In Vivo Administration of Stem Cell Factor to Mice Increases the Absolute Number of Pluripotent Hematopoietic Stem Cells

By David M. Bodine, Nancy E. Seidel, Krisztina M. Zsebo, and Donald Orlic

We have examined the effects of administration of stem cell-factor (SCF) on the number and distribution of pluripotent hematopoietic stem cells (PHSC) in normal mice. Using the competitive repopulation assay we found that in vivo administration of SCF increases the absolute number of PHSC per mouse threefold. The increased numbers of PHSC are found in the peripheral blood and spleen of the SCF-treated animals. The spleen and peripheral blood stem cells completely repopulated the erythroid, myeloid, and lymphoid lineages of irradiated or W/W\(^+\) hosts, similar to bone marrow PHSC. PHSC from the peripheral blood of SCF-treated mice have a lineage marker—negative, c-kit—positive phenotype that is indistinguishable from that of bone marrow PHSC. The increase in the absolute number of spleen PHSC is associated with efficient gene transfer to these cells without prior treatment with 5-fluorouracil. This is a US government work. There are no restrictions on its use.

ALL CELLS in the peripheral circulation of normal individuals are derived from a pool of pluripotent hematopoietic stem cells (PHSC), which are largely confined to the bone marrow of humans, and the bone marrow and spleen of mice. Recent purification experiments have shown that the most primitive murine hematopoietic stem cells express the c-kit receptor on their cell surface (c-kit\(^+\))\(^3,5\) but do not express any hematopoietic lineage markers (Lin\(^-\)).\(^4,5\) Similarly, the most primitive human bone marrow hematopoietic stem cells express the CD34 and c-kit antigens (CD34\(^+,\) c-kit\(^+\)), but are Lin\(^-\).\(^6,7\)

Although most primitive hematopoietic stem cells reside in the marrow, low numbers of these cells can be isolated from the peripheral blood of normal humans and much higher numbers from the peripheral blood of patients treated with cytokines or with cytotoxic drugs.\(^2,5\) In human trials, treatment of patients with either granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), high doses of myeloablative drugs, or a combination of these, causes a dramatic increase in the number of CD34\(^+\) cells and hematopoietic colony-forming cells (CFU-C) in the peripheral blood.\(^8,9\) Peripher al blood stem cells are becoming an important component of many bone marrow transplantation protocols, and have been shown to significantly shorten the period of neutropenia following bone marrow transplantation.\(^10,12\) It is not clear from these studies whether the absolute number of PHSC in the patient is increased along with the mobilization of PHSC in the peripheral circulation. If the absolute number of PHSC does not increase, collection of PHSC from peripheral blood could drain the pool of PHSC.

The biology of the PHSC is most easily studied in the mouse. Increased numbers of hematopoietic progenitor cells were observed in the peripheral blood of mice treated with either GM-CSF or G-CSF.\(^1,13,14\) Peripheral blood from mice receiving G-CSF contained PHSC capable of reconstituting irradiated recipients.\(^13\) Other studies have shown that peripheral blood from mice treated with cyclophosphamide also contained PHSC.\(^16\)

Previous studies have shown that stem cell factor (SCF), the product of the Steel (S\()\) locus in the mouse,\(^1,17-19\) is an important hematopoietic growth factor for the growth and proliferation of primitive hematopoietic cells.\(^20,25\) The combination of SCF and interleukin-6 (IL-6) and/or IL-3 dramatically increases the number and size of multipotential hematopoietic colony formation in vitro,\(^20,24\) and has been shown to increase the number of colony-forming units-spleen (CFU-S) and PHSC in liquid suspension culture.\(^25\)

Administration of SCF to genetically anemic Sl/SI\(^b\) mice increases the red blood cell count and bone marrow cellularity.\(^20,25\) Treatment of normal mice with SCF produces a neutropenia and an increase in spleen cellularity similar to that of G-CSF administration,\(^26\) but to a lesser degree.

Based on these observations, we investigated the effects of in vivo administration of SCF on the number and distribution of PHSC in mice. Our results show that SCF treatment increased the absolute number of PHSC per mouse threefold. The greatest increases in PHSC number were seen in the peripheral blood and spleen, in which the absolute number of PHSC increased 10-fold or more, whereas the number of PHSC in the bone marrow of SCF-treated mice decreased threefold. The PHSC from bone marrow and spleen were shown to be phenotypically c-kit\(^+\), Lin\(^-\), identical to bone marrow stem cells. In addition, spleen PHSC from SCF-treated mice were transduced with a retrovirus containing the human multidrug resistance gene with an efficiency comparable to that of 5-FU–treated bone marrow.

MATERIALS AND METHODS

**Mice.** All mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6J (single hemoglobin; 10-kb EcoRI fragments containing \(\beta\) and \(\beta\) genes) or B6.C-H-1\(^b\)/ByJ (abbreviated HW80; diffuse hemoglobin; 7.0- and 14.0-kb bands representing \(\beta\) and \(\beta\) genes, respectively) mice were used as donor animals in CFU-S and competitive repopulation assays. Recipients for CFU-S assays were WBB6F\(_1\), +/+-/+ mice irradiated with a dose of 900 R. In some competitive repopulation assays WBB6F\(_1\), +/+-/+ mice were used as recipients, and in others irradiated (1,000 R) WBB6F\(_1\), +/+ mice were used as recipients. Identical results were obtained with both recipients.

**SCF injections.** Polyethylene glycol (PEG)-complexed recombinant rat SCF was prepared (Amgen Corp, Thousand Oaks, CA) and shown to contain less than 2 pg endotoxin/\(\mu\)g of protein. The PEG-SCF was supplied at a concentration of 1 mg/mL and stored at 4°C.

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until use. PEG-SCF was diluted to a concentration of 20 μg/mL in phosphate-buffered saline BioFluids, Rockville, MD) containing 0.1% bovine serum albumin (BSA; Miles Laboratories, Kankakee, IL). Donor mice (average weight, 20 g) were injected subcutaneously with 100 μL (dosage, 100 μg/kg/d) of this solution, or with 100 μL of 0.1% BSA, daily for 7 consecutive days. Analysis of the relative amount of hemoglobin in circulating red blood cells was performed 120 days after transplantation.20 At this point, the animals were killed and DNA was prepared from bone marrow and thymus. After digestion with EcoRI, the relative amount of the β and ρm mouse allelic genes in myeloid and lymphoid cells was determined by Southern blotting using a 611-bp fragment BamH1/Pst fragment from the IVS2 of the mouse ρm gene as a probe. Secondary competitive repopulation assay recipients were injected with 2 × 10⁷ cells from primary recipients and analyzed as above.

### Purification of pluripotent hematopoietic stem cells.** Peripheral blood cells from PEG-SCF–treated C57BL/6 mice were collected as described above, and the mononuclear fractions separated by centrifugation through LSM (Organon Teknika) according to the manufacturers instructions. These cells were incubated with a mixture of rat monoclonal antibodies (MoAbs) directed against murine hematopoietic lineage-specific surface markers. The fluorescein isothiocyanate (FITC)-conjugated MoAbs were directed against B lymphocytes (B220), T lymphocytes (L3/T4 and Ly2), granulocytes (Gr-1), and myelomonocytic cells (Mac-1) (purchased from Caltag Laboratories Inc, San Francisco, CA), macrophages (macroph; Coulter Immunology). During FACS sorting, FITC-labeled Lin⁻ cells were discarded. Cells expressing c-kit among the lineage-depleted (Lin⁻) cells were identified with the phycoerythrin (PE)-conjugated MoAb A61-4 (a gift of Dr S.I. Nisikawa, Kumamoto, Japan). Three populations of Lin⁻ cells were observed, c-kit⁻ (≈90%), c-kit⁺/thin (≈9%), and c-kit⁺/thick (≈1%). We and others have shown that bone marrow stem cells have a phenotype of Lin⁻, c-kit⁺/thick. Either 100 or 1,500 Lin⁻ c-kit⁺/thick cells were injected into W/W⁺ recipients. As a control, 1,500 c-kit⁺/thin or 2 × 10⁷ c-kit⁻ cells were injected into W/W⁺ recipients.

### Retrovirus-mediated gene transfer. The cell line producing the retrovirus containing the human multidrug resistance (MDR) gene has been described previously, as has the procedure for retrovirus-mediated gene transfer. Briefly, peripheral blood or spleen cells from PEG-SCF–treated mice, or bone marrow cells from 5-fluorouracil–treated mice were isolated as above and placed into suspension culture for 48 hours in the presence of 100 ng/mL 8-tRNA, 100 ng/mL human IL-6, and 50 ng/mL mouse IL-3 (all provided by Amgen Corp). The cells were then cultured for an additional 48 hours on plates containing a monolayer of retrovirus producer cells in the same growth factors and 6 μg/mL polybrene (Sigma, St Louis, MO). Recipient mice were either BWB6F1/W/W⁺ or irradiated (1,000 R) WB6F1/+/+ . Recipients of bone marrow and spleen cells were injected with 2 × 10⁷ cells, whereas, because of the smaller number of cells harvested from the peripheral blood, recipients of peripheral blood cells were injected with 1 × 10⁷ cells.

### Table 1. Effects of SCF Injection Into Normal Mice

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>Carrier</th>
<th>PEG-SCF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hematology</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBC*</td>
<td>5.8 ± 1.0</td>
<td>6.44 ± 1.8</td>
<td>27.9 ± 6.3f</td>
</tr>
<tr>
<td>(22)</td>
<td>(11)</td>
<td>(30)</td>
<td></td>
</tr>
<tr>
<td>RBC*</td>
<td>8.4 ± 0.2</td>
<td>8.39 ± 0.32</td>
<td>8.51 ± 0.39f</td>
</tr>
<tr>
<td>(22)</td>
<td>(11)</td>
<td>(30)</td>
<td></td>
</tr>
<tr>
<td>HCTt</td>
<td>49.6 ± 1.1</td>
<td>49.9 ± 1.5</td>
<td>49.6 ± 2.5</td>
</tr>
<tr>
<td>(22)</td>
<td>(11)</td>
<td>(30)</td>
<td></td>
</tr>
<tr>
<td><strong>Cellularity: cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>×10⁷/mouse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marrow</td>
<td>6.93 ± 1.4</td>
<td>6.53 ± 1.9</td>
<td>6.92 ± 1.3</td>
</tr>
<tr>
<td>(2 femur + 2 tibia)</td>
<td>(22)</td>
<td>(11)</td>
<td>(30)</td>
</tr>
<tr>
<td>Spleen</td>
<td>25.8 ± 1.9</td>
<td>22.9 ± 3.0</td>
<td>59.0 ± 14.0f</td>
</tr>
<tr>
<td>(22)</td>
<td>(11)</td>
<td>(30)</td>
<td></td>
</tr>
<tr>
<td>CFU-S/10⁷ cells injected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marrow</td>
<td>8.73 ± 2.4</td>
<td>9.86 ± 3.4</td>
<td>8.5 ± 2.9</td>
</tr>
<tr>
<td>(20)</td>
<td>(16)</td>
<td>(16)</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>0.27 ± 0.15</td>
<td>0.32 ± 0.22</td>
<td>2.9 ± 0.56f</td>
</tr>
<tr>
<td>(10)</td>
<td>(12)</td>
<td>(18)</td>
<td></td>
</tr>
<tr>
<td>Peripheral blood</td>
<td>0.0 (7)</td>
<td>0.0 (5)</td>
<td>5.4 ± 1.6f</td>
</tr>
<tr>
<td>(22)</td>
<td>(11)</td>
<td>(10)</td>
<td></td>
</tr>
</tbody>
</table>

Values represent mean ± standard deviation; the number of animals or spleens from which the data is derived is shown in parentheses. Each data point represents data from 3 to 6 independent experiments.

* White blood cell count; number of white blood cells/μL × 10³.  
† Red blood cell count; number of erythrocytes/L × 10¹².  
‡ Hematocrit.  
§ P < .001 relative to untreated or carrier.
Analysis of gene transfer was performed by polymerase chain reaction (PCR) analysis of DNA extracted from peripheral blood 120 days after transplantation as described previously.71

Statistical analysis. The mean and standard deviation of all blood cell counts, CFU-S frequencies, and the proportion of single and diffuse hemoglobins were calculated. Where appropriate, the data were analyzed using the Student’s t-test. In Table 1, the highly significant values (P < .001) are highlighted. Otherwise there was no statistical difference (P > .05) between the control and the experimental values.

RESULTS

Hematology of PEG-SCF–treated normal mice. Normal C57BL/6J or congenic HW80 mice were treated for 7 days with 100 µg/kg/d of PEG-SCF, or with carrier alone. On day 7, the peripheral blood counts of these animals were compared with those of un.injected control animals. For all parameters tested, control animals were identical to those animals injected with carrier alone. In addition, there was no difference in the red blood cell count, hematocrit, bone marrow cellularity, or frequency of bone marrow CFU-S between the un injected or carrier injected animals and those injected with PEG-SCF (Table 1). SCF treatment was associated with a significant elevation in the peripheral blood white blood cell count (Table 1) and a shift in the differential from 20% neutrophils in the normal animals to 85% neutrophils in the SCF-treated animals (Fig 1A and B). SCF treatment was also associated with a threelfold increase in spleen cellularity (Table 1). Although the bone marrow cellularity was unchanged by SCF treatment, there was a significant increase in the number of immature granulocytes and a decrease in the number of immature erythroid cells in the bone marrow (Fig 1C and D). This situation was reversed in the spleen (data not shown). Significant increases in the frequency of CFU-S in peripheral blood mononuclear cells and in the spleen were associated with SCF treatment (Table 1).

Effects of SCF treatment on PHSC frequency. The increase in the frequency of CFU-S in the peripheral blood and spleen suggested that SCF treatment might be associated with a concurrent increase in stem cell frequency. Stem cell frequency was measured with a competitive repopulation assay.72 In this assay, equal numbers of hematopoietic cells from congenic strains of mice that have different alleles at the β-globin locus are combined and injected into irradiated or W/W recipients (Fig 2). After 120 days, the amount of hemoglobin derived from each donor is com-
pared to estimate the relative frequency of stem cells. This ratio can be confirmed in nucleated lymphoid and myeloid cells by Southern blot analysis, using the EcoRI polymorphism between the two β-globin alleles. Harrison et al have shown that the competitive repopulation assay demonstrates a direct correlation between the ratio of single and diffuse hemoglobin in recipient mice and the ratio of bone marrow cells injected throughout a range of 1:10 to 10:1. Recent work has shown that, when 5 × 10^6 or more cells are injected, the precision of the assay is increased to the limit of experimental error (±3%). In all of the experiments described below, hemoglobin content was analyzed every 2 weeks. The hemoglobin content completed a linear progression to its final value at 50 days after transplantation, and remained stable to the termination of the experiment at 120 days after transplantation.

The relative frequency of PHSC in bone marrow cells from normal and carrier-treated mice was identical (Fig 3), but the relative PHSC frequency in PEG-SCF–treated bone marrow cells was approximately one-third that of untreated bone marrow (P < .001; Fig 3 and Table 2).

The relative frequency of PHSC in peripheral blood mononuclear cells from untreated mice was approximately one-tenth that of normal bone marrow (P < .001; Fig 4), whereas the relative PHSC frequency in peripheral blood mononuclear cells from PEG-SCF–treated mice was greater than that of untreated bone marrow (Fig 4). The relative frequency of PHSC in peripheral blood mononuclear cells from PEG-SCF–treated mice was more than 10-fold greater than that of untreated peripheral blood mononuclear cells (P < .001; Fig 4 and Table 2).

The relative frequency of PHSC in spleen cells from untreated mice was approximately one-fifth that of normal bone marrow (P < .001; Fig 5), whereas the relative frequency of PHSC in spleen cells from PEG-SCF–treated mice was greater than that of untreated bone marrow (Fig 5). The relative frequency of PHSC in spleen cells from PEG-SCF–treated mice was more than fivefold greater than that of untreated spleen cells (P < .001; Fig 5 and Table 2).

*Analysis of SCF mobilized peripheral blood and spleen PHSC.* Complete hematopoietic repopulation of both lymphoid and myeloid lineages was shown by Southern
of at least 25 mice. The donor BM from donor mice injected with carrier alone for 7 days; SCF BM, bone marrow from donor mice injected with SCF-PEG (100 μg/kg/d) for 7 days; Untreated BM, bone marrow from untreated donor mice injected with carrier alone for 7 days; SCF BM, bone marrow from donor mice injected with SCF-PEG (100 μg/kg/d) for 7 days; Untreated BM, bone marrow from untreated donor mice.

Fig 3. Competitive repopulating ability of bone marrow from normal and SCF-PEG-treated mice. The bars represent the mean percentage (± standard deviation) of hemoglobin derived from the indicated donor cells 120 days after transplantation. Each pair of bar graphs represents cumulative data representing three independent experiments including a total of at least 25 mice. The donor cells are indicated below the bar graphs. Carrier BM, bone marrow from donor mice injected with carrier alone for 7 days; SCF BM, bone marrow from donor mice injected with PEG-SCF (100 μg/kg/d) for 7 days; Untreated BM, bone marrow from untreated donor mice.

analysis. DNA was isolated from the bone marrow and thymus of animals repopulated with peripheral blood mononuclear cells or spleen cells from SCF-treated C57BL/6 mice and digested with EcoRI. EcoRI generates a 10-kb band in C57BL/6 DNA (both β-globin genes are on similar sized fragments), or a 10-kb and a 7-kb band (1 gene each) in DNA from the heterozygous WBB6F1 recipients. Bone marrow and thymus DNA from all 10 animals examined (6 repopulated with spleen cells and 4 repopulated with peripheral blood mononuclear cells) had almost exclusively the 10-kb band of the donor mouse, indicating multilineage repopulation (Fig 6). A similar analysis conducted on DNA isolated from the bone marrow and thymus of competitive repopulation assay recipients showed concordance between the relative amounts of single and diffuse hemoglobin in peripheral blood red cells and the relative amounts of the 10-kb and 7-kb bands (data not shown).

One hypothesis for the increase in repopulating activity in response to SCF treatment is that, rather than increasing the absolute number of PHSC, PEG-SCF treatment "activates" or recruits a small number of PHSC to contribute more progeny to a transplant recipient. A second hypothesis is that PHSC from the peripheral blood or spleen of PEG-SCF-treated mice preferentially home to one organ (eg, the spleen), rather than the entire hematopoietic microenvironment. To address these points, we performed secondary transplant experiments. If a small number of PHSC are preferentially activated in the original donor, secondary recipients should have a different hemoglobin content from the primary recipient because of either dilution of the activated PHSC or reactivation of quiescent PHSC. Likewise, if the bone marrow is not seeded with PHSC from PEG-SCF-treated mice, secondary recipients should have a different hemoglobin content from the primary recipient. Competitive repopulation assay recipients of a mixture of either peripheral blood cells from SCF-treated mice and bone marrow cells from untreated mice or spleen cells from SCF-treated mice and bone marrow cells from untreated mice were selected. Bone marrow cells from each of these animals were injected into 5 W/W' recipients. After 120 days post-transplantation, the relative amount of single and diffuse hemoglobin in these recipients was nearly identical to that of the original donor (Table 3).

Phenotype of peripheral blood PHSC. Bone marrow PHSC are negative for the expression of surface markers for B cells, T cells, granulocytes, monocytes, and erythroid cells (Lin'), as well as strongly positive for c-kit.5,3,3 Using the same criteria, Lin', c-kithigh cells were isolated from peripheral blood mononuclear cells from PEG-SCF-treated C57BL/6 mice. Either 100 or 1,500 of these cells were injected into W/W' mice. All mice receiving 1,500 Lin', c-
kit$^{bright}$ cells were repopulated, as evidenced by conversion to 100% single hemoglobin (Table 4). In addition, one of five mice receiving 100 cells also converted to single hemoglobin, indicating a stem cell frequency of approximately 1/500 cells. In contrast, no repopulation was observed in six recipients of up to $2 \times 10^5$ Lin$^-$c-kit$^{dull}$ or $^-$ cells. These data indicate that the PHSC in the peripheral blood and spleen of PEG-SCF-treated mice have the same phenotype as those from bone marrow.

SCF treatment increases the absolute number of CFU-S and PHSC per mouse. The absolute number of CFU-S was calculated by multiplying the frequency of CFU-S observed by the mean cellularity per mouse of a particular hematopoietic organ. For the number of bone marrow cells per mouse the estimate of $2.4 \times 10^8$ cells made by Chervenick et al. was chosen. The average number of peripheral blood mononuclear cells in both normal and PEG-SCF-treated mice was calculated from the differentials and found...
to be approximately $7 \times 10^6$, and the average cellularity of the spleen of normal and PEG-SCF-treated mice is shown in Table 1. Untreated mice had an average of 20,952 CFU-S in the bone marrow and 618 CFU-S in the spleen (Table 5). PEG-SCF–treated mice had an average of 20,400 CFU-S in the bone marrow, 17,110 CFU-S in the spleen, and 378 in the peripheral blood (Table 5). The estimated absolute number of CFU-S increased from 21,570 in untreated mice to 37,888 (1.8-fold) in PEG-SCF–treated mice.

The frequency of PHSC was calculated from the ratio of the hemoglobin observed in a competitive repopulation assay. These ratios have been previously shown to accurately reflect the relative contribution of donor bone marrow cells in mixing experiments within the ranges that we observed. For example, spleen cells from PEG-SCF–treated C57BL/6 mice (single hemoglobin) were competed against bone marrow cells from untreated HW80 mice (diffuse hemoglobin). The ratio of single to diffuse hemoglobin was 1.34. When this ratio is multiplied by the estimated frequency of 1 PHSC/10⁵ normal murine bone marrow cells, derived independently by Harrison et al. and by Jordan and Lemischka, it gives a frequency of 1.34 PHSC/10⁵ cells from the spleen of an SCF-treated mouse (Table 2). Multiplying this number by the observed cellularity gives an estimate of the absolute number of PHSC in that organ. Using these calculations, untreated mice had an average of 2,400 PHSC in the bone marrow, 10 PHSC in the peripheral blood, and 550 PHSC in the spleen (Table 2). PEG-SCF–treated mice had an average of 936 PHSC in the bone marrow, 96 PHSC

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**Table 3. Repopulation of Secondary Hosts With Bone Marrow From Competitive Repopulation Assay Recipients**

<table>
<thead>
<tr>
<th>Primary Recipient No.</th>
<th>Secondary Recipient No.</th>
<th>% Hemoglobin Normal Marrow</th>
<th>% Hemoglobin Normal Marrow</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-1</td>
<td>54.5</td>
<td>46.4</td>
</tr>
<tr>
<td></td>
<td>1-2</td>
<td>52.7</td>
<td>47.3</td>
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<td></td>
<td>1-3</td>
<td>53.5</td>
<td>46.5</td>
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<tr>
<td></td>
<td>1-4</td>
<td>52.3</td>
<td>47.7</td>
</tr>
<tr>
<td></td>
<td>1-5</td>
<td>53.2</td>
<td>46.8</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>53.2 ± 0.38</td>
<td>46.8 ± 0.38</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2-1</td>
<td>54.8</td>
<td>45.2</td>
</tr>
<tr>
<td></td>
<td>2-2</td>
<td>59.9</td>
<td>41.5</td>
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<td>2-3</td>
<td>58.5</td>
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<tr>
<td></td>
<td>2-5</td>
<td>51.1</td>
<td>48.9</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>58.2 ± 2.62</td>
<td>41.8 ± 2.62</td>
<td></td>
</tr>
</tbody>
</table>

* Peripheral blood cells from SCF-treated donor.
† Spleen cells from SCF-treated donor.

**Table 4. Repopulation of W/W<sup>+</sup> Mice With Purified PHSC From the Peripheral Blood of PEG-SCF–Treated Mice**

<table>
<thead>
<tr>
<th>Type of Cells Injected</th>
<th>No. of Cells Injected</th>
<th>No. of Mice Repopulated (100% Hbb&lt;sub&gt;W/W&lt;sup&gt;+&lt;/sup&gt;&lt;/sub&gt;) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lin&lt;sup&gt;+&lt;/sup&gt;; c-kit&lt;sup&gt;PR&lt;/sup&gt;</td>
<td>1,500</td>
<td>4 (100)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1 (20)</td>
</tr>
<tr>
<td>Lin&lt;sup&gt;+&lt;/sup&gt;; c-kit&lt;sup&gt;Pull&lt;/sup&gt;</td>
<td>1,500</td>
<td>4 (0)</td>
</tr>
<tr>
<td>Lin&lt;sup&gt;+&lt;/sup&gt;; c-kit&lt;sup&gt;R&lt;/sup&gt;</td>
<td>$2 \times 10^6$</td>
<td>2 (0)</td>
</tr>
</tbody>
</table>
in the peripheral blood, and 7,906 PHSC in the spleen (Table 2). The estimated absolute number of PHSC increased from 2,959 in untreated mice to 8,938 (3.0 fold) in PEG-SCF-treated mice.

Retrovirus-mediated gene transfer to spleen and peripheral blood PHSC from PEG-SCF–treated mice. Previous studies have shown that efficient retrovirus-mediated gene transfer to PHSC requires 5-FU treatment of the donor mice before harvest.37,38,39 The 5-FU treatment may reduce the pool of progenitor cells and induce originally quiescent PHSC to enter the cell cycle to replenish the pool.40,41 Gene transfer efficiency is increased because cycling cells are the preferred targets for retroviral integration.42,43 We hypothesized that the expansion of the stem cell pool induced by PEG-SCF treatment would also increase the number of PHSC susceptible to retrovirus integration similar to 5-FU treatment. Peripheral blood mononuclear cells and spleen cells from PEG-SCF–treated mice were collected and put into suspension culture with IL-3, SCF, and IL-6. As a positive control, bone marrow from mice treated with 5-FU 48 hours previously was collected at the same time and placed into identical suspension cultures. After 48 hours of culture, cells were collected and cocultured with a cell line producing a retrovirus containing the MDR gene for an additional 48 hours.32,33 In two separate experiments, a total of 12 (7 in experiment 1, 5 in experiment 2) mice were injected with 5-FU–treated bone marrow cells, and 13 (7 and 6) and 17 (6 and 11) mice were injected with peripheral blood or spleen cells, respectively, from PEG-SCF–treated mice.

Four months after the cells were injected into recipients, peripheral blood DNA was analyzed for the presence of the MDR provirus using the PCR. As expected, 12 of 12 mice injected with 5-FU–treated bone marrow cells contained the MDR provirus (Fig 7). In contrast, only 3 of 13 animals repopulated with peripheral blood mononuclear cells from PEG-SCF–treated mice contained the MDR provirus (Fig 7). Fourteen of 17 mice repopulated with spleen cells from SCF–treated mice contained the MDR provirus (Fig 7).

DISCUSSION

In this report, we show that PEG-SCF treatment of normal mice expands the absolute number of PHSC per mouse, particularly in the peripheral blood and spleen. The effects of PEG-SCF on peripheral blood counts, spleen cellularity, and CFU-S number are identical to the results of Molineux et al.26 Previous work had shown that treatment of normal mice with endotoxin stimulates hematopoiesis and increases the number of peripheral blood progenitor cells.54 Because the PEG-SCF we injected was shown to be nearly endotoxin-free and animals injected with carrier alone were identical to un.injected mice, we feel that the effects we observed are direct effects of the injected PEG-SCF. Molineux et al13 described the mobilization of PHSC into the peripheral blood of G-CSF–treated mice. In these experiments, the PHSC content of the spleen was not investigated, and the magnitude of the increase in the relative frequency of PHSC in the peripheral blood was not calculated. More recent studies have shown that in vivo administration of PEG-CSF to 5/ST38 mice increased the read blood cell count and the cellularity and CFU-S content of the bone marrow and spleen, while decreasing the MCV of the recipients.50

The PHSC in the peripheral blood and spleen of PEG-SCF–treated mice were identical to untreated bone marrow PHSC by many criteria. Functionally, PHSC from PEG-SCF–treated mice repopulated the lymphoid and myeloid compartments of both W/W51 and lethally irradiated recipients. In addition, PHSC from both the peripheral blood and spleen of PEG-SCF–treated mice had the same phenotype with regard to the expression of cell-surface markers as bone marrow PHSC.23,31 We estimate that we have achieved a 200-fold purification of PHSC from the peripheral blood of PEG-SCF–treated mice.

One important difference between normal bone marrow PHSC and the PHSC from the spleens of PEG-SCF–treated mice is in their susceptibility to retroviral transduction. Conventional retrovirus-mediated gene transfer protocols all use 5-FU treatment of donor mice before bone marrow harvest and exposure to the retrovirus-producing cells.37,38,39 5-FU treatment has been shown to increase the number of primitive progenitor cells in the cell cycle, a condition shown to improve retrovirus integration. In support of this hypothesis, studies have shown that untreated bone marrow PHSC are rarely transduced by retroviruses.37,39 Our results show that spleen PHSC from PEG-SCF–treated mice are transduced nearly as efficiently as PHSC from 5-FU–treated mice. We propose that many PHSC in the spleen of PEG-SCF–treated animals are in the cell cycle. An increase in the number of cycling PHSC would lead to both greater numbers of PHSC in the spleen and efficient gene transfer without prior conditioning. Gene transfer efficiency to peripheral blood PHSC was low, similar to that of untreated bone marrow. This observation may be artificially low because fewer cells were injected into the recipient animals; however, it appears that few PHSC in the peripheral blood are in the cell cycle.

The competitive repopulation assay has several advantages over limiting dilution assays for showing changes in the number of PHSC in response to cytokine treatment. The use of genetically distinguishable bone marrow cells clearly shows that the repopulation we observed was attributable to the transplanted peripheral blood or spleen cells.

Table 5. Estimation of the Absolute Number of CFU-S in Normal and SCF-Treated Mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Frequency of CFU-S</th>
<th>Cellularity</th>
<th>Absolute Number of CFU-S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal PB</td>
<td>0.0/10⁶ cells</td>
<td>7 × 10⁴†</td>
<td>0.0</td>
</tr>
<tr>
<td>Normal spleen</td>
<td>0.27/10⁶ cells</td>
<td>2.29 × 10⁴*</td>
<td>618.3</td>
</tr>
<tr>
<td>Normal marrow</td>
<td>8.73/10⁶ cells</td>
<td>2.4 × 10⁵‡</td>
<td>20,952.0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>21,570.3</td>
</tr>
<tr>
<td>SCF PB</td>
<td>5.4/10⁶ cells</td>
<td>7 × 10⁴†</td>
<td>378.0</td>
</tr>
<tr>
<td>SCF spleen</td>
<td>2.9/10⁶ cells</td>
<td>5.9 × 10⁵*</td>
<td>17,110.0</td>
</tr>
<tr>
<td>SCF marrow</td>
<td>8.5/10⁶ cells</td>
<td>2.4 × 10⁵‡</td>
<td>20,400.0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>37,888.0</td>
</tr>
</tbody>
</table>

* From Table 1.
† Calculated from differential count, based on the assumption of a blood volume of 1.8 ml/mouse.
‡ Data of Chervenick et al.24
not to endogenous recovery. The changes in repopulating ability we observed are well within the limits of the assay as defined in the original description, highly significant, and the experiments performed with adequate numbers of cells to ensure reproducibility and precision. The competitive repopulation assay assumes that PHSC from SCF-treated mice and untreated mice contribute equally to the repopulation of the recipient. If a small population of PHSC stimulated by PEG-SCF treatment are more efficient at establishing hematopoiesis in a transplant recipient, the results of the assay would not reflect the relative number of PHSC injected. In addition, the competitive repopulation assay assumes that PHSC from SCF-treated mice and untreated mice seed the hematopoietic microenvironment with equal efficiency. Our secondary transplantation experiments addressed both of these issues. We observed that the relative numbers of cells derived from PEG-SCF-treated PHSC and untreated PHSC were identical in primary and secondary recipients, and the standard deviations were relatively small. We conclude that the increase in repopulating ability was not attributable to “activation” of a small number of PHSC, which would be transferred randomly among the recipients generating large standard deviations. Furthermore, we conclude that PHSC from normal and PEG-SCF-treated animals seeded the bone marrow with equal efficiency.

Our results support the conclusions of others who have examined the effects of peripheral blood cells collected after cytokine and/or cytotoxic drug treatment on the recovery after bone marrow ablation and transplantation. We
hypothesize that PEG-SCF treatment of normal individuals would allow for collection of peripheral blood PHSC for future bone marrow transplantation without diminishing the pool of PHSC in these individuals. In view of the synergistic effects on the hematologic parameters of G-CSF and SCF administered in vivo, it is possible that this combination would lead to an even greater increase in PHSC number.

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REFERENCES

5. Orlic D, Bodine DM: Pluripotent hematopoietic stem cells of low and high density can repopulate W/W + mice. Exp Hematol 20:1291, 1992
17. Copeland NG, Gilbert DJ, Cho BC, Donovan PJ, Jenkins NA, Cosman D, Anderson D, Lyman SD, Williams DE: Mast cell growth factor maps near the Steel locus on mouse chromosome 10 and is deleted in a number of Steel alleles. Cell 63:175, 1990
and characterization of low and high density pluripotent hematopoietic stem cells. Blood (in press)


43. Miller DG, Adam MA, Miller AD: Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. Mol Cell Biol 10:4239, 1990


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DM Bodine, NE Seidel, KM Zsebo and D Orlic