, although apoptotic cells are rarely seen within the thymus, probably because they are rapidly phagocytosed. Mature medullary thymocytes contain much more Bcl-2 protein than do immature cortical cells (Pezzella *et al.* 1990; Hockenbery *et al.* 1991), suggesting that modulation of *bcl*-2 expression plays an important role in T cell selection.

Surprisingly, however, the *bcl*-2 transgene had no major impact on T lymphoid homeostasis. The numbers and relative proportions of all major subpopulations of T cells were normal and thymic involution with age was unaffected (Sentman *et al.* 1991; Strasser *et al.* 1991*a*; Siegel *et al.* 1992). Furthermore, negative selection was apparently still effective, as no self-reactive T cells were detected in the peripheral lymphoid organs, as judged by analysis of the response to endogenous Mls 'superantigens'. Nevertheless, the $CD4^+8^+$ cells of *bcl*-2 mice were refractory to *in vivo* or *in vitro* treatment with anti-CD3 antibody (Strasser *et al.* 1991a; Sentman *et al.* 1991), which kills conventional $CD4^+8^+$ cells and is believed to mimic negative selection (Smith *et al.* 1989; Shi *et al.* 1991).

To resolve this paradox, we crossed *bcl*-2 transgenic mice with mice in which T cells express a transgene encoding a T-cell receptor recognizing the male HY antigen in the context of the $H\text{-}2D^b$ allele of the MHC (von Boehmer 1990). Male $H\text{-}2D^b$ anti-HY TCR transgenic mice have a very small thymus, due to deletion of the self-reactive T cells. Expression of *bcl*-2 reduced the efficiency of deletion, since *bcl*-2/TCR transgenic male mice accumulated four- to sixfold more thymocytes than TCR transgenic male littermates (Strasser *et al.* 1994*b*). Anti-HY TCR-expressing cells were also more numerous in the peripheral lymphoid tissues, but these cells expressed abnormally low levels of CD8 co-receptor and were not responsive to the HY antigen. Thus, although *bcl*-2 expression hampered deletion of immature self-reactive cells in the thymus, tolerance to self was maintained.

Analysis of female anti-HY mice established that bcl-2 expression prolonged the survival of thymocytes in a non-selecting background. This suggests that upregulation of Bcl-2 could be a consequence of positive selection (Strasser *et al.* 1994*b*).

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4. BCL-2 EXPRESSION PROMOTES B- BUT NOT T-LYMPHOID DEVELOPMENT IN SCID MICE

Lymphoid ontogeny depends upon the rearrangement and expression of antigen receptor genes. If productive rearrangement is achieved, a developing B lymphoid cell first expresses a surface μ heavy chain in association with the λ5 and VpreB surrogate light chains (Melchers *et al.* 1993), while an immature T cell initially expresses a TCR β chain complexed to a gp33 polypeptide (Groettrup *et al.* 1993). Signals from these receptors apparently are required both for survival and further differentiation, since lymphoid development is arrested in scid, Rag-1 or Rag-2 deficient mice, which cannot productively rearrange antigen receptor genes (Bosma & Carroll 1991; Mombaerts *et al.* 1992; Shinkai *et al.* 1992).

To test whether *bcl*-2 expression could substitute for receptor engagement, we bred scid mice with transgenic mice expressing *bcl*-2 in both the T- and B-cell compartments (Strasser *et al.* 1994*a*). The number of B lymphoid cells in scid/*bcl*-2 mice was strikingly higher than in conventional scid littermates. As expected, these cells lacked surface Ig, but they expressed other markers of mature B cells. As most were quiescent, the increase apparently reflected enhanced survival rather than proliferation. These results suggest that upregulation of Bcl-2 may be a signal for positive selection of immature B lymphoid cells in the bone marrow. By analogy with findings for peripheral B cells in germinal centres (Liu *et al.* 1991), we hypothesize that Bcl-2 synthesis is induced as a result of engagement of the antigen receptor. Thus the survival of cells that have achieved productive rearrangement of receptor genes would be ensured.

Surprisingly, T cell development in scid mice was unaffected by the *bcl*-2 transgene. Thymocytes were no more numerous in *bcl*-2/scid mice than in scid littermates and died rapidly when cultured (Strasser *et al.* 1994*a*). There were no detectable peripheral T cells. The failure to remove the block to T cell development was not due to a failure of the pro-T cells to express the transgene, because the level of Bcl-2 detected in sorted (Thy-1^+) *bcl*-2/scid thymocytes was comparable to that found in more mature thymocytes from non-scid *bcl*-2 mice, which do display enhanced survival (Strasser *et al.* 1991*a*). Thus, inability to benefit from Bcl-2 (at least at this level of expression) seems to be confined to very early T cells.

Immature T cells acquire responsiveness to Bcl-2 after they display the antigen receptor and, significantly, this ability does not depend on signalling through the TCR. This conclusion was reached by analysis of scid mice which bear the anti-HY TCR transgene in addition to the *bcl*-2 transgene (Strasser *et al.* 1994*a*). To avoid immunological selection by TCR engagement, we analysed female H-$2D^{d/d}$ mice. Scid/anti-HY TCR mice with this MHC background normally have very few thymocytes. However, the *bcl*-2 transgene increased the number of thymocytes 3.5-fold and greatly increased their ability to survive in culture.

Why do immature B and T lymphoid cells differ in their response to Bcl-2? We are struck by the fact that pro-B cells express antigen co-receptor but pro-T cells do not. The B-lymphoid co-receptor, mb-1/B29 (also known as Ig-α/Ig-β (Reth 1992)), is present on pro-B cells in association with surrogate heavy (X) and light chains (VpreB and 5) (Melchers *et al.* 1993). However, the T-lymphoid co-receptor, CD3, is not expressed on pro-T cells; it appears only after rearrangement and expression of TCR genes permits the formation of CD3 complexed with β-gp33 or α-β heterodimers. The T cells in the scid/anti-HY TCR mice bear the transgenic TCR (although it is ineffectual for signalling in the H-$2D^d$ background and cannot promote further differentiation) and would therefore express CD3. Conceivably, therefore, a function essential for realization of the cell survival-promoting activity of Bcl-2 is induced in lymphoid cells via the co-receptor when it first appears at the cell surface (Strasser *et al.* 1994*a*).

5. BCL-2 AND LEUKAEMIC TRANSFORMATION

To explore the role of *bcl*-2 in lymphomagenesis, we determined the frequency of spontaneous tumours over a 12-month period for four independent lines of *bcl*-2 mice expressing the transgene in B lymphoid cells and three lines with expression in T lymphoid cells (Strasser *et al.* 1993). The incidence of T lymphomas was barely, if at all, higher than that in non-transgenic mice of the same genetic background. The incidence of B lymphoid tumours was significant (around 10%), although the long latency (more than 40 weeks) argued that somatic mutation had played a major role in their onset. The tumours were predominantly plasmacytomas and novel early B lymphoid tumours. The *myc* gene was commonly rearranged in the plasmacytomas, as reported for *bcl*-2 tumours designated large cell lymphoma by others (McDonnell & Korsmeyer 1991). A minority of aggressive human lymphomas display both a *myc* and a *bcl*-2 translocation (Mufti *et al.* 1983; Pegoraro *et al.* 1984; de Jong *et al.* 1988; Gauwerky *et al.* 1988; Lee *et al.* 1989).

We concluded from these observations that, by itself, activation of *bcl*-2 expression is relatively innocuous. This conclusion is consistent with the indolent course of follicular lymphoma (median survival from diagnosis is more than 10 years) (Horning & Rosenberg 1984) and the fact that a low level of *bcl*-2/J_H recombination can be detected even in non-lymphomatous lymph nodes and tonsils during a strong immune response (Limpens *et al.* 1991). The primary role of *bcl*-2 in human follicular lymphoma probably is to enable a cell that has acquired the 14;18 translocation to resist apoptosis in the lymphoid germinal centre. Because the bcl-2 expressing clone can survive adverse circumstances, any chance somatic mutation which conferred a proliferative advantage to a cell within the resistant population would provoke a potent drive toward malignancy (Vaux *et al.* 1988; Strasser *et al.* 1990*a*).

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The presence of *myc* rearrangements within the plasmacytomas arising spontaneously in *bcl*-2 transgenic mice and in certain blast crises of follicular lymphoma implied a synergistic role for *myc* and *bcl*-2 in the aetiology of these tumours. Direct evidence for synergy was obtained by breeding Eμ-*bcl*-2 transgenic mice with Eμ-*myc* mice (Strasser *et al.* 1990*a*). Three-week-old *bcl*-2/*myc* mice exhibited astoundingly high white blood cell counts (around 5×10^8 cells per ml), and flow cytometry established that the excess cells were predominantly cycling pre-B and B cells. Despite their conspicuous overproduction, these cells were not malignant when injected into non-irradiated histocompatible mice. Thus, constitutive expression of *bcl*-2 and *myc* is insufficient to transform these cell types, as also concluded from our earlier studies on Eμ-*myc* bone marrow cells infected with a *bcl*-2 retrovirus (Vaux *et al.* 1988).

The bi-transgenic mice were, however, highly susceptible to tumour development. They all developed transplantable tumours within 7 weeks, significantly faster than their E-*myc* littermates. Surprisingly, these tumours were not the pre-B or B lymphomas typical of Eμ-*myc* mice (Harris *et al.* 1988). Their phenotype suggested that they were derived from a primitive progenitor or stem cell. When cultured *in vitro* with appropriate growth factor combinations, the tumour cells could differentiate down either the macrophage or B lymphoid pathways (Strasser & Cory, unpublished results).

The realization that *myc* can promote apoptosis as well as proliferation has provided insight into the basis for the synergy between *bcl*-2 and *myc* in lymphoma development. Myeloid cells and cells constitutively expressing *myc* are highly prone to apoptosis when maintained under sub-optimal growth conditions (Askew *et al.* 1991; Evan *et al.* 1992). Apoptosis of the *myc*-expressing cells can be blocked by antisense *myc* oligonucleotides (Shi *et al.* 1992) or by high levels of intracellular Bcl-2 protein (Fanidi *et al.* 1992; Bissonette *et al.* 1992). Cells mutated to constitutively express *bcl*-2 would also presumably have a survival advantage *in vivo* under limiting growth conditions, giving them an enhanced probability of acquiring oncogenic changes that confer a proliferative advantage.

Enhanced susceptibility to apoptosis in the absence of growth factors provides an explanation for the observation that pre-B cells from Eμ-*myc* mice died much faster than normal pre-B cells when cultured *in vitro* in simple medium (Langdon *et al.* 1988). As expected, both the pre-B and B cells from *bcl*-2/*myc* mice displayed enhanced survival under these conditions but, surprisingly, the *bcl*-2/*myc* tumour cells did not (Strasser *et al.* 1990*a*). Instead, they died even more rapidly than pre-B cells expressing only the *myc* transgene. The failure of the tumour cells to survive *in vitro* was not due to poor expression of the transgene, as the level of Bcl-2 protein was as high in these cells as in the pre-B and B cells from the *bcl*-2/*myc* mice (Strasser & Cory, unpublished results). It is apparent from these results that constitutive high expression of Bcl-2 is inadequate for the survival of primitive lympho-myeloid progenitor cells. As the tumour cells are readily transplantable and remain undifferentiated *in vivo*, they must receive an effective survival signal in lymphoid tissues. We surmise that this is due to the presence of a relevant growth factor. We have cultured the tumour cells in an extensive range of growth factors, which includes all the colony stimulating factors, stem cell factor and interleukins 1 to 7 (both singly and in many combinations) but to date have been unable to maintain viable undifferentiated cells. Certain stromal cell lines will, however, sustain them for up to two weeks.

Why is a high concentration of Bcl-2 protein inadequate for the survival of certain cell types under conditions where it so effectively protects closely related cells? We speculate that cell survival may require input from more than one signal transduction pathway, as depicted in figure 1. Signal 1 may elevate the concentration of Bcl-2 (or Bcl-x_L), while signal 2 may either elevate the concentration of a function essential for Bcl-2 (or Bcl-x_L) activity or reduce the level of inhibitors such as Bax or Bcl-x_S. In lymphocytes, we and others have provided data which implies that signal 1 is mediated via activation of the antigen receptor but in other cell types activation of a growth factor receptor may serve this function. The second signal may emanate from a co-receptor, as suggested for pro-B and pro-T cells (Strasser *et al.* 1994*a*); alternatively it may instead involve a second cytokine pathway, as we have speculated for the *bcl*-2/*myc* progenitor cell tumours.

6. IMPACT OF BCL-2 ON CELL DEATH INDUCED IN LYMPHOCYTES BY DNA DAMAGE

After exposure to DNA damaging agents such as γ-radiation, cells undergo cell cycle arrest (Hartwell & Weinert 1989). Certain mammalian cell types such as fibroblasts then undergo DNA repair but lymphocytes rapidly die by apoptosis (Wyllie 1980). It has recently become clear that the transcription factor encoded by the p53 tumour suppressor gene is a crucial regulator of the cellular response to DNA

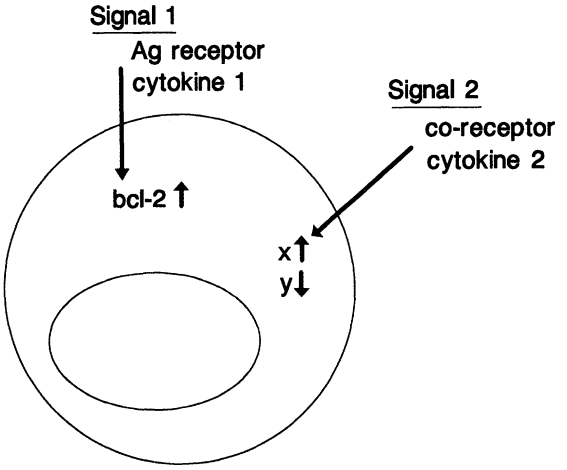

Figure 1. Does cell survival require two signals?

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damage. The level of p53 protein rises rapidly after DNA damage (Maltzmann & Czycyk 1984; Kastan *et al.* 1991). Analysis of mice rendered nullizygous for p53 by targeted homologous recombination has established that p53 is essential for both G_1 arrest of fibroblasts (Kastan *et al.* 1992; Livingstone *et al.* 1992) and apoptosis of thymocytes (Lowe *et al.* 1993; Clarke *et al.* 1993). Functional inactivation of p53 is one of the most frequently observed mutations in human tumours. Furthermore, inheritance of either one or two copies of an inactivated p53 gene strongly predisposes toward cancer development (Malkin *et al.* 1990; Donehower *et al.* 1992; Jacks *et al.* 1994). Loss of p53 function is believed to lead to malignant transformation because damaged DNA is neither adequately repaired nor eliminated via apoptosis (Lane 1992).

Because both p53 and *bcl*-2 mutations contribute to neoplastic transformation by blocking apoptosis, we are currently studying the relationship between these two regulators of cell survival and death. By enforcing high levels of Bcl-2 in an erythroleukaemia cell line with inducible wild-type (wt) p53 protein, we have demonstrated that Bcl-2 can block apoptosis induced by wt p53 but fails to prevent the block to proliferation. This observation implies that Bcl-2 acts downstream of the regulator(s) establishing growth arrest or that p53 mediates growth arrest and apoptosis via independent pathways. In a collaboration with Dr Tyler Jacks, we have crossed our *bcl*-2 transgenic mice with $p53^{-/-}$ animals and found that loss of p53 function and gain of *bcl*-2 function are not synergistic for the survival of lymphoid cells. However, *bcl*-2 can inhibit a p53-independent cell death pathway invoked by DNA damage (Strasser *et al.* 1995).

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