

## Defects of Granulopoiesis in Patients with Severe Congenital Neutropenia

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### ABSTRACT

To confirm the abnormalities of primitive myeloid progenitor cells in patients with severe congenital neutropenia (SCN), we studied their responsiveness to hematopoietic factors including granulocyte colony-stimulating factor (G-CSF). In all SCN patients studied no abnormalities of granulocyte colony-stimulating factor receptor (G-CSFR) gene were detected by polymerase chain reaction-single-strand conformation polymorphism analysis and sequence analysis. A flow cytometric analysis of bone marrow cells based on the expression of CD34, Kit receptor, and G-CSFR demonstrated a reduced frequency of CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> cells in patients with SCN. The granulocyte/macrophage (GM)-colony formation of CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> cells in patients was markedly decreased at all concentrations of G-CSF in serum-deprived semisolid culture. The responsiveness of CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> cells in patients showed a reduced response to the combination of stem cell factor, the ligand for flk2/flt3, and interleukin-3 with or without G-CSF in serum-deprived semisolid and liquid suspension cultures. In contrast, no difference in the responsiveness of CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>-</sup> cells was noted between SCN patients and normal subjects. The bone marrow cells from a patient who underwent bone marrow transplantation showed a restoration of both the reduced frequency and the decreased level of GM-colony formation of CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> cells. These results demonstrate that the presence of qualitative and quantitative abnormalities of primitive myeloid progenitor cells expressing G-CSFR may play an important role in the impairment of granulopoiesis in patients with SCN.

**Key words:** *Granulocyte colony-stimulating factor, Granulocyte colony-stimulating factor receptor, Fluorescence activated cell sorting, Severe congenital neutropenia*

Severe congenital neutropenia (SCN), also known as Kostmann-type neutropenia, is characterized by onset in early childhood, recurrent life-threatening infections, and profound neutropenia with less than 200 per  $\mu$ l of absolute neutrophil count (ANC) in the peripheral blood<sup>15,33,34,56,57</sup>. The bone marrows usually show a paucity of mature myeloid cells with an arrest of maturation of neutrophil precursors at the promyelocyte-myelocyte stage of differentiation. In the majority of patients with SCN, pharmacological doses of recombinant human granulocyte colony-stimulating factor (G-CSF) induce a marked increase in circulating neutrophil counts, resulting in both a significant reduction in serious infections and improved quality of life<sup>4-6,13,23,24,58,59</sup>.

To date, the underlying pathophysiology of SCN remains unclear. The hypotheses include seeing it

as a genetic defect resulting in a defective production of G-CSF or as a defective response of the neutrophil precursors to G-CSF or other hematopoietic growth factors<sup>15</sup>. The role of G-CSF and G-CSF receptor (G-CSFR) in the stimulation of granulopoiesis has been documented through the analysis of the G-CSF-deficient and G-CSFR-deficient mice<sup>3,8,37,38</sup>. However, the deficiency of G-CSF, the lack of G-CSFR, or the G-CSFR mutation by itself may not be a sufficient contribution to a severe neutropenia and the SCN phenotype<sup>19,37-39</sup>. In patients with SCN, the production of biologically active G-CSF appears to be normal, and the serum levels of G-CSF are often increased<sup>16,28,40,45</sup>. In addition, it has been shown that a defect in the expression of receptors or the affinity of G-CSF for its receptor does not occur in the majority of SCN patients<sup>35</sup>. Data obtained from sequencing the

reverse transcriptase polymerase chain reaction (PCR) products of G-CSFR mRNA or performing single-strand conformational analysis of the cytoplasmic or transmembrane portion of the G-CSF receptor from SCN patients has not shown any point mutations in the majority of patients<sup>15,16,32,48,56,57</sup>. However, a few patients have demonstrated acquired spontaneous mutations of the G-CSF receptor DNA<sup>9-12,25,53</sup>. Recently, a patient with SCN hyporesponsive to G-CSF having a novel point mutation in the extracellular domain of the G-CSFR was reported, further emphasizing the importance of G-CSFR mutations in the pathophysiology of SCN patients<sup>54</sup>. Thus far, no defect has been identified in the signaling pathways related to the granulopoiesis in myeloid cells from patients with SCN<sup>17,46,52</sup>.

Bone marrow cells from patients with SCN frequently show a reduced or complete lack of responsiveness to G-CSF in *in vitro* culture<sup>20,26,31</sup>. We have recently reported a defective proliferation of primitive myeloid progenitor cells from patients with SCN in response to hematopoietic factors including G-CSF<sup>32</sup>. From the results of further purification of primitive myeloid progenitor cells based on the expression of G-CSFR on their cells, we have shown the abnormalities of G-CSFR-positive cells in SCN patients<sup>42</sup>. In this report we have extended the number of patients studied to confirm the abnormalities of primitive myeloid cells expressing G-CSFR by *in vitro* colony and proliferation assays.

## MATERIALS AND METHODS

### Patients

Table 1 summarizes the clinical and laboratory data from seven patients with SCN enrolled in this study. The hematological findings presented were from the time of diagnosis before the administration of G-CSF. None of the patients had a family history of neutropenia. The diagnosis of SCN or Kostmann's syndrome was according to accepted criteria such as an ANC below 200 per  $\mu\text{l}$  in the peripheral blood, maturation arrest at the promyelocyte or myelocyte level in the bone marrow, absence of circulating antineutrophil antibodies as determined by a granulocyte indirect immunofluorescence test, and the onset of severe infections at an age of less than 12 months<sup>15,56,57</sup>. All patients had a history of recurrent life-threatening infections and were receiving recombinant human G-CSF except for one (Patient 7), with monitoring for hematologic problems including myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML). None of the patients developed MDS and/or AML during the administration of G-CSF. Prophylactic sulfamethoxazole-trimethoprim or G-CSF has been administered to all patients since the diagnosis was made. Patient 1 continued to have recurrent skin abscesses and chronic gingivitis, and he has been maintained on daily subcutaneous administration of G-CSF for the last 7 years. The other four patients have received intermittent administration of G-CSF when infections were observed. Patient 3 received a BMT from an unrelated HLA-matched donor. Hematological and clinical improvements without

**Table 1.** Clinical and laboratory characteristics of patients

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
Age (months) / Sex	11/M	3/M	6/M	6/M	11/F	3/F	2/M
White Blood Cells ( $\mu\text{l}$ )	5,400	4,200	5,800	3,500	7,300	4,900	4,000
Neutrophil (%)	0	1	0	2	3	2	0
Monocyte (%)	18	15	12	41	17	46	10
Eosinophil (%)	12	16	11	9	0	2	7
Lymphocyte (%)	69	68	75	43	77	49	77
Hemoglobin (g/dl)	12.8	11.9	12.8	11.8	10.4	8.8	10.0
Red Blood Cells ( $\mu\text{l}$ )	$4.80 \times 10^6$	$4.75 \times 10^6$	$4.80 \times 10^6$	$4.53 \times 10^6$	$4.31 \times 10^6$	$4.90 \times 10^6$	$4.43 \times 10^6$
Platelets ( $\mu\text{l}$ )	$350 \times 10^3$	$240 \times 10^3$	$320 \times 10^3$	$280 \times 10^3$	$360 \times 10^3$	$690 \times 10^3$	$360 \times 10^3$
Bone Marrow Findings							
Nucleated Cell Counts ( $\mu\text{l}$ )	$188 \times 10^3$	$155 \times 10^3$	$192 \times 10^3$	$54 \times 10^3$	$69 \times 10^3$	$75 \times 10^3$	$139 \times 10^3$
Myeloblast (%)	1	2	1	2	3	1	6
Promyelocyte (%)	2	6	4	6	0	2	2
Myelocyte (%)	3	1	0	0	2	3	0
Metamyelocyte (%)	0	0	0	0	0	0	0
Mature Neutrophil (%)	1	0	0	2	2	0	0
Lymphocyte (%)	46	35	30	46	70	33	37
Erythroid Series (%)	35	48	55	23	21	47	12
G-CSF Treatment	daily	intermittent	intermittent	intermittent	intermittent	temporary	Not done
Age when G-CSF Started	3 yr	1 yr		16 yr	1 yr	3 mo	
Months of G-CSF Therapy	120	60		72	1	1	
Dose of G-CSF ( $\mu\text{g}/\text{kg}/\text{day}$ )	4.5	2.0		3.0	8	13	

Age and hematological data show the findings at the time of diagnosis.

any complications were observed after the patient underwent the BMT.

### **Cytokines**

Recombinant human G-CSF, recombinant human interleukin-3 (IL-3) with a specific activity of  $1.0 \times 10^8$  units/mg, and recombinant human stem cell factor (SCF) were supplied by the Kirin Brewery Co. Ltd. (Tokyo, Japan). The recombinant human ligand for flk2/flt3 (FL) was purchased from PeproTech Inc. (Rocky Hill, NJ). Unless otherwise specified, the concentrations of factors used were as follows: G-CSF, 100 ng/ml; SCF, 100 ng/ml; FL, 20 ng/ml; IL-3, 100 U/ml.

### **Bone marrow cell separation**

In accordance with the Institutional Review Board for Human Research, Hiroshima University School of Medicine, bone marrow samples were obtained with informed consent in all cases (patients, their guardians, and healthy adult volunteers). The bone marrow cells used in this study were taken at a time when G-CSF had not been administered to six SCN patients (patients 2 to 7). Bone marrow samples were diluted with an equal volume of an  $\alpha$ -modification of Eagle's medium ( $\alpha$ MEM; ICN Biomedicals, Inc., Aurora, OH) and centrifuged over Lymphoprep (1.077 g/ml; Nycomed Pharma AS, Oslo, Norway). The light density bone marrow cells (LDBMC) were carefully harvested with a Pasteur pipette, washed three times with PBS containing 2% human AB serum (Sigma Chemical Co., St. Louis, MO), and 0.1 mg/ml of DNase I (type II-S; Sigma Chemical Co.) and resuspended in  $\alpha$ MEM containing 10% FBS (ICN Biomedicals, Inc.). Cells were incubated in plastic culture flasks (Becton Dickinson Labware, Lincoln Park, NJ) at 37°C for 1 hour to remove adherent cells. Nonadherent cells were used in the described purification or cryopreserved by a standard procedure using 10% dimethylsulfoxide and stored in liquid nitrogen until use. Cells, fresh or thawed, were washed and resuspended in PBS-human serum-DNase solution containing 0.1% sodium azide for subsequent immunofluorescence staining.

### **Reverse-transcriptase PCR and single-strand conformation polymorphism (SSCP)**

Total cellular RNA was extracted from bone marrow mononuclear cells using the guanidinium thiocyanate extraction method. RNA was converted into cDNA by reverse transcriptase. PCR amplification of cDNA was performed including [ $\alpha$ -<sup>32</sup>P] dCTP (3,000 Ci/mmol, Amersham Pharmacia Biotech, Inc., Piscataway, NJ) according to the method of Guba et al<sup>16)</sup> and Ward et al<sup>54)</sup>. The primers used for the extracellular, transmembrane, and cytosolic portions of the G-CSFR were as follows: extracellular (nucleotides 623 to

641, sense) CTACCCACCAGCTTCACTC and (nucleotides 959 to 978, antisense) TGGCGCAGCTCACACTTCTG to amplify nucleotides 623 to 978, and (nucleotides 906 to 924, sense) CAGGCTGCC-TACAGCTGTG and (nucleotides 1229 to 1247, antisense) GCACTGGCTTCCAGAACAG to amplify nucleotides 906 to 1247, transmembrane, (nucleotides 1970 to 1993, sense) ATGGCTGCCAGCCAGGCTGGGGCC and (nucleotides 2317 to 2338, antisense) CAGAGTGGGGAGGC-CACAGGTC to amplify nucleotides 1970 to 2338, cytosolic, (nucleotides 2315 to 2336, sense) GAGACCTGTGGCCTCCCCACTC and (nucleotides 2656 to 2680, antisense) CTAGAAGCTCCCAGCGCCTCCATC to amplify nucleotides 2315 to 2680. The PCR products were applied to electrophoresis in 6% polyacrylamide gel for 3 to 5 hours at 30 watts at room temperature. After electrophoresis, the gel was dried on filter paper and exposed to X-ray film at -80°C<sup>16,49)</sup>.

### **Sequence of PCR products**

Mutational analysis was performed by directly sequencing PCR-amplified cDNA with an ABI/PE Biosystems PRISM Big Dye terminator chemistry on an ABI/PE Biosystems 310 Analyzer (Applied Biosystems, Foster City, CA). The primers used for SSCP were also used for the sequence.

### **Flow cytometric analysis of bone marrow cells**

One million cells were simultaneously incubated with FITC-labeled monoclonal anti-CD34 antibody (clone 581, Beckman Coulter, Inc., Fullerton, CA), phycoerythrin (PE)-conjugated anti-c-Kit (clone 95C3, Beckman Coulter, Inc.), and biotin-conjugated anti-G-CSFR (clone LMM741, PharMingen, San Diego, CA) for 30 to 40 min at 4°C. Cells were then washed twice and stained with streptavidin labeled with allophycocyanin (APC, Caltag Laboratories, San Francisco, CA) for 15 min at 4°C. After the addition of propidium iodide (PI, Sigma Chemical Co.) at a concentration of 1  $\mu$ g/ml, cells were washed twice and resuspended in PBS-human serum-DNase-sodium azide solution. The appropriate isotype controls, FITC-, PE-, and biotin-conjugated mouse IgG1a, were used to identify background staining. The cells were immediately analyzed by FACS Vantage (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with a 4-W argon laser and a 35-mW helium neon laser. More than  $3 \times 10^5$  events were collected and then analyzed by CellQuest software (Becton Dickinson Immunocytometry Systems).

### **Purification of bone marrow cells**

Cell purification was performed according to the methods reported previously with

modification<sup>29,30</sup>). Cells ( $2 \times 10^7$ /ml) were incubated with FITC-labeled monoclonal anti-CD34 antibody for 30 min at 4°C. FITC-conjugated mouse IgG1a was used as an isotype control. After the addition of PI at a concentration of 1 µg/ml, cells were washed twice and resuspended in PBS-human serum-DNase-sodium azide solution. The initial enrichment of CD34<sup>+</sup> was carried out by setting the FACS Vantage equipped with a 4-W argon laser and a 35-mW helium neon laser to recognize only FITC-positive cells. Low to medium forward scatter and low side scatter as well as negative PI fluorescence gates were used. The resulting cell population contained 30% to 50% CD34<sup>+</sup> cells. Enriched CD34<sup>+</sup> cells were further stained with PE-conjugated anti-c-Kit and biotin-conjugated anti-G-CSFR (PharMingen) for 30 to 40 min at 4°C. Cells were then washed twice and stained with streptavidin labeled with APC for 15 min at 4°C. After the addition of PI at a concentration of 1 µg/ml, the cells were washed twice and resuspended in PBS-human serum-DNase-sodium azide solution. The appropriate isotype controls were used to identify background staining. Forward and orthogonal light scatter signals as well as the specific fluorescences of FITC, PE, APC, and PI excited at 488 nm and 633 nm were used to establish sort windows. Cells were separated into fractions expressing positive CD34, positive c-Kit and positive or negative G-CSFR. Data acquisition and analysis was performed with CellQuest software.

### Clonal cultures

The clonal cell culture was performed in 35-mm Falcon suspension culture dishes (Becton Dickinson Labware). In the serum-deprived culture, 1 ml of the culture mixture contained purified cells, 1% deionized crystallized BSA (Sigma Chemical Co.), 300 µg/ml fully iron-saturated human transferrin (98% pure; Sigma Chemical Co.), 10 µg/ml soybean lecithin (Sigma Chemical Co.), 6 µg/ml cholesterol (Sigma Chemical Co.), 1

$10^{-7}$  M sodium selenite (Sigma Chemical Co.), 10 µg/ml insulin (Sigma Chemical Co.), 4.5 mM L-glutamin (Sigma Chemical Co.), 1.5 mM glycine (Sigma Chemical Co.), 1.2% 1,500-centipoise methylcellulose (Shinetsu Chemical, Tokyo, Japan), and designated cytokines<sup>29,30,51</sup>. The cultures were incubated at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>/95% air. On day 14 of incubation, GM colonies were scored on an inverted microscope using the criteria described previously<sup>36</sup>. A GM colony contains pure granulocyte colonies consisting primarily of neutrophils and their precursors, and mixed granulocyte-macrophage colonies consisting mainly of neutrophils, macrophages/monocytes, and their precursors. The numbers of colonies represent the mean of triplicate cultures.

### Liquid suspension cultures

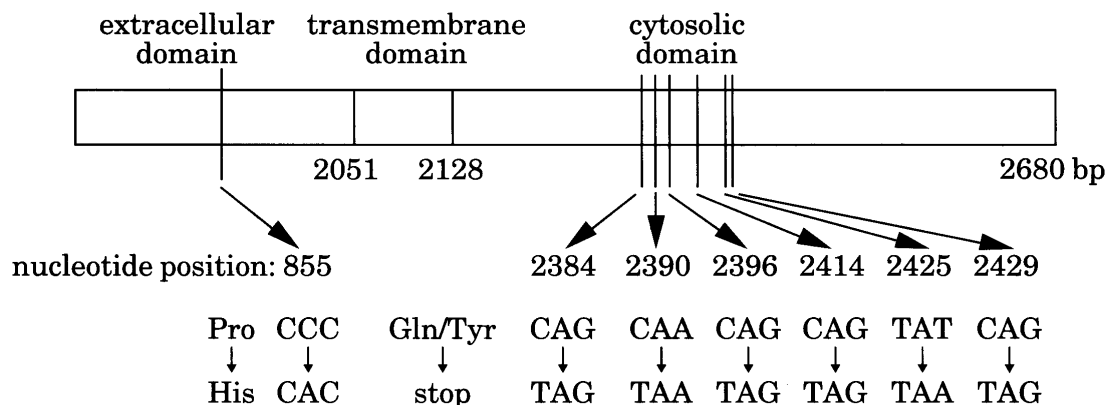
One hundred purified cells were cultured in serum-deprived liquid suspension media containing SCF, FL, and IL-3 with or without G-CSF in 96-well round-bottom microtrays (Corning Coaster Inc, Corning, NY). Incubation was carried out at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>/95% air for 10 days. The number of cells in each well was serially scored, and the reported values represent the mean of triplicate wells. In some experiments, some of the proliferated cells were individually picked, centrifuged onto slides using Shandon's Cytospin 2 Centrifuge (Shandon Inc, Pittsburgh, PA), and stained with Wright-Giemsa.

### Statistical analysis

Statistical significance was determined by t tests using StatView software (version 4.5; SAS Institute, Inc., Cary, NC).

## RESULTS

### Analysis of the structural conformation of the G-CSFR gene

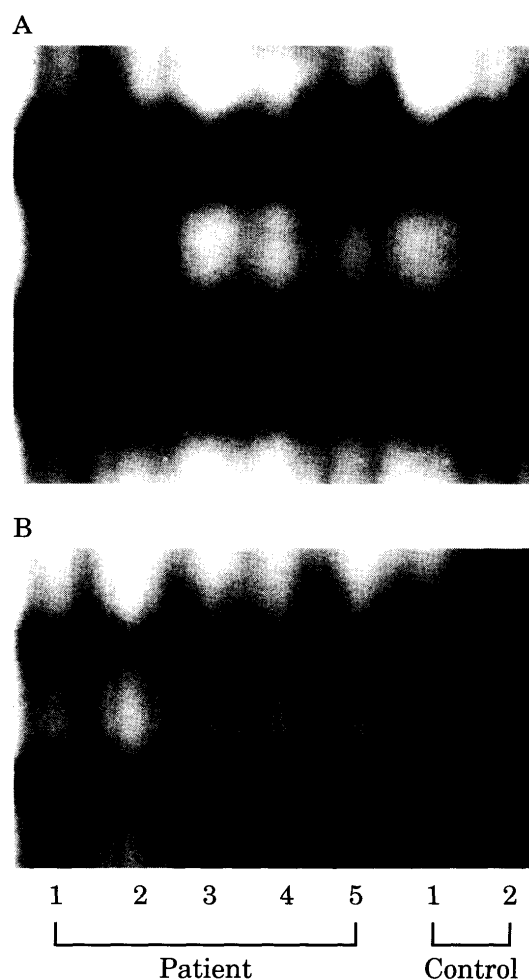


**Fig. 1.** Schematic structure of the G-CSF receptor cDNA and regions of reported mutations. The nucleotide positions given below indicate the reported point mutations detected in patients with severe congenital neutropenia.

Mutations in the gene for G-CSFR have been described in subgroups of patients with SCN<sup>9-12,25,46,54</sup>. The representative mutations reported are shown in Fig. 1. Nonsense mutations in cytosolic portions of the G-CSFR were reported in the majority of patients. These are considered to be acquired mutations and to be related to leukemogenesis in patients with SCN. Therefore, we first examined the presence of abnormalities in the gene encoding G-CSFR in the patients with SCN enrolled in this study. The structures of the extracellular, transmembrane and cytosolic portions of the G-CSFR were analyzed for conformational polymorphisms by SSCP (Patients 1 to 5). As shown in Fig. 2, SSCP analysis of the transmembrane (A) and cytosolic (B) portions of G-CSFR revealed that the patterns of morbidity of PCR products of the five patients with SCN were indistinguishable from those of the normal subjects. No difference was also observed in the SSCP analysis of the extracellular portion of G-CSFR (data not shown). To confirm the results of SSCP, mutational analysis was also performed by the direct sequence of PCR products. No mutations were observed in all patients by the direct sequence of PCR products (date not shown).

#### Flow cytometric analysis of bone marrow cells

We analyzed the expression of CD34, c-Kit, and G-CSFR on bone marrow cells in SCN patients to study the abnormalities of primitive myeloid progenitor cells. Table 2 summarizes the quadrant percentage of Kit<sup>+</sup>/G-CSFR<sup>+</sup>, Kit<sup>+</sup>/G-CSFR<sup>-</sup>, Kit<sup>-</sup>/G-CSFR<sup>+</sup>, and Kit<sup>-</sup>/G-CSFR<sup>-</sup> cells in CD34<sup>+</sup> cells in seven patients with SCN and nine normal subjects. The frequency of G-CSFR<sup>+</sup>/Kit<sup>+</sup> expression on CD34<sup>+</sup> cells in patients with SCN was apparently decreased as compared with that of normal subject. Although there were some variations in the frequency of quadrant cells, the frequency of Kit<sup>+</sup>/G-CSFR<sup>+</sup> cells was significantly



**Fig. 2.** PCR-SSCP analysis of the G-CSFR transmembrane (A) and cytosolic (B) portions. The RT-PCR was performed with primers as described in Materials and Methods. The PCR products from 5 patients (Patients 1 to 5) and 2 normal subjects (Controls 1 and 2) underwent polyacrylamide gel electrophoresis for 3 to 5 hours. After electrophoresis, the bands were visualized by autoradiography with X-ray film.

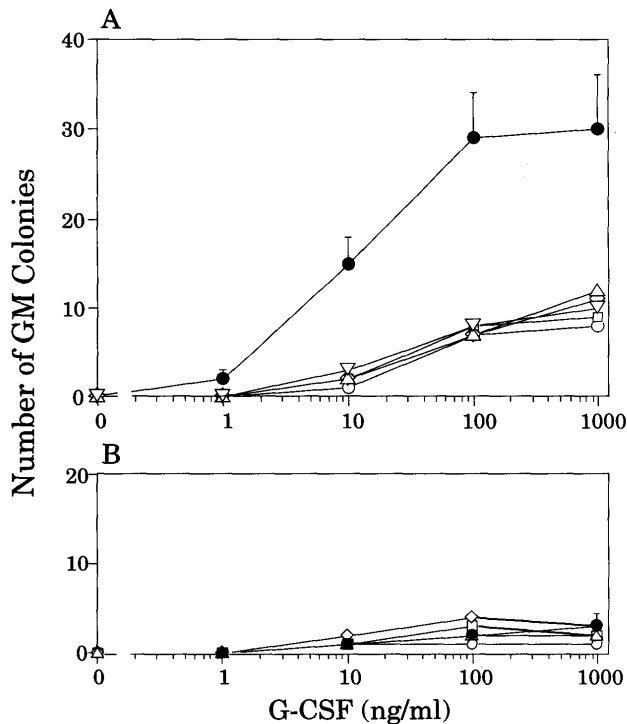
**Table 2.** Frequencies of the expression of Kit and G-CSFR on CD34 cells

Cells	Quadrant Percentages of Cells								Patients (Mean ± SD)	Normal Subjects (range)
	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7			
Kit <sup>+</sup> /G-CSFR <sup>+</sup>	3.5	4.8	4.9	12.1	4.2	8.8	4.9	6.2 ± 3.1*	33.4 ± 8.8 (20.6 – 44.8)	
Kit <sup>+</sup> /G-CSFR <sup>-</sup>	5.5	17.5	11.6	29.3	13.1	21.5	22.6	17.3 ± 7.9	16.1 ± 6.2 (9.0 – 20.9)	
Kit <sup>-</sup> /G-CSFR <sup>+</sup>	76.2	46.5	51.2	22.1	30.0	40.2	29.8	42.3 ± 18.1	26.4 ± 9.5 (12.5 – 40.3)	
Kit <sup>-</sup> /G-CSFR <sup>-</sup>	14.8	31.2	32.3	36.5	52.8	29.6	42.8	34.3 ± 11.8	24.1 ± 13.6 (9.7 – 53.8)	

Data show the quadrant percentage of each fraction within low to medium forward scatter and low side scatter, negative for PI fluorescence, and positive for CD34 gates.

Data of normal subjects represents mean ± SD (n = 9).

\*p < 0.001, compared with normal subjects



**Fig. 3.** GM-colony formation of CD34<sup>+</sup>/Kit<sup>+</sup> cells. Two hundred and fifty CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> cells (A) or CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>-</sup> cells (B) were plated in serum-deprived methylcellulose culture, with the concentrations of G-CSF being varied. Data represent the mean  $\pm$  SD of nine normal subjects (●) and mean of triplicate culture of patients 1 (○), 2 (△), 3 (□), 4 (◇), and 5 (▽).

decreased in all patients with SCN ( $p < 0.001$ ). To exclude the possibility that endogenous and/or exogenous G-CSF *in vivo* affects the binding of G-CSFR antibody to G-CSFR, CD34-positive cells from normal subjects were incubated with G-CSF at concentrations of 1,000 to 5,000 pg/ml, which is comparable to the maximal serum levels of patients before the staining of biotin-conjugated G-CSFR antibody. However, the preincubation of G-CSF did not affect the expression of G-CSFR on

CD34<sup>+</sup>/Kit<sup>+</sup> cells (data not shown). These results indicate that the decrease in the frequency of CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> expression in patients with SCN is not due to either high serum concentrations of G-CSF or the administration of G-CSF to patients.

#### GM colony formation of CD34<sup>+</sup>/Kit<sup>+</sup> cells

According to the expression of CD34, Kit, and G-CSFR, the CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> and CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>-</sup> cells were purified, and we then studied the proliferative capability of the purified cells in response to hematopoietic factors including G-CSF using semisolid culture under serum-deprived conditions. As shown in Fig. 3, the formation of GM colonies of CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> in normal subjects was supported with G-CSF dose-dependently, whereas CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>-</sup> failed to respond to various concentrations of G-CSF. The formation of GM colonies of CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> in patients with SCN was significantly reduced as compared with that in normal subjects at all concentrations of G-CSF. Recently we reported the decreased colony formation of CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> cells in patients with SCN in response to G-CSF alone and to the combination of various hematopoietic factors<sup>42</sup>. To confirm such findings in more patients, the formation of GM colonies of CD34<sup>+</sup>/Kit<sup>+</sup> cells with or without the expression of G-CSFR was studied in response to various hematopoietic factors primarily involved in myelopoiesis such as SCF, FL, IL-3, and G-CSF<sup>14,41,43,47</sup>. As shown in Table 3, GM colonies were supported by SCF, FL, and IL-3 in both CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> and CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>-</sup> cells. The addition of G-CSF to SCF, FL, and IL-3 augmented the number of GM colonies irrespective of the expression of G-CSFR in CD34<sup>+</sup>/Kit<sup>+</sup> cells. In CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> cells, the GM colony formation in patients with SCN showed a significant decrease in response to combinations of SCF, FL, and IL-3, both with and

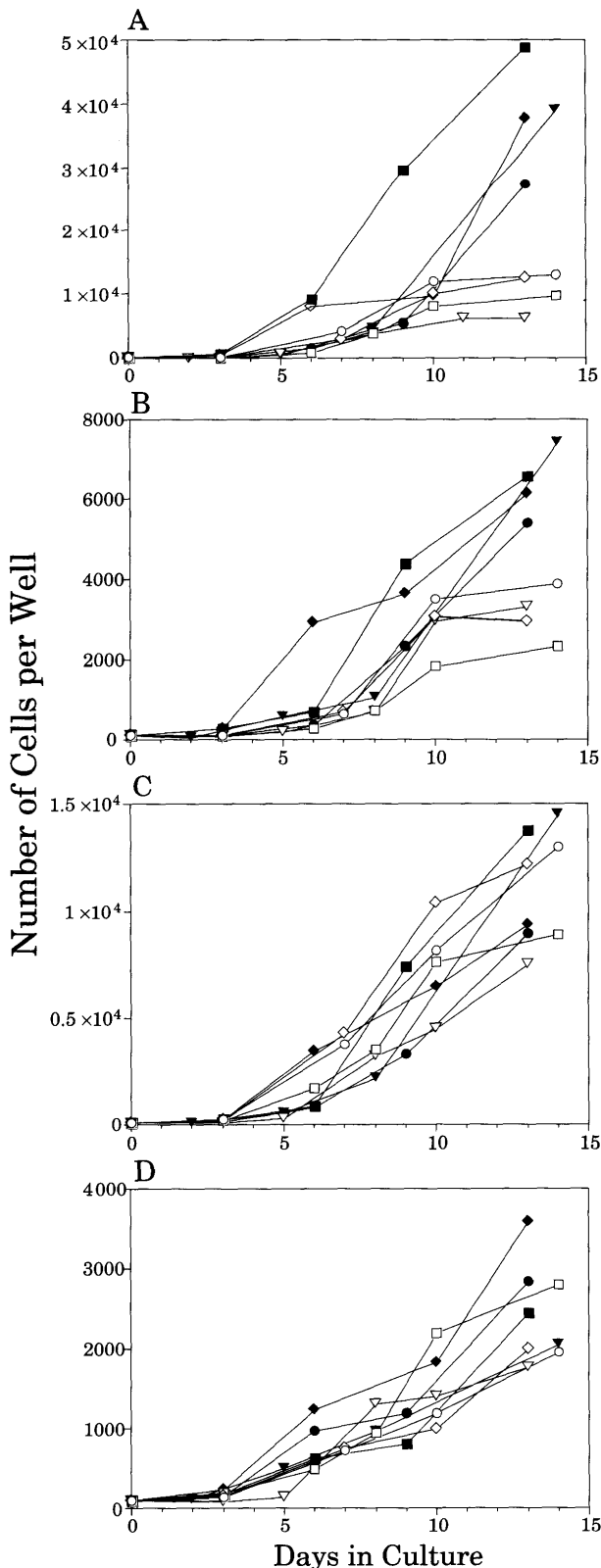
**Table 3.** Formation of GM colonies of purified cells supported by various factors

Factors	No. of GM Colonies									
	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patients (Mean $\pm$ SD)	Normal Subjects (Mean $\pm$ SD, n = 9)	Normal Subjects (Range, n = 9)
<b>A. CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup></b>										
SCF, FL, IL-3	11	11	18	13	16	14	13	14 $\pm$ 3*	30 $\pm$ 8	25–46
SCF, FL, IL-3, G-CSF	23	27	30	28	30	28	31	28 $\pm$ 3†	67 $\pm$ 8	56–76
<b>B. CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>-</sup></b>										
SCF, FL, IL-3	17	17	27	22	30	16	30	23 $\pm$ 6	18 $\pm$ 6	14–29
SCF, FL, IL-3, G-CSF	46	28	48	40	42	28	48	40 $\pm$ 9	36 $\pm$ 5	31–48

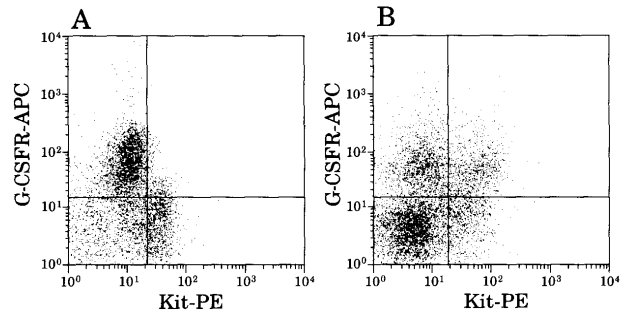
Cultures were performed under serum-deprived conditions in media containing 250 cells and designated factors. The CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> and CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>-</sup> cells were purified according to the flow cytometric analysis based on the expression of Kit and G-CSFR on CD34 cells.

Data of patients represent the mean of triplicate cultures.

\* $p < 0.01$  and †  $< 0.001$ , compared with normal subjects, respectively.



**Fig. 4.** Proliferation of CD34<sup>+</sup>/Kit<sup>+</sup> cells in liquid suspension culture. One hundred CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> cells (A, B) and CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>-</sup> cells (C, D) were cultured in serum-deprived media containing SCF, FL, and IL-3 (B, D) with G-CSF (A, C). Data represent the mean of the cell number of triplicate culture well in four normal subjects (●, ■, ◆, ▼) and four patients with SCN (○, □, ◇, ▽).



**Fig. 5.** Flow cytometric analysis of CD34, Kit, and G-CSFR expression of bone marrow cells before (A) and after (B) BMT in patient 3. Bone marrow cells were analyzed according to the method described in Table 2.

without G-CSF when compared with that of normal subjects. In contrast, there was no difference in the number of GM colonies of CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>-</sup> cells supported with SCF, FL, and IL-3 with or without G-CSF between normal subjects and SCN patients. These findings suggest that abnormal responsiveness to hematopoietic factors in patients with SCN lies in primitive myeloid progenitor cells expressing G-CSFR.

#### *Proliferation of CD34<sup>+</sup>/Kit<sup>+</sup> cells in liquid suspension culture*

The proliferation of CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> and CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>-</sup> cells in response to various hematopoietic factors including G-CSF was examined in a serum-deprived liquid suspension culture. The numbers of total cells in wells were serially recorded. As shown in Fig. 4A and 4B, the CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> cells in patients with SCN showed a markedly reduced proliferation compared with those in normal subjects in response to SCF, FL, and IL-3, both with and without G-CSF. The impaired proliferation of CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> in patients was more remarkably observed in response to SCF, FL, and IL-3 with G-CSF than without G-CSF. In contrast, no difference in the proliferation of CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>-</sup> cells was noted between normal subjects and SCN patients (Fig. 4C and 4D). These results are consistent with the data showing a reduced level of GM colony formation in CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> cells and a comparable level in CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>-</sup> cells in semisolid culture.

#### *The results after bone marrow transplantation in a patient*

Patient 3 had recently received a BMT from an unrelated HLA-matched donor. After the complete engraftment of donor cells was observed, bone marrow cells from the patient were aspirated and examined. The bone marrow picture revealed normocellularity of the myeloid series without any maturation arrest. Fig. 5 shows the results of the

**Table 4.** GM colony formation in a patient before and after BMT

Factors	No. of GM Colonies	
	Before BMT	After BMT
A. CD34 <sup>+</sup> /Kit <sup>+</sup> /G-CSFR <sup>+</sup> Cells		
G-CSF	10 ± 3	20 ± 3
SCF, FL, IL-3	18 ± 5	30 ± 5
SCF, FL, IL-3, G-CSF	30 ± 3	60 ± 8
B. CD34 <sup>+</sup> /Kit <sup>+</sup> /G-CSFR <sup>-</sup> Cells		
G-CSF	2 ± 1	1 ± 1
SCF, FL, IL-3	27 ± 5	25 ± 4
SCF, FL, IL-3, G-CSF	48 ± 3	44 ± 6

Cultures were performed under serum-deprived conditions in media containing 250 cells and designated factors.

Data represent the mean ± SD of triplicate cultures.

flow cytometric analysis of the expression of CD34, Kit, and G-CSFR of bone marrow cells before (A) and after (B) BMT. The decreased frequency of CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> cells observed before BMT was restored to the comparable range shown in normal subjects. Furthermore, the number of GM colonies of CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> cells in response to SCF, FL, IL-3, with or without G-CSF was also restored to levels comparable to those seen in normal subjects (Tables 3, 4).

## DISCUSSION

Studies on granulopoiesis in patients with SCN have consistently shown a reduced responsiveness of LDBMC to G-CSF under various culture conditions<sup>5,20,31</sup>. We have recently reported the defective proliferation of LDBMC and purified CD34<sup>+</sup>/Kit<sup>+</sup> cells in patients with SCN in response to G-CSF under both serum-supplemented and serum-deprived cultures<sup>32</sup>. To further define the role of G-CSF and G-CSFR in the proliferation of myeloid progenitor cells of SCN patients, primitive myeloid progenitor cells, CD34<sup>+</sup>/Kit<sup>+</sup> cells, were purified on the basis of the expression of G-CSFR and we have shown the abnormalities of G-CSFR-positive cells<sup>42</sup>. In this study we have confirmed the abnormalities of primitive myeloid cells expressing G-CSFR by *in vitro* colony and proliferation assays.

Quantitatively, the frequency of cells expressing Kit and G-CSFR on the CD34 cells was remarkably reduced in all patients with SCN compared with that of normal subjects. The proportion of CD34 cells of nonadherent LDBMC in SCN patients was comparable to that in normal subjects, resulting in a reduction in the absolute number of primitive myeloid progenitor cells expressing G-CSFR in patients with SCN. The significance of the expression of CD34 antigen in human hematopoietic stem cells has been under discussion. However, the CD34 antigen and Kit

receptor identify cell populations that are enriched for pluripotent and lineage-restricted hematopoietic progenitor cells *in vitro*, and some of them are capable of bone marrow reconstitution *in vivo*<sup>27,44,50,60</sup>. The decreased number of CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> cells may be compatible with a defective myeloid progenitor origin for neutropenia in SCN patients. Qualitatively, the proliferation of CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> cells was also defective in the present study, showing a remarkably reduced number of GM-colonies and a decreased proliferation in response to SCF, FL, and IL-3 with or without G-CSF. In contrast, there was no significant difference in the proliferation of CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>-</sup> cells between normal subjects and SCN patients. We have reported direct evidence of the defective proliferation of CD34<sup>+</sup>/Kit<sup>+</sup> cells using a single-cell proliferation assay<sup>32</sup>. These observations suggest that the qualitative abnormality seen in SCN patients mainly lies in the CD34<sup>+</sup>/Kit<sup>+</sup> cells expressing G-CSFR. Interestingly these abnormalities were completely resolved after BMT. Thus, the presence of both qualitative and quantitative abnormalities in CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> cells might contribute significantly to the defective granulopoiesis in patients with SCN.

The role of G-CSF and G-CSFR in the stimulation of granulopoiesis has been well documented through the analysis of G-CSF-deficient and G-CSFR-deficient mice<sup>37,38</sup>. The mice carrying a homozygous null mutation for G-CSF exhibit approximately 20% of normal circulating neutrophils and a corresponding decrease in granulocytic precursors in their bone marrow<sup>37</sup>. The production of G-CSF and serum levels of G-CSF were normal or increased in patients with SCN<sup>16,28,40,45</sup>. Mice carrying a homozygous null mutation in the G-CSFR gene show decreased numbers of normal circulating neutrophils and a modest reduction in the number of hematopoietic progenitors in the bone marrow<sup>38</sup>. Mutations in the G-CSFR gene truncating the c-terminal region of the receptor protein are found in a minority (approximately 9%) of SCN patients<sup>15,56,57</sup>. The role of mutations of G-CSFR has been examined by the generation of mice carrying a targeted mutation of the G-CSFR that reproduces the mutation found in patients with SCN. Mice heterozygous or homozygous for this mutation have normal or decreased levels of circulating neutrophils and no evidence of a block in myeloid maturation, indicating that the resting granulopoiesis is normal. Rather, the mutations of the G-CSFR gene may play a role in leukemogenesis in a subgroup of patients with SCN<sup>18,19,22,34,55</sup>. SSCP and sequence analysis of extracellular, transmembrane and cytosolic portions of G-CSFR in the present study demonstrated no abnormalities in any of the SCN patients enrolled. Taken together, a



deficiency in G-CSF, the lack of G-CSFR, or a G-CSFR mutation by itself may not constitute a sufficient contribution to severe neutropenia and the SCN phenotype. However, our current data demonstrate that abnormalities *in vitro* granulopoiesis are present in CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> cells but not in CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>-</sup> cells. This finding provides evidence that the granulopoietic defect in SCN patients may be closely related to the expression of G-CSFR and its function in primitive myeloid progenitor cells.

In addition, G-CSFR-deficient mice show a modest but significant reduction in the total number of hematopoietic colonies formed in response to pokeweed mitogen-stimulated conditioned media, IL-3, GM-CSF, or SCF<sup>38</sup>). These data demonstrate that G-CSFR is required for the maintenance of a normal number of hematopoietic progenitor cells. In this study, CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> cells of patients with SCN showed a reduced responsiveness to a combination of SCF, FL and IL-3, irrespective of the presence or absence of G-CSF. This evidence also suggests that functional G-CSFR is necessary for the full stimulation of hematopoietic cells in response to hematopoietic factors. Alternatively, the decrease in the responsiveness of CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> cells might reflect the functional abnormality of the G-CSFR or G-CSFR-mediated signal pathway in patients with SCN.

The colony formation and proliferation of CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>-</sup> cells as well as those of CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> cells were enhanced by the addition of G-CSF to SCF, FL, and IL-3. The precise mechanism of the enhancement of the proliferation and the differentiation of CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>-</sup> cells by the addition of G-CSF is unclear. We could not completely exclude the possibility of the existence of a small number of CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> cells or of cells with a low-level expression of G-CSFR not being recognized by the antibody used in the CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>-</sup> cells. Furthermore, to confirm the possibility of the induction of G-CSFR from CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>-</sup> cells, the expression of G-CSFR was examined after the liquid suspension culture of CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>-</sup> cells for 10 days in the presence of SCF, FL, IL-3 with or without G-CSF. However, the expression of G-CSFR on the cells after the culture of CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>-</sup> cells was not observed (data not shown). Thus, the role of CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>-</sup> cells in myelopoiesis remains to be elucidated. In the majority of patients with SCN, treatment with pharmacological doses of recombinant human G-CSF leads to a significant increase of ANC and results in dramatic clinical improvement. This *in vivo* finding supports the hypothesis that the G-CSF-mediated granulopoiesis is relatively conserved in patients with SCN. Our data in culture show that normal phenotypic neutrophils are produced from

CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>-</sup> cells in response to the addition of G-CSF to SCF, FL, and IL-3 (data not shown). The production of neutrophils and their precursor cells from CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>-</sup> cells may play an important role in the favorable responsiveness of G-CSF administration *in vivo* in the majority of SCN patients. The studies of G-CSFR-deficient mice suggest the existence of G-CSFR-independent granulopoiesis.

Recently, mutations in *ELA2*, which encodes neutrophil elastase, have been reported to be responsible for the pathophysiology in patients with cyclic neutropenia<sup>21</sup>). In addition, mutations of the gene encoding neutrophil elastase differ from those in patients with cyclic neutropenia in some of patients with SCN<sup>7</sup>). An acceleration of the apoptosis of neutrophils, their precursor cells, and the progenitor cells of bone marrow has been documented in patients with cyclic neutropenia and in those with SCN<sup>1,2</sup>). The proliferative kinetics of CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> cells in liquid suspension culture showed remarkable differences between normal subjects and SCN patients (Fig. 4). This finding may reflect both a defect in proliferation and a decreased survival of cells. Although the precise mechanism of the involvement of neutrophil elastase in the acceleration of apoptosis remains unclear, the decreased number and impaired proliferation of primitive myeloid progenitor cells expressing G-CSFR as well as accelerated apoptosis may play a pivotal role in reducing the supply of neutrophils in patients with SCN.

Finally, on the basis of abnormalities in CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> cells in SCN patients, the key to detecting the underlying pathophysiology of SCN is to clarify the significance of G-CSFR expression on primitive myeloid progenitor cells in the development of hematopoietic progenitor cells associated with cell survival. It is likely that the quantitative abnormality of CD34<sup>+</sup>/Kit<sup>+</sup>/G-CSFR<sup>+</sup> cells might be a consequence of an underlying primary cellular defect of patients with SCN. Further studies are required to search for the origin of the quantitative and qualitative abnormalities of primitive myeloid progenitor cells expressing G-CSFR in patients with SCN.

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