Severe Congenital Neutropenia: Abnormal Growth and Differentiation of Myeloid Progenitors to Granulocyte Colony-Stimulating Factor (G-CSF) But Normal Response to G-CSF Plus Stem Cell Factor

By Kjetil Hestdal, Karl Welte, Sverre O. Lie, Jonathan R. Keller, Francis W. Ruscetti, and Tore G. Abrahamsen

Several mechanisms have been proposed to explain the pathogenesis of severe congenital neutropenia (SCN); however, the mechanism(s) still remains unknown. In particular, clinical observations suggest that abnormal responsiveness of myeloid progenitors to hematopoietic growth factors (HGFs) is a possible mechanism. Therefore, to better define the status of hematopoietic progenitors in the bone marrow (BM) of patients with SCN, the responsiveness of myeloid progenitors to HGFs from two SCN patients was compared with the responsiveness of progenitors from healthy individuals. BM cells (BMCS) from the first SCN patient required higher (10 - 100-fold) concentrations of granulocyte colony-stimulating factor (G-CSF) to achieve maximal and half-maximal colony growth in vitro compared with BMCS from controls. In contrast, the dose-response of interleukin-3 (IL-3) and granulocyte-macrophage-CSF (GM-CSF) in colony formation was normal. Interestingly, IL-3, GM-CSF, and G-CSF at optimal doses showed reduced ability to induce neutrophil differentiation of BMCS from a SCN patient compared with BMCS from controls. Despite an abnormal responsiveness of mature myeloid progenitors to G-CSF in this SCN patient, myeloid progenitors responsive to the combination of stem cell factor (SCF) and G-CSF showed normal dose-response. In contrast to G-CSF alone, the combination of G-CSF and SCF induced the formation of neutrophils almost to the same extent compared with cultures of normal BMCS. Furthermore, on BM progenitor cells obtained from the second patient with SCN, SCF highly synergized with G-CSF to promote neutrophil progenitor cell growth and differentiation in vitro. Thus, these results indicate that one mechanism of the pathogenesis in SCN patients is reduced responsiveness of neutrophil progenitor cells to G-CSF and that SCF can enhance the responsiveness of these cells to G-CSF.

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KÖSTMANN DESCRIBED in 1956 an autosomal recessive disorder that results in severe neutropenia, frequent infections, and a high mortality rate.1,2 The Kostmann syndrome has been included in the group of severe congenital neutropenia (SCN), characterized by neutropenia (<0.2 x 10^9 neutrophils/L), frequent bacterial infections during the first year of life, but variable inheritance. The bone marrow (BM) morphology of patients with SCN shows a maturational arrest in the development of neutrophils at the promyelocyte stage.1,4 Therapeutic alternatives today are either allogeneic BM transplantation or injections of recombinant human granulocyte colony-stimulating factor (G-CSF), and both have resulted in correction of the neutropenia.7,12 Several mechanisms have been proposed to explain the pathogenesis of SCN, including (1) endogenous deficiency of G-CSF; (2) enhanced levels of inhibitors of BM progenitor growth; and (3) altered ability of progenitor cell growth caused by hematopoietic growth factor (HGF) receptor defects or postreceptor signal abnormalities. However, normal serum levels of colony-stimulating activity and G-CSF have been reported,4,10,13-15 and no inhibitors in serum from patients with SCN have been detected.4,10,16 Normal, increased, and reduced progenitor cell growth of BM cells (BMCS) from SCN patients has been reported.4,6,10,13,17-22 The discrepancies in these reports may be due to the differences in the source of HGF used in the colony assays (recombinant HGFs or conditioned medium) or differences in the dose of the growth factors used in the assays. Interestingly, the therapeutic doses of G-CSF needed to correct the neutropenia in most patients with SCN are higher than those doses required to reduce neutropenia after cytotoxic chemotherapy in cancer patients.8,12 Taken together, these results suggest that an altered responsiveness of hematopoietic progenitors to HGFs may be one mechanism to explain the pathogenesis of SCN. Therefore, to better understand the status of hematopoietic progenitors in the BM of patients with SCN, we examined responsiveness of BMCS from two patients with SCN to several HGFs important in the formation of neutrophils.

PATIENTS AND METHODS

Case reports. The first patient was a 1.5-year-old white male who suffered from frequent skin infections starting on his second day of life, and who experienced several episodes of otitis and oral mucositis. He was referred to our hospital for diagnosis and treatment. His father had a known severe chronic neutropenia. On admission, our patient had a slightly elevated total white blood cell count of 10.9 x 10^9 cells/L, with a differential count showing 1% neutrophils, 51% monocytes, 17% eosinophils, and 17% lymphocytes. This profound neutropenia was accompanied by a microcytic anemia with hemoglobin level of 10.4 g/dL and also a thrombocytosis with platelet count of 629 x 10^9/L. BM aspirate showed hyperplasia of all cell lineages, particularly of myelopoiesis, with an apparent maturation block between promyelocytes and myelocytes. Only a few bands and mature neutrophils were present. Antigranulocyte

From the Department of Pediatric Research and Department of Pediatrics, Rikshospitalet, The National Hospital, Oslo, Norway; the Department of Pediatric Hematology and Oncology, Children's Clinic, Medical School of Hannover, Hannover, Germany; the Biological Response Modifiers Program and Biological Carcinogenesis and Development Program, Program Resources, Inc/DynCorp, Inc, Frederick Cancer Research and Development Center, National Cancer Institute, Frederick, MD.

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Address reprint requests to Kjetil Hestdal, MD, Department of Pediatric Research, Rikshospitalet, The National Hospital, 0027 Oslo, Norway.

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antibodies were not found. Therefore, the diagnosis of SCN was established on the basis of the following criteria: (1) a low number of blood neutrophils (<0.2 x 10^9/L); (2) maturation arrest of the neutrophil lineage in the BM; (3) frequent bacterial infection starting before 3 months of age.

The patient was treated with G-CSF beginning with 2.5 μg/kg/d subcutaneously (SC) and escalated by doubling the dose every 14 days. At 20 μg/kg/d of G-CSF, the absolute granulocyte count reached 1.5 x 10^9/L. The absolute granulocyte count remained above this level when the G-CSF dose was decreased to 15 μg/kg/d. The patient is now experiencing far fewer infections and less need for antibiotic therapy.

The second patient with SCN was a 1-year-old female who experienced frequent episodes of severe bronchitis, pneumonitis, and skin abscesses. She had a total white blood cell count of 11.0 x 10^9/L, with a differential count showing 82% lymphocytes, 9% monocytes, and 9% eosinophils. This profound neutropenia was accompanied by an anemia with hemoglobin level of 10.7 g/dL and a platelet count of 450 x 10^9/L. BM aspirate showed a maturation arrest of myelopoiesis at the level of promyelocytes (12% promyelocytes), with a complete lack of myelocytes and neutrophils. Furthermore, the BM was normocellular and did not show any cytogenetic abnormalities or dysplasia.

She was treated with G-CSF. However, because of only partial response to ultra-high doses of G-CSF (144 μg/kg/d SC), she received a successful transplant of BMCs from an HLA-identical sibling.

**BMCs.** BMCs were obtained from the first SCN patient twice before and once after the onset of G-CSF therapy and from the second SCN patient once before and after G-CSF treatment. Furthermore, BMCs were obtained from four healthy BM donors after informed consent. BMCs were separated by Lymphoprep (density, 1.077 g/mL; Nycomed Pharma AS, Oslo, Norway) and the light-density fraction was obtained. The light-density BMCs (LDBMCs) were used in described hematopoietic assays or cryopreserved by a standard procedure using 20% dimethylsulfoxide (DMSO) and stored in liquid nitrogen for later in vitro use.

**Cytokines.** Purified recombinant human G-CSF, recombinant human granulocyte-macrophage CSF (GM-CSF), and recombinant stem cell factor (SCF; c-kit ligand) were kindly provided by Dr. I. McNiece (Amgen Corp, Thousand Oaks, CA). Purified recombinant human interleukin-3 (IL-3) was a gift from Dr. S. Gillis (Immunex Corp, Seattle, WA). Recombinant human transforming growth factor-β1 (TGF-β1) was provided by Dr. Tony Puchio (Bristol-Myers Squibb, Seattle, WA).

**Soft agar colony-forming assay.** LDBMCs, fresh or thawed, were resuspended in Iscove's modified Dulbecco's medium (IMDM) supplemented with 30% fetal calf serum (GIBCO, Life Technologies Ltd, Paisley, UK), 1% detoxified bovine serum albumin (Sigma Chemical Co, St Louis, MO), 1% L-glutamine, and antibiotics (complete IMDM). The cells were plated at 5 x 10^5 cells/aggregate) after 7 or 14 days of incubation. In addition, the dose-response curves for G-CSF-, GM-CSF-, and IL-3-induced colony formation were graphed and the growth factor concentrations (in nanograms per milliliter) at which 50% of maximal growth (ED_50) occurred were estimated.

**Morphologic characterization.** LDBMCs (8 to 10 x 10^9 cells/mL) were cultured for 10 days in liquid culture containing optimal concentrations of cytokines, 100 ng/mL of G-CSF, 10 ng/mL of GM-CSF, 10 ng/mL of IL-3, and 100 ng/mL of SCF. The cells were harvested, and cytocentrifuge preparations of these cells were stained with Diff-Quick Stain Solution (Baxter Deutschland, Unterschleissheim, Germany). Differential cell counts were performed and the cells were differentiated into blasts, eosinophils, monocytes, neutrophils, and bands. At least 100 cells were identified morphologically.

**RESULTS**

**Colony formation of BMCs from a patient with SCN.** To examine the responsiveness of hematopoietic progenitors in SCN, we plated LDBMCs from the first SCN patient in soft agar colony assays in the presence of increasing doses of recombinant G-CSF, IL-3, or GM-CSF and compared the results with those obtained with LDBMCs from four control individuals. The G-CSF-stimulated day-14 colony formation of LDBMCs from this patient showed a dose-dependent response, with maximal colony growth observed at 100 ng/mL of G-CSF (Fig 1A). In contrast, maximal CFU-c formation in response to G-CSF of LDBMCs from controls was observed at 1 to 10 ng/mL (Fig 1B). The IL-3-induced colony formation showed the same dose-response curve both in LDBMCs from this SCN patient and the controls, with plateau CFU-c formation achieved at 1 to 10 ng/mL of IL-3 (Fig 1A and B). Additionally, the colony formation of LDBMCs from the SCN patient and controls in response to GM-CSF showed the same dose-response curve, with maximal colony growth observed at 1 to 10 ng/mL of GM-CSF in both the SCN patient and controls (data not shown). As previously reported, we observed that the maximal day-14 colony growth in response to both optimal doses of G-CSF and IL-3 was higher in LDBMCs from this SCN patient compared with LDBMCs from controls (Fig 1A and B). This correlated with the morphology of BM aspirate from the SCN patient that showed hyperplasia, especially of immature myeloid cells (data not shown). Furthermore, although the ED_50 of IL-3 and GM-CSF for BM colony formation from control individuals was similar to the ED_50 of the patient's BMCs, the ED_50 of G-CSF was approximately 24-fold higher compared with the ED_50 of G-CSF on BMCs from controls (Table 1).

Thus, myeloid progenitors from this SCN patient required increased G-CSF concentration to promote maximal and half-maximal colony formation compared with controls, whereas the dose-response of colony formation in response to IL-3 and GM-CSF was approximately the same compared with BMCs from control individuals.

**Neutrophil differentiation of BMCs stimulated with G-CSF, GM-CSF, and IL-3 in suspension culture.** Because the myeloid progenitors from this SCN patient showed normal proliferative responsiveness to both IL-3 and GM-CSF, which promote neutrophil differentiation, we compared these cytokines to G-CSF in their ability to promote neutrophil differentiation in liquid culture. LDBMCs from the SCN patient and controls were cultured in suspension cultures in optimal concentrations of IL-3 (10 ng/mL), GM-CSF (10 ng/mL), and G-CSF (100 ng/mL) for 10 days. After 10 days in culture, the cell viability in the different treat-
Fig 1. Comparison of the effect of (G) G-CSF and (e) IL-3 on colony formation of LDBMCs from an SCN patient with control LDBMCs. LDBMCs were obtained from the first SCN patient (A) or control individuals (B). The BMCs (5 × 10⁴ or 1 × 10⁵ cells/plate) were plated in soft agar assay in the presence of G-CSF or IL-3 (0.05 to 1,000 ng/mL) and scored for colony formation (CFU-c) after 14 days of incubation in a fully humidified atmosphere at 37°C and 5% CO₂ as described in Patients and Methods. The results are presented as colony growth (mean ± SD) and are representative of at least three separate experiments. ND, not determined.

Table 1. Dose-Response of CSFs on Colony Formation of BMCs From an SCN Patient Versus Controls

<table>
<thead>
<tr>
<th>Stimulator*</th>
<th>Patient</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-3</td>
<td>0.66 ± 0.27</td>
<td>0.80 ± 0.10</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>0.45 ± 0.15</td>
<td>0.62 ± 0.20</td>
</tr>
<tr>
<td>G-CSF</td>
<td>11.10 ± 2.00</td>
<td>0.46 ± 0.02</td>
</tr>
</tbody>
</table>

* IL-3, G-CSF, and GM-CSF (0.05 to 1,000 ng/mL) were used to promote colony formation in vitro.

** LDBMCs were obtained from the first SCN patient or control individuals. The BMCs (5 × 10⁴ or 1 × 10⁵ cells/plate) were plated in soft agar assay in the presence of CSFs and scored for colony formation (CFU-c) after 14 days of incubation as described in Patients and Methods. The dose at which 50% of maximal CFU-c occurred (ED₅₀) was obtained from a dose-response curve. The results are presented as ED₅₀ (mean ± SD) of each CSF and are the mean of three separate experiments.

The effect of SCF (c-kit ligand) on hematopoietic progenitor growth in both SCN patients. SCF (c-kit ligand) has been shown to act on primitive hematopoietic progenitors and is a potent synergistic factor with other CSFs in promoting hematopoietic progenitor cell growth and differentiation. Therefore, to determine whether BM progenitors from SCN patients were altered in their response to the combination of SCF plus G-CSF, LDBMCs from the first SCN patient were incubated with different doses of G-CSF with or without a predetermined optimal dose of SCF (100 ng/mL) in soft agar colony assays. In the presence of SCF, the numbers of G-CSF-induced colonies were enhanced sevenfold to 10-fold in LDBMCs from one SCN patient and twofold to fourfold in controls (Fig 3A and B). The maximum synergistic effect of SCF in colony formation assays was observed at suboptimal doses of G-CSF both in LDBMCs from the patient and in controls (Fig 3A and B). At optimal doses of G-CSF, the same percentage of enhancement of
G-CSF-induced colony formation (30% to 40%) in response to SCF was observed both in this SCN patient and in controls (Fig 3A and B). Furthermore, in the presence of SCF, maximal G-CSF-induced colony formation of both LDBMCs from the SCF patient and controls was achieved at 1 ng/mL of G-CSF, whereas in the absence of SCF, maximal G-CSF-induced colony formation was again observed at 100 ng/mL of G-CSF in the SCF patient versus 1 ng/mL in control BMCs (Fig 3A and B). Thus, immature myeloid progenitors responding to the combination of SCF and G-CSF from this SCN patient showed a normal dose-response to G-CSF compared with controls.

Next, liquid cultures of LDBMCs stimulated with G-CSF plus SCF from the first patient and controls were morphologically examined. Cytocentrifuge preparations of the patient's BMCs cultured with optimal concentration of G-CSF (100 ng/mL) and SCF (100 ng/mL) for 10 days showed that this combination induced almost the same percentage of bands and neutrophils compared with cultures of control cells (55% to 60% v 70% to 75%). Taken together, in contrast to the response to G-CSF alone (Fig 2), LDBMCs from this SCN patient and control individuals showed comparable neutrophil differentiation in response to the combination of SCF and G-CSF.

Furthermore, to better define the synergistic effects of SCF on G-CSF-induced neutrophil proliferation and differentiation, we examined the neutrophil colony formation of LDBMCs from the second patient with SCN in the presence of G-CSF and SCF. Comparable to the first patient, this 1-year-old female suffered from a severe congenital neutropenia with a maturation arrest of myelopoiesis at the level of promyelocytes, with complete lack of myelocytes and neutrophils in BM. This patient responded partial to high doses of G-CSF in vivo. LDBMCs obtained from this patient before G-CSF treatment were plated (1 × 10⁶ cells/mL) in CFU-c assays in the presence of G-CSF (50.0 ng/mL), SCF (500 ng/mL), or a combination of G-CSF and SCF. Neutrophil granulocyte colony formation was determined after 14 days of incubation. Again, G-CSF alone showed low ability to promote neutrophil colonies, whereas SCF highly (12.0-fold) enhanced G-CSF-induced neutrophil progenitor cell growth and differentiation from this second SCN patient (Table 2). Thus, SCF highly synergized with G-CSF in neutrophil formation of progenitor cells from two patients with SCN in vitro.

The effect of TGF-β1 on the G-CSF-induced hematopoietic progenitor growth. TGF-β1 has bidirectional effects on hematopoietic progenitor cell growth in that it inhibits the growth of multipotent hematopoietic progenitors, and stimulates neutrophil colony formation in combination with GM-CSF or G-CSF. Therefore, to examine whether BM progenitors in an SCN patient were altered in their response to TGF-β1 plus G-CSF, LDBMCs from the first patient and controls were plated in soft agar in the presence of an optimal concentration of G-CSF (100 ng/mL) and different doses of TGF-β1 (0.1 to 10 ng/mL). The CFU-c formation was measured after 7 days in culture. As

![Figure 3. Comparison of the effect of SCF plus G-CSF on the colony formation of LDBMCs from an SCN patient with control LDBMCs.](image)

**Table 2. Synergistic Effect of SCF on G-CSF—Induced Neutrophil Colony Formation of BMCs From an SCN Patient**

<table>
<thead>
<tr>
<th>Stimulator*</th>
<th>Colony No.†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>0</td>
</tr>
<tr>
<td>SCF</td>
<td>0</td>
</tr>
<tr>
<td>G-CSF</td>
<td>2</td>
</tr>
<tr>
<td>G-CSF + SCF</td>
<td>25</td>
</tr>
</tbody>
</table>

* G-CSF and SCF were used at 50 ng/mL and 500 ng/mL, respectively.
† LDBMCs were obtained from the second SCN patient. The BMCs (1 × 10⁶ cells/plate) were plated in soft agar assay in the presence of CSFs as indicated and scored for neutrophil granulocyte colony numbers after 7 days in culture. The number of neutrophelial colonies was determined after 14 days of incubation as described in Patients and Methods.
previously reported, G-CSF–induced colony formation of normal BMCs was enhanced by low doses of TGF-β1 (0.1 ng/mL) compared with G-CSF alone, whereas high concentration of TGF-β1 (10 ng/mL) reduced the formation of day-7 CFU-c (Fig 4). In contrast, TGF-β1 showed a dose-dependent reduction of G-CSF-induced day-7 CFU-c of LDBMCs from this SCN patient (Fig 4). The maximal inhibition, 90% of the control level, was observed with 10 ng/mL of TGF-β1 (Fig 4). In addition, whereas the day-14 CFU-c formation in response to G-CSF (100 ng/mL) was enhanced in BMCs from the patient compared with controls (Fig 1), the G-CSF–induced colony formation after 7 days of incubation was reduced (50%) in the SCN patient compared with control BMCs (data not shown). Thus, in contrast to myeloid progenitors from control individuals, there were no G-CSF-responsive progenitors that were enhanced in response to TGF-β1 at day 7 in this patient with SCN; however, progenitors that were inhibited in response to TGF-β1 were shown both in LDBMCs from the patient and controls.

**DISCUSSION**

The mechanism(s) responsible for SCN is still unknown. Interestingly, a majority of these patients need higher doses of G-CSF to promote neutrophil formation compared with the doses used in patients with chemotherapy-induced neutropenia. Therefore, possible mechanisms for the neutrophil maturation defect observed in these patients may be related to reduced ability to produce G-CSF or reduced cellular response to G-CSF. The first possibility may be excluded because normal levels of G-CSF have been detected in the serum of patients with SCN, and monocytes from these patients produce normal amounts of G-CSF in vitro. In addition, allogeneic BM transplantation has also been shown to correct the neutropenia, indicating that a cellular defect in the progenitor cells is most likely. It has previously been shown that hematopoietic progenitors obtained from SCN patients either had normal, increased, or reduced ability to form colonies in response to conditioned media or CSFs in vitro. The discrepancy between these results may be due to the heterogeneity within the SCN disease group or to differences in the source and dose of CSFs used in these studies to promote colony formation, especially the use of conditioned media containing several CSFs that would induce a synergistic growth on progenitor growth and differentiation.

In this study we showed that BMCs from an SCN patient had reduced responsiveness to G-CSF compared with controls despite an increase in the total number of myeloid progenitors in the SCN BM compared with controls. We showed that a 10- to 100-fold higher dose of G-CSF was necessary to promote maximal colony growth of BMCs from an SCN patient compared with control BMCs. Furthermore, LDBMCs obtained from two patients with neutropenia that either was associated with an immune defect or autoimmune antineutrophil antibodies showed normal responsiveness to G-CSF in CFU-c assays compared with the SCN patient (K. Hestdal, unpublished observations). In addition, the reduced responsiveness of progenitors to G-CSF in vitro correlated with the clinical observation in this patient, who had to receive a high dose (15 μg/kg/d) of G-CSF to gain an absolute granulocyte count of 1.5 × 10⁹/L. Moreover, LDBMCs obtained from this SCN patient after G-CSF treatment showed the same reduced responsiveness to G-CSF in colony assay as before treatment (K. Hestdal, unpublished observations). Thus, the progenitor abnormality persisted also after the onset of G-CSF treatment.

The reduced responsiveness of SCN progenitors to G-CSF might be due to a decrease in G-CSF receptor numbers or receptor affinity on the progenitors. Kyas et al. have shown that blood neutrophils from SCN patients treated with G-CSF showed normal or enhanced numbers of G-CSF receptors with the same receptor affinities as compared with neutrophils from control individuals. The reduced responsiveness of hematopoietic progenitors to G-CSF in our patient may still be due to reduced G-CSF binding properties on progenitor cells. Therefore, G-CSF binding capabilities on BM progenitors have to be examined from untreated SCN patients to rule out whether receptor expression might be one possible mechanism of the reduced responsiveness of hematopoietic progenitors to G-CSF.

GM-CSF and IL-3 showed the same dose-response for colony formation of BMCs from an SCN patient compared
with controls; however, their ability to promote neutrophil differentiation was markedly reduced compared with normal progenitor cells in vitro. This indicates that administration of IL-3 or GM-CSF might not correct the neutropenia in these patients. In agreement with this, SCN patients who were treated with GM-CSF did not show increased neutrophils in peripheral blood after GM-CSF administration, although an increase in the numbers of monocytes and eosinophils were observed.6,10 Taken together, this is consistent with the defect observed in SCN patients being specific for a committed neutrophil progenitor.

SCF (c-kit ligand) has been shown to act on primitive hematopoietic progenitors and is a potent synergistic factor with other CSFs to promote hematopoietic progenitor cell growth and differentiation.28-30 We observed that myeloid progenitors responding to the combination of SCF and G-CSF showed normal responsiveness to G-CSF compared with progenitors that responded to G-CSF alone, suggesting that more primitive progenitors have normal responsiveness to G-CSF in SCN patients. In addition to the synergistic effect of SCF on G-CSF-induced progenitor growth observed in the two SCN patients, SCF also markedly enhanced the G-CSF-induced neutrophil differentiation compared with G-CSF alone in these SCN patients.

Interestingly, TGF-β1 at low doses did not enhance the formation of G-CSF-induced day-7 CFU-c in an SCN patient compared with progenitors from control BM. It has previously been shown that G-CSF-induced day-7 granulopoietic CFU-c formation is enhanced by TGF-β1,35 whereas CSF-induced primitive progenitor cell growth such as day-14 colony-forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte are inhibited by TGF-β1.29,31,35 These results again indicate that the defect observed in SCN patients is specific for a committed neutrophil progenitor.

In conclusion, the present study suggests that the neutropenia in patients with SCN is caused by a reduced cellular responsiveness of a neutrophil progenitor population to G-CSF as determined by the change in dose-response curve of G-CSF. The normal growth responsiveness to IL-3, GM-CSF, and SCF plus G-CSF and the ability of TGF-β1 to inhibit rather than stimulate the G-CSF progenitors suggest that more immature multilineage progenitors have normal growth properties in SCN patients. The reduced responsiveness of the neutrophil progenitor in SCN patients may be caused by reduced G-CSF receptor binding or a defect in the postreceptor signal transduction pathway. Regardless of the cellular defect, SCF may reduce the impaired responsiveness of neutrophil progenitor cells to G-CSF and therefore the combination of SCF and G-CSF may prove to be more effective in treatment of SCN patients compared with G-CSF alone.

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**REFERENCES**


RESPONSIVENESS OF BM PROGENITORS IN SCN


Severe congenital neutropenia: abnormal growth and differentiation of myeloid progenitors to granulocyte colony-stimulating factor (G-CSF) but normal response to G-CSF plus stem cell factor

K Hestdal, K Welte, SO Lie, JR Keller, FW Ruscetti and TG Abrahamsen