Stem Cell Factor Directly Stimulates the Development of Enriched Granulocyte-Macrophage Colony-Forming Cells and Promotes the Effects of Other Colony-Stimulating Factors

By Clare M. Heyworth, Anthony D. Whetton, Sian Nicholls, Krisztina Zaebbo, and T. Michael Dexter

The effects of the c-kit ligand (stem cell factor [SCF]) on the development of a highly enriched population of granulocyte-macrophage colony-forming cells (GM-CFC) were assessed. In soft agar assays, both in serum-containing and in serum-deprived cultures, SCF promoted the formation of colonies that contained predominantly granulocytic cells with some blast cells also present. The size of these colonies was far smaller than observed in the presence of interleukin-3 (IL-3). In serum-deprived conditions, no colonies were formed in the presence of macrophage colony-stimulating factor (M-CSF), but when M-CSF was combined with SCF, a marked change was noted in that large colonies were produced containing predominantly macrophages. When GM-CFC were cultured in the presence of IL-3 and SCF, colonies were formed that contained blast cells, granulocytes, and macrophages. A synergistic interaction was also seen using a combination of G-CSF plus SCF in either serum-containing or serum-deprived cultures. The addition of SCF to colony-forming assays markedly reduced the concentration of IL-3 or G-CSF required for optimal levels of colony formation. Furthermore, SCF was capable of promoting the survival of GM-CFC for several days, after which large colonies containing mature cells were formed upon the addition of a secondary growth factor such as G-CSF or IL-3. Thus, SCF can directly act on highly enriched committed progenitor cells in serum-deprived conditions to promote survival, proliferation, and development.

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Preparation of a population highly enriched in GM-CFC. GM-CFC from murine bone marrow were prepared as previously based on the method described by Williams et al. Cells from the bone marrow of cyclophosphamide-treated mice were initially enriched for GM-CFC using density gradient centrifugation; the GM-CFC-rich fraction was then subjected to elutriation centrifugation and a subset of cells obtained that were highly enriched for GM-CFC prepared. This cell population, which has been shown to consist of greater than 90% GM-CFC, was used for the experiments described.

Colony-forming assays. Soft agar colony-forming assays in the presence of serum were performed, as previously reported, in Iscove’s medium plus 20% (vol/vol) foetal calf serum. Serum-deprived colony-forming assays were performed with the following substitutes for serum in the assays, iron-saturated transferrin (0.3 mg/mL), soya-bean lipids (0.025 mg/mL), sodium pyruvate (1 mmol/L), α-thioglycerol (0.1 mmol/L), glutamine (2 mmol/L), linoleic acid (5.6 µg/mL), and cholesterol (7.8 µg/mL). Colonies that consisted of more than 50 cells were counted after 7 days and also 14 days’ incubation. The results obtained were essentially the same for both incubation periods and, unless otherwise stated, only the 7-day data are shown here.

For morphological studies, cyto spun preparations were made from individual colonies and the cells stained using May-Grünewald-Giemsa.

Hematopoietic growth factors. The growth factors used in these studies were all recombinant proteins with the exception of M-CSF, which was purified from L-cell-conditioned medium to stage 4 of the procedure described by Stanley and Heard. The other growth factors investigated were human G-CSF (106 U/mg protein; Amgen, Thousand Oaks, CA), mGM-CSF (1.25 × 107 U/mg protein; Biogen, Geneva, Switzerland), IL-3 (2.1 × 107 U/mg protein; Biogen), and rat SCF 1-164 (1.56 mg/mL; Amgen).

RESULTS

Influence of SCF on colony formation from GM-CFC in serum. The relative abilities of GM-CSF, G-CSF, M-CSF, IL-3, and SCF to stimulate colony formation from GM-CFC were compared in soft gel assays in the presence of serum. To facilitate comparison between the groups, the results are expressed as a percentage of the number of colonies produced in experiments where IL-3 (100 U/mL) was present as the colony-stimulating factor; the colony-forming efficiency of the elutriated cells observed with IL-3 (100 U/mL) was approximately 30% (see Cook et al). In the absence of other growth factors, maximal levels of SCF-stimulated colony formation were observed at doses of 100 ng/mL or greater. The maximum number of colonies observed with SCF (100 ng/mL) was equivalent to 44% of that observed when IL-3 alone (100 U/mL) was present (Fig 1). However, the size and the morphology of the colonies produced were significantly different in the presence of these two growth factors: SCF stimulated the formation of small colonies, which generally contained blast cells, early granulocytic cells, mature granulocytes, and occasionally a few macrophages (Tables 1 and 2); IL-3 promoted the formation of neutrophil/macrophage colonies, with few if any blast cells present after 7 days. After 14 days, the number of blast cells present in cultures supplemented with SCF alone decreased markedly and the colonies consisted of predominantly neutrophilic cells.

When IL-3 (100 U/mL) and SCF (250 ng/mL) were combined, colony number was not significantly increased (P > .6) over that seen in IL-3 alone, and the size of the colonies and number of cells present therein were markedly increased (Table 3).

<table>
<thead>
<tr>
<th>Colony Type Formed (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCF</td>
</tr>
<tr>
<td>G-CSF</td>
</tr>
<tr>
<td>IL-3</td>
</tr>
<tr>
<td>GM-CSF</td>
</tr>
<tr>
<td>M-CSF</td>
</tr>
<tr>
<td>SCF + G-CSF</td>
</tr>
<tr>
<td>SCF + IL-3</td>
</tr>
<tr>
<td>SCF + GM-CSF</td>
</tr>
<tr>
<td>SCF + M-CSF</td>
</tr>
</tbody>
</table>

Table 1. Morphology of Colonies Formed From GM-CFC in the Presence of Various Hematopoietic Growth Factors
The addition of various combinations of growth factors to GM-CFC. The colony (from 40 colonies) is shown below to give some indication of the mean value (as a percentage of the total number of cells per colony) from 40 colonies is shown below to give some indication of the proportion of cells formed in soft gel assays as a consequence of the addition of various combinations of growth factors to GM-CFC. The concentrations of growth factors used to obtain these colonies were IL-3, 100 U/mL; GM-CSF, 50 U/mL; SCF, 250 ng/mL; G-CSF, 5,000 U/mL; M-CSF, 100 U/mL.

The number of each cell type present in each colony was assessed, and the mean value (as a percentage of the total number of cells per colony) from 40 colonies is shown below to give some indication of the proportion of cells formed in soft gel assays as a consequence of the addition of various combinations of growth factors to GM-CFC. The concentrations of growth factors used to obtain these colonies were IL-3, 100 U/mL; GM-CSF, 50 U/mL; SCF, 250 ng/mL; G-CSF, 5,000 U/mL; M-CSF, 100 U/mL.

<table>
<thead>
<tr>
<th>Growth Factors</th>
<th>Blast Cells</th>
<th>Early Granulocytes</th>
<th>Late Granulocytes</th>
<th>Macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCF</td>
<td>20</td>
<td>30</td>
<td>45</td>
<td>5</td>
</tr>
<tr>
<td>G-CSF</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>IL-3</td>
<td>0</td>
<td>17</td>
<td>55</td>
<td>28</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>0</td>
<td>0</td>
<td>23</td>
<td>77</td>
</tr>
<tr>
<td>M-CSF</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>SCF + G-CSF</td>
<td>8</td>
<td>30</td>
<td>60</td>
<td>2</td>
</tr>
<tr>
<td>SCF + IL-3</td>
<td>20</td>
<td>33</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>SCF + GM-CSF</td>
<td>33</td>
<td>20</td>
<td>12</td>
<td>35</td>
</tr>
<tr>
<td>SCF + M-CSF</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

The size of the colonies formed in the presence of SCF plus any of the colony-stimulating factors was far greater than that observed when either cytokine was added alone (Table 3).

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Table 2. Morphological Analysis of Cells Present in Colonies Formed After 7 Days From an Enriched Population of GM-CFC in the Presence of Various Combinations of Growth Factors

The ability of SCF (0 to 250 ng/mL) to promote the formation of colonies from highly enriched GM-CFC in serum-free conditions was evaluated. Colony-forming efficiency in the presence of IL-3 was 15%, approximately half of the value observed when both IL-3 and serum were present in the assays (see above). At an optimal concentration (100 ng/mL), SCF stimulated the formation of 20% ± 4% (mean ±SD, n = 8) of the colonies observed when IL-3 alone was present in the colony-forming assays; however, the size of the colonies was smaller than observed when the CFC assays contained IL-3 or GM-CSF. The combination of SCF and IL-3 had an additive effect on the number of colonies produced, giving a 147% ± 22% (mean ±SD, n = 8) of the colonies observed when IL-3 was added alone. Furthermore, the size (ie, the number of cells per colony) was markedly increased compared with assays in the presence of IL-3 alone. The morphology of the colonies formed in the presence of SCF was similar to those seen in serum-containing cultures (see Tables 1-3).

When G-CSF was combined with SCF, there was a greater than additive increase (P < .0002) in the number of colonies produced compared with the addition of either growth factor alone, and colony size was also increased.

Marked synergism was also seen with a combination of M-CSF and SCF. As has been reported elsewhere using enriched progenitor cell populations,21 in the absence of serum, M-CSF is unable to stimulate the formation of colonies from GM-CFC in soft gel assays (Fig 1). However, the addition of SCF to M-CSF stimulated the development of colonies containing predominantly macrophages. Thus, SCF, to a large degree, can replace the serum that is required for the colony development of GM-CFC in the response to M-CSF.

The addition of IL-1 (2 U/mL) or IL-6 (25 U/mL) did not affect the number of colonies produced from GM-CFC in the presence of SCF (0 to 250 ng/mL) (results not shown).

SCF can decrease the concentration of hematopoietic growth factor(s) required for colony formation. While GM-CFC cultured in optimal concentrations of the hematopoietic growth factors can in some cases exhibit an increase in colony-forming efficiency upon the further addition of SCF (Fig 1), this effect is far greater when lower doses of these growth factors are used. In the presence of SCF (250 ng/mL) plus IL-3 (5 U/mL), the number of colonies formed was equivalent to that seen in cultures where IL-3 (100 U/mL) was the sole growth stimulus (Fig 2). The morphology of the cells formed in colonies containing suboptimal doses of IL-3 plus SCF was similar to that described in Tables 1 through 3 for this combination of growth factors at optimal doses.

Similarly, SCF can not only increase the number and size of colonies formed in the presence of G-CSF, but also reduce the dose of G-CSF at which 50% of optimal colony formation was observed from greater than 1,000 U/mL to approximately 100 U/mL (Fig 2).

Results shown are the mean value from three experiments where 50 colonies were pooled.

Table 3. The Effect of Combinations of Hematopoietic Growth Factors on Cell Number per Colony Formed From GM-CFC

Influence of SCF on colony formation from GM-CFC in serum-deprived conditions. The ability of SCF (0 to 250 ng/mL) to promote the formation of colonies from highly enriched GM-CFC in serum-free conditions was evaluated. Colony-forming efficiency in the presence of IL-3 was 15%, approximately half of the value observed when both IL-3 and serum were present in the assays (see above). At an optimal concentration (100 ng/mL), SCF stimulated the formation of 20% ± 4% (mean ±SD, n = 8) of the colonies observed when IL-3 alone was present in the colony-forming assays; however, the size of the colonies was smaller than observed when the CFC assays contained IL-3 or GM-CSF. The combination of SCF and IL-3 had an additive effect on the number of colonies produced, giving a 147% ± 22% (mean ±SD, n = 8) of the colonies observed when IL-3 was added alone. Furthermore, the size (ie, the number of cells per colony) was markedly increased compared with assays in the presence of IL-3 alone. The morphology of the colonies formed in the presence of SCF was similar to those seen in serum-containing cultures (see Tables 1-3).

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SCF can maintain the colony-forming potential of GM-CFC. To establish if there was a period during the colony-forming assays when the developing GM-CFC were particularly sensitive to SCF, the addition of this growth factor was delayed for a period of 0 to 7 days (Table 4). In the absence of other growth factors (and in serum-deprived conditions), the majority of the GM-CFC were incapable of forming colonies when IL-3, G-CSF, or SCF, respectively, were added to the cultures after 24 hours, demonstrating the absolute dependence of these cells on hematopoietic growth factors for their survival, as has previously been reported for GM-CFC. However, when IL-3 was present throughout the assay and SCF was added after 0 to 6 days, there was a significant increase in the number of colonies observed 7 days after the SCF addition, except when SCF was added after 2 days. The reason for the lack of effect of SCF when added after 48 hours is not clear; however, the results do suggest that IL-3 is capable of maintaining a population of SCF-responsive cells. The response to SCF varied in that the size of the colonies formed progressively diminished as a function of the delay in the SCF addition. When G-CSF was present before the addition of SCF, there was a marked decrease in the number of colonies formed if the SCF addition was delayed for 1 or more days, indicating that G-CSF was incapable of maintaining an SCF-responsive population of colony-forming cells viable in these assays. When the reverse order of additions was made (ie, SCF was constantly present in the culture and the addition of either G-CSF or IL-3 was delayed), then the results were markedly different. SCF was capable of stimulating the formation of some colonies when added alone (Figs 1 and 2), but when IL-3 was added after several days (Table 4), there was an increase in the number of colonies formed compared with cells cultured in SCF alone. The SCF present in the colony-forming assays maintained the clonogenic potential of a proportion of the GM-CFC such that the further addition of IL-3 led to the formation of colonies 7 days later. Similarly, the delayed addition of G-CSF to SCF-containing cultures causes a significant increase in the number of colonies formed for up to 2 days, indicating that there is a discrete subset of GM-CFC maintained by SCF that can form colonies on the further addition of SCF.

To investigate this further, the ability of SCF to maintain colony-forming cells in liquid culture was studied. GM-CFC were seeded in serum-deprived or serum-containing cultures for a period of 5 days, during which time there was a marked change in the number of cells present in the cultures depending on the hematopoietic growth factor(s) added (when no growth factor was added the cells rapidly

<table>
<thead>
<tr>
<th>First Addition</th>
<th>Second Addition</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA</td>
<td>G-CSF</td>
<td>4 ± 1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SCF</td>
<td>G-CSF</td>
<td>57 ± 3</td>
<td>71 ± 3</td>
<td>49 ± 8</td>
<td>38 ± 10</td>
<td>35 ± 9</td>
<td>42 ± 1</td>
<td>44 ± 5</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>NA</td>
<td>SCF</td>
<td>32 ± 2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>NA</td>
<td>IL-3</td>
<td>96 ± 7</td>
<td>20 ± 0</td>
<td>1 ± 1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SCF</td>
<td>IL-3</td>
<td>144 ± 10</td>
<td>93 ± 1</td>
<td>70 ± 4</td>
<td>60 ± 14</td>
<td>54 ± 8</td>
<td>60 ± 12</td>
<td>58 ± 6</td>
<td>52 ± 2</td>
</tr>
<tr>
<td>G-CSF</td>
<td>SCF</td>
<td>44 ± 9</td>
<td>20 ± 4</td>
<td>3 ± 2</td>
<td>14 ± 4</td>
<td>8 ± 1</td>
<td>9 ± 1</td>
<td>9 ± 2</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>IL-3</td>
<td>SCF</td>
<td>130 ± 7</td>
<td>166 ± 8</td>
<td>70 ± 6</td>
<td>135 ± 24</td>
<td>138 ± 10</td>
<td>189 ± 10</td>
<td>178 ± 14</td>
<td>133 ± 22</td>
</tr>
</tbody>
</table>

Cells were plated in serum-deprived soft agar cultures in the presence of the first growth factor (= day 0), and, after the number of days shown, a solution containing the second growth factor was added to the plate to give the final concentrations shown in Table 2. The plates were then incubated for a further 7 days before scoring the resultant colonies. Results shown are the mean of three experiments ± SD and are expressed as a percentage of the total number of colonies (counted at 7 days) formed in the presence of IL-3 from the same enriched GM-CFC population used in each experiment.
culturing an enriched population of GM-CFC with various combinations of growth factors for a 5-day period. Elutriated cells were cultured at a concentration of $2 \times 10^3$/mL in a total volume of 1 mL of fetal calf serum. After incubation for 5 days, the cells were counted and also washed, and taken for GM-CFC assays to determine their cell number.

Mature neutrophils and macrophages with some blast cells died; Fig 3). When IL-3 alone was added to serum-containing cultures, there was a 110-fold increase in cell number (morphologically the cells were predominantly granulocytic colonies from an unfractioned population of normal murine bone marrow cells. 

Our results confirming the observation that neutrophilic colonies can be formed in response to SCF using a highly enriched population of GM-CFC further suggest that the SCF is acting directly on the GM-CFC and not by stimulating cytokine production from other cell types present in the bone marrow.

Unlike M-CSF (which binds to a receptor that is related to c-kit and stimulates the production of macrophage colonies from elutriated, enriched GM-CFC) the colony-stimulating activity of SCF does not require the presence of serum, suggesting that SCF acts directly to promote GM-CFC development into neutrophils without the requirement for comitogens or other developmental stimuli (although the possibility that SCF stimulates autocrine production of colony-stimulating factors in GM-CFC cannot be excluded at present). Indeed, one of the intriguing aspects of our results is the finding that two growth factors (M-CSF and SCF) that bind to highly related tyrosine kinase receptors can elicit such different developmental responses in the same progenitor cell population.

The synergistic interactions reported to occur between SCF and other hematopoietic growth factors on primitive hematopoietic progenitor cells were not evident when elutriated GM-CFC were used in soft agar assays. There was a small increase in the number of colonies formed when SCF was added with optimal concentrations of IL-3, GM-CSF, or M-CSF for colony formation (Fig 1). When suboptimal concentrations of G-CSF or IL-3 were added to colony-forming assays in the presence of SCF, the concentration of growth factor such as IL-3 or G-CSF required for optimal levels of colony formation was markedly reduced (Fig 2). Thus, SCF can increase the efficacy of these colony-stimulating factors and also modify the biological response by increasing the size of the colonies derived, as has previously been reported by McNiece et al and Metcalf and Nicola. Thus, SCF can clearly promote (albeit modestly) the proliferation and development of GM-CFC to make neutrophils; perhaps just as important in the actions of this cytokine is the ability to potentiate the response of progenitor cells to other colony-stimulating factors. There is evidence from in vivo studies with SCF to support this: when infused into mice, the combination of SCF plus G-CSF gives a greater than additive increase in the number of circulating neutrophils. Furthermore, SCF markedly decreases the dose of G-CSF required to

DISCUSSION

SCF, or kit ligand, has a profound effect on the proliferation and development of primitive hematopoietic cells such as those defined in the high proliferative potential-CFC (HPP-CFC), colony-forming unit-spleen (CFU-S), and blast cell colony-forming cell assays and also on the proliferation and development of erythroid and mast cell progenitor cells. Recently, SCF has also been shown to promote the formation of predominantly granulocytic colonies from an unfractioned population of normal murine bone marrow cells. 

Fig 3. The effect on cell number and clonogenic potential of culturing an enriched population of GM-CFC with various combinations of growth factors for a 5-day period. Elutriated cells were cultured at a concentration of $2 \times 10^3$/mL in a total volume of 1 mL of fetal calf serum. After incubation for 5 days, the cells were counted and also washed, and taken for GM-CFC assays to determine their cell number.
achieve the observed neutrophilia in vivo,26 just as there is a decreased requirement for G-CSF to stimulate colony formation in the presence of SCF (Fig 2). The experiments we have described using liquid cultures of GM-CFC in the presence of SCF (Fig 3), and those where the addition of growth factor to cells was delayed for 1 to 5 days, demonstrate that SCF can maintain a population of cells in soft agar that do not form colonies until a second stimulus is added (Table 4). This is a further indication that SCF acts as a survival factor with limited mitogenic effects, which can, in part, maintain the clonogenic potential of GM-CFC (see Table 4 and Fig 2). It is noteworthy that SCF has also been shown to facilitate the survival of primordial germ cells without promoting proliferation.28,29

The combination of SCF plus M-CSF stimulates the development of macrophages and not neutrophils, suggesting that the M-CSF developmental signal is dominant over the SCF-mediated signal to the GM-CFC. Another intriguing feature of the response of GM-CFC to SCF is the fact that neutrophilic colonies are formed, although there are no detectable SCF receptors present on metamyelocytes, even though these receptors are expressed on monocytic cells.15 The fact that mature neutrophilic cells are present in both liquid and soft agar cultures of enriched GM-CFC treated with SCF certainly implies that this growth factor can act at every stage of neutrophilic development or, alternatively, that the cells are producing a second stimulus (eg, G-CSF) in response to SCF. Recent data suggest that c-kit expression is suppressed by IL-3, GM-CSF, and G-CSF.30 It is possible that the immeasurably low levels of c-kit expression believed to be present on metamyelocytes may be due to the exposure of these cells to one or more of these cytokines before their isolation from normal bone marrow. The exact nature of the mechanism whereby SCF can promote terminal maturation of granulocyte/macrophage progenitor cells awaits further investigation.

Recent experiments with a neutralizing antibody to c-kit have demonstrated the importance of this receptor to hematopoiesis in vivo.31 The experiments we have described on neutrophil/macrophage progenitor cells show that this may in part be due to the ability of kit ligand to potentiate the response of hematopoietic progenitor cells to second growth factors in terms of the dose required to achieve a biological response and also to amplify the proliferative potential of the committed progenitor cell population. Furthermore, our data indicate that SCF can stimulate survival, proliferation, and development in an enriched neutrophil/macrophage progenitor cell population in the absence of other growth stimuli.

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REFERENCES


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