Assessment of the normal or leukemic nature of CD34+ cells in acute myeloid leukemia with low percentages of CD34 cells

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Background and Objectives. The percentages of CD34+ cells in the bone marrow of patients with acute myeloid leukemia (AML) vary widely. Especially in the low range (<5% CD34+ cells), the nature (normal or malignant) of the CD34+ cells is uncertain. Since only in a minority of cases are molecular techniques applicable, in this study we explored a multiparameter approach using phenotypic and functional characteristics to discriminate normal CD34+ cells from malignant ones.

Design and Methods. CD34+ cells from 24 AML patients with <5% CD34+ cells and from 3 patients with >50% CD34+ cells were studied immunophenotypically for aberrant phenotypes, CD133 and CD90 expression and for P-glycoprotein activity.

Results. In the low (0.02-0.7%) CD34+ range, our approach offered strong evidence for a normal origin of the CD34+ cells in 18/19 cases, which was confirmed by interphase fluorescent *in situ* hybridization on sorted CD34+ cells in 3 cases, which had concomitant presence of cytogenetic abnormalities in the CD34– blasts. In contrast, in the intermediate (1.6-3.5%) CD34+ range, the CD34+ cells appeared as normal in only 1/5 cases. In the high (51-67%) CD34+ range, as expected the majority of CD34+ cells were malignant, although in 2/3 cases a small subpopulation (i.e. 0.15% and 0.20%) of CD34+ cells were of normal origin.

Interpretation and Conclusions. Our multiparameter approach enabled us to define the nature of CD34+ cells in AML. This has implications for studies dealing with the characterization of primitive malignant cells. Moreover, it enabled identification of truly CD34 negative AML, which would be eligible for CD34-based immunologic purging of autologous stem cell transplants.

Key words: P-glycoprotein, acute myeloid leukemia, immunophenotyping, CD34.

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Relapses frequently occur in acute myeloid leukemia (AML) because of the persistence and subsequent outgrowth of minimal residual disease (MRD). The immunophenotypic assessment of MRD at follow-up is a powerful predictor of survival.[1,2] Resistance mechanisms responsible for the emergence of MRD include the presence of ABC-transporters, such as P-glycoprotein (Pgp),[3] and apoptosis resistance.[4] Resistance mechanisms in AML are generally studied on the total blast population, whereas it might be more informative to investigate the more primitive, clonogenic, AML blasts. Since the stem cell marker CD34 has been shown to include both normal[5] and leukemic[6,7] stem cells, this marker might be very useful to incorporate in AML studies. The relevance of investigating clonogenic AML blasts is illustrated by a recent study which showed that CD34+ AML blasts are more resistant to apoptosis than their CD34– counterpart.[8]

Cases of AML are considered CD34+ using rough cut-off levels of 5-20%.[9] Since small numbers of residual normal CD34+ progenitors co-exist with CD34+ and CD34– AML blasts in the bone marrow (BM),[10,11] it is of importance to elucidate the nature of the CD34+ cells in those cases of AML with low percentages of CD34 cells (<5% CD34+) for several reasons: firstly, to investigate putative resistance mechanisms in the clonogenic AML blast population, secondly to be able to interpret outgrowth characteristics and lastly, to ensure that a particular AML is truly CD34-negative.

The aim of the present study was to investigate the nature, i.e. normal or malignant, of CD34+ cells present in AML samples in which the percentages of CD34 cells were low. The presence of cytogenetic abnormalities in the blast population would, in principle allow, an accurate determination of the nature of these CD34+ cells, after cell sorting. Although cytogenetic abnormalities are relatively common in AML patients,[12] the major limitation to using genetic approaches to assess the nature of CD34+ cells in AML patients is related to the absence of detectable specific genetic abnormalities in most cases. For example, DNA probes for fluorescence *in situ* hybridization (FISH) are currently available only for the most frequent genetic abnormalities described in AML. Therefore, other more generally applicable methods are necessary to investigate the nature of CD34+ cells present at diagnosis.

It was hypothesized that malignant CD34+ cells might be distinguished from normal CD34+ cells on the basis of aberrant marker expression present on the majority

of AML blasts,[1,2,13] in contrast to on normal CD34+ BM cells.[14] The levels of CD133 and CD90 expression might also allow discrimination, since a primitive subset of normal CD34+ cells expresses CD90 and CD133,[15-17] whereas most AML samples are negative for CD90,[18,19] while CD133 is expressed only in a subset of patients.[19,20] Furthermore, Pgp activity often discriminates between normal and malignant cells: normal CD34+ BM cells show a narrow range of Pgp activity,[13,21] whereas generally no or low activity is found in CD34+ AML with low percentages of CD34 cells.[22,25] Furthermore, within the CD34+ (>5% CD34+) AML samples, the CD34+ fraction showed only slightly higher Pgp activity, if any, than the corresponding CD34– fraction, and importantly, when CD34– blasts did not display Pgp activity in these samples, neither did the CD34+ blasts.[23] Based on these observations, we explored the feasibility of a multiparameter approach, using phenotypic and functional characteristics, to discriminate normal CD34+ from malignant CD34+ cells in AML BM samples with low percentages of CD34 cells.

Design and Methods

Patients and controls

After informed consent, BM samples were obtained from non-AML patients undergoing cardiovascular surgery and from 24 patients with newly diagnosed AML. Inclusion was based on the presence of low numbers of CD34+ cells, i.e. <5% CD34+ cells, and on sufficient amounts of cells to investigate these small numbers. The patients' characteristics are outlined in Table 1. Additionally, three AML cases with more than 50% CD34+ cells were studied.

For functional studies, mononuclear cells were isolated via a Ficoll gradient and erythrocytes were lysed afterwards.[13] Fresh samples were preferentially used for the experiments, but were occasionally supplemented with cryopreserved samples. When cryopreserved samples were used, these were rapidly thawed in a water bath (37°C) and immediately diluted in RPMI 1640 (Life Technologies, Paisley, UK) supplemented with 40% fetal calf serum (Life Technologies), prewarmed to 37°C, and centrifuged. The use of cryopreserved samples for both immunophenotyping and detection of Pgp function was validated in earlier studies.[13,26]

Immunophenotyping

BM samples from AML patients and healthy controls were studied immunophenotypically as described previously.[13,23] In order to accurately establish the immunophenotype of small numbers of CD34+ cells present in a sample, a total of at least 2×10^6 cells were analyzed on the flow cytometer. For sources of fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP) and allophyocyanin (APC) conjugated monoclonal antibodies (Moabs) see our previous study.[13]

Functional Pgp assay

For the detection of Pgp function, a green fluorescent probe assay was used with Syto16/PSC833 as the specific substrate/modulator combination as was previously described.[13,23] Again, in order to accurately establish the Pgp function of small numbers of CD34+ cells present in a sample, a total of at least 2×10^6 cells were analyzed. Cells were subsequently labeled with combinations of fluorochrome conjugated Moabs which define aberrant antigen expression, or labeled with CD133-PE and CD34-APC.[13,23] 7-amino actinomycin D (7-AAD, Pharmingen, San Diego, CA, USA) staining was always included in frozen-thawed samples to gate out dead and apoptotic cells in the final analysis.[26] Pgp activity was expressed as a ratio of drug fluorescence with modulator present and drug fluorescence without modulator present after subtraction of the fluorescence of the control (cells in medium alone).[13] A ratio >1.0 indicates activity.

Flow cytometry

Flow cytometry was performed using a FACScalibur (Becton Dickinson, San José, CA, USA) equipped with a 488 nm argon laser and a 635 nm red diode laser. Data acquisition and analysis were performed using Cell Quest software.

FISH analysis of isolated CD34– and CD34+ cells

Mononuclear cells were incubated with anti-CD34 Moab directly coupled to magnetic beads from Miltenyi (Sanquin Reagents, Amsterdam, The Netherlands) according to the manufacturer's instructions. After passing the cells over a 25 μm filter, CD34+ and CD34– cell fractions were isolated using an Automacs system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The purity of the isolated CD34+ and CD34– cell fractions was determined by FACS analysis.

For interphase FISH, the isolated CD34+ and CD34– cell fractions were washed three times with 3 mL of 3:1 methanol/acetic acid fixative and resuspended in 1 mL fixative. Subsequently, one droplet was dropped gently onto an object slide and air-dried. Next, slides were incubated for 30 min at 60°C on a slide warmer, denaturated in 70% formamide/2× standard saline citrate (SSC) for 5 min at 72°C and dehydrated in series of 2 min ethanol washes (70%, 80%, 96% and 100%) at room temperature. Dual-color (spectrum green and spectrum orange fluorophores) labeled LSI® DNA probes (Vysis Inc, Downers Grove, IL, USA) were

Table 1. Patients' characteristics.

Pat.	*FAB*	*Age*	*Karyotype*[a]	*Blast (%)*[b]	*Aberrant antigen expression*		*CD34 (%)*[e]
					on CD34⁻ blasts[c]	*on CD34⁺ cells*[d]	
1	M2	43	46,XX	66	CD117⁺HLADR⁻CD13⁺	Absent	0.02
2	M3	56	46,XY,t(15;17)	8	CD117⁺CD15⁺CD4⁺	Absent	0.03
3	M4	48	46,XX	46	CD117⁺CD14⁺	Absent	0.03
4	M1	68	46,XY,t(5;6)(q31;q21)	87	CD45dCD33⁺CD56⁺	Present (5%)	0.03
5	M5a	23	46,XX	92	None	ND	0.03
6	M2	39	46,XX,del(6)(q15;q21)	57	CD133⁺CD34⁻	ND	0.05
7	M5b	70	46,XX,del(11)(q23;q23)	57	CD45dCD56⁺CD14⁺	Absent	0.07
8	M3	22	46,XX,t(15;17)	43	None	ND	0.07
9	M2	54	46,XY	36	None	ND	0.07
10	M2	67	46,XY	64	CD45dCD33⁺CD7⁺	Absent	0.08
11	M4	64	46,XY	88	CD133⁺CD34⁻	ND	0.10
12	M2	65	46,XY	61	CD45dCD33⁺CD56⁺	Absent	0.13
13	M5	53	46,XX	80	CD117⁺CD13⁺CD7⁺	Absent	0.14
14	M5a	60	47,XX,+8	97	CD133⁺CD34⁻CD65⁺	Absent	0.18
15	M5a	76	No metaphases	60	None	ND	0.18
16	M2	55	46,XY	65	CD117⁺CD33⁺CD13⁻	Absent	0.20
17	M5	42	46,XX	53	CD133⁺CD34⁻	ND	0.20
18	M4	35	46,XX,t(8;21)(q22;q22)	75	CD117⁺CD56⁺	Absent	0.41
19	M4	71	46,XX	43	CD117⁺CD15⁺CD4⁺	Absent	0.70
20	RAEB-T	59	46,XX	8	None	ND	1.55
21	M5	55	46,XY,del(9)(q11;q22)	67	CD133⁺CD34⁻CD7⁺	Present (46%)	1.58
22	M5a	42	46,XX	94	CD117⁺CD33⁺CD7⁺	Present (87%)	1.68
23	M6	33	47,XY,+8	8	CD133⁺CD34⁻	ND	2.51
24	M2	70	46,XY	43	CD117⁺CD13⁺CD7⁺	Absent	3.47

FAB: French-American-British leukemia classification system; [a]karyotypes were established on whole BM by metaphase analysis; [b]percentages of blasts were scored in May-Grünwald-Giemsa stained BM smears; [c]aberrant antigen expression on CD34⁻CD45d(dim) blasts; [d]Aberrant antigen expression on CD34⁺CD45d cells, in brackets the percentage of aberrant antigen expression on the CD34⁺ cells; [e]CD34 percentages were scored with immunophenotyping; ND: not determined, due to absence of aberrant antigen expression on CD34⁻ blasts or inappropriate aberrant antigen expression (i.e. CD133⁺CD34⁻) for investigation on the CD34⁺ cells.

denaturated for 5 min at 72° C and applied to the denaturated slides as recommended by the manufacturer. The following probes were used: the LSI PML/RARA dual color translocation probe for t(15;17), the LSI MLL dual color break apart rearrangement probe for del 11(q23) and the LSI AML1/ETO dual color dual fusion translocation probe for t(8;21). Following hybridization for 16-20 hours at 37°C in a humidified chamber, samples were washed three times for 10 min with 50% formamide/2×SSC, then washed with 2×SSC for 10 min, and finally washed with 2×SSC/0.05% Tween for 5 min at 42°C. After dehydration in respectively 70%, 80%, 96% and 100% ethanol for 2 min at room temperature, slides were air-dried and mounted in Vectashield (Vector Laboratories Inc.,

Burlingame, CA, USA) containing DAPI (4,6-diamino-2-phenyl-inol, 0.10 μg/mL; Sigma, St Louis, MO, USA) counter stain. Hybridization signals were scored, after incubation for 30 min at 4°C, in 100 interphase nuclei with an Axioscop 20 (Carl Zeiss Jena GmbH, Jena, Germany) fluorescence microscope with three single-band pass filters and one triple band pass filter. Nuclei were scored positive for the fusion gene, when a green and orange spot were less than one spot diameter apart. The images were captured with a digital camera using CytoVision 4.1 software (Applied Imaging Corporation, Newcastle, UK).

Statistical analysis

Spearman's rank correlation test was used to assess correlations, Wilcoxon's rank-signed test to determine differences between paired samples and the Mann-Whitney U test to determine differences between different groups. p values <0.05 were considered statistically significant. Statistical analyses were performed using SPSS software (version 9.0; SPSS, Chigaco, IL, USA).

Results

Aberrant antigen expression on CD34$^-$ and CD34$^+$ cells

De novo AML samples with a low percentage of CD34 cells were studied immunophenotypically for aberrant antigen expression in the malignant CD34$^-$ blast compartment as well as on the CD34$^+$ cells. In 19 out of the 24 patients, the malignant CD34$^-$ blasts showed aberrant antigen expression. In 15 patients the CD34$^+$ cells could be studied for these aberrant phenotypes. In only 2 patients identical aberrant antigen expression was found on a considerable part of the CD34$^+$ cells, suggesting a leukemic origin, while in one patient only a small subpopulation of the CD34$^+$ cells showed aberrant antigen expression (Table 1).

Pgp activity of CD34$^-$ and CD34$^+$ cells

Next we investigated whether the nature of CD34$^+$ cells could be established based on differences in Pgp activity between normal and malignant cells. Figure 1 shows an example of the Pgp activity in normal CD34$^+$ cells and Figure 2 shows an example of the Pgp activities found in an AML patient's malignant CD34$^-$ blasts and CD34$^+$ cells of unknown origin. In normal CD34$^+$ BM cells a narrow range of Pgp activities was found (range 1.7-3.7) with higher activities in the more primitive CD34$^+$CD133$^+$ cells than in the more mature CD34$^+$CD133$^-$ cells (Figure 1 and Table 2). In the AML samples with low percentages of CD34 cells it was found that the malignant CD34$^-$ blasts showed much lower levels of Pgp activity (mean±sd of 1.2±0.2, range: 1.0-1.5, n=24) than did normal CD34$^+$ cells. On the other hand, the CD34$^+$ cells present in these AML samples, in 19/24 cases showed much higher Pgp activity than the corresponding malignant CD34$^-$ blasts, and moreover, the Pgp activities found were comparable with those found in normal CD34$^+$ cells (Figure 3A). As for normal CD34$^+$ cells, the Pgp activities were generally (14/21 cases) higher in the CD34$^+$CD133$^+$ cells than in the corresponding CD34$^+$CD133$^-$ cells (Figure 3B): in these cases the median factor difference was 1.9 (range: 1.3-9.8), compared to 2.5 (range: 1.6-3.0) for normal CD34$^+$ BM cells (p=0.31).

In addition, we studied 3 samples from patients with more than 50% of CD34$^+$ cells. The majority of these CD34$^+$ blasts showed aberrant CD7 or CD2 co-expression, whereas a minority of the CD34$^+$ cells lacked this aberrant antigen expression (0.15%, 0.37% and 0.20%). We investigated whether these CD34$^+$CD2$^-$ and CD34$^+$CD7$^-$ cells were of normal or leukemic origin based on their Pgp activity. In the first patient, the CD34$^+$CD2$^-$ cells showed aberrantly low Pgp activity, similarly to that found for the CD34$^+$CD2$^+$ blasts (1.1 and 1.0, respectively), which indicates a leukemic origin. In the second patient the CD34$^+$CD7$^+$ blasts had a low Pgp activity (1.1), while the CD34$^+$CD7$^-$ cells had high Pgp activity (7.0). In the third patient the CD34$^+$CD7$^+$ blasts had very high Pgp activity (16.0) while the CD34$^+$ CD7$^-$ cells had a lower Pgp activity (5.0). The data from the latter two patients indicate that low percentages of normal CD34$^+$ cells can exist in cases of AML with high CD34 percentages.

Expression of the stem cell markers CD133 and CD90 on CD34$^-$ and CD34$^+$ cells

Next we investigated whether the nature of CD34$^+$ cells could be established based on differences in CD133 and CD90 expression between normal and malignant cells. Table 2 shows the level of CD133 and CD90 expression on normal CD34$^+$ BM cells. In the malignant CD34$^-$ AML blasts, lower expression of CD133 was found in all but one of the cases (mean±s.d. of 6±11%, range 0-43%, n=23). In contrast, the corresponding CD34$^+$ cells showed relatively high CD133 expression in 18/24 cases (82±10%, range: 60%-97%, n=18, Figure 3C). For CD90, extremely low expression was found on the malignant CD34$^-$ AML blasts (18/21 cases showed <4% CD90$^+$). In contrast, the corresponding CD34$^+$ cells showed higher CD90 expression in 16/21 cases (23±22%, range 4.3-73%, n=16, Figure 3D). Thus in the majority of AML cases with low percentages of CD34 cells the CD34$^-$ blasts displayed low CD133 and CD90 expression, whereas the CD34$^+$ cells displayed higher expression of both antigens, more similar to that of normal CD34$^+$ BM cells.

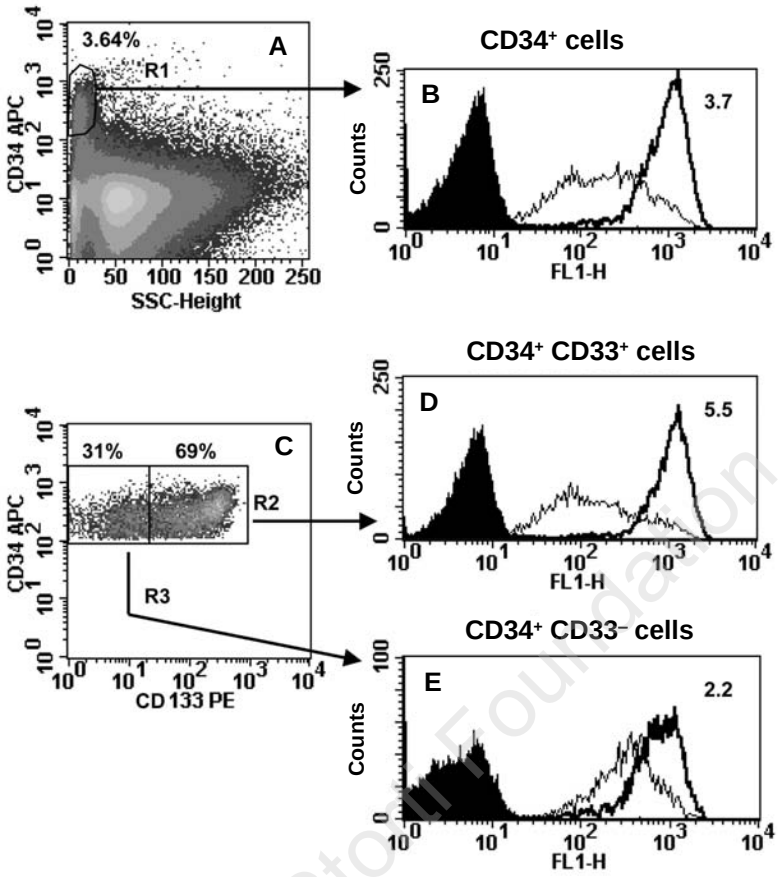

Figure 1. Representative example of Pgp function in normal CD34+ BM cells. The left column of figures represents CD34 expression (A) and CD133 expression of gated CD34+ cells (C). The CD34+ cells displayed CD45dim/SSClow expression (*not shown*). The right column of figures represents Pgp function of the gated cells as shown in the first column. The filled curve represents the autofluorescence of cells, the thin line represents the fluorescence of cells incubated with Syto16 and the thick line represents the fluorescence of cells incubated with Syto16+PSC833. A fluorescence shift >1.0 indicates activity. Regions were set around the CD34+ (A: R1), CD34+CD133+ (C: R2) and CD34+ CD133- (C: R3) cell populations, for which the corresponding Pgp function is shown in B (3.7), D (5.5) and E (2.2), respectively. Note the bimodal distribution of Syto16 fluorescence in the CD34+ cells (thin line in B) and the higher Pgp function in the CD34+ CD133+ cells (D) compared to the CD34+ CD133– cells (E). The CD34+ CD133+ cells (R2) can be further subdivided into a dim and bright CD133 population: the CD133dim population shows a Pgp activity in between the activity found for the CD133– and the CD133bright population (*not shown*).

FISH analysis of sorted CD34- and CD34+ cells

For patients #7, 8 and 18 we were able to determine the nature of the CD34+ cells with interphase FISH. Cytogenetic abnormalities were detected only in a minority of the CD34+ cells, whereas they were detected in the majority of the corresponding malignant CD34– blasts (Table 3 and Figure 4).

Final assessment of the nature of CD34+ cells in AML with low percentages of CD34

The results of immunophenotyping, Pgp activity and FISH analysis of the CD34+ cells present in cases of AML with low percentages of CD34 cells are summarized in Table 4. A final conclusion about

Table 2. Expression of the early stem cell markers CD133 and CD90 and Pgp activity in CD34+ normal bone marrow cells.

	CD34+	*CD34+CD133+*	*CD34+CD133-*
CD133%	55±8.0 (48-69)	–	–
CD90%	8.3±3.6 (4.0-13)	ND	ND
Pgp activity	2.3±0.7 (1.7-3.7)	3.8±1.3 (2.2-5.5)*	1.6±0.4 (1.3-2.2)

*The results are expressed as mean ± s.d (range) of 6 normal bone marrow samples, harboring 3.0±2.2% CD34+ cells; ND: not determined; *p=0.04 compared to CD34+CD133- cells.*

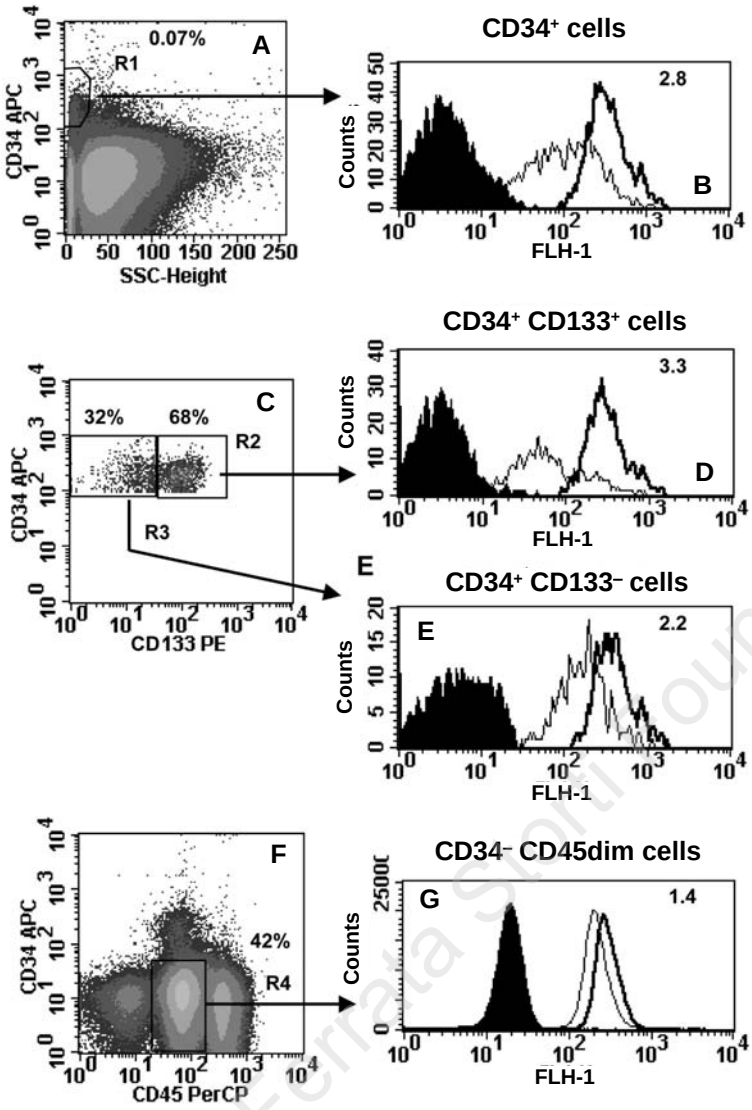

Figure 2. Pgp function in CD34⁻ and CD34⁺ cells present in a *de novo* AML sample. Results from patient 8 are given as an example. The left column of figures represents CD34 expression (A), CD133 expression of gated CD34⁺ cells (C) and CD34⁻CD45^{dim} expression of malignant blasts (F). The CD34⁺ cells also displayed CD45^{dim} expression (see F). For all cell fractions gating with low SSC has been included. The right column of figures represents Pgp function of the gated cells as shown in the first column (see legend to Figure 1 for explanation). Regions were set around the CD34⁺ (A: R1), CD34⁺CD133⁺ (C: R2), CD34⁺CD133⁻ (C: R3), and CD34⁻CD45^{dim} (F: R4) cell populations, for which the corresponding Pgp function is shown in B (2.8), D (3.3), E (2.2) and G (1.4), respectively. Note the low Pgp function in the CD34⁻CD45^{dim} malignant blasts (G) and the higher Pgp function in the CD34⁺ cells (B), CD34⁺CD133⁺ cells (D) and CD34⁺CD133⁻ cells (E). The CD34⁺ cells present in this *de novo* AML sample show Pgp function similar to that of normal CD34⁺ BM cells (compare histogram overlays B, D and E with those depicted in Figure 1). The normal nature of these CD34⁺ cells is shown by FISH analysis in Figure 4E.

the normal or leukemic nature of these CD34⁺ cells has been added in the last column. The criteria, in order of importance, used to define the normal or leukemic nature of CD34⁺ cells were as follows: i) FISH results, ii) the presence of aberrant antigen expression on the CD34⁺ cells, when present on the concomitant CD34⁻ cells, is regarded as evidence for a leukemic origin, iii) a Pgp activity that is outside the range found for normal CD34⁺ cells is regarded as evidence for a leukemic origin, iv) an abnormal pattern of Pgp activity in the CD133⁺ and CD133⁻ subset, i.e. higher activity in the CD133⁻ subset or similar activities in both subsets, is regarded as evidence for a leukemic origin, and v)

Table 3. FISH analysis of sorted CD34⁻ and CD34⁺ AML cell populations.

Patient	*Cytogenetic aberrations#*	*Purity of sorted cell fractions (%)*		*Cytogenetic aberrations (%) in 100 interphase nuclei*	
		CD34⁻	*CD34⁺*	*CD34⁻*	*CD34⁺*
7	del (11)(q23;q23)^	ND	ND	48	2
8	t(15;17)	99.7	60.7	72	10*
18	t(8;21)	99.8	85.1	72	16

*#Cytogenetic aberrations detected with conventional karyotyping; ^Aberrancy detected in 25% (5/20) of the metaphases; *50 nuclei were examined; ND: not determined.*

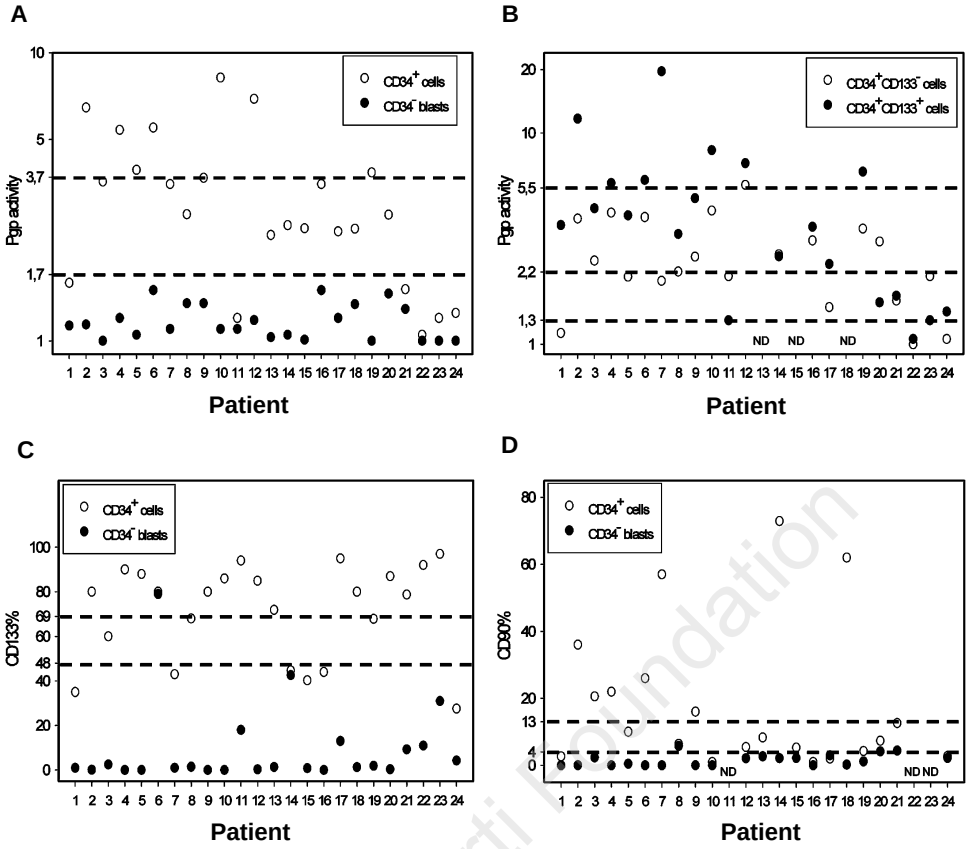

Figure 3. Comparison of Pgp function and expression of the stem cell markers CD133 and CD90 on CD34⁻ and CD34⁺ cells present in *de novo* AML samples. Patients were grouped according to the number of CD34⁺ cells present in the BM samples (see Table 1): from patient 1 with the lowest percentage of CD34 cells (0.02%) to patient 24 with the highest (3.5%). A: Pgp function of malignant blasts, i.e. CD34⁻CD45^{dim} cells with or without aberrant antigen expression (closed circles) and of CD34⁺ cells (open circles). A fluorescent shift >1.0 indicates Pgp activity. B: Pgp function of CD34⁺CD133⁺ cells (closed circles) and of CD34⁺CD133⁻ cells (open circles). The dotted lines in the figures indicate the range of Pgp activities found in normal CD34⁺ cells (A, range: 1.7-3.7, n=6), normal CD34⁺CD133⁺ cells (B, range: 2.2-5.5, n=6) and normal CD34⁺CD133⁻ cells (B, range: 1.3-2.2, n=6). C: Percentages of CD133 expression on the malignant CD34⁻blasts (closed circles) and on the CD34⁺ cells (open circles). D: Percentages of CD90 expression on the malignant CD34⁻ blasts (closed circles) and on the CD34⁺ cells (open circles). The dotted lines in the figures indicate the range of CD133 and CD90 co-expression on normal CD34⁺ BM cells (C, range: 48%-69%, n=6 and D, range: 4%-13%, n=6, respectively). ND: not determined.

a normal distribution of CD133 and CD90 on the CD34⁺ cells is indicative of normal origin.

This differential approach to diagnosis is summarized in Table 4 for all patients studied. It can be established that the CD34⁺ cells from patients 1-10 and 12-20 are of normal origin. The CD34⁺ cells from patients 11 and 21-24 were considered to be of malignant origin. This attribution was based primarily on the findings of i) aberrant antigen expression, in patients 21 and 22, and ii) abnormally low Pgp activities, in all five patients, and iii) abnormal Pgp activity distribution in the CD133⁺ and CD133⁻ cell populations, in all five patients.

Discussion

In the present study we explored the use of a multiparameter flow cytometric approach using differences in characteristics between normal and malignant cells, in order to determine whether CD34⁺ cells present in AML with low percentages (i.e. <5%) of CD34 cells are of normal or leukemic nature. As parameters for differential diagnosis we used: i) aberrant antigen expression, ii) Pgp activity on CD34⁺ cells, iii) Pgp activity of the CD34⁺CD133⁺ versus the CD34⁺CD133⁻ subset, iv) expression of CD133 and CD90, and v) when applicable, FISH analysis of sorted CD34⁺ cells. FISH analysis, aberrant antigen expression and Pgp activity were the most powerful

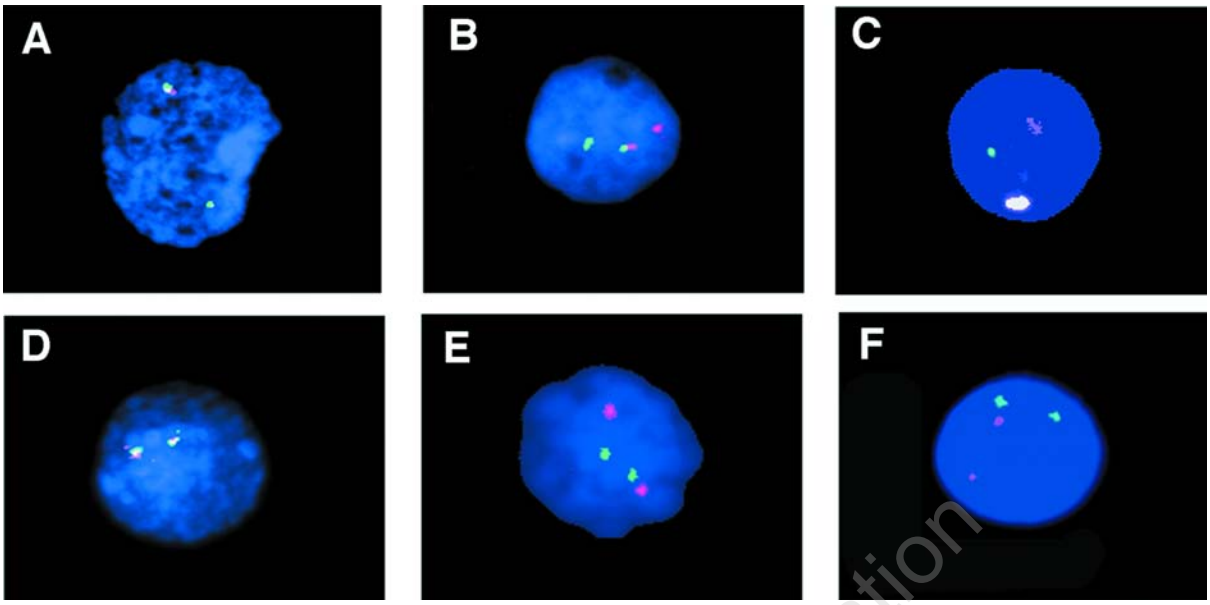

Figure 4. Dual color FISH analysis of isolated CD34⁻ and CD34⁺ cells from *de novo* AML samples. The upper part of the figures depicts the malignant CD34⁻ blasts and the lower part of the figures the CD34⁺ cells. A: Malignant CD34⁻ blasts from patient 7 with del(11), which is indicated by the absence of one orange signal; corresponding CD34⁺ cell fraction (D) with a normal pattern of two fusion signals. B: Malignant CD34⁻ blasts from patient 8 with t(15;17), indicated by a fusion signal; this is absent in the corresponding CD34⁺ cell fraction (E). C: Malignant CD34⁻ blasts from patient 18 with t(8;21), which is indicated by a yellow fusion signal; this is absent in the corresponding CD34⁺ cell fraction (F). See Table 3 for the percentages of cytogenetic aberrations in the isolated cell fractions.

parameters to establish the nature of the CD34⁺ cells.

We found that in the majority (18/19) of *de novo* AML patients, CD34⁺ cells present at low percentages (<1%: range 0.02%-0.7%) were of normal origin (Table 4). In contrast, the majority (4/5) of AML patients with intermediate numbers of CD34⁺ cells (range: 1.6-3.5%) present at diagnosis had CD34⁺ cells of malignant origin (Table 4). As expected in AML with high numbers of CD34⁺ cells (>50%) present at diagnosis, the majority of CD34⁺ cells were of leukemic origin, whereas in 2/3 patients a small sub-population of normal CD34⁺ cells could be detected.

These results suggest that CD34⁺ cells, when present at <1% in *de novo* AML samples, are most likely of normal origin. Such an AML can thus be considered as true CD34⁻. It cannot be excluded completely, however, that in some cases the CD34⁺ cells are of malignant origin, as illustrated by patient number 11 (0.10% CD34⁺). An exceptional case is that of patient 4, who displayed aberrant CD56 expression in 5% of the CD34⁺ cells, which is a higher level of expression than that found for normal CD34⁺ BM cells (mean±s.d. of 1.22±0.55%, range: 0.63-2.27%, n=5). Moreover, these CD34⁺ CD56⁺ cells showed strikingly lower Pgp activity than the corresponding CD34⁺ CD56⁻ cells (1.1 versus 5.4, respectively), a level very close to that observed in the corresponding malignant CD34⁻ CD45^{dim}CD56⁺ cells (i.e. 1.2). Thus, most of the CD34⁺ cells from patient 4 were of normal origin, whereas a small subpopulation (0.002% in total) was of malignant origin. Our findings, together with others that showed cytogenetic abnormalities in cases with <1% CD34⁺ cells,[27] suggest that it remains necessary to examine the CD34⁺ cells for a proper identification of their nature even when present at a very low percentage.

In addition to the parameters described, we investigated the use of mean fluorescence intensity (MFI) of the CD34 labeling. It was hypothesized that normal CD34⁺ cells in AML samples would show a relatively high CD34 MFI, similar to that found in normal CD34⁺ BM cells, whereas malignant CD34⁺ cells might display lower or higher CD34 MFI. However, it was found that in most cases the CD34 MFI overlapped too much for a proper discrimination to be possible between CD34⁺ cells of normal and malignant origin (*data not shown*).

We observed that in AML samples, the CD34⁺ cells of normal origin showed generally higher levels of CD133 and CD90 expression, i.e. in 11/19 and 8/19 cases, respectively, than those observed in

Table 4. Determination of the nature of CD34⁺ cells present in cases of AML with low percentages of CD34 cells.

Pat.	*CD34 (%)*	*Immunophenotype*			*Pgp activity*		*FISH*	*Nature of CD34⁺ cells[e]*
		Aberrant antigen expression	*CD133[a]*	*CD90[b]*	*All CD34⁺ cells[c]*	*CD133⁺/CD133⁻[d]*		
1	0.02	Normal	Normal[f]	Abnormal	Normal[f]	Normal	ND	Normal
2	0.03	Normal	Normal	Normal	Normal	Normal	ND	Normal
3	0.03	Normal	Normal	Normal	Normal	Normal	ND	Normal
4	0.03	Normal[g]	Normal	Normal	Normal[g]	Normal	ND	Normal[g]
5	0.03	ND	Normal	Normal	Normal	Normal	ND	Normal
6	0.05	ND	Normal	Normal	Normal	Normal	ND	Normal
7	0.07	Normal	Normal	Normal	Normal	Normal	Normal	Normal
8	0.07	ND	Normal	Normal	Normal	Normal	Normal	Normal
9	0.07	ND	Normal	Normal	Normal	Normal	ND	Normal
10	0.08	Normal	Normal	Abnormal	Normal	Normal	ND	Normal
11	0.10	ND	Normal	ND	Abnormal	Abnormal	ND	Leukemi
12	0.13	Normal	Normal	Normal	Normal	Normal	ND	Normal
13	0.14	Normal	Normal	Normal	Normal	ND	ND	Normal
14	0.18	Normal	Normal	Normal	Normal	Abnormal	ND	Normal
15	0.18	ND	Normal	Normal	Normal	ND	ND	Normal
16	0.20	Normal	Normal	Abnormal	Normal	Normal	ND	Normal
17	0.20	ND	Normal	Abnormal	Normal	Normal	ND	Normal
18	0.41	Normal	Normal	Normal	Normal	ND	Normal	Normal
19	0.70	Normal	Normal	Normal	Normal	Normal	ND	Normal
20	1.55	ND	Normal	Normal	Normal	Abnormal	ND	Normal
21	1.58	Abnormal	Normal	Normal	Abnormal	Abnormal	ND	Leukemic
22	1.68	Abnormal	Normal	ND	Abnormal	Abnormal	ND	Leukemic
23	2.51	ND	Normal	ND	Abnormal	Abnormal	ND	Leukemic
24	3.47	Normal	Abnormal	Abnormal	Abnormal	Abnormal	ND	Leukemic

ND: not determined; [a]Based on percentage of CD133 expression on CD34⁺ cells; [b]Based on percentage of CD90 expression on CD34⁺ cells; [c]Based on Pgp activity in CD34⁺ cells; [d]Based on Pgp activity in CD34⁺ CD133⁺ cells compared with that in CD34⁺ CD133⁻ cells; [e]Normal or leukemic nature of CD34⁺ cells based on results obtained from combinations of techniques (see final paragraph of results section); [f]slightly lower CD133 expression and Pgp activity than those of normal CD34⁺ cells; [g]5% of the CD34⁺ cells were of leukemic origin, showing aberrant antigen expression and abnormally low Pgp activity, whereas 95% were of normal origin, showing normal immunophenotype and normal Pgp activity (see text).

normal CD34⁺ BM cells (Figures 3 C, D). Although the reason for this is unclear, it is not unlikely that the maturation of the normal CD34 precursors is affected by high numbers of malignant blasts present at diagnosis.

Increases in CD34 percentages at relapse have been reported[28,29] in AML. If the small numbers of CD34⁺ cells in *de novo* AML were of leukemic origin, then a clonal outgrowth of these CD34⁺ cells at relapse might be expected. On the other hand,

such increases seem unlikely if the CD34+ cells at diagnosis are of normal origin. For three patients with low numbers of CD34+ cells at diagnosis, shown to be of normal origin based on our multiparameter approach (i.e. patients #10, 13 and 14), we were able to compare CD34 percentage at diagnosis and at relapse. No differences in CD34 percentages were observed, all patients showing <1% CD34+ cells at relapse, which confirms our hypothesis presented above for the normal origin of these CD34+ cells at diagnosis. No relapse samples were available for investigation from the other patients studied at diagnosis.

Altogether our results show that the described multiparameter approach allows discrimination between malignant and normal CD34+ cells in cases of AML with low percentages of CD34 cells. This may have implications for the phenotypic and functional characterization of primitive malignant stem cells and for studying mechanisms contributing to therapy resistance in AML. Moreover it allows the identification of truly CD34− AML, with a low likelihood of proceeding to CD34 positivity, thus enabling safe immunological purging via CD34+ selection of autologous stem cell transplants obtained at a later stage of disease.[30]

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Contributions

MAVDP was responsible for the data, analysis and wrote the manuscript, HJB, GJO and GJS designed and guided the study, NF and GW organized the immunophenotyping studies, MR carried out Pgp activity and immunophenotyping measurements and BM designed and performed the FISH analysis. All the authors contributed to the analysis, interpretation and drafting of the manuscript. We thank A. Nieuwint for providing karyotypic information on the patients.

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In the following paragraphs, Dr. Béné summarizes the peer-review process and its outcomes.

What is already known on this topic

CD34 is an early differentiation antigen, physiologically present on hematopoietic stem cells. It remains expressed in a large number of cases of leukemia, both myeloblastic and lymphoblastic, where the blasts are $CD34^+$. In a small number of cases of more mature AML, however, a small percentage of $CD34^+$ cells can be detected: these could be remaining normal cells or malignant cells. This poses a problem for therapies based on the administration of autologous $CD34^+$ stem cells collected when the patient has reached complete remission.

What this study adds

Here, a flow cytometry and FISH approach is proposed to assess whether, in AML with small numbers of $CD34^+$ cells, these are normal stem cells in truly $CD34^-$ neoplasia or potentially malignant cells.

Caveats

The techniques used in this manuscript are relatively sophisticated and may not be easily applicable. The most practical conclusion is that in AML patients with a low range of $CD34^+$ cells, these cells are likely to be normal residual stem cells.