bcl-2 Proto-oncogene Expression in Normal and Neoplastic Human Myeloid Cells

By Domenico Delia, Antonella Aiello, Davide Soligo, Enrico Fontanella, Cecilia Malani, Francesco Pezzella, Marco A. Pierotti, and Giuseppe Della Porta

The present study provides immunobiochemical and molecular data on the differentiation-linked expression of the bcl-2 proto-oncogene in normal and neoplastic myeloid cells. Using a recently developed monoclonal antibody (MoAb) to the bcl-2 molecule, staining of normal bone marrow myeloblasts, promyelocytes, and myelocytes, but neither monocytes nor most polymorphonuclear cells, was demonstrated. By two-color flow cytometric analysis, bcl-2 was evidenced in CD3^+ and CD3^-CD4^+ myeloid cells as well as in the more primitive CD3^-CD4^+ population. The leukemic cell lines HL-60, KG1, GM-1, and K562 were bcl-2 positive together with 11 of 14 acute myeloid leukemias (AML) and three of three chronic myeloid leukemias (CML) in blast crises; six of seven CML were negative. Among myelodysplastic cases, augmentation of the bcl-2 positive myeloblastic compartment was found in refractory anemia with excess of blasts (RAEB) and in transformation (RAEB-t). Western blots of myeloid leukemias and control lymphocytes extracts evidenced an anti–bcl-2 immunoreactive band of the expected size (26 Kd). Moreover, the HL-60 and KG1 cell lines, both positive for the bcl-2 protein, exhibited the appropriate size bcl-2 mRNA (7.5 Kb). These findings clearly indicate that the bcl-2 gene is operative in myeloid cells and that the anti–bcl-2 MoAb identifies its product and not a cross-reactive epitope. Induction of HL-60 differentiation toward the monocytic and granulocytic pathways was accompanied by a marked decrease in bcl-2 mRNA and protein levels; bivariate flow cytometric analysis showed that the fraction becoming bcl-2 negative was in the G1 phase of the cell cycle. These data establish that the bcl-2 proto-oncogene is expressed on myeloid cells and their progenitors and is regulated in a differentiation-linked manner.

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Fig 2. Two-color flow cytometric analysis of bcl-2 protein in CD33+ and CD34+ populations. The analyses were performed on paraformaldehyde-fixed/Triton-treated cells labeled with the specified MoAbs and with iso-type-specific, FITC- and PE-conjugated second antibodies. For each fluorescence histogram (log scale), the horizontal and vertical markers (set up on negative controls) separate the reactive from unreactive cells. Analysis on bone marrow (A) and on blood cells of an rhGM-CSF-treated patient (B) are shown. The size distribution (forward vs 90° light scattering) of the latter sample is depicted in top left histogram together with bitmaps 1 and 2, which encompass the small- and large-size cells, respectively; in this specimen most of CD34+ cells were found in bitmap 1.

Flow controls separate the reactive from treated cells labeled with the latter sample is depicted in top left histogram together with bitmaps 1 and 2, which encompass the small- and large-size cells, respectively; in this specimen most of CD34+ cells were found in bitmap 1.

blood, and spleen suspensions were enriched in mononuclear cells by centrifugation (30 minutes at 400g) on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). Erythrocyte-depleted bone marrows were prepared by treatment with an erythrocyte lysis solution (Coulter Electronics, Hialeah, FL). Granulocytic and monocytic differentiation of HL-60 was achieved by culturing the cell line in the presence of 1.2% dimethyl sulfoxide (DMSO) (Sigma Chemical Co, St Louis, MO) and 10 ng/mL of 12-O-tetradecanoyl phorbol-13-acetate (TPA) (Sigma), respectively. DMSO was added every 72 hours.

MoAbs, cell permeabilization, immunostaining procedures, and flow cytometric analysis. The mouse MoAb to bcl-2 protein used in this study (clone 124, IgG2a isotype) has been recently described7 and proven suitable for immunostaining of cryostat and paraffin-embedded tissue sections and for Western blotting analysis; it was raised against a synthetic peptide corresponding to amino acids 41 to 54 of the bcl-2 protein.

MoAbs to CD33 (MY9, IgG2b isotype)9 and CD34 (QBEND10, IgG1)7 were obtained from Coulter Immunology (Hialeah, FL) and N. Bradley (Quantum Biosystems, Cambridge, UK), respectively. The MoAbs specific for HLA-DR (MA6, IgM), CD11b (clone 44, IgG1), and CD11c (clone 3.9, IgG1)9 were obtained from the Fourth International Workshop on Human Leucocyte Antigens.

The fixation/permeation protocol for optimal immunodetection of bcl-2 internal molecule will be described in detail elsewhere. Briefly, a pellet of 10^8 cells was resuspended in 1 mL of 2% paraformaldehyde in phosphate-buffered saline (PBS) and kept for 10 minutes at 4°C; 100 μL of 0.05% Triton X100 (Sigma) in PBS was then added and 10 minutes later the cells were washed twice in PBS plus 1% bovine serum albumin (BSA) and used for labeling. Single- and two-color indirect immunofluorescence (IF) staining of fixed or viable cells was performed at 4°C on 96-well microtiter plates as described20; the cells were precultivated for 10 minutes with 1% human heat-inactivated AB-serum to prevent binding of the antibodies to Fc receptors.

For the single-color IF the cells were incubated (30 minutes) with saturating doses of the specified MoAb, washed four times in RPMI-C, and incubated again (30 minutes) with a 1:3 dilution of fluorescein (FITC)- or phycoerythrin (PE)-conjugated antiserum Ig antibodies purchased from Technogenetics (Trezzano SN, Italy; cat. 3900-2) and Southern Biotechnology Associates Inc (Birmingham, AL; cat. 1010-09), respectively.

For the two-color IF, combinations of primary MoAbs (or irrelevant mouse Igs for the negative controls) of different isotypes were simultaneously added to the cells; their binding was shown by FITC- and PE-conjugated goat antibodies specific for the mouse IgG1 isotype (cat. 1070-02; 1070-09), IgG2a (cat. 1080-02; 1080-09), IgG2b (cat. 1090-01; 1090-09), or IgM (1020-02; 1020-09) purchased from Southern Biotechnology.

Immunocytochemical staining of air-dried paraformaldehyde-fixed cytospin preparations was performed by the alkaline-antialkaline phosphatase (AAPAP) technique using a commercial kit (Dakopatts, Glostrup, Denmark); three AAPAP repeats were performed.
allowed optimal amplification of the reaction. Sections from paraffin-embedded bone marrow specimens were deparaffinized in xylene and graded ethanols, incubated with PBS containing 5% decomplemented human AB serum, and immunostained by the APAAP method; normal mouse serum and an MoAb specific for the leukocyte common antigen CD45 (Dako) were used as negative and positive controls of the APAAP reaction, respectively. Anti-bcl-2 reactive cells were quantified at 400× magnification by means of an ocular grid in more than 20 randomly selected fields (equivalent to 2 mm² of tissue).

Double-color staining for bcl-2 (green) and DNA (red) was performed on cells that had previously been paraformaldehyde-fixed/Triton-permeabilized and indirectly labeled for bcl-2 using an FITC-tagged secondary antibody; the cells were then incubated (30 minutes at room temperature) with 1 mg/mL RNAase A (Sigma) in PBS and finally in 1 mL of 10 μg/mL propidium iodide (Sigma) in PBS.

Flow cytometric analysis of single- and two-color stained samples was performed on an EPICS-C instrument (Coulter Electronics, Hialeah, FL) equipped with a 5-W argon-ion laser set at 488 nm. Bcl-2 reactive cells were quantified at 400× magnification by means of an ocular grid in more than 20 randomly selected fields (equivalent to 2 mm² of tissue).

**RESULTS**

**Detection of bcl-2 protein in myeloid cells.** bcl-2 positive myeloid cells were found in normal bone marrows as shown by the immunostaining of cytospin preparations (Fig 1); 70% of myeloblasts, 83% of promyelocytes, 40% of myelocytes, 12.5% of metamyelocytes, 12.5% of polymorphonuclear cells, and 60% of monoblasts were bcl-2+; whereas monocytes were totally negative. Although the localization of the protein was prevalently cytoplasmic, some perinuclear staining was observed.

The immunofluorescence flow cytometric analysis of bcl-2 in normal bone marrows evidenced 18% ± 5.3% of labeled cells on erythrocyte-depleted samples; however, in Ficoll-separated samples (and thus depleted of most of the granulocytes) 78% ± 8.6% were found positive. In the latter samples the CD33+ myeloid cells were 28.5% ± 21.2% and the CD34+ cells 5.3% ± 3.5%. Dual-color IF analysis showed that 89.5% ± 3.6% of the CD33+ and 75.7% ± 21.2% of the CD34+ fractions were bcl-2 positive (Fig 2A). Coexpression of bcl-2 and CD34 was also found in peripheral blood samples from patients treated with recombinant human (rh) GM-CSF and presenting elevated numbers of leukemic blasts.
bcl-2 IN MYELOID CELLS

Fig 5. bcl-2 mRNA in KG1 and HL-60 cells. Equal amounts of poly-A enriched mRNA (5 µg/lane) were size-fractionated in agarose, blotted onto nylon filter, hybridized with 32P-labeled DNA probe pFL1 specific for bcl-2, and exposed to autoradiographic film with intensifying screen. Poly-A RNA from the B-lymphoblastoid cell line Raji and the colon carcinoma HT29 were included as positive and negative controls, respectively. Exposure times were 60 hours (A) and 9 days (B).

The myeloid leukemic cell lines HL-60, KG1, GM1, and K562 were found, by IF and immunocytochemistry, positive for bcl-2; the mean fluorescence staining intensity values indicated KG1 as having the highest amount of protein per cell (not shown).

Western and Northern blot analysis. To verify that, on myeloid cells, the binding of bcl-2 MoAb was to the bcl-2 gene product rather than to a cross-reactive epitope, further studies at protein and mRNA level were performed.

On Western blots of myeloid leukemia lysates the anti-bcl-2 MoAb identified a protein of identical size (26 Kd) to that of spleen lymphocyte lysates (used here as positive control) and corresponding to bcl-2 (Fig 4). The 26-Kd protein was also demonstrated in the early myelo/erythroblastic cell line K562; this finding, which suggests that the bcl-2 gene may be expressed by erythroid progenitors, is supported by the reactivity of the anti-bcl-2 MoAb.

<table>
<thead>
<tr>
<th>Tab. 2. bcl-2 Protein Modulation in Chemically Induced HL-60 Cell Maturation</th>
<th>Days of Treatment</th>
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<tbody>
<tr>
<td></td>
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<tr>
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<tr>
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<tr>
<td>i</td>
<td>118</td>
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<tr>
<td>CG</td>
<td>1.5</td>
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<td>IN</td>
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For each specified marker, the percentages of positive cells after background subtraction are shown. In addition, the mean fluorescence intensity of bcl-2 positive cells, measured on a log scale, is indicated.

*Morphologic analysis of DMSO-treated cells was performed on May-Grünwald/Giemsa stained cytospin preparations; the percentage of cells bearing cytoplasmic granules (CG) and indented nuclei (IN) is reported.
with normal bone marrow erythroblasts (see Fig 1). Differences in the intensity of the bands indicated that each cell line presented variable amounts of bcl-2 protein that were, among the myeloid leukemias, highest in KG1, albeit lower than in spleen lymphocytes.

Results of Northern blot hybridizations are shown in Fig 5. The bcl-2 specific probe pFL1 detected a major mRNA transcript in HL-60 and KG1 of similar size (approximately 7.5 kb) to that found in the B-lymphoblastoid cell line Raji. Strikingly, the KG1 cell line expressed at least 50-fold more mRNA than Raji and this difference did not correspond to a similar augmentation in protein level.

Altogether, the concordance between the immunobiochemical and molecular results indicates that myeloid cells express the bcl-2 proto-oncogene, whose product is recognized by the anti-bcl-2 MoAb.

*bcl-2 on in vitro differentiating cells.* The pattern of bcl-2 staining in normal bone marrow (BM) myeloid cells is in accord with a differentiation-regulated expression of the bcl-2 gene. The bcl-2 positive promyelocytic cell line HL-60, which can be induced to differentiate along either the granulocytic or monocytic pathway when treated in vitro with specific chemicals, provides a suitable model to study this relationship.

Therefore, we have cultured the HL-60 cells in the presence of 1.25% DMSO and 10 ng/mL TPA and monitored at various intervals of time the levels of bcl-2 protein, in relation to other differentiation markers, morphology, and proliferation.

The results are listed in Table 2. It can be seen that the DMSO-induced granulocytic maturation was accompanied by a progressive decrease in bcl-2 positivity, from greater than 95% in the untreated control to 65% by day 8 (Fig 6). Similar downregulation of the 26-Kd protein was confirmed by Western blot analysis (Fig 7). Parallel morphologic changes (e.g., increased numbers of cytoplasmic granules and indented nuclei) as well as reduction of the proliferative rate (Fig 6) were observed. As expected, CD11b and CD11c became detectable on a small fraction of cells. Dual-color flow cytometric analysis (Fig 6) showed that most of the bcl-2 negative cells were in the G1 phase of the cell cycle. These cells were, in addition, characterized by a granulocytic morphology (Fig 8).

The TPA-triggered monocytic/macrophagic differentiation resulted in a rapid decrease in bcl-2 expression (Fig 6); within 24 hours the bcl-2 positivity dropped to 55% and by day 5 to about 30%. These changes were also observed on Western blots (Fig 7). The TPA-induced maturation was evidenced by the strong upregulation of CD11b and CD11c molecules. The majority of bcl-2 negative cells were in the G1 phase of the cell cycle.

Downregulation of bcl-2 gene was detected on Northern blots (Fig 5); the very faint bands present at day 2 became undetectable by day 5, despite measurable amounts of protein. This finding may actually suggest that the protein has a long half-life.

**Fig 6.** Relationship between bcl-2 and cell cycle in differentiating HL-60 cells. The left and middle histograms were obtained from the flow cytometric analysis of single-color labeled samples, whereas those on the right were from the analysis of bcl-2 (ordinate, green)/DNA (abscissa, red) double-labeled samples; the horizontal window, set up on a negative control, defines the threshold between bcl-2 reactive and unreactive cells.

**DISCUSSION**

The bcl-2 proto-oncogene, rearranged and deregulated in B-cell lymphomas carrying the t(14;18) translocation, is expressed in normal and neoplastic cells of the lymphoid lineage negative for the t(14;18) chromosomal abnormality, and the levels of transcription appear to correlate
with proliferation and differentiation stage. This relationship has been recently further elucidated by immunohistochemical studies of lymphoid tissues using a newly developed MoAb to bcl-2 gene product, showing strong levels of bcl-2 protein in many normal blood T and B lymphocytes, mantle-zone but not germinal center B cells, in medullary but not cortical thymocytes, and undetectable levels in proliferating lymphoid cells.

Although studies regarding the involvement of bcl-2 in human hematopoietic cells have been mostly limited to the lymphoid lineage, bcl-2 is not lineage restricted; indeed, the presence of minimal amounts of transcripts in a monoblastic cell line and the recent immunodetection of the protein in normal myeloid cells suggest that this protooncogene may also play a role in myeloid cell differentiation.

In this report we have provided novel data on the expression of bcl-2 in normal and malignant cells of the myeloid lineage. We based this evidence partly on analysis using an anti-bcl-2 MoAb whose specificity for the 26-Kd protein was confirmed by Western blot of a variety of myeloblastic cell lines.

To perform quantitative and multiparameter flow cytometric measurements of bcl-2 on single cells, we developed an appropriate fixation/permeabilization procedure for the detection of the protein, localized primarily in the inner mitochondrial and, to a lesser extent, on the perinuclear membrane.

The bcl-2 protein levels among normal myeloid cells are inversely related to maturation; thus, a large fraction of myeloblasts and promyelocytes are bcl-2+ whereas metamyelocytes and polymorphonuclear cells are mostly bcl-2 negative, and monocytes totally negative. These findings have been substantiated by two-color flow cytometric analysis showing a large percentage of normal bone marrow bcl-2+ cells expressing CD33, a marker for myeloid cells as well as CD34, a marker for myeloid progenitor cells including stem cells. Coexpression of bcl-2 and CD34 molecules has been further documented on circulating CD34+ hematopoietic progenitor cells present in blood samples of rhGM-CSF-treated patients. In particular, the bcl-2 protein has been found on CD34+/CD33+/HLA-DR+ cells characterized by low right angle and low forward light scattering properties; these features are consistent with a progenitor of most of the colony-forming cells in long-term marrow cultures. However, further work is needed to determine whether the hematopoietic stem cell is bcl-2+. In view of the fact that bcl-2 protein confers stress resistance to heat shock, ethanol, methotrexate and serum deprivation, and survival, it is attractive to hypothesize that bcl-2 on early hematopoietic progenitor cells, eg, CD33+/CD34+ (stem cells), favors their long-lived capacity and confers resistance to various external insults.

Overall, the pattern of bcl-2 distribution in normal bone marrow myeloid cells appears concordant with the in vitro findings on HL-60, whose differentiation toward the granulocytic and monocytic pathways results in a downregulation of bcl-2 transcription and protein levels. Correlations with cell growth and morphology have shown that the bcl-2 negative cells are mostly in G1 phase of the cell cycle and present features of mature cells.

We have assessed the expression of bcl-2 molecule on fresh myeloproliferative disorders; given that the anti-bcl-2 MoAb reacts with paraffin-embedded tissues, the study was extended to bone marrow biopsies. The results have shown that greater than 70% of AML are bcl-2 positive, whereas most CML are bcl-2 negative.

Increased numbers of bcl-2 positive blasts have been found in myelodysplastic syndromes. No clear-cut correlation between FAB classification and bcl-2 positivity was observed. Overall, it appears that the expression of bcl-2 in myeloid disorders reflects that found in their normal cell counterparts.

The expression of bcl-2 in myeloid leukemias raises the question of its potential prognostic value. In fact, because many anticancer agents also operate through the activation of programmed cell death and bcl-2 levels are positively associated with resistance to apoptosis, the possibility exists that bcl-2 positive leukemias are less responsive to chemotherapy than those that are negative. This speculation is now open to investigation.

In conclusion, the data presented here provide the basis for future studies on the role of bcl-2 in myeloid cell commitment, maturation, and survival.

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