The Effects on Hematopoiesis of Recombinant Stem Cell Factor (Ligand for c-kit) Administered In Vivo to Mice Either Alone or in Combination With Granulocyte Colony-Stimulating Factor

By G. Molineux, A. Migdalska, M. Szmitkowski, K. Zsebo, and T.M. Dexter

Stem cell factor (SCF) is the ligand for the receptor encoded by the c-kit proto-oncogene. Mutations of either c-kit or the SCF gene are responsible for the defects of W and SI mutant mice, which both suffer a macrocytic anemia, the former associated with defective stem cells and the latter with a defective hematopoietic microenvironment. Recombinant SCF was administered to normal or splenectomized mice for up to 21 days. SCF was found to be a modest stimulator of peripheral blood neutrophil numbers in both groups of animals. The peak in neutrophil numbers was higher and occurred earlier in splenectomized mice. Bone marrow and spleen cellularity changed little during treatment but the content of interleukin-3-responsive progenitor cells and spleen colony-forming cells (CFU-S) reached very high levels, particularly in the spleen. Using recombinant human granulocyte colony-stimulating factor (rhG-CSF), we have shown that SCF induces a greater than additive increase in both blood neutrophils and blood-borne CFU-S. This synergy was seen throughout the dose range and may indicate a clinical role for SCF either alone or in augmenting the activity of G-CSF upon blood neutrophils and transplantable stem cells.© 1991 by The American Society of Hematology.

STEM CELL FACTOR (SCF) or mast cell growth factor (MGF, Kit ligand [KL]) has been recently molecularly cloned1 and produced in a recombinant form.2,3 It is the ligand for the tyrosine kinase-associated receptor encoded by the c-kit proto-oncogene, and mutations of the gene coding for SCF or c-kit are responsible for the congenital macrocytic anemia seen in Steel and W mice.2,4 In addition to effects on the hematopoietic system, mutations at these loci also influence the migration of primordial germ cells and melanocytes,2,4 indicating that SCF has a multifactorial role to play in development of several cell systems. In vitro SCF has direct hematopoietic colony-stimulating activity,4 and can also synergize with granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), and erythropoietin (Epo) on human target cells5,6 in addition to its demonstrated synergy with IL-1, IL-3, IL-6, and IL-7 on murine bone marrow cells.5,7 These data suggest that SCF, either alone or in combination with other cytokines, can influence growth and development of primitive multipotential hematopoietic stem cells as well as their more developmentally restricted progeny.

Many studies both in the clinic and the laboratory have demonstrated the effectiveness of G-CSF for enhancing the production and activity of mature neutrophils,1,1-13 an effect that is exaggerated in splenectomized mice.14 Because SCF can synergize with G-CSF in vitro10 and as a prelude to developing therapeutic strategies that make use of the biologic activities of SCF, we have now examined the effects of SCF both alone and in combination with G-CSF, in normal and splenectomized mice.

MATERIALS AND METHODS

Mice and Growth Factor Injections

All mice were female B6D2F1 and were aged between 8 and 10 weeks at the beginning of the experiments. They were allowed standard rodent food and water ad libitum throughout the studies. Both PEGylated recombinant rat stem cell factor (SCF) and recombinant human G-CSF (rhG-CSF) were kindly supplied by Amgen (Thousand Oaks, CA). Both materials were highly purified and a single injection contained less than 8 pg of endotoxin. After routine storage at −70°C or 4°C, respectively, SCF or rhG-CSF was diluted in phosphate-buffered saline (Oxoid, Basingstoke, Hants, UK) plus 0.1% bovine serum albumin (PBS/BSA; Sigma, Poole, Dorset, UK) and stored at 4°C until injected. Mice were injected subcutaneously twice daily (9:00 AM and 9:00 PM) with half the daily dose of the growth factors. The injection volume was 200 μL. Where combinations were administered, solutions of each growth factor at twice the required concentration were mixed in equal volumes and again injected in 200 μL.

Blood Counts

Blood was collected from the tail vein of mice and either smeared immediately or diluted without anticoagulation in the diluent appropriate for determination of total nucleated cells (0.3% acetic acid), reticulocytes (1% New Methylene Blue [NMB]), or platelets (1% ammonium oxalate). Nucleated cells and platelets were counted in a hemocytometer (the latter under phase contrast). Reticulocytes were counted on blood smears after 2 hours of incubation in NMB at room temperature, methanol fixation, and tetrachrome staining. Further blood smears were fixed, stained, and counted differentially.

Cell Suspensions

Groups of three mice were killed by cervical dislocation, then the spleen and left femur were removed from each mouse. The epiphyses were removed from femora and bone marrow cells were flushed with a syringe and 21G needle into Iscove’s modified Dulbecco medium (IMDM; GIBCO, Paisley, Scotland). Spleens were disrupted in a teflon/glass tissue grinder in IMDM. These procedures yielded single cell suspensions that were counted in a Neubauer hemocytometer.

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Colony Assays

IL-3-responsive progenitor cells. Bone marrow cells (5 × 10⁶) or 5 × 10⁶ spleen cells were immobilized in 0.33% noble agar (Difco, East Molesey, Surrey, UK) in IMDM supplemented with 20% preselected donor horse serum (MVS, Botolph Claydon, Bucks, UK) and 2% murine IL-3-conditioned medium as a source of murine IL-3. This medium, conditioned by a murine cell line into which the murine IL-3 gene had been inserted, is devoid of detectable levels of other hematopoietic CSFs (Spooncer and Molineux, unpublished data). Triplicate plates were prepared for each assay point. After incubation for 11 days at 37°C in fully humidified nitrogen with 5% oxygen and 5% carbon dioxide, colonies of more than 50 cells were scored under a dissecting microscope.

Spleen colony assay. Mice were irradiated overnight with 60Co γ-rays to a dose of 15 Gy; this reduced endogenous spleen colonies to less than 0.1 colonies per spleen. Within 4 hours of completing the irradiation, 5 × 10⁴ bone marrow or 5 × 10⁶ spleen cells were injected intravenously into 20 mice per assay point. Eight or 11 days later 10 mice were killed, and the spleens removed and fixed in Telley’snicksky’s fixative. Colonies were then scored under a dissecting microscope.

Splenectomy

Under ethrane (Abbot Medical, Queensborough, Kent, UK) anesthetic a small incision was made in the flank and the inner body wall. The spleen was exteriorized and the blood vessels detached by pulling the spleen sharply with forceps to prevent hemorrhage. The wound was closed with stainless steel clips, which were left in place, and the mice left at least 3 months before being included in subsequent protocols.

Statistical Analysis

Student’s t-test was used to assess the significance of the difference between results. Two levels of significance were considered P < .01 and P < .05.

RESULTS

SCF Alone

Normal mice. Throughout the first 5 days of a regimen of continuous treatment of normal mice, SCF failed to elicit any detectable change in the number of peripheral blood leucocytes. Thereafter, however, a transient leukocytosis was induced reaching twofold to threefold control levels at day 7 and decreasing to slightly subnormal levels by day 18 despite continuing treatment (Fig 1A). During this period, the majority of recruited cells were neutrophilic granulocytes (Fig 2), though some increases in lymphoid cells were also seen. Reticulocyte counts were increased slightly in SCF-treated animals (Table 1), though no changes in hematocrit were seen. Platelet numbers were not changed by SCF administration (Table 1). Peripheral blood spleen colony-forming unit (CFU-S) numbers were increased dramatically during SCF therapy. Throughout the treatment period, blood-borne CFU-S numbers were between twofold and 30-fold higher than controls, with an apparent peak in the second week of treatment.

Bone marrow cellularity during treatment was not consistently altered (Fig 3), but a modest increase in spleen cellularity was seen after 10 days of treatment (Fig 4). In the bone marrow, IL-3-responsive progenitor cells were significantly increased on days 14, 15, and 21 of treatment, reaching approximately twofold control values (Fig 5). Between 1.5- and 3.5-fold increases in femoral day 8 CFU-S were also seen at these times, but there was little, if any, effect upon developmentally earlier day 11 CFU-S (Fig 6). In comparison with bone marrow, the twofold increase in spleen cellularity seen after 11 days of treatment (Fig 4) was associated with a 50-fold increase in the number of IL-3-responsive progenitor cells (Fig 5). Like the increase in cellularity, this was a transient response and normal levels of progenitor cells were reestablished. However, both day 8 and day 11 CFU-S in the spleen showed significant increases of between fourfold and sixfold for the duration of 15 days of treatment before returning to normal values by day 21 (Fig 7).

Effects of SCF in splenectomized mice. The leukocytosis induced by SCF was more marked and occurred earlier in splenectomized compared with normal mice and was clearly elevated after only 5 days of treatment (Fig 1B). However, like normal mice, circulating leukocyte count returned to normal after 11 days despite continuing treatment with SCF up to 3 weeks (Fig 1B). Neither bone marrow cellularity, the content of IL-3-responsive progenitor cells, or CFU-S were significantly changed following treatment with SCF (Figs 3, 5, and 6).
SCF + G-CSF IN VIVO

Fig 2. Absolute counts of blood cells during SCF administration (100 μg/kg/d) of neutrophils (upper), lymphocytes (middle) and monocytes (lower). Points that differ significantly are marked: **P < .01, *P < .05.

SCF in combination with rhG-CSF. Continuous treatment with G-CSF alone for 21 days did not induce a leukocytosis when used at a dose of 1,500 U/kg/d. Doses of 15,000 and 150,000 U/kg/d were, however, more effective in this respect (Fig 8), inducing 20 and 100 × 10⁶ cells/mL of blood, respectively. It should be noted that these doses correspond to 0.1, 1, and 25 μg/kg/d of the G-CSF used in our previous study, and that the clinically efficacious dose in terms of recruitment of neutrophils is about 10 to 20 μg/kg/d (120,000 U/kg/d). The main effect was on the production of neutrophils (Fig 8). Treatment with SCF by itself at 100 μg/kg/d also caused a mild leukocytosis (Fig 1). However, combined treatment with SCF plus rhG-CSF continued for 21 days exceeds the additive effect of both growth factors (Fig 9). SCF plus rhG-CSF at concentrations of 1,500, 15,000, and 150,000 U/kg/d yielded a maximum of 80, 125, and 240 × 10⁶ nucleated cells/mL of blood, respectively. In combination with SCF, G-CSF became threefold more effective when administered at a clinically relevant dose, five times more effective at a dose of 15,000 U/kg/d, and allowed a small dose of 1,500 U/kg/d to become effective when this dose administered alone induced little extra neutrophil production. In the mice that received combination therapy, the induction of leukocytosis also occurred more quickly (Fig 9).

Analysis of the numbers of CFU-S in the peripheral blood of mice treated for 5 days with G-CSF alone showed that multipotent cells increased 10-fold at a dose of 15,000 U/kg/d and 35-fold at the higher dose of 150,000 U/kg/d. In combination with SCF, which by itself induced a sixfold increase after 5 days of treatment, G-CSF induced a greater than additive increment in CFU-S numbers. Levels up to 100 times control numbers were seen.

Table 1. Response of Reticulocytes and Platelets to SCF Administration Into Normal Mice

<table>
<thead>
<tr>
<th>Days</th>
<th>1</th>
<th>4</th>
<th>7</th>
<th>11</th>
<th>13</th>
<th>15</th>
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</thead>
<tbody>
<tr>
<td>Reticulocytes (×10⁸/mL of blood)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Carrier</td>
<td>2 ± 0.12</td>
<td>3.3 ± 0.2</td>
<td>1.3 ± 0.4</td>
<td>2 ± 0.2</td>
<td>1 ± 0.6</td>
<td>4 ± 0.1</td>
</tr>
<tr>
<td>SCF</td>
<td>ND</td>
<td>ND</td>
<td>1.7 ± 0.3</td>
<td>1.5 ± 0.1</td>
<td>4 ± 0.2</td>
<td>5.5 ± 0.2*</td>
</tr>
<tr>
<td>Platelets (×10⁹/mL of blood)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Carrier</td>
<td>ND</td>
<td>ND</td>
<td>88.6 ± 13</td>
<td>103 ± 11</td>
<td>72 ± 14</td>
<td>74 ± 10</td>
</tr>
<tr>
<td>SCF</td>
<td>ND</td>
<td>ND</td>
<td>106 ± 12</td>
<td>134 ± 10</td>
<td>120 ± 17</td>
<td>92 ± 9</td>
</tr>
</tbody>
</table>

Figures represent the mean of data obtained from duplicate experiments with three donors per experiment, each assessed individually ±1 standard error.

Abbreviation: ND, no data.

*These values differ significantly from carrier alone (P < .001).
Fig 4. Spleen cellularity during continuous treatment with SCF at 100 μg/kg/d for up to 21 days. *P < .05.

**DISCUSSION**

Mutations and deletions at the Steel locus in mice are associated with profound defects in several cell systems manifesting as anemia, sterility, and defective migration of melanocyte precursor cells. Recently, it has been shown that this locus encodes a growth factor, SCF, that is the ligand for the membrane receptor encoded by the c-kit proto-oncogene. SCF stimulates the in vitro proliferation and development of the primitive hematopoietic progenitors that survive 5-fluorouracil treatment in mice. It also interacts synergistically with growth factors required for the development of erythroid cells in vitro and can temporarily "cure" the hematopoietic defect in S1/Sd mice when administered in vivo.

However, while the importance of SCF in hematopoietic cell development is inferred by its association with the macrocytic anemias in the S1 and W mutant mice, its role in "steady state" hematopoiesis is unclear. To examine this, we have investigated the effects of SCF in normal and in...
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significant changes in either the number of IL-3-responsive in vitro progenitor cells or the number of day 8 or day 11 CFU-S in the marrow. (However, it should be noted that there was a tendency toward increased numbers of progenitor cells and day 8 CFU-S in the marrow of normal mice treated with SCF.) The main effect of SCF was seen in the spleens of normal mice, where there was a 50-fold increase of IL-3-responsive in vitro progenitor cells and a fourfold to sixfold increase in the day 8 and day 11 CFU-S. It is of some interest that, in the spleen, the in vitro progenitor cell levels peak at day 10 and subsequently decrease to levels only fourfold to eightfold that seen in nontreated mice, whereas, in the marrow, day 8 and day 11 CFU-S are elevated for the first 16 days of treatment and subsequently decrease to normal levels by day 21. We have no clear explanation for either these differences or the transient leukocytosis seen in the blood following treatment with SCF. One possibility being investigated at present is that PEGylated rat SCF is immunogenic in mice. However we consider this unlikely

splenectomized mice. The latter animals were chosen in view of our earlier studies showing that certain effects of growth factors were more readily apparent in the absence of splenic hematopoiesis. In this study, we have found that SCF, administered to normal mice, has a significant though relatively modest and transitory stimulatory effect upon the number of circulating neutrophilic granulocytes, and that this effect is magnified in splenectomized mice. However, it is presently unclear if the effect seen is due to increased production of neutrophils in the bone marrow. Furthermore, no greatly significant changes were seen in spleen cellularity, an effect that contrasts with that seen using a variety of other growth factors.

In view of the in vitro data indicating a role for SCF in the proliferation and development of multipotential progenitor cells we examined these populations in both normal and splenectomized animals treated with SCF. In both of these groups of animals, administration of SCF did not lead to

Fig 7. The incidence of spleen-derived spleen colony-forming cells during SCF treatment at 100 µg/kg/d. Data are expressed as a percentage of age-matched carrier-treated controls. **P .01, *P .05. (A) Day 8 CFU-S per spleen. (B) Day 11 CFU-S per spleen.

Fig 8. The effects of rhG-CSF on blood leukocytes. Nucleated cells per milliliter of blood.

Fig 9. The changes in peripheral blood nucleated cell count during treatment with SCF (100 µg/kg/d) either alone or in combination with G-CSF at doses between 1,500 and 150,000 U/kg/d.
because when the SCF is used in combination with G-CSF, the synergistic effects are seen throughout the 21-day treatment period (see later). In view of the synergy seen in vitro when SCF is used in combination with other hematopoietic cell growth factors, we have begun to examine some of these combinations in vivo. We titrated G-CSF down to a point where the administered dose had little effect on the number of circulating neutrophils. When this small dose of 1,500 U/kg/d was administered in combination with a dose of SCF that was itself weakly stimulatory the effect was dramatic; peripheral neutrophil numbers were more than doubled, an effect equivalent to an administered dose of G-CSF around 10 times greater. Throughout the dose range, SCF was an effective adjunct to G-CSF, making it many times more effective, though not apparently affecting the nature of the response.

Our data also indicate that SCF is effective in increasing yet further the concentration of transplantable primitive cells in the blood of G-CSF-treated mice. Since the effect of G-CSF for enhancing the regeneration of neutrophils and for mobilizing stem cells into the blood has now been confirmed in clinical studies (unpublished data), it is possible that the combined treatment of SCF plus rhG-CSF could be useful in a clinical scenario.

REFERENCES


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The effects on hematopoiesis of recombinant stem cell factor (ligand for c-kit) administered in vivo to mice either alone or in combination with granulocyte colony-stimulating factor

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