Effects of BCL-2 Antisense Oligodeoxynucleotides on In Vitro Proliferation and Survival of Normal Marrow Progenitors and Leukemic Cells

By Lydia Campos, Odile Sabido, Jean-Pierre Rouault, and Denis Guyotat

Previous studies have shown that the BCL-2 protooncogene encodes a mitochondrial protein that promotes cell survival by blocking programmed cell death. Bcl-2 protein has been detected in normal immature myeloid cells and in acute myeloid leukemia (AML) cells. To assess its functional role in normal and leukemic hematopoiesis, we performed serum-free cultures of CD34+ normal marrow cells, of bcl-2-positive myeloid lines, and of AML cells in the presence of bcl-2 sense, nonsense, and antisense phosphorothioate oligodeoxynucleotides. In all antisense-treated cultures, we observed (1) an inhibition of bcl-2 protein expression by day 4 to 6 of culture; (2) a decrease in cell survival duration; and (3) a decrease in the number of clonogenic cells present in the culture. Moreover, exposure to chemotherapeutic drugs resulted in more effective killing of AML cells in the presence of antisense oligomers. We conclude that bcl-2 protein is necessary for the survival of myeloid cells in culture, and that it may be implicated in the resistance of AML cells to chemotherapy.

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MATERIALS AND METHODS

Cells. Three human leukemia/lymphoma cell lines were studied: Daudi, a Burkitt's lymphoma derived line used as bcl-2-negative control; and KG1a (myeloblastic) and HL-60 (promyelocytic), which are both bcl-2-positive.

Experiments with fresh AML cells were conducted on three samples obtained from consenting patients at diagnosis. The first two samples were selected because of their high percentages of bcl-2-positive cells (AML no. 1 and AML no. 2), and the third one (AML no. 3) served as negative control. According to French-American-British (FAB) recommendations, diagnoses were M1 (AML no. 1), M5 (AML no. 2), and M2 (AML no. 3). Leukemic cells were collected by bone marrow aspiration into heparinized syringes, separated by Ficoll density gradient, washed, and resuspended in RPMI-1640. Cytocentrifuge slides showed that all samples contained more than 95% blasts cells.

Normal marrow was obtained from allogeneic bone marrow transplant donors. CD34+ cells were prepared by the AIS MicroCELLector system (Applied Immune Sciences, Menlo Park, CA) according to the instructions of the manufacturer. Briefly, Ficoll-separated cells were incubated for 1 hour in a Soybean Cell Culture Flask (Applied Immune Sciences) at room temperature. Supernatant cells were then incubated for 1 hour in a CD34 culture flask selection. CD34+ cells were detached, washed, and resuspended in RPMI-1640. The percentage of CD34+ cells in the suspension was higher than 90% in all experiments.

Preparation of oligodeoxynucleotides. Sense and antisense 20-
mer phosphorothioate oligodeoxynucleotides directed to the translation initiation site of BCL-2 transcripts were purchased from Geneset SA (Paris, France). Preparation was performed as described by Reed et al14 according to the following sequences: 5'-GGGAAAGAT-GGCGCACGCTG-3' (sense) and 5'-CAGCGTGCGCCATCC-3' (antisense). In preliminary experiments performed with HL-60 cells, nonsense oligodeoxynucleotides with the same base composition (5'-TCGCCACTCGATCCTGCCCG-3') were used as well. Oligodeoxynucleotides were suspended in Hank's Buffered Salt Medium after control of purity and stored at -20°C.

Table 1. Expression of bcl-2 According to Day of Culture and Oligodeoxynucleotide Treatment

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Day 6</th>
<th>Day 11</th>
<th>Day 15</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>S</td>
<td>NS</td>
</tr>
<tr>
<td>CD34*</td>
<td>75</td>
<td>68</td>
<td>61</td>
</tr>
<tr>
<td>KGla</td>
<td>98</td>
<td>95</td>
<td>82</td>
</tr>
<tr>
<td>HL-60</td>
<td>92</td>
<td>89</td>
<td>90</td>
</tr>
<tr>
<td>AML no. 1</td>
<td>89</td>
<td>84</td>
<td>80</td>
</tr>
<tr>
<td>AML no. 2</td>
<td>78</td>
<td>75</td>
<td>69</td>
</tr>
</tbody>
</table>

Results are expressed as the percentage of bcl-2-positive cells. Cells were cultured without (C) or with sense (S), nonsense (NS), or antisense (AS) bcl-2 oligodeoxynucleotides. Each result is the mean of three different sets of experiments, except for AML no. 1 and AML no. 2.

Abbreviation: ND, not done (no viable cells).

Cell cultures. Cells were cultured in serum-free conditions in 96-well flat-bottomed microtiter plates (Falcon; Becton Dickinson Labware, Lincoln Park, NJ). A total of 5 x 10^4 viable cells (leukemic lines) or 1.5 x 10^5 cells (AML and CD34* cells) were placed into wells containing 200 μL of RPMI-1640 medium supplemented with 2 mmol/L glutamine, 50 U/mL of penicillin, 0.1 mg/mL of streptomycin, and 15% QSBF-51 concentrated supplement (TechGen International, Les Ullis, France). Cells were then incubated at 37°C in a fully humidified atmosphere with 5% CO2 for the appropriate time (8 to 15 days). On day 0, 0 μmol/L of oligodeoxynucleotides was added in cultures. On day 6, 5 μmol/L of the same oligomer was added.

In some experiments, daunorubicin (DNR) or L-β-D-arabinofuranosyl-cytosine ( Ara-C) were added on day 0 at respective concentrations of 0.05 to 5 μg/mL and 0.4 to 20 μg/mL, respectively.

All experiments were performed in duplicate or triplicate (according to the number of cells available).

Cell viability and progenitor assays. At intervals depending on the experiment, cells were resuspended and removed from each well for the analysis of number and viability, clonogenic properties, and antigen expression. Cell counts were performed on days 2, 4, 6, 8, 11, and 15 in the presence of trypan blue dye to determine the concentration of viable cells.

Normal (colony-forming unit granulocyte-macrophage [CFU-GM] and CFU-granulocyte, erythroid, monocyte, megakaryocyte [CFU-GEMM]) and leukemic (CFU-leukemia [CFU-L]) progenitor assays were performed on days 0, 4 (when cultures were treated with mitotoxic drugs), 6, and 11 as follows. Cells were washed three times with RPMI medium, resuspended at a concentration of 2.5 x 10^4 viable cells/mL in 200 μL Iscove's modified Dulbecco's medium supplemented with 0.8% methylcellulose (Sigma Chemical Co, St Louis, MO) and 30% fetal calf serum (GIBCO, Paisley, UK). 5637 bladder carcinoma cell line supernatant (HTB9) at a final concentration of 12% (for CFU-GEMM assay) 2 μU/mL of erythropoietin (Terry Fox Laboratories, Vancouver, Canada) were added as growth factors. Cells were plated onto 96-well flat-bottomed plates. Colonies (more than 50 cells) were counted on days 7 (CFU-L) and 14 (CFU-GM and CFU-GEMM).

Immunofluorescence assays. Bcl-2 staining was described in details elsewhere. Briefly, cells were washed, permeabilized for 30 minutes in phosphate-buffered saline (PBS) with 70% ethanol, washed twice, and resuspended in PBS with 1% human AB serum. Cells were then incubated for 30 minutes at 4°C with anti-bcl-2 monoclonal antibody (MoAb; Dakopatts, Glostrup, Denmark). After two washings in PBS, fluorescein-conjugated goat antimouse Fab', fragments (Biocart, Meudon, France) were added for 30 minutes at 4°C. Negative controls were performed by incubating cells with an irrelevant antibody of the same isotype (Immunotech, Marsille, France) instead of anti-bcl-2 MoAb. Fluorescence analysis was immediately performed with a FacsStar Plus cytometer (Becton Dickinson, Mountain View, CA). Fluorescence-activated cell sorter (FACS)
results included the percentage of bcl-2-positive cells and the fluorescence intensity (mean equivalent of soluble fluorescence [MESF]) in positive cells calculated by comparison to calibrated beads (QuickCal; Flow Cytometry Standard Corp, San Juan, CA).

In addition, two surface markers were studied by indirect immunofluorescence: CD34 (QBEND 10; Immunotech) and CD15 (CD15; Dakopatts).

Two-color analysis of bcl-2 and CD15 expression was performed in HL-60 cells. For this technique, cells were first incubated for 30 minutes at 4°C with a fluorescein-conjugated CD15 MoAb (Dako- patt), washed twice, and then incubated with the bcl-2 MoAb (Dakopatts) after permeabilization in 0.25% paraformaldehyde (for 15 minutes at room temperature) and 70% methanol (for 1 hour at 4°C). Phycoerythrin-conjugated antimouse F(ab')2 fragments were used as second layer.

RESULTS

Oligonucleotide concentration. These experiments were performed with HL-60 cells. As shown in Fig 1, antisense oligonucleotide decreased the number of viable cells in a concentration-dependent manner. The maximal effect was observed at day 11 (30% cell survival at a concentration of 50 μmol/L). Sense or nonsense oligonucleotides had no significant toxic effect on day 11. The addition of 10% of the initial dose on day 6 resulted in more efficient inhibition with no remaining cell on day 11 at an initial dose of 50 μmol/L. This addition did not significantly increase sense or nonsense oligonucleotide toxicity. Therefore, an initial dose of 50 μmol/L with an additional dose of 5 μmol/L on day 6 was used throughout the study.

Bcl-2 and surface marker expression. The initial percentages of bcl-2-positive cells were 75% for CD34+ cells, 98% for KG1a cells, 92% for HL-60 cells, and 3% for Daudi cells (negative control). In the three leukemia samples studied, these percentages were, respectively, 89%, 78%, and 8%. These values decreased moderately throughout the culture in controls. Treatment by sense or nonsense oligomers

Fig 2. Flow cytometry diagram of HL-60 staining at day 0 ([A] control; [B] anti-bcl-2 antibody) and day 6 (C) in the presence of bcl-2 antisense oligonucleotides. The cursor indicates positive cells. The percentage of positive cells and fluorescence intensity are those indicated in Table 1.

Fig 3. Double-color cytometry analysis of HL-60 staining by CD15 (horizontal) and bcl-2 (vertical) on day 0 and on day 6 of culture in the presence of bcl-2 antisense oligonucleotides.
did not modify the expression of bcl-2 when compared with controls. By contrast, the percentage of bcl-2-positive cells decreased dramatically by day 6 and we observed an almost complete disappearance of bcl-2-positive cells by day 11. Detailed results are presented in Table 1.

A quantification of mean fluorescence intensity in bcl-2-positive cell lines showed a decrease correlated to that of the percentage of bcl-2-positive cells. The calculated MESF values for HL-60 and KG1a are indicated in Table 2. An example of flow cytometry analysis is presented in Fig 2.

The evolution of CD34 expression was studied in a KG1a line, in CD34-sorted cells, and in the two CD34+ AML samples (AML no. 1 and AML no. 2). Initially, the respective percentages of CD34+ cells were 97%, 96%, 60%, and 78%. At day 11, these percentages remained similar (89%, 80%, 48%, and 68%, respectively) in sense-treated cultures, but were significantly decreased (29%, 25%, 37%, and 26%) in antisense-treated samples. CD15 expression was not modified by culture conditions. A representative diagram of CD15 versus bcl-2 staining of HL-60 cells before and after antisense treatment is provided in Fig 3.

Cell survival in liquid culture. Cells were cultured for 15 days or until death with or without sense or antisense oligodeoxynucleotides. Sense oligonucleotides had little effect on cell survival when compared with controls. However, the number of cells remaining at day 15 was 10% to 25% lower (median, 16%) in the sense-treated cultures than in controls. By contrast the number of viable cells in antisense-treated cultures was significantly decreased in cell samples that were initially bcl-2-positive (CD34+ cells, KG1a and HL60 lines, and 2 AML samples). The effect of antisense treatment was really noticeable at day 6 or 8, and no viable cells remained by day 15 except for CD34+ cells (22% of initial number). The bcl-2-negative line (Daudi) and AML sample (AML no. 3) were not affected by antisense treatment. Detailed results are presented in Fig 4 and 5.

The survival of clonogenic cells in liquid culture is presented in Table 3. At day 6 of liquid culture, the number of KG1a colonies and CFU-L from bcl-2-positive AML cases decreased in the presence of antisense oligomers, whereas it remained stable until day 11 in the cultures treated with sense oligomers. The number of CFU-L in the bcl-2-negative leukemia was initially high but rapidly decreased in the sense- as well as in the antisense-treated cultures.

Sensitivity to cytostatic drugs. AML cells were treated with two cytostatic drugs, DNR and Ara-C. The optimal concentration of each drug for this study was determined to decrease the number of day-8 cells in culture by approximately 50% (data not presented). These concentrations were 0.5 μg/mL for DNR and 4 μg/mL for Ara-C. Figure 5 shows that the cytotoxic effect of both drugs was more effective in antisense-treated cultures.

The sensitivity of CFU-L to chemotherapy was also highly increased in antisense-treated cultures, as shown in Table 4.

**DISCUSSION**

The BCL-2 gene encodes a membrane protein located in the inner mitochondrial membrane and probably in endoplasmic reticulum and perinuclear membrane. Although initially discovered in the lymphoid lineage, Bcl-2 protein can be detected in hematopoietic cells and myeloid leukemia cells. In this study, we investigated its functional role by blocking its expression. The possibility of using antisense oligomers as inhibitors of oncoproteins implicated in cell proliferation such as c-myc and c-myc has already been documented in hematopoietic cell lines and in chronic myeloid leukemia cells. We used here phosphorothioate oligodeoxynucleotides according to the results obtained by Reed et al in a bcl-2-transfected lymphoid line. Our data show that the effects of antisense oligomers could be observed at days 4 to 6, and that bcl-2 protein was no longer detectable by day 11. The delay in protein-expression decrease may be explained by the use of phosphorothioate rather than normal phosphodiester oligomers, because phosphorothioate oligomers attain their highest intracellular concentration after 4 or 5 days. This delay may also be explained by a long protein half-life, as can be observed in resting lymphocytes in which bcl-2 protein can be detected in the absence of RNA.

The decrease of bcl-2 protein content in antisense-treated cultures was followed by rapid cell death, whereas sense-treated cells survived for more than 15 days despite of the absence of growth factors. A limited toxicity of sense oligomers was observed, as already reported when phosphorothioate oligonucleotides are used. The effect on cell survival
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was observed for all cell types studied, except for the Daudi line and for the bcl-2-negative leukemia sample. In this last case, cell survival was short regardless of oligonucleotide treatment and similar to that of antisense-treated bcl-2-positive leukemia cells. On the contrary, Daudi cells could be maintained in the same culture conditions until day 15, which indicates that other survival mechanisms were implicated or that this line was not involved in programmed cell death.

Table 3. Effects of Oligodeoxynucleotide Treatment on Clonogenic Cell Number in Liquid Culture

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Day 0</th>
<th>Day 6</th>
<th>Day 11</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>S</td>
<td>AS</td>
</tr>
<tr>
<td>KG1a</td>
<td>128</td>
<td>132</td>
<td>118</td>
</tr>
<tr>
<td>AML no. 1</td>
<td>32</td>
<td>62</td>
<td>26</td>
</tr>
<tr>
<td>AML no. 2</td>
<td>42</td>
<td>62</td>
<td>51</td>
</tr>
<tr>
<td>AML no. 3</td>
<td>176</td>
<td>176</td>
<td>34</td>
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<tr>
<td>Day-14 CFU-GM</td>
<td>46</td>
<td>46</td>
<td>40</td>
</tr>
<tr>
<td>CFU-GEMM</td>
<td>10</td>
<td>10</td>
<td>8</td>
</tr>
</tbody>
</table>

Values are the number of colonies/10^6 cells grown in methylcellulose. Cells were plated after 0, 6, or 11 days of culture without (C) or with sense (S) or antisense (AS) BCL-2 oligodeoxynucleotides. Abbreviation: ND, not done (no viable cells).

Table 4. Effects of Oligodeoxynucleotides on Leukemic Clonogenic Cells After Exposure to DNR and Ara-C

<table>
<thead>
<tr>
<th>Type of Culture</th>
<th>AML No. 1</th>
<th>AML No. 2</th>
<th>AML No. 3</th>
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<tbody>
<tr>
<td>Control day 0</td>
<td>32</td>
<td>62</td>
<td>176</td>
</tr>
<tr>
<td>Day 4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>25</td>
<td>53</td>
<td>89</td>
</tr>
<tr>
<td>AS</td>
<td>5</td>
<td>8</td>
<td>79</td>
</tr>
<tr>
<td>DNR + S</td>
<td>2</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>DNR + AS</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ara-C + S</td>
<td>10</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>Ara-C + AS</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are the mean number ± SD of colonies/10^6 viable cells grown in methylcellulose. Each experiment was performed in triplicate. Cells were plated after 4 days of culture in the presence of DNR or Ara-C and sense (S) or antisense (AS) BCL-2 oligodeoxynucleotides.

The mechanism by which bcl-2 protein interferes with programmed cell death is not known. It has been recently demonstrated that it is implicated in oxidative metabolisms. Immunolocalization studies have shown that bcl-2 is expressed in nonlymphoid cells with long-term survival such as neurons and in tissues characterized by complex proliferation and differentiation processes such as skin and bone marrow. Our experiments show that the decrease of bcl-2 expression in cultured cells was associated with a decreased number of clonogenic cells and with cell maturation, as indicated by the loss of stem-cell marker CD34. This finding might argue that bcl-2 is necessary to maintain hematopoietic cells of the proliferating compartment possibly to facilitate the action of other mechanisms directly implicated in cell proliferation. Indeed, experiments with doubly transgenic mice have suggested that bcl-2 could cooperate with other oncogenes controlling proliferation such as c-myc. However, our data are in contradiction with the report by Veis et al. that bcl-2–deficient mice exhibit normal red blood cells and neutrophil counts and normal marrow cellularity, whereas lymphoid tissues are subject to rapid apoptosis after birth. It is likely that in vivo conditions can provide other mechanisms of hematopoietic cell survival. It is also possible that human hematopoiesis is more dependent on BCL-2 expression, or that the role of BCL-2 is more important in adult tissues.

Many drugs commonly used in the treatment of AML have been reported to induce DNA fragmentation and apoptotic cell death. Overexpression of BCL-2 gene in murine or human cell lines confers a high level of resistance to a variety of chemotherapeutic agents. Moreover, the clinical course of patients with lymphomas or acute leukemia seems to be worse when malignant cells express high levels of bcl-2. In this study, we examined the effects of two major antileukemic drugs on fresh leukemia cells. The inhibition of bcl-2 expression resulted in increased susceptibility of the two bcl-2–positive AML samples. Although the mechanism by which bcl-2 protects cells from chemotherapy damage is not known, our results confirm its implication in drug resistance of AML cells.
REFERENCES


9. Miyashita T, Reed JC: Bcl-2 gene transfer increases relative resistance of $S_{49}$.1 and WEHI17.2 lymphoid cells to cell death and DNA fragmentation induced by glucocorticosteroids and multiple chemotherapeutic drugs. Cancer Res 52:5407, 1992


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