Mobilization of Long-Term Hematopoietic Reconstituting Cells in Mice by the Combination of Stem Cell Factor Plus Granulocyte Colony-Stimulating Factor

By X-Q. Yan, R. Briddell, C. Hartley, G. Stoney, B. Samal, and I. McNiece

In this study, we have compared the ability of recombinant human granulocyte colony-stimulating factor (rhG-CSF) alone and the combination of low doses of recombinant rat pegylated stem cell factor (rrSCF-PEG) plus rhG-CSF to mobilize peripheral blood progenitor cells (PBPCs) with long-term engraftment potential. Female irradiated mice were transplanted with PBPCs from male mice that were mobilized with rhG-CSF alone (group A) or rrSCF-PEG plus rhG-CSF (group B). As previously shown, greater short-term survival resulted in group B compared with group A, with 80% and 40% survival at 30 days posttransplant, respectively. Both groups of animals showed long-term donor-derived engraftment in greater than 95% of animals, as determined by quantitative specific polymerase chain reaction amplification of a Y chromosome sequence from whole blood of the mice at 6 to 12 months posttransplantation. Analysis of individual granulocyte-macrophage colonies, picked up from semisolid methylcellulose culture of bone marrow cells from transplanted mice, resulted in detection of donor-derived DNA in 98% of colonies from group B mice compared with 81% from group A mice. These data show that cells with long-term potential are mobilized by rhG-CSF alone and the combination of rrSCF-PEG plus rhG-CSF. Furthermore, an increased number of cells with short-term and long-term engraftment potential was obtained with rrSCF-PEG plus rhG-CSF compared with rhG-CSF alone.

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GROWTH FACTOR-mobilized peripheral blood progenitor cells (PBPCs) are widely used for support of high-dose chemotherapy. More rapid recovery of neutrophils and platelets is achieved with PBPCs compared with bone marrow transplantation (BMT).1 In particular, granulocyte colony-stimulating factor (G-CSF) has been shown to be an effective mobilization factor.2 However, a significant period of neutropenia and thrombocytopenia still occurs after transplantation of recombinant human G-CSF (rhG-CSF)—mobilized PBPCs.2

Recent work has demonstrated the ability of stem cell factor (SCF) to also mobilize PBPCs in primates.3 When used in combination with rhG-CSF, low doses of SCF have been shown to give enhanced mobilization of PBPCs compared with rhG-CSF alone. In mice,4 primates,3 and canines,6 the combination of optimal doses of rhG-CSF with low doses of SCF (25 μg/kg/d) resulted in increased numbers of circulating white blood cells (WBCs) and both mature and primitive progenitors. Transplantation of irradiated mice or canines with these PBPCs resulted in enhanced radioprotection and recovery of platelets and WBCs posttransplant.4,6

An outstanding issue with the use of growth factor-mobilized PBPC is the potential of these cells to provide long-term engraftment. In the present study, we have examined both the short-term and long-term engraftment potential of rhG-CSF alone and rhG-CSF plus recombinant rat pegylated SCF (rrSCF-PEG)—mobilized PBPCs in irradiated mice.

MATERIALS AND METHODS

Mice. Ten- to 12-week-old female and splenectomized male (C57BL/6J × DBA/2J) F1 (BDF1) mice were purchased from Charles River Laboratories (Wilmington, MA) and housed under sterile conditions.

Mobilization and transplantation. Splenectomized male BDF1 mice were treated for 7 days with rrSCF-PEG or rhG-CSF, or a combination, as previously described.3 Both rrSCF-PEG and rhG-CSF were obtained from Amgen Inc (Thousand Oaks, CA).3,4 PB was harvested by cardiac puncture and collected into EDTA-containing tubes. The number of WBCs and platelets was determined using a Sysmex Microcellcounter (Baxter Healthcare Corp, Irvine, CA). Low-density cells (<1.167 g/mL) were obtained by layering whole PB into 90 μL of H2O. Samples were boiled for 10 minutes, and cooled on ice. Two microliters of proteinase K (10 mg/mL) was added to each tube and incubated at 60°C for 2 hours. Samples were boiled for another 10 minutes and then vortexed. Twenty-five microliters of the mixture were added into the PCR-reaction. For single-colony PCR analysis, GM colonies in PCR amplification. For single-colony PCR analysis, GM colonies containing 109 to 1010 cells were individual picked from methylcellulose culture in a total volume of 5 μL. Samples were then added into 95 μL of H2O, boiled for 10 minutes, and cooled on ice. Two microliters of proteinase K (10 mg/mL) was added to each tube and incubated at 60°C for 2 hours. Samples were boiled for another 10 minutes and then vortexed. Twenty-five microliters of the mixture was added into the PCR-reaction.

Mouse Sry locus sequences on the Y chromosome in whole blood cells and single GM colonies were amplified by PCR with a specific pair of primers, 5′ (ctg ctc tga aca gac act ac) and 3′ (gac tcc tct gac ttc act tg), corresponding to mouse Sry locus sequences 256 through 276 and 958 through 978, respectively.5 Samples were


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heated to 94°C for 4 minutes and then amplified for 28 cycles at
94°C for 1 minute, 62°C for 1 minute, and 72°C for 2 minutes in
50 μL of PCR reaction containing 20 pmol of primers, 10 mmol/L
Tris-HCl, pH 8.3, 1.5 mmol/L MgCl2, 50 mmol/L KCl, and 2.5 U
Taq polymerase (Boehringer Mannheim, Indianapolis, IN). Platelet-
derived growth factor (PDGF) B receptor sequences were amplified
simultaneously as a control for the PCR amplification with a pair
of murine PDGF B receptor-specific primers, 5’ (cat tgg ctc cat cct
gca ta) and 3’ (gga taa gcc tcg aac acc ac), corresponding to PDGF
B receptor cDNA sequence 948 through 968 and 1146 through 1166,
resulting in an 700- to 800-bp genomic DNA fragment (listed here
as 700 bp for convenience). Amplified materials were Southern
blotted and hybridized to 25-mer internal sequences of the
Sry locus and PDGF B receptor. The 25-bp sequences, cta gag atc
gga gct tgg ctc agg t, which corresponds to Sry locus sequence 627 through 652,
and gca acg tgt cag tga gtg tga acg a, which corresponds to PDGF
B receptor cDNA sequence 1007 through 1032, were P3'-end-la-
beled and hybridized to the amplified materials at 60°C using a
conventional hybridization protocol.

RESULTS

Transplantation of 5 × 10^5 low-density mononuclear cells
mobilized with rhG-CSF plus rrSCF-PEG into lethally irradiated
mice resulted in 80% survival at 60 days. Only 40% survival occurred for mice transplanted with the same num-
ber of cells mobilized with rhG-CSF alone (Fig 1). Control
mice received no transplantation of cells and all animals
were dead by day 14 posttransplantation. All mice that were
alive at 60 days have survived long-term with observation
up to 1 year.

WBC and platelet recovery after transplantation of cells
from rhG-CSF alone (group A) or rrSCF-PEG plus rhG-CSF
(group B) mobilized mice was evaluated. Irradiated mice
were transplanted with 1 × 10^6 cells and routine blood analy-
isis was performed. As shown in Fig 2, both WBC and plate-
let numbers recovered more rapidly in mice in group B.
Furthermore, only 1 of 20 mice in group B reached platelet
numbers less than 50,000/μL, whereas 19 of 20 animals in
group A had platelet levels less than 50,000/μL for 1 or more
days.

To address whether PBPCs were able to provide long-
term engraftment, male mice were treated with cytokines
for 7 days. Peripheral low-density cells were collected and
transplanted into lethally irradiated female recipients. Donor-
derived hematopoiesis was analyzed by quantitation of Y-
chromosome sequences in the whole blood of recipients by
PCR. A 720-bp Y-chromosomal sequence was amplified by
a specific pair of primers that correspond to the mouse Sry
locus. A PDGF B receptor sequence, which is present in
both the donor and recipient genome, was amplified simulta-
neously, yielding a 700-bp fragment. The proportion of do-
nor-derived hematopoiesis was estimated by the ratio of the
amount of amplified Y-chromosome sequence to the amount
of amplified PDGF B receptor sequence. PCR results are
shown in three categories in Fig 3: (A) donor-derived hema-
topoiesis was less than the detection level; (B) greater than
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Fig 3. Detection of donor-derived hematopoiesis in mice reconstituted with cytokine-mobilized PBPCs by Y-chromosome–specific PCR. Blood DNA was amplified with Y-chromosome–specific primers (sequence at mouse Sry locus) and with PDGF B receptor–specific primers, resulting in a 720-bp fragment and a 700-bp fragment, respectively. The amplified materials were Southern blotted and hybridized to a 25-mer P<sup>32</sup>-labeled Sry locus internal oligo (top) and a PDGF B receptor internal oligo (bottom). Results are presented in three categories: (A) undetectable level of donor-derived hematopoiesis; (B) greater than 5% donor-derived hematopoiesis; (C) greater than 95% donor-derived hematopoiesis. Controls were female and male DNA samples.

5% of donor-derived hematopoiesis; and (C) greater than 95% of donor-derived hematopoiesis.

To determine the long-term reconstituting ability of PBPCs mobilized with rhG-CSF, a plateau dose of rhG-CSF of 200 μg/kg/d was chosen. The long-term reconstituting ability of PBPCs mobilized with rhG-CSF alone was evaluated in recipients by transplantation of different cell doses (Table 1). The number of long-term (22 to 47 weeks) surviving animals and the number of donor-positive animals correlated with the number of cells transplanted. Transplantation of 2.5 × 10<sup>6</sup> cells resulted in 40% survival and all 8 mice had greater than 95% donor-derived hematopoiesis determined by whole blood quantitative PCR. Transplantation of 1 × 10<sup>6</sup> cells provided 100% survival with greater than 95% donor-derived hematopoiesis.

We next tested whether the combination of low-dose rrSCF-PEG plus rhG-CSF was more efficient in mobilizing long-term reconstituting cells, because we have recently shown a low dose of rrSCF-PEG synergizing with rhG-CSF in mobilizing primitive hematopoietic precursors, high proliferative potential–colony-forming cells (HPP-CFC).<sup>4</sup>

Donor mice were treated with rhG-CSF (200 μg/kg/d) plus rrSCF-PEG (25 μg/kg/d) for 7 days. Lethally irradiated recipients were transplanted with different doses of low-density cells, as shown in Table 2. Mice were analyzed at 22 to 47 weeks by whole blood PCR. The addition of low-dose rrSCF-PEG to rhG-CSF in mobilizing PBPCs increased the long-term survival of mice from 35% and 40% (group A) to 47% and 70% (group B) at transplanted cell doses of 1 × 10<sup>5</sup> and 2.5 × 10<sup>5</sup> cells, respectively (Tables 1 and 2). The most striking results were observed in mice transplanted with 2.5 × 10<sup>5</sup> cells, in which 28 of 40 (70%) mice had long-term survival and all of the mice had greater than 95% donor-derived hematopoiesis.

The observation that mobilized PBPCs contained long-term reconstituting cells was based on whole blood PCR analysis designed to detect the donor-derived Y-chromosomal sequences in female recipient mice. It was possible that some donor-positive mice with a lower percentage of donor-derived hematopoiesis might result from cotransplantation of lymphocytes, which are thought to be long-lived in vivo.<sup>13,14</sup> Secondly, quantitative PCR is limited by the primer concentrations, pipetting errors, and amplification

### Table 1. Long-Term Reconstitution Ability of PBPCs Mobilized With rhG-CSF

<table>
<thead>
<tr>
<th>No. of Cells Injected (x10&lt;sup&gt;6&lt;/sup&gt;)</th>
<th>No. of Mice Transplanted</th>
<th>Time (wks)</th>
<th>No. of Donor-Positive/Long-Term Survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>20</td>
<td>38-39</td>
<td>6/7</td>
</tr>
<tr>
<td>2.5</td>
<td>20</td>
<td>38-39</td>
<td>8/8</td>
</tr>
<tr>
<td>5.0</td>
<td>20</td>
<td>38-47</td>
<td>13/14</td>
</tr>
<tr>
<td>10.0</td>
<td>10</td>
<td>22-25</td>
<td>10/10</td>
</tr>
</tbody>
</table>

Male BDF<sub>1</sub> mice were treated with rhG-CSF at 200 μg/kg/d for 7 days. PB low-density cells were harvested and transplanted into lethally irradiated female recipients. Donor-positive mice were determined by whole blood PCR using Y-chromosomal–specific primers.

### Table 2. Long-Term Reconstitution Ability of PBPCs Mobilized With the Combination of rhG-CSF and rrSCF-PEG

<table>
<thead>
<tr>
<th>No. of Cells Injected (x10&lt;sup&gt;6&lt;/sup&gt;)</th>
<th>No. of Mice Transplanted</th>
<th>Time (wks)</th>
<th>No. of Donor Positive/Long-Term Survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>40</td>
<td>37-47</td>
<td>18/19</td>
</tr>
<tr>
<td>2.5</td>
<td>40</td>
<td>37-47</td>
<td>28/28</td>
</tr>
<tr>
<td>5.0</td>
<td>30</td>
<td>38-47</td>
<td>20/21</td>
</tr>
<tr>
<td>10.0</td>
<td>10</td>
<td>22-25</td>
<td>10/10</td>
</tr>
</tbody>
</table>

Mice were transplanted with PBPCs mobilized with rhG-CSF at 200 μg/kg/d plus rrSCF-PEG at 25 μg/kg/d. Donor-positive mice were determined by whole blood PCR using Y-chromosome–specific primers.
cells were cultured in methylcellulose for 9 days. Eight to 10 PBPCs for up to 5 years or more,15 with no reports of long-term failure. In animal models, long-term engraftment of donor PBPCs mobilized by rhG-CSF alone have been demonstrated.16 Other studies have shown similar results from SCF-mobilized PBPCs and IL-1–mobilized PBPCs.17,18 In recent mouse studies, we have been evaluating the potential of SCF to synergize with rhG-CSF to mobilize primitive progenitor cells (HPP-CFC) and cells with short-term engraftment potential.5 To further evaluate the mobilization of PBPCs with long-term engraftment potential by SCF plus G-CSF, we performed sex-mismatched transplantation of mobilized PBPCs into lethally irradiated mice and evaluated donor engraftment at 6 to 12 months posttransplantation. As we have previously shown,4 mice transplanted with PBPCs mobilized by rrSCF-PEG plus rhG-CSF had increased survival compared with mice transplanted with rhG-CSF–mobilized PBPCs. This may be in part caused by faster recovery of WBC and platelet levels with decreased nadirs in the mice transplanted with rrSCF-PEG plus rhG-CSF–mobilized PBPCs (Fig 2). Mice that survived to 30 days postransplantation in both groups were alive at 6 months or longer. In addition, greater than 90% of mice showed donor-derived engraftment at these later times postransplantation. Analysis of individual GM-CFC colonies demonstrated a higher level of donor-derived engraftment (98%) in mice transplanted with PBPCs mobilized by rrSCF-PEG plus rhG-CSF when compared with mice transplanted with rhG-CSF–mobilized PBPCs (81%). These data show the mobilization of cells with long-term engrafting potential by rhG-CSF alone or in combination with rrSCF-PEG. A higher percentage of donor-derived engraftment was achieved with rrSCF-PEG plus rhG-CSF–mobilized PBPC. Because these experiments were performed by transplantation of equivalent numbers of low-density mononuclear cells, the absolute number of long-term engrafting cells in rrSCF-PEG plus rhG-CSF–mobilized PBPCs is at least twofold or higher than the number from rhG-CSF–mobilized PBPCs. Also, considering that faster WBC and platelet recovery occurred in these animals at equivalent transplant cell numbers, PBPCs mobilized by rrSCF-PEG plus rhG-CSF–mobilized PBPCs contain at least twofold more short-term engrafting cells.

Recent data have been presented for clinical analysis of mobilization of breast cancer patients with rhSCF plus rhG-CSF and show increased mobilization of GM-CFC, burst-forming unit-erythroid, and CD34+ cells compared with rhG-CSF alone.19 Also, more rapid platelet recovery was reported for patients transplanted with rhSCF plus rhG-CSF–mobilized PBPCs in this study. Another report by Bodine et al20 suggests that PBPCs mobilized with rrSCF-PEG plus rhG-CSF are a better target cell population for gene transfer compared with rhG-CSF–mobilized PBPCs. This is consistent with the data presented in this study showing that PBPCs mobilized with rrSCF-PEG plus rhG-CSF have increased numbers of short- and long-term repopulating cells.

In summary, the combination of SCF plus G-CSF enhances mobilization of PBPCs compared with G-CSF alone. These PBPCs are more efficient in engraftment of myelosuppressed animals and provide long-term durable engraftment of 1 year or longer. Preliminary data from clinical studies evaluating the potential of rhSCF to enhance rhG-CSF mobilization of PBPCs are consistent with the data from several animal studies.4,6 However, optimization of harvesting schedules and doses of rhSCF still remain to be determined and are currently being explored.

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**Table 3. Long-Term Engraftment in Mice Transplanted With rhG-CSF Alone and rhG-CSF Plus rrSCF-PEG–Mobilized PBPCs**

<table>
<thead>
<tr>
<th>Factors (µg/kg/d)</th>
<th>Time (wks)</th>
<th>No. of Donor-Positive/ Survivors (whole blood PCR)</th>
<th>No. of Donor-Positive/ Colonies Analyzed (single-colony PCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rhG-CSF 200</td>
<td>38-48</td>
<td>8/8</td>
<td>34/42 (0.81)</td>
</tr>
<tr>
<td>rhG-CSF 200 + SCF 25</td>
<td>37-60</td>
<td>28/28</td>
<td>49/50 (0.98)</td>
</tr>
</tbody>
</table>

Mice were transplanted with PBPCs mobilized with growth factors as indicated. Ali mice received 2.5 x 10^6 cells. Five Y-positive mice determined by whole blood PCR were killed and bone marrow cells (2 x 10^4/mL) were cultured in methylcellulose containing SCF, IL-3, IL-1, IL-6, and Epo. Eight to 10 GM colonies from each mouse were individually picked and amplified by Y-chromosome–specific and PDGF B receptor-specific primers.

The use of rhG-CSF–mobilized PBPCs for support of high-dose chemotherapy has gained wide acceptance in clinical practice. The rapid neutrophil and platelet engraftment with high-dose chemotherapy has gained wide acceptance in clinical practice.
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Mobilization of long-term hematopoietic reconstituting cells in mice by the combination of stem cell factor plus granulocyte colony-stimulating factor

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