Differences between the CD34+ and CD34– blast compartments in apoptosis resistance in acute myeloid leukemia

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Background and Objectives. Altered expression of members of the Bcl-2 family might account for the observed apoptosis resistance to chemotherapy in acute myeloid leukemia (AML). Given the poor prognosis associated with CD34+ expression in AML, we studied the role of spontaneous apoptosis and apoptosis regulatory proteins in sorted CD34+ and CD34- primary AML fractions.

Design and Methods. The expression levels of apoptosis regulatory proteins and spontaneous apoptosis were measured in primary AML samples by Western blot analysis and flow cytometry. To determine the role of CD34+ cells in apoptosis resistance, spontaneous apoptosis in serum-free conditions and apoptosis regulatory protein levels were measured in CD34+ and CD34– sorted cells from CD34+ primary AML samples.

Results. We show that CD34+ AML fractions are more resistant to apoptosis than are corresponding CD34– AML fractions, and that this is paralleled by higher Bcl-2, Bcl-x_L, Mcl-1, Pgp and lower Bax expression levels. Interestingly, as the percentage of CD34 cells increased in the primary AML sample, so too did the apoptosis resistance in the corresponding CD34– fraction, which was reflected by an increasing anti-apoptosis protein profile.

Interpretation and Conclusions. The data show that the CD34+ fraction is more resistant to apoptosis than is the corresponding CD34– fraction and secondly that the AML as a whole is more apoptosis resistant with increasing CD34 percentage.

Key words: apoptosis, Bcl-2, Bcl-x_L, Bax, AML, CD34a.

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Acute myeloid leukemia (AML) is a malignancy of immature cells. Intensive chemotherapy is the mainstay of treatment, but primary resistance and relapse after an apparent initial remission are frequently observed. Various factors are considered to be responsible for the difference in response to chemotherapy and to serve as prognostic parameters predicting outcome of therapy. Biological heterogeneity lies at the bottom of drug resistance. CD34 expression,[1-9] Bcl-2 expression,[10–13] the Bcl-2/Bax ratio,[14] apoptosis resistance *in vitro*,[15–17] autonomous proliferation *in vitro*,[11, 18-20] and cell cycle perturbations[21] have been associated with poor prognosis. The classical multidrug transporter, Pgp, has been measured in large series of AML patients and has been established as a major indicator of poor prognosis.[22–28]

Contradictory results have been reported concerning the role of CD34 in AML, and in an attempt to establish the clinical significance of CD34 expression in AML, Kanda *et al*. overviewed 22 studies, encompassing 2,483 patients.[29] Twelve of these studies showed that AML patients with CD34– blasts had a significantly better complete remission (CR) rate than did CD34+ patients, whereas such a difference in CR rate was not apparent in the other ten studies. Given the significant heterogeneity between the studies, Kanda *et al*. were not able to pinpoint CD34 expression as an adverse prognostic marker. Bcl-2, the best-known anti-apoptosis member of the Bcl-2 family of proteins, is heterogeneously expressed in AML and its expression often upregulated compared to levels in non-malignant CD34+ counterparts.[17;30–34] Down-regulation of Bcl-2 has been shown to reverse resistance to chemotherapy in several experimental systems.[35] High expression of Bcl-2 in AML, which is often correlated with high CD34 expression, is associated with primary resistance to chemotherapy.[14;36-39] Pgp activity has also been shown to be associated with AML cases with high CD34 expression.[17] Besides its role in multidrug resistance in AML, Pgp has recently been described to be an inhibitor of caspase-mediated apoptosis.[40–42]

Apoptosis resistance is a central theme in the heterogeneous response of AML blasts to treatment. Although most studies only measure proteins that regulate apoptosis, it seems more informative to combine measurements of apoptosis-related proteins with the ability of AML blasts to undergo apoptosis and die. The reports of Smith *et al*.[15] and Wuchter *et al*.[17] describe that measurements of spontaneous apoptosis of *de novo* AML blasts can discriminate between patients who will enter

CR and those who will not. The former study showed the relevance of spontaneous apoptosis measurement, but no attempts were made to identify possibly more resistant blast subsets. The study, by Banker *et al.*,[33] demonstrated that AML samples with high Bcl-2 expression generally showed low apoptosis *in vitro*. In order to investigate the mechanism of apoptosis resistance in AML we considered the putative contribution of $CD34^+$ AML cells to apoptosis resistance in AML by examining $CD34^+$ and $CD34^-$ subsets of cells from patients with AML in relation to spontaneous apoptosis and expression of apoptosis-related proteins. In addition, an enlarged set of apoptosis-related proteins, i.e. Bcl-2, Bax, Bcl-xL, Mcl-1 and Pgp, was used for these experiments.

Design and Methods

AML patients, normal bone marrow donors and sampling

After informed consent, bone marrow aspirates from newly diagnosed AML patients or healthy donors were isolated by Ficoll gradient (1.077 g/mL) according to standard procedures. The patients' characteristics are summarized in Table 1. For Western blot analysis, mononuclear cells were pelleted, frozen in liquid nitrogen and stored at -80°C until analysis. For the apoptosis assays and flow cytometric detection of Bcl-2, Bcl-x_L, Bax, Mcl-1 and Pgp activity, mononuclear cells were processed immediately and analyzed.

Reagents

Iscove's modified Dulbecco's medium, folic acid, penicillin and streptomycin were from Gibco BrL, (NY, USA), 2-mercapto-ethanol, human transferrin, saponin, Tween 20, bromophenolblue and BSA were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Annexin V fluoroscein isothiocyanate (FITC) was purchased from Nexins Research (Kattendijke, The Netherlands), 7-amino actinomycin D (7-AAD) Via-Probe™ from Pharmingen (San Diego, CA, USA) and Triton X-100 was from Brunschwig (Amsterdam, The Netherlands). PSC833 was a gift from Novartis (Basel, Switzerland).

Antibodies

The p53 (DO-7) and Bcl-2 (clone 124) mouse monoclonal antibodies were obtained from Dako (Diagnostics B.V., The Netherlands). The mouse monoclonal antibody c-myc (9E10), and the rabbit polyclonal antibodies Bax (P-19), Bcl-$x_{S/L}$ (S-18) and Mcl-1 (S-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PerCP-conjugated CD45 (clone 2D1) and APC-conjugated CD34 (clone 8G12) were from Becton Dickinson (San José, CA, USA) and FITC-conjugated mouse anti-human Bcl-2 (clone 124) and FITC-conjugated rat anti-rabbit IgG was from Dako.

Table 1. Patients' characteristics.

FAB classification[1]	*n (%)*
M0	5 (11)
M1	8 (17)
M2	9 (19)
M4	10 (21)
M5	5 (11)
M6	4 (9)
MDS	5 (11)
Unclassified	1 (2)
Cytogenetics[2]	*n (%)*
Favorable	4 (9)
Intermediate	33 (70)
Poor	4 (9)
Not tested	6 (13)

[1]French-American-British leukemia classification system; [2]Grouped according to Grimwade et al.[62]

Cell culture and apoptosis assay

To assess spontaneous apoptosis *in vitro*, 0.5×10^6 cells/well were cultured at 37°C in 24-well culture plates (Costar, Corning, NY, USA) in serum-free Iscove's modified Dulbecco's medium supplemented with 7.5% BSA, 100 IU/mL penicillin, 100 μg/mL streptomycin, 10 μg/mL folic acid, 5×10^{-5} M 2-mercapto-ethanol, and 300 μg/mL human transferrin. The cells were harvested at set times (0, 2, 4, 6, and 24 hours). The percentage of apoptotic cells in each harvested sample was assessed with the annexin V assay.[43-46] Cells were stained with FITC-labeled annexin V according to the manufacturer's instructions. In short, after washing the cells in phosphate-buffered saline (PBS) the cell pellet was resuspended in 1 mL of binding buffer (at a concentration of 10^5-10^6 cells/mL). Next, 5 μL of annexin V-FITC (1:10 dilution in binding buffer) and 5 μL of 7-AAD were added to 490 μL of the cell suspension. The cells were incubated for 10 minutes in the dark on ice. The annexin V and 7-AAD fluorescence were measured using a FACScalibur (Becton Dickinson, Mountain View, CA, USA) equipped with an argon and red iodide laser. Quadrants were set in the annexin V/7-AAD dot plot according to the settings of t=0 hrs of culture. Corrections were made when small shifts of the annexin V dot plot occurred during incubation. According to Vermes *et al.*[43,46] the annexin $V^-/7^-AAD^-$ cells (lower left quadrant) were designated as intact (or non-apoptotic), the annexin $V^+/7^-AAD^-$ cells (lower right quadrant) as apoptotic, and the annexin $V^+/7^-AAD^+$ cells (upper right quadrant) as (secondary) necrotic cells. For each sample 10^4 cells

were analyzed and all measurements were performed using the same instrument settings.

CD34 determination, CD34 positivity and CD34+-cell selection

The CD34 percentage was measured with an APC-conjugated MoAb against CD34. A gate was set around the population of cells exhibiting an APC fluorescence equal to or higher than the second log. The median CD34 percentage of the set of 22 AML samples was 14.5% (range 0%-88%). From these samples, seventeen samples contained 10% or more $CD34^+$ cells (range: 10%-88%, median: 38%) and were separated into $CD34^+$ and $CD34^-$ subsets. Five samples contained less than 10% of $CD34^+$ cells (range 0%-4%, median: 0.3%).

In an additional set of 25 AML patients in whom multiparametric flow cytometry assay was used to detect apoptosis-related proteins, the median CD34 percentage was 27% (range: 0.04%-81%). The CD34 mean fluorescence intensity ratio (MFI) was calculated by the following formula: MFI [CD34-APC]/ MFI [IgG1-APC] of the blast cell population.

The mononuclear cells were separated into $CD34^+$ and $CD34^-$ fractions using a MiniMacs magnetic cell sorting system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's recommendations. The mean purity of the separated $CD34^+$ fraction was 92% (79%-98%) and that of the $CD34^-$ fraction, 90% (52%-98%). Morphologic inspection of cytospin slides after Ficoll purification and before CD34 selection was performed when blast percentages were lower than 90%. This inspection showed that the majority of cells other than blasts were mainly (pro-) myelocytes and a few eosinophils, monocytes and lymphocytes.

Apoptosis-related protein content by Western blot analysis

Cells were pelleted by centrifugation at 300g for 5 minutes. Cell pellets of $1\text{-}5\times10^6$ cells were immediately frozen in liquid nitrogen and stored at –80° C until analysis. Frozen-thawed cell pellets were lysed in 50 µL /2×10^6 cells of lysing solution containing 0.06 mM $NaVO_3$, 176 mM NaCl, 11.8 mM Tris-HCl pH 7.8, 5.9 mM EDTA, 1.2% Triton X-100 and protease inhibitors (complete mini protease inhibitors from Boehringer Mannheim, Germany) at 4°C for 30 minutes. An aliquot of the lysate was diluted 1:400 with demi water and used for protein level estimation using the Bio Rad protein assay (Bio Rad Laboratories Ltd, Hertfordshire, UK) that employs the Bradford method.[47] Sample buffer containing 0.19 M Tris HCl, pH 6.8, 30 mM DTT, 2% SDS, 0.1% bromophenolblue and 30% glycerol was added to the lysate in a lysate to sample buffer ratio of 2:1. The samples were heated at 100°C for 5 minutes. The HL60 cell line was used both as a positive control for Bcl-2, Bax, Bcl-x_L, Mcl-1 and c-myc expression as well as a calibrator. The KG1 cell line was used as a positive control and calibrator for p53 expression. Cells were lysed as described above and diluted to give a five point standard curve for each blot by loading 5, 10, 15, 20 and 25 µg (HL60) or 1, 3, 5, 7 and 10 µg (KG1) of total cellular protein. Each sample was subjected to SDS-polyacrylamide gel electrophoresis according to the method of Laemmli[48] using the mini-protein II system supplied by Bio-Rad Laboratories Ltd. The amount of total protein loaded for each sample varied from 1-80 µg depending on the protein to be detected. All proteins were analyzed on 15% acrylamide gels. For an individual experiment five AML protein concentrations, as well as a molecular weight marker (Bio-Rad rainbow marker, Bio-Rad Laboratories Ltd, Hertfordshore, UK), were loaded on the same gel. Proteins from the reference cell lines were stored at -20°C in a large number of fractions and for each experiment four different concentrations of these were included on the same gel. After electrophoresis, proteins were transferred to nitrocellulose membranes (Hybond-C; Amersham, Little Chalfont, UK) using the mini-protein II system. After transfer, the blots were immersed for 1 hour in a blocking buffer of 2% BSA in PBS containing 0.1% Tween (PBS-T). Primary antibody was diluted in blocking buffer and incubated with the blots for 1 hour as follows: c-myc at 1:300, Bcl-2, Bax, Bcl-x_L and Mcl-1 at 1:500 and p53 at 1:2,000. Blots were washed thoroughly with PBS-T and incubated in secondary antibody (horseradish peroxidase-labeled anti-mouse or anti-rabbit, diluted 1:1000 in PBS-T) for 1 hour. After a final wash in PBS-T, horseradish peroxidase was detected using Lumi-Light reagent (Boehringer Mannheim, Mannheim, Germany) as described in the manufacturer's instructions. Chemiluminescence was detected by autoradiography using X-ray film. The integrated optical density (IOD) of the resulting bands was determined by densitometry using a Bio-Rad densitometer. The identity of the target protein was determined on the basis of the molecular weight marker. A linear reference line was produced by plotting the IODs of the specific protein bands of the samples with different total protein concentrations against these total protein concentrations. The specific protein value of each AML sample was generated by extrapolating the IODs of the five concentrations of the AML to the reference line. This avoids misinterpretation due to single measurements or to small differences in the amount of loaded AML protein. The experimental value of the AML protein is thus represented as a ratio relative to the positive reference control cell

line, which allows interpatient comparisons even when there are experimental differences in blotting efficiency, exposure time, and cell volume.

Apoptosis-related protein expression detected by flow cytometry

The detection of apoptosis-related protein expression by flow cytometry has previously been described in detail.[49] In brief, 1×10^6 AML or normal BM cells were incubated with PerCP-conjugated CD45 and APC-conjugated CD34 for 15 minutes at room temperature. Cells were washed with PBS/0.1% BSA/0.05% sodium azide and fixed in 1% paraformaldehyde for 5 minutes, again at room temperature. After washing, cells were permeabilized with 100 μL 0.1% saponin for 15 minutes at room temperature and washed. The cells were incubated for 30 minutes at 4°C with either 50 μL FITC-conjugated mouse anti-human Bcl-2 monoclonal antibody (1:20) or the polyclonal unconjugated rabbit anti-human antibodies against Bax (1:20), or Bcl-x_L (1:20) or Mcl-1 (1:20). For Bcl-2, cells were washed and analyzed immediately. For Bax, Bcl-x_L and Mcl-1, cells were washed, and subsequently incubated with FITC-conjugated anti-rabbit IgG (1:20). Differential staining of CD45 was used to distinguish blasts from lymphocytes, monocytes and neutrophils.[50] The levels of apoptosis-related protein expression in $CD34^+$ and $CD34^-$ blasts were expressed as ratios:

Protein expression [$CD34^+$ or $CD34^-$]=$MFI_{protein}$ [$CD34^+$ or $CD34^-$]/$MFI_{Isotype\ control}$ [$CD34^+$ or $CD34^-$]

in which MFI is the mean fluorescence intensity.

Pgp function

Detection of Pgp function using multiparameter flow cytometry has also been described in detail elsewhere.[51] The cells were washed in accumulation medium (DMEM without bicarbonate and phenol red but with 20 mM Hepes and 10% FCS), and then $0.3\text{x}10^6$ cells were incubated in 1 mL accumulation medium with 0.6 nM Syto16 with or without 1 μM PSC833 in a 37°C water bath and carefully shaken. After 45 minutes of incubation, by which time an almost steady state cellular accumulation has been reached for Syto16,[52] the cells were immediately diluted with ice-cold accumulation medium and centrifuged. Cells were then resuspended in PBS/0.1% BSA/0.05% sodium azide and analyzed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). Pgp activity is expressed as the ratio of drug fluorescence with PSC833 and drug fluorescence without PSC833 after subtraction of the fluorescence of the control cells (cells in medium, without drugs present). A ratio >1.0 is taken to represent Pgp activity.

Statistical analysis

Spearman's correlation was performed in correlation analyses with apoptosis-related proteins (WB and flow cytometry) and progenitor markers or spontaneous apoptosis. t-tests of paired samples were used to determine the differences in apoptosis-related proteins in the $CD34^+$ and $CD34^-$ AML subsets. The Wilcoxon sign rank test was used to test the differences in apoptosis between $CD34^-$ and $CD34^+$ populations. All statistical analyses were done with the SPSS software program. A p-value <0.05 was considered as statistically significant.

Results

Constitutive levels of expression of Bcl-2, Bcl-x_L, Mcl-1, Bax, c-myc, and Pgp function in relation to CD34 expression

A central issue in this study is the role of CD34 expression in resistance to chemotherapy in AML. We, therefore, first verified that earlier described correlations between CD34 and Bcl-2, Bcl-x_L and Pgp could be confirmed in the two groups of patients investigated in this study. The first group of 22 AML samples were analyzed for Bcl-2, Bcl-x_L, Mcl-1, Bax, p-53 and c-myc expression by Western blotting (Figure 1A) and Pgp function using flow cytometry, as described previously.[51] This revealed that CD34 percentage correlated significantly with Bcl-2, Bcl-x_L and Pgp (p=0.02, p=0.04 and p<0.0001, respectively). Mcl-1, c-myc and Bax expression as well as Bcl-2/Bax, Bcl-x_L/Bax and Mcl-1/Bax ratios did not directly or inversely correlate with CD34 percentages. The same proteins were analyzed by flow cytometry in the second group of 25 AML samples. Table 2 shows the correlations of CD34 percentage and CD34 MFI with apoptosis-related protein expression and function, which were significant for Bcl-2 and Pgp. In addition (*not shown*), Bcl-2/Bax, Bcl-x_L/Bax and Mcl-1/Bax ratios correlated significantly both with CD34 percentage (p=0.006, p=0.018 and p=0.003, respectively) and CD34 MFI (p=0.01, p=0.02 and p=0.03, respectively).

Spontaneous apoptosis and apoptosis-related protein expression in AML

Blast cells from the first group of AML patients were cultured for 0, 2, 4, 6, or 24 hours in the absence of serum to induce apoptosis (Figure 1B). The percentages of apoptotic cells measured at the indicated time points after serum-free culture were then correlated with expression levels of the apoptosis-related proteins at t=0 hrs. p53 was undetectable in all AML samples and was not further included in the correlation analyses. Spearman's analysis revealed several associations between

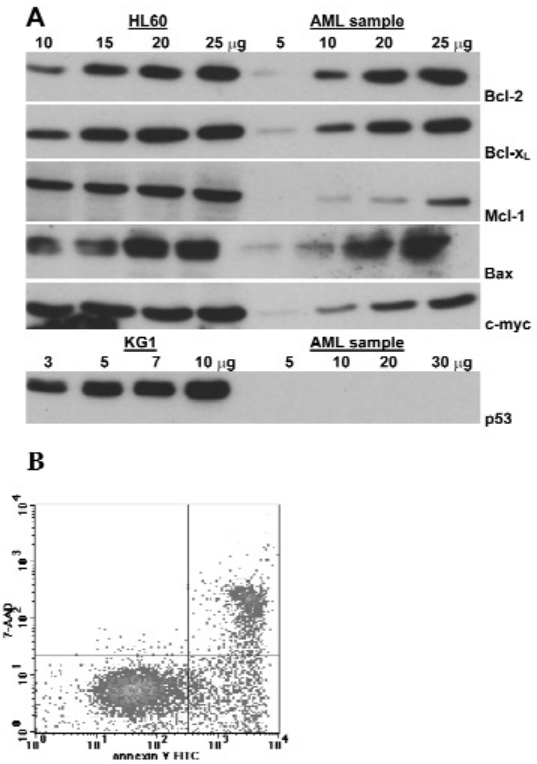

Figure 1. Detection of apoptosis-related proteins and spontaneous apoptosis in AML. A) Western blot analysis (performed as described in Design and Methods) of Bcl-2, Bcl-x_L, Mcl-1, Bax, c-myc and p53 in a case of AML. Different amounts of HL60 and KG1 were included in every run and used as a reference for the amount of total protein and specific protein loaded on the gel. B) An example of flow cytometric analysis using annexin V and 7-AAD after 4 hours of serum-free culture. Populations representing viable (lower left), apoptotic (lower right), and secondary necrotic (upper right) cells can be distinguished. Primary necrotic cells, if present, would appear in the upper left quadrant.

both protein expression and Pgp function with apoptosis resistance. In Figure 2, the *p*-values for these correlations have been indicated at different time periods of serum-free culture. At one or more time points, CD34, Bcl-2, Bcl-xL and Pgp correlated significantly and inversely with the percentage of apoptosis, while Mcl-1, Bax, and c-myc expression levels, as well as Bcl-2/Bax and Bcl-xL/Bax and Mcl-1/Bax ratios (*not shown*) showed either no (inverse) correlation or only a correlation at t=24 hrs with the percentage of apoptosis. However, at the latest time point, t=24 hrs, apoptosis could not be reliably detected in 7 cases because of extensive cell loss and only those samples in which 10,000 events could be measured were

Table 2. Correlation analysis of primitive markers and apoptosis-related parameters, measured by flow cytometry, in AML.

		CD34%	*CD34 MFI*	*CD133%*	*CD117%*
Bcl-2	R	0.61	0.48	0.60	0.44
	p	0.002	0.02	0.003	0.04
Bax	R	-0.29	-0.36	-0.19	-0.26
	p	0.16	0.07	0.37	0.22
Bcl-xL	R	0.36	0.26	0.46	0.48
	p	0.08	0.21	0.03	0.02
Mcl-1	R	0.36	0.001	0.59	0.41
	p	0.08	0.99	0.003	0.05
Pgp	R	0.46	0.46	0.14	0.07
	p	0.03	0.03	0.54	0.75
Anti/pro[1]	R	0.45	0.57	0.56	0.52
	p	0.02	0.009	0.004	0.009
Anti+pgp/pro[2]	R	0.65	0.58	0.57	0.50
	p	0.002	0.007	0.009	0.02

[1]Anti/pro= (Bcl-2+ Bcl-x_L+Mcl-1)/Bax; [2]Anti+pgp/pro= (Bcl-2+ Bcl-x_L+Mcl-1+Pgp)/Bax.

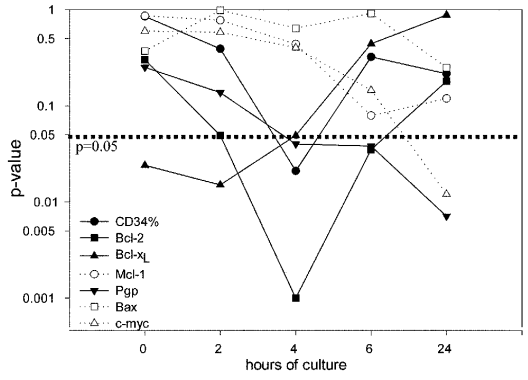

Figure 2. Expression of apoptosis-related proteins and spontaneous apoptosis. Bcl-2, Bcl-x_L, Pgp, Mcl-1, Bax, c-myc and CD34% were correlated with the percentage of apoptosis as detected by annexin V and 7-AAD after 0, 2, 4, 6, and 24 hours of serum-free culture. The *p* values of the correlations (Bax, c-myc) or inverse correlations (CD34%, Bcl-2, Bcl-x_L, Mcl-1, Pgp) with percentage apoptosis at the indicated time points are plotted; the horizontal dotted line shows the *p*=0.05 cut-off. Parameters with values below this line are thus indicated as significant predictors of apoptosis or inhibitors of apoptosis.

included. The statistical analysis at that time point is, therefore, not reliable. AML samples with a high CD34 percentage, a high Bcl-2 or Bcl-x_L level or high Pgp activity are thus associated with apoptosis resistance.

Spontaneous in vitro apoptosis of CD34⁺ and CD34⁻ subfractions

Taking together the two observations that 1) the CD34 percentage, Bcl-2, Bcl-x_L expression and Pgp activity correlate with apoptosis resistance in AML *in vitro,* and 2) Bcl-2 and Bcl-x_L expression and Pgp activity correlate with CD34 percentage in our group of AML patients, led to the following hypothesis: the CD34⁺ fraction within CD34⁺ AML is apoptosis resistant and increasing percentages of CD34⁺ cells causes the AML as a whole to become more resistant to apoptosis. To investigate this hypothesis, we sorted CD34⁺ AML samples into CD34⁺ and CD34⁻ cell populations and analyzed spontaneous apoptosis in both fractions. Two samples could not be included in this analysis since the yield of separated CD34⁻ cells was too low for analysis. Figures 3A and B present an example of an AML sample after 2 hours of serum-free culture and show a higher percentage of apoptotic and necrotic cells in the CD34⁻ fraction (A) than in the CD34⁺ fraction (B). In 11/13 AML samples, the CD34⁻ fractions showed significantly more apoptosis than the corresponding CD34⁺ fractions at all time points measured. Mean percentages of spontaneous apoptosis (Figure 3C) and total cell injury (apoptosis plus secondary necrosis, Figure 3D) in the CD34⁻ fraction were significantly higher than in CD34⁺ samples at several time points.

After MiniMacs separation of the CD34⁺ and CD34⁻ fractions, non-blast cells that might be present in the CD34⁻ fraction could interfere with the results. Morphologic examination of the AML samples after Ficoll separation revealed that besides AML blasts and a minor percentage of lymphocytes, neutrophils and monocytes, only (pro)-myelocytes were present, without any evidence of further maturation, indicating that the CD34⁻ fraction consisted mainly of malignant cells. For further confirmation that cells other than the leukemic ones in the CD34⁻ fraction had not interfered with the apoptosis results, in an additional set of experiments we performed simultaneous labeling of annexin V, CD45 and CD34 in whole AML samples: this, together with scatter characteristics, enabled the detection of apoptosis in CD34⁺ blasts and CD34⁻ blasts, in lymphocytes, monocytes and in neutrophils. Apoptosis plus secondary necrosis in these subpopulations is presented in Figure 4 showing that cell injury in lymphocytes (in 16/20 cases), monocytes (in 12/14) and neutrophils (12/14) was lower than in the malignant CD34⁻ cells. The effect, if any, of these low percentages of non-malignant cells might be a

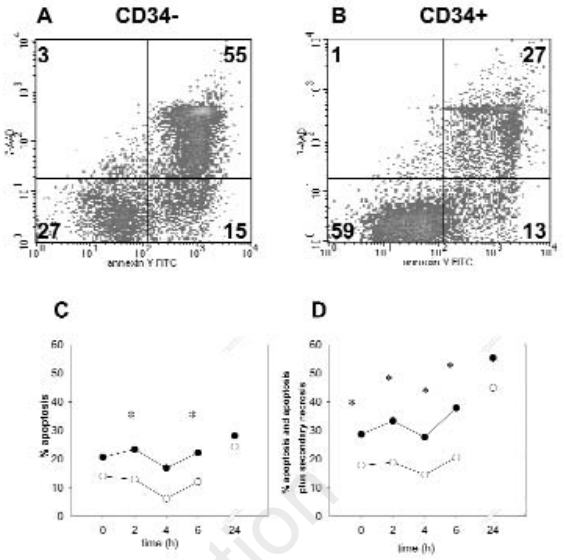

Figure 3. Apoptosis and apoptosis plus secondary necrosis in CD34⁺ and CD34⁻ AML samples. Combined annexin V and 7-AAD staining was used to detect apoptosis and secondary necrosis in sorted CD34⁻ (A) and CD34⁺ (B) AML fractions. Mean percentages of apoptosis (C) and apoptosis plus secondary necrosis (D) in 13 corresponding CD34⁺ (open circles) and CD34⁻ (closed circles) AML fractions, cultured for different periods in serum-free medium. *Indicates significant difference between CD34⁺ and CD34⁻ fractions (apoptosis: t=2, *p*=0.005; t=4, *p*=0.021 and apoptosis plus necrosis: t=0, 2, 4, and 6, *p*=0.022, *p*=0.001, *p*=0.007 and *p*=0.002, respectively).

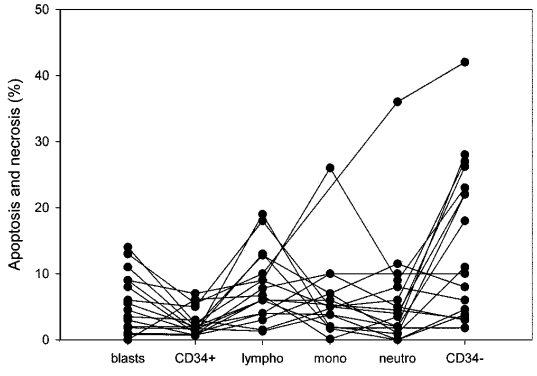

Figure 4. Flow cytometric detection of spontaneous apoptosis plus secondary necrosis in whole blast populations, CD34⁺ blasts, CD34⁻ blasts, monocytes, neutrophils/(pro-) myelocytes and lymphocytes. Percentages of apoptosis plus secondary necrosis measured in 6 unseparated AML cell samples at t=0h using annexin V FITC in whole blast populations (CD45^{low}/SSC^{low}), CD34⁺ blasts (CD34⁺/CD45^{low}/SSC^{low}), CD34⁻ blasts (CD34⁻/CD45^{low}/SSC^{low}), monocytes (CD34⁻/CD45^{high}/SSC^{intermediate}), neutrophils/(pro-) myelocytes (CD34⁻/CD45^{low}/SSC^{high}) and lymphocytes (CD34⁻/CD45high/SSC^{low}). Solid lines connect subpopulations within the same AML.

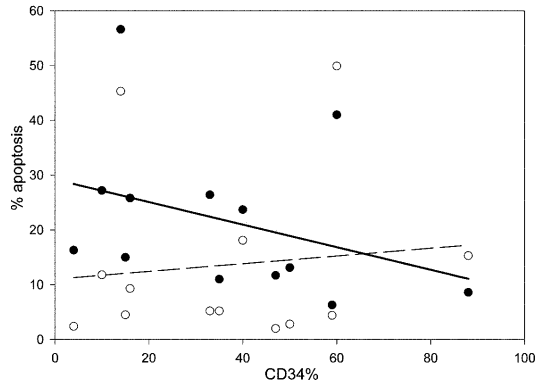

Figure 5. Apoptosis in separated CD34⁺ (open circles) and CD34⁻ (closed circles) fractions in relation to the CD34% of the corresponding whole AML sample. Apoptosis was determined after 2 hours of serum-free culture with annexin V and 7-AAD. Decreased apoptosis with increasing CD34 percentages in the whole AML sample was seen in the CD34⁻ fractions (R=-0.484: p=0.09) but not in the CD34⁺ fractions (R=0.113: p=0.71). The dashed line represents the CD34⁺ fractions, the solid line represents the CD34⁻ fractions. Other time points after serum-free culture show the same phenomenon.

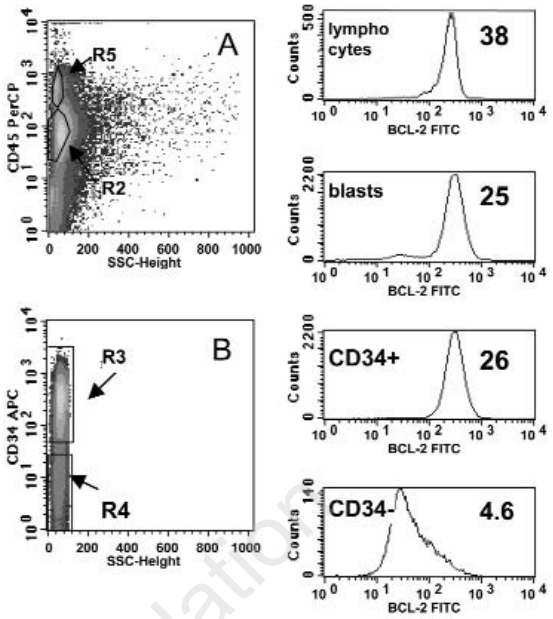

Figure 6. Bcl-2 expression in subfractions of an AML sample as identified by flow cytometry. After surface marker labeling, fixation and permeabilization, cells were stained for Bcl-2, Bcl-x_L, Mcl-1 or Bax (see Design and Methods). Bcl-2 is shown here as an example. CD45^{low}/SSC^{low} are blasts (R2), CD45^{high}/SSC^{low} are lymphocytes (R5). Cells in plot B were gated on the CD45^{low}/SSC^{low} cells shown in A: R3 shows CD34⁺/CD45^{low}/SSC^{low} blasts and R4 shows the CD34⁻/CD45^{low}/SSC^{low} blasts. Bcl-2 fluorescence of lymphocytes and blasts (as gated in A) and in CD34⁺ and CD34⁻ blasts as gated in A and B are shown. The ratio of Bcl-2 of the subpopulations was calculated relative to the appropriate isotype control as described in the Design and Methods and is indicated in the upper right part of the graphs.

slight underestimation of apoptosis in the CD34⁻ fraction. Furthermore, the CD34⁻ fraction can be contaminated by CD34⁺ cells, while the CD34⁺ fraction is usually of high purity. The total effect of impurities in both fractions would again lead to an underestimation of apoptosis in the CD34⁻ fraction and therefore of the difference between CD34⁺ and CD34⁻ blasts. So, in agreement with the hypothesis, the CD34⁺ AML fractions was shown to be more resistant to apoptosis than was the CD34⁻ fraction.

When plotting the percentage apoptosis found in both the CD34⁺ and CD34⁻ fractions against the initial percentage of CD34 cells in the AML sample (Figure 5) an inverse correlation was found between apoptosis in the CD34⁻ fraction and CD34 percentage. No such phenomenon was seen for the CD34⁺ fractions. Although Figure 5 only shows apoptosis after 2 hours of serum-free culture, the same was seen for apoptosis and secondary necrosis at all time points after serum-free culture (*not shown*). It thus appears that apart from the reproducible differences in apoptosis resistance between CD34⁺ and CD34⁻ fractions, there is also an overall increase of apoptosis resistance in CD34⁻ fractions as the CD34 percentage of the whole AML sample increases.

In the next series of experiments, we investigated whether this is directly related to the absolute level of protein expression or function in the CD34⁻ fractions.

Flow cytometric evaluation of CD34⁻ and CD34⁺ cells for Bcl-2, Bcl-xL, Mcl-1 Bax content and Pgp function

Since in a considerable part of the previous AML cases the number of cells after sorting would have been too low for Western blot analysis, in an additional series of 25 AML samples we performed a flow cytometric analysis that enabled detection of Bcl-2, Bcl-x_L, Mcl-1, Bax expression or Pgp activity in the whole blast population but also in CD34⁺ and CD34⁻ blast subsets without needing to use purified subpopulations. An example of an AML sample is shown in Figure 6, demonstrating the gating strategy and showing heterogeneous Bcl-2 expression in the whole blast population (CD45^{low}, R2). This seems to be due to high Bcl-2 expression in CD34⁺CD45^{low} blasts (R2 and R3) but low expression of the CD34⁻CD45^{low} blasts (R2 and R4). In

Table 3. Apoptosis-regulating protein expression in primary AML subsets and normal BM.

		Bcl-2 ratio	*Bcl-x_L ratio*	*Mcl-1 ratio*	*Bax ratio*
nBM		Mean (±SD)	Mean (±SD)	Mean (±SD)	Mean (±SD)
n=10	CD34+	6.9 (±2.6)	2.1 (±0.8)	6.8 (±3.5)	6.1 (±4.0)
AML	CD34+	15.0 (±10.1)*	3.2 (±2.4)	9.6 (±7.2)	6.9 (±6.6)
n=25	CD34-	9.5 (±9.4)#	2.9 (±2.3)	8.6 (±7.7)#	8.1 (±8.5)¶

*nBM: normal bone marrow; AML: bone marrow from patients with AML; *significantly different from nBM: p=0.015. #significantly lower than in the AML CD34+ compartment: Bcl-2: p<0.0001; Bcl-xL: p=0.022; Mcl-1: p=0.048. ¶higher than in the AML CD34+ compartment AML: Bax: p=0.08. The values given are mean (±SD), determined as in Figure 6.*

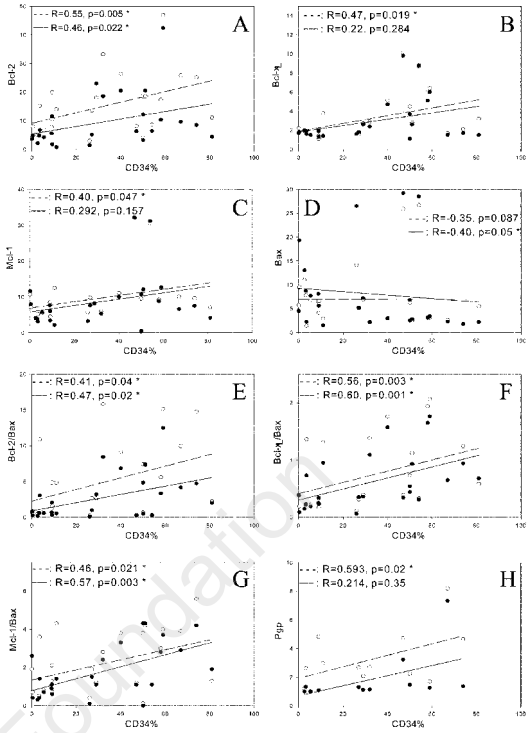

Figure 7. Apoptosis-related proteins in CD34+ (open circles) and CD34– (closed circles) fractions. CD34+ and CD34– blast fractions were identified and protein expression measured in 25 AML samples as exemplified in Figure 6. The gating strategy for Bcl-x_L, Mcl-1 and Bax was the same as that described for Bcl-2. Differences in protein expression between CD34– and CD34+ cells were significant for: Bcl-2 ($p<0.0001$), Bcl-x_L ($p=0.022$), Mcl-1 ($p=0.048$) and Pgp activity (n=14: $p=0.007$). The difference in Bax expression in CD34+ and CD34– fractions was of borderline significance ($p=0.08$). Linear regression of apoptosis-related protein expression in CD34– (solid line) and CD34+ (dotted line) fractions is plotted against the CD34 percentage of the whole AML sample. The significance of the correlation analysis is indicated in the upper part of each graph. *Marks significance.

addition, lymphocytes (CD45^{high}, R5) showed high expression of Bcl-2. The same strategy was applied to establish Bcl-x_L, Mcl-1, Bax expression and Pgp activity in these populations. The results of the 25 AML samples show that 1) Bcl-2 expression in CD34+ blasts differs significantly from that in CD34– blasts and is higher in CD34+ blasts (Table 3) in 23/25 cases; 2) similar results were found for Bcl-x_L and Mcl-1 although the differences were much smaller than for Bcl-2 (Table 3); 3) in contrast, the pro-apoptosis protein, Bax, was expressed to a borderline significantly lower level in the CD34+ fraction in 20/25 cases; 4) Pgp activity, measured in 14/25 patients, was higher in the CD34+ fraction in 11 of these 14 cases (p=0.007, not shown in Table 3).

In order to compare the expression levels of AML blasts with those of normal BM, 10 normal BM samples were also evaluated. This showed that all anti-apoptosis proteins were expressed at higher levels in AML samples (Table 3), and that this difference was significant for Bcl-2.

When plotting protein expression of the AML samples against CD34 percentage (Figure 7) it was observed that the expression of the anti-apoptosis proteins, Bcl-2, Bcl-x_L and Mcl-1, tended to increase as the CD34 percentage increased and that the pro-apoptosis protein, Bax, tended to decrease, as illustrated by the regression lines in the graphs in Figure 7. In detail, Spearman's correlation analysis showed that i) these trends were significant for all proteins (that for Bax was of borderline significance) for the CD34+ fractions (Figure 7 A-D) and for Bcl-2 and Bax in the CD34– fraction (Figure 7A, D); ii) Bcl-2/Bax, Bcl-x_L /Bax and Mcl-1/Bax ratios in both fractions significantly correlated with CD34 percentage (Figure 7E-G); iii) Pgp activity increased with increasing CD34 percentages (Figure 7H) and this increase was statistically significant for the CD34+ fraction.

Antigenic determinants of the degree of immaturity of AML samples

Because CD133, CD117 and CD90 are also, alongside CD34, primitive markers of the hematopoietic system, we investigated whether these markers correlated with a profile of resistance to apoptosis. This investigation revealed that expression of anti-apoptosis proteins correlated not only with CD34 percentage and MFI of CD34 but also with other progenitor markers such as CD117 and CD133

(Table 2). We did not find CD90 expression in any of the analyzed cases (*not shown*). Overall, percentages of primitive markers correlated with expression of anti-apoptosis proteins, and these correlations reached statistical significance in most cases. The expression of the pro-apoptotic protein, Bax, showed an inverse trend with percentages of primitive markers. In addition, formulae representing an apoptosis-resistant phenotype, comprising all the parameters, showed strong correlations with CD34 as well as with CD133 and CD117 expression.

Discussion

This study was designed to unravel some of the properties of $CD34^+$ blast cells in AML because the background of the role of CD34 as a prognostic marker in AML is still not fully understood. A high CD34 percentage in AML has been reported to be associated with a poor prognosis in many studies.[1-9] However, these results could not be confirmed in other studies.[53-57] CD34 positivity, besides its suggested clinical implications, has also been associated with apoptosis-resistance markers such as Bcl-2.[13,17] Pgp, the classical multidrug resistance ABC transporter, is of particular interest since it has recently also been described as an anti-apoptotic protein inhibiting caspase-dependent cell death. Antibodies or modulators inhibiting the function of Pgp increased the apoptotic sensitivity in leukemia cell lines and primary AML cells.[40-42] Furthermore, Pgp has also been shown to correlate with CD34. These observations raised the question of whether CD34 and apoptosis-resistance markers are causally linked. The $CD34^+$ population in AML is of special interest since the leukemogenic cell likely resides in the $CD34^+$ population.[58,59] The analysis of the $CD34^+$ population with respect to its apoptotic behavior and expression of proteins involved in apoptosis should, therefore, give more insights into the mechanisms of chemotherapeutic resistance. A better understanding of the mechanisms by which the $CD34^+$ AML population withstands the chemotherapeutic insult might be helpful in designing new therapeutic interventions towards the best target cell in AML.

This study had three parts: in the first part, function and expression of apoptosis-related proteins were investigated by Western blotting and flow cytometry in Ficoll-separated AML samples and related to spontaneous apoptosis *in vitro*; in the second part, $CD34^+$ and $CD34^-$ fractions, purified from $CD34^+$ AML samples, were investigated for putative differences in spontaneous apoptosis; and in the third part, apoptosis-related proteins in $CD34^+$ and $CD34^-$ fractions from another series of AML samples were investigated by multiparametric flow cytometric measurements.

In the first part, apoptosis was measured in a serum-free apoptosis induction assay to allow investigation of the well-known apoptosis-related proteins, Bcl-2, Bcl-x_L, Mcl-1 and Bax, but also the study of Pgp as an anti-apoptotic protein without the interference of its role as an efflux pump of chemotherapeutic drugs. We first found that Bcl-2, Bcl-x_L, and Pgp correlated with the CD34 percentage in the samples from patients with AML. These correlations are in accordance with studies reporting higher levels of anti-apoptosis proteins in $CD34^+$ AML samples[10] and indicate that our study group is similar to other groups described in the literature.

The percentages of apoptosis and secondary necrosis correlated in this assay with the $CD34^+$ percentage as well as with Bcl-2 and Bcl-x_L expression levels, and Pgp function. Banker *et al.*[33] also found a correlation between Bcl-2 expression and reduced spontaneous apoptosis. However, they were not able to demonstrate that all AML samples with low spontaneous apoptosis frequencies had high Bcl-2 expression. This indicates that other proteins besides Bcl-2 also contribute to resistance to apoptosis. In our study a larger panel of apoptosis-resistance markers was investigated, demonstrating that not only Bcl-2, but also, although to a less extent, Bcl-x_L and Pgp activity might contribute to apoptosis resistance. The correlations of these anti-apoptosis markers with a reduced ability to undergo spontaneous apoptosis and with CD34 percentage might reflect the increased resistance to apoptosis of $CD34^+$ AML cells. From these results it was hypothesized that the $CD34^+$ subpopulation is the most apoptosis-resistant subpopulation in AML. In order to prove this, sorted $CD34^+$ and $CD34^-$ AML fractions were tested in the spontaneous apoptosis assay in the second part of the study. This approach was chosen to ensure that at each time point the fate of the correct population was studied: loss of expression, which is common in apoptosis and cell death, might at some point during the assay characterize cells erroneously as being $CD34^-$ whereas these were originally $CD34^+$ cells. Using this approach the $CD34^+$ fraction turned out to be significantly more resistant than the $CD34^-$ fraction. This would support the idea that $CD34^+$ AML samples with high CD34 percentages are resistant to apoptosis by virtue of a relatively higher number of resistant $CD34^+$ cells.

Remarkably, we found that $CD34^-$ fractions derived from AML samples with higher percentages of CD34 cells were more resistant to apoptosis than those derived from samples with a low CD34 percentage. So, one would expect that apoptosis-related proteins would also be different. To establish such measurements in $CD34^+$ and $CD34^-$ cell fractions, we relied on flow cytometry since in general not enough cells were available for Western blot analysis after cell sorting. Multiparametric flow cytometry enabled CD34 assessment to be

combined with analysis of the expression of the apoptosis-related proteins Bcl-2, Bcl-x_L, Mcl-1, and Bax and also Pgp function, thus allowing them to be specifically assessed in the $CD34^+$ and $CD34^-$ subpopulations. The results showed significantly higher expression of Bcl-2, Bcl-x_L, and Mcl-1 and a borderline significantly lower Bax expression in $CD34^+$ versus $CD34^-$ subpopulations and are thus in agreement with the finding that $CD34^+$ cells are more apoptosis-resistant than $CD34^-$ cells. Secondly, we found a higher apoptosis-resistance protein profile in the $CD34^-$ as well as in the $CD34^+$ fractions with increasing CD34 percentage. Garrido *et al.*[16] recently described higher Bcl-2 levels and lower apoptosis in $CD34^+$ AML samples but did not pinpoint the $CD34^+$ fraction as the most resistant fraction within CD34 positive AML samples.

Regarding the clinical significance of spontaneous apoptosis in AML, Smith *et al.*[15] and Norgaard *et al.*[60] found that spontaneous apoptosis was predictive for entering complete remission, which supports the prognostic relevance of apoptosis measurements in AML. We did not investigate the clinical response in this relatively small group of patients but such an analysis could, when applied to a larger series of patients and in combination with analysis of spontaneous apoptosis and apoptosis-related proteins, lead to a refinement of the prognostic index for AML patients.

So far, little is known about the expression levels of apoptosis-related proteins in subpopulations in AML. Very recently Suarez *et al.*[61] reported that Bcl-2 and Bax differed according to different stages of maturation: $CD34^+$ AML blast cells were defined as the most immature subpopulation, $CD34^-CD32^{-/+}CD33^+$ as a less immature subpopulation and $CD34^-CD32^+/^{++}CD33^{++}$ as the most mature subpopulation. Bcl-2 expression level was the highest in the most immature subpopulation whereas Bax was lowest in that subpopulation. Our data on $CD34^+$ and $CD34^-$ cells are in agreement with this. No data, however, were reported for Mcl-1 and Bcl-x_L or protein expression in relation to CD34 percentage. Furthermore, we also investigated Bcl-x_L and Mcl-1 expression and Pgp function and showed higher expression and/or function in the most immature subpopulations than in the mature population. The correlation of the other progenitor markers, CD133 and CD117, with an apoptosis-resistant protein profile supports the finding that immature cells are more apoptosis-resistant than mature cells. This warrants further investigations into the use of combinations of these markers.

In conclusion, we hypothesize that $CD34^+$ fractions are the most apoptosis-resistant AML cells and that the $CD34^-$ AML cells are likely derived from the $CD34^+$ AML cells by further maturation and thus exhibit a lesser resistance to apoptosis. Moreover, in AML with high percentages of CD34 cells, the $CD34^+$ compartment might be more immature. In that case, the $CD34^-$ fraction from a case of AML with high CD34 percentages (presence of more immature AML cells) would likely have similar properties regarding Bcl-2 family members and therefore also similar functional apoptosis properties as do $CD34^+$ subpopulations. The apoptosis-resistant protein profile and the reduced ability to undergo apoptosis in cases of AML with high CD34 percentages indicate that the poor clinical outcome in $CD34^+$ AML patients is, at least, partly caused by a higher percentage of cells with increased resistance to apoptosis, contributing to a cellular resistance which includes both apoptosis resistance and multidrug resistance.

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Pre-publication Report & Outcomes of Peer Review

Contributions

AvS: principal investigator, MvdP: acquisition of data, Pgp activity; AK, PMB: acquisition of apoptosis data; RB: contribution to design; GO, GJS: contribution to concept and design, conducting the work, drafting and revising the article. All authors contributed to analyzing and interpreting the data and revising the manuscript. Primary responsibility for the paper: GJS; primary responsibility for all Tables and Figures: AvS.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Dr. Craig Jordan, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Dr. Jordan and the Editors. Manuscript received November 12, 2002; accepted March 24, 2003.

In the following paragraphs, the Associate Editor summarizes the peer-review process and its outcomes.

What is already known on this topic

It has been speculated that primitive AML cells might be relatively resistant to apoptosis. If true, this could at least partially explain why relapse after conventional chemotherapy is common.

What this study adds

The study by van Stijn *et al.* demonstrates that $CD34^+$ cells are more resistant to apoptosis than $CD34^-$ cells. This finding suggests that leukemic stem and progenitor cells may also be relatively resistant to apoptosis.