

The cell death inhibitor Bcl-2 and its homologues influence control of cell cycle entry

Lorraine A.O'Reilly, David C.S.Huang and Andreas Strasser¹

The Walter and Eliza Hall Institute of Medical Research,
PO Royal Melbourne Hospital, Victoria 3050, Australia

¹Corresponding author

The effect of the cell death inhibitor Bcl-2 and its homologues on cell cycle regulation was explored in lymphocytes and cell lines. Expression of a *bcl-2* transgene reduced proliferation of thymocytes and delayed cell cycle entry of mitogen-stimulated B and T lymphocytes. Overexpression of Bcl-2, Bcl-x_L or adenovirus E1B19kD substantially delayed serum stimulation-induced S phase entry of quiescent NIH 3T3 fibroblasts. Bcl-2-mediated cell survival and growth inhibition are both antagonized by Bax. Bcl-2, Bcl-x_L and E1B19kD, but not Bcl-2 mutants that are defective in blocking apoptosis, suppress growth of colon carcinoma cells. This evidence that regulation of cell survival is coupled to control of cell growth has implications for normal cell turnover and tumorigenesis.

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Introduction

The cell content of an organ is determined by the rates of input, proliferation, differentiation, emigration and death of cells. These processes must be coordinated with respect to each other to assure proper development and functioning of an organism. During differentiation in primary lymphoid organs and after activation in the periphery, B and T lymphocytes undergo successive rounds of cell division and growth arrest. Intermittent selection on the basis of antigen receptor specificity facilitates the generation and maintenance of an immune repertoire with useful antigen receptor specificities and the death of cells that are defunct or potentially dangerous (Rolink and Melchers, 1991; Nossal, 1994; Sprent, 1994; von Boehmer, 1994).

Apoptosis is an evolutionarily conserved physiological process for killing unwanted cells (Wyllie *et al.*, 1980; Ellis *et al.*, 1991). It can be triggered by diverse physiological and experimentally applied stimuli and can in many but not all instances be inhibited by Bcl-2 or a functional homologue, such as Bcl-x_L or adenovirus E1B19kD protein (Vaux *et al.*, 1994; Cory, 1995; Korsmeyer, 1995; White, 1996). It has therefore been argued that multiple independent signalling routes to apoptosis converge upon a common final effector mechanism which can be antagonized by Bcl-2 (Cory *et al.*, 1994; Hengartner and Horvitz, 1994b; Korsmeyer, 1995; White, 1996). Some insight into the molecular nature of this

effector mechanism has derived from genetic studies on the nematode *Caenorhabditis elegans* (Vaux *et al.*, 1992; Yuan *et al.*, 1993; Hengartner and Horvitz, 1994a). In the nematode, developmentally programmed cell death requires the combined activities of Ced-3 and Ced-4, whereas Ced-9 is essential for cell survival (Ellis and Horvitz, 1986; Ellis *et al.*, 1991; Hengartner *et al.*, 1992; Yuan *et al.*, 1993). Ced-3 is related to a family of mammalian cysteine proteases (Kumar, 1995) and Ced-9 is a functional and structural homologue of Bcl-2 (Vaux *et al.*, 1992; Hengartner and Horvitz, 1994a). It therefore appears that apoptosis is precipitated by proteolytic cleavage of one or several critical substrates and Bcl-2 may function by blocking a step that leads to the activation of cysteine proteases, by inhibiting active proteases or by protecting the proteolytic substrate(s) (Cory *et al.*, 1994).

The control of cell death appears more complex in mammals than in nematodes. Mice and humans contain several genes encoding cysteine proteases (Kumar, 1995) and multiple *bcl-2* relatives (Cory *et al.*, 1995; Korsmeyer, 1995), which may result in some functional redundancy, whereas only a single essential copy of each type of gene has been identified in *C.elegans* so far (Ellis *et al.*, 1991). Vertebrates possess two classes of Bcl-2 related proteins: one including Bcl-2 and Bcl-x_L inhibits apoptosis, while the other, including Bax, Bad, Bak, Bik and Bcl-x_S promotes cell death and can antagonize the survival function of Bcl-2 (Cory, 1995; Korsmeyer, 1995; Thompson, 1995). Proteins like Bax have so far not been found in nematodes and it is still unknown whether they are essential for cell killing or merely act as antagonists of proteins such as Bcl-2.

It is also unclear how signals from surface receptors are coordinated to regulate proliferation, growth arrest, differentiation and death of cells. Signals which impinge on the cell cycle machinery are known to also affect differentiation and the cell death programme. For example, expression of the proto-oncogene *c-myc* does not only stimulate cell proliferation but also inhibits differentiation and can predispose cells to apoptosis when growth factors are limiting (Askew *et al.*, 1991; Evan *et al.*, 1992). Might there also be hitherto unrecognized signals which act in the opposite direction; more precisely, do the cell death inhibitors Bcl-2 and its functional homologues affect cell proliferation?

There are some indications that this may be the case. Whenever expression of Bcl-2, Bcl-x_L or adenovirus E1B19kD inhibits apoptosis of proliferating cells, the surviving cells generally undergo cell cycle arrest. They usually accumulate in G₀/G₁, but under some circumstances are blocked in S phase or G₂/M (Vaux *et al.*, 1988; Hockenbery *et al.*, 1990; Miyashita and Reed, 1992; Chiou *et al.*, 1994; Marvel *et al.*, 1994; Strasser *et al.*, 1994a). However, it is unclear whether the stimuli that

Table I. The numbers of cycling thymocytes are reduced in *bcl-2* transgenic mice

Mice	Age	No.	Cellularity ($\times 10^7$)	Cell cycle distribution		
				G ₀ /G ₁	S	G ₂ /M
Control	6 weeks	3	16.5 \pm 2.5	83.5 \pm 0.5	8.8 \pm 0.7	7.9 \pm 0.3
<i>bcl-2</i>	6 weeks	3	18.8 \pm 1.3	89.4 \pm 1.9	5.2 \pm 0.5	5.5 \pm 0.7
Control	8 days	5	4.5 \pm 0.3	81.1 \pm 0.8	12.4 \pm 0.5	6.9 \pm 0.7
<i>bcl-2</i>	8 days	3	4.6 \pm 0.3	86.1 \pm 0.9	8.3 \pm 0.4	5.3 \pm 0.7

DNA content of thymocytes from control littermates and *bcl-2* transgenic mice (strain E μ -*bcl-2-36*) was determined by flow cytometry. Differences in numbers of cells residing in S phase are statistically different by Student's *t*-test to a probability of $P < 0.001$. Similar results were obtained with another strain, E μ -*bcl-2-25*, expressing the transgene in T cells.

induce apoptosis are solely responsible for growth arrest and Bcl-2 merely allows normally doomed cells to complete this process by blocking their death, or whether Bcl-2 can inhibit cell cycle progression. Another hint that Bcl-2 might influence cell cycle regulation is that the peak of a humoral immune response is delayed by ~ 2 – 3 days in mice expressing a *bcl-2* transgene in B cells (Strasser *et al.*, 1991b). This indicates that Bcl-2 might delay proliferation and differentiation of antigen-stimulated B lymphocytes.

We have explored the effects of Bcl-2 and some of its relatives on cell cycle regulation in immortalized cell lines and normal lymphocytes, both *in vivo* and *in vitro*. Our results demonstrate that constitutive overexpression of Bcl-2 or its functional homologue Bcl-x_L or adenovirus protein E1B19kD can inhibit cell growth at the transition from the quiescent G₀ stage into S phase. This effect of Bcl-2, like its survival function, can be reduced by co-expression of Bax. These data provide evidence for a link between cell death and cell cycle regulation and have implications for tumorigenesis and normal cell turnover.

Results

Bcl-2 reduces proliferation of T cell precursors *in vivo*

Since most ($\sim 97\%$) thymocytes are fated to die within the thymus (Egerton *et al.*, 1990) and since overexpression of Bcl-2 protects thymocytes against many physiological and experimentally applied death stimuli, it is puzzling that thymus cellularity is not increased in *bcl-2* transgenic mice (Table I and Sentman *et al.*, 1991; Strasser *et al.*, 1991a, 1994b; Siegel *et al.*, 1992; Linette *et al.*, 1994). A possible rationale is that increased survival of thymocytes is balanced by decreased proliferation. Consistent with this possibility, we found that adult as well as very young (8 days of age) *bcl-2* transgenic mice contained significantly fewer (1.5- to 1.6-fold; $P < 0.001$) thymocytes in the S phase of the cell cycle than control littermates (Table I). To characterize the cellular basis of this difference, we performed cell cycle analysis on purified CD4⁻CD8⁻ and CD4⁺CD8⁺ thymocytes, the sub-populations in which most cell division occurs. Compared with control littermates, *bcl-2* transgenic mice contained 1.8- to 2.2-fold fewer CD4⁻CD8⁻ and 2.0- to 2.5-fold fewer CD4⁺CD8⁺ thymocytes in the S phase of the cell cycle (Figure 1).

Proliferation is a principal determinant of cell turnover within an organ. In view of the differences in the number of cycling thymocytes (Table I and Figure 1), we compared

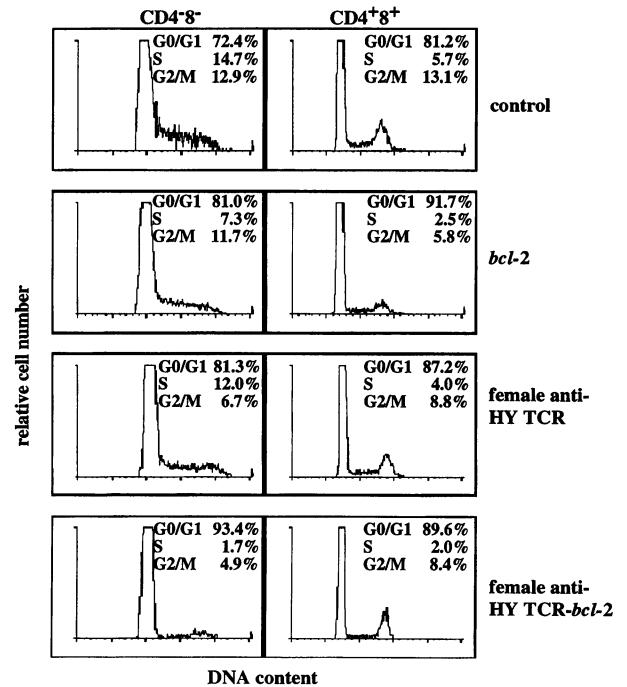


Fig. 1. The number of proliferating thymocytes is reduced in *bcl-2* transgenic mice. Cell cycle analysis from DNA content flow cytometry is shown for CD4⁻CD8⁻ (left column) and CD4⁺CD8⁺ (right column) thymocytes from control mice (top row), *bcl-2* transgenic mice (second row), female anti-HY TCR transgenic mice (third row) and female anti-HY TCR-*bcl-2* bi-transgenic mice (bottom row). CD4⁻CD8⁻ and CD4⁺CD8⁺ thymocyte subsets were purified by immunofluorescence staining and fluorescence activated cell sorting (FACS). The purity of the sorted subpopulations was 95–98%. Data shown here are from 3–5 week-old mice and are representative of results obtained from three to five mice of each of the four genotypes. Similar results were obtained with two independent *bcl-2* transgenic strains (E μ -*bcl-2-25* and E μ -*bcl-2-36*).

the rate of thymocyte turnover in control and *bcl-2* transgenic mice. The thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) was administered continuously for 1, 3, 5 or 7 days to mice via their drinking water to label cells undergoing DNA synthesis. Three-colour immunofluorescence staining with monoclonal antibodies to CD4, CD8 and BrdU, followed by flow cytometry, was used to identify BrdU-labelled cells within the CD4⁻CD8⁻ and CD4⁺CD8⁺ thymocyte sub-populations (Figure 2). After 3 days of labelling, the frequency of BrdU-positive CD4⁻CD8⁻ and CD4⁺CD8⁺ thymocytes was 1.3- to 1.8-fold lower in *bcl-2* transgenic mice than in control littermates (see Figure 2 for a representative example). Kinetic

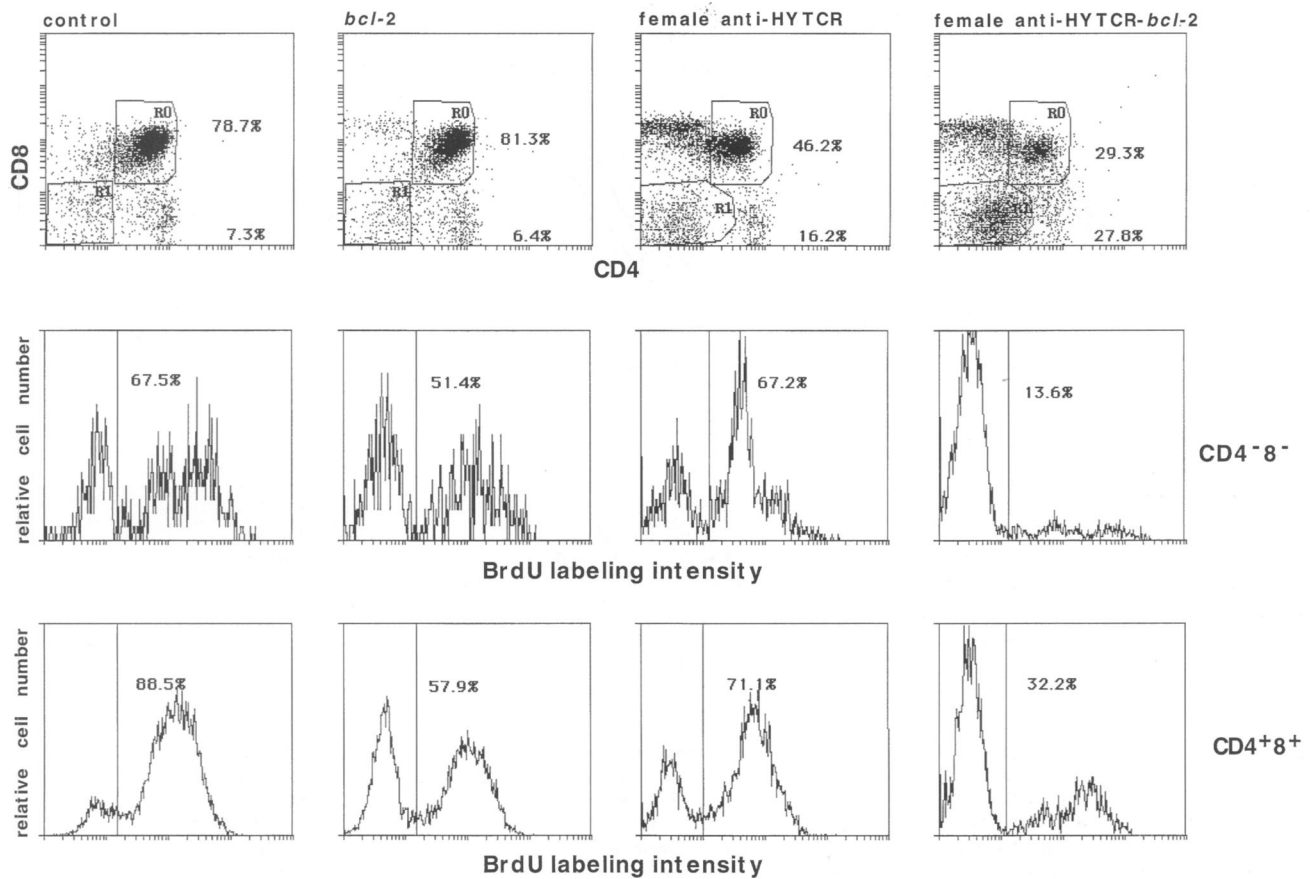


Fig. 2. The rate of thymocyte turnover is reduced by expression of a *bcl-2* transgene. Representative examples from an analysis of cell turnover in a control mouse (first column), *bcl-2* transgenic mouse (second column), female anti-HY TCR transgenic mouse (third column) and female anti-HY TCR-*bcl-2* bi-transgenic mouse (fourth column). The mice were fed continuously for 3 days with the thymidine analogue BrdU to label dividing cells. The number of BrdU-labelled CD4⁺CD8⁻ and CD4⁺CD8⁺ thymocytes was determined by 3-colour immunofluorescence staining and flow cytometric analysis using a FITC-labelled antibody to BrdU, an R-PE-labelled antibody to CD8 and a biotinylated antibody to CD4 plus Tricolor-labelled streptavidin. 10 000 cells were analysed and the thymic subpopulations identified on CD4 versus CD8 2-colour dot plots on which is indicated the electronic gating for the CD4⁺CD8⁻ (gate R1) and CD4⁺CD8⁺ (gate R0) subsets (top row). The fraction of BrdU-labelled cells amongst CD4⁺CD8⁻ (middle row) and CD4⁺CD8⁺ thymocytes (bottom row) is shown on histograms.

analysis revealed that the rate of accumulation of BrdU-labelled CD4⁺CD8⁻ and CD4⁺CD8⁺ thymocytes was significantly slower in *bcl-2* transgenic mice than in control littermates (Figure 3A and B).

The rate of accumulation of BrdU-labelled thymocytes is influenced not only by proliferation but also by cell death. In order to minimize the contribution of cell death, we used female C57BL/6 anti-HY TCR transgenic mice. The restriction specificity of this transgenic TCR $\alpha\beta$ in combination with the genetic background prevent the extensive cell death normally caused by lack of expression of a TCR β chain or failure to undergo positive selection (von Boehmer, 1990). In this system the inhibitory effect of Bcl-2 on cell proliferation and turnover was even more pronounced. Compared with littermates carrying only the TCR transgene, *bcl-2*-TCR bi-transgenic mice contained on average 5- to 6-fold fewer CD4⁺CD8⁻ and 1.6- to 2.2-fold fewer CD4⁺CD8⁺ thymocytes in the S phase of the cell cycle (Figure 1). The fraction of CD4⁺CD8⁻ and CD4⁺CD8⁺ thymocytes labelled with BrdU over 3 days was ~5-fold and 2.5-fold lower in *bcl-2*-TCR bi-transgenic mice compared with TCR transgenic littermates (Figure 2), and turnover was considerably slowed (Figures 3C and D). These results demonstrate that constitutive overexpress-

sion of Bcl-2 reduces the rate of cell division and turnover in thymocytes *in vivo*.

The effect of Bcl-2 on thymocyte proliferation is cell autonomous

It is possible that Bcl-2 inhibits cellular proliferation directly. Alternatively, the reduction in dividing cells may be an indirect effect, reflecting homeostatic feedback. To resolve this question, we generated chimeric mice containing approximately equal numbers of normal and *bcl-2* transgene expressing thymocytes by reconstituting lethally irradiated mice with mixtures of bone marrow from Ly5.1 control mice and Ly5.2 *bcl-2* transgenic mice (Table II). In control mice reconstituted with a single source of bone marrow (from normal mice only or from *bcl-2* transgenic mice only), the numbers of *bcl-2* transgene expressing thymocytes residing in S phase was similar to that of thymocytes in unmanipulated *bcl-2* transgenic mice and significantly lower (1.4- to 1.8-fold, $P < 0.002$) compared with control cells (Table II). Tellingly, in chimeric animals, the number of *bcl-2* transgene expressing thymocytes residing in S phase was also significantly lower (1.4- to 1.7-fold, $P < 0.002$) compared with that of control cells (Table II). These results provide evidence

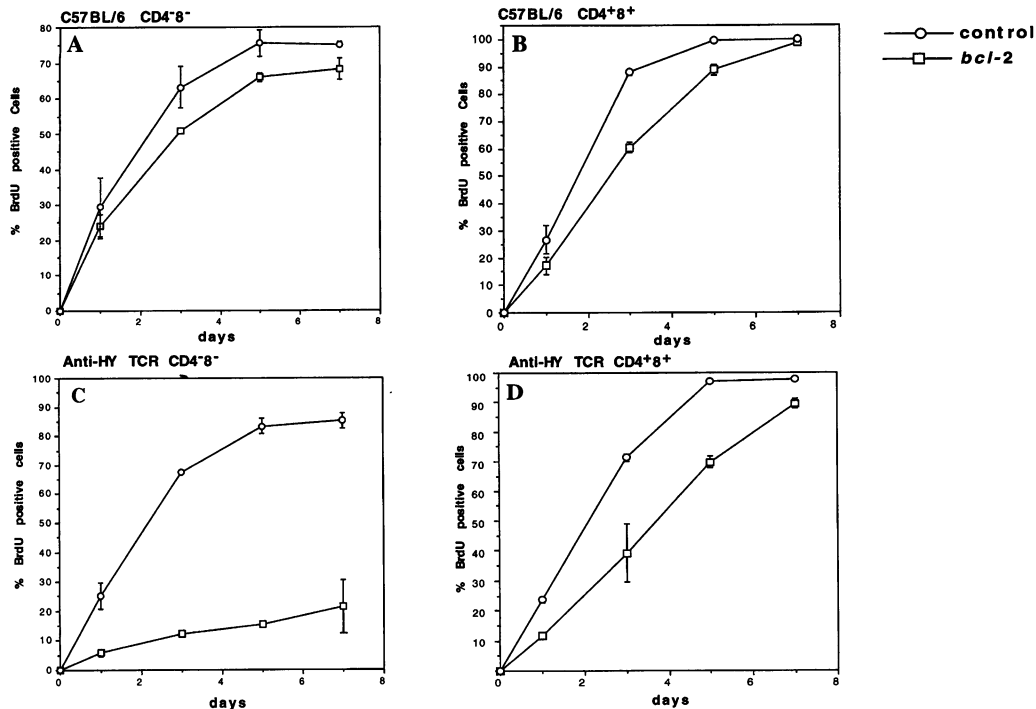


Fig. 3. Expression of a *bcl-2* transgene slows thymocyte turnover in normal mice and in anti-HY TCR transgenic mice. Control mice and *bcl-2* transgenic mice (A and B) as well as female anti-HY TCR transgenic mice and female anti-HY TCR-*bcl-2* bi-transgenic mice (C and D), all on a C57BL/6 background and aged 5–8 weeks, were continuously fed for 1, 3, 5 or 7 days with the thymidine analogue BrdU to label dividing cells. The fraction of BrdU-labelled CD4⁺CD8⁻ (A and C) and CD4⁺CD8⁺ (B and D) thymocytes was determined as shown in Figure 2. Data are presented as the fraction of BrdU-labelled cells as a function of time of continuous labelling. Values are arithmetic means \pm SD from three mice of all genotypes. For several data points (for example CD4⁺CD8⁻ thymocytes from *bcl-2* transgenic mice fed for 3 days with BrdU), error bars are not visible because the standard deviation is so small.

Table II. The numbers of cycling *bcl-2* transgenic thymocytes are reduced in chimeric mice

Bone marrow donor	Recipient	No.	Cell cycle distribution					
			Control			<i>bcl-2</i>		
			G ₀ /G ₁	S	G ₂ /M	G ₀ /G ₁	S	G ₂ /M
C57BL/6 Ly5.1	C57BL/6 Ly5.1	4	82.9 \pm 4.2	6.4 \pm 0.8	9.8 \pm 3.4	N/A	N/A	N/A
C57BL/6 <i>bcl-2</i> Ly5.2	C57BL/6 Ly5.1	4	N/A	N/A	N/A	88.2 \pm 4.3	4.1 \pm 0.7	6.8 \pm 3.8
C57BL/6 Ly5.1 + C57BL/6 <i>bcl-2</i> Ly5.2	C57BL/6 Ly5.1	4	86.5 \pm 2.9	7.2 \pm 1.4	6.0 \pm 1.7	90.7 \pm 3.3	4.9 \pm 2.0	4.1 \pm 1.5

DNA content of thymocytes from C57BL/6 Ly5.1 radiation bone marrow chimeras reconstituted with bone marrow from either control C57BL/6 Ly5.1, or C57BL/6 E μ -*bcl-2*-36 Ly5.2 mice only, or a mixture of these. Differences in the numbers of cells residing in S phase are statistically significant by a paired Student's *t*-test to a probability of $P < 0.002$, in the chimeric mice analysed. N/A, not applicable.

for a direct, cell autonomous effect of Bcl-2 on cell cycle control.

***Bcl-2* delays cell cycle entry of mitogen-stimulated B and T cells**

To avoid influences of homeostatic regulation we investigated whether *bcl-2* transgene expression could influence activation or proliferation of quiescent B and T lymphocytes upon mitogenic stimulation *in vitro*. B cells were activated with a monoclonal antibody to CD40 plus interleukins (IL) 2, 4 and 5, while T cells were stimulated with concanavalin A (ConA) plus IL-2. After 24, 48 and 72 h, the cells were analysed for DNA content and cell size by flow cytometry. Some of the cultures received a short pulse (2 h) of the thymidine analogue BrdU to

determine the frequency of DNA synthesising cells by immunofluorescence staining.

The amount of cell death did not differ between control and *bcl-2* transgenic lymphocytes under these culture conditions (30–50% killing during the initial 24 h of stimulation and <10% thereafter), but both the B and T cells from *bcl-2* transgenic mice were significantly delayed in their activation and initial entry into S phase. In comparison with normal lymphocytes, 1.5- to 2.5-fold fewer *bcl-2* transgenic B and T cells were found in the S, G₂ or M phases of the cell cycle after 24 and 48 h of stimulation (Figures 4A and 5A). Consistent with these results, the frequency of DNA synthesising cells was reduced ~2-fold in cultures of mitogen-activated *bcl-2* transgenic T cells (Figure 5B). The majority of *bcl-2*

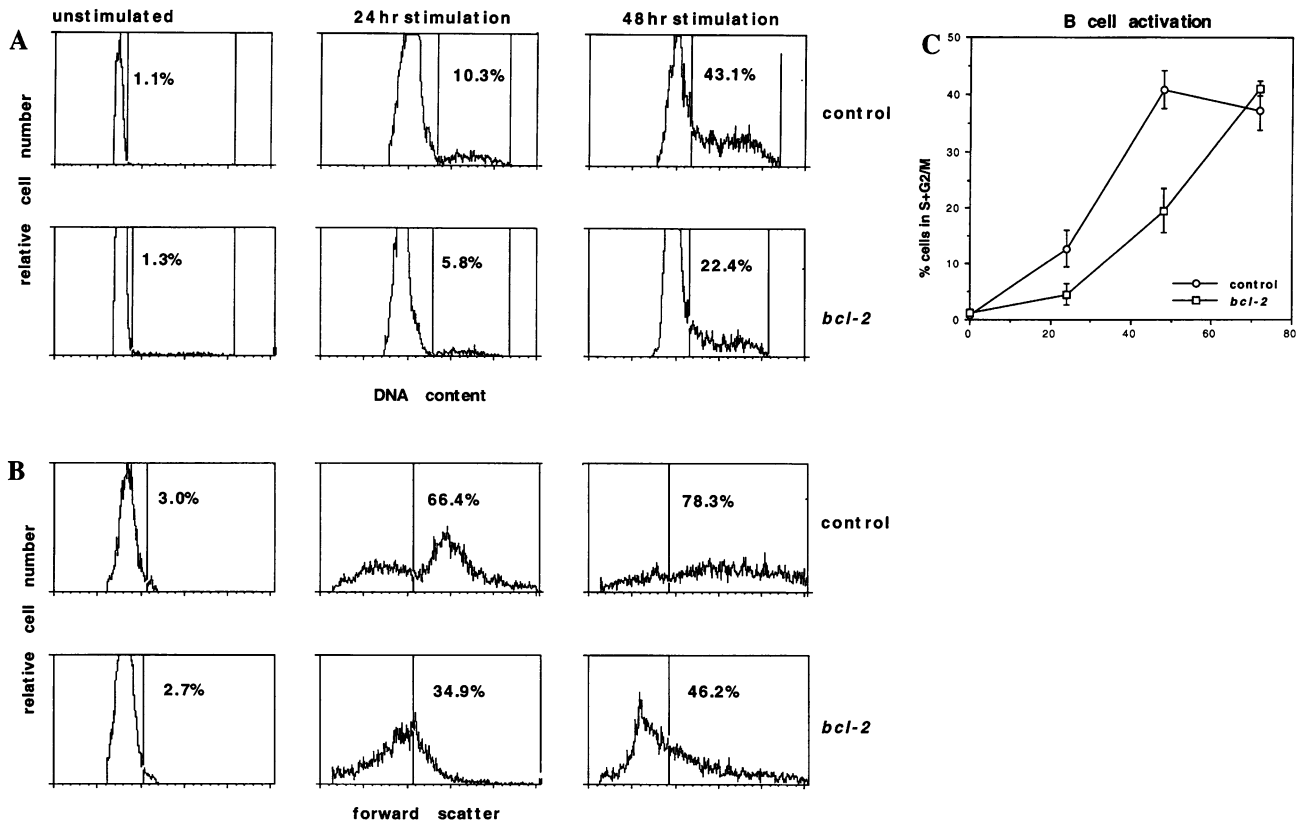


Fig. 4. Expression of a *bcl-2* transgene delays activation of B lymphocytes *in vitro*. Representative examples from a time course analysis of mitogenic stimulation of purified B cells from a control mouse (top row and third row) and a *bcl-2* transgenic mouse (second row and bottom row). B cells were either not stimulated (first column) or had been stimulated *in vitro* for 24 (second column) or 48 h (third column) with a monoclonal antibody to CD40 plus interleukins 2, 4 and 5 (IL-2, IL-4 and IL-5). The top two rows (A) present data on the DNA content (*x*-axis: PI fluorescence intensity on a linear scale) and the bottom two rows (B) present data on forward light scatter (*x*-axis: log scale), a measure of cell size of the cultured B cells. Data shown are electronically gated to exclude dead cells. The amount of cell death during stimulation was indistinguishable between control and *bcl-2* transgenic mice and ranged between 35 to 40% within the first 24 h and <10% thereafter. (C) Kinetic analysis of B cell activation after 0, 24, 48 and 72 h. Data are presented as the fraction of viable cells in the S, G₂ or M phase of the cycle as a function of time of stimulation. Data points represent the arithmetic means \pm SD from two or three control mice and two or three *bcl-2* transgenic mice. Similar results were obtained with B cells from two independent *bcl-2* transgenic strains (E μ -*bcl-2*-36 and E μ -*bcl-2*-22).

transgenic B cells (65 and 55%) and T cells (60%) remained small after 24 and 48 h of mitogen treatment, presumably because they were still quiescent (Figures 4B and 5C). In contrast, most normal B cells (66 and 78%) and T cells (78%) were already enlarged after 24 and 48 h, indicating that they became activated more rapidly (Figures 4B and 5C). After 72 h of stimulation, the *bcl-2* transgenic B and T cells were indistinguishable in cell cycle activity and cell size from their normal counterparts (Figure 5A and B and data not shown).

Expression of a *bcl-2* transgene also slowed activation of T cells after stimulation with antibodies to CD3 plus CD28, or phorbol ester plus ionomycin, and activation of B cells upon stimulation with antibodies to surface immunoglobulin M (IgM) plus IL-2, IL-4 and IL-5 (data not shown). These properties of *bcl-2* transgenic lymphocytes do not result from an increased activation threshold or abnormal accumulation of anergic cells since they are indistinguishable from their normal counterparts in their dose response to mitogens and in their cloning efficiency (Figure 5D and data not shown). Collectively, these results demonstrate that Bcl-2 can retard entry of mitogen-stimulated B and T cells into the cycle and indicate that Bcl-2 may exert its growth-inhibitory effect on cells predominantly during transition from the quiescent G₀

state into the initial S phase and may have little or no effect once cells are continuously cycling.

***Bcl-2* and its functional homologues retard cell cycle entry of quiescent fibroblasts but do not affect growth of cells that are continuously cycling**

To test the growth inhibitory effect of Bcl-2 in a different cell type and to ascertain the impact of two of its functional homologues, Bcl-x_L and the adenovirus protein E1B19kD, we turned to cultured cell lines. Expression constructs encoding Bcl-2, Flag-epitope-tagged Bcl-x_L or E1B19kD were stably introduced into the IL-3-dependent promyelocytic line FDC-P1, B6.2.16.BW2 T hybridoma cells and NIH 3T3 fibroblasts (Vaux *et al.*, 1988; Lithgow *et al.*, 1994; Strasser *et al.*, 1995). There was no discernible difference between the parental and the Bcl-2-, Bcl-x_L- or E1B19kD-overexpressing clones in cell growth or distribution between the various phases of the cell cycle. Cells of all sublines grew exponentially with a doubling time of ~14–16 h (Figure 6A, B and C) and 40 to 60% were in the S, G₂ or M phase of the cycle (data not shown).

The observations that Bcl-2 inhibits the growth of normal lymphocytes (Figures 4 and 5) but not that of cell lines (Figure 6A, B and C) are not necessarily at odds. Our experiments with mitogen-stimulated B and T lymphocytes

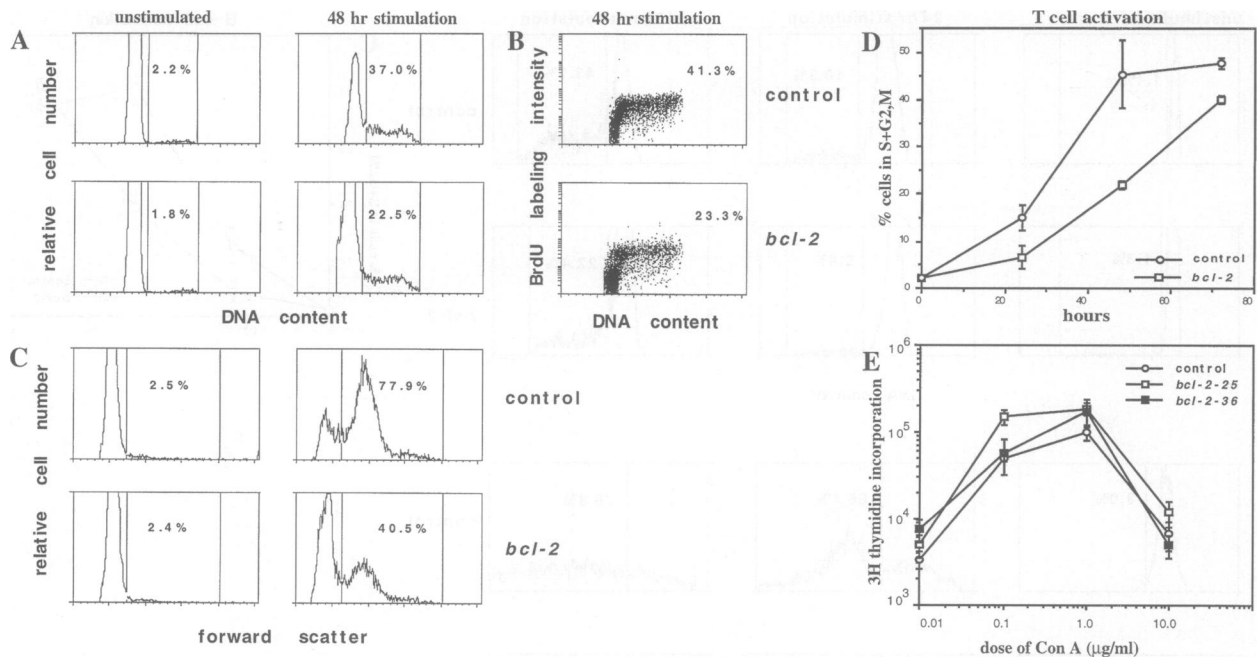


Fig. 5. Expression of a *bcl-2* transgene delays activation of T lymphocytes *in vitro*. (A, B and C) Representative examples from a time course analysis of mitogenic activation of purified T cells from a control mouse (top row and third row) and a *bcl-2* transgenic mouse (second row and bottom row). T cells were either not stimulated (first column) or had been stimulated for 48 h (second and third column) with ConA plus IL-2. (A) presents data on cell cycle distribution of the cultured T cells. Histogram analysis of cellular DNA content (*x*-axis: PI fluorescence on a linear scale) is shown in (A) and 2-colour fluorescence dot plots of cellular DNA content (*x*-axis, linear scale) versus BrdU fluorescence intensity (*y*-axis, log scale), as a measure of the activity of DNA synthesis, is presented in (B). Data on forward light scatter (*x*-axis: log scale), which is a measure of the size of the cultured T cells, are presented in (C). All data have been electronically gated to exclude dead cells. The amount of cell death during stimulation was indistinguishable between control and *bcl-2* transgenic cells and ranged between 30–40% in the first 24 h and <10% thereafter. (D) Kinetic analysis of T cell activation after 0, 24, 48 and 72 h. Data are presented as the fraction of viable cells in the S, G₂ or M phase of the cycle as a function of time of stimulation. Data points represent the arithmetic means \pm SD from 2–3 control mice and 2–3 *bcl-2* transgenic mice. Similar results were obtained with T cells from two independent *bcl-2* transgenic strains (E μ -*bcl-2-36* and E μ -*bcl-2-25*). (E) Dose-response of control, E μ -*bcl-2-25* and E μ -*bcl-2-36* T cells to the mitogen concanavalin A (ConA). Data are presented as arithmetic means \pm SD of [³H]thymidine incorporation after 3 days of stimulation from three mice of each genotype.

indicate that Bcl-2 may exert its growth-inhibitory effect predominantly during the transition from G₀ into the initial S phase (Figures 4 and 5). This situation is normally never encountered by cells growing exponentially in tissue culture, but it can be imposed experimentally. We therefore sought a system in which we could test the effect of Bcl-2 and its functional homologues on growth arrest in the absence of apoptosis. NIH 3T3 cells undergo growth arrest and remain viable (<20% apoptotic cells) when cultured in 0.5% serum for several days (Brancolini *et al.*, 1995). They do however undergo apoptosis upon culture in 0% serum and this death could be substantially inhibited by Bcl-2, Bcl-x_L or adenovirus protein E1B19kD, indicating that these proteins are functional in our NIH 3T3 clones (Figure 6D, Table III and Brancolini *et al.*, 1995). To investigate the impact of Bcl-2, Bcl-x_L and E1B19kD on entry into and emergence from growth arrest, NIH 3T3 clones overexpressing these proteins were starved for 120 h in medium containing only 0.5% serum and then returned to medium containing 10% serum. Throughout this time course we determined the DNA content of the cells by flow cytometry. Control NIH 3T3 cells entered the quiescent G₀ state within 72 h of serum depletion and after 16 h of re-addition of 10% serum essentially all of them had re-entered the cell cycle (85–95% residing in S phase, Figure 7A). Overexpression of Bcl-2, Bcl-x_L or E1B19kD slightly enhanced serum starvation-induced growth arrest of NIH 3T3 cells and significantly delayed

their re-entry into the cell cycle after re-addition of 10% serum (only 10–15% of cells residing in S phase after 16 h, Figure 7A).

The cell survival function of Bcl-2 can be inhibited by Bax (Oltvai *et al.*, 1993). To test whether Bax could also modulate the growth inhibitory effect of Bcl-2, we generated NIH 3T3 clones overexpressing Bax or both Bcl-2 plus Bax. The Bax and the Bcl-2/Bax clones were indistinguishable from parental NIH 3T3 cells in their growth kinetics in standard medium (Figure 6C), indicating that under these circumstances overexpression of Bax does not induce apoptosis or influence cell cycle control. Bax is however functional in these NIH 3T3 clones since it accelerates apoptosis induced by serum withdrawal (0%) and can reduce the survival effect of Bcl-2 (Figure 6D). Most importantly for our investigations, overexpression of Bax antagonizes the inhibitory effect of Bcl-2 on cell cycle entry of quiescent NIH 3T3 cells after serum stimulation (35–45% Bcl-2/Bax cells residing in S phase after 16 h, Figure 7A) although on its own, Bax did not cause proliferation of quiescent NIH 3T3 fibroblasts (Figure 7A and data not shown).

To investigate further the links between the anti-apoptotic activity and the growth suppressive effects of Bcl-2, NIH 3T3 fibroblasts were also transfected with expression constructs encoding mutant forms of Bcl-2 to assess their effect on cell cycle re-entry of quiescent cells. Point mutations within the conserved Bcl-2 homology domain

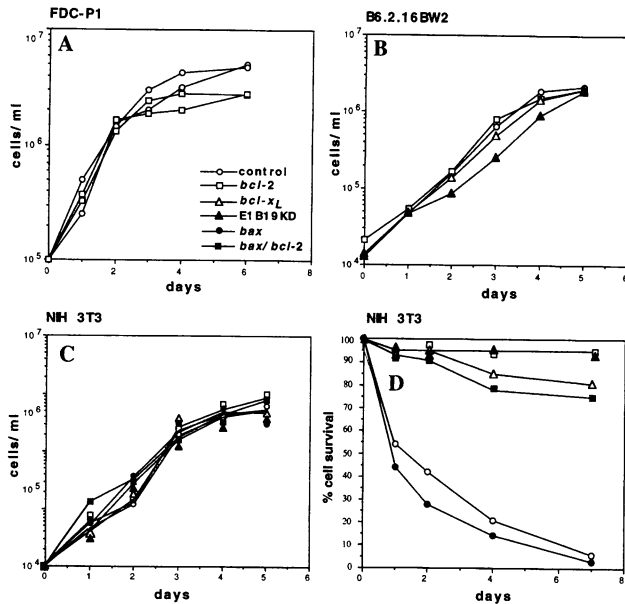


Fig. 6. Expression of Bcl-2, Bcl- x_L or E1B19kD does not affect the growth rate of immortalized cell lines under optimal conditions. The growth of the promyelocytic cell line FDC-P1 (A), B6.2.16BW2 T hybridoma cells (B) and NIH 3T3 fibroblasts (C) and their sublines overexpressing Bcl-2, Flag-epitope-tagged Bcl- x_L or adenovirus E1B19kD (and in the case of NIH 3T3 cells also Flag-epitope-tagged Bax and Bcl-2 plus Flag-epitope-tagged Bax) was studied in optimal tissue culture medium, containing 10% FCS, and in the case of FDC-P1 cells, saturating levels of IL-3. Cells growing exponentially were diluted to a starting concentration of 10^4 or 10^5 cells/ml and cell concentration was then determined in a Coulter counter after 10 min (to determine the precise starting concentration) and after 1, 2, 3, 4, 5 and 8 days. Data are presented as the cell concentration (total cells/ml) as a function of time. Figures A, B and C show data from one representative line of each genotype, but similar results were obtained with at least three sublines of each genotype. All experiments were repeated independently three times and produced similar results. The effect of Bcl-2, Bcl- x_L , adenovirus E1B19kD, Bax and Bcl-2 plus Bax on serum starvation-induced apoptosis was investigated in NIH 3T3 fibroblasts (D). Parental NIH 3T3 cells and sublines overexpressing Bcl-2, Flag-epitope-tagged Bcl- x_L , adenovirus protein E1B19kD, Flag-epitope-tagged Bax or Bcl-2 plus Flag-epitope-tagged Bax were cultured in serum free medium and cell viability was determined after 1, 2, 4 and 7 days by visual inspection under an inverted microscope using phase contrast. Data shown are from one representative clone of each genotype but similar results were obtained in three independent experiments with three independent clones.

S2 (for nomenclature see Cory, 1995), also called BH1 (for nomenclature see Korsmeyer, 1995), at amino acid residue 145 from glycine to glutamic acid (G145E) or within S3 (BH2) at amino acid residue 188 from tryptophan to alanine (W188A) disrupt the cell survival function of Bcl-2 and also abrogate its ability to interact physically with Bax (Yin *et al.*, 1994 and our unpublished observations). Deletion of the 36 amino-terminal residues ($\Delta N36$), encompassing the Bcl-2 homology domain S1, also obliterates the survival function of Bcl-2 but does not affect its ability to bind to Bax (Borner *et al.*, 1994 and our unpublished observations). Stable NIH 3T3 clones transfected with inactivating mutants of Bcl-2, in contrast to lines expressing wild-type Bcl-2, did not show delayed entry into or emergence from growth arrest (Figure 7B and Table III).

In summary, these results demonstrate that Bcl-2, Bcl- x_L and E1B19kD do not affect growth control of continuously

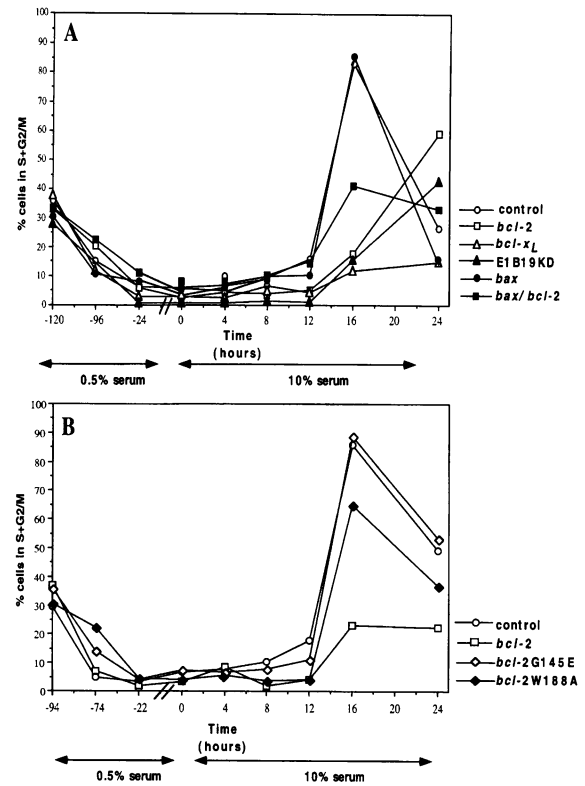


Fig. 7. Bcl-2, Bcl- x_L or E1B19kD delays serum stimulation-induced cell cycle entry of quiescent NIH 3T3 fibroblasts. The impact of Bcl-2, Bcl- x_L , adenovirus E1B19kD, Bax and Bcl-2 plus Bax (A) or mutant forms of Bcl-2: G145E or W188A (B) on serum stimulation-induced cell cycle entry was investigated in quiescent (G_0) NIH 3T3 fibroblasts. Cells were harvested from cultures in logarithmic growth, cultured for 94–120 h in medium containing 0.5% serum and then restimulated by addition of 10% serum. Cell cycle analysis was performed by flow cytometry at the start, after 24, 96 and 120 h of serum starvation and then 4, 8, 12, 16 and 24 h after restoring the serum concentration to 10%. Data are presented as the fraction of cells in the S, G_2 or M phase of the cycle (y-axis) as a function of time (x-axis, not drawn to scale). Data shown are from one representative clone of each genotype, but similar data were obtained in three independent experiments with three clones of each genotype. After 72, 96 and 120 h of starvation, all clones contained <5% cells with >2C DNA content. Symbols are drawn staggered above each other for the sake of clarity.

cycling cells under optimal conditions. However, Bcl-2 or its homologues but not inactivating mutants, can exert a growth inhibitory effect in situations of entry from the quiescent stage into the cell cycle. Moreover, our data show that Bax, while not directly promoting proliferation, reduces not only the survival function of Bcl-2 but also its effect on control of cell cycle entry, thus providing evidence that these two processes may be intricately connected.

Growth of a colon carcinoma derived tissue culture line is inhibited by wild-type Bcl-2 but not by mutants that abrogate its survival function

Although constitutive overexpression of Bcl-2 does not affect the growth of several tissue culture lines under optimal conditions (Figure 6A, B and C) it did have much more pronounced effects on some human colon carcinoma lines since it inhibited colony formation as efficiently as wild-type p53 (Pietenpol *et al.*, 1994). This might have

Table III. *bcl-2* or its homologues, but not mutant forms of *bcl-2*, suppress colony formation by SW480 colon carcinoma cells and delay cell cycle re-entry of quiescent NIH 3T3 fibroblasts

Construct	Relative cloning efficiency (% control) by SW480	Growth suppression of quiescent NIH 3T3	Protection against apoptosis in NIH 3T3
<i>puro</i>	100	-	-
<i>bcl-2/puro</i>	6 ± 5	+	+
G145E <i>bcl-2/puro</i>	94 ± 30	-	-
W188A <i>bcl-2/puro</i>	73 ± 24	-	-
ΔN36 <i>bcl-2/puro</i>	84 ± 30	-	-
<i>bcl-x_L/puro</i>	6 ± 5	+	+
E1B19kD/ <i>puro</i>	10 ± 7	+	+

Total number of colonies formed after transfection of the colon carcinoma derived tissue culture line SW480 with 20 µg of DNA from one of the following constructs: puromycin (*puro*), *bcl-2/puro*, *bcl-x_L/puro*, E1B19kD/*puro* or one of the mutant forms of *bcl-2*: G145E [point mutation within the S2 (BH1) domain], W188A [point mutation in the S3 (BH2) domain] or ΔN36 (deletion of the 36 amino-terminal residues encompassing the S1 domain). Cells were selected with the appropriate antibiotic (puromycin) and colonies counted by visual inspection 2–3 weeks later. These results were obtained with independent preparations of DNA. Data shown are arithmetic means ± SD from two to eight independent experiments for each construct. The growth suppression by *bcl-2/puro*, *bcl-x_L/puro* and E1B19kD/*puro* cannot be attributed to non-specific toxicity as the same batches of DNA allowed us to generate NIH 3T3 clones expressing high levels of Bcl-2, Bcl-x_L or adenovirus protein E1B19kD. All constructs promoted high level protein expression that were easily detectable by immunofluorescence staining.

resulted from the ability of Bcl-2 to inhibit cell cycle progression. We were able to reproduce and expand these results by demonstrating that not only Bcl-2 but also its homologues can suppress colony formation of the human colon carcinoma derived tissue culture line SW480 by a factor of 10- to 100-fold (Table III).

In order to investigate further whether the two functions of Bcl-2 to regulate cell survival and cell cycle control are linked, we transfected SW480 cells with expression constructs encoding mutant forms of Bcl-2 and assessed their impact on colony formation. Interestingly, transfection of three inactivating Bcl-2 mutants (G145E, W188A or ΔN36) did not suppress colony formation of SW480 cells (Table III). Thus, these data provide further evidence that Bcl-2 and its homologues can affect cell growth and that this function and the ability to promote cell survival may be linked.

Discussion

A link between cell survival and growth control is regulated by Bcl-2 and its homologues

Signal transduction pathways leading from cell cycle control to the cell death programme have been described but so far none are known that lead in the opposite direction: from cell survival control to the cell cycle machinery. The data presented in this paper provide evidence that regulators of the cell death programme can affect the control of the cell cycle machinery. Our results demonstrate that Bcl-2 and its functional homologues, Bcl-x_L and the adenovirus protein E1B19kD, can inhibit cell growth. Enforced expression of Bcl-2 reduced proliferation (Table I and Figure 1) and slowed turnover (Figures 2 and 3) of thymocytes *in vivo* and retarded cell cycle entry of quiescent lymphocytes after mitogen stimulation *in vitro* (Figures 4 and 5), consistent with a recent report (Mazel *et al.*, 1996). We demonstrate here that these effects of Bcl-2 on cell growth are cell autonomous and cannot be ascribed to homeostatic feed-back (Table II). Moreover, expression of Bcl-2 or its functional homologues delayed serum stimulation-induced cell cycle entry of quiescent fibroblasts (Figure 7A).

The stable introduction of expression constructs encod-

ing Bcl-2, Bcl-x_L or E1B19kD into several haematopoietic and other types of cell lines has been achieved in many laboratories (Vaux *et al.*, 1988; Nunez *et al.*, 1990; Rao *et al.*, 1992; White *et al.*, 1992; Boise *et al.*, 1993; Debbas and White, 1993; Lithgow *et al.*, 1994) and clearly, their overexpression did not affect their growth rate under optimal tissue culture conditions (Figure 6A, B and C) as has been shown for fibroblasts (Fanidi *et al.*, 1992). It therefore appears that Bcl-2 and its homologues can only affect cell cycle control at the transition from the quiescent G₀ state into the first S phase but has no effect on cells that are continuously cycling. This raises the question how transfection with Bcl-2, Bcl-x_L or E1B19kD expression constructs could inhibit colony formation of several human carcinoma derived tissue culture lines (Pietenpol *et al.*, 1994 and Table III). We speculate that the act of transfection induces transient growth arrest and that overexpression of Bcl-2 or its homologues suppresses colony formation by blocking re-entry into the cell cycle.

Potential physiological roles for Bcl-2-mediated growth inhibition

The physiological significance of Bcl-2, Bcl-x_L and adenovirus E1B19kD in cell death regulation is clearly established. The cellular proteins contribute to the selection and maintenance of useful cells by inhibiting apoptosis (Veis *et al.*, 1993; Linette *et al.*, 1994; Motoyama *et al.*, 1995), and viral homologues protect infected cells against various host defence systems (Rao *et al.*, 1992; White *et al.*, 1992; Debbas and White, 1993; Vaux *et al.*, 1994). What roles might growth inhibition mediated by Bcl-2 and its functional homologues play in normal physiology?

Cells possess mechanisms that monitor the cell cycle circuitry and trigger the death programme whenever abnormalities are sensed. Apoptosis ensues when cell cycle activators, such as c-Myc, cyclin D1 or E2F1 are inappropriately induced in growth arrested cells (Evan *et al.*, 1992) or when cell cycle inhibitors such as p53 are introduced into cycling cells (Yonish-Rouach *et al.*, 1991). However, not all mediators of cell survival inhibit cell growth and cell cycle entry. Indeed, cytokines such as IGF and PDGF prevent not only cell death induced by overexpression of *c-myc* in serum-deprived rat embryo

fibroblasts but promote their sustained proliferation (Harrington *et al.*, 1994). By inhibiting apoptosis and cell cycle progression simultaneously, Bcl-2 and its homologues may be ideally suited to promote cell cycle entry of quiescent cells and may also facilitate growth arrest during differentiation into a post-mitotic state (Vairo *et al.*, 1996).

Transition of quiescent NIH 3T3 fibroblasts into S phase is inhibited not only by Bcl-2 but also by Bcl-x_L and E1B19kD (Figure 7A). It therefore appears that the survival and the growth inhibitory functions are both conserved amongst at least some of the functional homologues of Bcl-2. Several viruses carry Bcl-2-related genes and use their anti-apoptotic activity to promote host cell survival (Vaux *et al.*, 1994). What benefit could viruses derive from the ability of Bcl-2 homologues to inhibit cell cycle progression? Some viruses have mechanisms to activate the host cell cycle machinery, which they use to promote replication of their genome. Cell cycle activation by viral proteins, such as adenovirus E1A, in the absence of costimulatory signals, triggers apoptosis which can be blocked by Bcl-2 or its adenovirus homologue E1B19kD (White *et al.*, 1992). It is possible that viral homologues facilitate infection not only by preventing cell death but also by delaying host cells from entering the cycle under inappropriate circumstances.

Both activities, enhancing survival and growth inhibition, may be evolutionarily conserved within the Bcl-2 family, since Ced-9 deficient nematodes displayed abnormalities in cell division (Hengartner *et al.*, 1992). During development of this organism, Ced-9 might play a role not only by blocking cell death but also by facilitating cell cycle exit when dividing cells undergo growth arrest and terminal differentiation. Cell cycle analysis in doubly-mutant nematodes deficient in Ced-3, to block programmed cell death, and also lacking Ced-9 is expected to provide an interesting insight into the role of Bcl-2 homologues in cell cycle control.

Potential mechanisms of Bcl-2-mediated growth inhibition

Bcl-2 and its functional homologues are thought to inhibit apoptosis by blocking either activation or function of cysteine proteases (see Introduction) but their biochemical action is still unclear. It is therefore difficult to predict how these proteins affect cell cycle control, but the two functions may well be intricately linked since both are antagonized by Bax (Figures 6D and 7A) and obliterated by the same mutations within conserved regions of Bcl-2 (Figure 7B and Table III).

Could Bcl-2 inhibit the function of a protein which is essential for cell cycle progression? Reduced levels of cdc2 have been detected in growth arrested Bcl-2 over-expressing cells that were deprived for several days of essential growth factors (Marvel *et al.*, 1994). In this experiment it was however impossible to determine whether this was the cause or effect of prolonged cell cycle arrest since all control cells had died within 2 days of starvation. Further studies from our own laboratory have shown that when T cells from *bcl-2* transgenic mice are stimulated with mitogens, the product of the retinoblastoma gene (RB) remains in the hypophosphorylated state longer compared with control T cells (B. Warner,

personal communication). This indicates that after mitogen stimulation, the G₁ cyclin–cyclin dependent kinase complexes remain inactive for a prolonged period in *bcl-2* transgenic T cells. However, it is unclear whether the persistence of the active form of RB is the cause or consequence of prolonged arrest in G₀. Furthermore, Bcl-2-mediated growth inhibition does not require the presence of p53 since SW480 colon carcinoma cells, that show reduced colony formation upon transfection with Bcl-2, Bcl-x_L or E1B19kD expression constructs (Table III), lack this tumour suppressor (Pietenpol *et al.*, 1994).

Another possible explanation may be that low level activity of some cell death effector molecule is essential for cell cycling and that much higher levels are needed for apoptosis. By blocking apoptosis, Bcl-2 may lower the activity of this effector below the threshold needed for cell cycling and thereby promote growth arrest. The concept that a single component is essential for both of these processes is attractive, as it would guarantee that mutations which abrogate physiological cell death would also prevent further cell division. It has been proposed that cyclin dependent kinase cdc2 may carry out such a function (Shi *et al.*, 1994), but this is now controversial (Martin *et al.*, 1995). Could Ced-3-like cysteine proteases and Ced-4 homologues be alternative candidates? Prima facie it would seem not, since cell proliferation and differentiation appear normal in *ced-3* and *ced-4* mutant nematodes (Ellis and Horvitz, 1986; Ellis *et al.*, 1991), but it is unclear whether these mutations annihilate the function of the corresponding proteins or merely reduce it.

Another hypothesis is that Bcl-2 and its functional homologues are part of a signalling pathway that affects cell cycle control. Such a signal might be generated constitutively or might only be triggered when Bcl-2 is actively inhibiting cell death. The signal leading to growth inhibition may interact directly with the cell cycle machinery or intercept a growth stimulatory pathway further upstream. All the proteins that have been found to interact with Bcl-2, namely Bax, Bak, Bag-1, Bik, Nip-1, Nip-2 and Nip-3 might be involved in generating such a signal (Cory, 1995; Korsmeyer, 1995). However, the GTPase R-ras, functioning as a molecular switch, stands out as an attractive candidate (Fernandez-Sarabia and Bischoff, 1993). Since the ability to associate with Bax is insufficient for Bcl-x_L and Bcl-2 to block apoptosis (Cheng *et al.*, 1996 and our unpublished observations), it is possible that members of the Bcl-2 family function in cell survival and cell growth control not only as dimers (Korsmeyer, 1995) but as part of a higher-order multi-protein complex. Clearly more work is needed both in mammals and nematodes to fully understand the molecular mechanisms of cell death and cell cycle control to uncover the connections between these regulatory networks.

Implications for tumorigenesis and cancer therapy

The notion that Bcl-2 expression can inhibit growth under some circumstances is compatible with the finding that it is only poorly oncogenic on its own in transgenic mice (McDonnell and Korsmeyer, 1991; Strasser *et al.*, 1993; Linette *et al.*, 1995). It fits with the low frequency of cycling tumour cells in human follicular centre B lymphoma and chronic lymphocytic leukemia, two

indolent cancers in which the *bcl-2* gene was found to be activated by chromosomal translocation (Korsmeyer, 1995). Cells with an activated *bcl-2* oncogene may only become proliferating tumour cells if appropriate cell cycle stimulatory signals are also activated, either by normal external stimuli or by synergistic mutations in other oncogenes. This might explain why expansion of many follicular lymphomas appears to be driven by antigenic stimulation (Levy *et al.*, 1987) and why *bcl-2* synergizes so potently with *c-myc* and *pim-1* in oncogenesis (Strasser *et al.*, 1990; Acton *et al.*, 1992). Mutations which abrogate the growth inhibitory effect and leave cell survival function intact would thus be expected to dramatically increase the transforming potential of these oncogenes. Conversely, a drug which specifically inhibits the anti-apoptotic function of Bcl-2 and/or its functional homologues, but does not affect, or even augments their ability to retard entry into the cell cycle, may be useful for cancer therapy.

Materials and methods

Mice

The generation of the E μ -*bcl-2* transgenic mice (strain *bcl-2-25*, expressing the human *bcl-2* cDNA only in T cells, strain *bcl-2-36*, expressing the transgene in B and T cells and strain *bcl-2-22*, expressing the transgene only in B cells) and the anti-HY TCR transgenic mice has been described previously (von Boehmer, 1990; Strasser *et al.*, 1991a,b). These strains had all been backcrossed for >10 generations with C57BL/6 mice before they were used in these studies. *bcl-2*/anti-HY TCR bi-transgenic mice were generated by inter-crossing the two parental strains. Inheritance of the *bcl-2* transgene was identified by PCR using specific oligonucleotide primers, by peripheral blood analysis [only in strain *bcl-2-36* by looking for B cell excess; (Strasser *et al.*, 1990, 1991a,b)] or by immunofluorescence staining of lymphocytes with a human Bcl-2 protein specific monoclonal antibody. Inheritance of the anti-HY TCR transgene was identified either by PCR using specific oligonucleotide primers or by immunofluorescence staining and flow cytometric analysis of peripheral blood cells, using monoclonal antibody F23.2 which recognizes the transgene encoded TCR β (v β 8.2) chain (von Boehmer, 1990). All animals were used at 5–10 weeks of age, unless stated otherwise. Chimeric mice containing both control as well as *bcl-2* transgenic thymocytes were generated by reconstituting lethally irradiated (9 Gy) C57BL/6 Ly5.1 mice with mixtures of bone marrow from control C57BL/6 Ly5.1 mice and C57BL/6 Ly5.2 *bcl-2* transgenic mice. We only analysed chimeric animals which had roughly equal portions of control and *bcl-2* transgenic thymocytes and this was achieved with a bone marrow mixture of control versus *bcl-2* transgenic bone marrow of 4:1 or 8:1. As controls we reconstituted lethally irradiated C57BL/6 Ly5.1 mice either with bone marrow from control C57BL/6 Ly5.1 mice alone or with bone marrow from C57BL/6 Ly5.2 *bcl-2* transgenic mice alone. These animals were fed with the antibiotic neomycin (1.6 g/l in the drinking water) and were analysed 4 weeks after reconstitution.

Cell lines and expression constructs

FDC-P1 is a mouse IL-3-dependent promyelocytic cell line. B6.2.16BW2 is a mouse T cell hybridoma expressing the same T cell receptor as anti-HY TCR transgenic T cells. NIH 3T3 is a mouse fibroblast line. These tissue culture lines were either infected with a control *neo* or a *bcl-2*/*neo* retrovirus (Vaux *et al.*, 1988; Lithgow *et al.*, 1994) or transfected by electroporation with expression vectors (pEFpGK*neo*) or (pEFpGK*puro*) derived from pEFBos (Mizushima and Nagata, 1990) and containing cDNA encoding human Bcl-2, Bcl-x_L, adenovirus E1B19kD or mouse Bax with or without the Flag-epitope-tag (Blanan and Rutter, 1992) or with control *neo* or *puro* constructs (Strasser *et al.*, 1995). In addition, NIH 3T3 fibroblasts were also transfected with expression constructs encoding the following mutants of Bcl-2: point mutation at either amino acid residue 145 from glycine to glutamic acid (G145E) or at amino acid residue 188 from tryptophan to alanine (W188A) or deletion of the 36 amino-terminal residues (Δ N36). The mutant constructs were generated by PCR and fully sequenced. Transfectants were selected by growth in G418 (1–2 mg/ml) or puromycin

(2–5 μ g/ml). Cell lines were single cell cloned using limiting dilution culture or the single cell deposition unit of the FACStar Plus (Becton Dickinson). Clones expressing high levels of the protein of interest were identified by immunofluorescence staining and flow cytometric analysis (see below). The human colon carcinoma derived tissue culture line SW480 (ATCC) was transfected by lipofection with pEFpGK*puro* expression constructs encoding wild-type human Bcl-2 or the following mutants: G145E *bcl-2*, W188A *bcl-2* or Δ N36 *bcl-2*. Cells were transfected in 35 mm Petri dishes with 20 μ g of DNA and 100 μ g of lipofectAMINE (Gibco BRL). Drug selection (400 μ g/ml G418 or 5 μ g/ml puromycin) was commenced 2 days after transfection and colony formation was scored by visual inspection under an inverted microscope after an additional 14 days.

Tissue culture

All cell lines and normal, purified B and T cells were cultured in the high glucose version of Dulbecco's modified Eagle's (DME) medium supplemented with 10% fetal bovine serum, 50 μ M 2-mercaptoethanol, 13 μ M folic acid and 100 μ M L-asparagine. Growth rates of cell lines were determined by plating cells at a starting concentration of 10⁴/ml or 10⁵/ml and counting cell concentrations at 24 h intervals over a 5–8 day period. B cells were stimulated *in vitro* with either 20 μ g/ml Fab₂ goat anti-mouse IgM antibodies (Jackson Immuno Research), 2 μ g/ml FGK45, rat anti-mouse CD40 monoclonal antibody (A.Rolink and F.Melchers, manuscript submitted) or 20 μ g/ml LPS (Difco). All stimulations were performed in the absence or presence of saturating concentrations of recombinant mouse interleukins, IL-2 plus IL-4 plus IL-5, produced by transfected X63/0 hybridoma cells (Karasuyama and Melchers, 1988). T cells were stimulated *in vitro* either with 2 ng/ml phorbol-12-myristate-acetate (PMA) plus 2 μ g/ml ionomycin (both from Sigma), 2 μ g/ml concanavalin A (ConA, Pharmacia) plus IL-2 (at saturating concentration) or with anti-CD3 (KT3) plus anti-CD28 (37N51) monoclonal antibodies bound to the tissue culture plates (concentration of both antibodies in the coating solution: 10 μ g/ml).

Cell turnover and cell cycle analysis

Cell turnover *in vivo* was determined by labelling proliferating cells with the thymidine analogue BrdU (Sigma), which was provided continuously for 1, 3, 5 or 7 days in the drinking water (1 mg/ml plus 2% glucose to overcome taste aversion). Drinking water bottles were shielded from light and exchanged at 3 day intervals. Thymus, spleen and bone marrow cellularity did not drop during the course of this treatment, indicating that it was not toxic. BrdU was detected by immunofluorescence staining with a specific FITC-labelled monoclonal antibody, BU-1 (Becton Dickinson) and flow cytometric analysis according to published procedures (Fulcher and Basten, 1994). Cells were fixed in 0.5% paraformaldehyde in PBS (20 min at room temperature). DNA was denatured using 3 N HCl, containing 0.5% Tween-20 to permeabilize cell membranes (20 min at room temperature), in order to facilitate detection of BrdU incorporated into DNA. The acid was neutralized with 0.1 M di-sodium-tetra-borate and cells were then stained with a fluorescein-isothiocyanate (FITC)-labelled anti-BrdU monoclonal antibody (Becton Dickinson) in the presence of 0.5% Tween-20. Thymocyte subsets were identified by staining with R-phycoerythrin (R-PE)-labelled monoclonal antibody to CD8 (Caltag) and a biotinylated monoclonal antibody to CD4 (clones YTA321 or H129.19.6.8) plus Tricolor-conjugated streptavidin (Caltag). Flow cytometric analysis (10 000 cells per sample) was performed on a FACScan (Becton Dickinson). As a negative control for the BrdU staining we included in all experiments thymocytes from a mouse that had not received BrdU. To verify that mice had incorporated BrdU and as a positive control for the BrdU staining we always checked that cells with a high turnover rate, such as granulocytes within bone marrow, were labelled strongly.

The rate of cell division of cell lines and mitogen-stimulated B and T lymphocytes *in vitro* was determined by pulsing cultures for 1–2 h with BrdU (3 mg/ml). Cells that had incorporated BrdU were detected by immunofluorescence staining as described above. Cell cycle analysis was performed as described previously (Taylor, 1980). Cells were fixed (>8 h at 4°C) in 70% ethanol, treated with 0.5 mg/ml DNase-free RNase A (20 min at room temperature) and then stained with 69 μ M propidium iodide (PI, Sigma) in 0.1 M sodium citrate (pH 7.4). Alternatively, DNA was stained by staining cells for 30 min at 4°C in 50 μ g/ml PI in 0.1% sodium acetate with 0.2% Triton X-100 (BDH Chemicals). Flow cytometric analysis (10 000 cells per sample) was performed on a FACScan. Cell cycle distribution was determined with the Cellfit programme or manual gating. Immunofluorescence staining with FITC-labelled anti-BrdU antibodies and with PI could be combined

to simultaneously assess the rate of DNA synthesis and total cellular DNA content.

Immunofluorescence staining, flow cytometric analysis and cell sorting

Surface immunofluorescence staining of thymocytes, splenocytes, lymph node and bone marrow cells was performed with cell surface marker specific monoclonal antibodies as described previously (Strasser *et al.*, 1991a). Antibodies were conjugated to either FITC, R-PE or biotin (which was detected with R-PE- or Tricolor-streptavidin; both from Caltag) to allow two or three colour flow cytometric analysis using a FACScan. Expression of transgene-encoded human Bcl-2, E1B19KD and Flag-epitope-tagged Bcl-x_L was studied as previously described (Strasser *et al.*, 1995) by cytoplasmic immunofluorescence staining of 1% para-formaldehyde fixed, 0.3% saponin permeabilized cells using specific monoclonal antibodies to human Bcl-2 (Bcl-2-100; Pezzella *et al.*, 1990), adenovirus E1B19KD (Oncogene Science) or FLAG (IBI). B or T lymphocytes were purified from spleen or lymph nodes by negative sorting to prevent delivering a signal to cells via binding of antibodies to surface molecules. All unwanted cells within these tissues (i.e. granulocytes, macrophages, erythroid cells and lymphocytes from the unwanted lineage) were stained with FITC-labelled surface marker specific monoclonal antibodies (8C5 anti-Gr-1, MI/70 anti-Mac-1, Ter119 and for purification of B cells, plus T24.31.2 anti-Thy-1, KT3 anti-CD3, GK1.5 anti-CD4 and 53.6.72 anti-CD8 or for purification of T cells, plus 5.1 anti-IgM, HB58 anti-Igκ, JC5 anti-Igλ and RA3-6B2 anti-CD45R-B220) and the vital dye PI (1 μg/ml). Viable cells that were not stained with FITC-labelled monoclonal antibodies (FITC⁻, PI⁻) were purified on a FACSSII or a FACStar Plus. The purity of sorted B and T cells was verified by staining cells with R-PE- or Tricolor-labelled monoclonal antibodies specific to B220 or CD4 and CD8 (all from Caltag); purity was 95–99%. Viable (PI-excluding) Thy-1⁺CD4⁻CD8⁻, Thy-1⁺CD4⁺CD8⁺ and Thy-1⁺CD4⁻CD8⁺ thymocytes were FACS purified after staining with R-PE-labelled anti-CD4, FITC-labelled anti-CD8 (both from Becton Dickinson) and biotinylated anti-Thy-1 (clone T24.31.2) plus Tricolor-streptavidin (Caltag). The purity of the sorted populations was 92–98%. Control (Ly5.1) and *bcl-2* transgenic (Ly5.2) thymocytes were purified from bone marrow reconstitution chimeric mice (see above) after staining with a biotinylated anti-Ly5.2 monoclonal antibody plus R-PE streptavidin. Cytoplasmic immunofluorescence staining with a human Bcl-2 specific monoclonal antibody (Bcl-2-100) was routinely performed to verify that all Ly5.2 and none of the Ly5.1 thymocytes expressed the *bcl-2* transgene. The purity of the sorted populations was >98%.

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