A c-kit Ligand, Recombinant Human Stem Cell Factor, Mediates Reversible Expansion of Multiple CD34+ Colony-Forming Cell Types in Blood and Marrow of Baboons


The ligand for the human c-kit, recombinant human stem cell factor (SCF), was administered to baboons at doses of 200, 100, 50, 25, and 10 μg/kg/d. SCF induced a dose-dependent expansion of hematopoietic colony-forming cells (CFU) of multiple types in both blood and marrow, including colony-forming unit (CFU) granulocyte-monocyte, burst-forming unit-erythroid, CFU-MIX, and high proliferative potential-CFC. These changes were associated with a dose-dependent leukocytosis, involving all leukocyte lineages, a reticulocytosis, and increases in marrow cellularity. At 200 μg/kg/d of SCF, CFC in blood were increased 10-fold to greater than 100-fold. This correlated with an increased frequency of CD34+ cells in blood. The frequency of CFC in blood approached that of marrow in some animals. These changes were reversed within 7 to 14 days of stopping SCF. The results of these studies suggest a role for the c-kit ligand in stimulating the expansion of multiple CFC types in blood and marrow for potential therapeutic purposes.

MATERIALS AND METHODS

Animals. Healthy juvenile baboons (Papio cynocephalus or P. anubis) were housed at the University of Washington Regional Primate Research Center, under American Association for Accreditation of Laboratory Animal Care approved conditions. Studies were conducted under Institutional Review Board and Animal Care and Use Committee approved protocols. All animals were provided with water, biscuits, and fruit ad libitum throughout the study. Baboons administered intravenous (IV) SCF had indwelling central venous catheters inserted via the femoral vein for use with a tether system previously described, and were administered prophylactic antibiotics to prevent catheter-related infections. All study blood samples were drawn through the line. All procedures, including bone marrow biopsies, bone marrow aspirates, and blood draws for baboons administered subcutaneous injections (SQ) were performed after animals had been anesthetized with a combination of ketamine-HCl (Aveco, Fort Dodge, IA) and Xylazine (Haver, Shawnee, KS). Marrow samples were obtained from sites on the iliac crest, distal femur, proximal humerus, and proximal tibia. Biopsies were obtained using an 11-gauge Jamshidi biopsy needle and aspirates with a 13-gauge marrow aspiration needle. Marrow aspirates and peripheral blood samples for studies of CFC and expression of CD34 antigen were collected in 3-mL heparinized syringes. Marrow biopsies were fixed in 10% formalin and processed in paraffin and sectioned for histology. Marrow biopsies and peripheral blood samples for studies of CFC were stained with Wright-Giemsa and counted by a hematopathologist without prior knowledge of the treatment the animals had received. Sections from normal cellular baboon marrows had a cell to fat ratio of 1:1.

Growth factors for in vivo administration. A total of 15 animals were studied before and during SCF treatment. Ten were administered SCF at 200 μg/kg/d, three by continuous IV infusion, four by twice daily, and three by single daily SQ injections. Two were administered SCF at 100 μg/kg/d, one by twice daily and the other by single daily SQ injection. Single animals were administered SCF at 50, 25, and 10 μg/kg/d as twice daily SQ injections. None of the animals had evidence for adverse reactions, skin rashes, or skin changes at the injection sites.

Recombinant human SCF as either the 164 (SCF164) or the 165 (SCF165) amino acid form was produced in Escherichia coli as pre-

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viously described and was used as either the unmodified protein or modified, polyethylene glycol-conjugated protein (PEG-SCF).

Purified material used for IV infusions was stored at −70°C and then thawed and diluted in 50 mL normal saline (Abbott Laboratories, Chicago, IL) containing 0.8% (vol/vol) sterile (0.2 km filtered) autologous baboon plasma immediately before starting the infusions. SCF used for SQ injection was stored at 4°C and the volumes per injection were ≤1.0 mL at a single site. The doses of both unmodified SCF and PEG-SCF were based on the molecular weight of the unmodified protein, resulting in equal molar quantities per kilogram of body weight being administered to all animals.

Monoclonal antibodies (MoAbs) and immunofluorescent staining. MoAb 12-8 (CD34) and the isotype-matched control antibody H12C12 (antimouse Thy 1.2) were used to label peripheral blood cells, and were stained with phycoerythrin (PE)-conjugated goat antimouse IgM (chain specific) antisera (Calbiochem, La Jolla, CA, or Biomed, Foster City, CA), as previously described.

CFU assays. Baboon marrow and peripheral blood buffy coat cells were isolated, residual red blood cells were lysed with ammonium chloride buffer, and the cells were cultured in 35-mm culture dishes (Nunc, Naperville, IL) at 5.0 × 10⁴/mL for marrow cells and 1 × 10⁷/mL for peripheral blood cells. All cells were cultured in two-layer agar cultures. The lower layer (1.0 mL) consisted of α-medium in 0.5% (wt/vol) agar (FMC, Rockland, ME, or Difco, Detroit, MI, for high proliferative potential-CFC [HPP-CFC] assays) supplemented with 25% fetal bovine serum (FBS; Hyclone, Logan, UT), 2% (wt/vol) bovine serum albumin (BSA; Fraction V; Sigma, St Louis, MO), gentamicin sulfate 100 μg/mL, and recombinant human growth factors IL-3 (100 ng/mL), granulocyte-macrophage-CSF (GM-CSF) (100 ng/mL), IL-6 (100 ng/mL), erythropoietin (Epo) (4 U/mL), and SCF (100 ng/mL) (purified material supplied by Amgen, Thousand Oaks, CA), or for HPP-CFC cultures, GM-CSF (30 ng/mL), IL-1 (20 ng/mL), IL-3 (20 ng/mL), and SCF (100 ng/mL). After the lower layer had gelled, cells in 0.5 mL α-medium, 25% FBS, 2% BSA, gentamicin, and 0.3% (wt/vol) agar were layered on top. Cultures were incubated at 37°C in a 5% O₂, 5% CO₂ humidified incubator. At day 14 of culture, CFU-GM, BFU-E, and CFU-MIX were enumerated using an inverted microscope. Macroscopic BFU-E were defined as those greater than 0.5 mm in diameter. HPP-CFC, defined after the criteria of Bradley et al., and similar to those described by McNiece et al using human marrow, were enumerated after 21 days of culture and defined as unicentric colonies of ≥1.0 mm in diameter that had a dense center, while colonies lacking the dense center were defined as ≥1.0 mm colonies.

All cultures were performed in triplicate, unless otherwise indicated. Data are reported as the mean ± 1 standard deviation per 5 × 10⁶ marrow cells, or per 1 × 10⁹ peripheral blood buffy coat cells, and as colonies per milliliter of blood ([colonies per 10⁵ cells] × (total white blood cells [WBC]/mL blood))/10³.

RESULTS

Effect of SCF on peripheral blood counts. SCF was administered at doses of 200, 100, 50, 25, and 10 μg/kg/d (see Materials and Methods). Baboons administered 200 μg/kg/d of SCF displayed an increase in their peripheral blood WBC counts by day 7 to 17 of SCF administration, and achieved maximal increases between days 17 and 21 (Table 1). Afterwards, there was a slight decrease in the number of WBC, but they remained elevated above pretreatment levels. After the SCF administration was discontinued, the WBC counts returned to pretreatment values within 7 to 10 days, as previously observed. Animals administered 100 and 50 μg/kg/d of SCF also had increased WBC counts, but to a lesser extent than animals administered 200 μg/kg/d (Table 1). The two animals administered 25 and 10 μg/kg/d of SCF showed no changes in peripheral blood WBC counts throughout or after the period of SCF administration. Reticulocyte counts increased within 3 to 7 days of starting SCF, as previously observed, but the magnitude of the increase was less in the animals administered 10 and 25 μg/kg when compared with those administered 50, 100, and 200 μg/kg.

Effect of SCF on CFU in peripheral blood. We next asked if SCF influenced the number of CFU-GM, BFU-E, and CFU-MIX.
MIX, and HPP-CFC detectable in the circulation of baboons.

Before the administration of SCF, 5 ± 5 CFU-GM per 10^5 peripheral blood buffy coat cells (mean ± SD; median = 4; N = 15) were detectable in the blood. When animals were administered 200 µg/kg/d of SCF, striking increases in peripheral blood CFU-GM were manifested by day 7 (Table 2), representing 10-fold to greater than 50-fold increases in peripheral blood CFU-GM of circulating animals were administered 200 µg/kg/d of 10^5 peripheral blood buffy coat cells (mean values are different from those at day 0 as determined by two-sided Student’s t-test. At day 0 there were 5

CFU-GM/mL of blood

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Data are mean ± SD of triplicate cultures of 10^5 peripheral blood buffy coat cells (see Materials and Methods). The values per milliliter of blood are estimated by the formula (number of CFC/10^5 PBL buffy coat cells) x (total number of WBC per milliliter of blood x 10^-5). For each animal the P values are different from those at day 0 as determined by two-sided Student’s t-test. At day 0 there were 5 ± 5 CFU-GM and 0.2 ± 0.6 BFU-E per 10^5 cells (mean ± SD for N = 15 animals), and in the two control animals studied twice weekly for 3 weeks the number of CFU-GM and BFU-E per 10^5 cells in peripheral blood at any given time point was ± 2 and 0.1 ± 0.3, respectively. Three additional control animals tested at a single time point had 6 ± 2, 0, and 3 ± 1 CFU-GM and 0, 0, and 0 BFU-E per 10^5 peripheral blood buffy coat cells. Seven to 11 days after SCF was stopped, the number of CFU-GM per 10^5 blood cells was 1 ± 1 for T90209, 7 ± 2 for F88230, and 6 ± 1 for F88148, and the WBC counts had returned to pretreatment values.

Abbreviation: ND, not done.

*P < .01.
†P < .05.
‡P < .025.
one time point in one of two untreated animals studied twice weekly for a period of 3 weeks (Table 2). None of these BFU-E were macroscopic (≥ 0.5 mm in diameter) in size (see Materials and Methods). All animals administered 200 μg/kg/d of SCF had evidence for significant increases in BFU-E in their circulation. Significantly, 10% to 50% of these circulating BFU-E, as well as a proportion of the CFU-GM, were macroscopic (≥ 0.5 mm in diameter) in size, and the frequency of these colonies was dose-dependent (Fig 1). These changes in circulating BFU-E also were dose-related, although there was overlap in the values for animals administered 100 and 50 μg/kg. The animals administered 25 and 10 μg/kg had values for circulating BFU-E that were not statistically different from untreated control animals.

The kinetics of the increase in numbers of CFC in blood was examined in two animals (Fig 2). Administration of 200 μg/kg/d of SCF induced a significant change in CFC of all types after day 4. These changes occurred before the significant increase in the total WBC counts was observed. Furthermore, whereas mixed lineage colonies (CFU-MIX) were not detected in cultures of blood samples from baboons before treatment with SCF, they were consistently detected in the blood samples as early as day 5 and throughout the period of SCF administration.

We also assayed a colony type identified as HPP-CFC in the blood of six animals treated with SCF (five were administered 200 μg/kg and one was administered 100 μg/kg of SCF). The HPP-CFC were defined as macroscopic colonies (≥ 1.0 mm in diameter) that were present after 3 weeks when cells were cultured in the presence of SCF, IL-3, IL-1, and GM-CSF. Individual HPP-CFC contain up to 9 × 10^5 cells, consisting primarily of granulocytic, monocytic, and mast cell lineages, and a small portion of these colonies also contained cells of the erythrocytic lineage if Epo also was present in the cultures (Bartelmez SH, Andrews RG, Bernstein ID, unpublished observations). HPP-CFC were assayed immediately before starting SCF treatment in all six animals. For two animals, we also assayed the blood twice weekly for a period of 3 weeks before starting SCF. In these untreated animals, before SCF treatment, HPP-CFC were rarely detected in the circulation (mean ± SEM = 0.02 ± 0.02 HPP-CFC/10^5 cells). After starting treatment with SCF, the number of detectable HPP-CFC in blood began to increase within 3 to 4 days and increased to 5.8 ± 0.8 and 5.2 ± 0.7 (mean ± SEM) HPP-CFC/10^5 cells by days 6 and 11 of SCF treatment, respectively. The incidence of HPP-CFC in the circulation remained elevated throughout the period of SCF administration and then returned to virtually undetectable levels within 7 to 14 days after stopping SCF.

Effects of SCF on CD34+ cells in peripheral blood. CFC in both marrow and peripheral blood express the CD34 antigen. Therefore, we analyzed the frequency of CD34+ cells in the circulation of SCF-treated baboons. Before treatment with SCF, 0.1% ± 0.1% (N = 10) of the cells in
blood with light scatter properties of blast-like cells expressed high levels of CD34 antigen. After 7 days of SCF administered at 200 μg/kg/d, the frequency of CD34+ cells in the blast-sized population had increased to 3.1% ± 0.7% (mean ± SEM, N = 6), whereas for animals administered 100 μg/kg, it was 1.4% ± 0.2% (N = 2), and for the animals administered 25 and 10 μg/kg, it was 0.6% ± 0.2% (N = 2). The frequency of CD34+ cells in the blast-sized population was correlated with the frequency of CFC per 10^3 peripheral blood Buffy coat cells (Fig 3).

**Effects of SCF on bone marrow cellularity and morphology.** Marrow biopsies on baboons administered 200 μg/kg/d of SCF showed that the marrow became markedly hypercellular (180% ± 20% of normal) by day 7 (Fig 4), and remained hypercellular throughout SCF treatment. After treatment was stopped, the cellularity returned to pretreatment values within 14 days. No discernible changes in marrow cellularity were observed in the animals administered 50, 25, and 10 μg/kg of SCF, and intermediate levels of increased marrow cellularity were observed in animals administered 100 μg/kg of SCF.

The morphology of cells in marrow before and during SCF administration were determined by examination of Wright-Giemsa-stained cytospin preparations of marrow Buffy coat cells (Fig 5). After 7 days of SCF at 200 μg/kg/d, the frequency of myeloblasts and promyelocytes had increased 6.9- ± 2.4-fold (range, 3.2- to 14.0-fold; N = 4) and the frequency of erythroblasts and basophilic normoblasts had increased 3.0- ± 0.5-fold (range, 2.0- to 4.5-fold; N = 4). The morphology and differentials of cells in marrows of these SCF-treated animals returned to normal over the following 7 to 14 days, although the marrows remained hypercellular. These changes were not observed in animals administered SCF at doses of 10 and 25 μg/kg/d.

**Effects of SCF on marrow CFC.** The incidence of CFC in marrow increased slightly in animals administered 200 μg/kg/d of SCF (Table 3). The frequency of CFC in marrows of animals treated with lower doses of SCF also increased slightly, although these changes were less often significantly different from the pretreatment values. Given the hypercellular state of marrows of animals administered 200 μg/kg/d, this indicates that the absolute number of CFC in marrow was at least doubled.
C-KIT LIGAND EXPANDS CFC IN BLOOD AND MARROW

The ligand for c-kit is also a potent stimulator for mast cell proliferation in vitro. Mast cells were increased in number in the skin, marrow, and other tissues of these treated animals. However, we did not observe any adverse reactions to treatment suggesting mast cell activation.

The mechanisms by which SCF and other cytokines induce the circulation of hematopoietic progenitor cells and their precursors are presently unknown, nor is it known if the same or different classes of progenitor cells are induced to circulate by different cytokines. Understanding the basic biology of stem cell and progenitor cell trafficking may provide new approaches for clinical transplantation of both autologous and allogeneic hematopoietic stem cells.

ACKNOWLEDGMENT

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DISCUSSION

In the present study, treatment with recombinant human SCF induced increased numbers of hematopoietic CFC of multiple types, including CFU-GM, BFU-E, CFU-MIX, and HPP-CFC, in blood and marrow. In blood, the increases in both the relative number (per 10^5) and absolute number (per milliliter) of CFC were dose-dependent. In some animals, the frequency of CFC in blood approached that of marrow. Even so, these changes occurred before there were significant increases in the peripheral blood WBC counts and were not associated with declining numbers of CFC in marrow, and as such these effects differ from those reported for G-CSF and GM-CSF. The fate of these numerous circulating CFC is not known, and large numbers of cells with intermediate levels of differentiation were not detected in the circulation.

In blood, where CD34+ cells normally are virtually undetectable, treatment with SCF induced increased numbers of CD34+ cells. Initial experiments to assess the function of these CD34+ cells have indicated that unfractonated mononuclear cells isolated from the blood after 10 to 11 days of SCF treatment can engraft lethally irradiated baboons, while the same number of cells isolated from the blood of untreated animals fail to rescue irradiated baboons (Andrews RG, Bensinger WI, Knitter GH, Appelbaum FR, Bernstein ID, and Zsebo KM, submitted for publication). Thus, precursors capable of at least short-term marrow repopulation are increased in the circulation with SCF treatment, but it remains to be determined if cells capable of long-term marrow repopulation have also been increased in number. A small proportion (<10%) of the circulating CD34+ cells had low or undetectable expression of HLA-DR β chain, while the majority of these cells expressed high levels of HLA-DR. A similarly small proportion of these circulating CD34+ cells also expressed T- and B-cell-associated differentiation antigens (Andrews RG, unpublished observations). With the exception of CD34+ HLA-DR+ cells, it was not possible to identify cells with the phenotype of putative primitive precursor cells in human marrow because MoAbs that react with both human and baboon CD33, CD38, and 7B9 antigens were not available.

The results of the present study suggest that new CFC, of multiple types, are being generated in the hematopoietic tissues of SCF-treated animals, presumably from their precursors. This would be consistent with in vitro studies that showed that SCF can stimulate human marrow-derived precursors of CFC to generate CFC progeny. The effect of SCF on inducing increased numbers of CFC in blood also has been observed in mice administered recombinant rat SCF, and would be consistent with altered trafficking of CFC. Both G-CSF and GM-CSF have been reported to influence the circulation of not only CFC but also cells capable of at least short-term marrow repopulation in vivo, and it appears that SCF also may induce similar hematopoietic repopulating cells to circulate.

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Table 3. Effect of SCF on CFC in Bone Marrow

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<tr>
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The ligand for c-kit is also a potent stimulator for mast cell proliferation in vitro. Mast cells were increased in number in the skin, marrow, and other tissues of these treated animals. However, we did not observe any adverse reactions to treatment suggesting mast cell activation.

The mechanisms by which SCF and other cytokines induce the circulation of hematopoietic progenitor cells and their precursors are presently unknown, nor is it known if the same or different classes of progenitor cells are induced to circulate by different cytokines. Understanding the basic biology of stem cell and progenitor cell trafficking may provide new approaches for clinical transplantation of both autologous and allogeneic hematopoietic stem cells.
REFERENCES


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A c-kit ligand, recombinant human stem cell factor, mediates reversible expansion of multiple CD34+ colony-forming cell types in blood and marrow of baboons

RG Andrews, SH Bartelmez, GH Knitter, D Myerson, ID Bernstein, FR Appelbaum and KM Zsebo