American Journal of Pathology, Vol. 146, No. 2, February 1995
Copyright © American Society for Investigative Pathology

Regulation and Function of Bcl-2 During Differentiation-Induced Cell Death in HL-60 Promyelocytic Cells

Adalberto Benito,* Didier Grillot,† Gabriel Nuñez,† and Jose Luis Fernández-Luna*

From the Servicio de Inmunología, Hospital Universitario Marques de Valdecilla, 39008 Santander, Spain, and the Department of Pathology,† University of Michigan Medical School, Ann Arbor, Michigan*

Polymorphonuclear leukocytes are generated by differentiation of early myeloid precursors. Once fully differentiated, blood neutrophils are programmed to die rapidly and are removed by tissue macrophages. In normal myeloid cells, the death mechanism seems to be coupled to the differentiation pathway and is accomplished by a process termed apoptosis. In the present study, we have examined the role of Bcl-2 in the differentiation pathways of the promyelocytic cell line HL-60. Treatment of HL-60 with retinoic acid or phorbol ester, which induced neutrophil or macrophage-like cell differentiation, respectively, resulted in progressive loss of cellular viability and internucleosomal DNA degradation. In HL-60, differentiation and apoptosis were coupled to down-regulation of the Bcl-2 protein. Overexpression of Bcl-2 by gene transfer inhibited apoptosis triggered by terminal differentiation of HL-60. Yet, Bcl-2 did not alter the expression of surface markers or other phenotypic changes that are induced upon myeloid differentiation. In contrast to HL-60, another immature myeloid cell line, K562, did not produce Bcl-2 but expressed a related protein, Bcl-x_L, that functions as a repressor of apoptotic cell death. K562 has been shown to be relatively resistant to a variety of apoptotic stimuli. Incubation of HL-60 and K562 with inhibitors of macromolecular synthesis induced apoptosis, which appeared earlier in HL-60 than in K562. Interestingly, Bcl-2 overexpression protected K562 cells from apoptosis induced by inhibitor of macromolecular synthesis but it had little or no effect on HL-60 cells. We conclude that although differentiation and apoptosis proceed simultaneously, they can be uncoupled by expression of Bcl-2. Down-regulation of Bcl-2 appears to be part of the differentiation pathway and may serve to facilitate the apoptotic response. (Am J Pathol 1995, 146:481–490)

Cellular homeostasis in vertebrates is regulated by cell proliferation as well as by cell death. The demise of cells that occurs during embryogenic and postnatal development is referred to as programmed cell death and usually occurs by apoptosis, a morphologically defined process that ultimately leads to activation of endogenous nucleases and DNA degradation.[1–4] Although the mechanism that induces and executes apoptosis is poorly understood, it is thought that in most instances cell death is controlled by a genetic program that is activated within the dying cell. The requirement for new DNA transcription and protein synthesis for induction of apoptosis in many cellular systems suggested that the demise of cells represents activation of a suicide program.[5,6] However, it has been also found that inhibition of protein and RNA synthesis alone can induce apoptosis, arguing that in some cases apoptosis may be actively suppressed by proteins inside the cell.[7] Apoptosis can be triggered by a variety of stimuli. They include deprivation of survival factors, signaling via certain surface receptors, and treatment with hormones such as glucocorticoids or DNA-damaging agents.[8,9] In hematopoietic tissues, apoptosis is coupled to terminal differentiation of myeloid progeni-

Supported in part by Fondo de Investigaciones Sanitarias grant FISS-94/1415. DG is supported by a fellowship from the Fonds National Suisse de la Recherche Scientifique. GN is supported by a Research Career Development Award from the NIH.

Accepted for publication October 28, 1994.

Address reprint requests to Dr. Jose Luis Fernandez-Luna, Servicio de Inmunología, Hospital Universitario Marqués de Valdecilla, 39008 Santander, Spain.

tors.[10] Although the mechanism responsible for activation of apoptosis during myeloid maturation is poorly understood, the process serves to remove from the organism aging neutrophils. Moreover, apoptosis appears to play an important role in the disposal of polymorphonuclear leukocytes involved in the resolution of the acute inflammatory response.[11] In neutrophils, apoptosis appears to be suppressed by the stimulation of survival factors such as interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF).[12,13] It is generally thought that hematopoietic survival factors can maintain cell survival by inducing the synthesis of cellular proteins capable of suppressing the apoptotic mechanism.[14,15] A candidate protein that functions as a repressor of apoptosis is the product of the *Bcl*-2 proto-oncogene. *Bcl*-2 is deregulated in 85% of follicular lymphomas as a consequence of the chromosomal translocation t(14;18) which juxtaposes *Bcl*-2 and the Ig heavy chain locus.[16,17] *Bcl*-2 encodes a 26-kd integral membrane protein that localize to outer mitochondria, smooth endoplasmic reticulum, and perinuclear envelope.[18–20] Bcl-2 protein expression appears widely distributed on tissues characterized by apoptotic cell death[21] and protects hematopoietic cells from apoptosis induced by growth factor withdrawal and chemotherapeutic agents.[22–24] Within the myeloid lineage, Bcl-2 is expressed in early myeloid precursors but is absent in polymorphonuclear leukocytes of the blood.[25] Similarly, promyelocytic HL-60 cells induced to differentiate toward neutrophils down-regulate Bcl-2.[25] The correlation between expression of Bcl-2 and differentiation suggests that down-regulation of Bcl-2 may be part of the myeloid maturation pathway. To provide an experimentally useful model for examining the potential action of Bcl-2 in the myeloid lineage, we have stably transfected two myeloid leukemic cell lines HL-60 and K562 with a *Bcl*-2 expression vector.[26] Induction of HL-60 differentiation by retinoic acid or phorbol ester leads to down-regulation of Bcl-2 protein and apoptotic cell death. Overexpression of Bcl-2 inhibited differentiation-induced apoptosis but it failed to alter the maturation process.

Materials and Methods

Cell Cultures and Chemicals

K562 and HL-60 cells were maintained at 5–9 × 10^5 cell/ml in RPMI 1640 medium (Seromed Biochrom KG) supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories), nonessential amino acids, 2 mmol/L glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Viability and total cell counts were determined at various times by the trypan blue exclusion test by counting at least 200 cells from each individual culture. The following chemicals were used: actinomycin D (ActD) (Dactinomycin, MSD); cycloheximide (CHX) (Sigma Chemical Co., St. Louis, MO); arabinosyl-cytosine (Ara C) (Upjohn); puromycin (Sigma); phorbol-12-myristate-13-acetate (PMA) (Sigma) and all-*trans*-retinoic acid (RA) (Sigma). RA was dissolved and stored in DMSO at 5 mmol/L and diluted 1:5000 in culture medium to induce myeloid differentiation.

Cell Transfection

K562 and HL-60 cells were transfected by electroporation with the SFFV-Neo expression vector[26] containing the human *Bcl*-2 open-reading frame driven by the long terminal repeat of the splenic focus-forming virus (SFFV-*Bcl*-2). As a control, transfections were performed with empty SFFV-Neo plasmid. For transfections, 20 to 40 µg of the plasmid was linearized with *Not*I (Pharmacia) and resuspended in Hebs buffer (20 mmol/L HEPES, pH 7.0; 137 mmol/L NaCl; 5 mmol/L KCl; 0.7 mmol/L Na_2PO_4H; 6 mmol/L dextrose) or in PBS buffer. K562 cells (10^7) were subjected to electroporation at 400 V, 500 µF and selected for resistance to neomycin by growth in the presence of 650 µg/ml G418 antibiotic (GIBCO). HL-60 cells (10^6) were electroporated at 450 V, 75 µF and selected in medium containing 1 mg/ml G418.

Western Blot and Flow Cytometry Analysis

Cell suspensions (2.5 × 10^6 cells) were lysed in a buffer containing 25 mmol/L Tris-HCl pH 8.0, 1% NP-40 (Sigma), 0.5% deoxycholate (Sigma), 144 mmol/L NaCl, 0.1% sodium dodecyl sulfate (SDS), 5 mmol/L dithiothreitol, 10 mmol/L iodoacetamide, and 0.5 mmol/L phenylmethylsulfonylfluoride at 4 C. After a brief sonication, lysates were cleared by centrifugation at 17,000 rpm for 30 minutes and then boiled for 5 minutes in sample buffer (50 mmol/L Tris-HCl, pH 6.8; 8% glycerol; 1.6% SDS; 4% 2-mercaptoethanol; and 2% bromophenol blue) before being run on a SDS-12% polyacrylamide gel. Gels were transferred to nitrocellulose (Bio Rad), blocked with 3% BSA and then incubated for another hour with 25 µg/ml hamster anti-human Bcl-2 antibody (6C8)[18] or rabbit anti-Bcl-x antiserum (a gift of Dr. Craig Thompsom, University of Chicago). Blots were incubated with secondary rabbit anti-hamster IgG (Jackson ImmunoResearch Lab,

Inc.) or goat anti-rabbit IgG-horseradish peroxidase (Amersham), washed, and developed with 0.6 mg/ml 3,3′-diaminobenzidine tetrahydrochloride dihydrate (DAB) (Bio Rad) in 50 mmol/L Tris, pH 8.0, containing H_2O_2 (1:1000) for Bcl-2 or ECL substrate for Bcl-x (Amersham).

Differentiation of myeloid cell lines was induced by addition of 20 ng/ml of PMA (macrophage-like cell phenotype) or 1 μmol/L RA (granulocytic phenotype) and monitored by flow cytometry on a FACScan using antibodies to CD71, CD14, and CD11b antigens and a secondary FITC-conjugated goat anti-mouse IgG (Becton-Dickinson). To assess the expression of Bcl-2 by flow cytometric analysis, 10^6 cells were washed twice with PBS/1% BSA, fixed with 2% paraformaldehyde (Merck) for 10 minutes at room temperature, and permeabilized with PBS/0.03% saponin (Sigma). Staining for Bcl-2 protein was performed as previously described.[27] Flow cytometric analysis was performed in a FACScan apparatus equipped with the Lysis II program.

DNA Fragmentation Analysis

Cells (1–3 × 10^6) were washed with PBS and pelleted by centrifugation at 200 × *g* for 5 minutes. Cell pellets were resuspended in 750 μl of lysis buffer (50 mmol/L Tris, pH 8.0; 10 mmol/L EDTA, pH 8.0; 0.5% SDS) and frozen at −70 C for 15 minutes. The lysates were then centrifuged at 14.000 × *g* for 15 minutes and the supernatants were incubated with 0.5 mg/ml proteinase K (GIBCO-BRL) for 1 hour at 65 C. The DNA preparations were then extracted with buffered phenol, phenol:chloroform (1:1), and chloroform:isoamyl alcohol, and ethanol precipitated. Samples were air-dried and resuspended in 15 μl of water containing 0.25 mg/ml RNAse A (GIBCO-BRL). DNA samples were stained with 0.1% ethidium bromide and electrophoresed on a 2% agarose gel .

S1 Protection Analysis

Total RNA was prepared by the guanidinium thiocyanate method.[28] To construct a human *Bcl-x* probe, a 725-bp cDNA fragment containing the entire coding region of *Bcl-x*$_L$ was inserted into the *Eco*RI polylinker site of pBluescript.[29] The 3′ end of the antisense strand was labeled at the unique *Nco*I site of *Bcl-x*$_L$ by filling with [^{32}P]CTP (6000 Ci/mmol) (Amersham) and Klenow (Promega). A control probe specific for the human (GAPDH) was prepared as previously described.[30] To assess mRNA expression, both *Bcl-x*$_L$ and GAPDH-labeled probes (10^5 cpm each) were hybridized simultaneously with 10 μg of total RNA in the same tube for 16 hours at 55 C and then digested with 200 U of S1 nuclease for 1 hour at 37 C. Protected fragments were size separated on a 6% sequencing gel, dried, and autoradiographed as previously described.[31] Autoradiographs were quantified by densitometry scanning with a radioanalytic imaging system and AMBIS QuantProbe Software (AMBIS, Inc., San Diego, CA).

Results

Development of Myeloid Cell Lines Overexpressing Bcl-2

The Bcl-2 oncoprotein has been shown to function as a repressor of apoptosis in a variety of cellular systems.[22–24] To determine whether Bcl-2 can exert a protective effect against apoptosis within the myeloid lineage, the myeloid cell lines HL-60 and K562 were stably transfected with the SFFV-*Bcl-2* plasmid containing the human *Bcl-2* open-reading frame. Control cells generated by transfection with the SFFV-Neo vector lacking the *Bcl-2* sequence were also developed. Bulk and clonal cell lines transfected with *Bcl-2* and control plasmid were generated by growth in medium containing G418. The expression of Bcl-2 protein in transfected cells was determined by flow cytometry and Western blot analysis. As shown in Figure 1, C and D, HL-60-Neo cells express moderate levels of endogenous Bcl-2 protein, which were increased four- to fivefold after transfection with the *Bcl-2* vector. Untransfected HL-60 cells displayed an identical pattern of Bcl-2 expression than HL-60-Neo cells (data not shown). The K562 cells expressed no detectable levels of endogenous Bcl-2 protein (Figure 1, A and B). Even polymerase chain reaction (PCR) analysis failed to detect trace amounts of *Bcl-2* mRNA (data not shown). After transfection with the *Bcl-2* plasmid, K562 expressed uniform levels of the 26-kd Bcl-2 in practically all cells (Figure 1B).

Bcl-2 Inhibits Apoptosis Induced by Myeloid Differentiation in HL-60 Cells

Differentiation of HL-60 toward neutrophils or macrophage-like cells can be induced by stimulation with RA or PMA, respectively.[29,32,33] To assess the function of Bcl-2 in the differentiation pathway of HL-60 cells, control and Bcl-2-transfected HL-60 bulk cell lines were cultured in the presence of 1 μmol/L RA (granulocytic differentiation) or 20 ng/ml PMA (macrophage-like cell differentiation) for various

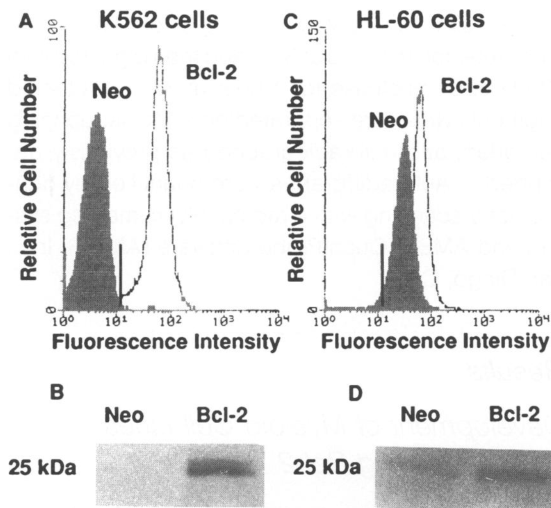

Figure 1. *Expression of Bcl-2 protein in Neo and Bcl-2-transfected HL-60 and K562 cells.* **A, C:** *flow cytometric analysis of cell populations was performed as described in Materials and Methods. Bulk cell lines were stained with hamster anti-human Bcl-2 MAb (6C8) followed by biotin-conjugated goat anti-hamster Ab and PE-conjugated streptavidin. The line "cut-off" represents the highest level of background fluorescence obtained by staining with an isotype matched control hamster MAb. In the experiment shown the mean fluorescence intensity of Bcl-2 staining for K562-Neo was 20 HL-60-Neo 330 HL-60-Bcl-2 661 and 630 for K562-Bcl-2.* **B, D:** *Western blot analysis for Bcl-2. Cell lysates from 2.5 × 10⁶ HL-60 or K562 cells were loaded onto a 12% polyacrylamide gel. After electrophoresis proteins were transferred to nitrocellulose and analyzed for Bcl-2 with 6C8 MAb followed by peroxidase-conjugated rabbit anti-hamster IgG.*

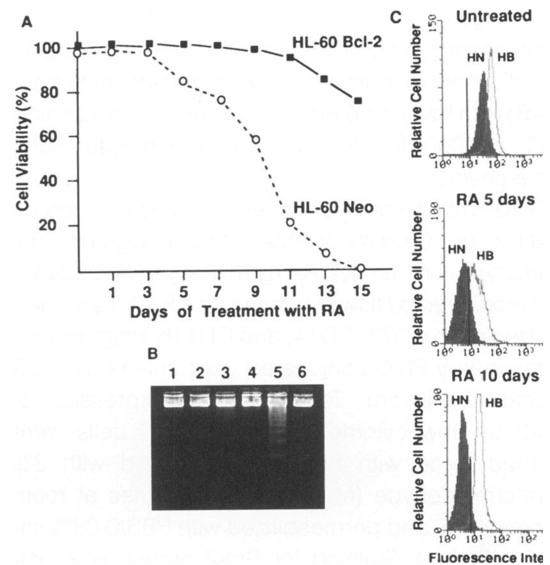

Figure 2. *Inhibition of granulocytic differentiation-induced apo sis in HL-60 by Bcl-2.* **A:** *Time course of cell viability as measure exclusion of trypan blue dye after incubation with 1 μmol/L RA Analysis of DNA fragmentation in HL-60-Neo cells (lanes 1, 3, an and HL-60-Bcl-2 cells (lanes 2, 4, and 6) after induction of diffe tiation with RA. Before treatment (lanes 1 and 2) and after tr ment for 5 days (lanes 3 and 4) and 10 days (lanes 5 and 6) DNA was prepared from cells as described in Materials and Meth and electrophoresed on a 2% agarose gel.* **C:** *Expression of Bcl- HL-60-Neo (HN) and HL-60-Bcl-2 (HB) cell populations after tr ment with RA. Histograms show staining with 6C8 MAb in the p ence of saponin. The markers indicate the staining background o isotype match MAb 3F11.*

times. The percentage of viable cells in the HL-60-Neo population fell dramatically by day 11 of RA treatment (20% viable) and dropped to less than 2% by day 15 (Figure 2A). In contrast, the great majority (94%) of the HL-60-Bcl-2 cells remained viable at day 11, and 76% of the cells continued viable by day 15 of RA treatment. Similarly, only 3% of the HL-60-Neo cells treated with PMA survived by 6 days (Figure 3A). By contrast cell death of HL-60-Bcl-2 was delayed, and about 45% of the cells were still viable after 6 days of incubation with PMA. Similar results were obtained using two separate HL-60-Bcl-2 clones (data not shown). This loss of viability observed after treatment with either RA or PMA was due to the activation of an apoptotic process. Thus, after treatment with RA or PMA, more than 70% of the cells became smaller and exhibited pyknotic and fragmented nuclei, which are morphological features of apoptosis (data not shown). Furthermore, as shown in Figures 2B and 3B, treatment with RA or PMA induced DNA Fragmentation of genomic DNA in an oligonucleosomal pattern after 10 days of RA treatment (Figure 2B, lane 5), or 3 and 5 days of PMA treatment (Figure 3B, lanes 3 and 5). In contrast, genomic DNA from HL-60-Bcl-2 cells remained relatively unfragmented (Figures 2B and 3B, lanes 4 and 6). These experiments indicate that in HL-60 cells, differentiation, and apoptosis occ simultaneously, and Bcl-2 can inhibit the ferentiation-induced death process.

Down-Regulation of Endogenous Bcl-2 during Myeloid Differentiation

To assess whether the endogenous levels of Bc may regulate the apoptotic mechanism that is tr gered by differentiation, the expression of Bcl-2 w examined before and after treatment with agents t induce myeloid differentiation. Incubation of HL with RA or PMA was accompanied by a progressi decrease in the endogenous levels of the Bcl-2 p tein in the SFFV-Neo-transfected HL-60 cells (HN) assessed by flow cytometric analysis (Figures 2C a 3C). In RA-treated cells, 39% remained Bcl-2 positi after 5 days and 11.4% after 10 days of treatme (Figure 2C). In PMA-treated HL-60 (HN), 74% of t cells remained Bcl-2 positive after 3 days, and o 1% of the cells were positive by 5 days of treatme (Figure 3C). In contrast, the expression of Bcl-2 mained positive in HL-60-Bcl-2 cells (HB) (Figures and 3C), indicating that SFFV-driven Bcl-2 is not si nificantly down-regulated during differentiation.

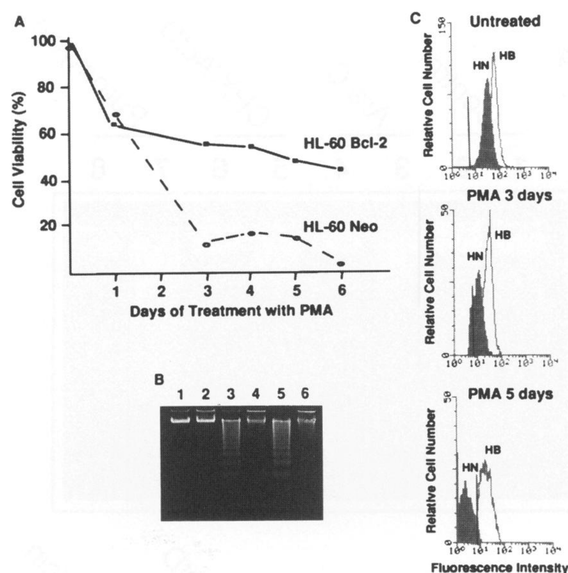

Figure 3. *Inhibition of macrophage-like cell differentiation-induced apoptosis in HL-60 by Bcl-2.* A: *Time course of cell viability as measured by exclusion of trypan blue dye after incubation with 20 ng/ml PMA. Values shown represent the mean of triplicate cultures. Standard deviation of the mean was less than 15% of the mean value.* B: *Analysis of DNA fragmentation in HL-60-Neo cells (lanes 1, 3, and 5) and HL-60-Bcl-2 cells (lanes 2, 4, and 6) after induction of differentiation with PMA. Before treatment (lanes 1 and 2) and after treatment for 3 days (lanes 3 and 4) and 5 days (lanes 5 and 6) total DNA was prepared from cells as described in Materials and Methods and electrophoresed on a 2% agarose gel.* C: *Expression of Bcl-2 in HL-60-Neo (HN) and HL-60-Bcl-2 (HB) cell populations after treatment with PMA. Histograms show staining with 6C8 MAb in the presence of saponin. The markers indicate the staining background of the isotype match MAb 3F11.*

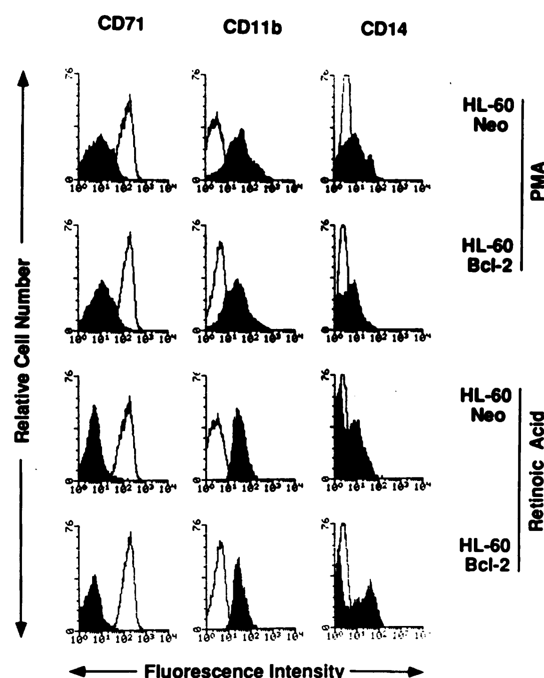

Figure 4. *Expression of differentiation markers in HL-60-Neo and HL-60-Bcl-2 cell populations treated with PMA or RA. Cells untreated* (open graphs) *and treated with PMA (4 days) or RA (10 days)* (shaded graphs) *were stained with a primary antibody anti-CD71 anti-CD11b or anti-CD14 followed by a FITC-conjugated goat anti-mouse IgG.*

Bcl-2 Overexpression Fails to Inhibit Differentiation in Myeloid Cell Lines

Because apoptosis is only a final step in the differentiation pathway of myeloid cells, we wished to determine whether Bcl-2 overexpression could have a more generalized effect on the differentiation process of HL-60 cells. To assess that, the expression of several cell surface markers (CD71, CD11b, CD14) known to be regulated during myeloid differentiation were examined in untreated and HL-60 cells incubated with RA for 10 days or PMA for 4 days. A common feature of HL-60 cells differentiating toward granulocytes or macrophage-like cells is the rapid internalization of surface transferrin receptor (CD71), which precedes the inhibition of proliferation and the acquisition of other differentiation markers (i.e., CD11b, CD14).[34,35] As shown in Figure 4, there was no detectable difference in the expression of CD71, CD11b, and CD14 between control HL-60-Neo and HL-60-Bcl-2 cell populations after differentiation. As an additional criteria for differentiation, HL-60-Neo and HL-60-Bcl-2 cells were stained with Wright-Giemsa at different intervals after treatment with RA or PMA. The percentage of mature cells in the cultures was similar in HL-60-Neo and HL-60-Bcl-2 cells further confirming that Bcl-2 has no significant effect on terminal differentiation (data not shown).

Effect of Bcl-2 Overexpression on Apoptosis Induced by Inhibitors of Macromolecular Synthesis in HL-60 and K562 Cells

In many cellular systems, there is a requirement for *de novo* protein synthesis in the activation of an apoptotic response. However, in other systems such as HL-60, inhibition of RNA or protein synthesis induces apoptosis[7] which argues for the presence of proteins within the cells that actively suppress the apoptotic mechanism. HL-60 and K562 cells were incubated with the protein synthesis inhibitor puromycin (6 µmol/L), a combination of protein and RNA synthesis inhibitors, CHX (25 µg/ml) and ActD (0.1 µg/ml), or Ara C (100 µmol/L), which interferes with DNA synthesis and has been shown to induce apoptosis in human myeloid leukemia cells.[36] Treatment of HL-60-Neo and K562-Neo with puromycin, the combination of CHX/Act D or Ara C induced a time-dependent loss

of cell viability (Figure 5). Noticeably, cell death induced by all drugs examined was detected much earlier in HL-60-Neo than in K562-Neo (Figure 5). During the first 3–6 hours, overexpression of Bcl-2 in HL-60 provided little or no protection from cell death induced by all drugs examined (Figure 5, F–H). In contrast, K562 overexpressing Bcl-2 were much more resistant than control cells to apoptosis triggered by the same inhibitors (Figure 5, B–D). To determine the cell death mechanism, genomic DNA was isolated and examined by agarose gel electrophoresis. Incubation of HL-60-Neo and K562-Neo cells with inhibitors of macromolecular synthesis induced degradation of DNA in an oligonucleosomal pattern which is characteristic of apoptosis (Figure 6, A and B, lanes 3, 5, and 7). Furthermore, the morphology of the cells treated with inhibitors of macromolecular synthesis revealed features which are characteristic of apoptosis (data not shown). Consistent with the viability results shown in Figure 5, overexpression of Bcl-2 did not inhibit significantly the degradation of genomic DNA in HL-60 cells incubated for 6 hours with either puromycin or

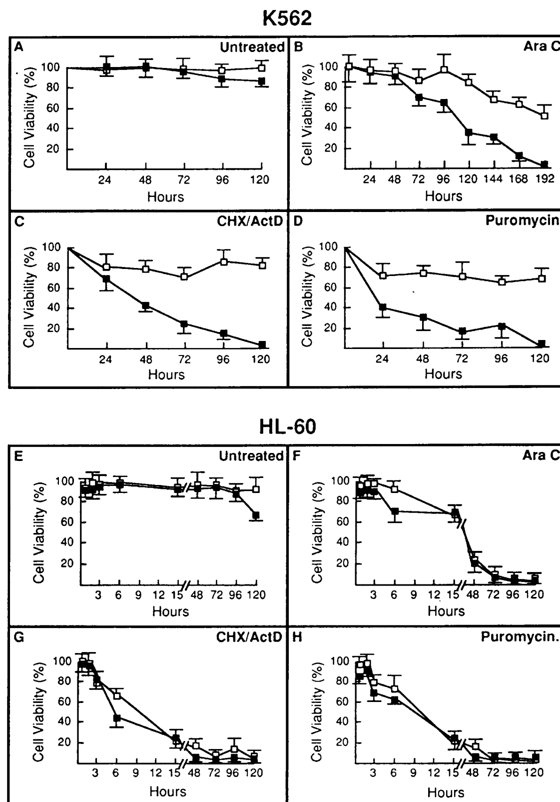

Figure 5. *Viability of K562 and HL-60 cells incubated in the presence of macromolecular synthesis inhibitors Neo- (■) and Bcl-2- (□) transfected cells were incubated with Ara C (**B** and **F**), CHX/ActD (**C** and **G**) or puromycin (**D** and **H**) and at the indicated time points cell viability was measured by exclusion of trypan blue dye. All data points represent the mean of triplicate cultures ± standard deviation.*

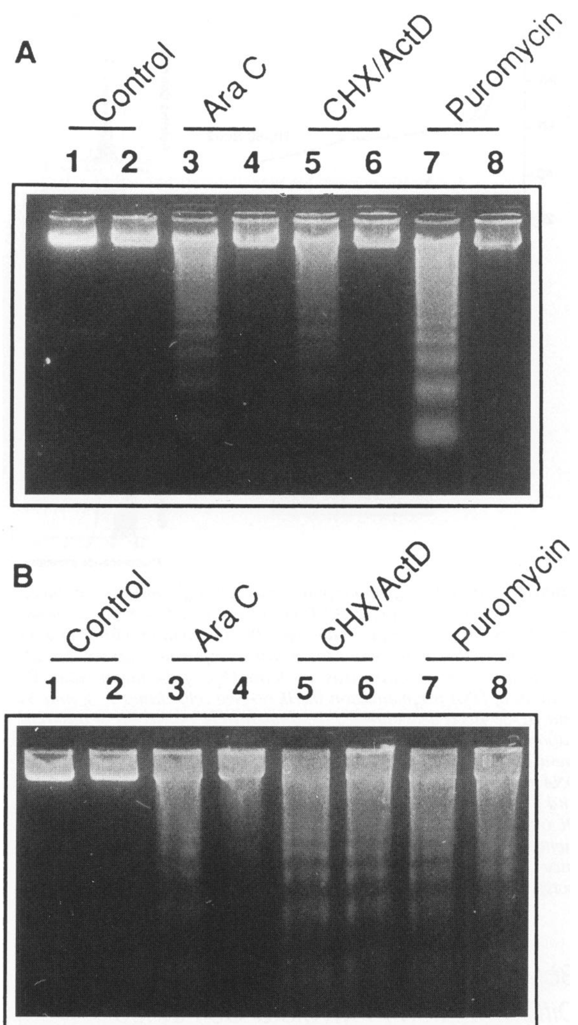

Figure 6. *Analysis of DNA fragmentation in K562 and HL-60 cells following treatment with macromolecular synthesis inhibitors.* **A:** *K562-Neo (lanes 1, 3, 5, 7) and K562-Bcl-2 (lanes 2, 4, 6, 8) cells were incubated for 120 hours with Ara C 72 hours with CHX/ActD and 72 hours with puromycin.* **B:** *HL-60-Neo (lanes 1, 3, 5, 7) and HL-60-Bcl-2 (lanes 2, 4, 6, 8) cells were incubated for 6 hours with the same agents. DNA fragmentation was monitored by electrophoresis in a 2% agarose gel.*

CHX/ActD (Figure 6B, lanes 6 and 8), although some protection was observed after incubation with Ara C for 6 hours (Figure 6B, lane 4). In contrast, the DNA from K562 overexpressing Bcl-2 remained uncleaved after treatment with the same macromolecular synthesis inhibitors (Figure 6A, lanes 4, 6, and 8).

Myeloid Cell Lines Can Express Bcl-x_L, a Bcl-2-Related Gene that Inhibits Apoptosis

The cell line K562 is relatively more resistant than HL-60 to a variety of stimuli capable of inducing apoptosis[37,38] (Figure 5). The relative resistance of K562

to apoptosis is surprising, since it does not express endogenous Bcl-2 protein. One reasonable possibility is that the survival of K562 is maintained by anti-apoptotic genes other than *Bcl*-2. A candidate gene is *Bcl-x*, whose *Bcl-x$_L$* form is a powerful inhibitor of apoptosis in growth factor-dependent hematopoietic cell lines.[39] Because two mRNA species of *Bcl-x* with opposite biological function (*Bcl-x$_L$* and *Bcl-x$_S$*) have been described,[39] we developed a S1-nuclease assay capable of discriminating between both mRNA forms (Figure 7A). Hybridization of cellular RNA to an end-labeled *Bcl-x* probe protected a fragment of 455 nucleotides corresponding to *Bcl-x$_L$*, which was expressed threefold greater in K562 than in HL-60 (Figure 7B). The *Bcl-x$_S$* mRNA was not detectable in K562 and HL-60 (Figure 7B). The *Bcl-x$_L$* product was detected in whole cell lysates from K562 but not in HL-60 by Western blot analysis (Figure 7C), which is consistent with the expression pattern of *Bcl-x$_L$* mRNA shown in Figure 7B.

Discussion

The proto-oncogene *Bcl-2* localizes to membranes of the outer mitochondria, endoplasmic reticulum, and nuclear envelope and promotes cell survival by inhibiting the apoptotic mechanism. Although much of the work has been focused on the involvement of *Bcl*-2 in lymphoid differentiation, this proto-oncogene is not lineage restricted. Bcl-2 is expressed in early hematopoietic progenitors and within myeloid cells is regulated in a differentiation-associated manner.[25] Because Bcl-2 is absent in polymorphonuclear leukocytes, it has been postulated that diminished expression of Bcl-2 may be a mechanism that triggers or facilitates the apoptotic mechanism that is coupled to terminal differentiation of myeloid progenitors.

In the present study we have examined the function of Bcl-2 in two myeloid leukemic cell lines HL-60 and K562. Induction of differentiation in HL-60 by RA or PMA leads to progressive loss of cell viability by activation of an apoptotic process. Thus, like in normal neutrophils, differentiation of HL-60 is coupled to apoptosis. Moreover, as it was previously reported by others, we find that differentiation of HL-60 is associated with a progressive diminution of Bcl-2 which is reminiscent of the developmental regulation of Bcl-2 observed in normal myeloid cells.[25] Importantly, we find that constitutive expression of Bcl-2 inhibits the apoptotic death triggered by differentiation. Significantly, however, enforced Bcl-2 expression did not affect the phenotypic changes that occur during myeloid maturation. These results suggest that although

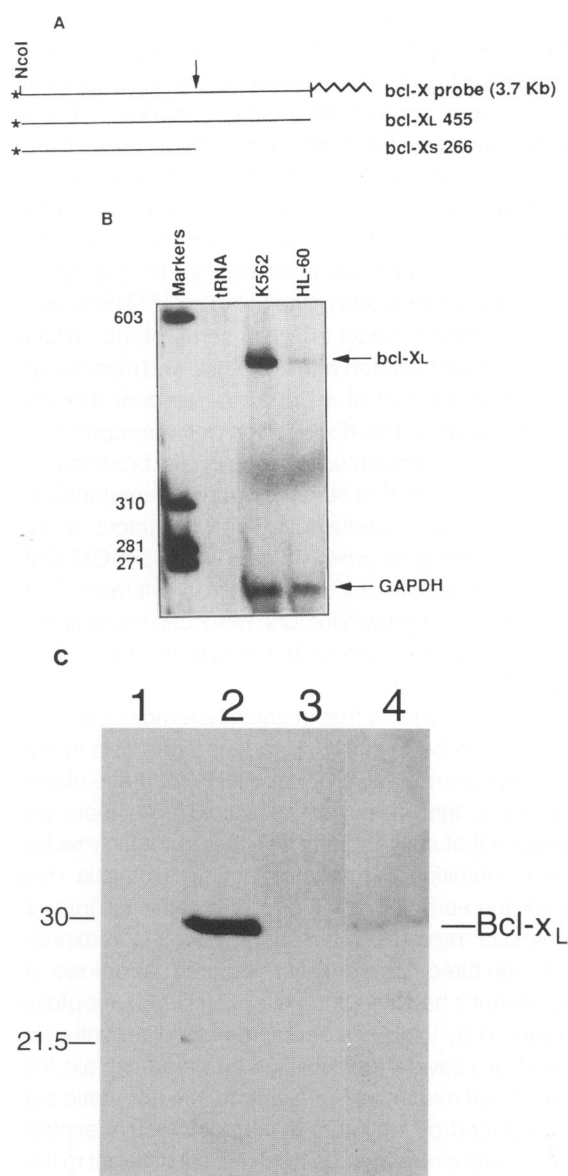

Figure 7. *Expression analysis of Bcl-x in K562 and HL-60 cell lines.* A: *The antisense strand of the Bcl-x fragment was end-labeled at the* NcoI *site located in the coding region of the human Bcl-x cDNA*[39] *pBluescript sequences are indicated by a wavy line. The splice site utilized for the generation of Bcl-x$_S$ is indicated by an arrow. Expected protected fragments for Bcl-x$_L$ and Bcl-x$_S$ mRNA forms are indicated in nucleotides.* B: *End-labeled Bcl-x and GAPDH S1-nuclease probes were simultaneously hybridized to total RNA samples (10 μg). Hybridization revealed a protected fragment of 455 (Bcl-x$_L$) nucleotides. The amount of Bcl-x$_L$ signal was threefold greater in K562 than in HL-60 as determined by scanning densitometry (relative values were corrected according to the internal GAPDH standard). An expected fragment of 266 nucleotides corresponding to the Bcl-x$_S$ form was not detected in K562 or HL-60. Size markers are in nucleotides.* C: *Detection of Bcl-x$_L$ protein by Western-blot analysis. Protein lysates (2×10^6 cells/lane) from murine FL512 cells (lane 1) FL512 transfected with a human Bcl-x$_L$ cDNA (lane 2) HL-60 (lane 3) and K562 (lane 4) are shown. Size markers are in kilodaltons.*

apoptosis and differentiation proceed simultaneously in the myeloid lineage, they can be uncoupled by expression of Bcl-2, which confirms results recently

published by others.[40,41] In other studies using a transgenic system of Bcl-2 overexpression, it was concluded that Bcl-2 inhibits apoptosis of neutrophils but not their engulfment by macrophages.[42] These observations are consistent with a model in which the mechanism that triggers engulfment of terminally differentiated myeloid cells can be separated from the apoptotic process inhibited by Bcl-2. Phagocytosis of apoptotic cells appears to be induced by changes in the plasma membrane of the dying cell.[43] These findings suggest a model in which some of the cellular processes associated with apoptosis and myeloid senescence are part of a Bcl-2-independent differentiation pathway. The dissociation of the apoptotic response and differentiation by signals provided by Bcl-2 is similar to that observed during hematopoietic cell proliferation mediated by growth factors. In the latter, signaling via growth factors like IL-3 or GM-CSF can promote both cell survival and proliferation.[22] In the absence of growth factors, Bcl-2 can maintain hematopoietic cell survival but it fails to induce cell-cycle progression.[22]

Chemical agents that inhibit macromolecular synthesis have been shown to induce apoptosis in myeloid cell lines.[7] A likely explanation for these observations is that in leukemic myeloid cells there are proteins that actively suppress the apoptotic mechanism. Inhibition of macromolecular synthesis may block the activity or synthesis of putative apoptosis-repressor proteins. Interestingly, Bcl-2 overexpression inhibited differentiation-induced apoptosis in HL-60 but it had little or no effect on HL-60 apoptosis triggered by inhibitors of macromolecular synthesis. There are several possibilities that may explain this differential response. For example, the apoptotic signal induced by inhibitors of macromolecular synthesis may be stronger or be qualitatively different to that generated during differentiation in HL-60 cells. These observations are not too surprising as the ability of Bcl-2 to inhibit apoptosis in lymphoid cells differs depending on the apoptotic stimulus.[44,45] The cellular proteins involved in controlling apoptosis in the myeloid lineage are not well understood. It is likely that down-regulation of Bcl-2 in myeloid cells facilitates the apoptotic process triggered by terminal differentiation. Recently, another *Bcl*-2-related gene, *mcl*-1 has been isolated from human myeloid cells treated with PMA and it appears to be regulated during differentiation.[46] In the present work, we provide evidence that another *Bcl*-2 family member, *Bcl-x*, is expressed in the early myeloid cell line, K562. The predicted Bcl-x_L protein shares a remarkable amino acid and structural homology to Bcl-2 and its expression suppresses apoptosis induced by growth factor withdrawal.[39] Our preliminary studies suggest that the expression of Bcl-x_L in K562 may account at least in part for the relative resistance of this cell line to apoptotic cell death.[37,38] However, additional experiments will be needed to firmly establish a role for *Bcl-x* within myeloid cells.

The mechanism by which Bcl-2 and other Bcl-2-related proteins inhibits apoptosis is poorly understood. Recent evidence suggests that Bcl-2 may prevent apoptosis through the regulation of an antioxidant pathway.[20] However, other experimental data indicate that Bcl-2 may inhibit apoptosis by modulating Ca^{2+} fluxes through intracellular organelles[47] or by regulating protein trafficking across the nuclear membrane.[48,49] Bcl-2 appears to be expressed as a part of an intracellular protein complex. For example, Bcl-2 has been shown to interact with at least two cellular proteins, BAX[50] and r-Ras p23,[51] although the significance of these protein interactions is not well understood. At least four genes of the *bcl*-2 family, *Bcl*-2, *Bcl-x*, *bax*, and *mcl*-1, can be expressed at some point during myeloid differentiation. Together, these results indicate that the regulation of apoptosis within myeloid cells is complex and modulated by multiple proteins.

Acknowledgments

We thank Dr. S. J. Korsmeyer and Dr. C. B. Thompson for providing antibodies, M. P. Lopez for expert help with flow cytometric analysis, and C. González-Pardo and L. Ding for expert technical assistance and M. Benedict for critical review of the manuscript.

References

1. Wyllie AH, Kerr JF, Currie AR: Cell death: the significance of apoptosis. Int Rev Cytol 1980, 68:251–306
2. Walker NI, Harmon BV, Gobe GC, Kerr JF: Patterns of cell death. Methods Achiev Exp Pathol 1988, 13: 18–54
3. Hamburger V, Oppenheim RW: Naturally occurring death in vertebrates. Neurosci Commun 1982, 1:39–55
4. Kerr JF, Wyllie AH, Currie AR: Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. Br J Cancer 1972, 26:239–257
5. Martin DP, Schmidt RE, DiStefano PS, Lowry OH, Carter JG, Johnson EM Jr: Inhibitors of protein synthesis RNA synthesis prevent neuronal death caused by nerve growth factor deprivation: J Cell Biol 1988, 106: 829–843
6. Oppenheim RW, Prevette D, Tytell M, Homma S: Naturally occurring and induced neuronal death in the

chick embryo in vivo requires protein and RNA synthesis:evidence for the role of cell death genes. Dev Biol 1990, 138:104–113

7. Martin SJ, Lennon SV, Bonham AM, Cotter TG: Induction of apoptosis (programmed cell death) in human leukemic HL-60 cells by inhibition of RNA or protein synthesis. J Immunol 1990, 145:1859–1867
8. Ellis RE, Yuan JY, Horvitz HR: Mechanisms and functions of cell death. Annu Rev Cell Biol 1991, 7:663–698
9. McConkey DJ, Aguilar-Santelises M, Hartzell P, Eriksson I, Mellstedt H, Orrenius S, Jondal M: Induction of DNA fragmentation in chronic B-lymphocytic leukemia cells. J Immunol 1991, 146:1072–1076
10. Martin SJ, Bradley JG, Cotter TG: HL-60 cells induced to differentiate toward neutrophils subsequently die via apoptosis. Clin Exp Immunol 1990, 79:448–453
11. Savill JS, Wyllie AH, Henson JE, Walport MJ, Henson PM, Haslett C: Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. J Clin Invest 1989, 83:865–875
12. Colotta F, Re F, Polentarutti N, Sozzani S, Mantovani A: Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. Blood 1992, 80:2012–2020
13. Brach MA de Vos S, Gruss H-J, Herrmann F: Prolongation of survival of human polymorphonuclear neutrophils by granulocyte-macrophage colony-stimulating factor is caused by inhibition of programmed cell death. Blood 1992, 80:2920–2924
14. Lotem J, Sachs L: Hematopoietic cytokines inhibit apoptosis induced by transforming growth factor β1 and cancer chemotherapy compounds in myeloid leukemic cells. Blood 1992, 80:1750–1757
15. Williams GT, Smith CA, Spooncer E, Dexter TM, Taylor DR: Haemopoietc colony stimulating factors promote cell survival by suppressing apoptosis. Nature 1990, 343:76–79
16. Bakhshi A, Jensen JP, Goldman P, Wrigth JJ, McBride OW, Epstein AL, Korsmeyer SJ: Cloning the chromosomal breakpoint of t(14:18) human lymphomas: clustering around JH on chromosome 14 and near a transcriptional unit on 18. Cell 1985, 41:899–906
17. Cleary ML, Smith SD, Sklar J: Cloning and structural analysis of cDNAs for Bcl-2 and a hybrid Bcl-2/immunoglobulin transcript resulting from the t(14;18)-translocation. Cell 1986, 47:19–28
18. Monaghan P, Robertson D, Amos TA, Dyer MJ, Mason DY, Greaves MF: Ultrastructural localization of Bcl-2 protein. J Histochem Cytochem 1992, 40:1819–1825
19. Jacobson MD, Burne JF, King MP, Miyashita T, Reed JC, Raff MC: Bcl-2 blocks apoptosis in cells lacking mitochondrial DNA. Nature 1993, 361:365–369
20. Krajewski S, Tanaka S, Takayama S, Schibler MJ, Fenton W, Reed JC: Investigation of the subcellular distribution of the Bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum and outer mitochondrial membranes. Cancer Res 1993, 53:4701–4714
21. Hockenbery DM, Zutter M, Hickey W, Nahm M, Korsmeyer SJ: Bcl-2 protein is topographically restricted in tissues characterized by apoptotic cell death. Proc Natl Acad Sci USA 1991, 88:6961–6965
22. Vaux DL, Cory S, Adams J: Bcl-2 gene promotes haematopietic cell survival cooperates with c-myc to immortalize pre-B cells. Nature 1988, 325:440–442
23. Nuñez G, London L, Hockenbery D, Alexander M, McKearn JP, Korsmeyer SJ: Deregulated Bcl-2 gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines. J Immunol 1990, 144:3602–3610
24. Miyashita T, Reed JC: Bcl-2 oncoprotein blocks chemotherapy-induced apoptosis in a human leukemia cell line. Blood 1993, 81:151–157
25. Delia D, Aiello A, Soligo D, Fontanella E, Melani C, Pezzella F, Pierotti MA, Della Porta G: Bcl-2 proto-oncogene expression in normal neoplastic human myeloid cells. Blood 1992, 79:1291–1298
26. Fuhlbrigge RC, Fine SM, Unanue ER, Chaplin DD: Expression of membrane interleukin 1 by fibroblasts transfected with pro-interleukin 1 α cDNA. Proc Natl Acad Sci USA 1988, 85:5649–5653
27. Merino R, Ding L, Veis DJ, Korsmeyer SJ, Nuñez G: Developmental regulation of the Bcl-2 protein and susceptibility death in B lymphocytes. EMBO J 1994, 13:683–691
28. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987, 162:156–59
29. Breitman TR, Selonik SE, Collins SJ: Induction of differentiation of the human promyelocytic cell line (HL-60) by retinoic acid. Proc Natl Acad Sci USA 1980, 77:2936–2462
30. Gratiot-Deans J, Ding L, Turka LA, Nuñez G: Bcl-2 proto-oncogene expression during human T cell development: evidence for biphasic regulation. J Immunol 1993, 151:83–91
31. McDonnell TJ, Nuñez G, Platt FM, Hockenberry D, London L, McKearn JP, Korsmeyer SJ: Deregulated Bcl-2-immunoglobulin transgene expands a resting but responsive immunoglobulin M and D-expressing B-cell population. Mol Cell Biol 1990, 10:1901–1907
32. Collins SJ, Ruscetti FW, Gallagher RE, Gallo RC: Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. Proc Natl Acad Sci USA 1978, 75:2458–2462
33. Rovera G, Santoli D, Damsky C: Human promyelocytic leukemia cells in culture differentiate into macrophage-like cells when treated with phorbol diester. Proc Natl Acad Sci USA 1979, 76:2779–2783

34. Ho PT, Ishiguro K, Sartorelli AC: Regulation of transferrin receptor in myeloid and monocytic differentiation of HL-60 leukemia cells. Cancer Res 1989, 49: 1989–1995
35. Trayner ID, Clemens MJ: Phorbol ester-induced macrophage-like differentiation of human promyelocytic leukemia (HL-60) cells occurs independently of transferrin availability. Cancer Res 1990, 50:7221–7225
36. Gunji H, Kharbanda S, Kufe D: Induction of internucleosomal DNA fragmentation in human myeloid leukemia cells by 1-β-D-arabinofuranosylcytosine. Cancer Res 1991, 51:741–743
37. Diomede L, Colotta F, Piovani B, Re F, Modest EJ, Salmona M: Induction of apoptosis in human leukemic cells by the ether lipid 1-octadecyl-2-methyl-rac-glycero-3-phosphocholine. A possible basis for its selective action. Int J Cancer 1993, 53:124–130
38. Martin SJ, Cotter TG: Ultraviolet B irradiation of human leukaemia HL-60 cells in vitro induces apoptosis. Int J Radiat Biol 1991, 59:1001–1016
39. Boise LH, González-García M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nuñez G, Thompson CB: Bcl-x a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell 1993, 74: 597–608
40. Naumovki L, Cleary ML: Bcl-2 inhibits apoptosis associated with terminal differentiation of HL-60 myeloid leukemia cells. Blood 1994, 83:2261–2267
41. Park RH, Robertson KHickstein DD, Tsai S, Hockenbery DM, Collins S: Dysregulated Bcl-2 expression inhibits apoptosis but not differentiation of retinoic acid-induced HL-60 granulocytes. Blood 1994, 84:440–445
42. Lagasse E, Weissman IL: Bcl-2 inhibits apoptosis of neutrophils but not their engulfment by macrophages. J Exp Med 1994, 179:1047–1052
43. Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM: Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J Immunol 1992, 148:2207–2216
44. Sentman CL, Shutter JR, Hockenbery DM, Kanagawa O, Korsmeyer SJ: Bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. Cell 1991, 67:879–888
45. Cuende E, Ales-Martinez JE, Ding L, Gonzalez-Garcia M, Martinez-A C, Nuñez G: Programmed cell death by Bcl-2-dependent and independent mechanisms in B lymphoma cells. EMBO J 1993, 12:1555–1560
46. Kozopas KM, Yang T, Bucham HL, Zhou P, Craig RW: MCL1 a gene expressed in programmed myeloid cell differentiation has sequence similarity to BCL2. Proc Natl Acad Sci USA 1993, 90:3516–3520
47. Baffy G, Miyashita T, Williamson JR, Reed JC: Apoptosis induced by withdrawal of interleukin-3 (IL-3) from an IL-3-dependent hematopoietic cell line is associated with repartitioning of intracellular calcium and is blocked by enforced Bcl-2 oncoprotein production. J Biol Chem 1993, 268:6511–6519
48. Meikrantz W, Gisselbrecht S, Tam S, Schlegel R: Activation of cyclin A-dependent protein kinases during apoptosis. Proc Natl Acad Sci USA 1994, 91:3754–3758
49. Ryan JJ, Prochownik E, Gottlieb CA, Apel IJ, Merino R, Nuñez G, Clarke MF: c-myc and Bcl-2 modulate p53 function by altering p53 subcellular trafficking during the cell cycle. Proc Natl Acad Sci USA 1994, 91:5878–5882
50. Oltvai ZN, Millimam CL, Korsmeyer SJ: Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax that accelerates programmed cell death. Cell 1993, 74:609–619
51. Fernández-Sarabia MJ, Bischoff JR: Bcl-2 associates with the ras-related protein R-ras p23. Nature 1993, 366:274–275