Apoptosis Induced by Erythroid Differentiation of Human Leukemia Cell Lines Is Inhibited by Bcl-XL.

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The induction of tumor cell differentiation represents an attractive strategy for the treatment of a wide range of malignancies. Differentiation of HL-60 promyelocytic leukemia cells towards neutrophils or monocytes has been shown to induce apoptotic cell death, which is inhibited by bcl-2 overexpression. However, the role of the bcl-2 gene family during erythroid differentiation of human leukemia cells remains unknown. We found that human erythroleukemia (HEL) and K562, two leukemia cell lines that undergo erythroid differentiation do not express Bcl-2, but express Bcl-XL, a related protein that functions as an inhibitor of apoptosis. Differentiation of HEL or K562 cells with inducers of erythroid differentiation (hemin, retinoic acid, or transforming growth factor-β) was accompanied by progressive cell death and degradation of genomic DNA into oligonucleosomal fragments.

Cellular homeostasis in vertebrates is regulated by several processes that include cell proliferation, differentiation, and cell death. The demise of cells is often accomplished by apoptosis, also referred to as programmed cell death. Apoptosis is a genetically regulated process that can be induced by a variety of stimuli, which include deprivation of growth factors, signaling via certain surface receptors, treatment with DNA-damaging agents or inhibitors of macromolecular synthesis.1 In hematopoietic tissues, the survival of progenitor cells and turnover of mature elements is controlled by apoptosis. For example, apoptosis is coupled to terminal differentiation of myeloid progenitor cells.2 In the erythroid lineage, the production of red blood cells appears to be regulated through the hormone erythropoietin, which has been shown to maintain the survival of erythroid progenitor cells by inhibiting apoptosis.3,4 Although it is generally thought that hematopoietic survival factors maintain cell survival by inducing the synthesis of cellular proteins capable of repressing the apoptotic mechanism, the signals involved in the regulation of hematopoietic cell survival are still poorly understood. Bcl-2 was the first described member of an expanding family of proteins that regulate apoptosis in vertebrates.5 Four of these proteins: Bcl-2, Mcl-1, A1, and Bcl-XL are expressed in hematopoietic cells and function as repressors of the apoptotic process.6 Beyond their role in the regulation of hematopoietic cell survival and homeostasis, Bcl-2 family members have been shown to contribute to tumorigenesis by inhibiting apoptotic signals that are generated during the activation of certain oncogenes, such as c-myc or the p53 tumor suppressor gene.7 Moreover, Bcl-2 and Bcl-XL can provide signals that rescue tumor cells from apoptosis induced by chemotherapeutic drugs, implicating these survival proteins in the resistance of cancer cells to chemotherapy.8

Leukemia cell lines such as HL-60 undergo differentiation after treatment with retinoic acid (RA) or phorbol ester.9,10 In this model, treatment of HL-60 promyelocytic leukemia cells with RA or phorbol ester induced neutrophil or macrophage-like cell differentiation, respectively, and resulted in progressive loss of cellular viability and internucleosomal DNA degradation. This differentiation-induced apoptosis was coupled to downregulation of Bcl-2.4 Because little is known about the regulation of apoptosis in tumor models of erythroid differentiation, we have studied the expression and regulation of Bcl-2 and Bcl-XL in human erythroleukemia (HEL) and K562 human leukemia cells. Exposure of HEL and K562 cells to hemin has been reported to induce erythroid differentiation.9,10 In addition, erythroid differentiation of HEL cells was found to be associated with decreased cell proliferation and viability and increased levels of tissue transglutaminase, a marker of apoptosis.11,12 Here, we show that HEL and K562 leukemia cells express Bcl-XL but not Bcl-2. Furthermore, erythroid differentiation of HEL and K562 cells was associated with downregulation of Bcl-XL and activation of apoptosis. Our results also demonstrate that constitutive expression of Bcl-XL blocks apoptotic death, but not the differentiation process triggered by RA, hemin, or transforming growth factor-β (TGF/β1). These results suggest that apoptosis and erythroid differentiation proceed simultaneously, but can be separated by Bcl-XL in human leukemia cells.

Materials and Methods

Cell culture. The human leukemia (HEL and K562) cell lines were maintained at 5 to 9 × 10⁴ cell/mL in RPMI 1640 medium.
(Seromed Biochrom KG) supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories, Irvine, CA), nonessential amino acids, 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. Viability and total cell counts were determined at various times by trypsin blue exclusion and counting of at least 200 cells from each individual culture. To induce differentiation, cells were cultured in the presence of 60 µM/L hemin (Sigma, St Louis, MO); 1 ng/mL TGFβ1 (GIBCO-BRL, Gaithersburg, MD), or 10 µM/L RA (Sigma) for various times. Fresh medium containing the differentiation inducers was added to the cultures every 48 to 72 hours.

Cell transfection. HEL and K562 cells were transfected by electroporation with the SFFV-Neo expression vector containing the human bcl-xL open reading frame driven by the long terminal repeat of the spleen focus forming virus (pSFFV-bcl-xL). As a control, transfection was performed with empty SFFV-Neo plasmid. For transfection, 20 µg of plasmid was linearized with Not I (Pharmacia) and resuspended in Hebs buffer (20 mM/L HEPES, pH 7.0; 137 mM/L NaCl; 5 mM/L KCI; 0.7 mM/L Na2PO4H; 6 mM/L Dextrose). HEL cells (108) were subjected to electroporation at 300 V, 25 µJ and selected by growth in the presence of 1.25 mg/mL of G418 (GIBCO). K562 cells (107) were electroporated at 400 V, 500 µJ and selected in medium containing 650 µg/mL of G418.

Western blot analysis and flow cytometric analysis. Expression of Bcl-X, and Bax was determined by Western blot analysis as previously described. The amount of protein in cell lysates was determined by the bicinchoninic acid method. 

RESULTS

HEL cells express Bcl-X, but not Bcl-2. The expression of Bcl-2 and Bcl-X was investigated in HEL and K562, two leukemic cell lines that undergo erythroid differentiation. Two species of bcl-x mRNA, bcl-xL, and bcl-xS, that exhibited distinct biological function have been described in the human. We, therefore, selected an RT-PCR method to discriminate between the two bcl-x forms. Figure 1A shows that bcl-xL, but not bcl-2 mRNA was expressed in K562 and HEL cells. Expression of the bcl-xL form of bcl-x mRNA was not detected in leukemia cells (data not shown). Flow cytometric analysis showed that HEL and K562 expressed Bcl-XL, but not the Bcl-2 protein (Fig 1B). In contrast, the promyelocytic cell line HL-60 expressed Bcl-2, but hardly any detectable levels of Bcl-X protein (Fig 1B) in agreement with previous results. As an additional control, the expression of the bcr/abl fusion gene that has been shown to inhibit apoptosis in leukemic cell lines was evaluated.

Erythroid differentiation of HEL cells is associated with downregulation of bcl-xL mRNA and protein. HEL cells can undergo erythroid differentiation in the presence of RA, hemin, or TGFβ. We next assessed the expression of bcl-x mRNA in HEL cells by a semiquantitative RT-PCR analysis after treatment with agents that induce erythroid differentiation. As shown in Fig 2A, bcl-xL levels in HEL-Neo cells remained unaltered after incubation for 3 days with RA, hemin, or TGFβ, but were clearly downregulated after 6 days of treatment with RA or after 9 days of treatment with hemin or TGFβ. As a control, the levels of GAPDH mRNA remained unchanged throughout the course of treatment with the same agents (Fig 2A). Western blot analysis showed that treatment with RA for 6 days and hemin or TGFβ for 9 days induced downregulation of the Bcl-XL protein, whereas the levels of Bax, another Bcl-2 family member remained unaltered (Fig 2B).

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Figure 3 shows that HEL and K562-Neo cells expressed endogenous Bcl-X<sub>L</sub> protein, which was increased about eightfold after transfection with the pSFFV-bcl-x<sub>L</sub> plasmid. Untransfected leukemia cells displayed an identical pattern of Bcl-X<sub>L</sub> expression as HEL-Neo and K562-Neo cells (data not shown). To assess whether both HEL-Neo and HEL-Bcl-X<sub>L</sub> cells could undergo erythroid differentiation, we analyzed the expression of the \( \alpha \)-globin mRNA, which has been shown to be increased in response to a number of inducers of erythroid differentiation. Untreated HEL-Neo and HEL-Bcl-X<sub>L</sub> cells expressed low levels of \( \alpha \)-globin mRNA (Fig 4). However, treatment with RA, hemin, or TGF\( \beta \) for 3 days resulted in a clear increase in the levels of \( \alpha \)-globin mRNA as determined by Northern blot analysis (Fig 4). To confirm this result, the same mRNA analysis was performed in two different HEL-Bcl-X<sub>L</sub> clones that yielded the same results as those obtained with the HEL-Bcl-X<sub>L</sub> bulk cell line (data not shown).

**Fig 1.** Expression of Bcl-2, Bcl-X<sub>L</sub>, and BCR-ABL in leukemic cell lines. (A) Total RNA was purified from HL-60, K562, and HEL cells and subjected to RT-PCR analysis with oligonucleotide primers specific for bcl-2, bcl-x, and bcr/abl genes. After 30 cycles, PCR products were electrophoresed onto a 2% agarose gel and stained with ethidium bromide. PCR amplification of GAPDH was used as an internal control. (B) Cells were permeabilized and labeled with hamster anti-Bcl-2 or mouse anti-Bcl-X monoclonal antibody (closed graphs) or irrelevant antibody as a background control (open graphs) followed by biotinylated anti-hamster or anti-mouse IgG and PE-labeled streptavidin, and analyzed by flow cytometry.

**Fig 2.** Expression of bcl-x<sub>L</sub> mRNA and protein in HEL cells treated with RA, hemin (Hem), or TGF\( \beta \). (A) Total RNA was purified from HEL cells and subjected to semiquantitative RT-PCR analysis with oligonucleotide primers specific for bcl-x. The PCR product of GAPDH was used as a control. After 22 cycles (shown to be at the linear phase of amplification), PCR products were electrophoresed onto a 2% agarose gel and stained with ethidium bromide. (B) Western blot analysis of lysates from untreated HEL cells or treated for 6 days with RA and 9 days with hemin or TGF\( \beta \). The blot was incubated with rabbit anti-Bcl-X, rabbit anti-Bax or anti-\( \beta \) Tubulin antibody as a loading control and developed as described in Materials and Methods. The levels of Bax did not significantly change during erythroid differentiation when compared with the levels obtained for \( \beta \) Tubulin, which were used as a loading control.

modified by overexpression of Bcl-X<sub>L</sub>. Because Bcl-X<sub>L</sub> is downregulated on erythroid differentiation of HEL cells, we studied next whether differentiation of leukemia cells could be altered by overexpression of Bcl-X<sub>L</sub>. HEL and K562 cells were stably transfected with the expression vector pSFFV-bcl-x<sub>L</sub> or control pSFFV-Neo plasmid. Bulk and clonal cell lines transfected with bcl-x<sub>L</sub> and control plasmid were selected by growth in medium containing G418.

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Western blot analysis of Bcl-X\textsubscript{L} protein in bcl-x\textsubscript{L}-transfected HEL and K562 cells. Lysates from cells stably transfected with SFFV-neo and SFFV-bcl-x\textsubscript{L} plasmids (2 x 10\textsuperscript{6} cells/lane) were loaded onto a 12% polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose and analyzed for Bcl-X\textsubscript{L} with rabbit anti-Bcl-X followed by peroxidase-conjugated goat anti-rabbit IgG. As a positive control, FL5.12 cells transfected with a human bcl-x\textsubscript{L} cDNA (BcI-XL) were used. Equal loading of the lanes was verified by staining of the upper part of the gel with Coomassie brilliant blue.

As an additional assessment of erythroid differentiation, the number of benzidine positive cells were measured before and after treatment with hemin. After treatment for 3 days with hemin, there was no significant difference in the percentage of benzidine-positive cells in HEL-Neo and HEL-Bcl-X\textsubscript{L} cells (23% \pm 2% vs 25% \pm 3%, respectively, mean of three experiments). These results indicate that constitutive expression of Bcl-X\textsubscript{L} does not alter the erythroid differentiation pathway of HEL cells.

Constitutive expression of Bcl-X\textsubscript{L} inhibits apoptosis induced by erythroid differentiation of HEL and K562 cells. Because it has been reported that there is a decrease in the proportion of viable cells on treatment of HEL cells with hemin,\textsuperscript{8} we assessed the viability of HEL-Neo and HEL-Bcl-X\textsubscript{L} cells cultured in the presence of RA, hemin, or TGF\textbeta. The percentage of viable cells in the HEL-Neo population began to decrease significantly after the first 3 days of treatment (Fig 5). The viability of HEL-Neo cells was 20%, 48%, and 83% by day 6 of treatment with RA, hemin, and TGF\textbeta, respectively. By day 9 of treatment, the viability of HEL cells dropped to 5% (RA), 14% (hemin), and 23% (TGF\textbeta). By contrast, this loss of viability was not observed in the HEL-Bcl-X\textsubscript{L} population. Thus, after treatment with RA, hemin, or TGF\textbeta for 9 days, between 72% and 85% of the Bcl-
TGFβ and DNA fragmentation was monitored by electrophoresis onto a 2% agarose gel and staining with ethidium bromide. HEL-Neo (N) and HEL-Bcl-XL (B) were incubated for 9 days with RA, hemin (Hem), or TGFD, which is characteristic of apoptotic cell death. In contrast, the genomic DNA from HEL-Bcl-XL cells treated with the same differentiation promoting agents remained unfragmented (Fig 6). Dose response experiments for RA (1 to 20 μmol/L), hemin (10 to 100 μmol/L), and TGFD (from 0.1 to 2 ng/mL) showed that differentiation of HEL cells as assessed by the percentage of benzidine-positive cells was always accompanied by loss of cell viability due to apoptosis. Thus, we were unable to separate erythroid differentiation and apoptosis in these leukemia cells.

We also analyzed K562, another leukemic cell line that undergoes erythroid differentiation by treatment with hemin. Figure 7A shows that expression of ε-globin mRNA was upregulated in both K562-Neo and K562-Bcl-XL cells in response to hemin. Furthermore, the number of benzidine-positive cells after treatment with hemin for 6 days was similar in both K562-Neo and K562-Bcl-XL cells (56% ± 4% vs 50 ± 6, respectively, mean of three experiments). As we found with HEL cells, expression of endogenous Bcl-XL protein was downregulated in K562 in a time-dependent manner during hemin-induced differentiation (Fig 7D). In contrast, the levels of Bax in K562 remained unaltered after hemin-induced differentiation (data not shown). The levels of bcl-x mRNA also declined in response to hemin, in agreement with the protein results (Fig 7C). RT-PCR analysis showed that bcl-x was not induced during erythroid differentiation of K562 cells (data not shown). Downregulation of Bcl-XL in K562 treated with hemin for 12 and 15 days was accompanied by progressive loss of cell viability that was associated with fragmentation of genomic DNA into oligonucleosomal fragments (Fig 7B). In contrast, constitutive expression of Bcl-XL in K562 cells inhibited the fragmentation of genomic DNA into oligonucleosomal fragments (Fig 7B). Thus, erythroid differentiation of both HEL and K562 cells is coupled to apoptosis, which is inhibited by constitutive expression of Bcl-XL.

DISCUSSION

bcl-x is a new member of the bcl-2 family of apoptosis regulatory genes. Two bcl-x cDNAs, bcl-xL and bcl-xS, have been isolated in the human. Bcl-XL exhibits remarkable structural homology with Bcl-2 and inhibits apoptotic cell death on growth factor withdrawal. Recent studies have reported that Bcl-X immunoreactivity is detected in a wide

**Fig 6.** Analysis of DNA fragmentation in HEL-Neo and HEL-Bcl-XL cells after induction of erythroid differentiation. HEL-Neo (N) and HEL-Bcl-XL (B) were incubated for 9 days with RA, hemin (Hem), or TGFD, which is characteristic of apoptotic cell death. In contrast, the genomic DNA from HEL-Bcl-XL cells treated with the same differentiation promoting agents remained unfragmented (Fig 6). Dose response experiments for RA (1 to 20 μmol/L), hemin (10 to 100 μmol/L), and TGFD (from 0.1 to 2 ng/mL) showed that differentiation of HEL cells as assessed by the percentage of benzidine-positive cells was always accompanied by loss of cell viability due to apoptosis. Thus, we were unable to separate erythroid differentiation and apoptosis in these leukemia cells.

**Fig 7.** Erythroid differentiation of K562-Neo and K562-Bcl-XL cells. (A) Cells were treated with hemin (HE) or not (C) for 3 days. Expression of ε-globin mRNA in cells was determined by Northern blot analysis. Hybridization with a GAPDH probe and ribosomal RNA (rRNA) staining with ethidium bromide were used as loading controls. (B) Analysis of DNA fragmentation in K562-Neo (N) and K562-Bcl-XL (B) cells after treatment with hemin for 3 days. DNA fragmentation was monitored by electrophoresis onto a 2% agarose gel and staining with ethidium bromide. (C) Total RNA was purified from K562 cells at the indicated time points and subjected to semiquantitative RT-PCR analysis with oligonucleotide primers specific for bcl-x. The PCR product of GAPDH was used as a control. After 22 cycles, PCR products were electrophoresed onto a 2% agarose gel and stained with ethidium bromide. (D) K562 cells were permeabilized and labeled with mouse anti-Bcl-X monoclonal antibody (closed graphs) or irrelevant antibody as a background control followed by biotinylated anti-mouse IgG and PE-labeled streptavidin, and analyzed by flow cytometry. The “cut off” line represents the highest level of background fluorescence obtained with control antibody. The percentage of cells that were negative for Bcl-X increased from less than 5% (day 0) to 45% (day 12) and 60% (day 15).
variety of human and mouse tissues. Among the hematopoietic and lymphoid tissues, Bcl-X was expressed in cortical thymocytes, activated lymphocytes, megakaryocytes, erythroid precursors, and differentiating myeloid cells. In this report, we have examined the regulation and function of bcl-x during erythroid differentiation of HEL and K562 leukemia cells. Both HEL and K562 undergo erythroid differentiation after treatment with RA, hemin, or TGFβ (and our observations). We found that erythroid differentiation of both HEL and K562 cells led to progressive loss of cell viability by activation of an apoptotic process. Moreover, differentiation of HEL and K562 was associated with diminished expression of bcl-x mRNA and protein. Downregulation of Bcl-XL in HEL and K562 leukemia cells is reminiscent of that observed for Bcl-2 during myeloid differentiation of leukemia cells. Because deregulated expression of Bcl-XL in HEL and K562 cells inhibited apoptosis that is triggered by differentiation, our results suggest that diminished Bcl-XL plays a role in initiating the apoptotic response. In another study, there was no evidence of apoptosis in HEL cells stimulated with RA. However, in that report, the investigators analyzed DNA degradation during the first 48 hours following RA treatment of HEL cells. In the present study, DNA fragmentation was observed after 6 to 9 days of treatment with RA, hemin, or TGFβ.

Treatment of erythroid leukemic cells with RA, hemin, or TGFβ is associated with induction of e-globin, which has been used as a marker of erythroid differentiation. In contrast to the effect of constitutive expression of Bcl-XL on the apoptotic response of HEL and K562 cells, enforced Bcl-XL expression did not affect the induction of e-globin or the percentage of benzidine positive cells, which are charactertistic markers of erythroid differentiation. These results indicate that apoptosis and erythroid differentiation proceed simultaneously, but they can be uncoupled by expression of Bcl-XL. This data is in agreement with that recently reported for HL-60 myeloid leukemia cells in that overexpression of Bcl-2 cells did not affect their maturation, but prevented apoptosis induced by myeloid differentiation. Thus, both myeloid and erythroid leukemia cells, apoptosis and differentiation events can be separated by Bcl-2 or Bcl-XL. Although the molecular machinery that regulates cell death in the erythroid lineage has yet to be fully elucidated, our results argue that Bcl-XL plays an important role by inhibiting the apoptotic response. The importance of bcl-x for the maintenance of normal hematopoietic cells has been definitively established in mice that are deficient in Bcl-x. These animals exhibited extensive apoptosis of immature hematopoietic cells in the embryonic liver and died by day 12 to 13 of development. However, it is intriguing that two structurally related proteins such as Bcl-2 and Bcl-XL that exhibit overlapping function are differentially regulated in myeloid and erythroid leukemic cell lines. It is possible that differential expression of two Bcl-2 family members in leukemic cells reflects a different regulation of Bcl-2 and Bcl-XL in the myeloid and erythroid lineages. Both Bcl-2 and Bcl-XL share remarkable structural homology and localize to similar intracellular sites suggesting that they inhibit cell death by a similar biochemical mechanism. However, it has recently shown that Bcl-2 and Bcl-XL interact differentially with some intracellular targets in vivo. Thus, it is formally possible that Bcl-2 and Bcl-XL differ in their functional ability to prevent cell death in a subtle manner. Consistent with this possibility is the observation that Bcl-XL appears more efficient than Bcl-2 in protecting the immature B-cell line WEHI-231 from apoptosis induced by certain chemotherapeutic agents.

In addition to Bcl-2 and Bcl-XL, the BCR-ABL fusion protein has been shown to play a role in protecting leukemic cells (those with t(9;22) (q34;q11) chromosomal translocation) from apoptotic cell death. Thus, deregulated expression of genes such as bcrabl, bcl-2 or bcl-x may result in an accumulation of leukemic cells by inhibiting apoptosis during tumor development and/or contributing to the resistance of cancer cells to apoptosis induced by therapeutic agents. In this regard, it has been recently suggested that deregulated Bcl-XL expression may be involved in resistance to cytotoxic drugs in human U937 clones that escaped death triggered by deoxorubicin and vincristine. Future studies will have to elucidate whether approaches aimed at inhibiting the activity of these antiapoptotic genes, either alone, or in combination with agents that induce apoptosis (eg, chemotherapeutic drugs, differentiation inducers) are useful in the treatment of malignant diseases.

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REFERENCES


Apoptosis induced by erythroid differentiation of human leukemia cell lines is inhibited by Bcl-XL

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