Characterization of CD34+, CD13+, CD33− cells, a rare subset of immature human hematopoietic cells

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Background and Objectives. Hematopoietic progenitor cells that express CD34 are heterogeneous in their lineage affiliation and degree of maturation. Expression of CD13 and CD33 antigens indicates myeloid lineage association, but the precise sequence of expression of these two markers during differentiation is unclear. We noted the presence of CD34+ cells expressing CD13 but lacking CD33, a subset of cells not yet well characterized. In this report we describe the prevalence and the immunophenotype of this cell subset.

Design and Methods. We studied the immunophenotype of immature myeloid cells in human bone marrow samples from 11 healthy transplantation donors and in 4 cord blood samples. We used four-color flow cytometry and a large panel of monoclonal antibodies directed against lineage and differentiation-associated antigens. Three additional bone marrow samples were analyzed after immunomagnetic sorting of CD34+ cells. We focused our analysis on the subset of cells defined by the expression of CD34 and CD13 and the lack of CD33.

Results. We found CD34+, CD13+, CD33− cells in all 11 bone marrow and 4 cord blood samples studied. These cells represented 0.5±0.5% (mean ± SD) and 0.8±1.2% of mononucleated cells, respectively. CD34+, CD13+, CD33− cells appeared to be more immature than those expressing CD33 because of their light scatter characteristics (smaller size and lower granularity), the expression of markers associated with early hematopoietic cells (CD90, CD133 and CD117), and the absence of lineage-associated markers.

Interpretation and Conclusions. These findings suggest that the expression of CD13 precedes that of CD33 during myeloid differentiation, and that CD34+, CD13+, CD33− cells are at an early stage of human myeloid cell differentiation.

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Key words: CD34+ cells, flow cytometry, hematopoiesis, minimal residual disease, leukemic myeloblasts.
after centrifugation on a density gradient using Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden) and washed three times in phosphate-buffered saline (PBS) and resuspended at 0.5-1.0×10⁶/mL in PBS containing 0.5% bovine serum albumin.

Immunomagnetic cell sorting

CD34+ cells from 3 bone marrow samples were isolated by incubating 10⁶ cells in 300 µL of PBS with 100 µL of Fc receptor blocking reagent and 100 µL of CD34 (QBEND10)-conjugated magnetic beads (CD34 direct isolation kit; Miltenyi Biotec, Bergisch, Gladbach, Germany) followed by incubation for 30 min at 4-8°C. After washing with PBS, cells were processed through a MS+ separation column for 30 min at 4-8°C. After washing with PBS, cells were treated with Fix and Perm™ (F/P, An der lands). For intracellular staining, surface labeled (IgG1, ImmunoQuality, Groningen, The Netherlands) and labeled cells were retained. For higher purity, a second run with a new column was applied to the recovered CD34+ fraction. Viable isolated CD34+ cells were enumerated by trypan blue dye exclusion and purity was checked by flow cytometry after double staining with anti-CD34 PE and CD45 PerCP (Becton Dickinson, San Jose, CA, USA).

Immunostaining and flow cytometric analysis

One hundred microliters of cell suspension were incubated with antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP) and allophycocyanine (APC) for 10 min at 20°C. After two washes, cells were resuspended in 0.5 mL of 0.5% paraformaldehyde and analyzed with a FACSscalibur flow cytometer equipped with two lasers (488 and ~635 nm) using CellQuest™ and PAINT-a-GATE plus™ software programs (Becton Dickinson). Four-color antibody combinations (Table 1) included anti-CD13 FITC (Dako, Glostrup, Denmark) or PE (Becton Dickinson), CD34 PerCP (Becton Dickinson), CD33 APC (Becton Dickinson), and one of the following FITC or PE monoclonal antibodies: anti-MPO, CD2, CD79a (Dako); anti-CD3, CD4, CD45, CD117, anti-CD90 (Coulter/Immunotech, Miami, FL, USA); anti-CD65 (Caltag Laboratories, San Francisco, CA, USA); anti-CD41, CD11b, CD14, CD15, CD38, CD45, CD56 (Becton Dickinson), anti-CD133 (Miltenyi Biotec); anti-CD65 (Caltag Laboratories, San Francisco, CA, USA); anti-CD41a, CD117, anti-CD90 (Coulter/Immunotech, Miami, FL, USA). Three-color antibody combinations (Table 1) included anti-CD13 FITC (Dako), CD34 PerCP (Becton Dickinson) and one of the following anti-CD33 PE antibodies: clone p-67-6 (IgG1, Becton Dickinson), and clone WM S3 (IgG1, Immunoquality, Groningen, The Netherlands). For intracellular staining, surface labeled cells were treated with Fix and Perm™ (F/P, An der Grub, Vienna, Austria) according to the manufacturer's instructions. Isotype-matched non-reactive antibodies conjugated to FITC, PE, PerCP and APC (Becton Dickinson) were used as negative controls. All the antibodies used in this study were titrated, and used at optimal concentration to avoid non-specific staining.

To define the immunophenotype of CD34+, CD34+ cells we used the following protocol: first, 10,000 non-gated events were acquired. These were then gated as follows: gate R1 surrounded the light-scatter dot plot of mononucleated cells, excluding debris and mature granulocytes; gate R2 was drawn on the fluorescence dot plot to include all cells expressing either CD13 or CD33. Electronic events falling within both gates 1 and 2 were selectively recorded. At the time of the analysis, we drew a gate (R3) surrounding CD34+ cells with low/intermediate SSC characteristics.

Table 1. Monoclonal antibody combinations used to analyze the immunophenotype of CD34+/CD13+/CD33+ and CD33− cell subsets.

<table>
<thead>
<tr>
<th>Combination</th>
<th>Description</th>
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<tr>
<td>IgG FITC/CD13 PE/CD34 PerCP/CD33 APC</td>
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Results

Prevalence and light scatter characteristics of CD34+, CD13+, CD33− cells in normal bone marrow and cord blood.

During the immunophenotypic characterization of normal myeloid cell differentiation, we identified a subset of cells that expressed CD34 and CD13 but lacked CD33. In bone marrow (n = 11), cells with this phenotype represented 0.5±0.5% (mean ± SD) of mononucleated cells and 22.0±10.3% of CD34+ cells. Interindividual variations in the distribution of this subset did not appear to be age-related (data not shown). In cord blood (n = 4), cells with the CD34+, CD13+, CD33− phenotype formed 0.8±1.2% of mononucleated cells and 11.8±11.5% of CD34+ cells (Table 2). CD13+, CD33− cells had light scattering properties that were reminiscent of those of early myeloid cells or small monocytes. When compared to the CD33+ counterpart, this population contained cells of smaller size and lower granularity as demonstrated by forward (FSC) light scatter and SSC measurements (Figure 2). The intensity of CD34 expression was indistinguishable from that measured in CD33+ cells; mean fluorescence intensity (MFI) was 194.7 in CD13+, CD33− cells versus 192.7 in CD13+, CD33+ cells. Expression of CD13 was lower in these cells than in their CD33+ counterpart (MFI: 122.1 versus 460.9).

Immunophenotype of CD34+, CD13+, CD33− cells

To characterize the immunophenotype of CD34+, CD13+, CD33− cells in normal bone marrow and cord blood, we used four-color flow cytometry and a variety of lineage and maturation-associated markers in combination with CD34, CD13 and CD33. The median number of CD34+ cells analyzed (gate R3 in Figure 1) was 1,500 (range 770–5,728). Both CD34+, CD13+, CD33− and CD33+ bone marrow subpopulations expressed either low levels or an absence of surface antigens associated with T (CD7 and CD2) or B (CD19) cell lineages (Table 3).
Myeloid-associated antigens such as CD65, CD15 and CD14 were not expressed in CD34+, CD13+, CD33− cells, while CD65 and CD15 were clearly detectable (mean 22.3±11.9% and 28.7±15.8%, respectively) among CD34+, CD13+, CD33+ cells. Expression of myeloperoxidase (MPO) was markedly higher in the CD13+, CD33+ population than in the CD33− cells (46.2±13.3% versus 7.5±6%) suggesting a more advanced myeloid commitment within the former cell subset. Expression of CD11b was low in both CD33− and CD33+ cell populations (14.4±16.6% and 6.3±3.9%, respectively). Natural killer (CD56), megakaryocytic (CD41a) and erythroid (glycophorin-A) antigens were not expressed in either CD34+ subsets. Maturation-associated antigens such as CD45, HLA-DR, CD38, CD133 and CD117 were expressed in the great majority of CD34+, CD13+, CD33− and CD33+ cells (Table 3). A lower expression of HLA-DR was observed in the CD13+, CD33− subset compared to that in the CD13+, CD33+ one (MFI 151±70 versus 398±180, respectively). No significant differences were observed in the levels of expression of CD45, CD38, CD133 and CD117 (MFI 84.5±93 versus 85±53.5; 50.3±13.4 versus 53.2±24; 67 versus 68 and 827±424 versus 399±181, respectively).

We also studied 4 cord blood samples and found that the immunophenotype of CD34+, CD13+, CD33− cells was similar to that of their marrow equivalent. However, no CD34+, CD13+, CD33− cells expressing lineage-associated antigens were found in cord blood (Table 3).

To ensure that our observations were not peculiar to the anti-CD33 antibody clone used (p-67-6), the fluorochrome conjugated to it (APC), or the gating strategy applied, we analyzed the immuno-

<p>| Table 2. Prevalence of CD34+/CD13+/CD33+ and CD34+/CD13+/CD33− cell subsets in normal bone marrow and cord blood. |</p>
<table>
<thead>
<tr>
<th>CD34+/CD33+ subset</th>
<th>CD34+/CD33− subset</th>
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<tbody>
<tr>
<td>% of mono-nucleated cells</td>
<td>% of CD34+ cells</td>
</tr>
<tr>
<td>Bone marrow (n = 11)</td>
<td>1.8±1.2</td>
</tr>
<tr>
<td>(0.5-4.0)</td>
<td>(23.0-67.1)</td>
</tr>
<tr>
<td>Cord blood (n = 4 )</td>
<td>1.0±0.5</td>
</tr>
<tr>
<td>(0.5-1.7)</td>
<td>(38.0-91.1)</td>
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Abbreviations: n, number of donors tested.
Characterization of CD34+, CD13+, CD33− hematopoietic cells

We purified CD34+ cells from three bone marrow samples by immunomagnetic sorting and labeled them with two triple antibody combinations: 1) anti-CD13 FITC, CD34 PerCP, and either CD33 PE p-67-6 or WM53 clones; 2) anti-CD13 PE, CD34 PerCP and CD33 APC (Figure 3). We detected the CD13+, CD33− cell subset in all three cases, although the proportion of these cells among CD34+ cells was higher in samples labeled with the APC-conjugated p-67-6 clone (mean 14.3±6.2%) than in those labeled with the PE-conjugated p-67-6 antibody or with the WM53 antibody (mean 8.2±3.3% and 5.7±2.2%, respectively).

### Table 3. Phenotype of CD34+/CD13+/CD33− and CD34+/CD13+/CD33+ cell subsets in normal bone marrow and cord blood.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Bone marrow</th>
<th>Cord blood</th>
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<tr>
<td></td>
<td>CD34+/CD33− cells mean %±SD (range)</td>
<td>CD34+/CD33+ cells mean %±SD (range)</td>
</tr>
<tr>
<td>CD2</td>
<td>4</td>
<td>neg</td>
</tr>
<tr>
<td>CD7</td>
<td>6</td>
<td>&lt;10.0</td>
</tr>
<tr>
<td>CD3+</td>
<td>3</td>
<td>&lt;10.0</td>
</tr>
<tr>
<td>CD19</td>
<td>7</td>
<td>&lt;10.0</td>
</tr>
<tr>
<td>CD39+</td>
<td>3</td>
<td>&lt;10.0</td>
</tr>
<tr>
<td>CD65</td>
<td>6</td>
<td>22.3±11.9 (11.9-45.3)</td>
</tr>
<tr>
<td>CD11b</td>
<td>6</td>
<td>14.4±6.6 (0.0-47.8)</td>
</tr>
<tr>
<td>CD15</td>
<td>4</td>
<td>28.7±18.8 (20.0-52.5)</td>
</tr>
<tr>
<td>CD117</td>
<td>6</td>
<td>91.6±4.9 (82.0-94.9)</td>
</tr>
<tr>
<td>CD14</td>
<td>3</td>
<td>neg</td>
</tr>
<tr>
<td>MPOa</td>
<td>8</td>
<td>46.2±13.3 (33.0-73.6)</td>
</tr>
<tr>
<td>CD56</td>
<td>7</td>
<td>neg</td>
</tr>
<tr>
<td>CD41a</td>
<td>4</td>
<td>&lt;10.0</td>
</tr>
<tr>
<td>Glycophorin A</td>
<td>4</td>
<td>neg</td>
</tr>
<tr>
<td>CD90</td>
<td>3</td>
<td>3.1±1.5 (1.8-5.2)</td>
</tr>
<tr>
<td>CD38</td>
<td>4</td>
<td>98.6±7 (97.6-99.4)</td>
</tr>
<tr>
<td>CD45</td>
<td>5</td>
<td>98.3±9 (97.6-99.7)</td>
</tr>
<tr>
<td>CD133</td>
<td>3</td>
<td>79.3±3 (78.6-80.7)</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>4</td>
<td>95.8±1.7 (93.2-96.9)</td>
</tr>
</tbody>
</table>
| Abbreviations: n, number of donor tested; neg, negative (expression of the antigen was considered negative when less than 10 positive events were detected by flow cytometry); nd, not determined. *means cytoplasmic antigen expression; †means that in three patients, of those tested, the antigen expression was determined after immuno-magnetic purification of CD34+ cells.

Figure 3. Flow cytometric analysis of normal bone marrow CD34+ cell subsets defined according to their CD13 (x axis) and CD33 (y axis) expression. CD34+ cell selection was carried out as described in the Methods. Four-color immunofluorescence staining was performed with IgG1 FITC, CD13 PE, CD34 PerCP, and either CD33 PE p-67-6 or WM53 clones; 2) anti-CD13 PE, CD34 PerCP and CD33 APC (Figure 3). We detected the CD13+, CD33− cell subset in all three cases, although the proportion of these cells among CD34+ cells was higher in samples labeled with the APC-conjugated p-67-6 clone (mean 14.3±6.2%) than in those labeled with the PE-conjugated p-67-6 antibody or with the WM53 antibody (mean 8.2±3.3% and 5.7±2.2%, respectively).
To investigate the degree of maturation of CD34+, CD13+, CD33− cells further, we tested the expression of CD90, a marker that has been reported to be expressed by the most immature fraction of hematopoietic precursors.39-41 The mean percentage of CD34+ cells expressing CD90 was 2.3±0.2%; a higher proportion of CD90+ cells was found among CD13+, CD33− cells than among CD13+, CD33+ cells (13.3±6.3 versus 3.1±1.5%, Table 3 and Figure 4).

Cytoplasmic expression of the lineage specific-associated markers MPO, CD79a and CD3 was analyzed in three different CD34+ cell subsets defined according to their CD13 and CD33 reactivity (Figure 5), using four-color staining as indicated in Table 1. CD34+ cells lacking CD13 and CD33 expression had high levels of the B-cell marker CD79a (mean 60.3% ± 29.2%), but did not express MPO (less than 1% positivity), whereas CD34+, CD13+, CD33− cells expressed high levels of MPO (mean 46.2±13.3 %), but did not contain B-cells (less than 1% of CD79a expression, Table 3). Some CD34+, CD13+, CD33− cells expressed, although in a low percentage, both CD79a and MPO (mean 6.0±3.2% and 7.5±6.0%, respectively), suggesting that this cell subset includes some B-cell progenitors in addition to myeloid progenitors. Cytoplasmic CD3, a marker of T-cell differentiation, was negative on all CD34+ cells, irrespective of their CD13 and CD33 expression. The distribution of B-cell progenitors among these three cell subsets was further assessed by CD19, CD13, CD34 and CD33 surface staining of sorted CD34+ cells from one bone marrow sample; as shown in Figure 6, most of the CD13−, CD33− cells expressed the CD19 antigen (71%). The expression of this marker was clearly detectable in a minority of CD13+, CD33− cells (13%), while it was absent in CD13+, CD33+ cells.

In all samples studied, CD34+ cells with the phenotype CD13−, CD33− were extremely rare (Figure 3), had no homogeneous light scatter distribution, and were negative for CD90 (data not shown).

Discussion

We characterized the prevalence and the immunophenotype of a subset of immature hematopoietic cells which express CD34 and CD13 but lack CD33. These cells were reported to contain primitive hematopoietic progenitors with pluripotent and vigorous, albeit delayed, in vitro cell growth.28 We took advantage of four-color flow cytometry to characterize the immunophenotype of CD34+, CD13+, CD33− cells further. The majority of CD34+, CD13+, CD33− cells lacked markers associated with the myeloid lineage, such as CD65 and CD15, but a minor proportion (approximately 7-8%) were reactive for MPO. They also lacked reactivity for the T-lineage (CD7 and cytoplasmic CD3), megakaryocytic lineage (CD41a) and erythroid lineage (glycophorin A). Expression of CD79a and CD19, indicative of B-cell commitment, on these cells was generally low (less than 10%). Cord blood CD34+, CD13+, CD33− cells were even more phenotypically immature than those found in bone marrow, and did not express any lineage-associated marker. Expression of CD133 and CD117 was compatible with the relative immaturity of this cell subset,41-43 although no significant differences were observed in the level of expression of these two markers between CD34+, CD13+, CD33− and CD34+, CD13−.
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Figure 5. Flow cytometric analysis of MPO (top panels), CD79a (middle panels) and CD3 (bottom panels) cytoplasmic expression in bone marrow CD34+ cells. Four-color immunofluorescence staining of purified CD34+ cells was performed as described in the Methods and Table 1. The histograms shown are from one representative case of three studied. Reactivity of each antibody was analyzed in CD13−, CD33− (left panels), CD13+, CD33− (central panels), and CD13+, CD33+ (right panels) cell subsets. Open areas represent fluorescence distribution of the antibodies tested and solid areas represent that of the isotype-matched control antibody. Numbers within the histograms are percentages of positive cells. The cell number is shown on the y axis.

Figure 6. Flow cytometric analysis of CD19 surface expression in bone marrow CD34+ cells. Staining of purified CD34+ cells was performed with anti-CD19 FITC, CD13 PE, CD34 PerCP and CD33 APC antibodies. The reactivity of anti-CD19 was analyzed in CD13−, CD33− (panel A); CD13+, CD33− (panel B); and CD13+, CD33+ (panel C) cell subsets. Dot plots, subdivided into four quadrants, represent the fluorescence distribution of anti-CD34 (y axis) and anti-CD19 (x axis) antibodies. Percentages of CD19+ cells are indicated. Isotype-matched control antibodies were used to set up the background level.

CD33− cells. Likewise, CD38 expression was indistinguishable between these two cell populations. However, the CD33− subset had increased levels of CD90, a feature of immaturity. In addition, CD34+, CD13+, CD33− cells had lower levels of HLA-DR and CD13 compared to those measured in the CD13+, CD33+ compartment. It has been previously observed that there is a positive correlation between the degree of maturation of myeloid progenitors and the level of HLA-DR and CD13.

Expression of CD13 and CD33 antigens is commonly associated with cells committed to the myeloid lineage and occurring very early during myeloid cell maturation. Nevertheless, it has been a matter of debate whether expression of CD13 pre-
cedes that of CD33 during this process. Several authors who studied the immunophenotype of CD34+ cells in bone marrow, in cord blood and in peripheral blood have reported an increased expression of CD13 compared to that of CD33, implying the existence of CD34+ cells expressing CD13 but not CD33. By contrast, others did not find substantial differences in the percentage of CD34+ cells expressing these two markers. The interpretation of these results is, however, complicated by the fact that none of the above studies simultaneously examined CD13 and CD33 expression on CD34+ cells with three-color staining; the simple comparison of percentages may be misleading especially when small cell populations are examined. Our demonstration of a CD34+, CD13+, CD33− cell subset was confirmed using purified CD34+ cells and was not dependent on a particular clone or fluorochrome used for CD33 detection. This result suggests that CD13 is expressed earlier than CD33 during myeloid differentiation. In line with this notion are the findings of Boezeman et al. who reported that sorted CD13+, CD33− cells gave rise to large colonies of mixed, erythroid and myeloid morphology, while CD13+, CD33+ cells yielded smaller colonies predominantly of myeloid origin.

San Miguel et al. and Terstappen et al. found that the CD34+, CD13+, CD33− phenotype occurred in approximately 20% of cases of acute myeloid leukemia studied. The high proportion of leukemic cases with this phenotype is in sharp contrast with the rarity of equivalent normal cells, and these authors used this immunophenotype to monitor the presence of residual leukemic cells in bone marrow. Although in some cases with small numbers of normal cells with the CD34+, CD13+, CD33− phenotype this approach may be informative, our study indicates that investigators should be aware of the possibility of false-positive results caused by the mistaken identification of normal myeloid progenitors as leukemic myeloblasts.

Contributions and Acknowledgments

GG contributed to drafting the article, perform laboratory experiments and conception; DC contributed to conception and design. GG and DC should be considered as the principal authors. The remaining authors have taken specific responsibility for the following parts: E C-S and ET for analysis and interpretation of data; OM for laboratory experiments; AB for critically revising the manuscript. We thank Mrs. E. Paccagnini for secretarial support.

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Disclosures

Conflict of interest: none.
Redundant publications: no substantial overlapping with previous papers.

References


**What is already known on this topic**
Conflicting results about the existence of CD34+/CD13+/CD33− cells have been provided by previous studies.

**What this study adds**
This study confirms the presence of CD34+/CD13+/CD33− cells in adult bone marrow and in cord blood and describes their antigenic features of early myeloid progenitors.

**Manuscript processing**
This manuscript was peer-reviewed by two external referees and by Dr. Dario Ferrero, who acted as an Associate Editor. The final decision to accept this paper for publication was taken by the Dr. Ferrero and the Editors. Manuscript received November 5, 2001; accepted February 19, 2002.

**Potential implications for clinical practice**
The presence of CD34+/CD13+/CD33− cells in normal bone marrow must be considered in studies aimed to identify residual AML cells during remission in patients with CD13+/33− blasts.

Dario Ferrero, Associate Editor