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Expression of the Bcl-2 Homologue Mcl-1 Correlates with Survival of Peripheral Blood B Lymphocytes[1]

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Abstract

Normal peripheral blood B lymphocytes undergo spontaneous apoptosis *in vitro*, and this process is regulated positively and negatively by several immunomodulatory stimuli. We have shown previously that Bcl-2 protein levels are unaltered by these factors, suggesting a Bcl-2-independent regulation of apoptosis in this system. Here, we have investigated the possibility that the three recently identified Bcl-2 homologues, Bax, Bcl-x, and Mcl-1, could be involved instead. Freshly isolated cells expressed both Bax and Mcl-1 protein, but only low levels of Bcl-x_L and no detectable Bcl-x_S, as determined by Western blot analysis. Upon culture of cells with apoptotic or survival stimuli, Bax and Bcl-x_L protein levels remained relatively unchanged. By contrast, Mcl-1 levels decreased markedly in cells undergoing apoptosis in medium and, even more dramatically, after treatment with the apoptotic stimuli transforming growth factor β1 and forskolin. This decrease was rapid and preceded cell death. Furthermore, all the survival stimuli tested (interleukin 4, anti-IgM antibodies, and the phorbol ester 12-O-tetradecanoylphorbol-13-acetate) prevented the decline in Mcl-1 levels. This striking correlation between cell survival and Mcl-1 expression in peripheral blood B cells suggests the possible involvement of Mcl-1, instead of Bcl-2, in the regulation of apoptosis in these cells. The present study is the first one linking this novel Bcl-2 homologue to the control of cell death in normal cells.

Introduction

Apoptosis, or programmed cell death, is a fundamental biological phenomenon that plays a crucial role in processes such as embryogenesis, immune regulation, and general tissue homeostasis. Like cell proliferation, it is subject to complex control, involving a set of specific regulatory genes. One of the most studied genes is *bcl-2*, which encodes a M_r 26,000 protein localized to intracellular membranes that prevents cell death by a still unknown mechanism (1). The important role of *bcl-2* in supporting cell survival has been well demonstrated, in particular by studies with *bcl-2* transgenic (2, 3) or knockout mice (4). However, despite the frequent correlation between Bcl-2 levels and cell survival, no absolute interdependence exists. For example, in some cases apoptosis may be modulated without any regulation of Bcl-2 expression (5–7). Moreover, the forced overexpression of *bcl-2* by transfection into cell lines does not always affect the rate of apoptosis (8). Importantly, studies with *bcl-2-/-*mice have shown that, despite the lack of *bcl-2*, embryonic development proceeds normally, and phenotypic aberrations occur only in selected tissues postnatally (4). These findings show that Bcl-2 may be redundant in many situations and suggest Bcl-2-independent strategies for the regulation of cell death.

Good candidates in this respect are to be found in the growing family of Bcl-2-related proteins, which share many of the essential characteristics of Bcl-2, such as intracellular localization and the ability to modulate apoptosis. Here we have studied the role of three of these, Bax, Bcl-x, and Mcl-1, in a cell system in which the regulation of cell death is Bcl-2 independent. Bax, which heterodimerizes with Bcl-2, induces cell death when overexpressed in cell lines (9). Bcl-x exists in two forms; the long form Bcl-x_L inhibits apoptosis, whereas the short form Bcl-x_S promotes cell death (10). Mcl-1 is a new member of this family that was identified by differential screening of cDNA libraries derived from a human myeloid leukemia cell line induced to undergo differentiation in culture (11). It was recently shown that transfection of Mcl-1 into chinese hamster ovary cells leads to inhibition of apoptosis induced by *c-myc* overexpression (12), implying that it is an inhibitor of cell death. Similar to the anti-apoptotic proteins Bcl-2 and Bcl-x_L, the Mcl-1 protein heterodimerizes with Bax (13). However, no studies exist regarding the role of Mcl-1 in the regulation of apoptosis of normal cells. Here we report a distinct correlation between cell survival and Mcl-1 expression in normal PB[3] B lymphocytes, suggesting a possible function of this novel Bcl-2-related protein in the regulation of PB B-cell death. The results are discussed in relation to apoptosis control in the B-cell compartment and the role of the Bcl-2 family.

Materials and Methods

Reagents. rhuTGFβ1 was kindly provided Dr. Tony Purchio (Oncogene Corp., Seattle, WA), and rhuIL-4 was a gift of Dr. Paul P. Trotta (Schering-Plough Research Corp., Bloomfield, NJ). F(ab'$_2$) fragments of rabbit polyclonal antibodies to human IgM heavy chain (anti-μ) were from Dako (Copenhagen, Denmark). Forskolin (Calbiochem-Behring Corp., La Jolla, CA) was dissolved in ethanol and stored at 10 mM concentration. *O*-tetradecanoylphorbol-13-acetate (TPA) was purchased from Sigma Chemical Co. (St. Louis, MO).

B Cell Purification and Culture. Normal human B lymphocytes were isolated from PB buffy coats by positive selection using anti-CD19 coated magnetic beads (Dynabeads M-450 Pan B; Dynal, Oslo, Norway) as described previously (14). Cells (1×10^6 ml) were cultured in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with penicillin, streptomycin, and 1% fetal bovine serum.

Determination of Cell Death. Viability (or cell death) was measured by vital dye exclusion test using PI. The cells were stained directly with 5 μg/ml PI (Calbiochem Corp., La Jolla, CA) for 5–30 min on ice before running at least 5000 cells/sample on a FACScan flow cytometer (Becton Dickinson, Lincoln Park, NJ) and analyzing the data using Lysys II software (Becton Dickinson). Apoptosis was detected by TUNEL and was quantitated using FACScan analysis as described previously (7).

Western Blot Analysis. Ten million cells were washed twice in PBS, resuspended in 50 μl of sample buffer [10% glycerol, 5% mercaptoethanol, 0.0625 M Tris-HCl (pH 6.8), 2.5% SDS], and boiled for 15 min. Ten μg of total protein from each sample were run on 12% SDS-polyacrylamide gels and

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[3] The abbreviations used are: PB, peripheral blood; CD40L, CD40 ligand; GC, germinal center; hu, human; PI, propidium iodide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; TPA, O-tetradecanoylphorbol-13-acetate; TBS, Tris buffered saline; IL-4, interleukin 4; TGF-β1, transforming growth factor β1.

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Table 1 *Regulation of apoptosis in PB B lymphocytes*

	% of PI-positive cells			% of TUNEL-positive cells		
	0 h	24 h	48 h	0 h	24 h	48 h
Medium	5 (1)	22 (2)	47 (3)	0.6 (0.3)	21 (4)	34 (0.6)
IL-4		14 (2)	23 (2)		13 (3)	19 (3)
Anti-μ		10 (1)	16 (0.6)		9 (2)	12 (1)
TPA		12 (0.6)	23 (3)		6 (1)	7 (0.6)
Forskolin		30 (4)	57 (1)		28 (6)	49 (2)
TGF-β1		27 (1)	61 (3)		34 (4)	54 (5)

PB B cells were cultured with the indicated stimuli, and cell death was measured by direct staining with PI and by TUNEL labeling. The following concentrations were used: 40 ng/ml IL-4, 37.5 μg/ml anti-μ antibodies, 10 nM TPA, 100 μM forskolin, and 10 ng/ml TGF-β1. Data are presented as means (SEM) of three separate experiments.

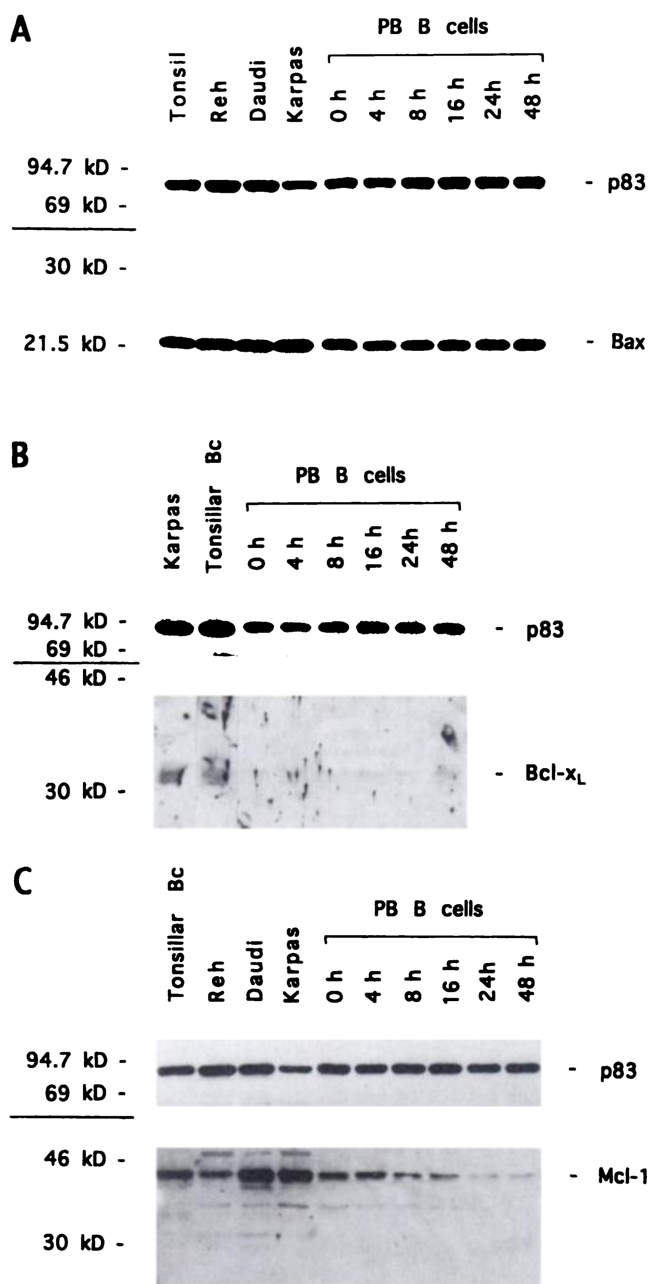

Fig. 1. Expression of Bax, Bcl-x, and Mcl-1 in spontaneously dying PB B cells. PB B cells were cultured *in vitro* for the indicated time periods, and relative protein expression of Bax-α (A), Bcl-x_L (B), and Mcl-1 (C) was determined by Western blotting. Each lane was loaded with 10 μg of total protein, and as a control for equal loading, the expression of the nuclear antigen p83 was used. For comparison, levels of Bcl-2 homologues in whole tonsil cell suspension *(Tonsil)*, tonsillar B cells *(Tonsillar Bc)*, and the B cell lines Reh, Daudi, and Karpas 422 are shown. kD, molecular weight in thousands.

blotted onto nitrocellulose filters. The filters were pretreated with TBS containing 0.1% Tween-20 and 5% dry milk and incubated at room temperature overnight in TBS buffer (TBS plus 0.1% Tween-20, 0.5% dry milk, and 0.1% fetal bovine serum) containing 0.05% (v/v) anti-Bcl-2 mAb (kindly provided by D. Y. Mason, Oxford, United Kingdom), or 0.1% rabbit antiserum against Mcl-1 (15), Bax (Santa Cruz Biotechnology, Heidelberg, Germany, or as in Ref. 16), or Bcl-x (17) proteins. After washing, the blots were incubated for 1 h with 0.033% (v/v) goat anti-mouse IgG (Bcl-2) or 0.025% goat anti-rabbit IgG (Mcl-1, Bax, and Bcl-x) antibodies conjugated to horseradish peroxidase (Bio-rad, Herkules, CA), and the respective proteins were visualized using ECL Western blotting kit (RPN 2106; Amersham, Buckinghamshire, United Kingdon) according to the manufacturer's protocol. To control protein loading of the gels, Western blots were also hybridized with the mAb 34C1 (18), which detects a protein of M_r 83,000. p83 is distributed diffusely and abundantly throughout the nucleoplasm of all human cells tested, and the expression per cell does not appear to vary with growth rate or tissue origin (18).

Results and Discussion

We have shown previously that human B lymphocytes isolated from PB have the capacity to die by apoptosis *in vitro*. Furthermore, cell death may be regulated in a positive and negative manner by several important factors known to affect other B-cell responses (7). Table 1 shows the effect of some of the most potent of these stimuli on cell death measured by PI exclusion or by the apoptosis-specific TUNEL assay, which detects DNA fragmentation in individual cells. As demonstrated, the activation of B cells with IL-4, cross-linking of surface IgM using anti-IgM antibodies (anti-μ), and protein kinase C stimulation using the phorbol ester TPA lead to inhibition of spontaneous apoptosis in medium by at least 50% at 48 h. In contrast, TGF-β1 and forskolin, an activator of the cyclic AMP-generating enzyme adenylyl cyclase, both enhanced apoptosis relative to medium control. Both survival and apoptotic stimuli had little effect at 24 h, whereas effects were clearly manifested at 48 h.

Previous studies showed that relative Bcl-2 protein levels measured by Western blotting for as long as 48 h, were not altered by any of the above stimuli (7), suggesting that the regulation of apoptosis by these factors did not involve Bcl-2. To search for other possible mechanisms we studied the expression of three recently identified Bcl-2 homologues, Bcl-x, Bax, and Mcl-1. First, the relative protein levels in cells cultured in medium alone were determined and were compared to those of control cells (total tonsillar cells or tonsillar B cells, as well as B-cell lines Reh, Daudi, and Karpas 422). As shown in Fig. 1, fresh PB B cells expressed appreciable levels of the M_r 21,000 Bax-α and the $\approx M_r$ 41,000 Mcl-1 protein. In contrast, Bcl-x_L (a doublet of proteins at M_r 29,000–31,000) was only weakly expressed and Bcl-x_S (M_r 20,000) was not detectable (data not shown). There was no change in Bax-α and Bcl-x_L levels within the time period studied (48 h). In contrast, Mcl-1 levels showed a marked decline that was apparent within the first 24 h, previous to cell death. As seen in Fig. 1*C*, the Mcl-1 protein appears as a doublet around M_r 40,000 in tonsillar cells and the Daudi cell line, whereas in PB B cells the upper

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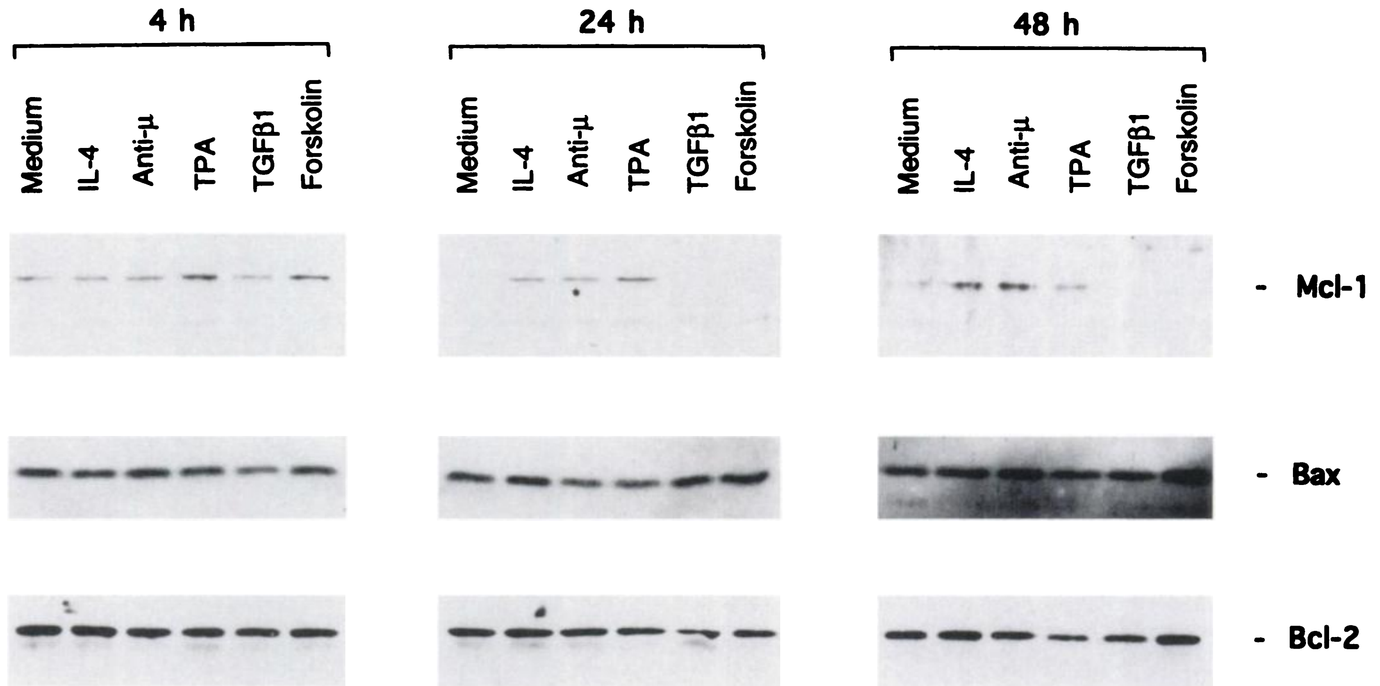

Fig. 2. Survival stimuli maintain Mcl-1 expression. PB B cells were cultured with the indicated stimuli for 4, 24, and 48 h, and relative protein expression of Mcl-1, Bax, and Bcl-2 was measured by Western blotting. Each lane was loaded with 10 μg of protein. The following concentrations of survival and apoptotic factors were used: 40 ng/ml IL-4, 37.5 μg/ml anti-μ polyclonal antibodies, 10 nM TPA, 100 μM forskolin, and 10 ng/ml TGF-β1.

band is the more prominent. Splitting of the Mcl-1 band has also been reported by others (19), but the significance is unknown.

Next, the effect of the various apoptotic and survival stimuli on the expression of Bcl-2, Bax, Bcl-x, and Mcl-1 was studied (Fig. 2). In agreement with previous findings (7), Bcl-2 levels remained unaltered by IL-4, anti-μ, TPA, forskolin, and TGF-β1 both at 4, 24, and 48 h. The same was also observed for Bax-α. In contrast, dramatic changes in Mcl-1 levels were observed. Levels decreased markedly in cells cultured for 24 h or more in medium, and repeated experiments showed that Mcl-1 expression was reduced even more by the apoptotic stimuli forskolin and TGF-β1. On the other hand, all three survival stimuli studied sustained Mcl-1 protein levels. Essentially no regulation of Bcl-x_L protein levels by the apoptosis regulators could be seen (data not shown), and Bcl-x_S was not detected under any of the culture conditions (data not shown).

The consistent correlation of Mcl-1 expression with cell survival observed in this system, where a number of important immunomodulators have been used, is striking. This, together with the finding that down-regulation of Mcl-1 in dying cells precedes cell death, could indicate that Mcl-1 is involved mechanistically in the control of apoptosis in PB B cells. The results also support the notion that Mcl-1 functions as an inhibitor of cell death, rather than an inducer of apoptosis such as Bax. They are, therefore, in agreement with the recent study by Reynolds *et al.* (12), demonstrating that overexpression of Mcl-1 blocks c-*myc*-induced cell death, as well as studies in yeast where Mcl-1 was reported to block Bax-induced lethality (13, 20). In apparent contrast, an immunohistochemical study provided suggestive, albeit indirect, evidence that Mcl-1 could be a death-inducing molecule because Mcl-1 expression was high in normal apoptosis-prone germinal center B cells, whereas the long-lived cells of the follicular mantle lacked Mcl-1 (15). However, as indicated in the study, Mcl-1 might still be involved in the rescue of GC B cells, in that it could provide a transient survival stimulus until other survival genes, notably Bcl-2, are switched on (15).

The rapid kinetics of Mcl-1 down-regulation is notable. Unlike Bcl-2, the Mcl-1 protein contains two PEST motifs that have been associated with fast protein turnover (11); accordingly, the estimated half-life of Mcl-1 is only 1–3 h (19). This property of Mcl-1 is especially relevant to apoptosis resulting from lack of survival factors, probably an important cause of cell death *in vivo.* Raff *et al.* (21) have postulated that all cells constitutively express the essential components of the apoptotic machinery, and that cells remain alive as long as the suicide program is suppressed by survival stimuli (21). One effective mechanism by which these stimuli prevent apoptosis could be the maintenance of Mcl-1 expression; the withdrawal of these factors would then lead to the rapid downregulation of Mcl-1 resulting in cell death. As our study suggests, this mechanism could be relevant to PB B cells.

The finding that Mcl-1 is regulated by survival and apoptotic stimuli in PB B lymphocytes sheds light on the mechanisms of apoptosis control in the B-cell compartment and offers a possible explanation in cases where the regulation of apoptosis is Bcl-2-independent. Apart from PB B lymphocytes, Bcl-2-independent regulation of apoptosis also occurs in B-cell lines. For example, in the immature B lymphoma cell line WEHI-231 Bcl-2 levels are unaltered when cells are induced to die with anti-μ; moreover, Bcl-2 overexpression fails to prevent anti-μ induced death. Recently, Bcl-x_L was implicated as an alternative regulator of cell death in these cells (6, 22) because Bcl-x_L overexpression inhibited anti-μ-induced apoptosis, and stimulation of CD40, which inhibits anti-μ-induced death, up-regulated Bcl-x_L while Bcl-2 remained unaltered. The human B lymphoma cell line BL-41 provides yet another example (5). Here, Bcl-2 expression is unaffected when cells are induced to die with anti-μ. Instead, the induction of cell death by anti-μ treatment is preceded by the up-regulation of Bax, implicating this death-inducing molecule in the apoptosis pathway.

Whether Bcl-2 is involved appears to depend not only on the cell type but on the type of stimulus regulating apoptosis. For example, when GC B cells are rescued by anti-immunoglobulin antibodies *in vitro,* Bcl-2 is induced rapidly (23). In contrast, CD40L, also a potent

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survival signal for these cells, does not induce Bcl-2 expression until very late in the process, suggesting that Bcl-2 is not the key regulator, at least not initially (24). Moreover, this result suggests alternative survival mechanisms during CD40L-mediated rescue. One interesting possibility is that Mcl-1 is involved instead, given the central role of CD40-CD40L interactions in the germinal center reaction (25), and the finding that Mcl-1 expression is high in GC B cells *in vivo* (15).

An important question in current apoptosis research is why so many Bcl-2-related proteins exist, and what are their individual roles in normal cell death control. Here, for the first time, we have identified a normal cell type in which the novel homologue Mcl-1 is a possible regulator. Additional studies, in particular using transgenic and knockout approaches, will be required to elucidate the exact role of Mcl-1 in the regulation of apoptosis in this system and others.

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