Bcl2 Inhibits Apoptosis Associated With Terminal Differentiation of HL-60 Myeloid Leukemia Cells

By Louie Naumovski and Michael L. Cleary

The Bcl2 protein inhibits apoptosis (programmed cell death) induced by a variety of noxious stimuli. However, relatively little is known about its effect on apoptosis that occurs after terminal differentiation. Bcl2 protein levels decrease during differentiation of myeloid cells into granulocytes that subsequently undergo apoptosis, but the potential role of Bcl2 in coupling survival and differentiation remains undefined. To ascertain the relationship between decreasing Bcl2 levels and the onset of apoptosis in differentiating myeloid cells, Bcl2 was hyperexpressed in the HL-60 cell line after retroviral gene transfer. After treatment of HL-60/BCL2 cells with all-trans retinoic acid or phorbol myristic acid, Bcl2 levels did not decrease as in normal HL-60 cells but, rather, increased because of activation of the viral promotor. Differentiation of the Bcl2-overexpressing cells was similar to that of normal HL-60 cells, but they showed little evidence for apoptosis and had a prolonged survival. These studies show that the survival-enhancing properties of Bcl2 counteract programmed cell death that accompanies terminal differentiation; however, Bcl2 has no significant effect on differentiation itself, suggesting that apoptosis and differentiation are regulated independently in myeloid cells.

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MATERIALS AND METHODS

Reagents. ATRA and PMA were obtained from Sigma Chemical Co (St Louis, MO). ATRA was dissolved in ethanol at 1 mmol/L and used at 1 μmol/L to induce differentiation. PMA was dissolved in DMSO at 10 μmol/L and used at 16 nmol/L to induce differentiation.

Cell culture. HL-60 cells were grown in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum and 100 U/mL penicillin, 290 μg/mL L-glutamine, and 100 μg/mL streptomycin. Cells were maintained at 37°C in a 5% CO2/95% air incubator. HL-60 stock cultures infected with the control neomycin or BCL2 retroviruses were grown as above in the presence of 500 μg/mL.

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Fig 1. Western blot analysis of Bcl2 protein in HL-60, HL-60/neo, and HL-60/BCL2 cell lines before and after differentiation. UND, undifferentiated cells; ATRA, cells exposed to ATRA for 7 days; PMA, cells incubated with PMA for 7 days.

G418. Cells used for induction with ATRA or PMA were in exponential phase of growth in the above medium lacking G418. Retroviral constructs and infection of cells. The BCL2 gene was cloned between the EcoRI-HindIII sites of the cos MSV-tk-Neo plasmid vector.28 Retroviruses were generated by cotransfections of MSV-tk-Neo and amphotropic packaging plasmids into COS7 cells. Culture supernatants containing viral stocks were collected from day 3 to 5 after transfection, centrifuged, and filtered. Viruses were titrated by infection of NIH 3T3 or Rat-1 fibroblasts, followed by detection with anti-Bcl2 antibody and visualization with horseradish peroxidase substrate. For infections of HL-60 cells, 1 × 10⁷ cells/mL were treated with viral supernatants added at 12-hour intervals for 3 days in the presence of polynucle 6 μg/mL. After 1 more day, G418 was added to 500 μg/mL. Media were changed weekly for approximately 1 month, at which time the cultures began to grow well. Cultures were then maintained by diluting 1:20 into fresh medium one to two times per week. Pools of cells were used for subsequent experiments to minimize differences that might have resulted from viral integration.

Immunohistochemistry and Western blots. Bcl2 protein in cells was detected with a monoclonal anti-Bcl2 antibody (Dako, Carpentaria, CA). Briefly, cells were collected by cytopsin and fixed with 50% methanol-50% acetone for 2 minutes. The slides were treated with 0.18% H₂O₂ in methanol for 30 minutes to block endogenous peroxidase activity. The cells were rehydrated in phosphate-buffered saline (PBS) and then blocked with PBS 5% nonfat dry milk containing 0.1% Tween-20 for 30 minutes. Anti-Bcl2 antibody was added at 1:100, followed by 1 hour of incubation. The slides were washed with PBS and then treated with the secondary antibody, biotin-labeled goat antimouse (1:500), for 1 hour. Horseradish peroxidase-avidin complex was then added (1:500) and visualized with the AEC substrate kit (Biomeka Corp, Foster City, CA). Bcl2 was detected by Western blotting performed according to standard techniques.29 Cells (1 × 10⁷) were solubilized in 250 μL of loading buffer and boiled, and 10-μL samples of proteins were separated on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels. After transfer to Immobilon-P membrane (Millipore Corp, Bedford, MA), proteins were visualized with Ponceau S (Sigma) to confirm equal loading of protein. Membranes were blocked with 5% nonfat dry milk in PBS and anti-Bcl2 monoclonal antibody was added (1:300) to the blocking solution for 2 to 16 hours. The secondary antibody was alkaline phosphatase goat antimouse (Promega, Madison, WI) and was visualized on x-ray film after treatment with nitroblock reagent (Tropix, Bedford, MA), followed by application of the chemiluminescence agent Lumiphos 530 (Boehringer Mannheim, Indianapolis, IN).

Assessment of differentiation. Differentiation was determined by several techniques. For ATRA-induced differentiation, cytopsinss were stained with Wright-Giemsa and differentiation was quantitated by morphology. Alternatively, nitroblue tetrazolium (NBT; Sigma) was used to more accurately quantitate differentiation.20 Eliminating observer subjectivity associated with morphological assessment alone.21 In ATRA-treated cultures, we assessed differentiation at 7 days, because our HL-60 line continues to differentiate until this time, as others have noted.21 For PMA-induced differentiation, cells were observed morphologically and quantitated by staining for nonspecific esterase (Sigma).

Cell viability. For suspension cultures of untreated or ATRA-induced cells, viable cells were counted after the addition of an equal volume of 0.4% trypan blue in 0.9% saline on an improved Neubauer hemocytometer. Cells induced to differentiate with PMA were detached from the plastic plates by the addition of 10 mmol/L EDTA, pH 7.4, directly to the media and incubation at 37°C for 30 minutes followed by the use of a cell scraper.

DNA fragmentation assay. Cells (1 to 5 × 10⁷) were harvested and washed with PBS. Total DNA was isolated by incubating washed cells in 400 μL lysis buffer containing 50 mmol/L Tris, pH 7.5, 10 mmol/L EDTA, 0.5% SDS, 100 μg/mL RNase, and 4 μg/mL proteinase K for at least 2 hours at 60°C. After the addition of 75 μL of 8 mol/L KAc, the DNA was extracted with phenol/chloroform, precipitated with ethanol, and resuspended in TE with 100 μg/mL of heat-treated RNase. The DNA from 0.5 to 1 × 10⁷ cells was analyzed on a composite 1% agarose/0.5% Nusieve gel.26

RESULTS

Characterization of HL-60 cells. HL-60, HL-60 infected with the control retrovirus (HL-60/neo), and HL-60 infected with the BCL2 retrovirus (HL-60/BCL2) were grown in culture and several characteristics were assessed. Bcl2 protein expression was analyzed by Western blotting. HL-60 and HL-60/neo cells had similar low-level expression of Bcl2; however, the HL-60/BCL2 cells had levels that were fivefold to 10-fold higher than baseline (Fig 1). The morphology of the three cell lines, as assessed microscopically after Wright-Giemsa staining, was similar, except that the BCL2 virus-infected cells appeared somewhat larger and had larger nuclei with a more open chromatin pattern. A Coulter counter (Coulter, Hialeah, FL) was used to quantitate cell size and showed that HL-60/BCL2 cells were approximately 20% larger by volume than HL-60 or HL-60/neo cells.

Overexpression of Bcl2 protein blocks apoptosis of HL-60

Table 1. Assessment of Granulocyte Differentiation

<table>
<thead>
<tr>
<th>Culture</th>
<th>% Capable of Reducing NBT</th>
<th>% Granulocytes</th>
</tr>
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<tbody>
<tr>
<td>HL-60</td>
<td>60 ± 7</td>
<td>48.5 ± 6.5</td>
</tr>
<tr>
<td>HL-60/neo</td>
<td>71.5 ± 1</td>
<td>62 ± 4</td>
</tr>
<tr>
<td>HL-60/BCL2</td>
<td>49 ± 12.5</td>
<td>44.5 ± 8</td>
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Cells were analyzed after treatment with ATRA for 7 days. Morphology was assessed after staining with Wright-Giemsa. Values shown are the average (± standard deviations) of two experiments counting 1 to 200 cells each.
Fig 2. Immunohistochemical detection of Bcl2 protein in HL-60/neo and HL-60/BCL2 cell lines before and after differentiation. (A) HL-60/neo cells treated with ATRA for 7 days. (B) HL-60/BCL2 cells treated with ATRA for 7 days. (C) HL-60/neo cells treated with TPA for 3 days. (D) HL-60/BCL2 cells treated with TPA for 3 days. Original magnification × 1,000 under oil immersion.

Fig 5. Differentiation of HL-60 and HL-60/BCL2 cell lines treated with PMA. Photomicrographs of cells taken through an inverted microscope at × 200 original magnification. (A) HL60 cells on day 2; (B) HL-60/BCL2 cells on day 2; (C) HL-60 cells on day 7; (D) HL-60/BCL2 cells on day 7.
Exponentially growing cultures of HL-60, HL-60/neo, and HL-60/BCL2 were induced to differentiate into granulocytes by the addition of ATRA to 1 μmol/L. Differentiation was assessed by morphology after staining with Wright-Giemsa and by reaction with NBT, which is reduced to an insoluble blue deposit by differentiated cells. The time course and extent of differentiation in all cultures was similar but with slightly fewer differentiated cells in HL-60/BCL2 cultures at day 7 (Table 1).

After treatment with ATRA, the levels of Bc12 protein decreased in HL-60 and HL-60/neo cells (Fig 1), as expected from previous experiments that showed a similar significant decrease in Bc12 levels with differentiation of HL-60 cells. Bc12 levels are probably decreasing shortly after the onset of differentiation and before cells are mature enough to reduce NBT. This is consistent with data that show a progressive decrease of Bc12 protein in normal myeloid maturation in human bone marrow. Bc12 levels increased significantly in ATRA-treated HL-60/BCL2 cells as compared with ATRA-treated HL-60/neo cells because of activation of the retroviral promoter, as assessed by Western blotting (Fig 1). Several smaller molecular weight bands are also detected by this highly specific anti-Bc12 antibody; these may represent breakdown products of Bc12 or translation from alternatively spliced transcripts and have been seen previously.

Immunocytochemistry was used to confirm the relative difference in Bc12 expression between HL-60/neo cells and HL-60/BCL2 cells after differentiation with ATRA (Fig 2). Viability, as assessed by trypan blue exclusion, was initially similar in all cultures for 4 days after induction, but by day 7 started to decline more rapidly in HL-60 and HL-60/neo cultures than in HL-60/BCL2 (Fig 3). Morphologic examination showed some cells with condensed chromatin and fragmented nuclei consistent with apoptosis. With prolonged in vitro culture, there were few viable cells left in the HL-60 and HL-60/neo cultures, whereas the HL-60/BCL2 cultures showed a slower decline in viability. Examination of stained cytospins of HL-60/BCL2 cells treated with ATRA for 1 month showed that the majority of cells appeared differentiated and were capable of reducing NBT, indicating that they had differentiated along the granulocytic lineage. Some cells that appeared undifferentiated (<10% of the total viable cells) and did not reduce NBT were noted in the ATRA-treated HL-60/BCL2 cultures (see Discussion).

To determine whether DNA degradation consistent with apoptosis was occurring, total DNA was isolated from these cultures at days 8 and 11. This analysis showed degradation of DNA into nucleosomal-sized fragments in HL-60 and HL-60/neo cells (Fig 4). Although there were dead cells in the HL-60/BCL2 cultures, DNA fragmentation was not observed.

**Overexpression of Bcl2 blocks apoptosis of HL60-derived macrophage-like cells.** Exponentially growing cultures of HL-60, HL-60/neo, and HL-60/BCL2 were induced to differentiate into macrophage-like cells by the addition of PMA to 16 nmol/L. Morphologic assessment showed that all three cell lines responded to PMA by adherence of the majority of the cells to the plastic dish within 24 to 48 hours (adherence shown for HL-60 and HL-60/BCL2 in Fig 5), as previously described. Along with morphologically apparent differentiation, a similar number of cells in all three cultures stained positively for nonspecific esterase, a marker for macrophage differentiation. With differentiation, the levels of Bc12 protein decreased in HL-60 and HL-60/neo cells, but increased in HL-60/BCL2 cells, as assessed by Western blotting (Fig 1). Immunocytochemistry was used to confirm the relative difference in Bc12 expression between HL-60/neo cells and HL-60/BCL2 cells after differentiation with PMA (Fig 2). This increase in Bc12 protein was expected because PMA induces transcription of viral promoters in HL-60 cells.

Three days after differentiation with PMA, cells in the HL-60 and HL-60/neo cultures began to detach and fragment and most cells were detached by day 7 (Fig 5). In contrast, the macrophage-like cells in the HL-60/BCL2 culture did not begin to detach at day 3 and many remained attached until days 7 to 10 (Fig 5). Morphologic features of apoptosis, including chromatin condensation and nuclear fragmentation, were evident on staining of day-3 cultures of HL-60 and HL-60/neo cells, but not HL-60/BCL2 cells. Viability, as assessed by trypan blue exclusion, decreased significantly in HL-60 and HL-60/neo cultures, but not as rapidly in HL-60/BCL2 (Fig 6). DNA analysis of combined floating and adherent cells showed degradation of DNA into nucleosomal-sized fragments in HL-60 and HL-60/neo cells by 3 days after differentiation, but not in HL-60/BCL2 cells (Fig 7). HL-60/BCL2 cells did not show detectable DNA degradation until at least 7 days after adding PMA. Taken together, these results indicate that overexpression of Bc12 does not inhibit morphologic differentiation of HL-60 cells, but does prolong cell survival by preventing apoptotic cell death.

**DISCUSSION**

**BCL2** was initially discovered at a translocation breakpoint in B-cell follicular lymphomas and was subse-
BCL2 BLOCKS DIFFERENTIATION-INDUCED APOPTOSIS

Fig 4. DNA fragmentation in ATRA-differentiated cells. Cultures were treated with ATRA for 8 or 11 days before isolation of total DNA.

The protein is expressed in various fetal tissues destined for long-term survival and adult tissues in which apoptosis serves an important homeostatic role. Bcl2 is expressed in the myeloid lineage at the myeloblastic and promyelocytic stages of differentiation, diminishing with maturation of cells into granulocytes. Overexpression of Bcl2 blocks apoptosis in the factor-dependent myeloid cell line FDC-P1, indicating that Bcl2 protein can function in myeloid cells.

A baseline level of Bcl2 protein is detected in the HL-60 cell line that has been extensively characterized because of its ability to undergo differentiation into granulocytes when induced with ATRA or into macrophage-like cells when induced with PMA. Differentiated cells show a reduction in Bcl2 protein. From these results it is not possible to determine whether Bcl2 levels need to decrease for differentiation to proceed or whether the decrease is a consequence of differentiation. Furthermore, differentiated cells undergo apoptotic death, possibly because of low levels of Bcl2 protein.

To determine whether overexpression of Bcl2 protein would alter the differentiation pathway or the life span of differentiated cells, we infected HL-60 cells with a retrovirus capable of overexpressing Bcl2 protein. Although HL-60 cell differentiation can be perturbed by overexpression of transcription factors such as MYC, PML-RARα, or HOX 2,3,33,34 HL-60 cells overexpressing Bcl2 protein by fivefold to 10-fold undergo relatively normal differentiation when treated with 1 μmol/L of ATRA or 16 nmol/L of PMA. An interesting finding is that long-term cultures of HL-60/BCL2 treated with ATRA contain a small number of undifferentiated cells, which can be explained by the fact that HL-60/BCL2 cells (which modestly overexpress Bcl2) can survive under adverse conditions such as serum deprivation that normally induce apoptosis in HL-60 cells (unpublished observations). Not all cells in a culture of HL-60 cells that are treated with ATRA will differentiate. In cultures of normal HL-60 cells, the undifferentiated cells will eventually die, but in cultures of HL-60/BCL2, we postulate that the undifferentiated cells will survive because of Bcl2. Treatment with ATRA at the concentration used in this study inhibits cell growth after 3 to 4 days and leads to inability of cells to grow when transferred to fresh media. Because we do not see an increase in the number of undifferentiated cells with prolonged culturing in media with ATRA, these cells appear not to be proliferating but are quiescent. In general, Bcl2 keeps cells in a nonproliferative state when they are subjected to agents that cause apoptosis. The small number of cells that appear undifferentiated could, in theory, differentiate at later times, but they would not add a significant number of differentiated cells to the population to influence our conclusions.

The viral LTR used in these studies is induced by differentiating agents; therefore, HL-60/BCL2-differentiated cells have very high levels of Bcl2 protein, as compared with normal differentiated cells that downregulate Bcl2 expression. Concomitant with the high levels of Bcl2 protein, cultures differentiated into granulocytes or macrophage-like cells have a greatly increased viability, as determined by trypan blue exclusion. After differentiation into granulocytes, the cells did not undergo apoptosis, as evidenced by...
lack of DNA degradation into nucleosomal-sized fragments; however, although Bcl2 blocked apoptotic death, significant cell death was still evident, indicating that other modes of death, such as necrosis, may also be occurring. Notably, after differentiation into macrophage-like cells, DNA fragmentation was delayed, but not completely abolished. This may be explained by the fact that PMA induction led to lower levels of Bcl2 protein as seen on Western blots than did ATRA induction and that lower levels of Bcl2 protein may be less protective. Alternatively, because the PMA-induced macrophage-like cells form clumps during differentiation, death signals may be more readily relayed from dying cells, making surviving cells more sensitive to apoptosis. Another possibility is that programmed cell death in these macrophage-like cells may occur through Bcl2-dependent and -independent mechanisms, as has been described for B-lymphoma cells.35

Our results show that Bcl2 protein is functionally active late in the myeloid lineage both in granulocytes and macrophages. Recently, MCL1, a gene with sequence similarity to BCL2, was identified as an early induction gene in phorbol ester-treated human myeloid leukemia cell line ML-1.36 Overexpression of the protein product of this gene could potentially have similar effects on the survival of differentiated myeloid cells.

Our studies suggest that, although differentiation and apoptosis occur concomitantly, they are regulated independently in differentiating myeloid cells. This conclusion is consistent with recent observations that differentiation of the multipotent hemopoietic cell line FDCP-Mix can proceed in the absence of growth factors if cell viability is maintained by constitutive Bcl2 expression.37 The HL-60/BCL2 cultures have been maintained for more than 1 year. Spontaneous differentiation into NBT-positive cells has been analyzed on several occasions and has not been significantly higher than in control cultures. Visual examination of these cells does not disclose the presence of increased numbers of macrophage-like cells. Thus, the HL-60/BCL2 cells are stable and are not undergoing substantial amounts of spontaneous differentiation. HL-60 is a myeloid leukemia cell line that requires pharmacologic stimuli to differentiate significantly, whereas the FDCP-Mix cells are more primitive cells that are nonleukemic and can undergo multilineage differentiation when cocultured with bone marrow stromal cells. HL-60 cells are clearly different in their differentiation requirements from FDCP-Mix cells, but each of these models shows that Bcl2 does not inhibit differentiation and allows differentiated cells to survive.

Granulocytes and monocytes (the precursors of normal macrophages) have a limited life span, but certain cytokines, such as granulocyte-monocyte colony-stimulating factor, granulocyte colony-stimulating factor, IL-1β, tumor necrosis factor, interferon-γ, nerve growth factor, and bacterial lipopolysaccharides or inactivated streptococci can extend their life span significantly by preventing apoptotic death.38-42 It is not known whether any of these factors directly regulate or activate BCL2 either through induction of the gene or modulation of protein activity. Alternatively, and most likely, these factors function independently of Bcl2 in a separate cell survival pathway or pathways. Differentiated cells that do not receive the appropriate survival signals may activate an apoptotic death pathway. In the presence of inappropriately high amounts of Bcl2 protein or related proteins, the cells are prevented from activating this pathway, and they continue to survive in the absence of physiologic survival signals.

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