Long-Term Treatment of Canine Cyclic Hematopoiesis With Recombinant Canine Stem Cell Factor


Grey collie dogs have cyclic fluctuations in their blood cell counts caused by a regulatory defect in hematopoietic stem cells. To examine the role of stem cell factor (SCF) or its receptor in this disorder, we investigated the stimulatory effects of recombinant canine SCF (rc-SCF) on in vitro marrow cultures, cloned and sequenced the grey collie SCF gene, and treated three grey collies with rc-SCF, either alone or in combination with recombinant canine granulocyte colony-stimulating factor (rc-G-CSF). Colony-forming unit granulocyte-macrophage formation from grey collie or normal dog marrow showed similar dose-response curves for rc-G-CSF. Cloning and sequencing the SCF gene for two grey collies showed no evidence of mutations in the coding region of the SCF gene. Treatment with rc-SCF (10 to 100 μg/kg/d) did not induce neutrophilia except at the highest dose (100 μg/kg/d), but daily rc-SCF abrogated the neutropenic periods in doses of 20 μg/kg/d or greater. Combination of rc-G-CSF (0.5 to 1.0 μg/kg/d) with rc-SCF treatment (20 to 50 μg/kg/d) suggested a synergistic effect, i.e., the neutrophil levels on combined therapy were higher than the sum of the levels when these two cytokines were given separately. Long-term treatment of these dogs with rc-SCF in doses of 10 to 30 μg/kg/d was generally well tolerated, suggesting that SCF may be useful as a therapy for some chronic hypoproliferative disorders of hematopoiesis.

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Materials and Methods

Dogs and Blood Sampling

One male and two female adult grey collies (ages 3 to 7 years) were housed in accredited animal care facilities as previously described. Blood cell counts were performed daily, including white blood cells with differential, hematocrit, reticulocyte, and platelet counts by standard methods.

In Vitro Colony-Forming Assays

In vitro BM colony formation was used to assess the potency of rc-SCF to stimulate canine marrow cell growth, used singly or in combination with other growth factors, by methods previously described. For these studies BM samples were aspirated into heparinized syringes, separated by density gradient centrifugation, and the adherent cells removed by incubation in plastic flasks for 1 hour at 37°C. Mononuclear cells were plated at 1 × 10^4 cells per mL, in the presence of 10% fetal calf serum and 5% normal dog serum, with added hematopoietic growth factors in the following concentrations: rc-CSF (Amgen, Thousand Oaks, CA) 282 pmol/L; rc-GM-CSF (Genetics Institute, Cambridge, MA) 100 pmol/L; rhIL-3 (Amgen) 40 pmol/L; rc-SCF (Amgen) 2,350 pmol/L; or a combination of all four factors in these same concentrations. Colonies (≥50 cells) were counted at 8 to 10 days and results recorded for duplicate culture plates.

RNA Purification

RNA was isolated from canine fibroblasts (two grey collies and a normal beagle) using RNAzol (Cinna/Biotex, Friendswood, TX) based on the method of Chirgwin et al.

cDNA Synthesis, Cloning, and Sequence

cDNA was prepared by reverse transcription of purified RNA with random hexamers using Moloney murine leukemia virus reverse transcriptase. The cDNA was cloned into the pBluescript vector and sequenced using Sequenase (U.S. Biochemicals, Cleveland, OH) or Sequenase (United States Biochemicals, Cleveland, OH) with M13 primers and Sequenase (United States Biochemicals, Cleveland, OH) as sequencing kit.

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transcriptase (BRL, Gaithersburg, MD). SCF cDNA was amplified from the total cDNA by polymerase chain reaction (PCR). Primers 5’-CGCAAG CTT GGA TCG CAG CGC TGC TCC-3’ and 5’-GTG GCC GGC GC GAC GAA GCA AATG AAC TGT TAC C-3’ were derived from the cDNA sequence of canine SCF to amplify the entire coding region. After 30 cycles, further amplification was performed with nested primers (20 cycles). The amplified DNA was sequenced by a dideoxynucleotide chain termination method using an Applied Biosystems automated electrophoresis DNA sequencing system (Applied Biosystems, Foster City, CA). For each reverse transcription product, the entire process was repeated to verify sequence and exclude the possibility of PCR-related artifacts.

In Vivo Studies

rc-SCF (3.5 mg/mL) and rcG-CSF (1.0 mg/mL) (Angen, Thousand Oaks, CA) were administered subcutaneously at the base of the neck posteriorly on a daily basis unless otherwise indicated as in prior studies. All dogs had previously received hematopoietic growth factor treatment, but before beginning these trials each had a period off all treatments long enough to demonstrate cycling of their blood cell counts with the characteristic periodicity of approximately 13 days. During this study the dogs were regularly observed by experienced personnel with physical examinations, daily temperature readings, blood counts, and periodic renal and liver function tests.

Statistical Analysis

Student’s t-test was used to determine statistical difference between means. An analysis of variance (ANOVA) was used to examine the effects of rc-SCF and rcG-CSF alone and in combination on the blood neutrophil counts.

RESULTS

In Vitro Effects of SCF

Preliminary studies of colony formation by normal dog and grey collie marrow cells showed that addition of rc-G-CSF, rhGM-CSF, rhIL-3, and rc-SCF alone and a combination of these factors stimulated colony growth; the greatest growth regularly occurred with the combination of factors similar to published results. Dose-response studies for rc-SCF alone or in combination with a stimulatory dose of rcG-CSF (28 pmol/L rcG-CSF) showed a steep increase in colony formation between 10^{-10} and 10^{-8} g/mL (2.7 pmol/L to 270 pmol/L), with no additive or synergistic effects of the two factors in this serum containing culture system (Fig 1).

Cloning and Sequencing of SCF

Duplicate clones were sequenced from two grey collies and a normal beagle. Sequence data that were obtained from each of these dogs spanned the entire translated region of the cDNA using overlapping primers in both sense and antisense directions. Sequence data were then compared with the previously determined sequences of canine SCF. We found no evidence of mutations that might cause function impairment of the SCF protein. Occasional single-base substitutions were identified; none were consistently present in both clones from the same animal. Therefore, these were interpreted as being caused by artifacts of the PCR method. However, the regulatory sequences for SCF expression were not studied.

In Vivo Effects of SCF

The first dog treated with rc-SCF (100 µg/kg/d subcutaneously) did not show an immediate neutrophil increase or discernible effects on other blood cell counts. However, there was an abrogation of the next expected period of neutropenia (Fig 2). However, with continued treatment at this dose level, the dog developed fever, muzzle edema, loss of appetite, and general malaise, which resulted in discontinuation of rc-SCF approximately 5 weeks after it was started. This allergic-type response subsided as soon as the treatment was discontinued. On the basis of this experience, dogs were subsequently treated with rc-SCF in lower dosages.

Three collies were then treated with rc-SCF initiated at 10 µg/kg/d and increased to 30 µg/kg/d over the next several months (Fig 3). With this dose and schedule, the dogs were clinically well, without fever, muzzle edema, or other signs of toxicity. The characteristic neutrophil nadirs were progressively less severe, but no frank neutrophilia developed, as was seen in previous long-term trials in grey collies using other growth factors. Hematocrit values were maintained in a normal range. Regular oscillations of other blood element counts became less distinct with SCF treatment but continued to show considerable day-to-day variation (Fig 4).

Two dogs were treated with 4-day pulses of rc-SCF timed to attempt to prevent the anticipated periods of neutropenia. These pulses of SCF at 50 µg/kg/d appeared to alter the periodicity of the neutrophil cycles slightly. However, seven cycles before SCF the mean cycle length was 12.6 ± 0.5 (SD) days (range 12 to 13 days); for 10 cycles with pulse SCF the mean cycle length was 13.6 ± 2.5 (SD) days (range 11 to
Fig 2. The neutrophil response of a grey collie to injections of rc-SCF, 100 µg/kg/d, subcutaneously. This dog developed fever and muzzle edema leading to discontinuation of treatment (see text).

19 days) (Fig 5). To examine for in vivo synergy of rc-G-CSF and rc-SCF, two dogs were first treated for several months with rc-G-CSF in relatively low doses (0.5 or 1.0 µg/kg/d). They showed large amplitude neutrophil oscillations with abbreviated neutropenic periods, as described previously.2 rc-SCF (10, 20, and then 30 µg/kg/d) was then added (Fig 6) and treatment continued. Comparisons of mean neutrophil counts (days with absolute neutrophils less than 0.5 x 10⁹/L) and days of severe neutropenia showed that both rc-SCF and rcG-CSF could prevent neutropenia and that rcG-CSF but not rc-SCF caused neutrophilia (Table 1). For one dog on therapy for the longest period, rc-G-CSF and rc-SCF were synergistic in vivo to cause neutrophilia, i.e., the mean neutrophil count with rcG-CSF (0.5 to 1.0 µg/

Fig 3. The neutrophil response of a grey collie to gradually increasing doses of rc-SCF.

Fig 4. A comparison of blood counts for a collie before and on treatment with rc-SCF (30 µg/kg/d). The oscillations of reticulocytes and eosinophils were also abrogated (data not shown).

Fig 5. The response of a grey collie to pulse treatment with rc-SCF (50 µg/kg/d). This figure shows greater variation in the cycle length (nadir to nadir) on treatment with rc-SCF than for the period before rc-SCF (see text).
kg/d) plus rc-SCF (20 to 50 µg/kg/d) was greater than the sum of these counts when these agents were used singly in the same dosages. An ANOVA for the data in Table 1 showed that the mean neutrophil counts for treatment with rc-SCF (> 20 µg/kg/d), rcG-CSF (> 0.5 µg/kg/d), or rc-SCF plus rcG-CSF (same doses) were significantly different (P < .001) from the no-treatment level. There also appeared to be an interaction of rc-SCF and rcG-CSF to prevent neutropenia, i.e., days with neutrophils less than 0.5 × 10^9/L, but the differences were not statistically significant (P > .05).

Adverse effects. One dog that was administered rc-SCF (50 µg/kg, subcutaneously, daily) gradually developed thickening of the skin in the area of the injections. There were no overt sign of illness in this animal, but the peculiarly thickened skin was biopsied. It showed dense accumulations of mast cells with an increased dermal collagen. The rc-SCF

Table 1. Effects of rcSCF and rcG-CSF Singly and in Combination on Blood Neutrophil Levels in a Grey Collie

<table>
<thead>
<tr>
<th>SCF Dose µg/kg/d</th>
<th>Responses</th>
<th>None</th>
<th>0.5 µg/kg/d</th>
<th>1 µg/kg/d</th>
<th>≥0.5 µg/kg/d</th>
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<tbody>
<tr>
<td>None</td>
<td>ANC*</td>
<td>2.328 ± 230</td>
<td>8.562 ± 318</td>
<td>11.452 ± 1,509</td>
<td>8.822 ± 323</td>
</tr>
<tr>
<td>Day &lt; 0.5†</td>
<td>4</td>
<td>26</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>N (%) 220</td>
<td>130 (20%)</td>
<td>223 (2%)</td>
<td>22 (0%)</td>
<td>245 (1.6%)</td>
<td></td>
</tr>
<tr>
<td>ANC</td>
<td>2.632 ± 506</td>
<td>9.532 ± 987</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 µg</td>
<td>Days &lt; 0.5</td>
<td>4</td>
<td>0</td>
<td>ND</td>
<td>—</td>
</tr>
<tr>
<td>N (%) 220</td>
<td>18 (22%)</td>
<td>32 (0%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANC</td>
<td>3.715 ± 400</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 µg</td>
<td>Days &lt; 0.5</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>—</td>
</tr>
<tr>
<td>N (%) 220</td>
<td>56 (0%)</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>ANC</td>
<td>2.232 ± 206</td>
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<tr>
<td>30 µg</td>
<td>Days &lt; 0.5</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>—</td>
</tr>
<tr>
<td>N (%) 220</td>
<td>30 (0%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANC</td>
<td>2.563 ± 107</td>
<td></td>
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<tr>
<td>50 µg</td>
<td>Days &lt; 0.5</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>—</td>
</tr>
<tr>
<td>N (%) 220</td>
<td>128 (0%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANC</td>
<td>3.126 ± 342</td>
<td>15.413 ± 2,668</td>
<td>16.801 ± 1,547</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 µg/kg3x/wk</td>
<td>Days &lt; 0.5</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N (%) 220</td>
<td>148 (4%)</td>
<td>19 (0%)</td>
<td>30 (0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANC</td>
<td>2.944 ± 160</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥20 mg</td>
<td>Days &lt; 0.5</td>
<td>6</td>
<td>—</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>N (%) 220</td>
<td>362 (2%)</td>
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</table>

* ANC = PMN × 10^9/L.
† Days < 0.5 = no. days PMN < 0.5 × 10^9/L.
‡ N = total days of observation on this therapy.
§ % = % observed days with PMN < 0.5 × 10^9/L.
therapy was discontinued with resolution of this change. In another animal on intermittent rc-SCF (50 μg/kg/d) long-grade fevers developed after several weeks that correlated with the days of rc-SCF injection and resolved with discontinuation of rc-SCF. Otherwise treatment was well tolerated without apparent ill effects or changes in routine laboratory tests.

DISCUSSION

Canine cyclic neutropenia has proven to be a useful model for preclinical examination of the effectiveness of hematopoietic growth factor treatment for human cyclic neutropenia and other severe chronic neutropenic states. The trial shows that rc-SCF can be used as a long-term therapy and serves to characterize the hematopoietic effects with long-term use of this cytokine. Consistent with studies in other species, rc-SCF in the grey collies had far less effect on blood neutrophil counts than rcG-CSF or rhGM-CSF.

These studies also show that SCF can stimulate early precursor cells as manifest by the decrease in the depth of the neutrophil nadirs and the blunting of the oscillations of other blood cell counts. Its effect on early hematopoietic cells may be inferred from the observation that intermittent treatment appeared to alter the cycle length in at least one of these dogs (Fig 5). In other canine studies, Schuening et al observed marked neutrophilia after rc-SCF in normal dogs, but their dogs received much larger doses (200 μg/kg/d) than were administered in treating these grey collies.

Previously, several investigators have examined the in vitro effects of combining SCF with other hematopoietic growth factors. Synergy has been repeatedly demonstrated. Although we did not observe a synergistic effect of rc-SCF and rcG-CSF in vitro in the dose-response studies using a culture system containing fetal calf serum and normal dog serum, the in vivo studies suggested synergy when we combined these stimulants. Based on careful long-term studies of one dog (Table 1), combining rc-SCF and rcG-CSF appeared to increase the mean neutrophil count to a greater degree than the additive effect of these cytokines used alone. In previous studies, such synergy has also been shown in rats treated with SCF plus G-CSF or GM-CSF as well as in neonatal mice treated with SCF plus G-CSF. Because G-CSF is so potent for treatment of cyclic neutropenia both in the grey collies and in humans, this demonstration of synergy probably is of limited import for the treatment of these conditions, but may be of greater importance for treatment of refractory forms of congenital cytopenias, aplastic anemia, and other conditions causing marrow failure.

The long-term treatment of these dogs with rc-SCF was effective in maintaining hematopoiesis and preventing fever and infections associated with the canine cyclic neutropenia, but important adverse events were observed that provide useful information for future trials. We observed acute muzzle edema in the animal treated with the highest doses of SCF, chronic dermal induration with basophil accumulation in another animal, and intermittent fever associated with intermittent SCF therapy in a third dog, similar to previous reports. All of these events were avoided by dose reduction or interruptions of SCF therapy. We did not investigate pharmacologic agents to modify these allergic type reactions; their occurrence now in several species suggests that they may be a limiting factor for clinical use of SCF on a long-term basis.

We began these studies both to examine the use of SCF as a therapy for cyclic neutropenia (cyclic hematopoiesis) and also to consider the possibility that abnormalities of SCF or c-kit itself may be the cause of this disorder. However, cloning and sequencing of SCF from the affected dogs showed no mutations in the coding region of the SCF gene. We have not cloned and sequenced the regulatory sequences for SCF or c-kit, but believe on the basis of the treatment responses we observed and our in vitro dose-response studies that an abnormality of the receptor for SCF is unlikely to be the cause of this disorder. These results, together with our recent report of normal G-CSF receptor number and function on the mature neutrophils in the grey collies, suggest that a postreceptor abnormality is the most likely level for the primary defect in this disease.

We have previously shown that G-CSF is a very effective therapy for human and canine cyclic neutropenia. This treatment trial indicates that SCF also is an effective long-term treatment for this condition in the grey collies. SCF may prove to be an effective therapy, in combination with other growth factors, for other chronic hypoproliferative hematopoietic disorders. Based on our studies in these dogs, it appears that SCF should be used in relatively low doses or perhaps on an intermittent basis in the initial investigations.

ACKNOWLEDGMENT

We gratefully acknowledge the advice and assistance of Dr Frank Martin (Amgen, Thousand Oaks, CA) and Dr W.R.A. Osborne (University of Washington, Seattle) for aid in cloning and analyzing the grey collie SCF; Ron Ling for statistical assistance; Phyllis Child for manuscript preparation; and Linda Weber, Tony Norman, and the staff of the University of Washington Vivarium for the care of the grey collies.

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Long-term treatment of canine cyclic hematopoiesis with recombinant canine stem cell factor

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