Diagnostic Potential of CD34+ Cell Antigen Expression in Myelodysplastic Syndromes

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Key Words: Myelodysplastic syndromes; Acute myeloid leukemia; Bone marrow; CD34+ cells; Flow cytometry; Antigen expression

Abstract

The World Health Organization introduced flow cytometry as an additional criterion for diagnosis of myelodysplastic syndromes (MDS). Aberrant antigen expression on bone marrow (BM) blasts may identify “low-grade MDS.” This study aimed to examine differences in antigen expression on CD34+ BM cells between patients with MDS and those with secondary cytopenia. BM aspirates of 175 patients with cytopenia were classified as MDS or secondary cytopenia. Expression of stem cell antigens (CD34, CD133), myeloid antigens (CD13, CD33), B-cell antigens (CD19, CD10), growth factor receptors (CD117, CD123), and chemokine receptor (CD184) was examined. Thirty-two normal adults and 49 patients with CD34+ acute myeloid leukemia (AML) were also examined. High percentage of CD34+ cells, CD117 and CD123 overexpression, and abnormal CD45 expression on these cells are the best markers for MDS. These phenotypic aberrancies correlate with number of blasts and degree of dysplasia, and were similar to those in CD34+ AML, thus reflecting the relationship between these disorders.

Sustained cytopenia involving 1 or more hematopoietic lineages can occur secondary to nutritional deficiencies, prior infections, autoimmune diseases, bleeding episodes, toxic exposure, or bone marrow (BM) infiltration by hematologic or other neoplasms. Cytopenia is also found in myelodysplastic syndromes (MDS). These are clonal hematopoietic stem cell disorders characterized by peripheral cytopenia(s), ineffective hematopoiesis, morphologic dysplasia, genetic heterogeneity, a varying percentage of blast cells, and an enhanced risk to evolve toward acute myeloid leukemia (AML).1,2 In 2008, the World Health Organization (WHO) revised its criteria for the diagnosis of MDS.3 The extent of dysplasia, the number of blasts and ring sideroblasts, and the presence of karyotypic abnormalities are important parameters for classification. In patients with sustained cytopenia without known cause and without dysplasia or cytogenetic aberrations, the diagnosis of idiopathic cytopenia of undetermined significance (ICUS) can be used.

Peripheral blood counts, BM examination with iron stain, and cytogenetic analysis are generally performed in patients with unexplained cytopenia. Flow cytometry may add significantly to the diagnosis of MDS. The pattern of cell surface antigen expression is tightly regulated during hematopoiesis. Detection of aberrant marker expression on blasts and identification of abnormal maturation patterns of differentiating myelomonocytic cells with flow cytometric analysis may help distinguish MDS from secondary cytopenia.3,6 The lineage infidelity markers correlate with the severity of morphologic dysplasia, the cytogenetic abnormalities, and with the WHO classification of MDS. Flow cytometry could be especially useful to identify MDS cases without elevated blast counts, ring sideroblasts, or cytogenetic abnormalities.
However, standardization is needed to improve the data comparison among the different studies. Differences in reagents, expression of results, definition of positivity, choice of the reference population, and design of scoring systems are hindering factors.7

To evaluate the potential of flow cytometry in the diagnosis of MDS, we compared the antigen expression on the CD34+ BM cells in MDS with that in secondary cytopenia, acute myeloid leukemia (AML), and normal BM. BM aspirates of 175 consecutive patients with cytopenia seen at the University Hospital Brussels, Belgium, were included in this retrospective analysis. Patients were diagnosed as having MDS, secondary cytopenia, or ICUS based on clinical data, BM morphology, and cytogenetics, as described by WHO.3 The patients with MDS were further categorized as “low-grade MDS” (blast count in BM smear <5%) or refractory anemia with excess of blasts (RAEB). The expression of stem cell antigens (CD34, CD133), myeloid antigens (CD13, CD33), B-cell antigens (CD19, CD10), growth factor receptors (CD117, CD123), and a chemokine receptor (CD184) on the CD34+ cells was examined with flow cytometry. The results were compared with those found in BM aspirates of 32 hematologically normal adults and 49 patients with CD34+ AML. The ranges of the results obtained in the patients with secondary cytopenia were used as cutoff values in a scoring system. The robustness of our findings was evaluated by applying these cutoff values to a validation cohort of 62 consecutive patients with cytopenia. Finally, the flow cytometric characteristics of the different WHO subsets of low-grade MDS were examined.

Materials and Methods

Patients

Our study included 237 consecutive adult patients with cytopenia who were seen at our center and underwent BM aspiration as a part of the diagnostic procedure for suspected MDS. Final diagnosis was made independently by the clinical hematologist, based on the medical history, physical examination, screening laboratory studies (including blood cell count, reticulocyte count, vitamin B12 and folate levels, renal/hepatic function, and autoimmune/infectious serology if indicated), BM aspiration for morphologic and cytogenetic analysis, and BM biopsy findings; the hematologist was blinded with respect to the immunophenotyping results. The patients were separated into 2 cohorts: a study cohort and a validation cohort.

The study cohort consisted of the first 175 patients: 66 with MDS, 88 with secondary cytopenia, and 21 with ICUS. MDS was classified according to the WHO 2008 criteria.3 The MDS group consisted of 43 patients with low-grade MDS (<5% blasts in BM smear) and 23 with RAEB (10 with RAEB-1 and 13 with RAEB-2). The low-grade MDS group was further subdivided into 8 patients with refractory cytopenia with unilineage dysplasia (RCUD, 7 with refractory anemia and 1 with neutropenia), 10 patients with refractory anemia with ring sideroblasts (RARS), 24 patients with refractory cytopenia with multilineage dysplasia (RCMD) of whom 6 had more than 15% ring sideroblasts, and 1 patient with isolated del(5q). The secondary cytopenia could be attributed to immune mechanisms (30 patients), infectious diseases (13 patients), drug exposure (10 patients), vitamin deficiencies (8 patients), chronic renal failure (7 patients), and various other diseases (20 patients). BM aspirates were also examined in 32 hematologically normal adults (BM donors, cardiosurgical patients) and 49 patients with AML with CD34+ blasts.

For validation, an additional cohort of 62 consecutive patients with cytopenia was used: 26 with secondary cytopenia, 25 with MDS (18 low-grade MDS and 7 RAEB), and 11 with ICUS. The low-grade MDS group further consisted of 1 patient with RCUD, 5 patients with RARS, 11 patients with RCMD (of whom 4 had more than 15% ring sideroblasts), and 2 patients with isolated del(5q).

Our study was performed with full respect for patients’ rights to confidentiality and in accordance with local ethical research guidelines.

Monoclonal Antibodies

Samples were labeled with monoclonal antibodies directed against antigens on progenitor cells (CD34, CD133), B cells (CD19, CD10), and myeloid cells (CD13, CD33); and against growth factor (CD117, CD123), chemokine receptors (CD184), or common leukocyte antigen (CD45) Table 1. An IgG1 antibody was used as negative control. The CD34 antibody was conjugated to fluorescein isothiocyanate (FITC), whereas all other antibodies were conjugated to phycoerythrin (PE). All antibodies were used following the manufacturer’s instructions.

Preparation and Labeling of the Cell Suspensions

BM aspirates were collected in ethylenediaminetetraacetic acid and stored at 4°C for no longer than 48 hours until processing. Mononuclear cell suspensions were prepared using Ficoll-Hypaque centrifugation (Lymphoprep, Fresenius Kabi AG, Bad Homburg, Germany). Cell suspensions of 20 × 10⁶ cells/mL were made in phosphate-buffered saline (PBS), pH 7.4, supplemented with 1% bovine serum albumin. A cytocentrifuge preparation, made from the cell suspension, was stained with May–Grunwald-Giemsa and examined microscopically. The percentage of blasts was counted.

Cells were labeled by incubating 50 µL of the cell suspension with 10 µL of CD34-FITC and 5 µL of a second monoclonal antibody for 10 minutes in the dark. For
enumerating the CD34+ cells, CD45-PE was used as the second antibody. Cells were rinsed with FACSFlow (Dulbecco PBS, BD Biosciences, San Jose, CA) and resuspended in 0.4 mL of this buffer. For immunophenotyping the CD34+ cells, 0.5 mL of ammonium chloride lysis buffer was added and left for 10 minutes in the dark to lyse remaining RBCs. Cells were then rinsed and resuspended as described before.

Flow Cytometric Analysis

All labeled samples were examined with a Beckman Coulter XL flow cytometer (Beckman Coulter, Miami, FL). Forward and side scatter detection was calibrated with unlabeled normal lymphocyte suspensions. Fluorospheres (Dako, Glostrup, Denmark) were used to set the fluorescence detection voltages. A mixture of normal lymphocytes stained separately with CD4-FITC and CD4-PE was used for compensation. Settings were checked each week. All labeled samples were examined within 4 hours of labeling. The percentage of CD34+ cells was determined on all mononuclear cells after exclusion of the CD45−/CD34− populations (erythroblasts, nonlysed RBCs).

The phenotype of the CD34+ cells was determined on all mononuclear cells after exclusion of the CD45−/CD34− populations by setting a gate for cells with medium to high CD34 fluorescence intensity and low side scatter. Data on at least 1,000 CD34+ cells were collected. Dubious and autofluorescent cells were excluded in forward and 90° light scatter and fluorescence 1 to fluorescence 2 dot plot of the cells. Backgating was then performed toward a forward and side scatter dot plot. Results were expressed as percentage of antigen-positive cells or as relative mean fluorescence intensity (RMFI, defined as the mean fluorescence intensity [MFI] of each antigen staining divided by the MFI of isotype-matched negative control staining).

Statistics

Statistical analysis was performed with Analyse-it software, version 1.73 (Analyse-it Software, Leeds, England) and Microsoft Excel 2003 (Microsoft, Redmond, WA). Most parameters were not normally distributed so that nonparametric methods had to be applied. For each parameter, median value and range were determined. Patients with secondary cytopenia were used as the reference population and statistical differences with the different patient populations were assessed using the Mann-Whitney U test for continuous variables. A P value of less than .05 was considered statistically significant. Spearman rank correlation was used to investigate the relationship between the percentage of CD34+ cells and the percentage of blasts in the BM smear and the cytocentrifuge preparation.

Results

Percentage of CD34+ Cells

The percentage of CD34+ cells in low-grade MDS and RAEB as well as in AML was significantly higher than that in secondary cytopenia, whereas that in patients with ICUS and normal BM was not different. A good correlation was found between the percentage of CD34+ cells and the blast counts in the cytocentrifuge preparations ($r_s = 0.87$, 95% confidence interval [CI], 0.82-0.90). These percentages were not statistically different ($P = .6512$). However, both were slightly higher than the percentage of microscopic blasts in the BM smears ($P < .0001$) and showed good correlation ($r_s = 0.82$; 95% CI, 0.76-0.86 and $r_s = 0.89$; 95% CI, 0.85-0.92, respectively). This was probably because of the enrichment of the mononuclear cells by the density gradient centrifugation.

Immunophenotype of CD34+ Cells

The immunophenotypic characteristics of the CD34+ cells were listed (Table 2) as an example. Low-grade MDS showed a lower expression of B-lymphoid antigens (CD19, CD10) and a higher expression of myeloid antigens (CD117, CD13)
**Figure 1** Gating strategy used for the detection of antigen expression on the CD34+ cells. The cell suspension was labeled with CD34-FITC (fluorescence 1) and CD19-PE (fluorescence 2). CD34+ cells were selected based on fluorescence 1 intensity and side scatter (A). Debris and autofluorescent cells were excluded in forward and 90% side scatter (B) and in a fluorescent 1–fluorescent 4 dot plot (C). The gate was then narrowed around the CD34+ cell cluster (D). Back-gating was performed in a forward and side scatter dot plot (E). The positivity for CD19 was measured in a fluorescence 1 (CD34)–fluorescence 2 (CD19) dot plot (F). The mean fluorescence intensity for CD19 in the CD34+ population was determined (G). Finally the positivity in the total cell population was visualized (H). FITC, fluorescein isothiocyanate; PE, phycoerythrin.

**Table 2** Percentage and Immunophenotype of CD34+ Cells From Patients in the Study*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal BM</th>
<th>Secondary Cytopenia</th>
<th>Low-Grade MDS</th>
<th>RAEB</th>
<th>AML</th>
<th>ICUS</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34, %</td>
<td>1.5 (0.6-3.2)</td>
<td>1.5 (0.3-7.0)</td>
<td>1.7 (0.2-8.8)†</td>
<td>10.5 (3.0-35.5)‡</td>
<td>35.5 (12.8-95.5)¶</td>
<td>1.2 (0.3-4.0)</td>
</tr>
<tr>
<td>CD34+ cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD19 (RMFI)</td>
<td>1.5 (0.9-4.7)</td>
<td>1.1 (0.5-12.8)</td>
<td>1.0 (0.5-3.4)‡</td>
<td>0.9 (0.6-14.7)‡</td>
<td>1.0 (0.7-74.9)¶</td>
<td>1.5 (0.9-10.5)</td>
</tr>
<tr>
<td>CD10 (RMFI)</td>
<td>2.4 (1.1-8.9)</td>
<td>1.4 (0.9-28.2)</td>
<td>1.2 (0.7-3.3)‡</td>
<td>1.1 (0.7-2.6)‡</td>
<td>1.0 (0.9-5.3)¶</td>
<td>1.5 (1.0-28.7)</td>
</tr>
<tr>
<td>CD133 (RMFI)</td>
<td>6.1 (2.4-10.1)†</td>
<td>3.7 (1.2-16.1)</td>
<td>4.7 (1.6-15.0)†</td>
<td>6.6 (2.7-26.2)†</td>
<td>12.4 (1.4-71.8)¶</td>
<td>3.0 (1.6-8.9)</td>
</tr>
<tr>
<td>CD13 (RMFI)</td>
<td>12.9 (3.6-27.0)†</td>
<td>10.5 (2.6-82.8)</td>
<td>14.8 (3.1-59.6)†</td>
<td>36.7 (4.3-112.9)‡</td>
<td>43.6 (0.9-330.3)¶</td>
<td>7.5 (2.6-27.0)</td>
</tr>
<tr>
<td>CD33 (RMFI)</td>
<td>6.5 (2.1-16.3)†</td>
<td>5.4 (1.2-42.2)</td>
<td>5.5 (1.2-35.9)</td>
<td>4.4 (1.0-75.3)</td>
<td>5.8 (1.1-131.3)</td>
<td>3.9 (1.2-16.3)</td>
</tr>
<tr>
<td>CD45 (RMFI)</td>
<td>24.3 (9.8-68.8)</td>
<td>30.6 (11.0-61.5)</td>
<td>24.7 (6.1-104.8)</td>
<td>22.9 (2.2-117.6)</td>
<td>24.4 (2.8-190.1)</td>
<td>25.9 (14.7-53.6)</td>
</tr>
<tr>
<td>CD117 (RMFI)</td>
<td>39.4 (13.8-78.3)†</td>
<td>26.8 (4.7-80.1)</td>
<td>73.3 (8.3-255.4)†</td>
<td>128.1 (43.1-303.7)†</td>
<td>130.0 (4.2-404.3)†</td>
<td>23.1 (4.1-124.1)</td>
</tr>
<tr>
<td>CD123 (RMFI)</td>
<td>10.1 (5.6-21.5)†</td>
<td>9.5 (3.3-19.4)</td>
<td>13.1 (4.0-83.8)†</td>
<td>16.4 (2.5-81.3)†</td>
<td>26.6 (3.2-384.6)†</td>
<td>7.1 (3.7-25.0)</td>
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<tr>
<td>CD104 (RMFI)</td>
<td>3.2 (1.3-8.5)†</td>
<td>1.9 (1.0-4.9)</td>
<td>1.7 (1.0-5.9)</td>
<td>1.2 (0.7-3.6)¶</td>
<td>1.3 (0.9-15.5)¶</td>
<td>2.4 (1.3-6.6)</td>
</tr>
<tr>
<td>CD117/CD19 (RMFI)</td>
<td>25.2 (3.5-77.7)†</td>
<td>23.0 (0.4-92.5)</td>
<td>65.3 (2.4-257.1)†</td>
<td>136.2 (8.7-333.9)†</td>
<td>130.7 (4.2-442.5)†</td>
<td>11.0 (0.4-124.1)†</td>
</tr>
</tbody>
</table>

AML, acute myeloid leukemia; BM, bone marrow; ICUS, idiopathic cytopenia of undetermined significance; MDS, myelodysplastic syndrome; RAEB, refractory anemia with excess of blasts; RMFI, relative mean fluorescence intensity.

* Data are given as median (range).
† Significantly higher than in secondary cytopenia (< .05).
‡ Significantly lower than in secondary cytopenia (< .05).
than secondary cytopenia. The myeloid/B-lymphoid ratio (CD117/CD19) was increased. These changes in MDS were mainly because of a decrease in the B-cell progenitors and an increase in the myeloid progenitors in the CD34+ cell population. For B-cell progenitors (CD19), a median value of 1% (range, 0.1%-26.7%) was found in low-grade MDS compared with 5.2% (range, 0%-56.2%) in secondary cytopenia; for myeloid progenitors (CD117), values of 91.9% (50.6%-99.9%) and 81.3% (34.9%-95.2%), respectively, were found.

Figure 3. A higher positivity was also found for the stem cell antigen CD133 and the interleukin 3 (IL-3)-receptor α (CD123). The RMFI of CD33, CD45, and the chemokine receptor CD184 was not statistically different from that in secondary cytopenia.
Similar but more pronounced changes compared with secondary cytopenia were found in RAEB and AML. The results in RAEB were significantly different from those in low-grade MDS for CD19 (lower) and for CD133, CD13, CD117, and CD117/CD19 (higher). In addition, CD184 had decreased positivity.

The results in AML were not statistically different from those in RAEB with the exception of a higher positivity for CD123.

Patients with ICUS showed no significant differences in the expression of individual markers on CD34+ cells compared with secondary cytopenia. However, there was a

(F) CD33.  (G) CD45.  (H) CD117.  (I) CD123.  (J) CD184. Bars are medians.  \( P < .05 \) considered as statistically different (Mann-Whitney \( U \) test).  AML, acute myeloid leukemia;  BM, bone marrow;  ICUS, idiopathic cytopenia of unknown significance;  MDS, myelodysplasia;  RAEB, refractory anemia with excess blasts.
tendency for a higher expression of B-cell antigens (CD34+, CD19+) and a lower expression of myeloid antigens (CD34+, CD117+). This led to a significantly decreased myeloid/B-lymphoid ratio in the CD34+ precursors. These changes were contrary to those found in MDS.

Normal BM also showed significant differences from secondary cytopenia. Some of them were similar, though sometimes less pronounced, than those seen in MDS, such as the higher positivity for CD133 and CD117 and a slightly higher CD117/CD19 ratio. The increase of the CD184 positivity, however, was the opposite of that found in MDS.

Sensitivity of the Different Markers for the Detection of MDS

The sensitivity of the different markers for the detection of MDS was then calculated as the percentage of patients with a result outside the RMFI ranges found in secondary cytopenia. However, the direction of the change was also taken into account. When the results in MDS were significantly higher (percentages of CD34, CD117, CD133, CD13, CD123) or lower (CD19, CD10, CD184) only the results above or below the lower limit of the reference range were taken into account. When no significance was found in MDS (CD33, CD45), higher as well as lower results were taken into account. The percentages of positive patients are listed in Table 3.

The best markers for the detection of low-grade MDS were an increase of CD117 and the CD117/CD19 ratio. A high CD123 and a low or high CD45 positivity were present in a significant minority of the samples.

The percentage of CD34+ cells in the mononuclear cell suspension was increased in 65.2% of the RAEB samples. In addition, the abnormalities found in low-grade MDS were more frequently observed (CD117, CD117/CD19, CD123, CD45). High levels of CD133 and CD13 or abnormal levels of CD33 were found in 10% to 20% of the RAEB samples.

### Table 3

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Outside RR When</th>
<th>Low-Grade MDS</th>
<th>RAEB</th>
<th>AML</th>
<th>ICUS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mononuclear cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD34, %</td>
<td>&gt;7.0</td>
<td>2.3</td>
<td>65.2</td>
<td>100.0</td>
<td>0</td>
</tr>
<tr>
<td>CD34+ cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD19 (RMFI)</td>
<td>&lt;0.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>CD10 (RMFI)</td>
<td>&lt;0.9</td>
<td>2.8</td>
<td>9.1</td>
<td>6.1</td>
<td>0</td>
</tr>
<tr>
<td>CD133 (RMFI)</td>
<td>&gt;16.1</td>
<td>0</td>
<td>13.0</td>
<td>40.8</td>
<td>0</td>
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<tr>
<td>CD13 (RMFI)</td>
<td>&gt;82.8</td>
<td>0</td>
<td>8.7</td>
<td>24.5</td>
<td>0</td>
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<tr>
<td>CD33 (RMFI)</td>
<td>&lt;1.2 or &gt;42.2</td>
<td>0</td>
<td>17.4</td>
<td>14.3</td>
<td>0</td>
</tr>
<tr>
<td>CD45 (RMFI)</td>
<td>&lt;11.0 or &gt;61.5</td>
<td>16.2</td>
<td>31.8</td>
<td>47.9</td>
<td>0</td>
</tr>
<tr>
<td>CD117 (RMFI)</td>
<td>&gt;80.1</td>
<td>41.9</td>
<td>78.3</td>
<td>69.4</td>
<td>9.5</td>
</tr>
<tr>
<td>CD123 (RMFI)</td>
<td>&gt;19.4</td>
<td>28.6</td>
<td>39.1</td>
<td>62.8</td>
<td>7.1</td>
</tr>
<tr>
<td>CD184 (RMFI)</td>
<td>&lt;1.0</td>
<td>0</td>
<td>4.3</td>
<td>2.3</td>
<td>0</td>
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<tr>
<td>CD117/CD19 (MFI)</td>
<td>&gt;92.5</td>
<td>31.0</td>
<td>69.6</td>
<td>63.3</td>
<td>9.5</td>
</tr>
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</table>

AML, acute myeloid leukemia; ICUS, idiopathic cytopenia of undetermined significance; MDS, myelodysplastic syndrome; MFI, mean fluorescence intensity; RAEB, refractory anemia with excess of blasts; RMFI, relative mean fluorescence intensity; RR, reference range.
All AML samples showed an increase in the percentage of CD34+ cells. The number of samples with high CD117 and CD117/CD19 was similar to RAEB, but more samples showed aberrant values for CD133, CD13, CD45, and CD123.

Approximately 10% of patients with ICUS showed high values for CD117, CD117/CD19, and CD123. All other results were within the reference ranges of secondary cytopenia.

MDS Flow Cytometry Score in the Study Cohort

Each patient of the study cohort was scored based on the 10 parameters used in our panel (percentage of CD34 and RMFI of CD19, CD10, CD133, CD13, CD33, CD45, CD117, CD123, and CD184). One point was given for each result outside the defined reference ranges. Scores for the different patient populations are shown in Table 4. The scores varied between 0 and 3 in low-grade MDS, between 1 and 5 in RAEB, and between 1 and 6 in AML. A few patients with ICUS had a score of 1. The mean score was 1.0 for low-grade MDS, 2.6 for RAEB, 3.6 for AML, and 0.2 for ICUS. A score of 1 or higher was found in 70.4% of patients with low-grade MDS and in 100% of patients with RAEB and AML. Of the total MDS group (low-grade MDS and RAEB), 83.7% showed a score of 1 or higher. Because the scoring system was based on the range of secondary cytopenia, specificities for detection of low-grade MDS, RAEB, and total MDS were 100% (95% CI, 93.6-100). Sensitivities of the scoring system for low-grade MDS, RAEB, and total MDS were 70.4% (95% CI, 49.8-86.3), 95.5% (95% CI, 77.2-99.9), and 81.6% (95% CI, 68.0-91.2), respectively, whereas specificities were 100% (95% CI, 93.6-100).

Analysis of the Validation Cohort Using the Scoring System

We analyzed the results of the validation cohort by applying the same scoring system and using the same reference ranges that had been determined in the study cohort. No flow cytometric abnormalities were found for the patients with secondary cytopenia. Overexpression of CD117 was again the most frequent immunophenotypic aberrancy among patients with MDS: 38.9% of low-grade MDS and 57.1% of RAEB. Only 1 patient with ICUS showed increased expression of CD117. The frequencies of aberrancies of the other markers for MDS were comparable with those obtained in the study cohort, and they were also higher in RAEB than in low-grade MDS (results not shown).

Application of the extensive scoring system (10 parameters) in the validation cohort (Table 4) revealed that all patients with secondary cytopenia had scores of 0. The scores varied from 0 to 4 for low-grade MDS and RAEB. The mean scores were 0.3 for ICUS, 0.7 for low-grade MDS, and 2.1 for RAEB. Sensitivities of the alternative scoring system for low-grade MDS, RAEB, and total MDS were respectively 70.4% (95% CI, 49.8-86.3), 100% (95% CI, 84.6-100), and 56.0% (95% CI, 34.9-75.6), respectively.

Table 4

<table>
<thead>
<tr>
<th>Score</th>
<th>Study Cohort</th>
<th>Validation Cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low-Grade MDS</td>
<td>RAEB</td>
</tr>
<tr>
<td>0</td>
<td>29.6</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>44.4</td>
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<tr>
<td>3</td>
<td>3.7</td>
<td>36.4</td>
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<tr>
<td>4</td>
<td>0</td>
<td>13.6</td>
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<tr>
<td>5</td>
<td>0</td>
<td>9.1</td>
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<td>6</td>
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<td>7-10</td>
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</tbody>
</table>

AML, acute myeloid leukemia; ICUS, idiopathic cytopenia of undetermined significance; MDS, myelodysplastic syndrome; RAEB, refractory anemia with excess of blasts.
whereas specificities were 100.0% (95% CI, 86.8-100).
Again, these sensitivities and specificities did not differ statistically from those obtained in the study cohort because of the overlap in 95% CIs.

When data from both cohorts were combined, application of the extensive scoring system (10 parameters) yielded sensitivity of 62.2% (95% CI, 46.5-76.2), 96.6% (95% CI, 82.2-99.9), and 75.7% (95% CI, 64.3-84.9) for detection of low-grade MDS, RAEB, and total MDS, respectively. Specificity was 100% (95% CI, 95.6-100) for all patient groups.

Application of the alternative scoring system (4 parameters) on the combined data from both cohorts yielded similar results. The sensitivities for detection of low-grade MDS, RAEB, and total MDS were 60.0% (95% CI, 44.3-74.3), 93.1% (95% CI, 77.2-99.2), and 73.0% (95% CI, 61.4-82.7), respectively, whereas specificity was 100% (95% CI, 95.6-100).

Immunophenotype of WHO Subsets in Low-Grade MDS

When the study cohort (n = 43) and the validation cohort (n = 18) were combined, 9 patients had RCUD (8 with refractory anemia and 1 with refractory neutropenia), 14 had RARS, and 35 had RCMD, of whom 10 had 15% or more ring sideroblasts (previous RCMD-RS). Abnormalities in the marker expression and scores of these WHO subsets are listed in Table 5.

Overexpression of CD117 was the most frequent immunophenotypic aberrancy among all WHO subsets. Subsets with a higher degree of morphologic aberration (multilineage vs unilineage dysplasia) showed higher percentages of patients with CD117 overexpression and aberrant CD45 expression. Higher expression of CD123 was found in both unilineage and multilineage dysplasia subsets, but not in the subsets with ring sideroblasts (RARS and RCMD with ≥15% ring sideroblasts).

Flow cytometry scores were higher in the subsets with multilineage dysplasia than unilineage dysplasia (P < .05, Figure 4). Mean scores were 0.4 for RCUD, 0.3 for RARS, 1.1 for RCMD with less than 15% ring sideroblasts, and 1.5 for RCMD with 15% or more ring sideroblasts. Higher percentages of patients with multilineage dysplasia had a score of 1 or higher (Table 5).

The sensitivities of the scoring system for detection of low-grade MDS without (RCUD and RCMD with <15% ring sideroblasts) and with ring sideroblasts (RARS and RCMD with ≥15% ring sideroblasts) were 64.0% (95% CI, 42.5-82.0) and 58.8% (95% CI, 32.9-81.6), respectively, whereas specificities were 100.0% (95% CI, 95.6-100).

Discussion

Flow cytometric analysis of BM CD34+ cells may be of added value for diagnostic workup and prognostication of

<table>
<thead>
<tr>
<th>Table 5</th>
<th>Percentage of Patients From Different WHO Subsets of Low-Grade MDS (From Study and Validation Cohort) With Abnormal Marker Expression Outside the Indicated Reference Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter</td>
<td>Outside RR When RA RARS RCMD &lt;15% RS RCMD ≥15% RS</td>
</tr>
<tr>
<td>Mononuclear cells</td>
<td>CD34, %</td>
</tr>
<tr>
<td>CD34+ cells</td>
<td>CD19 (RMFI)</td>
</tr>
<tr>
<td>CD10 (RMFI)</td>
<td>&gt;0.9</td>
</tr>
<tr>
<td>CD133 (RMFI)</td>
<td>&gt;16.1</td>
</tr>
<tr>
<td>CD13 (RMFI)</td>
<td>&gt;82.8</td>
</tr>
<tr>
<td>CD33 (RMFI)</td>
<td>&lt;1.2 or &gt;42.2</td>
</tr>
<tr>
<td>CD45 (RMFI)</td>
<td>&lt;11.0 or &gt;61.5</td>
</tr>
<tr>
<td>CD117 (RMFI)</td>
<td>&gt;80.1</td>
</tr>
<tr>
<td>CD123 (RMFI)</td>
<td>&gt;19.4</td>
</tr>
<tr>
<td>CD184 (RMFI)</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Score</td>
<td>≥1</td>
</tr>
</tbody>
</table>

MDS, myelodysplastic syndrome; RA, refractory anemia; RARS, refractory anemia with ring sideroblasts; RCMD, refractory cytopenia with multilineage dysplasia; RMFI, relative mean fluorescence intensity; RR, reference range.

Figure 4 | Frequency of flow cytometry scores in low-grade myelodysplasia subsets of study and validation cohorts. RARS, refractory anemia with ring sideroblasts; RCMD, refractory cytopenia with multilineage dysplasia; RCUD, refractory cytopenia with unilineage dysplasia; RS, ring sideroblasts.

However, multiple variables hamper the comparison of the results of different studies, and standardization is needed. To better understand the diagnostic potential of flow cytometry in MDS, we studied the immunophenotypic characteristics of the CD34+ BM cells in a cohort of consecutive patients with cytopenia, including secondary cytopenia, MDS, and ICUS. Patients with secondary cytopenia were used as the reference population, because these patients have to be differentiated from MDS in clinical practice.9-11 We compared the results to those found in CD34+ AML, a possible end stage
in the evolution of MDS. In addition, we examined the differences between secondary cytopenia and normal BM, which is used as the reference population in a number of studies.

The antigen expression on CD34+ cells was studied in mononuclear cell suspensions prepared via density gradient centrifugation. This technique was often applied at the time that this study was started (2002) to enrich the CD34+ cells and to reduce the effect of peripheral blood contamination by removing mature granulocytes. Density gradients were also applied in other studies on CD34+ cells.10,12,13 Today leukocyte suspensions prepared using RBC lysis are often preferred to reduce potential changes in the cellular composition of the BM aspirate. A limited study using 10 BM samples in our laboratory did not show significant changes in the CD34+ cell subsets after Ficoll-Hypaque centrifugation compared with RBC lysis (results not shown).

Data were collected from 2002 to 2007. The stability of the results over time was ensured by regularly checking the fluorescence detection voltages and the compensation settings. Each lot of antibodies was compared with the previous lot before application. During the whole period of the study, the same flow cytometer was used. We retrospectively compared the results for the most important markers (CD117, CD45, and CD123) from the patients in the secondary cytopenia group for the periods 2002 to 2004 (44 patients) and 2004 to 2007 (44 patients), and no significant differences were found (Mann-Whitney U test). This proved that the results were comparable over the whole period of the study.

The percentage of CD34+ cells was significantly higher in MDS than in secondary cytopenia and increased from low-grade MDS to RAEB and further to AML. The majority of patients with RAEB and all patients with AML had high values. The percentage in ICUS and normal BM was not different from that in secondary cytopenia. The percentage of CD34+ cells was not different from the blast counts in the cytocentrifuge preparations of the mononuclear cell suspensions. This proves that the large majority of the blasts in nearly all patients were CD34+. These findings are in line with those of other reports.5,8,10,12,14-16 These percentages were, however, slightly higher than the blast count in the BM smears. This is because we prepared mononuclear cell suspensions, which led to an enrichment of the CD34+ cells. If whole BM were used, after RBC lysis, the percentages of CD34+ cells could be lower because of dilution of the BM aspirate by peripheral blood.

The stem cell factor receptor as detected by the CD117 antibody is a good marker for the myeloid differentiation of CD34+ cells. High CD117 positivity (RMFI) of the CD34+ cells was the most frequent immunophenotypic abnormality in MDS, with higher RMFI and more patients with levels above the reference range in RAEB than in low-grade MDS. The CD117 positivity in AML was similar to that in RAEB. Similar findings have been described elsewhere.6,12,16,17

The expression of the B-cell antigen CD19 was significantly lower in MDS and AML than in secondary cytopenia, but none of the patients showed values below the reference range. Low levels of CD34+ B-cell progenitors have been reported by others.5,6,9 Ogata et al5 found that low levels of CD34+ B-cell progenitors was the most frequent immunophenotypic abnormality in low-grade MDS. The discrepancy with our study may be because of the composition of the reference population and the definition of the reference range.

A high expression of CD117 and a low expression of CD19 lead to a high CD117/CD19 (myeloid/B-lymphoid) ratio, which was a good marker for MDS with higher values in RAEB and AML. Similar findings were described by van de Loosdrecht et al.9

A high expression of the IL-3 receptor α was found on the CD34+ cells in 30% to 40% of patients with MDS and in 63% of patients with CD34+ AML. Data on the IL-3 receptor α expression in MDS are limited. The receptor is highly expressed on the CD34+ cells in AML,18 which correlates with a high proliferation of the blasts and a poor prognosis.19 CD123 is also strongly positive on AML stem cells (CD34+/CD38−) but rarely found on CD34+/CD38− cells of normal precursors.20 High levels were also found on CD34+/CD38− cells of patients with MDS and the levels were found to be increased with increased number of blasts at diagnosis.16,21 The high CD123 expression on the CD34+ cells in MDS could make these cells more sensitive to immunotoxins targeting CD123.22

No significant difference was found between the RMFI of common leukocyte antigen on the CD34+ cells, as detected by the CD45 antibody, in secondary cytopenia, MDS, and AML. However, values fell outside the reference range (low and high) in these disorders, and their frequency increased from low-grade MDS to RAEB and AML. Low and high levels were reported by Matarraz et al6 whereas others have reported only low levels.5,12

The RMFI levels for CD133 and CD13 were significantly increased in MDS and AML but values fell outside the reference ranges only in RAEB and AML. CD133 detects an antigen on CD34+ hematopoietic precursor cells as well in AML and acute lymphoblastic leukemia.23,24 High levels have been described in low-grade MDS and RAEB.5,16,21 An increase of the CD13 positivity was also found by others, more in RAEB than in low-grade MDS.5,6,12,21

Low and high values were found for CD33 only in RAEB and AML. Abnormal values have been described in the majority of patients with MDS.12,25 High5 as well as low8 values have been found.

A slight but significant decrease of the CD184 reactivity was found in RAEB and AML. Rare patients had values below the reference range. The chemokine receptor CXCR4 is expressed on CD34+ hematopoietic precursors and mediates
transendothelial migration induced by stromal cell-derived factor 1 (SDF-1). CXCR4 expression is mainly found on normal CD34+ lymphoid precursors and in blasts of acute lymphoblastic leukemia and acute myelomonocytic or monoblastic leukemia. The low CD184 RMFI in RAEB and AML could arise because of a decrease of the B-lymphoid precursors or downregulation of the CXCR4 expression on the myeloid precursors. The latter could contribute to the appearance of blasts in blood in these disorders. No data were found in the literature on the expression of CXCR4 in MDS.

In summary, the CD34+ cells in a minority of patients with low-grade MDS showed high values for CD117 and CD123 and low or high values for CD45. These abnormalities were more frequently present in RAEB and AML together with high values for CD133 and CD13, low or high values for CD33, and low values for CD184. Overexpression of CD133 and CD123 were particularly frequent in CD34+ AML. These findings were confirmed in the validation cohort.

Only 10% of the patients with ICUS had CD34+ cells with overexpression of CD117 or CD123, the most frequent aberrations seen in MDS and AML. An increased CD117 expression was found by Truong et al in patients with cytopenia without BM dysplasia who later developed MDS, whereas CD123 expression was not noted. Unfortunately, follow-up data on our patients were not available.

By combining the study and validation cohorts, the number of patients was adequate to get an idea of the immunophenotypic changes of the CD34+ cells in the WHO subtypes of MDS in the low-grade MDS group. As expected, overexpression of CD117 and of CD123 and a low or high expression of CD45 are the most frequent abnormalities. Multilineage dysplasia was associated with a higher frequency of abnormal CD117 or CD45 levels than unilineage dysplasia. Remarkably, overexpression of the IL-3 receptor α was not found in the MDS subsets with ring sideroblasts. Yue et al found that the CD123 expression on CD34+/CD38− cells in MDS showed a positive correlation with the number of cell lineages with dysplasia in the BM but no data were given about the subsets with and without ring sideroblasts. Our data have to be confirmed on a larger number of patients. Whether this finding has biological and clinical implications remains to be elucidated.

We developed a scoring system based on the percentage of CD34+ cells and the RMFI of the other 9 markers and with secondary cytopenia as the reference population. Scores of 1 or higher were obtained for 70.4% of patients with low-grade MDS and 100% of patients with RAEB and AML, and the mean scores were also higher with evolving disease category. Twenty-one percent of the patients with ICUS had a score of 1. The validation cohort showed similar scores for low-grade MDS, RAEB, and MDS.

Combining the data from both cohorts, our scoring system showed a sensitivity of 62.2%, 96.6%, and 75.7% for the detection of low-grade MDS, RAEB, and MDS, respectively, whereas the specificity was 100%. Similar performance was obtained with only 4 parameters percentage of CD34+ cells and RMFI of CD117, CD45, and CD123. The use of a reduced panel of antibodies could reduce the laboratory workload and the cost of these tests. The sensitivity of the 10-parameter scoring system for the detection of MDS without increase of the blasts and without ring sideroblasts was 64.0%.

The diagnostic performance of our scoring system is in line with previous reports, showing similar sensitivities and specificities for the detection of low-grade MDS, AML, and secondary cytopenia (results not shown). Forty percent of the patients with secondary cytopenia in our study had a score of 1 or more with this approach (mean score, 0.6). The mean scores for MDS and AML also increased. An acceptable sensitivity for low-grade MDS could only be obtained with a specificity of less than 100% (eg, sensitivity of 55.6% and specificity of 85.7% for a score ≥2).

When reference values were obtained by combining the normal BM and secondary cytopenia cohorts, the sensitivity of the detection of MDS decreased slightly (73.0% vs 75.7%) and the specificity was 100% (results not shown).

In conclusion, this study found that a high number of CD34+ cells, an overexpression of the growth factor receptors for c-kit (CD117) and IL-3 (CD123), and a variable expression of common leukocyte antigen are the best markers to differentiate MDS from secondary cytopenia. These phenotypic aberrances correlate with the number of blasts and the degree of dysplasia in the BM smears. They were similar to, but less pronounced than, those found in CD34+ AML, thus reflecting the relationship between these disorders. Adding other markers to our panel could probably increase the sensitivity of this approach. Although an overexpression of CD123 is a marker for MDS, it was not found in patients with ring sideroblasts. Further studies are needed to support this finding and to elucidate its biological significance. Finally, a follow-up study in a population of patients with ICUS is needed to fully elucidate the clinical usefulness of this approach.

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