Minimal Residual Disease in Acute Myeloid Leukemia Is Predicted by an Apoptosis-Resistant Protein Profile at Diagnosis

Amber van Stijn, Nicole Feller, Alice Kok, Marjoleine A. van der Pol, Gert J. Ossenkoppele, and Gerrit J. Schuurhuis

Abstract

Purpose: Apoptosis is an important mechanism regulating survival of acute myeloid leukemia cells. The apoptosis-related protein profile at diagnosis is important for achieving complete remission thereby affecting survival variables such as disease-free survival (DFS) and overall survival (OS). To investigate the role of the apoptosis protein profile in further response to therapy and outgrowth of disease.

Experimental Design: We studied whether Bcl-2, Bcl-xL, Mcl-1, Bax as well as the Bcl-2/Bax ratio and a combination of all (antiapoptosis index, AAI) are related to the frequency of malignant cells surviving the chemotherapy (i.e., minimal residual disease, MRD). MRD cells were identified by leukemia-associated aberrant phenotypes established at diagnosis by flow cytometry.

Results: We found that Bcl-2 (R = 0.55, P = 0.002), Bcl-2/Bax (R = 0.42, P = 0.02), and AAI (R = 0.47, P = 0.01) at diagnosis directly correlated with MRD after the first cycle of chemotherapy. In turn, MRD frequency after first cycle correlated with DFS (P = 0.04). Taken together, these results directly explain why Bcl-2/Bax and especially AAI (P = 0.007) at diagnosis correlate with DFS.

Conclusion: Our results show that apoptosis resistance plays an important role in the first stage of the therapy (i.e., to eliminate the bulk of malignant cells), in terms of achievement of complete remission and frequency of MRD after first cycle of therapy.

All currently available cytotoxic drugs induce cell death by activating the intrinsic apoptotic pathway. This pathway is tightly regulated by Bcl-2 family members, which consist of antiapoptosis members like Bcl-2, Bcl-xL, and Mcl-1 and proapoptosis members like Bax, Bak, and Bad. The dimerization pattern and relative quantities of these proteins are essential in the regulation of cytochrome c release from the mitochondria, thereby regulating the apoptotic machinery. Aberrant expression patterns and concomitant changes in function of these proteins in cancer may result in the inability of tumor cells to initiate apoptosis and therefore contribute to therapy resistance as well as the growth and persistence of the tumor. In acute myeloid leukemia (AML), a high Bcl-2 expression in leukemic blasts is generally found associated with apoptosis resistance in vitro (1, 2) and discriminates between patients who enter complete remission (CR) and those who do not (3, 4). Furthermore, overexpression of Bcl-2 or an increased Bcl-2/Bax ratio in AML is also associated with a shorter survival (4–6).

Outgrowth of minimal residual disease (MRD) cells is thought to be responsible for the occurrence of relapse, and in line with that, the prognostic value of MRD detection has been shown in bone marrow after induction and intensification therapy in the study of San Miguel (7, 8), after consolidation therapy in the study of Venditti (9) and in addition to induction and consolidation therapy also in peripheral blood stem cell transplants (10). It can therefore be hypothesized that there may be a direct relationship between the apoptosis-related protein profile at diagnosis and MRD frequency during treatment. Besides our previously presented preliminary results (11), there is, to the best of our knowledge, no report that has examined the role of apoptosis-related proteins in the development and persistence of MRD as the missing link between the established prognostic values of apoptosis-related proteins at diagnosis on the one hand and the prognostic value of MRD frequency on the other hand.

The techniques that we developed which combine expression of apoptosis-related proteins and MRD detection (12, 13) provide a unique opportunity to study this.

Materials and Methods

Patients and controls. A total of 57 bone marrow samples from patients with newly diagnosed AML and 73 bone marrow samples at different time points (after first, second, or third cycle of chemotherapy) were included in this study. AML patients aged ≤60 years were treated according to the Dutch HOVON 29 (during 1998-2000) and HOVON 42 (during 2001-2003) protocols, which have basically an identical design, consisting of two remission induction cycles with cytarabin plus idarubicin and cytarabin plus amsacrine and one consolidation cycle with either etoposide and mitoxantrone or a
myeloablative regimen with busulfan and cyclophosphamide followed by autologous stem cell transplantation. Details of the protocols have been described by Feller et al. (10). The protocols can also be found at http://www.hovon.nl.

Patients aged >60 years were treated at the HOVON 32 protocol. Patients received two cycles of remission induction chemotherapy consisting of cytarabine supplemented with granulocyte colony-stimulating factor (filgrastim, 5 μg/kg, day 0 until neutrophil recovery > 0.5 × 10⁹/L) with or without the addition of fludarabine (25 mg/m², days 1-5). After the remission induction therapy, patients received a third cycle of chemotherapy consisting of the combination of cytarabine and daunorubicin plus granulocyte colony-stimulating factor from day 0 until a neutrophil recovery > 0.5 × 10⁹/L. Details have been described by Feller et al. (10). The protocol can also be found at www.hovon.nl. Patient characteristics are shown in Table 1.

Control bone marrow samples not infiltrated by tumor cells were obtained from healthy individuals or from patients suffering from cardiac disease. Informed consent was obtained both from patients and healthy individuals.

**Antibodies.** FITC-conjugated mouse anti-human Bcl-2 (IgG1, clone 124) and FITC-conjugated anti-rabbit were from DAKO Diagnostics B.V. (Uithoorn, the Netherlands). The rabbit polyclonal antibodies Bax (P-19), Bcl-xL (S-18), and Mcl-1 (S-19) and normal rabbit immunoglobulins (nrIgG) were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CD7 PE was purchased from Immunotech (Marseille, France), anti-CD45 PerCP and anti CD34 APC were from Becton Dickinson (Mountain View, CA).

**Minimal residual disease detection.** The phenotypic analysis of de novo AML was done on whole bone marrow upon staining with FITC-, PE-, PerCP-, and APC-conjugated monoclonal antibodies. MRD cells were detected using aberrant antigen combinations, the so-called leukemia-associated phenotypes (LAP), that are not or in very low frequencies present in normal bone marrow (8, 9, 14, 15). Our detailed description of the establishment of a LAP and MRD detection has previously been published (10, 16, 17). In the present patient cohort, 87% of the patients displayed one or more LAPs, enabling the flow cytometrical detection of MRD. In the majority of cases, a LAP is not expressed on 100% of the leukemia blast population. For the calculation of the frequency of the total leukemia blast population in a follow-up bone marrow, a correction for the LAP expression on AML blasts at diagnosis was done as previously described (10). Based on the recurrence in MRD of the different phenotypes seen at diagnosis, such corrections have been shown in our previous work (17) to be valid.

**Apoptosis-related protein detection in diagnosis of acute myeloid leukemia by flow cytometry.** We have previously described procedures for detection and quantification of apoptosis-related protein expression (12, 13). In short, cells were stained with PE-, PerCP-, and APC-conjugated monoclonal antibodies defining the blast population. After washing, cells were fixed (1% PFA, 5 minutes, room temperature) and permeabilized (0.1% saponin, 15 minutes, room temperature). After washing, cells were incubated with either FITC-conjugated anti-Bcl-2 or unconjugated rabbit polyclonal anti-Bax, anti-Bcl-xL, and anti-Mcl-1 for 30 minutes at 4°C. For Bax, Bcl-xL, and Mcl-1 detection, a second incubation step with FITC-conjugated anti-rabbit (30 minutes, 4°C) was done. Cells were measured immediately at a FACScalibur (Becton Dickinson) equipped with a red diode laser.

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**Complete remission and relapse.** Remission either after the first or second cycle of induction chemotherapy was defined as CR if ≤5% blasts were present in bone marrow and with concomitant evidence of erythropoiesis, granulopoiesis, and megalakaryopoiesis. Granulocytes and platelets in peripheral blood should be at least 1.5 × 10⁹/L and 100 × 10⁹/L, respectively.

Relapse was defined as marrow infiltration by >5% blasts in previously morphologic normal bone marrow.

**Statistics.** The Spearman's ρ correlation test, nonparametric Mann Whitney U test, independent samples Student's t test, Kaplan Meier survival test, and Cox regression were done using the SPSS software program. P < 0.05 was considered as significant.

**Results**

**Apoptosis protein characteristics at diagnosis.** The apoptosis-related protein expression profile of malignant AML blasts was determined at diagnosis. From 57 patients, we measured the Bcl-2, Bcl-xL, Mcl-1, and Bax expression and integrated these proteins both in a Bcl-2/Bax ratio and in the so-called antiapoptosis index (AAI; ref. 2): (Bcl-2 × Bcl-xL × Mcl-1) / Bax. Mean Bcl-2 expression, Bcl-2/Bax ratio, and the AAI were significantly higher in AML blasts than in normal bone marrow CD34⁺ cells (Table 2), showing that AML blasts have a relatively apoptosis-resistant protein profile.

**An apoptosis-resistant protein profile correlates with failure to achieve complete remission.** To determine the effect of the apoptosis-related protein profile at diagnosis on the initial response to therapy, we correlated apoptosis variables at diagnosis on the initial response to therapy, we correlated apoptosis variables at diagnosis on the initial response to therapy, we correlated apoptosis variables at diagnosis on the initial response to therapy, we correlated apoptosis variables at diagnosis on the initial response to therapy, we correlated apoptosis variables at diagnosis on the initial response to therapy.
group of patients. The expression of the antiapoptosis proteins Bcl-2, Bcl-xL, and Mcl-1 were all lower in the responders than in the refractory patients, although differences were significant only for Mcl-1 (Table 3). The mean Bax expression was higher in the responders than in the refractory patients. Whereas the difference between responders and refractory patients in the Bcl-2/Bax ratio was borderline significant, the differences in AAI were significant (Table 3).

An apoptosis-resistant protein profile at diagnosis is associated with a high minimal residual disease frequency. Subsequently, we examined whether the apoptosis protein profile of the blasts correlated with the frequency of leukemic cells that survived chemotherapy. Figure 1 shows an example of the flow cytometric MRD detection using the so-called LAPs (see Material and Methods; for details, see Fig. 1 legend). Using this approach, we measured bone marrow samples from AML patients after first, second, and third cycle of chemotherapy, provided that a LAP was found at diagnosis and that enough patients after first, second, and third cycle of chemotherapy, this approach, we measured bone marrow samples from AML material was available (Table 4A). To allow Kaplan Meier survival analysis for the apoptosis proteins in the last paragraph of the results section and to guarantee an objective analysis, the diagnosis samples were split into two groups using the median values of Bcl-2/Bax (median = 5.75) and AAI (median = 98). MRD frequencies in the group with diagnosis Bcl-2/Bax ratio of ≥5.75 were significantly higher than in the group with Bcl-2/Bax ratio of <5.75 (Fig. 3A). Similarly, patients with an AAI ≥98 had more MRD than patients with an AAI of <98 (Fig. 3B).

On the other hand, in linear regression, none of the apoptosis-related proteins or ratio's correlated clearly with MRD frequency both after the second cycle of chemotherapy (n = 26; Bcl-2: R = 0.3, P = 0.09; Bcl-xL: R = 0.06, P = 0.7; Mcl-1: R = −0.1, P = 0.5; Bax: R = −0.07, P = 0.7; Bcl-2/Bax: R = 0.2, P = 0.3; AAI: R = 0.2, P = 0.3) and after the third cycle (n = 17; Bcl-2: R = 0.3, P = 0.1; Bcl-xL: R = 0.05, P = 0.8; Mcl-1: R = 0.03, P = 0.9; Bax: R = −0.2, P = 0.5; Bcl-2/Bax: R = 0.3, P = 0.3; AAI: R = 0.4, P = 0.1).

A high minimal residual disease frequency is associated with short disease-free survival. Next, the question was whether in this particular patient group the frequency of MRD in bone marrow had prognostic value. This was done in two ways. First, MRD frequency was directly correlated with survival variables by Cox regression analysis, showing that patients with higher MRD frequency have shorter DFS after all cycles and shorter OS after the third cycle (Table 4B). Second, for Kaplan-Meier survival analysis, after the first cycle, patients were divided in two groups by the mean MRD frequency (0.33%). Patients with low MRD (<0.33%) had a significantly longer median DFS than patients with high MRD (≥0.33%; 26 versus 5.1 months, P = 0.04, Fig. 4A). OS was borderline significantly different between the patients with low and high MRD (not reached versus 8 months, P = 0.07, Fig. 4B). Similar survival analysis revealed that patients

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<th>Table 2. Apoptosis-related protein expression in AML blasts at diagnosis and CD34+ cells in normal bone marrow</th>
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<td><strong>Protein expression</strong></td>
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*Protein expression is determined by dividing the protein MFI by the appropriate isotype control MFI as mentioned in Materials and Methods.

†nBM indicates bone marrow from hematologically healthy individuals.

‡Bcl-2/bax and AAI ratios were determined for each individual sample.

| Abbreviation: NS, not significant. |

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with low MRD frequencies (<0.22%) after second cycle of chemotherapy had longer DFS ($P = 0.007$) and OS ($P = 0.02$). Patients with low MRD frequencies (<0.83%) after third cycle of chemotherapy had a significantly longer DFS ($P = 0.0001$) and OS ($P = 0.05$).

An apoptosis-related protein profile at diagnosis is associated with short disease-free survival. Considering the established significant correlations between the apoptosis protein profile at diagnosis with MRD on one hand and between MRD with survival variables on the other hand, it was investigated whether the apoptosis variables at diagnosis had prognostic effect. To this end, the samples were divided into two groups defined by the individual median protein values similar to that described in Fig. 3. Using these median protein expression values, trends became clear for differences in DFS: 25.7 versus 6.9 months for Bcl-2 ($P = 0.06$); 11.8 versus 7.5 for Bcl-xL ($P = 0.3$); 11.2 versus 6.5 for Mcl-1 ($P = 0.3$); and 7.9 versus 11.8 for Bax ($P = 0.2$). Median DFS for Bcl-2/Bax of $\leq 5.75$ was 25.7 versus 7.5 for Bcl-2/Bax of $> 5.75$ ($P = 0.08$; Fig. 5A). However, patients with an AAI of $> 98$ showed a significantly shorter survival than the patients with low AAI (Fig. 5B). Median DFS for AAI of $\geq 98$ was 6.9 months whereas the median DFS for AAI of $< 98$ was not reached ($P = 0.007$). Multivariate Cox regression analysis showed that of all factors tested, including gender, age, French-American-British classification (a tumor classification), treatment protocol, WBC and cytogenetics, only AAI was a significantly unfavorable variable for DFS.

### Discussion

Most cytotoxic drugs exert their effects by inducing apoptosis in their target cells and this suggests that the chemosensitivity of an AML blast cell may be dependent on a critical balance between proapoptosis and antiapoptosis proteins within the cell. This hypothesis has been supported by the work of many authors who studied the prognostic value of antiapoptosis protein Bcl-2 in AML. In our study, we not only investigated an extended number of relevant apoptosis proteins which now showed to be important too, but we were also able to link the antiapoptosis protein profile with the emergence of MRD cells in AML patients in clinical and morphological remission.
First, with our sensitive and quantitative flow cytometric detection method (12, 13), we showed that the antiapoptosis Bcl-2, Bcl-xL, Mcl-1, and proapoptosis Bax in diagnosis AML blasts were heterogeneously expressed among patients. Bcl-2, the Bcl-2/Bax and the AAI (Bcl-2/C2 Bcl-xL/C2 Mcl-1 / Bax; ref. 2) were significantly higher in AML bone marrow than in normal bone marrow CD34+ cells suggesting a relatively apoptosis-resistant protein profile of AML blasts at diagnosis. This is in accordance with previously published results (4–6). In a previous study (2), we were able to relate a high Bcl-2 and Bcl-xL expression in AML bone marrow to a decreased sensitivity to cytotoxic drugs in vitro.

**Fig. 2.** Correlation of apoptosis variables at diagnosis with MRD in bone marrow obtained after the first cycle of chemotherapy. Linear regression line of (A) Bcl-2 expression, (B) Bcl-2/Bax ratio, and (C) AAI with MRD frequency in bone marrow obtained after first cycle of chemotherapy. Apoptosis-related protein expression was measured at diagnosis. MRD frequency was determined after completion of the first cycle of chemotherapy as illustrated in Fig. 1.

**Fig. 3.** Correlation between the apoptosis-related protein profile at diagnosis and MRD after the 1st cycle of chemotherapy. Patients (n = 30) were divided by median split of (A) Bcl-2/Bax (cutoff: 5.75) or of (B) AAI (cutoff: 98) at diagnosis. Box and Whisker plots of the mean (bold line) and median (thin line) MRD frequency. Differences between mean values are significant as shown.
Campos et al. (5) showed that AML patients with high levels of Bcl-2 had a lower CR rate compared with those with low levels of Bcl-2. In addition, Del Poeta et al. (18) included the proapoptosis Bax in the analysis and observed that the Bcl-2/Bax ratio had prognostic significance with regard to CR. Likewise, high levels of Bcl-2 or a high Bcl-2/Bax ratio translated into decreased OS (5, 18). In line with these studies, we observed that Bcl-2/Bax ratio was different between patients who reached CR and those who did not, and that a high Bcl-2/Bax ratio resulted in a borderline significantly shorter DFS ($P = 0.08$). However, including all proteins studied in one factor (i.e. the AAI), resulted in a significantly predictive value with regard to DFS, indicating that inclusion of Bcl-xL and Mcl-1 improves the prognostic value in terms of survival. As we determined the cutoff levels for Kaplan Meier survival analysis by an objective median split (Bcl-2/Bax: 5.75, AAI: 98) also when we used the Bcl-2/Bax cutoff level that was chosen in the study of Del Poeta (3.33; ref. 18) this resulted in a significant shorter DFS ($P = 0.02$, data not shown). Furthermore, in a clinical setting, the use of antisense Bcl-2 oligonucleotides as an adjuvant in AML treatment, tested in a phase 1 study (19), further emphasizes the well-established prognostic value of antiapoptosis proteins as a potential important resistance mechanism to chemotherapy. However, what remains unclear up to now, is how an up-regulated antiapoptosis protein profile at diagnosis exerts its effect throughout the course of the disease/treatment in terms of the number of malignant cells surviving chemotherapy (i.e., MRD cells).

To investigate that, we had to be able to reliably detect MRD cells. Therefore, we relied on the recently well-established flow cytometric MRD detection method for patients in clinical remission and we were able to show that patients with high levels of minimal residual disease cells had a shorter survival, especially DFS. It has, however, never been determined whether the prognostic value of apoptosis-related proteins is mediated by the frequency of MRD cells resulting from incomplete eradication of leukemic cells present in the bone marrow from the patient at diagnosis. To this end, we correlated the apoptosis-related proteins at diagnosis with the frequency of MRD cells and indeed found a strong correlation among Bcl-2, the Bcl-2/Bax, and the AAI with MRD after the first cycle of chemotherapy.
chemotherapy. Elaborating on this, we showed that MRD frequencies differed significantly between patient groups defined by a Bcl-2/Bax cutoff of 5.75 or an AAI cutoff of 98. These results show that the antiapoptosis protein expression level at diagnosis predicts the relative number of malignant cells in remission bone marrow, thereby in turn affecting the length of DFS. Furthermore, it was observed that the relationship between apoptosis profiles at diagnosis and MRD frequency were not obvious after the second and third cycle of chemotherapy. Overall, this would implicate that antiapoptosis proteins are important for the debulking effect during the first chemotherapy. Elaborating on this, we showed that MRD emergence of MRD and especially to its maintenance in the second and consolidation course. Multidrug resistance is likely to contribute to both processes (20). The findings that multidrug resistance characteristics at diagnosis are preserved throughout the course of disease (21), whereas in contrast, MRD cells acquire a more apoptosis-sensitive protein profile (22) support this view. The development of therapy strategies to decrease the apoptosis resistance at diagnosis should be stimulated and simultaneously be extended from Bcl-2 to other antiapoptosis and proapoptosis proteins for example those that are described in this study.

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References

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