Characterization of CD34+ Peripheral Blood Cells From Healthy Adults Mobilized by Recombinant Human Granulocyte-Colony-Stimulating Factor

By Geir E. Tjønnfjord, Rita Steen, Stein A. Evensen, Erik Thorsby, and Torstein Egeland

Primed peripheral blood hematopoietic stem cells (PBSC) generate and sustain lymphohematopoiesis in myeloablative animals, and recent reports indicate that allogeneic transplantation using PBSC grafts may be feasible in humans. A major concern with the use of PBSC transplants is that permanent engraftment may be limited because of lack of sufficient numbers of primitive progenitor cells in the graft. In the present study, in vitro colony formation and immunophenotype of CD34+ cells in PB of healthy adults during short-term granulocyte-colony-stimulating factor (G-CSF) administration were compared with that of CD34+ cells in normal bone marrow (BM). The number of CD34+ cells mobilized to PB peaked at day 4 or 5 of G-CSF administration. The phenotypic profile of CD34+ PB cells showed a substantial increase in the percentage of CD34+CD13+ and CD34+CD33+ cells (myeloid progenitors) and a corresponding decrease in the percentage of CD34+CD10+ and CD34+CD19+ cells (B lymphoid progenitors) compared with CD34+ BM cells. The other subsets studied, including CD34+CD38+ and CD34+HLA-DR+ cells, were present in both compartments in similar proportions. Furthermore, primed CD34+ PB cells were enriched for colony-forming cells (CFC) and displayed an increased clonogenicity when compared with their counterparts in BM. A comparison between a postulated PBSC graft and an average BM graft is presented, showing that such PBSC grafts will be enriched for CD34+ cells as a whole, CD34+CD33+ cells, and colony-forming cells (CFC), factors which have been shown to correlate to acceleration of hematologic reconstitution and reduction in requirements for supportive care in autografting. Hence, we predict that allogeneic transplantation using G-CSF-primed PBSC grafts will result in a more rapid hematologic reconstitution after myeloablative conditioning than BM grafting. The question of whether PBSC allografting will result in permanent engraftment and clinical benefits as observed in autografting has to be determined in prospective clinical studies.

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MATERIALS AND METHODS

Subjects

PB samples were collected from six healthy adults; four men and two women, 25 to 51 years old. They received rhG-CSF (Filgrastim, Roche Norge, Oslo, Norway), administered subcutaneously (sc) at 10 μg/kg per day*4 for 7 days. Hematologic indices and the number of CD34+ cells in PB were recorded at baseline and daily for a total of 7 to 10 days. Cells were collected for additional studies at day 4 or 5 of G-CSF administration corresponding to the peak of CD34+ cells in PB. BM samples were collected from five healthy adults, 25 to 50 years old. Informed consent was obtained using protocols approved by The Institutional Review Board at The National Hospital, University of Oslo, Norway.

Cell Preparation and Staining

Mononuclear cells (MNC) were prepared by gradient centrifugation (Lymphoprep, 1,077 g/mL, Nycomed Pharma, Oslo, Norway),
Table 1. Effect of Density Gradient Centrifugation on Coexpression of a Selection of Cell Surface Molecules on G-CSF-Mobilized CD34+ PB Cells From Three Donors

<table>
<thead>
<tr>
<th>Subsets</th>
<th>Mean Relative No. of CD34+ Cells (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole Blood</td>
</tr>
<tr>
<td>CD34+CD2+</td>
<td>2.0 ± 0.3 (1.7-2.3)</td>
</tr>
<tr>
<td>CD34+CD10+</td>
<td>1.9 ± 0.7 (1.2-2.5)</td>
</tr>
<tr>
<td>CD34+CD13+</td>
<td>87.8 ± 3.2 (84.2-90.1)</td>
</tr>
<tr>
<td>CD34+CD15+</td>
<td>3.7 ± 1.2 (2.4-4.7)</td>
</tr>
<tr>
<td>CD34+HLA-DR</td>
<td>4.1 ± 0.3 (3.8-4.4)</td>
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</tbody>
</table>

Results are the mean ± SD from three experiments.

and CD34+ cells were purified by Dynabeads (Dynal, Oslo, Norway) as previously described. Unfractionated PB and BM together with MNC from the same compartments were labeled with the following monoclonal antibodies: Fluorescein isothiocyanate (FITC)-conjugated anti-CD2 (Leu-2b), anti-CD7 (Leu-9), anti-CD10 (anti-CALLA), anti-CD19 (Leu-12), anti-CD20 (Leu-16), anti-CD14 (Leu-M3), anti-CD15 (Leu-M1), anti-CD34 (anti-HPCA-2) and anti-HLA-DR, phycoerythrin (PE)-conjugated anti-CD13 (Leu-M7), anti-CD33 (Leu-M9), anti-CD34 (anti-HPCA-2) and anti-CD38 (Leu-17) and Simultest control (IgGl FITC + IgG2a PE), all purchased from Becton Dickinson Immunocytometry Systems (BDIS), San Jose, CA, and FITC-conjugated anti-CD45 (T29/33) purchased from Dako a/s, Glostrup, Denmark. FACS Lysing Solution (BDIS) was used to lyse erythrocytes after staining.

Flow Cytometry

Flow cytometry was performed on a FACScan (BDIS) equipped with a 15 mW air-cooled argon-ion laser tuned at 488nm. Quantitation of CD34+ cells in unseparated PB and BM was performed by analysis of a total of 100,000 cellular events in a two-color cytogram of FITC–anti-CD45 and PE–anti-CD34. We used CD45 fluorescence to discriminate between the lymphohematopoietic cells and erythrocytes or debris. Cells were assessed for their light scatter properties, and only cells within the lymphoid blast region were accepted as CD34+ cells. The percentage of CD34+ cells was calculated according to the formula:

\[(CD34+ cells - cells staining positive with the control reagents) \times 100 \div CD45+ cells\]

The number of white blood cells/L was multiplied by the percentage of CD34+ cells to calculate CD34+ cells/L.

For phenotyping, an amorphous gate identifying the CD34+ cells was drawn in a dual-parameter cytogram of orthogonal light scatter and fluorescence, and events (greater than 5,000 events in all experiments) satisfying this gate were acquired, stored in list mode files, and analyzed for two-color fluorescence.

Hematopoietic Colony-Forming Assays

MNC and CD34+ cells were analyzed in triplicate dishes for CFU-GM and BFU-E in semisolid phase agar and methylcellulose cultures. MNC and CD34+ cells were seeded at 1 × 10^5 and 1 × 10^3 cells per dish, respectively. The cultures were stimulated with recombinant murine c-kit ligand (2 U/mL), rh interleukin-3 (80 U/mL), rhGM-CSF (80 U/mL) (Genetics Institute, Cambridge, MA), and rh erythropoietin (3 U/mL) (Cilag, Sandvika, Norway). Colony formation was assessed after 14 days.

Statistics

Growth ability and immunophenotype of CD34+ cells were compared using the Student’s t-test.

RESULTS

Flow-cytometric analysis of samples prepared from MNC allowed acquisition of a substantially higher number of relevant cellular events than samples prepared from lysed whole blood or BM. Because progenitor cells are lost during density centrifugation to some extent, we wanted to rule out the possibility that density centrifugation resulted in a selective bias. A comparative analysis of the CD34+ populations with
respect to coexpression of a selection of cell surface molecules in lysed whole blood and in the MNC fraction disclosed no difference between the two cellular preparations (Table 1). The relative proportion of CD34+ HLA-DR- cells in the MNC fraction appears to be increased compared with whole blood. However, this should be interpreted with great caution because of the low number of observations and the lack of differences between the other subsets studied. Hence, CD34+ cells in the MNC fraction were considered representative of the CD34+ cells as a whole, and the data presented on the phenotype of CD34+ cells are based on analyses of MNC. However, we did not perform experiments to evaluate the proportion of CD34+ cells lost with density gradient separation.

Flow-Cytometric Analysis of CD34+ Cells

Quantitation of CD34+ cells. The number of CD34+ cells peaked at day 4 or day 5 of G-CSF administration (Fig 1). Hence, the time to the peak of CD34+ cells from the start of G-CSF administration was found to be quite predictable. However, with respect both to the percentage (0.04% to 0.40% at peak) of CD34+ cells in PB and to the number (15.4 × 10^6/L to 100.4 × 10^6/L at peak) of CD34+ cells mobilized to PB, substantial interindividual variation was observed. The percentage of CD34+ cells in BM samples from unprimed healthy adults was 0.48% ± 0.30%.

Light scatter properties. According to light scatter properties, CD34+ cells of both compartments were confined in the lymphoid blast region, and they could be further divided into two populations (R1 and R2) (Fig 2). R1 contained cells of low forward and very low orthogonal light scatter and R2 contained cells of intermediate to high forward and very low orthogonal light scatter. Nearly all CD34+ PB cells were confined within R2, and cells within R1 made up less than 3% of the CD34+ cells (Fig 2B), whereas R1 contained 18.0% ± 7.4% of CD34+ BM cells (Fig 2A).

Coexpression of lymphoid-associated cell surface molecules on CD34+ cells. The CD2 and CD7 cell surface molecules are associated with T lymphocytes and were expressed on low proportions in both compartments (Table 2 and Fig 3B and C). The percentage of CD34+CD2+ and CD34+CD7- cells were lower in PB than BM, but the difference was found to be statistically significant only with re-

<table>
<thead>
<tr>
<th>Subsets</th>
<th>Peripheral Blood (n = 6)</th>
<th>BM (n = 5)</th>
<th>P Value</th>
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<tbody>
<tr>
<td>CD34+CD2-</td>
<td>2.0 ± 0.6 (1.3-2.7)</td>
<td>4.2 ± 1.2 (2.9-5.8)</td>
<td>&lt;.004</td>
</tr>
<tr>
<td>CD34+CD7-</td>
<td>2.9 ± 1.3 (0.6-5.3)</td>
<td>3.2 ± 1.0 (1.7-4.4)</td>
<td>NS</td>
</tr>
<tr>
<td>CD34+CD10-</td>
<td>2.9 ± 2.3 (1.4-7.6)</td>
<td>27.7 ± 16.5 (17.6-52.0)</td>
<td>&lt;.006</td>
</tr>
<tr>
<td>CD34+CD19-</td>
<td>1.7 ± 0.7 (1.2-2.8)</td>
<td>20.1 ± 6.9 (13.3-29.6)</td>
<td>&lt;.0002</td>
</tr>
<tr>
<td>CD34+CD20-</td>
<td>1.0 ± 0.2 (0.8-1.2)</td>
<td>4.1 ± 0.5 (3.6-4.4)</td>
<td>&lt;.0002</td>
</tr>
<tr>
<td>CD34+CD13-</td>
<td>86.7 ± 6.9 (76.3-90.7)</td>
<td>56.5 ± 13.1 (46.3-70.6)</td>
<td>&lt;.0002</td>
</tr>
<tr>
<td>CD34+CD14-</td>
<td>1.1 ± 0.3 (0.6-1.5)</td>
<td>0.9 ± 0.3 (0.3-0.9)</td>
<td>NS</td>
</tr>
<tr>
<td>CD34+CD15-</td>
<td>4.1 ± 2.4 (0.4-6.6)</td>
<td>11.3 ± 4.8 (4.9-15.5)</td>
<td>&lt;.02</td>
</tr>
<tr>
<td>CD34+CD33-</td>
<td>91.1 ± 2.3 (86.6-93.2)</td>
<td>63.5 ± 13.7 (48.4-78.2)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>CD34+HLA-DR</td>
<td>3.2 ± 2.7 (1.4-8.7)</td>
<td>2.4 ± 0.8 (1.6-3.2)</td>
<td>NS</td>
</tr>
<tr>
<td>CD34+HLA-DR</td>
<td>4.9 ± 2.0 (2.2-6.4)</td>
<td>3.7 ± 2.2 (2.1-7.3)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Results are the mean ± SD from the number of experiments indicated. Abbreviation: NS, not significant.
spect to CD34⁺CD2⁺ cells. Both subsets displayed similar light scatter properties in PB and BM, and the cells were localized within R2 among cells with intermediate forward light scatter. A comparison of the proportion of CD34⁺ cells coexpressing B-lymphocyte-associated cell surface molecules in PB and BM is shown in Table 2 (see also Fig 3, D to F). The percentage of CD34⁺CD10⁺ and CD34⁺CD19⁺ cells were 2.9% ± 2.3% and 1.7 ± 0.7%, respectively, in PB compared with 27.7% ± 16.5% and 20.1% ± 6.9% in BM. The percentage of CD34⁺CD20⁺ cells was 1.0% ± 0.2% in PB and 4.1% ± 0.5% in BM. The observed differences were statistically significant. The majority of BM B-lymphocyte progenitors displayed light scatter properties corresponding to cells confined within R1 and constituted the vast majority of cells in R1, whereas a minority were localized to R2. Equal proportions of CD34⁺CD10⁺ PB cells were localized to R1 and R2, whereas CD34⁺CD19⁺ and CD34⁺CD20⁺ PB cells were mainly (≈70%) localized to R1.
PRIMED PBSC IN ALLOGENEIC TRANSPLANTATION 2799

CD34+ BM cells 5,500
cells.

cated.

BM MNC (n = 5) 88 ± 37 (48-130)* 49 ± 14 (26-611)

CD34+ PB cells 14,000 ± 1,000 9,500 ± 1,300
(n = 3) (13,000-14,800) (8,500-11,000)

CD34+ BM cells 5,500 ± 2,400 4,500 ± 2,200
(n = 3) (3,800-8,300)* (2,100-6,300)t

Results are the mean ± SD from the number of experiments indicated.
* Differences in BFU-GM, P < .07 for MNC and P = .005 for CD34+ cells.
† Differences in BFU-E, P < .03 for MNC and P < .03 for CD34+ cells.

Table 3. Colony Formation. G-CSF-Mobilized Peripheral Blood Cells Compared With BM Counterparts

<table>
<thead>
<tr>
<th></th>
<th>Mean No. CFU-GM/106 Cells (range)</th>
<th>Mean No. BFU-E/105 Cells (range)</th>
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</thead>
<tbody>
<tr>
<td>PB MNC (n = 3)</td>
<td>137 ± 13 (128-151)</td>
<td>75 ± 11 (64-86)</td>
</tr>
<tr>
<td>BM MNC (n = 5)</td>
<td>88 ± 37 (48-130)*</td>
<td>49 ± 14 (26-611)</td>
</tr>
<tr>
<td>CD34+ PB cells (n = 3)</td>
<td>14,000 ± 1,000 (13,000-14,800)</td>
<td>9,500 ± 1,300 (8,500-11,000)</td>
</tr>
<tr>
<td>CD34+ BM cells (n = 3)</td>
<td>5,500 ± 2,400 (3,800-8,300)*</td>
<td>4,500 ± 2,200 (2,100-6,300)t</td>
</tr>
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</table>

Coexpression of myeloid-associated cell surface molecules on CD34+ cells. A comparison of expression of the myeloid-associated cell surface molecules CD13, CD14, CD15, and CD33 on CD34+ cells from the two compartments is shown in Table 2 (see also Fig 3, G, H, J, and K). The CD13 and CD33 molecules were expressed on a high proportion of CD34+ cells in both compartments. Nevertheless, these molecules were expressed on a significantly higher proportion of CD34+ PB cells than CD34+ BM cells, 86.7% ± 5.6% and 91.1% ± 2.3% versus 59.5 ± 13.7% and 63.5 ± 13.7%, respectively (P < .002). CD14 expression was very low (<2%) in both compartments, whereas the proportion of CD34+ expressing CD14 was higher in BM than in PB, 11.3% ± 4.8% and 4.1% ± 2.4%, respectively (P < .02). CD34+CD13+ and CD34+CD33+ cells from PB and BM displayed similar light scatter properties corresponding to cells broadly distributed within R2. The light scatter profiles of CD34+CD14+ and CD34+CD15+ cells disclosed no differences between the two compartments, and the cells were localized to R2.

Coexpression of CD38 and HLA-DR on CD34+ cells. The vast majority of CD34+ cells in both compartments stained positive for CD38 and HLA-DR. Nevertheless, a low but comparable proportion of CD34+HLA-DR and CD34+CD38 cells were identified in both compartments in all experiments (Table 2 and Fig 3, I and L), and they displayed similar light scatter properties in both compartments and were mainly localized to R2 among cells with intermediate forward light scatter properties.

Colony Formation

Colony formation (CFU-GM and BFU-E) of cells collected from PB during G-CSF administration was assessed in three donors and compared with that of BM cells from five donors (Table 3). Both MNC and CD34+ cells prepared from PB during G-CSF administration generated more colonies than the BM counterparts. A twofold to threefold enrichment of CFU-GM (P < .005) and a twofold enrichment for BFU-E (P < .03) were observed when CD34+ cells were compared.

DISCUSSION

We report that G-CSF are capable of mobilizing CD34+ cells to PB in healthy adults, and that these CD34+ cells display a phenotypic and functional profile different from that of nonprimed CD34+ BM cells. The percentage of myeloid progenitors (CD34+CD33+CD13-) cells was significantly increased at the expense of B-lymphocyte progenitors (CD34+CD10+CD19+) cells in PB as compared with BM, corresponding to a decreased percentage of CD34+ cells localized to R1 in PB. The relative number of putative T-lymphocyte progenitors showed no unequivocal difference between the two compartments. The percentage of CD34+HLA-DR- and CD34+CD38 cells were found to be comparable in the two compartments and in the range previously reported in BM and in PB during recovery from high-dose chemotherapy. Furthermore, we found CD34+ PB cells to be enriched for colony-forming cells (CFC) and to exhibit increased clonogenicity when compared with CD34+ BM cells.

In vitro studies on primed PBSC indicate that the capacity of these cells to generate and sustain hematopoiesis is comparable with that of BM cells on a cell to cell basis. Moreover, during administration of G-CSF, PB contains sufficient numbers of primitive stem cells to sustain lymphohematopoiesis in radiation ablated mice. Mounting evidence indicates that long-term reconstituting lymphohematopoietic stem cells are found among CD34+CD38+ and CD34+HLA-DR- cells in BM and PB umbilical cord blood. In the present study, no experiments were performed to particularly address the question of whether CD34+CD38- and CD34+HLA-DR- cellular populations in PB and BM exhibit the same functional properties in vitro. However, analysis of the light-scatter profiles of these cellular populations disclosed no difference between the two compartments. Together, this observation and those of others provide circumstantial evidence that cells, exhibiting the capacity to generate and sustain lymphohematopoiesis shown to be present in PB during G-CSF administration, are confined to CD34+CD38- and CD34+HLA-DR- cells. However, the question of permanent engraftment in humans after PBSC grafting can only be resolved by direct evaluation of allogeneic transplantation.

The most striking difference observed regarding the phenotypic profiles of CD34+ cells in PB and BM was the very high percentage of CD34+ cells coexpressing CD33 and CD13 molecules and the correspondingly low percentage coexpressing CD10 and CD19 molecules in PB as compared with BM. Similar findings have been reported in cancer patients. The low relative number of B-lymphoid progenitors in PB fully explains the low relative number of CD34+ cells localized to R1. In BM, Andrews et al have shown that most CFC display the CD34+CD33- phenotype, and that their precursors display the CD34-CD33- phenotype. This has also been shown to be the case in PB. This fits with our observation that G-CSF–primed CD34+ PB cells are enriched both for CD34+CD33+ cells and CFC. However, the relative number of CD34+CD33+ cells was in-
increased by 40% whereas CFU-GM and BFU-E were increased by 155% and 110%, respectively, indicating that primed CD34+ PB cells display substantially higher clonogenicity than CD34+ BM cells.

We observed substantial interindividual variation with respect to the number of CD34+ cells mobilized to PB during short-term G-CSF administration in line with previous reports in which rhG-CSF in the range of 5 to 16 µg/kg/d or other hematopoietic growth factors were used. The reason for this is unknown. It has been suggested that a substantial increase in the dosage of G-CSF will result in less variation between donors, but at present there is little to support such a notion. Dührsen et al. reported that administration of rhG-CSF resulted in a dose-dependent increase in mature neutrophils and CFC, and maximal effect were observed at 10 µg/kg/d of G-CSF with no further increase at higher doses. This study was performed in cancer patients, and dose-escalation studies in healthy adults are warranted.

Allogeneic transplantation is usually performed with BM grafts containing 100 to 300 x 10^6 nucleated cells/kg of the recipient’s body weight. Based on our data on the percentage of CD34+ cells in BM grafts, an average BM graft of 200 x 10^6 nucleated cells/kg contains approximately 1 x 10^6 CD34+ cells/kg. A minimum requirement of CD34+ PB cells for allografting has not been established. However, successful syngeneic transplantation with PBSC collected by leukapheresis during rhG-CSF administration was recently reported with grafts containing 1.6 to 12.6 x 10^6 CD34+ cells/kg. This study showed that the collection of 5 x 10^6 CD34+ cells/kg may be obtained from most donors when leukapheresis is performed on two consecutive days, which is in line with our unpublished observations. Based on the data presented in this study, a comparison between a PBSC graft containing 5 x 10^6 CD34+ cells/kg and an average BM graft was performed, and the results are summarized in Table 4. In PBSC autografting, the content of CFU-GM, CD34+ cells as a whole, and CD34+CD33+ cells have been shown to correlate to rapid hematologic reconstitution after myeloablative therapy, whereas the content of CD34+CD33+ cells do not. Furthermore, transplantation with PBSC grafts collected in steady-state hematopoiesis, i.e., not containing primed progenitor cells, do not shorten hematologic recovery time when compared with BM grafting. The postulated PBSC graft will contain primed progenitor cells and will be enriched for CD34+ cells as a whole, CD34+CD33+ cells, and CFU-GM as compared with an average BM graft. Hence, we predict that hematologic reconstitution will be more rapid after myeloablative conditioning using PBSC allografts than conventional BM allografts. Hopefully, this may result in reduced requirements for supportive care and reduced financial costs.

However, compared with BM grafts, PBSC grafts will contain a lower number of B-lymphocyte progenitors, the impact of which, with respect to lymphohematopoietic reconstitution, is elusive, and unmanipulated PBSC grafts will be enriched for mature T lymphocytes, which may influence severity of graft-versus-host disease.

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Characterization of CD34+ peripheral blood cells from healthy adults mobilized by recombinant human granulocyte colony-stimulating factor

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