Phenotypic and Functional Characterization of Long-Term Culture-Initiating Cells Present in Peripheral Blood Progenitor Collections of Normal Donors Treated With Granulocyte Colony-Stimulating Factor

By Felipe Prosper, David Stronge, and Catherine M. Verfaillie

Granulocyte colony-stimulating factor (G-CSF) mobilized peripheral blood progenitor cells (PBPC) have successfully been used as stem cells for both autologous and allogeneic transplants. However, little is known concerning the absolute number and phenotype of primitive progenitors, such as long-term culture-initiating cells (LTC-IC) in mobilized PBPC. The aim of our study was to evaluate the capacity of G-CSF to mobilize LTC-IC in the PB of normal individuals and to evaluate the phenotypic and functional characteristics of G-CSF mobilized LTC-IC. G-CSF was administered to 29 healthy volunteers at 7.5 μg or 10 μg/kg/d subcutaneously (SC) for 5 consecutive days and PBPC were harvested on day 6. Mobilization with G-CSF increased the absolute number of week 5 LTC-IC in PB 60-fold, while the number of CD34+ cells and committed colony forming cells (CFC) was increased sevenfold to 12-fold. The frequency of CFC and week 5 LTC-IC in CD34+ cells selected by fluorescence-activated cell sorter (FACS) from mobilized PBPC was 2 ± 0.3-fold and 9 ± 2.2-fold higher respectively than in CD34+ cells selected from un mobilized PBMNC. CFC were enriched in the CD34+ CD38+ and CD34+ HLA-DR+ populations. The absolute number of LTC-IC present in CD34+ CD38+ and CD34+ HLA-DR+ cells selected by FACS from either mobilized PBPC, un mobilized PBMNC or steady state bone marrow (BM) was similar (0.5% to 2%). In contrast to un mobilized PBMNC or steady state BM CD34+ CD38+ and CD34+ HLA-DR+ cells, which contain less than 0.1% LTC-IC, CD34+ CD38+ and CD34+ HLA-DR+ cells sorted from mobilized PBPC contained 0.5% to 5% of cells capable of sustaining hematopoiesis in long-term culture for 5 weeks. However, 90% to 95% of LTC-IC present in mobilized CD34+ CD38+ and CD34+ HLA-DR+ cells were not able to sustain hematopoiesis for 8 weeks, while 30% of CD34+ CD38+ and CD34+ HLA-DR+ LTC-IC present in mobilized PBPC could sustain hematopoiesis for at least 8 weeks. This suggests that the majority of CD34+ CD38+ and CD34+ HLA-DR+ LTC-IC present in mobilized PBPC contain similar numbers of primitive progenitors capable of sustaining hematopoiesis in LTC for both autologous and allogeneic transplants.

The yield of PBPC can be greatly enhanced by using growth factors, such as granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage (GM)-CSF, interleukin (IL)-3, or stem cell factor (SCF), either alone or in combination, chemotherapy or both. Growth factors alone, chemotherapy alone, or a combination of both increase the relative frequency of CD34+ cells in PBPC by fivefold to 10-fold and the content of CFC. As for steady state PBMNC, less is known on the effect of growth factors and/or chemotherapy on the mobilization of more primitive progenitors such as LTC-IC.

The aim of our study was to evaluate the capacity of G-CSF to mobilize primitive progenitors into the blood of normal donors and further characterize these progenitors. We administered G-CSF to normal individuals and determined the number of LTC-IC mobilized in the blood. We demonstrate that G-CSF mobilizes LTC-IC to a greater extent than CD34+ cells or committed CFC. CD34+ CD38+ and CD34+ HLA-DR+ cells selected from G-CSF mobilized normal PBPC contain similar numbers of primitive progenitors capable of sustaining hematopoiesis in LTC for both 5 and 8 weeks as CD34+ CD38+ and CD34+ HLA-DR+ cells present in steady state PBMNC or bone marrow (BM). However, a large number of progenitors at an intermediate stage of differentiation were present in mobilized PBPC.

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differentiation between CFC and LTC-IC can be found in the CD34+ CD38+ and CD34+ HLA-DR+ fraction of G-CSF mobilized PBPC that can sustain in vitro hematopoiesis for 5 weeks, but not for 8 weeks.

MATERIALS AND METHODS

Samples

Blood and PBPC donors. Normal healthy donors were selected using standard criteria of the American Association of Blood Banks for blood donors.24 Informed consent was obtained using guidelines approved by the Committee on the Use of Human Subjects for Research at the University of Minnesota. All donors had a negative serologic test for HBsAg, anti-hepatitis C virus (HCV) and anti-human immunodeficiency virus (HIV) and they had normal hemoglobin, white blood cell (WBC) counts, white cell differentials, and platelet counts.

PBPC. The PBPC donors received a daily dose of either 7.5 or 10 μg/kg/day of human recombinant G-CSF (Neupogen, Amgen, Inc, Thousand Oaks, CA) subcutaneously (SC) for 5 days (days 1 through 5). G-CSF was given as a single morning dose. PBPC collections were performed between 20 and 24 hours after the last dose of G-CSF (day 6) with a Fenwal CS-3000 PLUS (Baxter Healthcare Corporation, Round Lake, IL) blood cell separator using a granulocyte separation chamber and a small volume collection chamber with 50 to 60 mL of blood was obtained by venipuncture from each donor before the first injection of G-CSF (day 0) and 10 mL of PBPC from the leukapheresis product (day 6).

Normal BM donors. Heparinized bone marrow was obtained from normal, healthy volunteer donors after informed consent, using guidelines approved by the Committee on the Use of Human Subjects for Research at the University of Minnesota.

Evaluation of Samples

The blood and PBPC hematocrit, red blood cell (RBC) count, WBC count, and platelet count were determined using an automated blood counter (S + IV, Coulter Electronics Inc, Hialeah, FL). Flow cytometry, clonogenic assays, and LTC were performed on steady state PBMNC, mobilized PBPC, and steady state bone marrow samples.

Immunophenotyping. Enumeration of CD34+ cells in PBMNC or mobilized PBPC samples was performed as described by Sutherland et al.25 In short, PBMNC or mobilized PBPC were stained simultaneously with a fluorescein isothiocyanate (FITC)-conjugated CD45 antibody (HLe-1; Becton Dickinson, Mountain View, CA) and a phycoerythrin (PE)-conjugated CD34 antibody (HPCA-2-PE). The incidence of true CD34+ cells was determined by gating cells for low-density CD45 expression and low-side scatter characteristics.

Cell selection. Steady state PBMNC and BM, but not the leukapheresis products, were separated by Ficoll Hypaque centrifugation (specific gravity, 1.077; Sigma Chemical Co, St Louis, MO). CD34 enrichment was done using the MACS CD34 Isolation Kit (Miltenyi Biotec Inc, Sunnyvale, CA) according to the manufacturer’s instructions with modifications. Briefly, the PBMNC, mobilized PBPC, or BMNC cell pellet was resuspended in cold phosphate-buffered saline (PBS) and 0.3% bovine serum albumin (BSA) and incubated with modified CD34 antibody (QBEND/10, mouse IgG1) for 15 minutes at 4°C and then washed and incubated for 15 minutes with superparamagnetic MACS microbeads recognizing anti-CD34 antibodies. Concurrently, the separation column was flushed with cold PBS and positioned in the MACS magnetic field. The cell suspension was loaded onto the top of the separation column and nonadherent cells were collected. The CD34+ cells, which were retained in the column, were then recovered by removing the column from the magnetic field and flushing with buffer. The magnetic separation step was repeated once to obtain a CD34+ cell purity over 90% in every sample.

Fluorescence-activated cell sorting (FACS). For FACS selection, CD34 enriched cells were labeled with PE-conjugated mouse anti-CD38, FITC-conjugated mouse anti-HLA-DR (Becton Dickinson), and mouse anti-CD34 conjugated to biotin (Cellpro Inc, Bothell, WA), incubated for 30 minutes on ice then washed with cold PBS and incubated with Streptavidin-RED670 (GIBCO-BRL, Grand Island, NY). Cells were selected on a FACStar-Plus laser flow cytometry system equipped with a CONSORT 32 computer for low forward and side scatter properties and expression of CD34, CD38, and HLA-DR antigens using mouse IgG1-PE and IgG2-FITC antibodies and SA670 as control.

Short-term methycellulose progenitor culture. Cells were cultured at 2 × 10^4 cells/mL (for the different CD34 subpopulations) or 2 × 10^5 cells/mL (for unselected MNC) in methylcellulose containing Iscove’s modified Dulbecco’s medium (IMDM; GIBCO Laboratories, Grand Island, NY), supplemented with 30% fetal calf serum (FCS; HyClone, Logan, UT), 3 IU erythropoietin (Epoeitin; Amgen, Thousand Oaks, CA) and 10% 5637 bladder carcinoma-conditioned media as described.26 Cultures were incubated in a humidified atmosphere at 37°C and 5% CO2. The cultures were assessed at day 14 to 18 for the presence of burst-forming unit erythroid (BFU-E), colony-forming unit granulocyte-macrophage (CFU-GM), and CFU-Mix as previously described.25

LTC

Complete long-term culture medium. LTC medium consisted of IMDM with 12.5% FCS, 12.5% horse serum (Terry Fox Laboratories, Vancouver, Canada), 2 mmol/L glutamine (GIBCO), penicillin 100 U/mL, streptomycin 100 U/mL (GIBCO) and 10^-5 hydrocortisone.27

Stromal feeders. M2-10B4 cells, a generous gift from Dr C. Eaves (Vancouver, British Columbia, Canada), were maintained in RPMI (GIBCO Laboratories) + 10% fetal calf serum. We and others have shown that these murine marrow stroma-derived fibroblasts can support human hematopoiesis in stroma-contact and stroma-noncontact cultures to the same extent as normal human marrow feeders.28,29 M2-10B4 cells were subcultured in wells of 6-well or 96-well plates. Once confluent, plates were irradiated with 6,000 cGy and the medium was changed to LTC medium.

LTC. LTC were established by plating 5 × 10^3 mobilized PBPC or 5,000 FACS selected CD34+ subpopulations in direct contact with normal M2-10B4 stromal layers subcultured in 6-well plates. Cultures were maintained for 5 weeks in a humidified atmosphere, at 37°C and 5% CO2. Weekly media changes were performed by removing half of the cell free supernatant medium and replacing it with fresh LTC medium. To determine the number of CFC present in LTC, cells were harvested by digesting the stromal layers with trypsin and replated in methylcellulose progenitor culture.

Enumeration of LTC-IC by limiting dilution assays (LDA). Two million PBMNC (22 replicates: 61,224; 20,408; 6,802; 2,267 cells/well) and 10,000 cells from CD34+ subpopulations (22 replicates: 300, 100, 33, 11 cells/well) were plated in limiting dilutions onto previously irradiated M2-10B4 feeders. Cultures were maintained in a humidified atmosphere, at 37°C and 5% CO2. Weekly, half of the medium was replaced by fresh LTC medium. After 5 weeks, unless
Table 1. Effect of Mobilization With G-CSF on the Number of WBC, CD34+ Cells, Committed Progenitors, and LTC-IC in the Blood of Normal Donors (n = 26)

<table>
<thead>
<tr>
<th></th>
<th>Pre G-CSF</th>
<th>Post G-CSF</th>
<th>Fold Increase</th>
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</thead>
<tbody>
<tr>
<td>WBC (x10^9/L)</td>
<td>6.1 ± 0.3</td>
<td>40.3 ± 2.1</td>
<td>6.9 ± 0.47</td>
</tr>
<tr>
<td>CD34+ (%)</td>
<td>0.19 ± 0.02</td>
<td>1.04 ± 0.18</td>
<td>8.6 ± 1.2</td>
</tr>
<tr>
<td>BFU-E</td>
<td>29.5 ± 5.7</td>
<td>106.9 ± 13.4</td>
<td>3.73 ± 1.58</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>34 ± 5.4</td>
<td>201.2 ± 18.9</td>
<td>9.1 ± 1.1</td>
</tr>
<tr>
<td>CFU-Mix</td>
<td>2.37 ± 0.4</td>
<td>17.5 ± 2.4</td>
<td>12.4 ± 2.3</td>
</tr>
<tr>
<td>LTC-IC</td>
<td>0.74 ± 0.16</td>
<td>18.8 ± 4.2</td>
<td>59.3 ± 15.94</td>
</tr>
</tbody>
</table>

Number of colonies and LTC-IC is expressed per 10^6 cells plated as mean ± SEM.

* Ficoll separation.
† Apheresis product.
‡ Significantly higher than WBC, CD34+ cells, BFU-E, CFU-GM, or CFU-Mix, P < .01.

otherwise stated, all media was removed and the stromal layers overlaid with methylcellulose containing medium supplemented with EPO (3 IU/mL) and 10% supernatant of the bladder carcinoma cell line 5637. Wells were scored for the presence or absence of secondary CFC at day 14. The absolute number of LTC-IC present in the different cell populations was calculated as the reciprocal of the concentration of test cells that gives 37% negative cultures using Poisson statistics.39

Statistics. Results of experimental points obtained from multiple experiments were reported as the mean ± standard error of mean (SEM). Significance levels were determined by two-sided Student's t-test analysis. Correlations were calculated using the Pearson's correlation coefficient.

RESULTS

Mobilization of Committed Progenitors and LTC-IC With G-CSF

Twenty-nine normal donors were included in the study. Fifteen subjects received 10 μg/kg G-CSF sc for 5 days, while 14 subjects received 7.5 μg/kg for 5 days. Since the number of WBC, CD34+ cells, BFU-E, CFU-GM, CFU-Mix or LTC-IC mobilized in PBPC following administration of either 7.5 μg/kg or 10 μg/kg of G-CSF was not statistically different, analyses were done using results obtained from all 29 individuals.

Administration of G-CSF increased the total number of WBC sevenfold, the percentage of CD34+ cells eightfold, and the number of BFU-E, CFU-GM, CFU-Mix per 100,000 MNC sevenfold, ninefold, and 12-fold respectively over baseline (Table 1). In contrast, the absolute number of LTC-IC in G-CSF mobilized PBPC increased 59-fold over that in unmobilized PBMNC (Table 1). The fold increase in LTC-IC was significantly higher than the increase in BFU-E (P < .01), CFU-GM (P < .008), CFU-Mix (P < .01), and CD34+ cells (P < .01). Interestingly, the number of secondary CFC generated per LTC-IC present in G-CSF mobilized PBPC after 5 weeks in LTC in four different experiments was only 1.3 ± 0.3 CFC/LTC-IC (defined as the total number of CFC in LTC initiated with mobilized PBPC divided by the absolute number of LTC-IC determined by LDA in that population). This is significantly lower than the number of CFC per LTC-IC present in steady state BM or PBMNC21,32 which ranges between 4 and 6 CFC per LTC-IC.

Both the number of CFU-GM and CD34+ cells have been used as predictors for early engraftment after transplantation.33,34 Because recent studies suggest that primitive, rather than committed, progenitors may be responsible for early recovery of hematopoiesis after transplantation,35,36 we examined if the percent CD34+ cells or CFU-GM correlates with percent of LTC-IC present in unmobilized PBMNC or mobilized PBPC. The percent CD34+ cells (r = 0.22) or CFU-GM (r = 0.18) in unmobilized PBMNC did not predict the number of LTC-IC present in unmobilized PBPC. Following mobilization with G-CSF a positive correlation was seen between the CD34+ cell percentage and CFU-GM percentage (r = 0.55; P < .05). However, neither the percent of CD34+ cells (r = 0.28) nor CFU-GM (r = 0.45) present in G-CSF mobilized PBPC predicted the number of LTC-IC present in the mobilized product.

The mean volume of the G-CSF mobilized PBPC product obtained from a single leukapheresis was 371 ± 15 mL containing 92.6 ± 4.8 x 10^8 WBC/mL. The total number of CD34+ cells obtained varied between 91.3 and 1758 ± 10^6 or 1.2 x 10^9/kg to 23 x 10^6/kg for a 75-kg recipient. The total dose of CFU-GM varied between 17.1 ± 10^6 and 150 ± 10^6 or 1.78 x 10^9/kg and 20 x 10^6/kg for a 75-kg recipient (Table 2). In 21 of 26 (80.7%) donors, more than 2 x 10^6 CD34+ cells/kg for a 75-kg patient were obtained. The number of CFU-GM per kg for a 75-kg patient was higher than 2 x 10^5 in 25 of 26 (96%) donors.

G-CSF Significantly Increases Myeloid Progenitors Within the CD34+ Cells

FACS selected CD34+ cells (purity >99.2%) were evaluated for their content of CFC and LTC-IC before and after mobilization with G-CSF. There was a statistically significant increase in the clonogenic capacity for both the committed progenitors (BFU-E: 1.79-fold, P < .02; CFU-GM: 2.5-fold, P < .02; CFU-Mix: 2.54-fold, P < .02) (Fig 1A through C) and for the more primitive LTC-IC (ninefold, P < .01)

Table 2. Total Number of Progenitors Obtained in a Single 3-Hour Leukapheresis of G-CSF-Mobilized Normal Donors (n = 26)

<table>
<thead>
<tr>
<th></th>
<th>Median</th>
<th>Range</th>
<th>75-kg patient*</th>
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</thead>
<tbody>
<tr>
<td>WBC (x10^9)</td>
<td>34.6</td>
<td>17.3-74.7</td>
<td>4.7 x 10^9/kg</td>
</tr>
<tr>
<td>CD34+ (x10^6)</td>
<td>424</td>
<td>91.3-1758</td>
<td>6 x 10^6/kg</td>
</tr>
<tr>
<td>BFU-E (x10^6)</td>
<td>34</td>
<td>6-97.5</td>
<td>4.8 x 10^6/kg</td>
</tr>
<tr>
<td>CFU-GM (x10^6)</td>
<td>66.7</td>
<td>17.1-150</td>
<td>9.6 x 10^6/kg</td>
</tr>
<tr>
<td>CFU-Mix (x10^6)</td>
<td>5.2</td>
<td>0.9-18</td>
<td>8 x 10^6/kg</td>
</tr>
<tr>
<td>LTC-IC (x10^6)</td>
<td>5.1</td>
<td>0.26-22.1</td>
<td>9.5 x 10^6/kg</td>
</tr>
</tbody>
</table>

* Mean value of WBC, CD34+ cells, CFU, and LTC-IC obtained for a 75-kg recipient.
after mobilization with G-CSF (Fig 1D). The fraction of identifiable progenitors (equal to the percentage of cells that gave rise to CFU-GM, BFU-E, and CFU-Mix + the percentage of cells that could initiate LTC) in the unmobilized CD34+ cells was 13.9% ± 2.1%. After mobilization with G-CSF the percentage of identifiable progenitors present in FACS selected CD34+ cells increased to 28.7% ± 2.13% (P < .01) (Fig 2).

LTC-IC Can Be Found in the CD34+ CD38− or CD34+ CD38+ and CD34+ HLA-DR+ or CD34+ HLA-DR− Fraction of G-CSF Mobilized PBPC

We next analyzed the phenotype of CD34+ cells before and after mobilization with G-CSF. There were no statistically significant differences between the percentage of CD34+ cells that expressed CD38 before and after mobilization. However, the percentage of CD34+ cells expressing the HLA-DR antigen increased from 82.3% ± 2% in steady state PBMC to 89.9% ± 1% in PBPC obtained after G-CSF mobilization (P = .002) (Table 3). Mobilized PBPC were then sorted based on CD38 and HLA-DR antigen expression into CD34+ CD38−, CD34+ CD38+, CD34+ HLA-DR−, CD34+ HLA-DR− cells, all with a purity over 99.5%

A representative analysis of steady state PB, steady state BM, and G-CSF mobilized PBPC is shown in Fig 3A through C. As is seen in normal steady state BM or PBMC, clonogenic progenitors were highly enriched in the CD34+ CD38− and to a lesser extent in the CD34+ HLA-DR+ fraction of G-CSF mobilized PBPC (Table 4). The absolute number of LTC-IC per 100 sorted cells, determined by LDA, in G-CSF mobilized PBPC CD34+ CD38− cells was similar to that seen in steady state BM-derived CD34+ CD38− (Fig 4A and B) or unmobilized PBMC CD34+ CD38− cells (data not shown). Likewise, the frequency of LTC-IC in CD34+ HLA-DR− cells from BM and mobilized PBPC was equivalent (0.4% ± 0.1% LTC-IC in mobilized PBPC CD34+ HLA-DR− cells, n = 4 and 0.68% ± 0.16% LTC-IC in BM CD34+ HLA-DR− cells, n = 4). Surprisingly, 2.5% ± 0.9% mobilized CD34+ CD38− cells and 0.98% ± 0.2% mobilized CD34+ HLA-DR− cells were capable of initiating and sustaining hematopoiesis in stromal cultures for 5 weeks. This is significantly higher than what was observed for CD34+ CD38− and CD34+ HLA-DR− subpopulations from mobilized PBPC (P < .01) or that of CD34+ CD38− and CD38− or CD34+ HLA-DR+ and HLA-DR− cells present in steady state BM (P < .05) (Fig 4A and B).

CD34+ CD38+ and CD34+ HLA-DR+ LTC-IC Present in Mobilized PBPC Are More Mature Than CD34+ CD38− and CD34+ HLA-DR− LTC-IC

The definition of an LTC-IC is "a cell capable of initiating and sustaining long-term cultures for at least 5 weeks".
Fig 2. G-CSF increases the percent of identifiable progenitors in FACS selected CD34+ cells. FACS selected CD34+ cells from PBMC and mobilized PBPC were plated in methylcellulose culture and LTC to enumerate the number of CFU-GM, BFU-E, CFU-Mix and LTC-IC. The fraction of identifiable progenitors (equal to the percentage of cells that gave rise to CFU-GM, BFU-E and CFU-Mix + the percentage of cells that could initiate LTC) in the unmobilized CD34+ cells was: pre-G-CSF: 13.9% ± 2% (n = 4), post-G-CSF: 28.7% ± 2% (n = 4) (P = .01).

However, we and others have demonstrated that LTC-IC still represent a spectrum of progenitors. Some progenitors are capable of sustaining hematopoiesis for 8 or more weeks. To further examine the maturation stage of LTC-IC present in G-CSF mobilized CD34+ subpopulations, we performed additional experiments in which the LTC were maintained for 8 weeks. Three-fold less cells present in mobilized CD34+ CD38- cells were able to initiate and sustain hematopoiesis in LTC for 8 weeks compared with 5 weeks. However, 20-fold less cells present in the CD34+ CD38- fraction of mobilized PBPC were capable of sustaining hematopoiesis for 8 weeks (Fig 4B and C). Likewise, fourfoldless mobilized CD34+ HLA-DR- cells sustained hematopoiesis for 8 weeks compared with 5 weeks (0.68% ± 0.16% at 5 weeks versus 0.15% ± 0.02% at 8 weeks; P = .09, n = 4), but 10-fold less mobilized CD34+ HLA-DR+ cells sustained hematopoiesis for 8 rather than 5 weeks (1% ± 0.2% at 5 weeks versus 0.13% ± 0.04% at 8 weeks; P = .01, n = 4). Thus, when LTC were maintained for 8 weeks, the phenotype of LTC-IC was CD34+ CD38- and CD34+ HLA-DR- and no longer CD34+ CD38+ and CD34+ HLA-DR+. Consistent with the notion that CD34+ CD38- and CD34+ HLA-DR- LTC-IC are more mature than CD34+ CD38- and CD34+ HLA-DR- LTC-IC is our observation that the number of CFC generated per LTC-IC from CD34+ CD38+ and CD34+ HLA-DR- populations was only 2.6 ± 0.27 (n = 5), which was significantly lower than the number of CFC generated per CD34+ CD38+ and CD34+ HLA-DR- LTC-IC (13.2 ± 2.5, n = 5) (P < .01).

**DISCUSSION**

In this study, we demonstrate that G-CSF increases the absolute number of week 5 LTC-IC 59-fold in MNC of peripheral blood of normal healthy donors, which is significantly higher than the mobilization of either phenotypically defined CD34+ progenitors or functionally defined CFU-GM or CFC. Previous studies have documented the presence of LTC-IC in mobilized PBPC from patients with malignancies.5,6 Henschler et al showed that the frequency of LTC-IC in unseparated mobilized PBPC was 1:9,075 cells, while Pettengell et al found a frequency of LTC-IC in mobilized PBPC of 1:10,302. In our study, we demonstrate that the frequency of LTC-IC in G-CSF mobilized PBPC from normal donors is 1:6,000. The higher frequency of LTC-IC mobilized in PBPC from normal donors compared with patients with malignancies may be due to the fact that patients, but not normal donors, have previously been treated with chemotherapy. CFC and LTC-IC in steady state PB were enumerated in MNC obtained after Ficoll-Hypaque separation, while CFC and LTC-IC in mobilized PBPC were enumerated in the apheresis product without further processing. Although density centrifugation and the apheresis process may alter the proportion of different populations present in the MNC fraction, we do not believe that differences in CFC and LTC-IC frequencies can be attributed solely to differences in MNC preparation techniques.

Infusion of a minimum of 2 × 10^6 CD34+ cells per kg is required for timely multilineage engraftment after autologous transplantation and infusion of more than 5 × 10^6 CD34+ cells/kg has been associated with early trilineage engraftment after autologous transplantation.7 Likewise, transplantation with more than 5 × 10^6 CD34+ cells present in allogeneic PBPC results in engraftment between day 9 and day 25.13,14 We demonstrate here that administration of 7.5 µg/kg/day of G-CSF for 5 days followed by a single 3-hour leukapheresis provided more than 2 × 10^6 CD34+ per kg for a 75-kg recipient in more than 80% of the donors and more than 5 × 10^6 CD34+ cells per kg in 16 of 26 donors (61%). However, the number of CD34+ cells recovered from different G-CSF-treated normal individuals varied significantly. This is in accordance with recent reports demonstrating that the number of progenitors in PBPC varies not only between patients and normals, but also between different normal donors. Increasing the duration of the leukapheresis may be one way to increase the likelihood of obtaining sufficient CD34+ cells for transplant in a single leukapheresis from the majority of normal donors.

**Table 3. Phenotype of PBPC Before and After Mobilization**

<table>
<thead>
<tr>
<th>With G-CSF (n = 18)</th>
<th>Pre G-CSF</th>
<th>Post G-CSF</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34+ CD38-</td>
<td>92.8 ± 1.3</td>
<td>90.5 ± 2.1</td>
<td>NS</td>
</tr>
<tr>
<td>CD34+ DR-</td>
<td>7.2 ± 1.2</td>
<td>9.4 ± 2.1</td>
<td>NS</td>
</tr>
<tr>
<td>CD34+ DR-</td>
<td>82.3 ± 2</td>
<td>89.9 ± 1</td>
<td>.002</td>
</tr>
<tr>
<td>CD34+ DR-</td>
<td>17.6 ± 2</td>
<td>10 ± 1.3</td>
<td>.002</td>
</tr>
</tbody>
</table>

Phenotypes are expressed as mean ± SEM.

Abbreviation: NS, not significant.
Discordant results have been reported on the correlation between the number of CFU-GM per kg infused and time to neutrophil and platelet engraftment after PBPC transplant. Recent murine studies have suggested that the number of primitive rather than committed progenitors may be predictive for both early and long-term engraftment. Similar studies in humans examining the predictive value of the number of infused primitive progenitors and engraftment have not yet been done. We examined if a correlation exists between the frequency of CD34+ cells or committed progenitors and engraftment frequency of either CFU-GM or LTC-IC, which may be due to the inaccuracy in determining the very low percent of CD34+ cells present in unmobilized PBMNC. Although the frequency of CD34+ cells in G-CSF mobilized PBPC was predictive of the CFU-GM frequency, neither CD34+ cells nor CFU-GM frequency in mobilized PBPC correlated with the frequency of LTC-IC. Clinical studies will need to demonstrate whether LTC-IC rather than CFU-GM or CFC predict early engraftment.

CD34+ cells comprise a heterogeneous population including committed myeloid and lymphoid progenitors and also more primitive progenitors and stem cells. Differences in the relative frequency of these subpopulations underlie the
observation that CD34⁺ cells obtained from various sources express different surface antigens and have different proliferative and self-renewal capacities. Several studies have demonstrated phenotypic differences between CD34⁺ cells obtained from steady state blood and following mobilization. We demonstrate here a significant increase in committed myeloid precursors and LTC-IC in FACS selected PBPC derived CD34⁺ cells compared with steady state PBMC-derived CD34⁺ cells. This is consistent with the observation that significantly less mobilized PBPC-derived CD34⁺ cells coexpress lymphoid markers than CD34⁺ cells present in steady state BM or unmobilized PBMC and that a larger proportion of G-CSF mobilized CD34⁺ cells coexpress the HLA-DR antigen, commonly associated with committed myeloid progenitors.

Presence or absence of CD38 and HLA-DR antigens on CD34⁺ cells is commonly used to differentiate committed from more primitive progenitors. Steady state BM and PBMC CD34⁺ CD38⁻ and CD34⁺ HLA-DR⁻ populations are enriched in LTC-IC, while CD34⁺ CD38⁺ and CD34⁺ HLA-DR⁻ are enriched in more committed progenitors. Here, we demonstrate that the fraction of CD34⁺ CD38⁻ and CD34⁺ HLA-DR⁻ cells capable of initiating LTC is similar for mobilized PBPC, unmobilized PBMC, or steady state BM. Surprisingly, significantly more week 5 LTC-IC were present in mobilized CD34⁺ CD38⁻ and CD34⁺ HLA-DR⁺ cells than in mobilized CD34⁺ CD38⁻ and CD34⁺ HLA-DR⁻ cells.

That LTC-IC are a heterogeneous population of cells becomes clear when BM-derived CD34⁺ CD38⁻ and CD34⁺ HLA-DR⁻ cells are maintained in LTC for prolonged periods of time. We have demonstrated that the number of BM-derived CD34⁺ HLA-DR⁻ cells capable of sustaining hematopoiesis for 8 weeks is twofold to threefold lower than the number of cells capable of sustaining hematopoiesis for 5 weeks. This suggests that LTC-IC represent a spectrum of cells at different stages of maturation. The observation that the fraction of mobilized PBPC-derived CD34⁺ CD38⁻ and CD34⁺ HLA-DR⁻ cells that sustained hematopoiesis in LTC for 8 weeks was 10-fold to 20-fold lower than the fraction capable of sustaining LTC for 5 weeks supports the idea of LTC-IC representing a more mature progenitor. Further evidence that CD34⁺ CD38⁻ and CD34⁺ HLA-DR⁻ progenitors capable of sustaining hematopoiesis for 5 weeks may represent a more mature progenitor than CD34⁺ CD38⁻ and CD34⁺ HLA-DR⁻ LTC-IC comes from our own studies and studies from Sutherland et al which demonstrate that the number of secondary CFC per LTC-IC in mobilized PBMC is only 1 to 1.5, which is significantly lower than that of steady state BM or PBMC (4 to 6 CFC per LTC-IC). Since more than 85% of mobilized

Table 4. Phenotype of CFC Present in G-CSF-Mobilized PBPC

<table>
<thead>
<tr>
<th>Colony Type</th>
<th>CD34⁺ CD38⁻</th>
<th>CD34⁺ CD38⁺</th>
<th>P Value</th>
<th>CD34⁺ HLA-DR⁻</th>
<th>CD34⁺ HLA-DR⁺</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BFU-E</td>
<td>9,533 ± 1,626</td>
<td>668 ± 194</td>
<td>.001</td>
<td>10,117 ± 1,383</td>
<td>8,128 ± 1,674</td>
<td>.2</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>12,800 ± 1,776</td>
<td>4,241 ± 1,493</td>
<td>.002</td>
<td>15,857 ± 1,907</td>
<td>6,494 ± 1,156</td>
<td>.03</td>
</tr>
<tr>
<td>CFU-Mix</td>
<td>1,053 ± 186</td>
<td>170 ± 40</td>
<td>.05</td>
<td>987 ± 114</td>
<td>780 ± 173</td>
<td>.1</td>
</tr>
</tbody>
</table>

Colonies are expressed per 10⁶ cells as mean ± SEM. n = 10 (CD34⁺ CD38⁻ and CD34⁺ CD38⁺), n = 6 (CD34⁺ HLA-DR⁻ and CD34⁺ HLA-DR⁺).
CD34+ cells are CD38- and HLA-DR-, more than 85% of week 5 LTC-IC measured in unselected mobilized PBPC correspond to LTC-IC capable of sustaining hematopoiesis for 5 weeks, but not for 8 weeks. These results indicate also that the overall increase in LTC-IC frequency in mobilized PBPC as shown in Table 1 is an overestimation, since this is based on week 5 cultures, representing CD34+ CD38+ as well as more primitive CD34+ CD38- LTC-IC. The increase in CD34+ CD38- cells, containing primitive LTC-IC that can sustain hematopoiesis for more than 5 weeks is only sixfold to eightfold in the mobilized PBPC population compared with steady state blood. Further, only 15% of CD34+ cells are CD34+ CD38- indicating that the true increase in primitive LTC-IC that can sustain hematopoiesis for more than 5 weeks is only 5.1-fold (15% of 60-fold). It is thus, imperative to use the term LTC-IC with care when assessing mobilized PBPC collections, since 85% of progenitors that read out at 5 weeks in LTC cannot sustain hematopoiesis beyond 5 weeks and have decreased proliferative potential. These findings also have implications for the evaluation of ex vivo stem cell expansion cultures. Comparison of week 8 LTC-IC in uncultured and cultured products will be required to demonstrate ex vivo expansion of the more primitive progenitor pool.

In conclusion, we demonstrate that G-CSF mobilizes week 5 LTC-IC in normal donors to a greater extent than committed progenitors or CD34+ cells. However, the characterisics of more than 85% of mobilized week 5 LTC-IC differ from LTC-IC present in steady state BM or PBMC; they are enriched in the CD34+ CD38- and CD34+ HLA-DR+ subpopulations and cannot sustain hematopoiesis in LTC for 8 weeks. These results suggest therefore, that the administration of G-CSF to normal donors, mobilizes a large population of progenitors at an intermediate stage of differentiation between CFC and primitive LTC-IC. The contribution of CFC, progenitors at an intermediate stage of differentiation, and the most primitive LTC-IC progenitors to early and late hematopoietic recovery after transplant will now be examined in clinical trials of G-CSF mobilized PBPC transplant.

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Phenotypic and functional characterization of long-term culture-initiating cells present in peripheral blood progenitor collections of normal donors treated with granulocyte colony-stimulating factor

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