original paper
Haematologica 1995; 80:98-102

BCL-2 ONCOPROTEIN EXPRESSION IN ACUTE MYELOID LEUKEMIA

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ABSTRACT

Background. **In lymphoproliferative diseases the expression of Bcl-2, a mitochondrial oncoprotein capable of blocking apoptosis, is well-documented, while little research has been carried out on its distribution in myeloproliferative conditions.**

Methods. **Using immunocytochemical methods, 63 cases of acute myeloid leukemia (AML) at onset and 10 relapses were studied to investigate Bcl-2 expression and any possible correlations with subtypes of the FAB classification, sex, age or white cell peripheral blood count at onset.**

Results. **Bcl-2 is present in 87.3% of AML cases at onset and in 100% of relapses. In 68.3% of cases at onset and in 90% of relapses the protein is present in more than 20% of the blasts. Relapses always show higher percentages of positive expression than those seen at onset. Our results demonstrate no statistical correlations between the expression of the oncoprotein Bcl-2 and FAB subtypes, sex, age, or white cell peripheral blood count.**

Conclusions. **The majority of blasts from AML patients express the oncoprotein Bcl-2, which is able to protect leukemic cells from apoptosis. Since numerous chemotherapies are cytotoxic in that they induce apoptosis, we feel that *in vitro* studies of cells from AML patients are necessary in order to broaden our knowledge about the effects of the most common therapeutic drugs and of those substances which, alone or in association, can modulate Bcl-2 expression.**

Key words: Bcl-2, immunocytochemistry, apoptosis, acute myeloid leukemia, FAB subtypes

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Bcl-2 is an oncoprotein codified by the bcl-2 gene located on chromosome 18.[1-4] Studies of subcellular fractions and with confocal microscopy have indicated that Bcl-2 is an integral protein of the mitochondrial membrane.[5] This oncoprotein has also been identified on other membranes such as the endoplasmic reticulum and the nuclear envelope.[6-9] Bcl-2 was discovered in 1985 as a result of the involvement of the bcl-2 gene in the most common t(14;18) translocation found in follicular B lymphomas.[1-4] This translocation juxtaposes the bcl-2 gene, located in the 18q21 region, to the gene of the heavy chains of the immunoglobulin, in region 14q32, causing a deregulation of bcl-2 transcription.

In 1990, Pezzella et al.[10] showed that the expression of the oncoprotein Bcl-2 is not specific to lymphoproliferative diseases with t(14;18), but is also characteristic of some B and T lymphocytes in the peripheral blood. Other authors have reported the presence of Bcl-2 in a wide range of other normal human tissues.[11] In particular, Bcl-2 is expressed in 70% of the myeloblasts, 83% of the promyelocytes, 40% of the myelocytes and 12% of the polymorphonucleates in normal human bone marrow and peripheral blood. Monoblasts are Bcl-2 positive, while monocytes are Bcl-2 negative. Bi-parametric analysis of the immunophenotype of mononucleates in normal bone marrow has shown that Bcl-2 is expressed by 90% of CD33$^+$ and 76% of CD34$^+$ cells.[12] Bcl-2 is expressed by normoblasts, nucleated erythroblasts and megakaryocytes, but not by reticulocytes and platelets.[11] Little attention has been paid in the

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*Acknowledgements: the authors are grateful to Mr. Mike Hammersley for his linguistic revision of the text. *LB is the recipient of a grant from Lega Italiana per la Lotta contro i Tumori.*
Received September 22, 1994; accepted January 11, 1995.

past to its expression in myeloproliferative conditions, in particular acute myeloid leukemia (AML). The aim of the present study is to investigate Bcl-2 expression in a significant population of AML cases at onset and in relapse, and to identify any possible correlations with subtypes of the French American British (FAB) classification, sex, age and white cell peripheral blood count at onset.

Materials and Methods

Patients

The study was carried out on 63 patients, 26 males and 37 females, average age 51.8 years (range: 15-89), with AML at onset or in relapse seen in our clinic between January 1990 and January 1994. Diagnosis was made on peripheral and bone marrow blood smears using standard cytological and cytochemical procedures. Surface markers were analyzed and the results classified according to the FAB criteria.[13-15] Bcl-2 expression in the peripheral and bone marrow blood of 5 healthy volunteers was also assessed as a control.

Immunological reagents

The expression of the Bcl-2 oncoprotein was measured by using the anti-Bcl-2 clone-124 monoclonal antibody (IgG1k against amino acids 41-54 of the oncoprotein) (Dakopatt, Glostrup, Denmark). CD45 (Dako) and control IgG1a (Immunotech, Marseilles, France) were used as positive and negative controls, respectively. Rabbit anti-mouse immunoglobulin (Dako) was employed as the secondary antibody, and the reaction was revealed by APAAP (Dako).

Cells

Samples of peripheral and bone marrow blood from normal subjects (allogeneic bone marrow transplant donors) and peripheral blood from AML patients at onset and in relapse were examined. The mononucleated cells in the samples were enriched by centrifugation (400 g, 30 min) on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), and cytocentrifugates were prepared. At least 90% of the sample consisted of leukemic blasts in all AML cases.

Fixation, conservation and immunocytochemical methods

Cytospins were air-dried for 24 hours, fixed in acetone (+4°C, 5 min) and stored in the dark at –20°C. One hour prior to the immunocytochemical reaction, samples were thawed and fixed again in acetone (+4°C, 5 min). Immunocytochemistry was performed using the APAAP method, as originally described by Cordell and Falini.[16] Staining was amplified by repeating APAAP three times. Samples were counterstained with Gill's hematoxylin. There was no variation in Bcl-2 expression when freshly prepared or cryopreserved samples were utilized.

Sample analysis

Samples were assessed under an optical microscope (Standard 25, Zeiss, Germany) at a magnification of 400× in immersion. At least 500 cells in randomly chosen fields were examined for each normal and leukemic sample. In addition to calculating the percentage of positive blasts, cytoplasmic staining was also recorded as either diffuse or perinuclear. The data obtained were arbitrarily grouped in three classes of positivity: $< 20\%$, between 20 and 70%, and $> 70\%$.

Statistical analysis

Differences between Bcl-2$^+$ (%) positivity class and FAB subtype or sex were assessed using the chi-square test (χ^2 test). The relationship between Bcl-2$^+$ (%) (as a percentage of the cells) and leukocytes or age was determined by regression analysis. Differences between average Bcl-2$^+$ (%) values in the various FAB subtypes were evaluated by variance analysis after the data were normalized and the variance stabilized with the transformation arcsine. Finally, the t-test was used to evaluate differences between average Bcl-2$^+$ (%), leukocyte values and age in both sexes. The assumption of validity was checked for all tests performed.

Results

Analysis of Bcl-2 expression on normal bone marrow and peripheral blood smears revealed that approximately 75% of blasts, 90% of pro-

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Table 1. Bcl-2 expression in normal peripheral and bone marrow blood.

peripheral blood		*bone marrow blood*	
cell type	*positivity %**	*cell type*	*positivity %**
neutrophils	7	blasts	75
eosinophils	0	promyelocytes	90
basophils	0	myelocytes	57
lymphocytes	85	metamyelocytes	31
monocytes	0	normoblasts	94
erythrocytes	0	orthochromic erythroblasts	89
platelets	0	megakaryocytes	100

*Mean value obtained in five normal volunteers.

Table 2. Bcl-2 Expression in AML.

positivity %	*onset patient n° (%)*	*relapse patient n° (%)*
neg	8 (12.7)	–
1-20	12 (19)	1 (10)
20-70	18 (28.6)	2 (20)
>70	25 (39.7)	7 (70)

myelocytes, the majority of erythroblasts and megakaryocytes, 85% of lymphocytes and 7% of the granulocytes express Bcl-2 (Table 1).

Cytospins of peripheral blood from 63 cases of AML at onset showed that in 25 cases (39.7%) more than 70% of the blasts analyzed expressed Bcl-2, in 18 cases (28.6%) between 20% and 70% expressed it, and in 12 cases (19.0%) between 1% and 20%, while in 8 cases (12.7%) no positivity to Bcl-2 was observed (Table 2). The varying distribution of the oncoprotein in AML cases at onset is given with reference to FAB subtype in Table 3. The FAB subtypes M0, M3 and M4 (Figure 1) contained the greatest number of cases in which more than 70% of the blasts examined were positive: 3 cases out of 5 for M0 (60%), 8 out of 15 for M3 (53.4%), and 8 out of 18 for M4 (44.5%). Two M1 cases out of 5 (40%), 4 M5 cases out of 8 (50%), the 2 M4 eosinophil variant cases and the only M7 case presented between 20% and 70% positivity. Three M2 cases out of 7 (42.8%) gave a positivity between 1% and 20%, while the two M6 cases revealed no Bcl-2 positive blasts (Figure 2). For subtype M3, no significant differences in Bcl-2 expression were noted between the 6 patients with classical M3 and the 9 cases of M3 microgranular variant.

With regards to Bcl-2 distribution in the individual samples, only one M0 case and one M4 case revealed predominantly perinuclear staining, while in numerous other instances this was only sporadic and related to the very low number of blast elements. Relapses in all of the FAB subtypes consistently showed a higher level of Bcl-2 expression than at onset; indeed of 10 relapses, more than 70% of the cells were positive in 7 (70%) (Tables 2 and 4).

Our results demonstrated no statistical correlations between expression of the oncoprotein Bcl-2 and FAB subtypes, sex, age, or white cell peripheral blood count.

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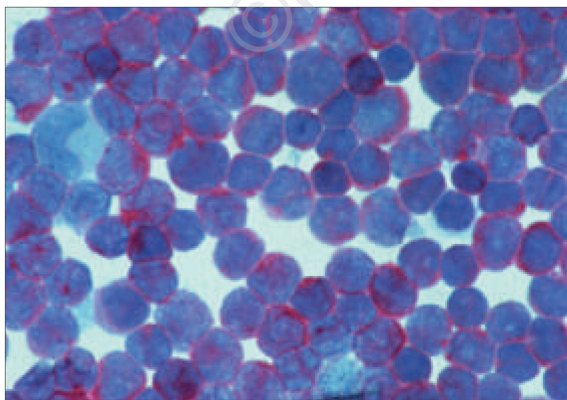

Figure 1. AML (FAB M4): cytospin from peripheral blood showing blast cells strongly reacting with anti-Bcl-2 antibody. APAAP 1000X.

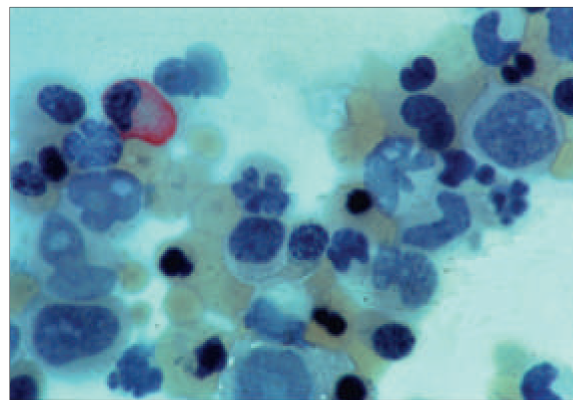

Figure 2. AML (FAB M6) peripheral blood cytospin: all leukemic blasts are Bcl-2 negative; only a plasma cell shows a strong pattern of positivity. APAAP 1000x.

Table 3. Bcl-2 expression in AML at onset.

	FAB subtypes: patient n° and percentage								
positivity	*M0*	*M1*	*M2*	*M3*	*M4*	*M4EO*	*M5*	*M6*	*M7*
neg		1 (20%)		2 (13.3%)	2 (11.1%)		1 (12.5%)	2 (100%)	
1-20%	1 (20%)		3 (42.8%)	3 (20%)	4 (22,2%)		1 (12.5%)		
20-70%	1 (20%)	2 (40%)	2 (28.6%)	2 (13.3%)	4 (22.2%)	2 (100%)	4 (50%)		1 (100%)
>70%	3 (60%)	2 (40%)	2 (28.6%)	8 (53.4%)	8 (44.5%)		2 (25%)		

Table 4. Bcl-2 expression in AML relapses.

	FAB subtypes: patient n° and percentage								
positivity	*M0*	*M1*	*M2*	*M3*	*M4*	*M4EO*	*M5*	*M6*	*M7*
neg									
1-20%							1 (50%)		
20-70%			1 (50%)			1 (100%)			
>70%	1 (100%)	2 (100%)	1 (50%)		2 (100%)		1 (50%)		

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Conclusions

Expression of the oncoprotein Bcl-2, which was originally thought to be characteristic of non-Hodgkin lymphoma with t(14;18), has recently been reported in various normal and neoplastic tissues without cytogenetic mutations affecting chromosome 18.[10] The results obtained in our population show that Bcl-2 is expressed in 55 of 63 (87.3%) cases of AML at onset and in all 10 relapses. At onset, in 25 cases (39.7%) more than 70% of blasts were Bcl-2 positive, as compared to 8 cases which were completely negative. FAB subtypes M0, M3 and M4 in particular revealed a greater number of strongly positive cases, while the two M6 cases gave no positivity at all. In relapses, the oncoprotein was consistently found in a greater number of cells than at onset. Our results are essentially in agreement with those of Delia et al.[12] who used immunohistochemical, biochemical and cytofluorimetric techniques to study Bcl-2 expression on bone marrow biopsies from cases of AML, myeloproliferative disease and myelodysplastic syndrome. In a population of 82 patients with AML at onset, Campos et al.[17] found positivity above 20% in only 34% of the samples examined, highlighting that FAB subtypes M4 and M5 in particular expressed high levels of Bcl-2, while none of the 6 M3 cases reported showed the oncoprotein. That study also reported that the results obtained with immunocytochemistry and flow cytometry corresponded perfectly. The discrepancies between these findings and those in the present study are probably due to: 1) the different fixing and staining methods used; 2) the different expression of Bcl-2 in peripheral and bone marrow blood in AML patients; and 3) the different incidence of the various FAB subtypes. Indeed our results show no statistical correlations between Bcl-2 oncoprotein expression and FAB subtypes, sex, age, and peripheral blood white cells. Interest in studying of Bcl-2 expression in neoplasms, in this case AML, is strictly linked to its function as an inhibitor of apoptosis. Campos et al.[17] reported a correlation between the level of Bcl-2 expression and the survival of leukemic cells in liquid culture in the absence of growth factors. An experiment by Miyashita et al.[18] demonstrated that Bcl-2 expression can protect pre-B cells of the 697 line transfected by the bcl-2 gene from the cytotoxic effects of various drugs such

as anti-metabolites, inhibitors of deoxynucleotide synthesis or DNA topoisomerase, alkylating and glucocorticoid agents, and those interfering with the microtubules, without, however, affecting cell proliferation. Lotem et al.[19] have also shown *in vitro* that AML with varying Bcl-2 expression exhibit varying susceptibility to chemotherapies. This differing response to chemotherapeutic agents is not correlated to differences in the activity of the mdr1 genes, which code for the P-170 protein and influence intracellular accumulation of cytostatic agents. The study of Bcl-2 expression could therefore identify a category of leukemias with low susceptibility to apoptosis-inducing drugs. In clinical practice, chemotherapies are used cyclically in order to reduce potentially fatal side effects. Thus some leukemic cells that express Bcl-2 could survive and begin to proliferate between cycles or acquire chemoresistance and proliferate even in the presence of anti-neoplastic drugs.[20-22] Experimental data have shown that *in vitro* all-trans retinoic acid induces differentiation of the HL60 promyelocytic cell line, which expresses the oncoprotein constitutively, and leads to a reduction in Bcl-2 expression.[12] We therefore feel that *in vitro* studies of cells from AML patients are necessary in order to broaden knowledge about the effects of the most common therapeutic drugs and of those substances which, alone or in association, can modulate Bcl-2 expression.[23]

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