Effect of Stem Cell Factor With and Without Granulocyte Colony-Stimulating Factor on Neonatal Hematopoiesis: In Vivo Induction of Newborn Myelopoiesis and Reduction of Mortality During Experimental Group B Streptococcal Sepsis

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Neonatal hematopoiesis and host defense are developmentally immature and under states of increased demand predispose the newborn to peripheral cytopenias and depletion of bone marrow storage pool reserves. We have previously demonstrated that recombinant human granulocyte colony-stimulating factor (rhG-CSF) can significantly modulate neonatal rat granulopoiesis and act synergistically with antibiotic therapy to reduce the mortality rate during experimental group B streptococcal sepsis. Stem cell factor (SCF) has been shown to stimulate early hematopoietic progenitor cells and, in the presence of lineage-specific CSFs, enhance committed progenitor cell proliferation. In the present study we examined the in vivo neonatal hematologic effects of recombinant rat (rr) SCF (14 days), simultaneous rrSCF + rhG-CSF (14 days), and sequential combination of rrSCF (7 days) + rhG-CSF (7 days). Sprague-Dawley newborn rats (≤24 hours) were injected intraperitoneal (IP) × 14 days with the above combinations. rrSCF (0 to 200 μg/kg/d) had a negligible effect on the peripheral platelet count and absolute neutrophil count (ANC) but the diminution in the hematocrit during the first 10 days of treatment was less pronounced (P = .0001). However, the simultaneous use of rrSCF + rhG-CSF synergistically increased the circulating day 6 to 13 ANC (P = .001). Similarly, sequential rrSCF + rhG-CSF also had a synergistic significant effect during the second week of therapy on the circulating ANC (P = .01). The bone marrow neutrophil storage and proliferative pools were also significantly increased in newborn rats treated with rrSCF + rhG-CSF versus rhG-CSF (P = .02). The bone marrow and liver/spleen CFU-GM pool was unchanged; however, the CFU-GM proliferative rates were significantly increased in the rrSCF + rhG-CSF group (P = .04). rrSCF also induced a significant increase in the bone marrow and liver/spleen mast cell pool (P = .002).

A new hematopoietic growth factor, determined to be the ligand for c-kit, a cell surface receptor with tyrosine kinase activity, has recently been identified by a number of groups. The ligand for the c-kit proto-oncogene product has been isolated and labeled by a number of names, including stem cell factor (SCF), mast cell growth factor (MGF), and kit ligand (kl). The ligand for c-kit has also been identified to be the product of the Steel (SL) locus in the mouse. Mutations at the Steel locus in the mouse are also associated with a similar phenotype including macrocytic anemia, mast cell deficiency, and abnormalities in melanogenesis and gametogenesis. SCF alone has failed to induce a significant increase in bone marrow colony formation when used in the presence of unseparated Ficol-Hypaque (St Louis, MO) low-density collected bone marrow. However, when used in combination with either rhGM-CSF, rhG-CSF, or rhIL-3, rhSCF has induced a significant synergistic increase in both myeloid and erythroid human progenitor cells. Recently, rhSCF has additionally been shown to significantly induce an increase in CFU-GM and BFU-E from purified CD34 human bone marrow cells in combination with rhG-CSF, rhGM-CSF, and rhIL-3. Therefore, SCF has been demonstrated to have direct in vitro activity in stimulating very early hematopoietic progenitor cells and, additionally, has been synergistic with later-acting cytokines such as G-CSF.
to stimulate more lineage-specific and more mature progenitor cells. However, the above-mentioned studies have been restricted to studying the effect of SCF on adult hematopoiesis. In the present study, we examined the effect of SCF either alone or in combination (simultaneously or sequentially) with rhG-CSF in modulating neonatal rat hematopoiesis and its effects during experimental group B streptococcal infection.

MATERIALS AND METHODS

m-SCF. Recombinant rat SCF (rr-SCF) was a generous gift from Krisztina Zsebo (Amgen Inc, Thousand Oaks, CA). It was produced in Escherichia coli, and purified to homogeneity before formulation in phosphate-buffered saline (PBS). The purified material contains less than 0.033 ng endotoxin per milligram of SCF protein.17 rr-SCF was diluted in PBS/0.01% bovine serum albumin (BSA) for in vivo studies at a dose of 1, 30, 100, or 200 µg/kg animal body weight.

rhG-CSF. rhG-CSF, a generous gift from Jeffrey Andreson (Amgen Inc), was also prepared from E coli. It was prepared to 95% purity before formulation in 0.025% human serum albumin. A biologic activity of 2 × 10^6 U/mg was determined by granulocyte colony formation (CFU-G) on human nonadherent bone marrow cells in semisolid media. The absence of measurable endotoxin contamination was demonstrated by the Limulus amebocyte lysate assay. Purified rhG-CSF was used at 5.0 pg/kg (diluted with PBS, pH = 7.4).

Organism. Group B streptococcus (GBS), type III, N. norris, was kindly provided by Dr Gerald Fisher (Bethesda, MD). The organism was isolated from an infected neonate and serotyped by the precipitin method using rabbit antisera. The organism was grown in Todd-Hewitt broth (THB) to logarithmic phase and then aliquoted and stored at −70°C until use. Aliquots were thawed and allowed to grow to maximum phase growth in fresh THB. Organisms were then sedimented by centrifugation and washed three times in sterile PBS. Concentration of bacteria was standardized by its optical density at 620 nm, and a suspension of 2.5 × 10^9 organisms/gram body weight/0.100 mL was prepared for injection.

Animal inoculation. Litters of albino Sprague-Dawley neonatal rats (Bantin-Kingman Laboratories, Fremont, CA) ≤24 hours (6 to 8 g) were used during this study. Mothers of the litters were received 1 week before delivery, and were housed at the vivarium at the University of California Irvine Medical Center. They were maintained at constant room temperature, with water and rodent feed (Purina Chow;Ralston Purina, St Louis, MO) ad libitum. Approval for this study was granted by the Animal Use Committee (UCI, Irvine, CA). Before inoculation of growth factor, the site of injection was washed with Betadine solution (povidone-iodine, 10%; Purdue Frederick, Norwalk, CT) and swabbed with 70% alcohol. Intraperitoneal injections of 0.100 mL were accomplished with a sterile tuberculin syringe fitted with a 27.5-gauge needle. Animals were injected daily for 14 days with rr-SCF at concentrations ranging from 0 µg/kg to 200 µg/kg body weight, or in combination with rhG-CSF at 5 µg/kg. Control animals received 0.100 mL injections of PBS/0.01% BSA.

Experimental sepsis. After receiving various combinations of cytokines for 14 days, 2.5 × 10^6 organisms/g of body weight of GBS was injected intraperitoneally (IP) into the groups of animals. Control animals received sham injections of PBS/0.01% BSA. Survival was monitored for the next 120 hours.

Quantification of circulating and storage neutrophils. Blood samples were obtained by nicking the jugular vein with a sterile 25-gauge needle, and 20 µL of free-flowing blood was collected. Samples were electronically counted (Sereno-Baker Diagnostics, Allentown, PA), and blood smears were prepared, stained with Wright stain, and a 100- to 200-cell differential was performed. Absolute neutrophil counts (ANCs) were determined by the multiplication of the nucleated cell count by the percentage of neutrophils in the differentials. Neutrophil bone marrow pools (NSP + NPP) were determined as previously described by Cairo et al7 (NSP = % PMNs + bands + metamyelocytes; NPP = % blasts + promyelocytes + myelocytes).

CFU-GM colony formation. Bone marrow or peripheral blood cells were collected as described above.7 Cells were suspended in Hanks’ balanced salt solution (GIBCO Laboratories, Grand Island, NY) and cultured in commercially prepared methylcellulose media containing 0.8% methylcellulose, fetal calf serum (30%), BSA (1%), and mercaptoethanol (10−3 mol/L) (Terry Fox Laboratories, Vancouver, BC, Canada). Cells (2 × 10^9) were stimulated by murine spleen cell conditioned medium (1%) (Terry Fox Laboratories) and erythropoietin (4 U/mL) (Amgen, Thousand Oaks, CA). Three replicates of the cell suspensions were plated in 10 × 35 mm tissue culture dishes (Nunc, Roskilde, Denmark) and incubated at 5% CO2, 37°C, in a high-humidity atmosphere. Cultures were evaluated at 10 days with aggregates of greater than 50 cells considered “colonies.”

CFU proliferative rate. Proliferative rates of CFU-GM were evaluated by the thymidine suicide as previously described.7 Briefly, light-density bone marrow mononuclear cells from neonatal rats were placed in 50-mL centrifuge tubes and nonradioactive thymidine and methyl-[3H]-thymidine (20 minutes) containing 0.1 mCi (specific activity, 75 Ci/mmol; ICN Radiochemicals, Irvine, CA) was added. The tubes were incubated and thymidine uptake was halted by adding excess nonradioactive thymidine in ice cold α-minimal essential media (α-MEM) with fetal calf serum. The cell suspensions were then washed twice with cold thymidine media, and plated in methylcellulose as described above. Colonies were allowed to develop for 10 days in a 5% CO2 incubator at 37°C, and thymidine suicide rate was determined by subtracting the average number of colonies formed per plate by cells exposed to [3H]-thymidine from the average number of colonies per plate formed by cells exposed to nonradioactive thymidine, divided by the average colonies per plate from cells exposed to nonradioactive thymidine.

Statistical analysis. All results are expressed as mean values plus or minus standard error of the mean (SEM) of 8 to 15 animals or multiple runs of 3 to 5 replicates of blood or bone marrow samples. The probability of significant differences when comparing two treated groups was determined with the use of the unpaired Student’s t-test, while the probability of significant differences when examining multiple treatments was determined by using the analysis of variance (ANOVA) followed by the Student-Newman-Keuls multiple range tests to define the unique subsets within the study. Statistical analyses were performed using the Biotest 1 statistical program (Sigma Soft, Placentia, CA) for the IBM (Boca Raton, FL) personal computer. P values <.05 are considered significant.

RESULTS

We first examined the in vivo effects of IP rrSCF (0 to 200 µg/kg/d × 14 days) on neonatal hematopoiesis in the newborn rat. rrSCF alone (at all doses) had a negligible effect on inducing an increase in the platelet count or absolute neutrophil count during the entire 14-day period compared with placebo-control-treated animals. The day 14 platelet count was 935.6 ± 80.0 versus 894.8 ± 81.0/10^9 mm3; day 14 ANC 1,820 ± 309 versus 1,368 ± 139/mm3 rrSCF versus PBS/BSA (P = not significant). However,
there was a significant improvement in the diminution of the hematocrit during the first 10 days of rrSCF therapy compared with PBS/BSA (Fig 1). However, by day 14, the hematocrit was similar in both groups. Additionally, there was no significant increase in the absolute lymphocyte, monocyte, eosinophil, or basophil count in animals treated with rrSCF versus placebo-control-treated animals. Similarly, during sequential and simultaneous administration of both rrSCF plus rhG-CSF, there was also no significant difference in the circulating hematocrit and platelet counts compared with placebo-control-treated animals on day 14 (Table 1).

However, during sequential administration of rrSCF or PBS/BSA × 7 days followed by rhG-CSF for 7 days, there was a significant increase in the circulating ANC. Animals pretreated with rrSCF for 7 days followed by rhG-CSF for 7 days had significantly higher ANC on days 10 through 13 compared to animals treated with PBS/BSA followed by 7 days of rhG-CSF (Fig 2). During simultaneous administration of rrSCF and rhG-CSF, there was also a significant increase in the circulating ANC during the second week of therapy (day 6 through day 13) as compared with rhG-CSF alone (Fig 3). However, during the first few days of therapy the simultaneous administration of rrSCF + rhG-CSF was not significantly different as compared with rhG-CSF therapy alone. Both the sequential and simultaneous administration of rrSCF + rhG-CSF had no significant influence on the circulating absolute lymphocyte, monocyte, eosinophil, or basophil counts during the 14-day treatment period as compared with placebo-treated animals.

| Table 1. Effect of SCF + G-CSF (sequentially or simultaneously) on Day 14 Hematocrit and Platelet Count in Newborn Rats |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Day 14 Hematocrit (%) | Day 14 Platelet Count (x 10^3/μL) |
| PBS/BSA         | 24.6 ± 0.8       | 894 ± 81        |
| SCF + G-CSF (seq) | 24.8 ± 0.7     | 963 ± 82        |
| SCF + G-CSF (sim) | 25.3 ± 0.4     | 947 ± 88        |

Abbreviations: SCF + G-CSF (seq), SCF × 7 days and G-CSF × 7 days; SCF + G-CSF (sim), SCF + G-CSF simultaneously × 14 days.

Neutrophil storage pools were determined on day 15 after 14 days of simultaneous administration of rrSCF + rhG-CSF compared with rhG-CSF alone. There was a significant increase in the bone marrow neutrophil storage pool between those animals treated simultaneously with both rrSCF + rhG-CSF versus rhG-CSF alone (P < .02) (Fig 4). However, the liver/spleen neutrophil storage pool was not significantly different between rrSCF + rhG-CSF compared with rhG-CSF (rrSCF + rhG-CSF v rhG-CSF 37,986 ± 3,712 v 34,727 ± 3,221 per liver/spleen) (P = not significant). Similarly, the bone marrow neutrophil proliferative pool (blasts + promyelocytes + myelocytes) was also significantly increased in the simultaneously treated animals (rrSCF + rhG-CSF) versus rhG-CSF alone (625 ± 51 v 425 ± 40/per femur) (P = .02).
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nSCF and rhG-CSF, rhG-CSF, or PBS/BSA by IP injection daily for 14 days. Samples were electronically counted and a 100- to 200-cell differential was performed on Wright's-stained blood smears. Values are the mean ± SEM of 20 animals. ANC: nSCF + rhG-CSF versus rhG-CSF days 6* and 12*** (P < .001), day 9** (P < .05). (■), SCF; (○), SCF + G-CSF; (△), G-CSF; (●), PBS/BSA.

There was, additionally, a significant difference in the mast cell pool in animals pretreated with nSCF ± rhG-CSF compared with rhG-CSF- or PBS/BSA-treated animals. The bone marrow mast cell pool was eightfold higher in the nSCF-treated group as compared with rhG-CSF- or PBS/BSA-treated animals (P < .002) (Fig 5). Similarly, there was also a significant increase in the mast cell pool in the liver/spleens of animals treated with nSCF as compared with all other groups (nSCF v PBS/BSA 5,629 ± 974 v 661 ± 231 per liver/spleen) (P < .02).

Bone marrow CFU-GM was determined on day 15 after 14 days of simultaneous administration of nSCF ± rhG-CSF. The day 14 bone marrow CFU-GM was similar in those animals treated with nSCF, nSCF + rhG-CSF, or rhG-CSF alone (Table 2). However, the bone marrow CFU-GM proliferative rate as determined by thymidine suicide studies was significantly higher between nSCF versus PBS/BSA (P = .001) and nSCF + rhG-CSF versus PBS/BSA (P = .04) (Table 2). Similarly, there was also no significant difference in liver/spleen CFU-GM colony formation, but there was a significant increase in liver/spleen CFU-GM rate in nSCF versus PBS/BSA (P = .005), and nSCF + rhG-CSF versus PBS/BSA (P = .03) (Table 2).

Lastly, we examined the prophylactic effect of nSCF ± rhG-CSF given × 14 days to modulate mortality during experimental GBS sepsis. Animals pretreated with either single (nSCF or PBS/BSA) or simultaneous combinations of nSCF + rhG-CSF were inoculated with lethal doses of...
GBS on day 14. At 48 and 120 hours there was a significant decrease in the mortality rate in animals receiving \( \text{rrSCF} \pm \text{rhG-CSF} \) \((P < .03 \text{ and } P < .05, \text{respectively})\) (Fig 6). At 48 hours, there was a significant decrease in the mortality rate in animals pretreated with \( \text{rrSCF} \) with or without \( \text{rhG-CSF} \) (Fig 6).

**DISCUSSION**

Neonatal neutropenia is a hallmark finding during bacterial sepsis in the newborn and has been associated with a poor prognosis.\(^{18-20}\) We have previously demonstrated an improved survival rate using adult donor granulocytes during neonatal neutropenia and overwhelming bacterial sepsis.\(^{21-23}\) The difficulty in obtaining and the potential morbidity of using adult donor granulocytes during neonatal bacterial sepsis has prompted us to develop alternative strategies with cytokine therapy for modulating neonatal myelopoiesis.\(^{24}\)

We have previously demonstrated that hematopoietic growth factor production from stimulated neonatal mononuclear cells is markedly reduced as compared with the adult.\(^{25}\) However, hematopoietic growth factor receptors on peripheral blood mature effector cells have been shown to be normal in number.\(^{25}\) Our recent studies have suggested that prophylactic administration of \( \text{rhG-CSF} \) to newborn rats during the first week of life results in a significant enhancement of neonatal myelopoiesis and a reduction in the mortality rate as compared with \( \text{rhG-CSF} \) or antibiotic therapy alone.\(^{7}\)

In the present study, we examined the in vivo effects of an early acting hematopoietic growth factor (SCF) on neonatal rat hematopoiesis. When neonatal animals were also pretreated with \( \text{rrSCF} \times 7 \text{ days} \) or both when CSFs were administered simultaneously, there was a significant increase in theANC during the second week of administration as compared with \( \text{rhG-CSF} \) therapy alone \((P = .001)\). Ulich et al\(^{26}\) has similarly demonstrated a synergistic increase in the circulating neutrophil count (day 4 through day 6) when adult rats were treated for 7 days with \( \text{rrSCF} + \text{rhG-CSF} \). However, \( \text{rrSCF} \) alone did not induce any significant increase in the neutrophil count in adult rats treated for 7 consecutive days. However, Andrews et al\(^{27}\) has recently demonstrated, in primates, that PEG-SCF administered by continuous infusion (200 \( \mu \text{g/kg/d} \times 28 \text{ days} \)) induced an increase in the circulating ANC by day 14 as compared with day 0.

Our present study also demonstrated an additive increase in the bone marrow neutrophil storage pool during simultaneous administration of \( \text{rrSCF} \) with \( \text{rhG-CSF} \) as compared with \( \text{rhG-CSF} \) therapy alone \((P = .02)\). Ulich et al\(^{26}\) also demonstrated an additive increase in the day 8 bone marrow neutrophil storage pool in adult rats treated with simultaneous \( \text{rrSCF} + \text{rhG-CSF} \) compared with \( \text{rhG-CSF} \) alone.

SCF alone has minimal hematopoietic activity as compared with its profound effect when used in combination with other CSFs. However, SCF alone has been shown to induce a significant increase in bone marrow mast cell pool both in neonatal rats, as demonstrated in the present study as well as in adult rats.\(^{28}\) However, the increase in the bone marrow mast cell pool in the neonatal rat did not appear to induce any toxic effects. Further, we speculate that the increase in the mast cell pool by SCF may be responsible, in part, for the increase in survival during experimental bacterial sepsis.

In summary, \( \text{rrSCF} \) administered for 14 days to neonatal rats does not induce a significant increase in the peripheral neutrophil or platelet count, but decreases the diminution of the circulating hematocrit. When \( \text{rrSCF} \) is administered simultaneously with \( \text{rhG-CSF} \), there is a significant increase in the peripheral neutrophil count during the second week of therapy and an increase in bone marrow neutrophil storage pool and bone marrow and liver/spleen proliferative rates as compared with \( \text{rhG-CSF} \) therapy alone. \( \text{rrSCF} \) also induced a significant increase in the neonatal rat bone marrow mast cell pool. Lastly, \( \text{rrSCF} \pm \text{rhG-CSF} \) decreased the mortality rate during experimental GBS infection. It remains to be seen whether SCF, with or without other cytokines, will play an important role in modulating human neonatal host defense and thereby influence survival during human newborn overwhelming bacterial infection.

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