CHAPTER 4

Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the Forkhead transcription factor FKHR-L1

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Introduction

The regulation of cell death is regulated mainly through an evolutionary conserved form of cell suicide termed apoptosis[1]. Deregulation of this process has been associated with cancer, autoimmune diseases and degenerative disorders. Many cells, particularly those of the hematopoietic system, have a default program of cell death and survival is dependent on the constant supply of survival signals. The Bcl-2 family, which has both pro- and anti-apoptotic members, plays a critical role in determining cell survival fate[2]. One family member, the Bcl-2 interacting mediator of cell death (Bim), contains only a protein-interaction motif known as the BH3 domain, allowing it to bind to pro-survival Bcl-2 members, neutralizing their function[3]. Disruption of the bim gene results in resistance to apoptosis following cytokine withdrawal in leukocytes, indicating that regulation of the pro-apoptotic activity Bim is critical for maintenance of the default apoptotic program[4]. Here we show that cytokine withdrawal results in upregulation of Bim expression concomitant with induction of the apoptotic program in lymphocytes. Activation of the forkhead transcription factor FKHR-L1, previously implicated in regulation of apoptosis in T lymphocytes[5], is sufficient to induce Bim expression. We propose a mechanism by which cytokines promote lymphocyte survival by inhibition of FKHR-L1, preventing Bim expression.

Results and Discussion

While Bim is expressed in many hematopoietic lineages, it is not known how its expression is affected after cytokine withdrawal[1]. Here, we determined whether changes in the levels of Bim expression could be responsible for regulation of cell survival. The mouse pro-B cell line, Ba/F3, has a default apoptotic pathway, which is repressed upon the addition of IL-3. We examined Bim protein levels in these cells following cytokine withdrawal. Interestingly, Bim expression increased steadily after IL-3 deprivation, correlating with induction of the apoptotic program (Fig. 1A; left panel). To determine whether this observation may represent a more general phenomenon, primary mouse fetal liver cells cultured in the presence or absence of survival factors[6] were analyzed for Bim expression. Indeed, fetal liver cells undergoing apoptosis following cytokine withdrawal exhibited elevated Bim protein levels (Fig 1A; right panel). To determine whether upregulation of Bim expression by IL-3 withdrawal was a result of enhanced transcription, bim mRNA was also analyzed. Bim has three isoforms (BimS, BimL and BimEL) that are generated by alternative splicing[3]. Northern blot analysis has shown several Bim transcripts, although the specific relationship between these transcripts and the three Bim isoforms is unclear[3]. We detected several transcripts as described previously, and importantly, expression of these mRNAs was significantly elevated upon IL-3 withdrawal (Fig. 1B).

To determine whether increased expression of Bim is sufficient to induce apoptosis,

we transiently expressed BimS or BimL, together with spectrin-linked GFP in Ba/F3 cells. This approach enables analysis of apoptosis in the transfected (GFP-positive) versus untransfected (GFP-negative). In cells that expressed only spectrin-GFP, cell survival was unaffected relative to control cells (Fig. 1C ; left panel). In cells expressing either BimL or BimS the level of apoptosis in GFP-positive cells was dramatically increased (Fig. 1C; middle and right panels).

Previous work has implicated phosphatidylinositol 3-kinase (PI3K) activity as being critical for cytokine-mediated rescue from apoptosis in lymphocytes[7]. To determine whether changes in Bim expression may be dependent on PI3K activity, Ba/F3 cells were either cytokine-starved, cultured with IL-3 or IL-3 in combination with the specific PI3K inhibitor LY294002. Bim protein levels were elevated in cells undergoing apoptosis induced either by IL-3 withdrawal or by inhibition of PI3K (Fig. 1D). As there was no change in Bax levels it appears that a general increase in pro-apoptotic Bcl-2 family members is not in itself a feature of cell death.

Protein Kinase B (PKB/Akt), a target of PI3K-activation, has recently been reported to inhibit transcriptional activity of a subfamily of forkhead transcription factors, which include FKHR-L1, AFX and FKHR[8]. FKHR-L1 activity, for example, is inhibited by PKB phosphorylation on three sites, resulting in an inability to translocate to the nucleus[5]. Mutation of these phosphorylation sites results in the generation of a constitutively active transcription factor and such a mutant, FKHR-L1(A3), has recently been shown to induce apoptosis in T cells through induction of Fas-L[5]. Ba/F3 cells, however, do not appear to be susceptible to Fas-L induced apoptosis (P.J.C. and P.F.D., unpublished observations). IL-3 withdrawal resulted in dephosphorylation of FKHR-L1 (Fig. 2A), which leads to nuclear translocation and activation of this transcription factor[5]. To determine whether activity of FKHR-L1 is linked to upregulation of Bim protein levels we generated a novel inducible FKHRL1(A3) expression construct. FKHR-L1(A3) was fused to the hormone-binding domain of the estrogen receptor[9], resulting in a 4-hydroxy tamoxifen (4-OHT) inducible protein, FKHR-L1(A3):ER*. We generated several stable clonal Ba/F3 cell lines in which expression levels of FKHR-L1(A3):ER* were approximately 3-5 times lower than of endogenous FKHR-L1 (data not shown). Addition of 4-OHT in the presence of IL-3 resulted in a dramatic induction of apoptosis, coinciding with an elevation of Bim protein levels (Fig. 2B, C). Levels of Bcl-2 and Bax were unaffected, demonstrating that the FKHR-L1 effects appear to be specific for Bim. Elevation of Bcl-2 levels, however, has previously been shown to counteract the pro-apoptotic activity of Bim[3]. To determine whether the increase in Bim levels may be a critical mechanism by which FKHR-L1 is able to induce apoptosis, Ba/F3 FKHR-L1(A3):ER* cells were electroporated with or without Bcl-2, together with spectrin-GFP as a marker for transfected cells. GFP-positive cells transiently expressing Bcl-2 were considerably more resistant to FKHRL1-induced apoptosis than GFP-negative control cells (Fig. 2C; lower panel). Finally, to examine whether the enhanced expression of Bim protein by FKHR-L1 activity was a result of transcriptional regulation, bim mRNA was analyzed. Similarly to IL-3 withdrawal (Fig. 1C), 4-OHT mediated FKHR-L1 activation significantly elevated Bim

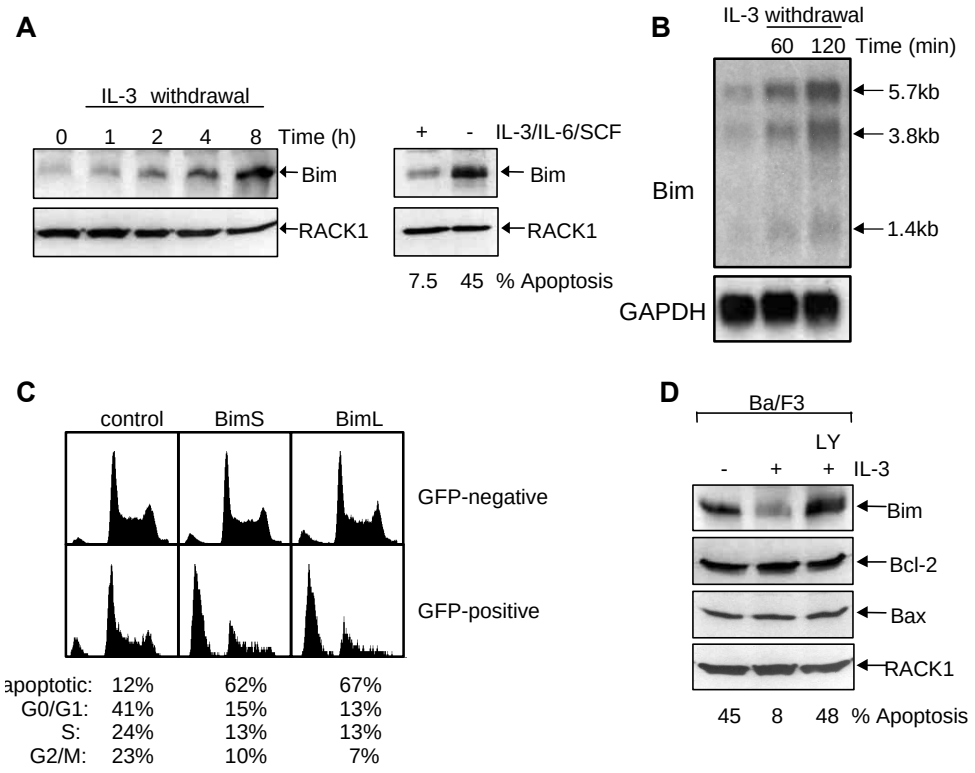

Figure 1 Bim levels are regulated by cytokines and determine cell survival fate.
(A) Left, Ba/F3 cells were IL-3-starved and lysed after the indicated times. Equal amounts of protein were loaded and Bim levels were determined by probing with anti-Bim. The blot was reprobed with anti-RACK1 to confirm equal protein loading. Right, Mouse fetal liver cultures were treated with or without cytokines for 24 hours, and the percentage of apoptotic cells were measured as well as levels of Bim and RACK1. (B) IL-3 withdrawal induces Bim mRNA expression. Ba/F3 cells were IL-3-starved for the indicated time points. PolyA+ RNA was isolated and Bim mRNA levels were analyzed using full length BimL cDNA as a probe. Equal RNA loading was confirmed by reprobing the blot with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (C) Bim expression is sufficient to induce cell death. Ba/F3 cells were electroporated with 18 μg empty vector, BimS or BimL, together with 2 μg spectrin-GFP. After 24 hours cells were fixed, stained with propidium iodide and the DNA content of 5000 GFP-positive or 20,000 GFP-negative cells was analysed by FACS. The data depicted is representative of several independent experiments. (D) PI3K activity is critical for cytokine-mediated repression of Bim. Ba/F3 cells were either cytokine-starved, cultured overnight with IL-3 without or with LY294002 (10 μM) and levels of Bim, Bcl-2 and Bax were determined. Blots were reprobed with RACK1 to confirm equal protein loading. The percentage of apoptotic cells was also determined by FACS analysis.

transcripts (Fig. 2D; left panel). To demonstrate that upregulation of Bim levels through FKHR-L1 occurs directly and does not require de novo protein synthesis, Ba/F3 FKHR-L1(A3):ER* cells were treated with 4-OHT for indicated timepoints in combination with the protein synthesis inhibitor cycloheximide. An elevation of Bim mRNA was also observed in cycloheximide treated cells (Fig. 2D, right panel), demonstrating that Bim transcription is indeed directly regulated by FKHR-L1.

Relatively little is known regarding transcriptional regulation of pro-apoptotic proteins in cells undergoing apoptosis. Our data identify cytokine-mediated inhibition of Bim expression as a novel mechanism of apoptotic regulation. In Caenorhabditis elegans, transcriptional repression of Egl-1, a pro-apoptotic protein related to Bim, is critical for regulating developmental cell death[10]. Recently, expression of the pro-apoptotic protein Hrk in hematopoietic progenitor cells was also found to be rapidly upregulated upon growth factor withdrawal[11]. Bim, like other BH3-domain proteins, exerts its pro-apoptotic activity through heterodimerization with anti-apoptotic Bcl-2 members[3]. Previously, regulation of pro-apoptotic activity of Bim has been reported to occur through its re-localization[12]. In this model, Bim is sequestered to the microtubular motor complex by binding to dynein light chain (LC8). Pro-apoptotic stimuli release LC8 together with Bim into the cytoplasm, allowing interaction of Bim with anti-apoptotic Bcl-2 members[12]. While this may indeed modulate the activity of Bim isoforms, we clearly demonstrate that the regulation of Bim expression by cytokines is very likely a contributory factor, defining the balance between cell survival and apoptosis. Moreover, the identification of Bim as a novel target of FKHR-L1 highlights the functional importance of this recently identified subfamily of forkhead transcription factors.

Bim levels are critical in regulating apoptosis since *Bim (-/-)* lymphocytes have an increased resistance to cell death induced by cytokine-withdrawal, surviving 10-30 times better than wild-type cells[4]. These results suggest that at least in lymphocytes Bim is the dominant transducer of death signals. By controlling the level of Bim through cytokine-mediated regulation of forkhead transcription factors, cell survival fate decisions can be rapidly made in response to changes in the local lymphocyte environment. This is analogous to the regulation of DAF-16, a *C. elegans* Forkhead transcription factor, which controls longevity in response to changes in environmental nutrient content[13]. Our data suggest that this evolutionary conserved signaling pathway has been exploited in mammals to regulate the lifespan of cytokine-dependent cells.

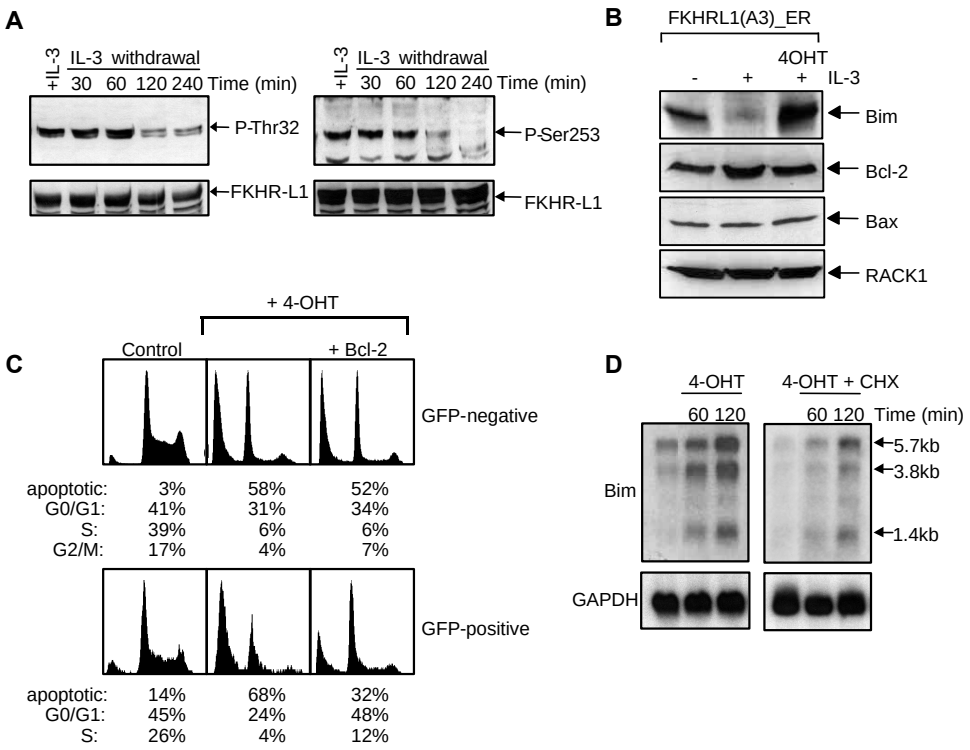

Figure 2. Bim levels are transcriptionally regulated by IL-3 through the forkhead transcription factor FKHR-L1.

(A) FKHR-L1 phosphorylation in Ba/F3 cells. IL-3 Ba/F3 cells were IL-3-starved and lysed at the indicated times. Levels of phosphorylated FKHR-L1 were analyzed by hybridizing with antibodies that detect the Thr32-phosphorylated (P-Thr-32) or Ser253-phosphorylated (P-Ser253) forms of FKHR-L1, and equal protein loading was verified by reprobing with FKHR-L1 antibody. (B) Activation of FKHR-L1 induces Bim expression. Ba/F3 cells stably expressing FKHR-L1(A3)-ER were treated with 4-OHT (100 nM) for 24 hours as indicated and levels of Bim, Bcl-2 and Bax were determined. Blots were reprobed with RACK1 to confirm equal protein loading. (C) Ba/F3 cells expressing FKHR-L1(A3):ER* were electroporated with 2 μg spectrin-GFP together with either 18 μg empty vector (control) or Bcl-2, and the percentage of apoptotic cells upon treatment with 4-OHT (100 nM) for 24 hours was measured as in 1C. (D) Bim mRNA levels are upregulated by FKHR-L1 activity. Left, Ba/F3 cells stably expressing FKHR-L1(A3):ER* were treated with 4-OHT (100 nM) for indicated time points. PolyA+ RNA was isolated and Bim mRNA levels were analysed using full length BimL cDNA as a probe. Equal RNA loading was confirmed by reprobing the blot with GAPDH. Right, same as left panel except that cells were pretreated with cycloheximide (CHX, 10 μg/ml) before addition of 4-OHT.

Materials and Methods

Plasmids

BimS and BimL constructs were generated by PCR from Ba/F3 cDNA isolated from IL-3 deprived cells, cloned into pSG5-MYC and verified by sequencing. pCDNA3- FKHR-L1(A3):ER* was generated by cloning FKHR-L1(A3) without the stop codon into pCDNA3 containing the hormone-binding domain of the estrogen receptor (pCDNA3-ER). Spectrin-linked GFP was a kind gift from Dr. A. Beavis and T. Sheck (Princeton, U.S.A.) and has been described previously[14]. pSG5-Bcl-2 was a kind gift from Dr. R.P. de Groot.

Western blotting

For determining protein levels, cells were lysed in a buffer containing (0.1% NP-40, 20 mM HEPES pH 7.5, 5 mM EDTA, 150 mM NaCl supplemented with 10 μg/ml aprotinin, 1 mM leupeptin, 1 mM PMSF, 1 mM Na_3VO_4, 40 mM β-glycerophosphate and 50 mM NaF). Protein content was determined and equal amounts of protein were analysed by SDS-PAGE and blots were probed with the appropriate antibodies. Bim polyclonal antibody was purchased from Affinity Bioreagents (Golden, CO, U.S.A.), Bcl-2 antibody from Santa Cruz (Santa Cruz, CA, U.S.A.); Bax mAb from BD PharMingen (San Diego, CA, U.S.A.); RACK1 mAb from Transduction Laboratories (Kentucky, U.S.A.); FKHR-L1, phospho-Thr32 FKHR-L1 and phospho-Ser253 FKHR-L1 were from UBI (Lake Placid, NY, U.S.A.).

Cell culture, transient electroporation and FACS analysis

Ba/F3 cells were cultured in RPMI 1640 supplemented with 8% Hyclone serum (Gibco) and recombinant mouse IL-3 (IL-3) produced in COS cells. Fetal liver-derived myeloid cultures were prepared from day 17 mouse embryos by culture of suspension cells in RPMI supplemented with IL-3, IL-6 and SCF as previously described[6].

For transient transfection, Ba/F3 cells were electroporated (0.28 kV; capacitance 960 μFD) and 2 hours after electroporation dead cells were removed by separating through a Ficoll gradient (2500 rpm for 20 minutes). After 24 hours cells were harvested, washed twice in PBS and fixed for at least 2 hours in 300 μl PBS and 700 μl ethanol. Cells were spun down gently and permeabilized in 200 μl 0.1% Triton X-100, 0.045 M Na_2HPO_4 and 0.0025 M sodium citrate at 37°C for 20 minutes. Next, 750 μl apoptosis buffer (0.1% Triton X-100, 10 mM PIPES, 2 mM $MgCl_2$, 40 μg/ml RNase, 20 μg/ml propidium iodide) was added and incubated for 30 minutes in the dark. The percentage of apoptotic cells was analysed by FACS as the percentage of cells with a DNA content of <2N, counting 5,000 cells. Thresholds were set to gate out cellular debris. Cell cycle profiles were determined using a FACS calibur (Becton and Dickson, Mountainview, CA, U.S.A.) and analysed using Cell Quest and MofFit software.

Northern blotting

Total RNA was isolated from Ba/F3 and 500 μg was used for the isolation of polyA+ RNA using polyA Tract mRNA isolation kit from Promega (Madison, WI, U.S.A.). Equal RNA loading was verified by reprobing the blots with a 1.4 kb cDNA fragment of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene.

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