CD34+ Cells from Acute Myeloid Leukemia, Myelodysplastic Syndromes, and Normal Bone Marrow Display Different Apoptosis and Drug Resistance–Associated Phenotypes

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ABSTRACT

Myelodysplastic syndromes and acute myeloid leukemia (AML) are heterogeneous disorders in which conflicting results in apoptosis and multidrug resistance (MDR) have been reported. We have evaluated by multiparameter flow cytometry the expression of apoptosis- (APO2.7, bcl-2, and bax) and MDR-related proteins [P-glycoprotein (P-gp), multidrug resistance protein (MRP), and lung resistance protein (LRP)] specifically on bone marrow (BM) CD34+ cells, and their major CD32- and CD32+ subsets, in de novo AML (n = 90), high-risk myelodysplastic syndrome (n = 9), and low-risk myelodysplastic syndrome (n = 21) patients at diagnosis, and compared with normal BM CD34+ cells (n = 6). CD34+ myeloid cells from AML and high-risk myelodysplastic syndrome patients displayed higher expression of bcl-2 (P < 0.0001) and lower reactivity for APO2.7 (P = 0.002) compared with low-risk myelodysplastic syndrome and normal controls. Similar results applied to the two predefined CD34+ myeloid cell subsets. No significant differences were found in the expression of P-gp, MRP, and LRP between low-risk myelodysplastic syndrome patients and normal BM, but decreased expression of MRP (P < 0.03) in AML and high-risk myelodysplastic syndromes and P-gp (P = 0.008) in high-risk myelodysplastic syndromes were detected. Hierarchical clustering analysis showed that low-risk myelodysplastic syndrome patients were clustered next to normal BM samples, whereas high-risk myelodysplastic syndromes were clustered together and mixed with the de novo AML patients. In summary, increased resistance to chemotherapy of CD34+ cells from both AML and high-risk myelodysplastic syndromes would be explained more appropriately in terms of an increased antiapoptotic phenotype rather than a MDR phenotype. In low-risk myelodysplastic syndromes abnormally high apoptotic rates would be restricted to the CD34− cell compartments.

INTRODUCTION

Myelodysplastic syndromes are a heterogeneous group of clonal hematopoietic stem cell disorders characterized by peripheral cytopenias despite normal or hypercellular bone marrow (BM; ref. 1), which could evolve into an overt acute myeloid leukemia (AML). Underlying excessive apoptosis or an apoptosis-associated phenotype of BM cells, including clonal CD34+ precursors, has been identified as a potential explanation for the ineffective hematopoiesis, especially prominent in early myelodysplastic syndrome cases (2–12). AML also represents a heterogeneous group of malignant stem cell diseases in which CD34+ blast cells are frequently identified (13).

Resistance of neoplastic cells to chemotherapy has been known to be multifactorial for some time. At present, it is well established that most chemotherapy drugs used in the treatment of hematologic malignancies produce cell death through apoptosis, and, as such, the increase of antiapoptotic mechanisms in tumor cells may represent a major factor responsible for resistance to chemotherapy (14–16). Among other proteins, the members of the bcl-2 family either accelerate or inhibit apoptosis in response to a variety of stimuli, and the ratio between pro- and antiapoptotic proteins apparently determines the susceptibility of individual cells to death (15, 16). In addition, expression of proteins implicated in drug transport, and/or inactivation, has also been directly involved in modulating sensitivity and resistance to multiple drugs (14). Among others, these include the P-glycoprotein (P-gp or MDR1), the multidrug resistance protein (MRP), and the lung resistance protein (LRP; ref. 14).

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MDR1 has been associated with CD34 expression, immature French-American-British (FAB) subtypes, and unfavorable karyotypes, as well as with low complete remission and/or survival rates (17–23). In turn, in myelodysplastic syndromes, proapoptotic features are frequently observed, and a multidrug resistance (MDR) phenotype has been associated with progression (22–23) and transformation into AML (24–27). Moreover, in both myelodysplastic syndromes and AML, MDR phenotypes have also been related to CD34 expression (23–26).

Interestingly, in AML and myelodysplastic syndromes, the number of CD34+ blast cells is higher in the advanced stages of the disease and at relapse (1, 28). This suggests that CD34+ blast cells are particularly resistant to chemotherapy and less susceptible to apoptosis (29, 30) as compared with their normal counterpart (2, 15, 22, 31–33). However, despite the increasing number of studies in which apoptosis and/or drug resistance phenotypes are analyzed in myelodysplastic syndromes and/or AML, conflicting results have frequently been reported (34–37), and few studies have been published in which multiparameter analyses of these phenotypes are specifically done on neoplastic CD34+ cells, in comparison to normal CD34+ BM progenitors.

In the present study, we have comparatively analyzed the expression of several apoptosis (APO2.7, bcl-2, and bax) and MDR-associated proteins (P-gp, MRP, and LRP) specifically in expression of several apoptosis (APO2.7, bcl-2, and bax) and progenitors.

In the present study, we have comparatively analyzed the expression of several apoptosis (APO2.7, bcl-2, and bax) and MDR-associated proteins (P-gp, MRP, and LRP) specifically in BM CD34+ cells and their major CD32dim and CD32bright subsets, in de novo AML and myelodyslastic syndromes patients at diagnosis, and compared them to normal CD34+ BM cells. Our results indicate that increased resistance to chemotherapy of CD34+ cells from both AML and high-risk myelodysplastic syndromes would most probably be related to increased resistance to apoptosis rather than to the mechanisms involved in MDR. In turn, in low-risk myelodysplastic syndromes, CD34+ myeloid cells display a high degree of similarity to normal precursors regarding the expression of apoptosis- and MDR-associated proteins, and high apoptotic rates would be restricted to the CD34+ cell compartments.

MATERIALS AND METHODS

Patients and Samples. BM samples were obtained from 120 patients with myeloid malignancies (90 de novo acute non-promyelocytic leukemia and 30 myelodysplastic syndromes) and from 6 healthy donors. In all of the cases, BM samples were collected in EDTA anticoagulant before the initiation of therapy and processed immediately or within the first 24 hours after collection, at the latest. In the de novo AML patient group, only cases in which a CD34+ blast cell subset was identified were included in this study.

The distribution of the de novo AML cases according to the FAB classification (38) was as follows: M0, 15%; M1, 20%; M2, 29%; M4, 15%; M5, 15%; M6, 5%; and M7, 1%. From the 30 myelodyslastic syndrome patients (39), 35% were classified as refractory anemia (RA), 19% as RA with ringed sideroblasts, 35% as RA with an excess of blasts, and 11% as RA with an excess of blasts in transformation. Myelodysplastic syndrome patients were grouped according to both the International Prognostic Scoring System (40) and the Spanish Prognostic Scoring System (41) into low-risk (International Prognostic Scoring System < 1.5; Spanish Prognostic Scoring System < 3)–21 cases (70%); and high-risk myelodysplastic syndromes (International Prognostic Scoring System > 1.5; Spanish Prognostic Scoring System > 3)–9 cases (30%).

Immunophenotypic Studies. Erythrocyte-lysed, freshly obtained BM samples were analyzed with a four-color immunofluorescence technique for the simultaneous staining of surface markers alone or surface markers together with cytoplasmic (cyt) antigens, according to well-established methods, which have been previously described in detail (15, 42). The following combinations of monoclonal antibodies conjugated to FITC/phycocerythrin (PE)/PE-cyanine 5 (Cy5)/allophycocyanine (APC) were used: Cy5bcl-2/CD34/CD34/CD34/CD34, Cy5bax/CD32/CD32/CD34/CD34, CD32/APO2.7/CD34/CD34, CD32/APO2.7/CD34/CD34, CD19/P-gp/CD34/CD34, Cy5MRP/CD34/CD19/CD33, and Cy5LRP/CD19/CD34/CD34. Each of these 4-color combinations allowed the specific identification of the whole CD34+ cell population present in the BM samples analyzed (Fig. 1A) and the discrimination within all of the CD34+ cells between B-lymphoid and myeloid CD34+ cells (Fig. 1B), providing identical results as regards the identification and enumeration of these cell subsets. Additionally, two subpopulations of CD34+ non-B cells could also be identified with the first three combinations, one corresponding to the more immature (CD34+/CD34dim) and the other to the more mature myeloid cells (CD34+/CD34bright; Fig. 1C). The specific monoclonal antibodies clones used and their source was as follows: anti-APO2.7–PE (clone 2.7A6A3), anti-bax (clone 4F11), CD19PECy5 (clone J4.119), and CD34PECy5 (clone 581) were purchased from Beckman-Coultier (Miami, FL); anti-Bcl-2–FITC (clone 124) was obtained from DakoCytomation (Glostrup, Denmark); anti-MRP (clone MRPM6) and anti-LRP (clone LRP-56) were from Chemicon (Temecula, CA); CD19–FITC (clone 4G7), CD34–PE (clone 8G12), CD34–APC (clone p67.6) and anti-P–gp (clone 15D3) were from Becton Dickinson Biosciences (San José, CA); CD32–FITC (clone AT 10) and CD32–PE (clone AT 10) were obtained from Serotec (Oxford, United Kingdom); and CD45–APC (clone H130) was purchased from Caltag Laboratories (San Francisco, CA). Appropriate isotype-matched negative controls were stained in parallel.

Data acquisition was done in a FACScalibur flow cytometer (Becton Dickinson Biosciences) with the CellQuest software program (Becton Dickinson Biosciences). In all of the cases analyzed, a minimum of 2 × 106 BM events corresponding to the whole BM cellularity were acquired for each four-color staining. In addition, a second acquisition step was done with an electronic “live gate,” in which only those cells displaying low/intermediate SSC and strong reactivity for CD34 were selected from a total of 3 × 108 events, to obtain information on high numbers of CD34+ cells for each combination of monoclonal antibodies. For data analysis, the Paint-a-Gate software program (Becton Dickinson Biosciences) was used.

The percentage of CD34+ ranged from 5 to 95% (median 42%) of all of the BM nucleated cells in de novo AML and between 0.3 and 22% (median 3.6%) of all of the nucleated cells in myelodysplastic syndromes, significantly (P < 0.0001) higher numbers of CD34+ cells were being found in high-risk as compared with low-risk myelodysplastic syndromes—median of 8.5% (range, 1.5 to 22%) versus 1.5% (range, 0.3 to 4.4%),
respectively. In normal BM, the median number of CD34+ cells was of 0.5% (range, 0.22 to 0.7%). In both de novo AML and high-risk myelodysplastic syndrome patients, all of the BM CD34+ cells corresponded to myeloid cells, whereas CD34+ B-lymphoid precursors were present in all of the normal BM samples and in 8 of 21 (38%) low-risk myelodysplastic syndromes patients, where they represented 0.06 ± 0.03% and 0.1 ± 0.4% of the overall cellularity, respectively (Table 1).

Among CD34+ non-B lymphoid progenitors, a clear predominance of the more immature CD34+/CD32−/dim precursors, as compared with the CD34+/CD32−/bright cells, was observed in all groups of individuals (high-risk myelodysplastic syndromes, 8.2 ± 7.7 versus 0.7 ± 0.8; P = 0.002; low-risk myelodysplastic syndromes, 1.1 ± 0.9 versus 0.3 ± 0.3; P < 0.0001; and normal BM, 0.32 ± 0.1 versus 0.09 ± 0.03; P = 0.004), except in the de novo AML cases who had similar proportions of both subpopulations (42 ± 22% versus 41 ± 24%, respectively; P > 0.05; Table 1).

The mean fluorescence intensity obtained for each individual apoptosis- and MDR-associated marker analyzed and the corresponding isotype-matched negative control were specifically recorded for each cell subpopulation. For each individual marker, results were reported as relative fluorescence intensity units (RFI) calculated as the ratio between the mean fluorescence intensity of the cells under analysis specifically stained for a given apoptosis- or MDR-associated protein and the mean fluorescence intensity obtained for the same cell subset in the corresponding isotype-matched negative control. In all of the cases, the consensus recommendations for staining and analysis of multidrug resistance-associated proteins were strictly followed (34–36).

Statistical Methods. For all of the phenotypic variables under study, median and mean values as well as their SD and range were calculated with the SPSS software program (SPSS 10.0, Inc., Chicago, IL). The Kruskal-Wallis and the Mann-Whitney U tests were used to estimate the statistical significance

![Image](https://clinicalcancerres.aacrjournals.org/content/7/6/1760/F1.large.jpg)

**Fig. 1** Representative SSC/CD45/CD34 three-dimensional dot plot corresponding to the whole cellularity of a BM sample from a patient with low-risk myelodysplastic syndromes, illustrating in A how total CD34+ cells (black dots) were identified based on their low/intermediate SSC and strong reactivity for CD34; B shows the distribution of the B-lymphoid (black dots) and myeloid (gray dots) precursor cells within the total (gated) CD34+ BM cells, based on their FSC/SSC characteristics. A representative bivariate dot plot of CD32− dim precursors corresponding isotype-matched negative control. In all of the samples and in 8 of 21 (38%) low-risk myelodysplastic syndromes patients, where they represented 0.06 ± 0.03% and 0.1 ± 0.4% of the overall cellularity, respectively (Table 1).
of the differences observed between groups. \( P < 0.05 \) was considered statistically significance.

For hierarchical clustering analyses, log-transformed RFI values of each apoptotic and MDR-related protein measured on BM CD34\(^+\) cells for each individual sample were used. Before the analysis, data were normalized dividing the RFI value of each apoptosis- and MDR-associated protein obtained for each individual sample by the mean RFI value observed for the same protein in normal BM samples; a logarithmic transformation was applied to the values of the ratio for individual data sets.

Hierarchical clustering analysis was done with the J-Express Pro V2.1 software (MolMine AS, Bergen, Norway). The average-linkage method, based on the analysis of Euclidean distance matrices, was used for the clustering of individual cases from each diagnostic group and normal controls. In this part of the study, the following variables were considered: relative number of CD34\(^+\) cells present in the BM and the bcl-2, APO2.7, bax, P-gp, MRP, LRP, and RFI ratios specifically obtained for BM CD34\(^+\) cells in each individual sample.

**RESULTS**

Concerning the expression of apoptosis-associated proteins (Fig. 2), BM CD34\(^+\) cells in AML and high-risk myelodysplastic syndrome cases showed a significantly (\( P < 0.0001 \)) higher bcl-2 expression than low-risk myelodysplastic syndrome patients and control subjects (mean RFI ± SD of 13.1 ± 7.2 and 12.4 ± 7.0 versus 7.0 ± 3.0 and 7.3 ± 1.5, respectively), no statistically significant differences were found between AML and high-risk myelodysplastic syndromes nor between low-risk myelodysplastic syndromes and control BM. As shown in Fig. 2, these differences

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**Fig. 2** RFI reflecting cyt expression of the bcl-2, APO2.7, and bax apoptosis-related intracellular proteins on CD34\(^+\) cells and their CD32\(^{-/}\)dim and CD32\(^+\) myeloid cell subsets from de novo AML (n = 90), high-risk (hr) myelodysplastic syndromes (n = 9), low-risk (lr) myelodysplastic syndromes (n = 21), and normal BM (NBM; n = 6), as analyzed by multiparameter flow cytometry. Boxes extend from the 25th to the 75th percentiles; the line in the middle and vertical lines represent median values and the 95% confidence intervals, respectively. The following statistically significant differences were observed: *, \( P \leq 0.0001 \) on comparing cyt bcl-2 RFI of CD34\(^+\) cells from AML and hr-myelodysplastic syndrome cases with lr-myelodysplastic syndrome cases and normal BM; **, \( P \leq 0.005 \) and ***, \( P \leq 0.01 \) on comparing the cyt APO2.7 RFI of CD34\(^-\) cells from de novo AML patients with low-myelodysplastic syndrome patients and normal BM; and ●, \( P \leq 0.005 \) on comparing the cyt APO2.7 RFI of CD34\(^+\) cells from hr-myelodysplastic syndrome cases with that of lr-myelodysplastic syndrome patients. Bars, ±SD.
similarly applied to the two predefined CD34⁺/CD32⁻dim (P < 0.0001) and CD34⁺/CD32²high (P < 0.0001) myeloid subsets. On comparing these two cell subpopulations, it was observed that in AML patients the expression of bcl-2 was significantly higher in the more immature subset (CD34⁺/CD32⁻dim) as compared with the more mature group (CD34⁺/CD32²high); mean RFI ± SD of 14.8 ± 7.9 versus 10.8 ± 5.2 (P = 0.011). By contrast, in myelodysplastic syndromes or normal individuals, no significant differences were observed between these two subpopulations according to bcl-2 expression. In line with bcl-2 results, CD34克斯 were observed between these two subpopulations according to bcl-2 expression. No statistically significant differences were found in the expression of these three MDR-proteins, we did not find any statistically significant differences between de novo AML and either low-risk or high-risk myelodysplastic syndrome patients. Regarding myelodysplastic syndrome patients, BM CD34克斯 cells from high-risk myelodysplastic syndrome cases showed a lower expression of both P-gp (P = 0.008) and MRP (P = 0.009), but not LRP, as compared with normal controls (mean RFI ± SD for P-gp of 1.9 ± 0.9 versus 4.0 ± 2.0 and for MRP of 1.5 ± 0.5 versus 3.1 ± 0.8, respectively). No statistically significant differences were found between low-risk myelodysplastic syndromes, high-risk myelodysplastic syndromes, and normal controls in terms of the expression of P-gp, MRP, and LRP on BM CD34克斯 cells (Fig. 3).

Hierarchical clustering analysis based on the relative number of CD34克斯 cells present in the BM and the amount expression of the different apoptosis- (bcl-2, APO2.7, and bax) and MDR-associated proteins (P-gp, MRP, and LRP) allowed the classification of all of the individual samples into two major groups (Fig. 4). In the first group, low-risk myelodysplastic syndrome patients were clustered next to normal BM samples, and in the second group, high-risk myelodysplastic syndromes were clustered together and mixed with de novo AML patients.

**DISCUSSION**

The CD34克斯 compartment of BM cells in both AML and myelodysplastic syndromes is believed to play a crucial role in the behavior of the disease because it potentially contains the neoplastic precursors with clonogenic capability. Until now, limited information based on conflicting results has been available concerning the expression of apoptosis- and MDR-related proteins on BM CD34克斯 cells from myelodysplastic syndrome and AML patients (3–12, 23–27). Such controversial results could be attributed, at least in part, to differences in the study design (34–37, 43). To avoid methodological pitfalls, in the present study we have restricted the analysis of apoptosis and MDR phenotypes to the study of the expression of several well-characterized proteins by multiparametric flow cytometry.
in fresh whole BM samples, obtained from untreated AML and myelodysplastic syndrome patients that contained a well-defined population of CD34 + cells, and compared them to normal BM CD34 + cells.

At present, it is well established that entry into the execution phase of apoptosis greatly depends on the balance between the cytoplasmic levels of several pro- and antiapoptotic proteins from which the members of the bcl-2 protein play an essential role (15, 16, 37). In the present study, we found high levels of the bcl-2 antiapoptotic protein and low amounts of the APO2.7 proapoptotic protein on BM CD34 + myeloid cells from AML and high-risk myelodysplastic syndrome patients. This concurs with previous studies (3, 5, 7–9). Of particular interest is the observation that high-risk myelodysplastic syndrome cases dis-

![Hierarchical clustering analysis of de novo AML (n = 90), high-risk myelodysplastic syndromes (n = 9), low-risk myelodysplastic syndromes (n = 21), and normal BM samples (n = 6) based on the number and phenotype of CD34 + BM cells for the apoptosis (bcl-2, APO2.7, and bax) and MDR-associated proteins (P-gp, MRP, and LRP) analyzed. Rows represent the different cases studied, labeled in a gray scale according to diagnosis, and columns represent each individual variable included in the analysis. The relative level of expression of each marker is represented by a color: red = expression > mean; green = expression < mean; color intensity represents the magnitude of the deviation from the mean. As indicated, the scale extends from −7.630 to +7.630 (in log2 units).]
played greater amounts of bcl-2 and reduced expression of APO2.7 as compared with low-risk myelodysplastic syndrome patients, resembling the phenotype of AML cases. In line with these findings, several authors (3–7, 9) have reported that in high-risk myelodysplastic syndromes, CD34+ cells are more resistant to apoptosis than they are in low-risk myelodysplastic syndromes, and that an increased ratio between proapoptotic and antiapoptotic proteins from the bcl-2 family is associated with disease evolution into aggressive forms of the disease (4, 7, 9). In contrast, Huh et al. (10) did not find any differences between RA with an excess of blasts in transformation and other subtypes of myelodysplastic syndromes, although when compared with AML patients, CD34+ cells from RA with an excess of blasts in transformation cases were more prone to apoptosis. Although we have not explored the expression of either bad or bcl-X in the present study, no statistically significant differences were observed in bax levels in any of the groups analyzed, supporting the notion (4, 9) that some proapoptotic proteins like bax do not play a crucial role in these myeloid malignancies.

Interestingly, we found that expression of the APO2.7, bcl-2, and bax proteins on BM CD34+ cells from low-risk myelodysplastic syndromes were within the normal range. These results are in line with those reported by Davis et al. (5) and Pecci et al. (12) who found no differences in the percentage of bcl-2 and the bcl-2 index or the degree of apoptosis between early myelodysplastic syndromes and normal BM. These findings are of particular interest in light of our previous observations (44) from a similar series of myelodysplastic syndrome patients, in which we showed that particularly in low-risk myelodysplastic syndromes, CD34+ nucleated red cells and myelomonocytic cells display a proapoptotic phenotype, probably reflecting the greater susceptibility of the cell to apoptosis. Such differences in the apoptotic phenotype of the CD34+ and CD34− compartments of BM cells in myelodysplastic syndromes concur with the clinical behavior of the disease, and they suggest that the more mature CD34− cells, but not the CD34+ cell compartment, could be responsible for the increased apoptosis observed in myelodysplastic syndromes (12, 44). On the basis of these findings, we aimed to investigate whether or not within the different maturational compartments of CD34+ myeloid precursors, there could also exist differences in the apoptotic-related phenotype. Our results show no significant differences in the expression of bcl-2, bax, and APO2.7 between the more immature CD34+/CD32−/dim and the more differentiated CD34+/CD32−/+dim myeloid cells from myelodysplastic syndromes patients nor from normal BM. In contrast, in AML, the CD34+/CD32−/+dim subpopulation displayed greater expression of bcl-2 than the more mature CD34+/CD32− cells, which is consistent with previous results (17, 18, 31) showing that expression of bcl-2 decreases with maturation of blast cells.

In contrast to AML (14, 20, 23), until now few studies have been reported (24–27, 45) in which the MDR phenotype of CD34+ cells is conserved during leukemic transformation. In addition, our results show that in low-risk myelodysplastic syndrome patients with normal BM and high-risk myelodysplastic syndromes together with de novo AML patients, thereby supporting our results.

These results would suggest that resistance to chemotherapy in both AML and high-risk myelodysplastic syndromes could most probably be related to the increased resistance in CD34+ leukemic cells to apoptosis than to the mechanisms evolved in intracellular or extracellular drug transport and extrusion. In addition, our results show that in low-risk myelodysplastic syndromes, CD34+ myeloid cells display a high degree of similarity to normal precursors regarding the expression of apoptosis- and MDR-associated proteins.

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