Stem Cell Factor Stimulates the In Vitro Growth of Bone Marrow Cells From Aplastic Anemia Patients

By Aleksandra Wodnar-Filipowicz, André Tichelli, Kristzina M. Zsebo, Bruno Speck, and Catherine Nissen

Aplastic anemia (AA) is a rare human bone marrow disorder of unknown etiology manifested by a strongly impaired growth of hematopoietic precursors. In this study, we examined the ability of recombinant human stem cell factor (SCF) to stimulate proliferation in vitro of bone marrow cells from 15 AA patients. All patients had been previously treated with antilymphocyte globulin (ALG), SCF, in combination with erythropoietin (Epo), interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF), increased the number of hematopoietic colonies formed in a semisolid medium by AA marrows. Maximal colony numbers reached 30% of the numbers observed with normal bone marrow cells. Proliferation of AA cells cultured in a liquid medium containing SCF together with Epo, IL-3, GM-CSF, and G-CSF approached 70% of the control level, as measured by 3H-thymidine incorporation. The effect of the combination of SCF with other growth factors was more than 10 times stronger than that of the growth factors alone. The most marked effect of SCF was on the generation of erythroid colonies by precursor cells. The results demonstrate synergy between SCF and other hematopoietic growth factors, resulting in the most efficient stimulation of in vitro growth of AA bone marrow cells described to date. Use of SCF, either alone or in combination with other factors, may be of potential value in treatment of AA.

© 1992 by The American Society of Hematology.

A PLASTIC ANEMIA (AA) is characterized by multilineage bone marrow failure resulting in pancytopenia. Although certain viruses, drugs, and chemical compounds have been implicated in the pathophysiology of AA, its actual cause remains unknown. Strongly impaired proliferative capacity of hematopoietic stem cells is a characteristic of the disease. In vitro, the hematopoietic colony formation by the bone marrow from AA patients is grossly reduced, the response to growth factors, like granulocyte-macrophage colony-stimulating factor (GM-CSF) or interleukin-3 (IL-3), is poor or absent, and stroma cells from some patients do not proliferate normally in culture. Absent or defective stem cells and an abnormal stromal microenvironment have, therefore, been postulated as the cause of AA, but the mechanism responsible for these abnormalities is not understood. Bone marrow transplantation offers virtually the only chance of cure. Immunosuppressive treatment induces a state of hematological remission that is fragile and prone to relapse. Hematopoietic growth factors, such as GM-CSF, granulocyte colony-stimulating factor (G-CSF), and IL-3, used recently in clinical trials for treatment of AA, although beneficial because of the transient increase of white blood cells they induce, cannot be considered curative treatment of aplasia.

Stem cell factor (SCF) is a recently identified hematopoietic growth factor, produced by marrow stromal cells. It is a ligand for c-kit, a cell surface receptor with tyrosine kinase activity. In the mouse, SCF is the product of the steel (S) locus, while c-kit is encoded by the white spotting (W) locus. In macrocytic anemia in S mice could be reversed by in vivo administration of SCF. A human homologue of the murine SCF has been shown to augment colony formation by normal human hematopoietic stem cells in culture and to synergize with other hematopoietic growth factors.

In this report, we describe the influence of recombinant human SCF alone, and in combination with other hematopoietic growth factors, on the growth of cultured bone marrow cells from AA patients. Results of the study, apart from suggesting the clinical value of SCF in treatment of AA, indicate that pathology at the level of SCF or its receptor c-kit may play a role in impaired hematopoietic proliferation in this disease.

MATERIALS AND METHODS

Patients. Fifteen patients with different stages of idiopathic acquired AA were included in the study (Table 1). All had been treated with horse antilymphocyte globulin (ALG; Lymphosar Berna, Bern, Switzerland) in combination with high doses of corticosteroids, according to a standard protocol. Before treatment, all patients were dependent on red blood cell and platelet transfusions. Eleven patients fulfilled the criteria for severe AA, as defined by Camitta et al; four patients had a hypoplastic marrow and severe thrombocytopenia. All patients, except for patient 15, became transfusion-independent as a primary response to ALG.

Bone marrow cells. Mononuclear cells were isolated from 10 mL of heparin-treated bone marrow by Ficoll-Hypaque (d = 1.077) density gradient centrifugation. Cells were cryopreserved in Iscove's modified Dulbecco's Medium (IMDM; Gibco, Paisley, UK) containing 40% fetal calf serum (FCS) and 20% dimethyl sulfoxide (DMSO). Before an experiment, cells were thawed (their viability was >80% in the trypan blue exclusion assay) and depleted of adherent cells by an overnight incubation in plastic adherent dishes with 25% FCS.

Hematopoietic colony formation assay. The nonadherent mono-
### Table 1. Patient Characteristics

<table>
<thead>
<tr>
<th>Patient No. (UPN)</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Neutrophils (× 10⁹/L)</th>
<th>Reticulocytes (× 10⁹/L)</th>
<th>Platelets (× 10⁹/L)</th>
<th>Time since ALG treatment (mo)</th>
<th>Hb (g/L)</th>
<th>Neutrophils (× 10⁹/L)</th>
<th>Reticulocytes (× 10⁹/L)</th>
<th>Platelets (× 10⁹/L)</th>
<th>Transfusion requirement</th>
<th>Current clinical condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (352)</td>
<td>71</td>
<td>F</td>
<td>0.3</td>
<td>15</td>
<td>72</td>
<td>12</td>
<td>14.1</td>
<td>14.0</td>
<td>11.7</td>
<td>254</td>
<td>No</td>
<td>REM</td>
</tr>
<tr>
<td>2 (356)</td>
<td>1</td>
<td>M</td>
<td>0.1</td>
<td>8</td>
<td>61</td>
<td>12</td>
<td>11.7</td>
<td>6.1</td>
<td>9.7</td>
<td>70</td>
<td>No</td>
<td>REM</td>
</tr>
<tr>
<td>3 (272)</td>
<td>19</td>
<td>A</td>
<td>0.2</td>
<td>61</td>
<td>38</td>
<td>12</td>
<td>11.7</td>
<td>6.1</td>
<td>9.7</td>
<td>70</td>
<td>No</td>
<td>REM</td>
</tr>
<tr>
<td>4 (217)</td>
<td>30</td>
<td>A</td>
<td>0.2</td>
<td>61</td>
<td>38</td>
<td>12</td>
<td>11.7</td>
<td>6.1</td>
<td>9.7</td>
<td>70</td>
<td>No</td>
<td>REM</td>
</tr>
<tr>
<td>5 (109)</td>
<td>9</td>
<td>A</td>
<td>0.2</td>
<td>61</td>
<td>38</td>
<td>12</td>
<td>11.7</td>
<td>6.1</td>
<td>9.7</td>
<td>70</td>
<td>No</td>
<td>REM</td>
</tr>
<tr>
<td>6 (350)</td>
<td>57</td>
<td>A</td>
<td>0.2</td>
<td>61</td>
<td>38</td>
<td>12</td>
<td>11.7</td>
<td>6.1</td>
<td>9.7</td>
<td>70</td>
<td>No</td>
<td>REM</td>
</tr>
<tr>
<td>7 (102)</td>
<td>11</td>
<td>A</td>
<td>0.2</td>
<td>61</td>
<td>38</td>
<td>12</td>
<td>11.7</td>
<td>6.1</td>
<td>9.7</td>
<td>70</td>
<td>No</td>
<td>REM</td>
</tr>
<tr>
<td>8 (183)</td>
<td>13</td>
<td>A</td>
<td>0.2</td>
<td>61</td>
<td>38</td>
<td>12</td>
<td>11.7</td>
<td>6.1</td>
<td>9.7</td>
<td>70</td>
<td>No</td>
<td>REM</td>
</tr>
<tr>
<td>9 (38)</td>
<td>14</td>
<td>A</td>
<td>0.2</td>
<td>61</td>
<td>38</td>
<td>12</td>
<td>11.7</td>
<td>6.1</td>
<td>9.7</td>
<td>70</td>
<td>No</td>
<td>REM</td>
</tr>
<tr>
<td>10 (240)</td>
<td>62</td>
<td>A</td>
<td>0.2</td>
<td>61</td>
<td>38</td>
<td>12</td>
<td>11.7</td>
<td>6.1</td>
<td>9.7</td>
<td>70</td>
<td>No</td>
<td>REM</td>
</tr>
<tr>
<td>11 (36)</td>
<td>7</td>
<td>A</td>
<td>0.2</td>
<td>61</td>
<td>38</td>
<td>12</td>
<td>11.7</td>
<td>6.1</td>
<td>9.7</td>
<td>70</td>
<td>No</td>
<td>REM</td>
</tr>
<tr>
<td>12 (202)</td>
<td>14</td>
<td>A</td>
<td>0.2</td>
<td>61</td>
<td>38</td>
<td>12</td>
<td>11.7</td>
<td>6.1</td>
<td>9.7</td>
<td>70</td>
<td>No</td>
<td>REM</td>
</tr>
<tr>
<td>13 (374)</td>
<td>40</td>
<td>A</td>
<td>0.2</td>
<td>61</td>
<td>38</td>
<td>12</td>
<td>11.7</td>
<td>6.1</td>
<td>9.7</td>
<td>70</td>
<td>No</td>
<td>REM</td>
</tr>
<tr>
<td>14 (258)</td>
<td>15</td>
<td>A</td>
<td>0.2</td>
<td>61</td>
<td>38</td>
<td>12</td>
<td>11.7</td>
<td>6.1</td>
<td>9.7</td>
<td>70</td>
<td>No</td>
<td>REM</td>
</tr>
<tr>
<td>15 (331)</td>
<td>33</td>
<td>A</td>
<td>0.2</td>
<td>61</td>
<td>38</td>
<td>12</td>
<td>11.7</td>
<td>6.1</td>
<td>9.7</td>
<td>70</td>
<td>No</td>
<td>REM</td>
</tr>
</tbody>
</table>

**Abbreviations:** A, androgens; CyA, cyclosporine A; Pr, prednisolone; REM, remission; partial relapse, relapse without transfusion requirement; PNH, paroxysmal nocturnal hemoglobinuria; MDS, myelodysplastic syndrome.
nuclear fraction of the bone marrow cells was incubated at 5 × 10⁴ cells/mL in IMDM containing 1% methylocellulose, 16% FCS, 1% (vol/vol) deionized, delipidated bovine serum albumin (BSA; Behring, Marburg, Germany), and 380 ng/mL iron-saturated human transferrin (TF, Behring), as described by Iscove et al.22 Human erythropoietin (Epo), 1.4 U/mL (Connaught Laboratories Ltd, Willowdale, Ontario, Canada), human recombinant hematopoietic growth factors (see below), or 10% of the conditioned medium (CM) from human peripheral blood mononuclear cells stimulated with 1% phytohemagglutinin (PHA) and 20% autologous serum, were added. After 14 days in culture, hematopoietic colonies were counted. Comparison of the results obtained with bone marrow cells before and after cryopreservation indicated that their ability to form hematopoietic colonies had been fully retained.

Recombinant human hematopoietic growth factors. SCF was supplied by AMGEN (Thousand Oaks, CA), and was tested at the concentrations given in the Figure legends. IL-3 and GM-CSF were gifts from Sandoz (Basel, Switzerland) and were each used at 25 U/mL. G-CSF was from Rhone-Poulenc (Paris, France) and was used at 2.1 ng/mL.

³H-thymidine incorporation assay. Bone marrow cells, 5 × 10⁴, were cultured in 96-well microtiter plates (in 100 μL) as described above for the colony formation assay, except that methylocellulose was omitted. Cells were pulsed with 6 hours with 0.5 μCi of ³H-thymidine (87 Ci/mmol, Amersham, Buckinghamshire, UK) per well, harvested, and ³H incorporation was determined, as described.23

Flow cytometric immunophenotyping. Cells were cultured for 12 days, washed extensively in phosphate-buffered saline (PBS), and analyzed with the FACSscan (Becton Dickinson, Heidelberg, Germany) after staining of 1 × 10⁶ cells with fluorescein-isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal mouse anti-human antibodies. Monoclonal antibodies included glycoporphin A/FITC (IgG1; Dako, Glostrup, Denmark), transferrin receptor (TFR)/FITC (CD71; IgG2a; Becton Dickinson), and Leu-M9/PE (CD33; IgG2b; Becton Dickinson). Control samples for fluorescence intensity were polyvalent IgG1/FITC and IgG2/PE-conjugated (Becton Dickinson).

RESULTS

Effect of growth factors on cell proliferation: formation of hematopoietic colonies. Bone marrow cells from 15 AA patients and, as a control, from five healthy bone marrow donors were cultured in methylocellulose and their ability to form colonies was tested using various stimulating conditions (Fig 1). The response to PHA-induced CM was reduced in all cultures from aplastic patients; the number of colonies varied between 9 and 27, compared with more than 50 formed by control cells (Fig 1A; AA marrows are arranged in the order of decreasing response to CM). The effect of the following human recombinant growth factors was assayed: SCF, a mixture of three hematopoietic growth factors (IL-3, GM-CSF, and G-CSF, called hereafter HGFs), and a combination of both (SCF + HGFs). All cultures contained Epo, since its presence is required for the colony-stimulating activity of SCF17 (data not shown). Control cells formed approximately 40 colonies per 5 × 10⁴ seeded cells in response to SCF, and approximately 120 colonies in response to SCF + HGFs. Aplastic marrows responded to SCF, although wide variations were observed. Colony formation by the majority of them improved in comparison to the response to CM; the increase was most apparent in cases where marrow responded poorly to CM. The highest colony numbers (13 to 31 colonies, corresponding to 12% to 27% of the control level; see patients 1 through 11, Table 1) were observed when both SCF and HGFs were present. In four cases (patients 12 through 15), the response was very weak, and at most six colonies were formed. Dose-response experiments (Fig 1B) confirm that the AA marrows respond to SCF, although their sensitivity is lower as compared with control cells. Colony formation by the control marrows reached a plateau at approximately 10 to 25 ng SCF/mL, while higher concentrations of SCF were required to stimulate efficient growth of the AA marrows.

Effect of growth factors on cell proliferation: ³H-thymidine incorporation in liquid culture. Measurements of the effects of SCF on bone marrow colony formation are semi-quantitative. SCF causes not only an increase of colony numbers, but also of colony size, leading to formation of more than 100 colonies at 100 ng SCF/mL, while only sparse colonies developed at 10 ng SCF/mL. A combination of SCF and HGFs further increased the number of colonies. The number of colonies formed in response to SCF + HGFs was significantly higher than those formed in response to SCF alone, especially at low concentrations of SCF.

Fig 1. Colony formation by bone marrow cells in response to different stimulating conditions. Colonies of more than 50 cells were scored on day 14. The values presented are the mean of duplicate determinations. All cultures contained Epo at 1.4 U/mL. CM and HGFs (IL-3, GM-CSF, and G-CSF) were used at concentrations given in Materials and Methods. (A) The number of colonies formed by marrows of 11 AA patients (no. 1 through 11) and five controls in the presence of 1, 10, 25, 50, and 100 ng SCF/mL. (B) The number of colonies formed by marrows of 11 AA patients (no. 1 through 11) and five controls in the presence of 1, 10, 25, 50, and 100 ng SCF/mL.
cultured in liquid medium was measured (Fig 2). The stimulatory effect of SCF, compared with that of HGFs, was more pronounced in liquid culture than in the colony assay, most likely reflecting the large number of cells present in the SCF-induced colonies. A combination of SCF with HGFs resulted in a further, often significant, enhancement of 3H-thymidine incorporation, which was markedly stronger in aplastic than in control cultures. In aplastic cultures, an average 13-fold higher level of incorporation was seen with SCF + HGFs, compared with HGFs alone; in the control cultures, the difference was only 2.5-fold. Three aplastic marrows (patients 12 through 14) responded poorly to the growth factors in liquid medium; the same marrows formed very few colonies in methylcellulose (see Fig 1A).

Based on the effect of SCF on cell proliferation in liquid cultures, AA marrow cells can be subdivided into three groups: (I) good responders (patients 2, 3, 4, 7, and 10), with the level of 3H-thymidine incorporation corresponding to 50% to 70% of control cells; (II) moderate responders (patients 1, 5, 6, and 8), with 10% to 20% 3H-thymidine incorporation of control cells; and (III) nonresponders (patients 12, 13, and 14). We have compared the time course and the effect of factor concentration on cell growth in the three groups of marrows and in control cultures (Fig 3). 

Effect of SCF on types of cells induced in cultured aplastic marrow. SCF stimulated the formation of similar colony types in aplastic and control marrows (Fig 4). Together with Epo, it led almost exclusively to the outgrowth of erythroid...
bursts. In cultures containing SCF in combination with Epo and HGFs, the erythroid bursts constituted 40% to 60% of all colonies; their number increased with increased SCF concentration. The absolute number of nonerythroid colonies induced by HGFs alone or SCF combined with HGFs was similar (data not shown). This indicates that the SCF-induced outgrowth of erythroid colonies from hematopoietic progenitors did not occur at the expense of other colony types.

Results of the colony assays were confirmed by immunophenotype analysis of the cells grown in liquid culture (Table 2). The erythroid-specific cell surface markers glycoporphin A and the transferrin receptor (TFR) were detected on 80% to 90% of the cells grown in the presence of SCF and Epo. A small fraction of CD33+ myeloid cells was present in cultures containing both SCF and HGFs. The proportion of cells belonging to the erythroid lineage, evaluated by FACS analysis, was higher than that estimated from the colony assay (Fig 4), most likely due to the formation of large colonies by erythroid, but not myeloid, precursors.

**DISCUSSION**

In severe AA, growth of hematopoietic cells ceases completely, and thus experiments concerning proliferation are not feasible. An alternative approach for the study of impaired cell proliferation is offered by the bone marrow cells from patients treated with high-dose immunosuppression. Therapy with ALG, cyclosporine A, or both induces a state of hematological remission, which rarely means "cure." Most patients continue to have mild clinical signs of impaired hematopoiesis and, in virtually all cases, hematopoietic colony formation remains grossly reduced. Assuming that the residual proliferation defect after immunosuppressive treatment reflects the pathophysiology of the original disease, we studied the effect of SCF and other hematopoietic growth factors using bone marrow from patients with AA who underwent therapy with ALG.

Colony formation by precursor cells from all 15 AA marrows tested in vitro was far below normal after stimulation with PHA-induced CM, reflecting a strongly reduced proliferation capacity of hematopoietic cells in AA. Addition of three recombinant hematopoietic growth factors, IL-3, GM-CSF, and G-CSF (HGFs), brought only a limited improvement of cell growth. Good recovery of proliferation of AA marrows was achieved by a combination of HGFs and Epo with a newly discovered hematopoietic growth factor, SCF, leading to an average 3.3-fold increase of colony formation as compared with the effect of HGFs. Despite differences in colony numbers formed by individual bone marrow cultures, the results represent the strongest improvement of the in vitro growth of AA cells observed so far. Synergism between SCF and several growth factors acting on early precursor cells has been established by previous studies on colony formation by normal human marrow cells, both unfractuated and the CD34+ fraction of immature progenitors. We have observed that the combination of SCF with other factors is also advantageous for growth of AA marrows compared with the effect of SCF alone (mean increase of 2.5-fold). However, under the conditions employed, none of the 15 AA marrows tested reached normal proliferation capacity. Moreover, cells from four patients virtually did not respond to stimulation. We have not observed any correlation between the in vitro response to SCF and the severity of the disease at presentation. However, we do currently observe a poor clinical status of those patients that did not respond to SCF in vitro (patients 12 through 15). Only one of these patients is in remission, one has partially relapsed, one developed paroxysmal nocturnal hemoglobinuria, and one died (see Table 1).

Improved hematopoietic growth of AA marrows was primarily reflected by formation of erythroid colonies, which was also confirmed by FACS analysis of the cell surface markers. The property of SCF to stimulate erythropoiesis has been described previously for normal marrow. Erythroid bursts appearing in the presence of SCF and Epo were composed of large spherical subcolonies, the sizes of

---

**Table 2. Flow Cytometric Analysis of Bone Marrow Cell Surface Markers**

<table>
<thead>
<tr>
<th>Erythroid markers</th>
<th>SCF + EPO</th>
<th>SCF + HGFs + EPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycoporphin A+</td>
<td>86</td>
<td>90</td>
</tr>
<tr>
<td>TFR+/CD33–</td>
<td>87</td>
<td>83</td>
</tr>
<tr>
<td>Myeloid markers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD33+</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>TFR+/CD33–</td>
<td>3</td>
<td>8</td>
</tr>
</tbody>
</table>

Bone marrow cells, control (C) and aplastic (AA), were cultured in liquid medium and analyzed by FACS as described in Materials and Methods. The results are expressed as percent of cells carrying a given cell surface antigen in a total cell population. No lymphoid markers (T, B, and non-T, non-B cells) were expressed (not shown).
which did not resemble bursts formed in the presence of other HGFs. For this reason, the stimulatory effect of SCF as measured by the amount of thymidine incorporated by growing cells outweighed the improvement of growth as judged by erythroid colony numbers. Among AA marrows, which did not resemble bursts formed in the presence of stem cell factor and aplastic anemia. SEMIN HEMATOLOGIC OPINIONS 3201

Other HGFs. For this reason, the stimulatory effect of SCF growing cells outweighed the improvement of growth as preferentially stimulated neutrophil colonies, which is in agreement with recent reports on high murine neutrophil counts in vitro and neutrophilia induced in vivo.

Our results support the idea, which has been suggested from animal models,6-9 of the therapeutic potential of SCF and indicate that SCF could be of value as a stimulator of erythropoiesis in vivo in human disease. SCF may have a place in the treatment of anemia due to aplasia not responding to current treatment. GM-CSF, G-CSF, and IL-3 stimulate neutrophoiesis in AA patients, provided they have minimal residual bone marrow function.12 Erythroid stimulation is described in some patients on long-term G-CSF treatment,13 but is not the primary goal of treatment with either G-CSF, GM-CSF, or IL-3. Like other HGFs, which do not cure AA, but are supportive in carrying the patient through infective complications, SCF may be of benefit in reducing red blood cell transfusion requirements in AA patients, without necessarily altering the disease course.

It should be emphasized that the bone marrow samples used in our experiments are derived from patients who had partially recovered from the disease, following ALG therapy. It remains to be determined whether cells derived from untreated patients show a response to SCF similar to that reported in this study. Nevertheless, despite improved clinical condition of the ALG-treated patients, cells from none of them responded to SCF with a sensitivity comparable to that of the normal bone marrow cells. It is therefore tempting to speculate that the hematopoietic defect in AA is associated with abnormalities affecting the SCF receptor, c-kit, or its intracellular transduction pathway. The differences in the in vitro responses to SCF observed with marrows from individual aplastic patients may reflect a heterogeneity in the pathogenesis of AA. It is reminiscent of the heterogeneity of murine W mutants carrying different mutations of the c-kit gene and, consequently, having the c-kit tyrosine kinase activity impaired to various degrees. Conclusive arguments as to the role of SCF and c-kit in deregulation of hematopoietic environment must await the molecular analysis of expression of these genes in AA. In view of a crucial function postulated for the c-kit receptor and SCF in normal hematopoiesis, AA—a disease of failing hematopoiesis—appears to be an interesting human model for studying the function of this ligand/receptor system.

ACKNOWLEDGMENT

We thank Dr. Nancy Hynes for critical reading of the manuscript and yolanda Moser and Verena dalle Carbonare for technical assistance.

REFERENCES


Stem cell factor stimulates the in vitro growth of bone marrow cells from aplastic anemia patients

A Wodnar-Filipowicz, A Tichelli, KM Zsebo, B Speck and C Nissen