**In Vitro Stimulation of Erythropoiesis by Stem Cell Factor Alone in Myelodysplastic Syndrome Patients with Elevated Endogenous Erythropoietin Serum Levels**

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INTRODUCTION

MDS\(^1\) represents a clonal disorder of an early hematopoietic progenitor cell, leading to abnormal maturation of all hematopoietic cell lines. With expansion of the malignant clone, progressive cytopenia reflecting ineffective hematopoiesis develops and the tendency for transition to overt leukemia increases. The course of MDS is highly variable (1), with some patients remaining stable or having slowly progressive disease and others with rapidly progressive disease.

The prognosis in MDS is closely correlated with the bone marrow blast cell count (2), cytogenetic abnormalities (3), and cytopenia, leading to an increased risk for lethal infections or bleeding (4). The majority of MDS patients usually demonstrate a hyperchromic, macrocytic anemia which often requires regular blood transfusions (5). Since MDS is a disease of mainly elderly individuals, these patients tend to also suffer from ischemic disorders, suggesting the need for consequent blood transfusions. RBC transfusions, however, are accompanied by the risk of transferring viral infections, iron overload, and allergic reactions.

Although many clinical trials have focused on differentiating agents, low-dose cytotoxic arabinoside (1β-D-arabinofuranosylcytosine) or intensive chemotherapy, no generally accepted treatment is available for MDS except allogeneic bone marrow transplantation (6). In the majority of cases, the need for therapy is dictated not by development of overt leukemia, but by dyserythropoiesis, leading to anemia, neutropenia, and/or thrombocytopenia, and therefore several clinical trials have investigated the potential effects of hematopoietic growth factors in MDS. G-CSF (7), GM-CSF (8, 9), and IL-3 (10–12) have proven to stimulate myelopoiesis, but usually do not stimulate erythropoiesis in MDS patients in vivo. Clinical trials with the application of Epo have demonstrated responses in only a limited number of MDS patients (13–15). This rather low response rate is not unexpected because the majority of MDS patients demonstrate significantly increased endogenous serum Epo levels (13), suggesting that other growth factors may be required to stimulate erythropoiesis further. A combination of Epo with G-CSF (16) or GM-CSF (17), although superior to a single treatment with Epo, significantly stimulates erythropoiesis in only up to 40% of the patients. Therefore, additional growth

\(^1\) The abbreviations used are: MDS, myelodysplastic syndrome; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage CSF; IL, interleukin; Epo, erythropoietin; SCF, stem cell factor; FAB, French-American-British; RA, refractory anemia; RAEB, refractory anemia with excess of blasts; RAEBt, refractory anemia with excess of blasts in transition; CMML, chronic myelomonocytic leukemia; MNC, mononucleated cell; mU, milliunits; SAA, severe aplastic anemia.
factors or their combinations are warranted to stimulate in vivo erythropoiesis in MDS patients.

SCF, also designated c-kit ligand or mast cell growth factor (18), has only limited colony-stimulating activity on its own but significantly stimulates in vitro colony formation of erythroid progenitor cells induced by Epo and myeloid progenitors induced by other hemopoietic growth factors like G-CSF, GM-CSF, or IL-3 (19, 20). Decreased endogenous SCF serum levels have been detected in aplastic anemia patients (21), suggesting that deficient production of SCF may contribute to hypoplasia in this disorder.

Aiming to characterize the potential of the bone marrow to release SCF in myelodysplasia and detect a possible role of SCF in the pathogenesis of MDS, we measured the endogenous SCF serum level in normal individuals and MDS patients. In view of the recent data on the in vitro stimulation of erythropoiesis with a combination of SCF and Epo (22, 23), we also studied the ability of SCF alone to stimulate erythroid colony formation in the presence of autologous serum obtained from MDS patients with a high endogenous Epo level.

PATIENTS AND METHODS

Patient Selection

To determine endogenous Epo or SCF serum levels, serum was collected from peripheral blood after centrifugation for 10 min at 2500 rpm. Samples from patients or volunteers were aliquoted and stored at −80°C until used; analysis was performed immediately after thawing.

For the colony assay, bone marrow cells aspirated from the iliac crest were obtained from 7 healthy volunteers and 20 MDS patients who had given informed consent. Patients were classified according to FAB criteria (2) into these groups: RA, refractory anemia with ring sideroblasts, RAEB, RAEBt, and CMML. Four patients presented with RA, five with RAEB, seven with RAEBt, and four with CMML. None of them suffered from infection, nor had they received blood transfusions, differentiating agents, cytotoxic drugs, and/or cytokines during the previous 6 weeks.

Cytokine Serum Levels

SCF. Serum samples were obtained from 17 healthy individuals and from 50 patients suffering from MDS (18 RA, 12 RAEB, 12 RAEBt, and 8 CMML). SCF serum concentrations (24) were determined using a commercially available quantitative sandwich enzyme immunoassay (Quantikine; R&D Systems, Inc., Cambridge, MA). With the assay described, the normal range of SCF had been determined by the manufacturer in the serum of 40 healthy blood donors and showed a range of 1164–1912 pg/ml with an average level of 1507 pg/ml. These values are likely to reflect the activation of SCF-producing cells like stromal cells, endothelial cells, and hepatocytes normally occurring upon physiological stimuli.

Epo. Endogenous Epo serum levels were measured in six patients (two RA, two RAEB, and two RAEBt) using a commercially available enzyme immunoassay (DRG Instruments, Freiburg, Germany). The normal range as indicated by the manufacturer is 6–25 mU/ml, with an average level of 12 mU/ml.

Cytokines

The following recombinant human hemopoietic growth factors/cytokines derived from Escherichia coli were used for stimulation of hemopoietic progenitor cell growth: SCF (provided by Amgen Ltd.) and Epo (provided by Cilag). All factors were derived from the same batch and applied at a pretested optimum stimulatory concentration determined for each factor (data not shown): SCF, 10 ng/ml and Epo, 2 mU.

Clonogenic Assay

Bone marrow MNCs were obtained by density gradient centrifugation as described previously (25). A soft agar culture system was used to cultivate hemopoietic progenitor cells (26). In brief, MNCs were suspended in McCoy’s 5A medium containing 10% FCS and 0.3% agar (Agar-Agar; Merck, Darmstadt, Germany). Subsequently, 250 μl of this suspension containing 1.5 × 10³ cells was plated into wells of multwell tissue culture plates (Nunc, Copenhagen, Denmark). The agar was allowed to solidify at room temperature and overlaid with 250 μl medium containing 10% FCS/5 × 10⁻³ M 2-mercaptoethanol and supplemented with cytokines to be tested. The culture plates were incubated for 14 days at 37°C in a fully humidified atmosphere containing 5% CO₂. At the end of the incubation, 1 ml 2.5% glutaraldehyde was injected into the well for 8 min to detach and fix the agar layer. The agar pellicle was then washed with distilled water, placed on a microscope slide, dried in an incubator (50°C), and stained according to Pappenheim.

Medium containing FCS without addition of any cytokines was used for negative control, whereas for positive control of colony growth, medium containing a pretested serum batch from patients with SAA with a high level of colony-stimulating activity was applied. The SAA serum was diluted in medium to a final concentration of 10%.

Cell aggregates of more than 60 hemoglobin-containing cells were scored as BFU-E. All specimens were tested in triplicate, and values represent the means of the results. Colony growth was evaluated by two independent blinded reviewers.

Progenitor Cell Growth after Incubation with Autologous Serum

In six MDS patients (three RA, two RAEB, and one RAEBt) with elevated endogenous Epo levels, bone marrow MNCs were cultured as described above in the presence of 10 ng/ml SCF, and FCS was completely replaced by autologous serum. Serum Epo levels were 50, 950, 1500 mU/ml in patients with RA, 210 and 4050 mU/ml with RAEB, and 320 mU/ml with RAEBt. MNCs from the patient with the serum Epo level of 1500 mU/ml were incubated with 10 ng/ml SCF and increasing concentrations of autologous serum to detect a possible dose dependency.

Statistical Analysis

Results of single groups are summarized as median and range values. For statistical evaluation, nonparametric tests were used and calculated with the StatView software package on an Apple Macintosh computer. Comparisons between the groups were performed with the Kruskal-Wallis test. A P value of 0.05 or less was estimated as significant correlation.
RESULTS

Cytokine Serum Levels

Endogenous SCF Serum Levels. Using the enzyme immunoassay described above, the SCF serum concentration was determined in 50 MDS patients (18 RA, 12 RAEB, 12 RAEBt, and 8 CMML), and the results were compared with the levels obtained in 17 healthy individuals. All sera demonstrated a detectable SCF level within the normal range as defined by the manufacturer (Fig. 1).

Broad interindividual variations were observed with SCF values ranging in healthy volunteers between 942 and 1987 (median, 1343) pg/ml and in MDS patients between 662 and 1220 (median, 1308 pg/ml; RA, 877–2280 pg/ml; median, 1240; RAEB, 990–1761 pg/ml; median, 1367.5; RAEBt, 834–2220 pg/ml; median, 1361; and CMML: 662–2200 pg/ml, median, 1230.5). Endogenous SCF serum levels did not significantly differ between healthy volunteers and MDS patients. No correlation between SCF serum levels and peripheral blood counts (neutrophils, platelets, and hemoglobin) nor with age, FAB status, or cytogenetic abnormalities (data not shown) was detected in our series.

Endogenous Epo Serum Levels. In all six MDS patients investigated, endogenous Epo serum levels were significantly elevated, with values ranging between 50 mU and 4050 mU (normal, 6–25 mU/ml).

Clonogenic Assay

Characteristics of the 20 MDS patients investigated are summarized in Table 1. No correlation between the presence of cytogenetic abnormalities or endogenous SCF levels and progenitor cell growth (BFU-E or colony-forming unit, granulocyte-monocyte) was detected regarding the effects of the cytokines investigated (data not shown). With the exception of CMML, where a usually significantly reduced progenitor cell growth was found as compared with the other FAB types, growth patterns could not be correlated with FAB classification but were usually decreased in MDS patients as compared with healthy controls.

Effect of Epo with or without SCF on Growth of BFU-E. When bone marrow MNCs obtained from healthy volunteers were stimulated with SCF alone, no significant stimulatory effect was determined, whereas stimulation with Epo alone led to a significant (P = 0.02) increase in BFU-E number but not of cells per colony. Addition of SCF plus Epo induced an additional significant increase of the BFU-E number (P = 0.04) and also a significantly increased number of cells per colony as compared with the addition of Epo alone (Fig. 2).

Similar results were obtained from 16 MDS patients suffering from RA, RAEB, or RAEBt (Fig. 3). In contrast to

Table 1 Patient characteristics

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* F, female; M, male; n.d., not done.
stimulation with Epo \( (P = 0.0003) \), SCF alone was not able to significantly increase the number of BFU-E as compared with controls. When Epo was combined with SCF, the number of erythroid colonies \( (P = 0.04) \) and the number of cells per colony showed a significant increase. Like in the assays performed on bone marrow from healthy individuals, in MDS patients only an estimate of the exact number of cells per colony was made because of how tightly packed the cells were.

Basically, the same data were obtained when the progenitor cell assay was performed with bone marrow MNCs obtained from four patients with CMML. Incubation with Epo resulted in a significant stimulation in the BFU-E number \( (P = 0.04) \) that was further increased \( (P = 0.05) \) by adding Epo plus SCF. The defect in erythroid colony formation, however, was obviously expressed more in CMML as compared with the other FAB types (Fig. 4) because even stimulation of BFU-E with Epo and SCF resulted in relatively low erythroid growth.

**Effect of SCF plus Autologous Serum on BFU-E.** In six patients with high endogenous Epo level between 50 and 4050 mU/ml, bone marrow MNCs were incubated in the presence of autologous serum and SCF. In all of these patients, an increase of the BFU-E number was detected. This increase, however, never reached the level of the stimulatory effect of the combination of Epo and SCF, but was significantly higher \( (P < 0.05) \) than for Epo alone (Fig. 5).

In one of these patients with an elevated endogenous Epo level \((1500 \text{ mU/ml})\), stimulation of erythropoiesis was shown to be dependent from the amount of autologous serum supplied (Fig. 6).

**Influence of Serum Obtained from Patients with Severe Aplastic Anemia.** Although the number of cells per colony after incubation with Epo plus SCF could not be counted exactly because they were too tightly packed, the increase in cell num-

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**Fig. 2** Effect of 10 ng/ml SCF, 2 mU/ml Epo, and combination of both cytokines on erythroid progenitor cell growth (BFU-E) in seven healthy individuals. Erythroid progenitor cell growth is expressed in absolute numbers of BFU-E obtained after plating \(1.5 \times 10^5\) MNCs. Compared to incubation with SCF alone, a significant increase of BFU-E was found after stimulation with Epo \( (P = 0.02) \) and an additional increase \( (P = 0.04) \) was found when Epo plus SCF was added.

**Fig. 3** Effect of 10 ng/ml SCF, 2 mU/ml Epo, and combination of both cytokines on erythroid progenitor cell growth (BFU-E) in MDS patients (four RA, five RAEB, and seven RAEBt). Erythroid growth was significantly \( (P = 0.0003) \) stimulated by Epo alone, and an additional increase \( (P = 0.04) \) was found when Epo plus SCF was added.

**Fig. 4** Effect of 10 ng/ml SCF, 2 mU/ml Epo, and combination of both cytokines on erythroid progenitor cell growth (BFU-E) in four CMML patients. Erythroid growth was significantly \( (P = 0.04) \) stimulated by Epo alone, and an additional increase \( (P = 0.05) \) was found when Epo plus SCF was added.

**Fig. 5** Effect of 10 ng/ml SCF, 2 mU/ml Epo, 10 ng/ml SCF plus autologous serum, and combination of 10 ng/ml SCF and 2 mU/ml Epo on erythroid progenitor cell growth (BFU-E) in MDS patients. Endogenous Epo serum levels (mU/ml) are indicated and refer to individual stimulation of erythroid progenitor cell growth.

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duce and secrete OM-CSF or IL-6 (29) quarter of MDS patients independent of their peripheral neutrophil or monocyte count (31), and endogenous serum IL-6 levels in MDS patients are correlated with blast cell count and prognosis (32). Thus, these cytokines may be involved in autocrine/paracrine growth stimulation, not only in acute myeloid leukemia but also in MDS.

With regard to SCF, which is mainly produced by bone marrow stromal cells (18), it cannot be ruled out that its overexpression might be involved in the pathogenesis of MDS by acting as a paracrine growth factor for the malignant clone. On the other hand, SCF deficiency may prevent later-acting hemopoietic growth factors by triggering cells along the maturation and differentiation pathways. The latter hypothesis is supported by the fact that bone marrow in MDS is hypercellular and usually contains an increased number of immature cells including myeloblasts.

Our data demonstrate that SCF serum levels in MDS patients are within the normal range produced by physiological stimuli and do not correlate with peripheral blood counts, FAB stage, or cytogenetic abnormalities. SCF overexpression or deficiency thus does not seem to be involved in the pathogenesis of MDS, although minor changes in the hemopoietic microenvironment cannot be completely ruled out.

In the vast majority of bone marrow obtained from MDS patients, in vitro erythroid colony formation is severely suppressed (26). Recently published results showed SCF in combination with Epo to have a stimulatory effect on erythroid progenitor cells obtained from patients with MDS, and SCF alone did not lead to an increase in BFU-E (22, 23). In one of these reports (22), a correlation of the BFU-E growth and FAB stage, or cytogenetic abnormalities. SCF overexpression or deficiency thus does not seem to be involved in the pathogenesis of MDS, although minor changes in the hemopoietic microenvironment cannot be completely ruled out.

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Because endogenous Epo serum levels are elevated in the majority of MDS patients, we aimed to work out whether the Epo serum concentration in these patients is sufficient to synergistically stimulate erythropoiesis with exogenously supplied

DISCUSSION

The management of anemia in MDS patients is a major clinical problem. Although RBC transfusions are often the basis of supportive care, frequent transfusions carry the risk of chronic iron overload or anaphylactic reactions, and transfer of viral infections cannot be completely ruled out. As shown in several clinical trials, the majority of MDS patients do not respond to Epo alone (13–15) nor in combination with G-CSF or GM-CSF (16, 17), since erythropoiesis in response to these factors is severely impaired in vitro (27) and in vivo. Recently, two reports have shown the ability of SCF to stimulate erythropoiesis in MDS patients in combination with Epo (22, 23). In view of this data, we attempted to investigate a potential role of SCF in the pathogenesis of MDS and tried to define further its possible role for in vivo application.

Despite extensive research during the past decade, the pathogenesis of MDS is still not completely understood. As a fascinating general concept for the origin and nature of cancer, the autocrine model envisages an affected cell producing an appropriate level of a growth factor to which it can respond, thus leading to self-stimulated proliferation (28). It has been argued that defects in hemopoietic growth factor regulation might be involved in myeloid malignant disorders. Recent studies have shown that malignant acute myeloid leukemia blasts may produce and secrete GM-CSF or IL-6 (29) in vitro and also express the receptors of these cytokines on their surface (29, 30). GM-CSF serum levels were found to be elevated in about one-quarter of MDS patients independent of their peripheral neutrophil or monocyte count (31), and endogenous serum IL-6 levels in MDS patients are correlated with blast cell count and prognosis (32). Thus, these cytokines may be involved in autocrine/paracrine growth stimulation, not only in acute myeloid leukemia but also in MDS.

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Fig. 7 Erythroid progenitor cell growth after incubation with FCS (a); with 10 ng/ml SCF plus 2 mU/ml Epo, ×100 (b); and with serum gained from SAA patients, ×500 (c). Optimal induction of differentiation is obtained after exposure to SAA serum.
SCF. Our data suggest that autologous serum in combination with SCF seems to exert a dose-dependent stimulatory effect on erythropoiesis which correlates with the endogenous Epo level. When the highest concentration of autologous serum is used, stimulation of erythroid growth is significantly more expressed than after the addition of Epo alone even at a pretested optimum concentration. An influence of cytokines other than Epo cannot be entirely excluded. Since SCF, however, has only limited hemopoietic growth factor activity and mainly primes erythroid growth for the later-acting Epo (20), the influence of other factors seems rather unlikely.

Although the combination of SCF and Epo is still more effective than SCF plus autologous serum, our data clearly suggest a possible in vivo role of SCF alone in MDS patients with high endogenous Epo serum levels. This may have important implications for the design of possible future clinical trials, which seem even more warranted because granulopoiesis is also significantly induced by SCF alone, with no correlation to the FAB group (23). Application of SCF alone to patients with high endogenous Epo serum levels should therefore enhance both erythropoiesis and myelopoiesis in vivo.

When SCF and Epo are replaced with serum obtained from patients with severe aplastic anemia with high endogenous hemopoietic growth stimulatory activity, no additional stimulation of erythroid growth is detected, but hemoglobinization is significantly more expressed. This suggests that besides Epo and SCF, either another factor or, what seems more probable, the correct balance of cytokines influencing erythropoiesis is required for optimum differentiation of erythroid progenitor cells.

When discussing the importance of clinical trials with SCF in MDS patients, it must be emphasized, however, that myeloblasts express receptors not only for hemopoietic growth factors like G-CSF, GM-CSF, and IL-3 (30, 33) but also for SCF (34). Although clinical trials performed to date with the application of SCF, either another factor or, what seems more probable, the correct balance of cytokines influencing erythropoiesis is required for optimum differentiation of erythroid progenitor cells.

When performing clinical trials with SCF in MDS, very close monitoring will be essential to detect a potential stimulation of the dysplastic clone as early as possible.

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In vitro stimulation of erythropoiesis by stem cell factor alone in myelodysplastic syndrome patients with elevated endogenous erythropoietin serum levels.
