The rate of apoptosis and expression of Bcl-2 and Bax in leukocytes of acute myeloblastic leukemia patients*

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The rate of apoptosis as well as expression of Bcl-2 and Bax was evaluated before and after induction therapy in leukocytes of 70 patients with acute myeloblastic leukemia (AML), retrospectively divided into group A (with longer survival) and group B (with shorter survival). We found, that leukocytes of untreated AML patients showed susceptibility to apoptosis similar to control cells. Marked increase in percentage of apoptotic leukocytes was observed after induction therapy exclusively in patients with longer survival, which was accompanied by better normalization of routine hematological parameters. In this group, the Bcl-2/Bax ratio was similar to the control and remained unchanged after treatment. In AML patients with shorter survival, a twofold increase in this ratio was observed both before and after the completion of induction therapy. In both groups of untreated patients, western blot analysis revealed the presence of prominent additional bands reacting with anti-Bcl-2 or anti-Bax antibody, which were undetectable in control leukocytes. After the therapy, these bands disappeared, especially in patients from group A. In conclusion, the lack of therapy-induced enhancement in leukocyte apoptosis, an increased ratio of Bcl-2/Bax as well as persistent presence of abnormal Bcl-2 and Bax protein bands after induction therapy in AML patients may be considered as factors associated with unfavorable clinicl outcome.

Key words: AML, apoptosis, Bax, Bcl-2, leukocytes.

Acute myeloblastic leukemias (AML) account for about 80% of adult leukemias [4]. Even with the introduction of new anti-leukemic agents and approaches, most adult patients do not recover fully. As apoptosis is the final common pathway of death for virtually all cytotoxic agents, the regulators of apoptotic mechanism of cell death may be considered as prognostic factors for the fate of leukemic patients [2, 20].

The role of apoptosis in oncogenesis and cell number control is now well established [12, 22, 27]. It is also known, that the susceptibility of an individual cell to apoptosis is determined by the balance of the well-studied Bcl-2 family members, some of which promote apoptosis (Bax, Bcl-x_S, Bad, Bak, and Bik/Nbk, Bid, Bag-1) and others which suppress it (Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, Blf-1/A1) [7, 8, 17]. The mechanism by which antiapoptotic members of Bcl-2 family promote survival is not yet understood. Current data suggest a role for competitive dimerization between selective pairs of antagonists and agonists. Homodimerization of Bcl-2 results in strong anti-apoptotic function, while heterodimerization of the proapoptotic members, eg. Bax with Bcl-2 counteracts this function and shifts the balance towards apoptosis [20, 24]. Bcl-2 dysregulation has been observed in several hematologic malignancies, including AML [1, 6, 18] and acute lymphocytic leukemia [5, 23]. Moreover, recent data suggest that defects in apoptosis induction are specifically associated with a poor response to chemotherapy [25]. In gene transfection experiments, overexpression of Bcl-2 and its homolog Bcl-x_L have been shown to render neoplastic cells resistant to the induction of apoptosis by a variety of chemotherapeutic drugs [13]. Likewise, down-

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regulation of Bcl-2 protein has been shown to reverse chemoresistance in several experimental systems [15]. Chemosensitization has also been achieved by overexpression of proapoptotic proteins such as Bcl-x_S and Bax.

In clinical studies, the data are not so unequivocal. On the one hand, high levels of Bcl-2 or high ratios of Bcl-2:Bax have been reported to correlate with failure to achieve complete remission and with shorter overall survival [11, 14]. On the other hand, no difference in Bcl-2 expression was found between patients who achieved complete remission and those who failed to do so. Moreover, low Bcl-2 content was associated with poor survival in a group of patients with poor prognosis cytogenetics, while high Bcl-2 expression was associated with good survival [16].

Despite extensive investigation, the interpretation of treatment benefit as a function of apoptosis regulators is difficult. The role of the Bcl-2 family protein expression on clinical outcome following chemotherapy is still under investigation and validation. Hence, the aim of our study was to evaluate the rate of spontaneous apoptosis along with the expression of Bcl-2 and Bax in leukocytes of AML patients who differ by time of survival in the course of treatment.

Material and methods

Peripheral blood from 70 patients (aged between 27 and 86; mean, 63 years) with newly diagnosed AML and from 30 age-matched healthy donors was obtained with their informed consent. Flow cytometric immunophenotyping was performed by Coulter EPICS XL. The treated group of patients had all received standard therapy (FRB, Cyt, Vep, DAC) [9]. The peripheral blood was collected on day 0 (before) and after completion of induction therapy (after). The leukocytes were isolated by density centrifugation on Histopaque 1077.

Monoclonal antibodies for immunophenotyping analysis were purchased from Becton Dickinson. Annexin V-FITC were purchased from Immunotech. Anti-Bax and anti-Bcl-2 antibodies as well as protein controls were obtained from Santa-Cruz Biotechnology. A prestained low molecular weight marker (Bio-Rad), the enhanced chemiluminescence (ECL) detection system, nitrocellulose membranes, secondary antibodies conjugated to horseradish peroxidase, and Hyperfilm ECL were from Amersham. All the other reagents were obtained from Sigma.

Determination of apoptotic index. The cellular rate of apoptosis was determined using Annexin V-Fluos and propidium iodide double staining [28]. The cytometric data were validated by fluorescence microscopic assay, based on acridine orange-ethidium bromide staining [10].

W*estern blotting*. Bcl-2 and Bax expression was determined by Western blotting. After separation, cells were washed once with 1.5 ml of ice-cold PBS containing 1 mM Na_3VO_4, pH 7.4, pelleted, and sonicated for 5 s in 0.4 ml of ice-cold lysis buffer [50 mM β-glycerophosphate, 1.5 mM ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na_3VO_4, 1 mM dithiotreitol (DTT), 10 μg/ml leupeptin, and 10 μg/ml aprotinin, pH 7.4]. The lysates were then centrifuged in a microcentrifuge at 4 °C for 30 min and 10 μl aliquot from each sample was removed for subsequent protein quantitation using the Bio-Rad protein assay reagent. The remaining supernatant was combined with an equal volume of 2x-concentrated SDS-PAGE loading buffer (0.1 M Tris-HCl [pH 7.6], 4% SDS, 200 mM dithiothreitol, 20% glycerol, and 0.2% bromophenol blue), and boiled for 5 minutes. Lysates (10 μg of total protein per lane) were subjected to SDS-PAGE followed by western blot analysis with the indicated antibody using the ECL detection system. Data from Western blotting were quantitated densitometrically with the use of Gel-Pro Analyzer™ from Media Cybernetics.

Statistical analysis. All results are reported, where applicable, as the mean $\pm$ SEM. Statistical analysis was performed using the Wilcoxon test. The level of significance was defined as $p<0.05$.

Results

Patients with AML were retrospectively segregated into two groups: group A – 40 patients, who survived at least 2 months after completion of induction therapy and group B – 30 patients who died within 1 month after induction therapy. A major cause of death were infections and hemorrhage into the central nervous system). Both groups were analyzed before and after induction therapy.

The mean total number of leukocytes before treatment was significantly elevated in group A as well as in group B (Tab. 1). After induction therapy, a significant decrease in the total number of leukocytes was observed in both groups (A: $p<0.05$; B: $p<0.005$). Before initiation of induction therapy, the mean percentage of blast cells was 61 ± 16% and

Table 1. The mean number of leukocytes and percentage of blast cells in the peripheral blood of AML patients

	Leukocyte number ($x10^3$)		% of blast cells	
	before	after	before	after
Group A	52 ± 36	$3.1 \pm 1.4^{*}$	61 ± 16	$6.5 \pm 6^{**}$
Group B	90 ± 28	$10 \pm 9^{**}$	70 ± 10	$27 \pm 11^{**}$

Before: sample collected before induction therapy. After: sample collected after completion of induction therapy. Group A: patients who survived at least 2 months after completion of induction therapy. Group B: patients who died within one month after induction therapy. * – $p<0.05$, ** – $p<0.005$.

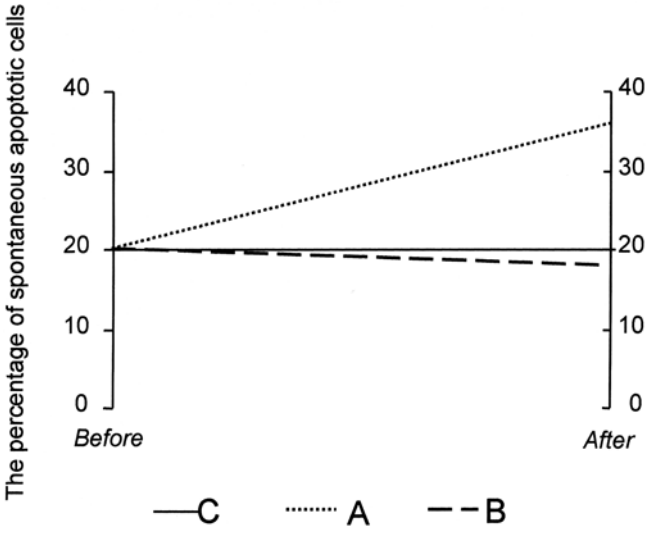

Figure 1. The percentage of apoptotic cells. Before: the peripheral blood collected before induction therapy. After: the peripheral blood collected after completion of induction therapy. Group A: patients who survived at least 2 months after completion of induction therapy. Group B: patients who died within one month after induction therapy. Group C: healthy donors.

$70 \pm 10\%$, respectively ingroup A and B. A marked reduction in the percentage of blast cells was observed during induction therapy in both groups; however they were still present after the completion of induction therapy.

The percentage of apoptotic cells. The measurement of susceptibility to apoptosis of AML leukocytes showed similar percentage of apoptotic cells in the control group ($20 \pm 10\%$) as well as in the untreated AML patients from A ($20 \pm 15\%$) and B group ($21 \pm 16\%$) (Fig. 1). After induction therapy an increase to $36 \pm 14\%$ of apoptotic cells percentage was noted exclusively in group A (Fig. 1).

Western blot analysis. The calculations of Bcl-2 and Bax expression rates were based on densitometry of Western blots. Levels of particular proteins in control cells were taken as 100%.

Bcl-2: The Bcl-2 level in leukocytes of untreated patients from group A and B ($160 \pm 70\%$ and $195 \pm 150\%$, respectively was not statistically different from control leukocytes (100%) (Fig. 2A). The levels of Bcl-2 protein were significantly elevated after induction therapy to $600 \pm 290\%$ ($p<0.04$), predominantly in the leukocytes of the survivors (group A). In group B, an insignificant elevation in the Bcl-2 level was found after treatment.

In healthy human leukocytes the immunoblot analysis demonstrated the presence of two bands in the molecular weight range expected for Bcl-2 (Fig. 2B). Comparison to the Bcl-2 control revealed that the lower band was specific to this protein. In the majority of western blots of AML patients from both groups (before), we observed additional

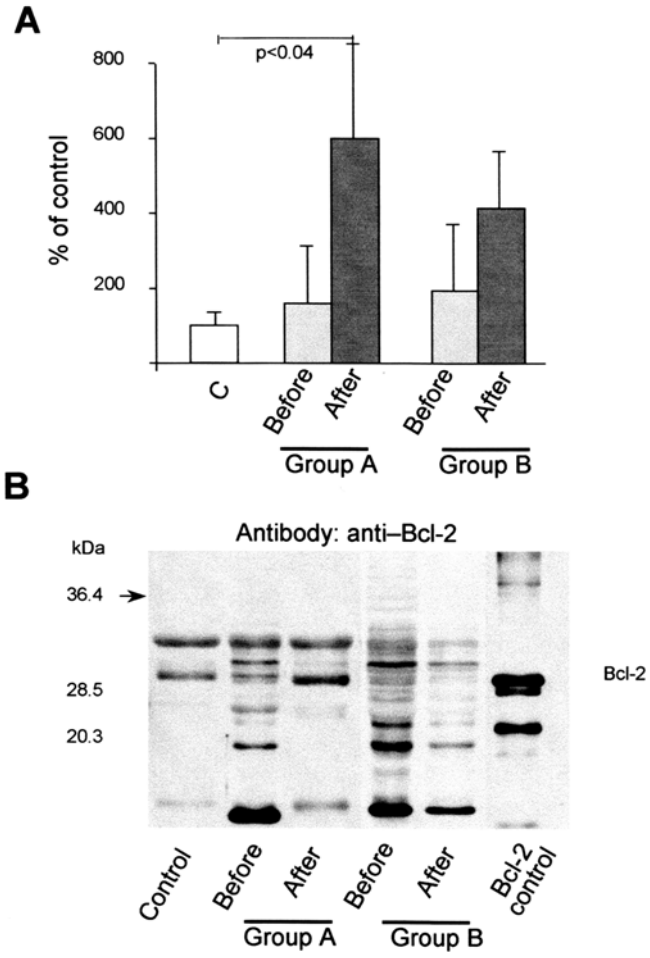

Figure 2. The Bcl-2 level in leukocytes of AML patients (A). Each bar represents the mean percentage of the control value ±SEM. Before: the peripheral blood collected before induction therapy. After: the peripheral blood collected after completion of induction therapy. Group A: patients who survived at least 2 months after completion of induction therapy. Group B: patients who died within one month after induction therapy. Group C: healthy donors.
Fragments of representative Western blots are shown (B). Left arrows, the position of molecular weight standards. Right arrow, the position of Bcl-2 band for Bcl-2 control protein.

anti-Bcl-2 immunoreactive bands in the position corresponding to slightly higher and much lower molecular weight. After induction therapy, in group A we observed fading or even disappearance of these bands, accompanied by an increased density in the band corresponding to normal protein. In many patients from group B, the band corresponding to the normal Bcl-2 was absent. Moreover, in most cases from group B, the additional forms of anti-Bcl-2 antibody reacting proteins persisted during therapy.

Bax: There was no significant difference in the Bax level between control and leukocytes from groups A ($196 \pm 90\%$) and B ($103 \pm 80\%$) (Fig. 3A). As in the case of Bcl-2, a significant increase in Bax expression was observed after induction

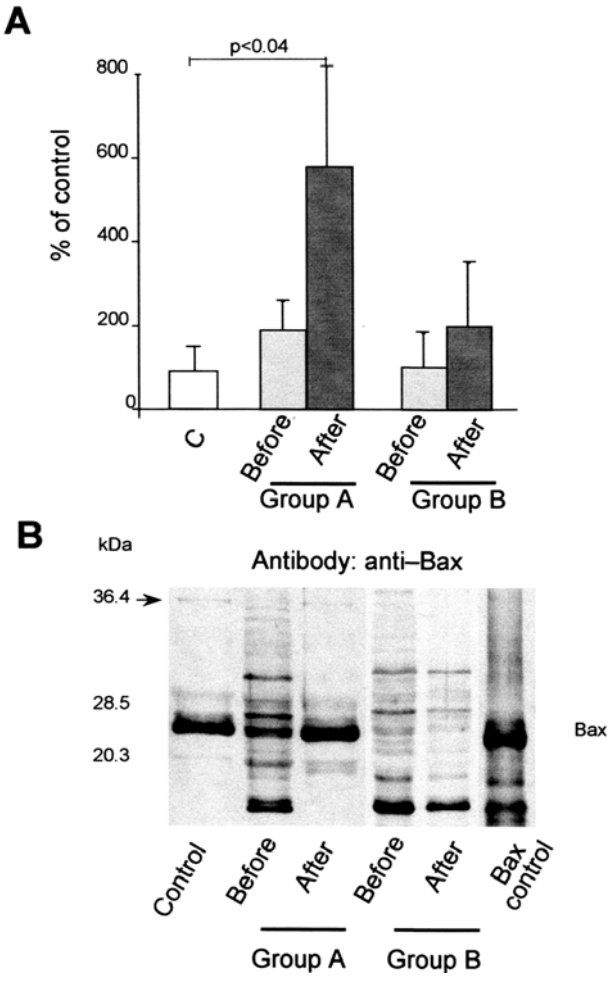

Figure 3. The Bax level in leukocytes of AML patients (A). Each bar represents the mean percentage of the control value ±SEM. Before: the peripheral blood collected before induction therapy. After: the peripheral blood collected after completion of induction therapy. Group A: patients who survived at least 2 months after completion of induction therapy. Group B: patients who died within one month after induction therapy. Group C: healthy donors.
Fragments of representative Western blots are shown (B). Left arrows, the position of molecular weight standards. Right arrow, the position of Bax band for Bax control protein.

therapy almost exclusively in group A (580 ± 240%; p<0.04).

In healthy human leukocytes, Western blot analysis demonstrated the presence of a single band in the position corresponding to the Bax control protein (Fig. 3B). The additional, anti-Bax antibody reacting proteins were observed in leukocytes of untreated AML patients from A and B groups. As in the case of Bcl-2, they faded or disappeared after therapy only in patients from group A. In many untreated patients from group B, the band corresponding to normal Bax was absent, too.

Bcl-2/Bax ratio: The Bcl-2/Bax ratio in leukocytes of untreated leukemic patients from group A (0.84) was similar

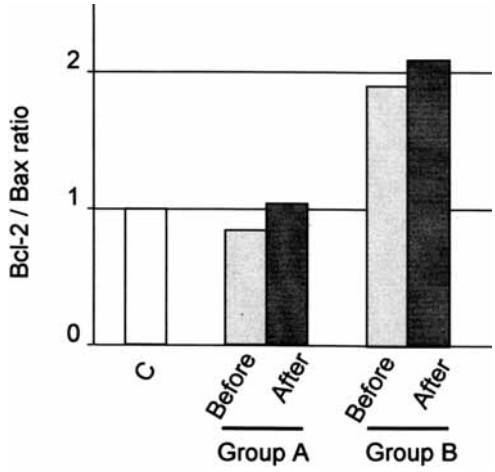

Figure 4. The Bcl-2/Bax ratio in leukocytes of AML patients. The ratio in control leukocytes was taken as 1.0. Before: the peripheral blood collected before induction therapy. After: the peripheral blood collected after completion of induction therapy. Group A: patients who survived at least 2 months after completion of induction therapy. Group B: patients who died within one month after induction therapy. Group C: healthy donors.

to the ratio in control cells (assumed as 1.0). In group B, a twofold increase in the Bcl-2/Bax ratio was observed before, as well as after, induction therapy (Fig. 4).

Discussion

In this study we compared the rate of apoptosis of leukocytes in AML patients and healthy donors. There was no difference in susceptibility of leukocytes to apoptosis among untreated AML patients and healthy control. The induction therapy caused marked increase of leukocyte apoptosis rate in patients with longer survival (group A) but not in patients with shorter survival (group B). It has been reported, that a low rate of apoptosis was correlated with both *in vitro* and *in vivo* pan-resistance to antileukemic chemotherapy [25]. The cause of inhibited apoptosis in AML is probably a function of interactions among multiple signals that influence apoptosis. Our results are in line with hypothesis, assuming that assessment of apoptosis may serve as an important prognostic factor for AML.

In our study, we found a significant increase of Bcl-2 expression (p<0.04) after induction therapy in the leukocytes of patients with longer survival times. Interestingly, in patients with poor response to induction therapy, despite some improvement in routine parameters, the increase of Bcl-2 was insignificant. So, marked elevation of Bcl-2 was found exclusively in leukocytes of patients with longer survival times, and was accompanied by normalization of routine hematological tests as well as by an increase in the percentage of apoptotic cells. The role of Bcl-2 expression as a good prognostic factor has been demonstrated in breast

cancer, where poor clinical outcome was strongly connected with downregulation of Bcl-2-expression [3]. In this case, the beneficial role of Bcl-2 expression is usually explained by the hypothesis that Bcl-2 may confer growth disadvantage to cancer cells by inhibition of cell cycle progression independently of its antiapoptotic function [19].

In our study, the pro-apoptotic protein – Bax revealed characteristics similar to Bcl-2, with a significant increase in Bax expression ($p<0.04$) restricted to the leukocytes of patients with longer survival times. Simultaneous increase of pro- and anti-apoptotic proteins during the treatment suggests that Bcl-2 or Bax alone are not reliable and independent predictors of sensitivity to apoptosis in AML patients. It is well known that the ratio of Bcl-2/Bax may be pivotal in determining the fate of cells [25]. In our study, we did not observe significant differences in leukocyte the Bcl-2/Bax ratio between controls and patients with longer survival times, before and after treatment. Within the limitations of this sample size we found no apparent correlation between Bcl-2/Bax ratio and FAB classification. In the group of patients with longer survival times the Bcl-2/Bax ratio seems to be independent of morphological parameters. It was in a similar range both before treatment – when the leukocyte number and the percentage of blast cells were significantly high, as well as after induction therapy, when leukocyte number was normalized and the percentage of blast cells was significantly lower ($p<0.005$). However, a normal (close to 1.0) Bcl-2/Bax ratio was accompanied by a slight increase in the percentage of apoptotic cells after induction therapy. On the other hand, the Bcl-2/Bax ratio was evidently elevated in the group of patients with the shortest survival times. A high Bcl-2/Bax ratio was also associated with no changes in leukocyte sensitivity to apoptosis after treatment. Therefore we assumed that the increased ratio of Bcl-2/Bax may be considered as an unfavorable feature in the context of the treatment process. Our results are in line with literature data showing that a high Bcl-2/Bax ratio is a significant marker of poor response to therapy in AML [26, 30] and may define a group of patients with therapy-resistant disease and poor clinical outcome.

In both groups of untreated patients, western-blotting analysis of Bcl-2 and Bax revealed the presence of prominent additional bands reacting with anti-Bcl-2 or anti-Bax antibody, which were undetectable in control leukocytes. The bands with greater molecular weight were usually more prominent than the native band of Bcl-2 or Bax. These bands may represent different forms of the Bcl-2 family members posttranslational modification, i.e. phosphorylation or glycosylation. It is well known, that Bcl-2 family proteins are regulated by phosphorylation, and depending on the site of phosphorylation, Bcl-2 function is either enhanced or abrogated [21]. Additional bands with lower molecular weight observed in the immunoblot of untreated AML patients in both groups may be the product of partial proteolytic degradation. In most cases all these additional bands faded or disappeared while normal Bcl-2 and Bax increased in the course of treatment, especially in patients with longer survival times. It was associated with the normalization of routine hematological tests and increase in the percentage of apoptotic cells. Therefore, these data may suggest that the treatment resulted in higher levels of normal protein by restoration of normal pathways of Bcl-2 and Bax posttranslational modification.

Taken together, our study found that after induction therapy leukocytes of AML patients with better normalization of routine hematological tests and longer survival, demonstrated an elevated rate of apoptosis, significant increase in Bcl-2 and Bax expression and disappearance of abnormal proteins reacting with anti-Bcl-2 and anti-Bax antibody. The leukocytes of AML patients with shorter survival showed an elevated Bcl-2/Bax ratio before and after induction therapy, lack of increased sensitivity to apoptosis after treatment as well as persistence of abnormal proteins reacting with anti-Bcl-2 or anti-Bax antibody. However, in order to assess whether these differences might represent clinically relevant predictive parameters in AML patients, careful evaluation of a greater number of FAB and cytogenetically classified patients will be necessary. This knowledge may provide the basis for defining new leukemia patient subgroups and new therapeutic concepts that target the cell death pathway to improve clinical outcome following cancer therapies.

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