Steel factor influences the distribution and activity of murine hematopoietic stem cells \textit{in vivo}\(^*\)

\textit{(growth factors/bone marrow transplantation/peripheral blood stem cells)}

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\textbf{ABSTRACT} To determine the effects of steel factor (SIF) on the number and distribution of phenotypically defined hematopoietic stem cells \textit{in vivo}, mice were treated with continuous s.c. infusions of SIF for up to 7 days. The bone marrow demonstrated a transient 5-fold increase in the number of c-kit-positive/lineage-negative/low cells with no change in cellularity. The radioprotective capacity of bone marrow cells was significantly reduced, and a 30% decrease in Thy\textsuperscript{b} Lin\textsuperscript{-}/Low Sca-1\textsuperscript{+} stem cells (Sca\textsuperscript{+} cells) was observed. In marked contrast, in the spleen a 2-fold increase in cellularity was accompanied by a 24-fold increase in c-kit-positive/lineage-negative/low cells. SIF-treated spleen cells provided increased radioprotection and a corresponding 4-fold increase in the number of Sca\textsuperscript{+} cells. In the peripheral blood, an increase in both neutrophils and lymphocytes resulted; however, the number of c-kit-positive/lineage-negative/low cells remained <1%. SIF produced a 25-fold increase in radioprotection capacity and a 20-fold increase in the number of Sca\textsuperscript{+} cells in the peripheral blood. The increased radioprotection capacity of both the spleen cells and peripheral blood cells was associated with donor-derived, long-term multilineage reconstitution of recipient mice. The total number of Sca\textsuperscript{+} cells isolated per mouse after SIF treatment was not significantly increased. These results show that exogenous SIF treatment causes a redistribution of Sca\textsuperscript{+} cells and stem cell activity while having little effect on the total number of stem cells in the mouse.

Hematopoietic stem cells (HSCs) maintain the continuous production of all cell lineages in the hematolymphoid system throughout the life time of higher vertebrates. To produce the requisite number of highly specialized cells in response to a variety of physiologic stimuli, these cells must be capable of differentiation into committed progenitor cells while retaining the capacity for self-renewal. Presently, the mechanisms that govern these processes are poorly understood. We have previously described a method for the isolation of phenotypically defined HSCs from murine bone marrow (BM) (1, 2). Subsequent studies have demonstrated that this population of cells, which exhibits low levels of Thy-1 expression, high levels of Sca-1 expression, and the absence or low levels of blood cell lineage marker expression (hereafter referred to as Sca\textsuperscript{+} cells), contains all the HSC activity found in normal BM (3). By using this highly enriched HSC population, it is now possible to study those factors that may regulate either the proliferation or the developmental programs of these primitive cells.

Steel factor (SIF), the c-kit ligand also called stem cell factor and mast cell growth factor, has been implicated in a wide variety of physiologic processes including melanocyte migration, primordial germ-cell development, mast cell function, and hematopoiesis (4–9). A number of recent studies have demonstrated the superadditive effect of SIF both \textit{in vitro} and \textit{in vivo} when administered in combination with other hematopoietic growth factors, such as granulocyte/macrophage colony-stimulating factor, granulocyte colony-stimulating factor, interleukin 3, and erythropoietin (10–15). In addition, antibodies that block the binding of SIF to its receptor have been shown to deplete the number of hematopoietic progenitors in murine BM dramatically (16). Treatment of enriched HSC populations \textit{in vitro} with SIF and interleukin 3 leads to proliferation of these cells and an increase in both colony-forming cells and colony-forming unit-spleen activity (17, 18).

Taken together these results suggest that SIF may act on a very primitive cell population at the earliest stages of hematopoiesis. Expansion and differentiation of this population could conceivably produce a large pool of committed progenitor cells responsive to the action of the lineage-specific growth factors, such as granulocyte colony-stimulating factor and erythropoietin. To test whether SIF can expand the HSC pool \textit{in vivo} we examined the effects of exogenous SIF on both HSC activity and Sca\textsuperscript{+} cell number in normal mice. Our results indicate that SIF produces a marked redistribution of HSCs while having little effect on their total number.

\textbf{MATERIALS AND METHODS}

\textbf{Mice.} The C57BL/Ka-Thy1.1 (Thy1.1, Ly5.2), C57BL/6Ly-5.1-Pep\textsuperscript{b} (Thy1.2, Ly5.1), and C57BL/J mice were used as either donors or recipients. Animals were maintained on acidified water (pH 2.5) in the mouse facility at Stanford University.

\textbf{SIF Administration.} Mice 5–7 weeks of age were anesthetized with methoxyflurane and implanted with 7-day osmotic minipumps (Alza) containing either recombinant rat SIF or empty pumps. SIF was conjugated to polyethylene glycol(PEG-SIF) (200 μg/kg per day) (Amgen Biologicals) diluted in phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (BSA) or PBS/BSA alone. After either 3 or 7 days, peripheral blood (PB), long bones (two femurs and two tibias per mouse), and spleen were harvested.

\textbf{Cell Preparations.} BM cell preparations were obtained by flushing the tibias and femurs of 5- to 7-week-old animals with Hanks' balanced salt solution/10 mM HEPES/3% fetal calf serum. Spleen cell suspensions free of connective tissue were obtained by passing spleen cells through a wire mesh. PB was obtained by retroorbital puncture with heparin at 30 units/ml. Nucleated cells were prepared by sedimenting erythrocytes in 2% dextran (T-500) followed by hypotonic lysis. Sca\textsuperscript{+} cells were obtained by using a series of enrichment steps as outlined (1).

\textbf{Abbreviations:} HSC, hematopoietic stem cell; Sca\textsuperscript{+} cells, Thy\textsuperscript{b} lineage \textsuperscript{-}/Low Sca-1\textsuperscript{+} cells; SIF, steel factor; PB, peripheral blood; BM, bone marrow; BSA, bovine serum albumin.

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5-fold increase in c-kit+/Lin−/b220 cell number was seen after 3 days of SIF treatment, which then returned to baseline by day 7 (Fig. 1A). In contrast, in the spleen c-kit+/Lin−/b220 cells represented <1% of total spleen cells in vehicle-treated controls but demonstrated a sustained increase reaching 12% of total spleen cells after 7 days of SIF treatment (Fig. 1B). This increase in Lin−/b220 cells was accompanied by a 2-fold increase in spleen cellularity resulting in a net 24-fold increase in these cells (Table 1).

After 7 days of SIF treatment, a 4-fold increase in total PB leukocytes was observed. This leukocytosis was primarily due to a 24-fold increase in neutrophils that was accompanied by a 1.7-fold increase in circulating lymphoid cells (Table 1). These changes in the PB are similar to those reported recently by Molineux et al. (19).

**SIF Enhances the Radioprotective Capacity of PB and Spleen**

To test the effect of SIF on cells that confer protection against lethal irradiation, various doses of unfractionated cells from the BM, spleen, and PB of SIF-treated mice were tested for their ability to rescue irradiated mice. Fig. 2 shows that 2 × 10⁴ vehicle-treated BM cells protected 43% of recipient mice, whereas only 3% of mice survived after receiving the same number of cells from SIF-treated donors. In contrast, 4 × 10⁴ spleen cells from vehicle-treated controls failed to provide detectable radioprotection, whereas 2 × 10⁴ SIF-treated donor spleen cells rescued 22% of recipients, and 4 × 10⁴ SIF-treated donor spleen cells rescued 58% of recipients (Fig. 3). Increased radioprotective capacity of PB cells from SIF-treated donors was also observed. Although 250 µl of unfractionated PB from vehicle-treated donors was required to rescue 21% of recipients, only 10 µl of PB from SIF-treated mice provided radioprotection to 30% of recipients (Fig. 4).

**Radioprotection Is Associated with Donor-Derived Reconstitution**

To determine whether the increased radioprotection observed with SIF treatment in both spleen and PB cells was due to transient engraftment of donor cells that permitted the subsequent recovery of radio-resistant host stem cells, or due to sustained donor-derived multilineage hematopoiesis, we used donor and recipient mice that were congenic at the Ly5 locus (1). The results of this multilineage long-term reconstitution assay in representative individual mice is shown in Fig. 5. Most nucleated cells in the PB of animals rescued with 4 × 10⁴ SIF-treated donor spleen cells were of donor origin 10 mo after lethal irradiation. Specifically, 99% of B cells (anti-B220), 89% of myelomonocytic cells (anti-Gr-1 and anti-Gr-1), and 73% of T cells (anti-CD3) were donor-derived (Fig. 5A). In mice rescued with 30 µl of PB cells from SIF-treated donors, the majority of circulating nucleated cells were found to be of donor origin, as were 96% of B cells, 97% of myelomonocytic cells, and 84% of T cells (Fig. 5B).

**SIF Increases the Number of Phenotypically Defined HSCs in the PB and Spleen**

To determine whether the increased radioprotection and multilineage reconstitution capacity of PB and spleen cells from SIF-treated mice could result from an increase in phenotypically defined HSC populations, we isolated Sca+ stem cells from the BM, spleen, and PB of mice treated with SIF. In the BM we observed a decrease in the number of Sca+ cells from 27,000 to 19,000 after 7 days of SIF treatment.
treatment (Table 2). In contrast, the number of Sca+ cells in the spleen increased nearly 4-fold with SIF treatment.

As a variety of hematopoietic growth factors are believed to mobilize early hematopoietic progenitor cells into the PB, we examined the effect of SIF on the number of Sca+ cells in this compartment. The PB demonstrated an increase in Sca+ cell number from 250 to 5000 per mouse. Although SIF treatment increased the number of Sca+ cells in both the spleen and PB, these changes were offset by a decrease in BM-derived Sca+ cells. The total number of Sca+ cells isolated per mouse did not significantly increase (Table 2).

DISCUSSION

The c-kit receptor is expressed on a variety of enriched HSC populations (16-18) including Sca+ cells (20). SI/SI mice homozygous for a deletion of the SIF gene die in utero in the setting of profound anemia, suggesting that this growth factor may have an important role in regulating the developmental potential of HSCs. However, the fetal liver of SI/SI mice contains Sca+ HSCs at ~40% of the level found in +/+ or +/+ SI littermates, indicating that SIF may not be necessary for the initial generation and early expansion of HSCs (19). SIF treatment of Sca+ cells in vitro had little effect other than as a survival factor, but SIF in combination with interleukin 3 or interleukin 6 led to a massive proliferation of cells involving ~40%-50% of input Sca+ cells, resulting in large numbers of myeloid progenitors (J. Friedman, S. Heimfeld, and I.L.W., unpublished work). It was therefore of interest to test whether SIF treatment in vivo could engage Sca+ cells in such activation pathways, and if so, how it would influence both the size and distribution of the stem cell pool. Here we demonstrate that the treatment of mice with exogenous SIF

FIG. 2. Radioprotective capacity of BM after SIF treatment. Groups of three mice were treated with either SIF at 200 μg/kg per day or PBS/BSA vehicle s.c. for 7 days. BM was then harvested and pooled; 2 × 10^6 cells were injected i.v. into lethally irradiated syngeneic recipients. Survival was monitored daily for 30 days. Percent survival over time is indicated: ---, 2 × 10^6 vehicle-treated BM cells (n = 33 mice); ○, 2 × 10^6 SIF-treated BM cells (n = 36 mice).

FIG. 3. Radioprotective capacity of spleen cells after SIF treatment. Groups of three mice were treated with either SIF at 200 μg/kg per day or PBS/BSA vehicle s.c. for 7 days. Spleen cells were then harvested, pooled, and injected i.v. into lethally irradiated syngeneic recipients. Survival was monitored daily for 30 days. Percent survival over time is indicated: ---, 4 × 10^6 vehicle-treated spleen cells (n = 20 mice); ○, 2 × 10^6 SIF-treated spleen cells (n = 33 mice); ●, 4 × 10^6 SIF-treated spleen cells (n = 33 mice).

FIG. 4. Radioprotective capacity of PB cells after SIF treatment. Groups of three mice were treated with either SIF at 200 μg/kg per day or PBS/BSA vehicle s.c. for 7 days. PB cells were then harvested, pooled, and injected i.v. into lethally irradiated syngeneic recipients. Survival was monitored daily for 30 days. Percent survival over time is indicated: ---, 2 × 10^6 vehicle-treated PB cells (n = 18 mice); ○, 250 μl of vehicle-treated PB cells (n = 18 mice); ●, 10 μl of SIF-treated PB cells (n = 18 mice).

FIG. 5. Donor-derived, long-term multilineage reconstitution. Ly5.2 congenic mice were used as donors with Ly5.1 congenic recipient mice. (A) PB leukocytes of lethally irradiated mice reconstituted 10 mo previously with 4 × 10^6 spleen cells from SIF-treated donors were analyzed by fluorescence-activated flow cytometry for the expression of donor-derived (Ly5.2) B cells (B220), myelomonocytic cells (MAC-1/GR-1), and T cells (CD3). (B) PB leukocytes from recipients reconstituted with 30 μl of unfractionated blood from SIF-treated donor mice 3 mo previously were analyzed, as described in A. Percentages indicated refer to % donor-Ly5.2 (upper right quadrant) and % host-derived (lower right quadrant) cells that also express the indicated lineage marker.
causes a marked redistribution of HSCs while having little effect on total stem cell number.

In the BM, both the radioprotection capacity and Sca+ cell number decrease after 7 days of SIF treatment. BM cells from vehicle-treated mice provided radioprotection to 42% of recipients, a result similar to that observed for BM from untreated donors. In contrast, BM from SIF-treated mice protected only 3% of recipients. A concomitant 30% decrease in Sca+ cells was seen in the SIF-treated mice. The loss of radioprotective capacity of the BM associated with SIF treatment is greater than would be anticipated from a 30% decrease in the number of Sca+ cells that should result in only a 15–20% decrease in radioprotection (1). Although the reasons for this difference remain unclear, possible mechanisms include a defect in the ability of Sca+ cells from SIF-treated animals to “home” to the BM of irradiated recipient mice, or possibly SIF treatment may produce changes in the developmental potential of these cells. Alternatively, a subpopulation of HSCs that primarily provides radioprotection may be inhibited by exogenous SIF.

In the mouse, the spleen remains a site of active hematopoiesis during adult life, contributing continually to the formed elements in the PB (21). Histologic evaluation indicates that after SIF treatment both erythropoiesis and megakaryopoiesis are increased (data not shown), a finding reported in other species (14). SIF treatment also results in the accumulation of a significant number of c-kit+/Lin−/Sca+ cells in the spleen accompanied by a 2-fold increase in spleen cell number. The appearance of spleen cells expressing c-kit is accompanied by a dramatic increase in the radioprotection capacity of spleen cells on a per cell basis. As few as 4 × 10^4 spleen cells from SIF-treated animals will rescue 58% of irradiated recipients, whereas 4 × 10^6 spleen cells from vehicle-treated donors fail to provide any degree of radioprotection. A corresponding 4-fold increase in the number of Sca+ cells is seen after SIF treatment. Sustained donor-derived, long-term multilineage reconstitution confirms the presence of stem cell activity in this population.

The presence of functional HSCs in the PB of mice, as defined by the capacity for radioprotection and long-term myeloid and erythroid reconstitution, was first reported >30 yr ago (22). A number of studies have since demonstrated that the colony-forming unit-spleen activity of PB in mice is increased in the setting of severe anemia (23), in response to bacterial endotoxin (24), and after treatment with SIF (19). The administration of granulocyte-colony-stimulating factor results in a dramatic increase in the radioprotection capacity of PB (25). Our results show that only 10 μl of PB from SIF-treated mice will rescue 30% of irradiated recipients, whereas 250 μl of PB from control mice is required to provide a similar degree of radioprotection. The results of these radioprotection studies correlate well with the 20-fold increase in the number of Sca+ cells seen in the PB of SIF-treated mice. The true stem cell activity of these PB cells is confirmed by their ability to provide long-term, donor-derived multilineage reconstitution. These findings are consistent with those of Andrews et al. (26), who have demonstrated that SIF treatment increases the number of CD34+ cells in the PB of baboons. This group has also recently demonstrated that PB mononuclear cells from SIF-treated donors have an increased capacity for both early engraftment and for the protection of lethally irradiated recipients (27). These studies, however, did not examine whether these SIF-mobilized PB cells have the capacity to provide long-term donor-derived hematopoiesis.

SIF treatment of mice reduces both the HSC activity and the number of Sca+ cells in the BM, which offsets increases in the number and activity of these cells in the PB and spleen. Consequently, no significant increase in the total number of Sca+ cells isolated per mouse was observed. These results indicate that the treatment of mice with exogenous SIF does not lead to significant expansion of Sca+ cells. These studies provide further evidence that factors other than SIF are likely to be required for both the expansion and self-renewal of HSCs in vivo.

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Table 2. Effect of SIF treatment on content of Sca+ cells

<table>
<thead>
<tr>
<th>SIF</th>
<th>BM</th>
<th>Spleen</th>
<th>PB</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>26,906 ± 5,406</td>
<td>3,272 ± 775</td>
<td>254 ± 65</td>
<td>30,432 ± 6,246</td>
</tr>
<tr>
<td>SIF</td>
<td>19,047 ± 4,495</td>
<td>11,734 ± 1,729</td>
<td>5,074 ± 1,189</td>
<td>35,855 ± 7,413</td>
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</table>

Mice were treated with SIF at 200 μg/kg per day or with PBS/BSA (control) by continuous infusion for 7 days. Sca+ cells were isolated from the tissues listed above. The mean ± SEM of the number of Sca+ cells per mouse is given (n = nine mice per group). The number of BM-derived Sca+ cells per mouse is calculated based on four long bones representing 15% of total BM. The number of Sca+ cells in the PB is based on a blood volume of 1.5 ml per mouse.


