Abnormal Responses of Myeloid Progenitor Cells to Recombinant Human Colony-Stimulating Factors in Congenital Neutropenia

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The effects of recombinant human interleukin-3 (IL-3) and recombinant human granulocyte colony-stimulating factor (G-CSF) on the growth of myeloid progenitor cells (CFU-C) in semisolid agar culture were studied in two patients with Kostmann-type congenital neutropenia. CFU-C growth in bone marrow cells from patients was significantly reduced in response to various concentrations of either IL-3 or G-CSF alone, compared with that from normal subjects. There was no inhibitory effect of bone marrow cells from patients on normal CFU-C formation supported by IL-3 or G-CSF. However, the simultaneous stimulation with IL-3 and G-CSF induced the increase of CFU-C formation in patients with congenital neutropenia. Furthermore, CFU-C growth in both patients was supported when bone marrow cells were preincubated with IL-3 in liquid culture followed by the stimulation with G-CSF in semisolid agar culture. In contrast, that was not supported by the preincubation with G-CSF and the subsequent stimulation with IL-3. This evidence suggests that the hematopoietic progenitor cells in patients with congenital neutropenia have the potential for developing CFU-C in the combined stimulation with IL-3 and G-CSF, and that this growth may be dependent on the priming of IL-3 followed by the stimulation with G-CSF. The level of mature neutrophils in peripheral blood was not fully restored to normal levels by the daily administration of G-CSF in doses of 100 to 200 $\mu g/m^2$ of body surface area for 20 to 25 days in both patients. These observations raise the possibility that the combination of IL-3 and G-CSF might have a potential role for the increase of neutrophil counts in patients with congenital neutropenia.

CONGENITAL NEUTROPENIAS are a heterogenous group of disorders. Kostmann-type neutropenia is a unique subgroup of these diseases and is characterized by the early onset of severe and recurrent infections, absence of neutrophils in the peripheral blood, and a bone marrow picture of maturation arrest of myeloid progenitor cells at the promyelocyte-myelocyte stage.[1-3] There have been several reports on in vitro granulopoiesis by semisolid colony formation and liquid-suspension culture, and the results from these reports have been variable.[4-10] However, in a majority of patients the proliferative and maturational potentials of myeloid progenitor cells in bone marrow have been considered normal in the presence of various colony-stimulating factors (CSFs), suggesting a deficiency in growth factor production as a possible cause of this phenomenon.[2,4,8,10]

Recently, CSFs that control the proliferation and the differentiation of hematopoietic progenitor cells have been purified, molecularly cloned, and expressed as recombinant proteins.[11-13] These factors are classified according to the types of hematopoietic progenitor cells with which they interact. It is generally accepted that recombinant human interleukin-3 (IL-3) induces the proliferation of stem cells and committed progenitors of multiple cell lineages, and that recombinant human granulocyte CSF (G-CSF) stimulates the terminal differentiation of granulocyte precursors.[14-16]

In this report, we study the effects of IL-3 and G-CSF on the proliferation of myeloid progenitor cells in patients with Kostmann-type congenital neutropenia. The results demonstrate that myeloid progenitor cells in patients proliferate in response to combined and sequential stimulation with IL-3 and G-CSF, although those fail to react with either IL-3 or G-CSF alone.

MATERIALS AND METHODS

Subjects. Two patients with Kostmann-type congenital neutropenia were studied. Patient 1 was a 3-year-old boy who contracted otitis media at the age of 6 months. At the age of 11 months he had a high fever of 2 weeks' duration due to a liver abscess that was treated by surgical drainage. At that time, the complete blood cell count showed a total white blood cell (WBC) count of 7.6×10^9/L, with no neutrophils. Red blood cell (RBC) and platelet counts were normal. Bone marrow aspiration showed the normal cellularity with a maturation arrest at the promyelocyte stage. After this event he was started on prophylactic sulfamethoxazole-trimethoprim and rifampin. However, he continued to have recurrent skin abscesses and chronic gingivitis. During the 22 months of follow-up, total WBC count ranged from 4.7 to 9.5×10^9/L, with an absolute neutrophil count (ANC) of 0 to 0.102×10^9/L. Patient 2 was a 2-year-old boy who presented at the age of 2 months with pneumonia. His WBC count was 5×10^9/L with an ANC of 0.05×10^9/L. RBC and platelet counts were normal. Bone marrow aspiration showed the normal cellularity with a deficiency of neutrophil precursors beyond the myelocyte stage. Since the age of 3 months, he has been maintained on daily administration of sulfamethoxazole-trimethoprim. Despite the prophylaxis, he had repeated hospitalizations due to respiratory tract infections and bacteremias. During the 24 months of follow-up, total WBC count ranged from 4 to 10.5×10^9/L, with an ANC of 0 to 0.25×10^9/L.

Recombinant human hematopoietic growth factors. IL-3, which was prepared by expressing in *Escherichia coli* followed by further purification, was the generous gift of Genetics Institute, Cambridge, MA. G-CSF that was produced with *E coli*, according to recombinant DNA techniques, was kindly supplied by Kirin-Amgen Co, Tokyo, Japan, and Sankyo Co Ltd, Tokyo, Japan.

Bone marrow cells. All bone marrow samples were obtained after informed consent of patients and/or their guardians. Normal bone marrow cells for this study were obtained from hematologically

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normal pediatric patients ranging in age from 9 months to 5 years who underwent the procedure for diagnostic purposes. Bone marrow aspirates from both patients and normal subjects for all experiments were performed during apparent infection-free periods.

The aspirated bone marrow cells were diluted with Iscove's modified Dulbecco's medium (IMDM; GIBCO, Grand Island, NY), layered over Ficoll-sodium metrizoate (1.077 g/mL, Nyegaard & Co, Norway), and centrifuged for 30 minutes at 400 × *g*. Interface cells were washed three times, resuspended in IMDM with 10% heat-inactivated fetal bovine serum (FBS; Hazleton, Lenexa, KS) at a concentration of 10^6 cells/mL and incubated in plastic dishes at 37°C over 3 hours to remove the adherent cells.

Semisolid cultures. Myeloid progenitor cell (CFU-C) assay was performed using a semisolid agar method originally described by Pike and Robinson[17] with modification in 35-mm plastic dishes (Falcon, Oxnard, CA).[8] Bone marrow nonadherent cells (5×10^4 or 1×10^5 cells) were plated in 1 mL IMDM containing 0.3% Bacto-agar (Difco Lab, Detroit, MI), 20% FBS, 1% bovine serum albumin (BSA; Sigma Chemical Co, St Louis, MO), 5×10^{-5} mol/L 2-mercaptoethanol (Nacalai Tesque, Kyoto, Japan), and various concentrations of IL-3 and/or G-CSF. The cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 7 to 10 days. Colonies were defined as clones of more than 40 cells. CFU-C counts represent the mean of duplicate or triplicate cultures. To determine the cellular composition of the resulting colonies, histochemical analyses using esterase double-staining methods in the agar gel as a whole plate preparation were performed and CFU-C were grouped into three classes, such as granulocyte-colony (G-colony), macrophage-colony (M-colony), and granulocyte/macrophage-colony (GM-colony), according to the previously described method.[8,18] In some experiments, 0.9% methylcellulose (Nacalai Tesque) cultures were performed and morphologic examinations of cells picked up in colony were determined by the Cytospin (Shandon Southern, Elliott, IL) preparations with Wright's stain and/or esterase double stain.

Liquid culture. For liquid culture experiments, bone marrow nonadherent cells prepared as described above (1×10^6/mL) were initially incubated in tissue culture flask in 5 mL IMDM plus 20% FBS with either IL-3 or G-CSF for up to 7 days. As for control, cells were incubated without any CSFs. The cultures were generally maintained by change of medium partially on the third or fourth day. After washing the cells completely, subsequent semisolid agar culture was performed in the presence of IL-3 or G-CSF for 7 days. CFU-C counts and cellular composition of the colonies on day 7 were determined as described above.

Study design for the administration of G-CSF. The patients received a sequential 10- to 14-day course of G-CSF in either daily 1-hour intravenous infusion or subcutaneous injections. The initial dose was 100 $\mu g/m^2$ of body surface area per day. If there was no obvious response, the daily dose increased to 200 $\mu g/m^2$ of body surface area for a subsequent 10 to 14 days. The responses were evaluated at that time.

RESULTS

Responsiveness of myeloid progenitor cells to IL-3 or G-CSF alone. The CFU-C formation stimulated by various concentrations of IL-3 or G-CSF in both normal subjects and patients with congenital neutropenia is presented in Fig 1 (IL-3) and Fig 2 (G-CSF), respectively. In normal bone marrow cells, CFU-C growth in response to IL-3 and G-CSF was dose-dependent, and maximal CFU-C growth was elicited by the concentration of 100 U/mL of IL-3 and 50 ng/mL of G-CSF, respectively. In contrast to normal subjects, CFU-C from two patients with congenital neutropenia

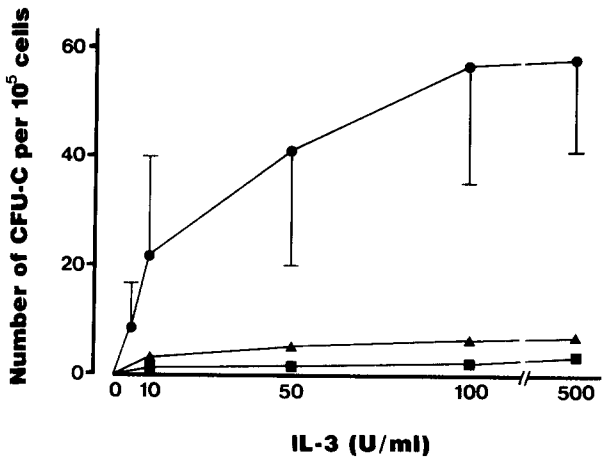

Fig 1. CFU-C formation in response to various concentrations of IL-3. Results are expressed as the mean ± SD from five normal subjects (●), the mean of three different experiments in patient 1 (▲), and the mean of two different experiments in patient 2 (■).

was markedly decreased in any concentrations of IL-3 and G-CSF. As shown in Tables 1 and 2, most colonies formed by G-CSF from normal subjects and patients were positive for chloracetate esterase and negative for nonspecific esterase, consistent with the colonies being granulocytic in type. On the other hand, those supported by IL-3 consisted of three different types, identified as G-, GM-, and M-colonies.

Effect of bone marrow cells on CFU-C formation supported by IL-3 or G-CSF. To study the presence of inhibitory activity from patient bone marrow cells, we used the double-layer agar method. As shown in Table 3, there was no inhibition of CFU-C formation from normal bone marrow cells in the presence of IL-3 or G-CSF when the bone marrow cells from patients were plated in the under layer. Alternatively, CFU-C formation supported by IL-3 was increased by the addition of the bone marrow cells in the

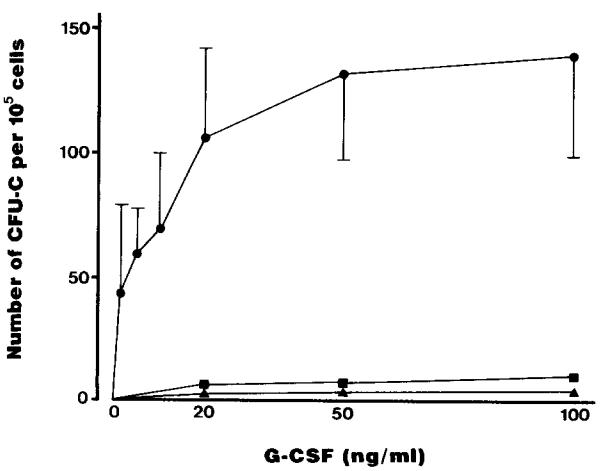

Fig 2. CFU-C formation in response to various concentrations of G-CSF. Results are expressed as the mean ± SD from five normal subjects (●), the mean of three different experiments in patient 1 (▲), and the mean of two different experiments in patient 2 (■).

Table 1. Types of CFU-C in Response to IL-3 and G-CSF in Normal Subjects

	No. of CFU-C	Type of CFU-C (%)		
		G	GM	M
IL-3 (U/mL)				
0	0			
10	22 ± 18	53	2	46
50	41 ± 21	43	15	42
100	57 ± 32	46	20	34
G-CSF (ng/mL)				
0	0			
10	69 ± 30	87	11	2
20	105 ± 37	88	10	2
50	130 ± 36	84	14	2
100	139 ± 41	91	7	2

Number of CFU-C per 1 × 10^5 bone marrow nonadherent cells is expressed as the mean ± SD from five normal subjects. Type of CFU-C is expressed as the mean of the same samples.

under layer. There was no difference between normal subjects and the patients in the enhancement of CFU-C growth in the presence of IL-3. These results imply that the decrease of CFU-C formation stimulated by IL-3 or G-CSF in patients with congenital neutropenia is not due to the production of diffusible inhibitors by patients' bone marrow cells.

Responsiveness of myeloid progenitor cells to the combination of IL-3 and G-CSF. We and others have previously shown that CFU-C are present in normal numbers in a majority of congenital neutropenias.[4-10] It seems likely that the discrepancy of CFU-C formation between our present observations, as shown in Figs 1 and 2 and the results from prior reports, is due to the difference of CSFs. As has been reported, there are different or overlapping effects between the synthetic CSFs, such as IL-3 and G-CSF, and they frequently display synergistic or additive effects in supporting hematopoietic progenitor cell colony formation in serum-containing culture.[19-23]

Table 2. Types of CFU-C in Response to IL-3 and G-CSF in Patients With Congenital Neutropenia

	Patient 1				Patient 2			
	No. of CFU-C	Type of CFU-C (%)			No. of CFU-C	Type of CFU-C (%)		
		G	GM	M		G	GM	M
IL-3 (U/mL)								
0	0				0			
10	3 ± 4	0	50	50	0			
50	5 ± 5	20	20	60	1 ± 1	50	0	50
100	6 ± 6	24	38	38	2 ± 1	50	0	50
500	7 ± 5	50	22	28	3 ± 2	71	20	9
G-CSF (ng/mL)								
0	0				0			
20	2 ± 2	67	16	17	9 ± 10	100	0	0
50	5 ± 4	62	20	18	12 ± 11	92	8	0
100	5 ± 3	100	0	0	16 ± 13	98	2	0
200	6 ± 2	98	0	2	12 ± 10	96	2	2

Number of CFU-C per 1 × 10^5 bone marrow nonadherent cells is expressed as the mean ± SD from three different experiments in patient 1 and from two different experiments in patient 2. Type of CFU-C is expressed as the mean of the same samples.

In normal subjects, increased CFU-C formation was found by the simultaneous addition of both IL-3 and G-CSF, as shown in Table 4. This effect was notably observed when a low concentration of IL-3 was used. The effect of the combined stimulation with IL-3 and G-CSF on CFU-C formation in patients with congenital neutropenia is also shown in Table 4. Although IL-3 or G-CSF alone was insufficient for CFU-C formation, G-colony formation was markedly induced by stimulation with the combination of factors in both patients. However, there was a difference between the responses observed with the two patients' samples: in patient 1 the frequency of CFU-C obtained was only one fourth of that from the sample of patient 2.

In some experiments, colonies formed by these factors from either patient were picked from methylcellulose culture and analyzed for their cellular composition. These colonies contained myeloid cells at various stages of maturation, from myeloblasts to segmented neutrophils (data not shown). This result is consistent with our previous report on suspension cultures of patient bone marrow cells.[8] These observations suggest that bone marrow cells from patients with Kostmann-type congenital neutropenia are capable of normal proliferation and differentiation to mature myeloid cells in response to appropriate CSFs.

Responsiveness of myeloid progenitor cells in patients to sequential stimulation with IL-3 and G-CSF. To study further the proliferative effect of the combination of IL-3 and G-CSF in patients, we performed secondary cultures in semisolid medium after primary liquid cultures in the presence of IL-3 or G-CSF. The results from both patients are presented in Fig 3. When bone marrow nonadherent cells were preincubated with IL-3 for up to 7 days, there was no CFU-C formation in the absence of subsequent stimulation with any CSFs (data not shown). However, the induction of CFU-C formation was observed with the subsequent stimulation with G-CSF but not with IL-3. There was no difference between the concentrations of subsequent G-CSF. Maximal CFU-C formation was seen at 4 or 5 days' preincubation with IL-3 in this condition (Fig 3A). Most of the colonies formed by the primary stimulation with IL-3 for 4 days and the subsequent stimulation with G-CSF were granulocytic (Table 5). These results were comparable with those from simultaneous addition of IL-3 and G-CSF in patient 2. In patient 1, the sequential stimulation was more conducive to CFU-C formation than the simultaneous stimulation with both factors. In contrast, preincubation in G-CSF led to little CFU-C formation in cultures subsequently supported by either IL-3 or G-CSF (Fig 3B).

Effect of the administration of G-CSF on absolute neutrophil counts of peripheral blood in patients. We administered recombinant human G-CSF in doses of 100 to 200 $\mu g/m^2$ of body surface area to both patients when they were apparently infection-free. As shown in Table 6, the absolute neutrophil counts in peripheral blood in both patients were slightly increased by the administration of G-CSF. However,

Table 3. Effect of Bone Marrow Cells From Patients and Normal Subjects on CFU-C Formation Supported by IL-3 or G-CSF

Cells in Under Layer	No. of CFU-C*	Type of CFU-C (%) G	GM	M	No. of CFU-C*	Type of CFU-C (%) G	GM	M
Exp 1		IL-3 (50 U/mL)				G-CSF (50 ng/mL)		
None	39	42	35	23	132	82	14	4
Patient 1	81	29	42	29	114	57	40	3
Normal subject	85	27	39	34	123	61	37	2
Exp 2								
None	42	43	15	42	130	84	14	2
Patient 2	86	25	45	30	145	75	22	3
Normal subject	76	32	34	34	157	81	16	3

Bone marrow nonadherent cells (1×10^5 cells) were plated in 1 mL IMDM containing 0.5% Bacto-agar, 20% FBS, and 1% BSA in the under layer. The composition of the upper layer and the experimental conditions were similar to those described in Materials and Methods. The representative results from two different experiments in each patient are shown.

*Number of CFU-C per 1×10^5 bone marrow nonadherent cells from normal subjects.

the dose of G-CSF used in this study design did not lead to an increase in normal levels of mature neutrophils.

DISCUSSION

The research on recombinant hematopoietic CSFs has recently been intensified in the re-examination and therapeutic management of congenital and acquired bone marrow disorders, including congenital neutropenia syndromes.[14-16,24-30] This study demonstrated that myeloid progenitor cells in the bone marrow from two patients with Kostmann-type congenital neutropenia displayed little responsiveness to either IL-3 or G-CSF alone in a semisolid colony assay. However, the decrease of CFU-C formation in the same samples was restored by the combined stimulation of IL-3 and G-CSF. Most of the colonies formed in this condition were granulocytic and consisted of myeloid cells at various stages of maturation. These results indicate that bone marrow cells from patients with congenital neutropenia have the capacity for normal proliferation and differentiation to mature myeloid cells. This conclusion is consistent with prior reports using crude conditioned medium, such as phytohemagglutinin-stimulated conditioned medium, human placenta-condi-

Table 4. Effect of Simultaneous Stimulation With IL-3 and G-CSF on CFU-C Formation

IL-3 + G-CSF (U/mL) (ng/mL)	No. of CFU-C	Type of CFU-C (%) G	GM	M
Normal subjects (n = 5)				
0 + 20	104 ± 31	90	8	2
0 + 50	120 ± 34	93	4	2
10 + 0	22 ± 18	53	2	45
50 + 0	43 ± 21	43	15	42
10 + 20	182 ± 58	91	4	5
10 + 50	192 ± 47	91	3	6
50 + 20	210 ± 67	86	4	10
50 + 50	211 ± 58	90	4	6
Patient 1				
0 + 20	2	67	0	33
0 + 50	5	94	0	6
10 + 0	3	50	50	0
50 + 0	5	20	20	60
10 + 20	15	93	4	3
10 + 50	20	90	5	5
50 + 20	22	95	2	3
50 + 50	34	93	3	4
Patient 2				
0 + 20	9	100	0	0
0 + 50	12	92	8	0
10 + 0	0			
50 + 0	1	50	0	50
10 + 20	125	99	1	0
10 + 50	123	99	1	0
50 + 20	125	99	0	1
50 + 50	144	96	2	2

Number of CFU-C per 1×10^5 bone marrow nonadherent cells is expressed as the mean ± SD from five normal subjects and the mean of two different experiments from patients. Type of CFU-C is expressed as the mean of the same samples.

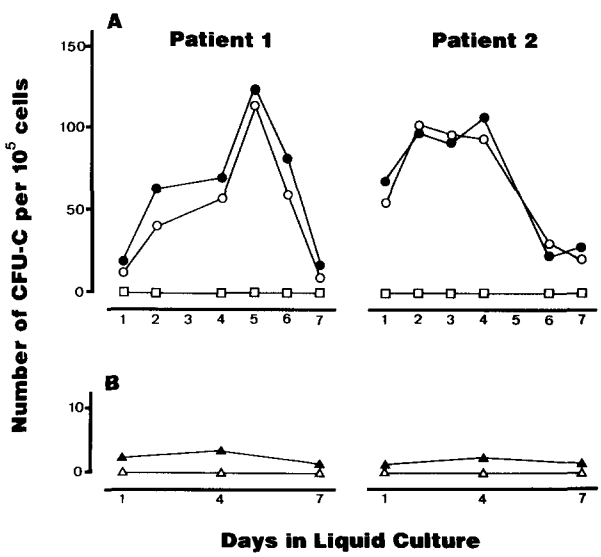

Fig 3. CFU-C formation induced by the sequential stimulation of IL-3 and G-CSF in patients. Bone marrow nonadherent cells were cultured in liquid suspension in the presence of 50 U/mL IL-3 (A) or 50 ng/mL G-CSF (B) for up to 7 days. Cells were washed completely and plated in semisolid agar cultures in the presence of 20 ng/mL G-CSF (○), 50 ng/mL G-CSF (●,△), and 50 U/mL IL-3 (□,▲), respectively. Results are expressed as the mean of two different experiments from both patients.

Table 5. CFU-C Induced by the Sequential Stimulation With IL-3 and G-CSF in Patients

CSF	Patient 1				Patient 2			
	No. of CFU-C*	Type of CFU-C (%)			No. of CFU-C*	Type of CFU-C (%)		
		G	GM	M		G	GM	M
IL-3 (50 U/mL) + G-CSF								
(20 ng/mL)	59	97	3	0	92	98	2	0
(50 ng/mL)	70	89	11	0	108	87	11	2

Bone marrow nonadherent cells were cultured in liquid suspension in 50 U/mL IL-3 for 4 days and plated in semisolid agar cultures stimulated with 20 ng/mL or 50 ng/mL G-CSF for 7 days. Results are expressed as the mean of two different experiments from both patients.

*Number of CFU-C per 1×10^5 cells after liquid culture.

tioned medium, and human omentum-conditioned medium.[4-10]

IL-3 is capable of supporting the growth of multilineage erythroid, myeloid, and megakaryocytic colonies, as well as that of mast cells.[14,15,19-22] G-CSF, on the other hand, is generally believed to be a lineage-specific hematopoietic factor that directly supports the proliferation of neutrophil colonies.[12,14,15] However, there are somewhat overlapping effects between IL-3 and G-CSF, and they frequently display synergistic or additive effects in supporting hematopoietic progenitor cell colony formation in serum-containing condition.[19-23,31] The ability of the simultaneous stimulation of IL-3 and G-CSF to restore the granulocyte-colony formation in patients was remarkable. The relationship between IL-3– and G-CSF–mediated growth was further studied after preculture of bone marrow cells in patients with congenital neutropenia. Granulocyte colonies were supported when the bone marrow cells were preincubated in suspension culture in the presence of IL-3 with subsequent culture in semisolid medium in the presence of G-CSF, but not when the order of exposure to the factors was reversed. These observations provide the possibilities that granulocyte colony formation in patients requires both stimulation of IL-3 and G-CSF, and that this may be dependent on the priming of IL-3 followed by the stimulation with G-CSF. These data from patients are in accordance with those that IL-3 has been capable of maintaining the number of CFU-C in liquid culture of normal human bone marrow cells, but G-CSF has not.[32-34] A similar effect of IL-3 has also been found in the proliferation of erythroid progenitors, suggesting that IL-3 either stimulates self-renewal of both erythroid burst-forming unit and CFU-C, or alternatively, drives erythroid and myeloid commitment from antecedent pluripotent cells.[35,36]

Table 6. Effect of the Administration of G-CSF on Absolute Neutrophil Counts of Peripheral Blood in Patients With Congenital Neutropenia

	Absolute Neutrophil Count ($\times 10^{-9}$/L)		
	Base Line	Pre	Post
Patient 1	0–0.102	0	0.474 (day 25)
Patient 2	0–0.25	0.062	0.396 (day 20)

Patient 1 received 60 μg of G-CSF in daily 1-hour intravenous infusions for 11 days and 120 μg of G-CSF for 7 days. Subsequently, 120 μg of G-CSF was administered by subcutaneous injection for 7 days. Patient 2 initially received 50 μg of G-CSF for 10 days and 100 μg of G-CSF for 10 days more in daily 1-hour intravenous infusion.

Although there was a difference in the frequency of CFU-C formation supported by the simultaneous addition of IL-3 and G-CSF between patients (Table 4), similar numbers of CFU-C were induced by the sequential stimulation with these factors (Table 5). At present, the basis for this phenomenon is not known. However, these differences may in part be due to the cellular variety related to the heterogeneity of this disorder[4-10] or to the cellular responsiveness to G-CSF in the liquid culture after the stimulation with IL-3. It has been reported that there should be a potentiating effect on CFU-C development supported by the combined stimulation with IL-3 and G-CSF.[19] Sieff et al[20] found the specific induction of granulocyte colonies by the combined stimulation with IL-3 and G-CSF, suggesting that distinct subsets of granulocytic precursors exist that respond to IL-3, G-CSF, or both factors combined.

It remains unclear why CFU-C formation stimulated by either IL-3 or G-CSF alone is reduced in patients with congenital neutropenia. One interpretation is that the inability of IL-3 or G-CSF alone to induce CFU-C may be caused by a decrease in the frequency of cells responsive to each CSF. As for G-CSF, enriched populations of normal human promyelocytes and myelocytes have demonstrated specific labeling with ^{125}I–G-CSF and clonal proliferation when stimulated with G-CSF.[37,38] The proportion of promyelocytes and myelocytes or chloracetate esterase-positive cells in light-density adherence depleted marrow cells from both patients was clearly smaller than that from normal subjects; patient 1 was 0.5% to 1.3% and patient 2 was 1.2% to 2.8%, respectively (normal control, 13% to 27%). However, the progenitor cells that respond to IL-3 in the bone marrow are still not well-defined in serum-supplemented culture. In serum-free culture, Sonoda et al[34] suggested that the primary targets of IL-3 are multipotent progenitors at the early stages of development.

Another explanation is that CFU-C growth from patients is more dependent on exogenously added CSFs than that from normal subjects because of possible underlying disturbances in the responsiveness to IL-3 or G-CSF. In our culture condition, CFU-C development may be affected by the potential factors in the serum and/or from accessory cells in the bone marrow. Wright et al[30] suggested these possibilities from the data of mathematical models of the regulation of myelopoiesis in response to granulocyte-macrophage CSF in patients with childhood onset cyclic neutropenia. With highly purified marrow progenitors, Bot et al[39] demonstrated that IL-3, although it did not stimulate granulocyte colony

formation by itself, regulated the survival and proliferative rate of granulocytic progenitors. Furthermore, multi-CSF activity of IL-3 for CFU-G, CFU-GM, and CFU-M has been dependent on the availability of accessory cells that are present in our conditions.[40] The increase of CFU-C formation in the presence of IL-3 was observed when the bone marrow cells from patients and normal subjects were plated in the under layer. This observation suggests that the bone marrow cells in patients may be capable of supporting the increase of CFU-C formation by IL-3.

Therefore, further examinations with more highly enriched hematopoietic progenitor cells and with defined, serum-depleted culture conditions should be necessary to clarify the growth factor requirements for the myeloid progenitors in patients with congenital neutropenia.

Recently, clinical trials of G-CSF to patients with a variety of different types of neutropenias have been started.[24-29] Bonilla et al[26] reported the G-CSF could lead to an increase of functional neutrophils in five patients with congenital agranulocytosis. However, the pattern of hematopoietic response differed from that previously reported after administration of growth factor to normal subjects, patients with myelodysplasia, or immunosuppressed patients. The effective dose of G-CSF varied among five patients, and some patients required high-dose administration of G-CSF to increase peripheral blood neutrophils. Weston et al[27] reported the rise of neutrophil counts and therapeutic advance by G-CSF during the infectious period in patients with congenital neutropenia. On the other hand, the level of mature neutrophils in peripheral blood was not fully restored to normal levels in our patients by the administration of daily doses of G-CSF. Thus, the effect of G-CSF on the induction of mature neutrophils would not be expected to be uniform because of the heterogeneity of this disorder in which both the in vitro responsiveness of myeloid progenitor cells and the in vivo efficacy of G-CSF have already been reported to be variable.[4-10,26] In view of our in vitro results, increases of mature neutrophils in patients with congenital neutropenia might best be obtained using a combination of the terminal differentiating factor G-CSF with exogenous or endogenous factors for early hematopoietic progenitor cell proliferation. Finally, further in vitro and in vivo analyses of more cases with congenital neutropenia using recombinant hematopoietic CSFs could define the pathogenesis and indicate a more suitable therapeutic approach to this disorder.

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REFERENCES

1. Kostmann R: Infantile genetic agranulocytosis (agranulocytosis infantalis hereditaria): A new recessive lethal disease in man. Acta Paediatr Scand 45:1, 1956 (suppl 105)

2. Kostmann R: Infantile genetic agranulocytosis: A review with presentation of ten new cases. Acta Paediatr Scand 64:362, 1975

3. Curnutte JT, Boxer LA: Disorders of granulopoiesis and granulocyte function, in Nathan DG, Oski FA (eds): Hematology of Infancy and Childhood, vol 1. Philadelphia, PA, Saunders, 1987, p 797

4. Barak Y, Paran M, Levin S, Sachs L: In vitro induction of myeloid proliferation and maturation in infantile genetic agranulocytosis. Blood 38:74, 1971

5. Amato D, Freedman MH, Saunders E: Granulopoiesis in severe congenital neutropenia. Blood 47:531, 1976

6. Falk PM, Rich K, Feig S, Stiehn ER, Golde DW, Cline MJ: Evaluation of congenital neutropenic disorders by in vitro bone marrow cultures. Pediatrics 59:739, 1977

7. Parmley FT, Crist WM, Ragab AH, Boxer LA, Malluh A, Lui VK, Darby CP: Congenital dysgranulopoietic neutropenia: Clinical, serologic, ultrastructural, and in vitro proliferative characteristics. Blood 56:465, 1980

8. Kawaguchi Y, Kobayashi M, Tanabe A, Hara M, Nishi Y, Usui T, Nagai S, Nishibayashi Y, Nagao K, Yokoro K: Granulopoiesis in patients with neutropenia. Am J Hematol 20:223, 1985

9. Ishiguro A: Complement-mediated cytotoxicity against marrow late neutrophilic cells in Kostmann's syndrome. Acta Haematol Jpn 50:1196, 1987

10. Coulombel L, Morardet N, Veber F, Leroy C, Mielot F, Fisher A, Teillet-Thiebaud F, Tchernia G, Griscelli C, Parmentier C: Granulopoietic differentiation in long-term bone marrow cultures from children with congenital neutropenia. Am J Hematol 27:93, 1988

11. Sieff CA, Emerson SG, Donahue RE, Nathan DG, Wang EA, Wong GG, Clark SC: Human recombinant granulocyte-macrophage colony stimulating factor: A multilineage hematopoietin. Science 230:1171, 1985

12. Souza LM, Boone TC, Gabrilove J, Lai PH, Zsebe KM, Murdock DC, Chazin VR, Bruszewski J, Kenneth HL, Chen K, Barendt J, Blatzer E, Moore MA, Mertelsmann R, Welte K: Recombinant human granulocyte colony-stimulating factor: Effect on normal and leukemic myeloid cells. Science 232:61, 1986

13. Yang YC, Ciarletta AB, Temple PA, Chung MP, Kovacic S, Witek-Giannotti JS, Leary AC, Kriz R, Donahue RE, Wong GG, Clark SC: Human IL-3 (multi-CSF): Identification by expression cloning on a novel hematopoietic growth factor related to murine IL-3. Cell 47:3, 1986

14. Sieff AS: Hematopoietic growth factors. J Clin Invest 79: 1549, 1987

15. Clark SC, Kamen R: The human hematopoietic colony-stimulating factors. Science 236:1229, 1987

16. Sachs L: The molecular control of blood cell development. Science 238:1374, 1987

17. Pike BL, Robinson WA: Human bone marrow colony growth in agar-gel. J Cell Physiol 76:77, 1970

18. Kubota K, Mizoguchi H, Miura Y, Suda T, Takaku F: A new technique for the cytochemical examination of human hemopoietic cells grown in agar gel. Exp Hematol 8:339, 1980

19. Messner HA, Yamasaki K, Jamal N, Minden MM, Yang YC, Wong GG, Clark SC: Growth of human hemopoietic colonies in response to recombinant gibbon interleukin 3: Comparison with human recombinant granulocyte and granulocyte-macrophage colony-stimulating factor. Proc Natl Acad Sci USA 84:6765, 1987

20. Sieff CA, Niemeyer CM, Nathan DG, Ekern SC, Bieber FR, Yang YC, Wong GG, Clark SC: Stimulation human hematopoietic colony formation by recombinant gibbon multi–colony-stimulating factor or interleukin 3. J Clin Invest 80:818, 1987

21. Migliaccio G, Migliaccio AR, Adamson JW: In vitro differentiation of human granulocyte/macrophage and erythroid progenitors:

Comparative analysis of the influence of recombinant human erythropoietin, G-CSF, GM-CSF, and IL-3 in serum-supplemented and serum-deprived cultures. Blood 72:248, 1988

22. Ikebuchi K, Clark SC, Ihle JM, Souza LM, Ogawa M: Granulocyte colony-stimulating factor enhances interleukin 3-dependent proliferation of multipotential hemopoietic progenitors. Proc Natl Acad Sci USA 85:3445, 1988

23. Paquette RL, Zhou JY, Yang YC, Clark SC, Koeffler HP: Recombinant gibbon interleukin-3 acts synergistically with recombinant human G-CSF and GM-CSF in vitro. Blood 71:1596, 1988

24. Gabrilove JL, Jakubowski A, Scher H, Sternberg C, Wong G, Grous J, Yagoda A, Fain K, Moore MAS, Clarkson B, Oettgen HF, Alton K, Welte K, Souza L: Effect of granulocyte colony-stimulating factor on neutropenia and associated morbidity due to chemotherapy for transitional-cell carcinoma of the urothelium. N Engl J Med 318:1414, 1988

25. Morstyn G, Campbell L, Souza LM, Alton NK, Keech J, Green M, Sheridan W, Metcalf D: Effect of granulocyte colony stimulating factor on neutropenia induced by cytotoxic chemotherapy. Lancet 1:667, 1988

26. Bonilla MA, Gillio AP, Ruggeiro M, Kernan NA, Brochstein JA, Abbound M, Fumagalli L, Vincent M, Gabriolove JL, Welte K, Souza LM, O'Reilly J: Effects of recombinant human granulocyte colony-stimulating factor on neutropenia in patients with congenital agranulocytosis. N Engl J Med 320:1574, 1989

27. Weston B, Locey B, Todd RF III, Hutchinson RJ, Boxer LA: Treatment of congenital neutropenia. Pediatr Res 25:158a, 1989 (abstr)

28. Jakubowski AA, Souza L, Kelly F, Fain K, Budman D, Clarkson B, Bonilla MA, Moore MAS, Gabrilove J: Effects of human granulocyte colony-stimulating factor in a patient with idiopathic neutropenia. N Engl J Med 320:38, 1989

29. Hammond WP, Price TH, Souza LM, Dale DC: Treatment of cyclic neutropenia with granulocyte colony-stimulating factor. N Engl J Med 320:1306, 1989

30. Wright DG, LaRussa VF, Salvado AJ, Knight RD: Abnormal responses of myeloid progenitor cells to granulocyte-macrophage colony-stimulating factor in human cyclic neutropenia. J Clin Invest 83:1414, 1989

31. Ferrero D, Tarella C, Badoni R, Caracciolo D, Bellone G, Pileri A, Gallo E: Granulocyte-macrophage colony-stimulating factor requires interaction with accessory cells or granulocyte-colony stimulating factor for full stimulation of human myeloid progenitors. Blood 73:402, 1989

32. Kobayashi M, Van Leeuwen BH, Elsbury S, Martinson ME, Young IG, Hapel AJ: Interleukin-3 is significantly more effective than other colony-stimulating factors in long-term maintenance of human bone marrow-derived colony-forming cells in vitro. Blood 73:1836, 1989

33. Migliaccio AR, Migliaccio G, Adamson JW: Effect of recombinant hematopoietic growth factors on proliferation of human marrow progenitor cells in serum-deprived liquid culture. Blood 72:1387, 1988

34. Sonoda Y, Yang YC, Wong GG, Clark SC, Ogawa M: Analysis in serum-free culture of the targets of recombinant human hemopoietic growth factors: Interleukin 3 and granulocyte/macrophage-colony-stimulating factor are specific for early developmental stages. Proc Natl Acad Sci USA 845:4360, 1988

35. Emerson SG, Yang YC, Clark SC, Long MW: Human recombinant granulocyte-macrophage colony stimulating factor and interleukin 3 have overlapping but distinct hematopoietic activities. J Clin Invest 82:1282, 1988

36. Halperin DS, Estrov Z, Freedman M: Diamond-Blackfan anemia: Promotion of marrow erythropoiesis in vitro by recombinant interleukin-3. Blood 73:1168, 1989

37. Begly CG, Metcalf D, Nicola NA: Binding characteristics and proliferative action of purified granulocyte colony-stimulating factor (G-CSF) on normal and leukemic human promyelocytes. Exp Hematol 16:71, 1988

38. Begly CG, Nicola NA, Metcalf D: Proliferation of normal human promyelocytes and myelocytes after a single pulse stimulation by purified GM-CSF or G-CSF. Blood 71:640, 1988

39. Bot FJ, van Eijk L, Schipper P, Löwenberg B: Effects of human interleukin-3 on granulocytic colony-forming cells in human bone marrow. Blood 73:1157, 1989

40. Bot FJ, Dorssers L, Wagenmaker G, Löwenberg B: Stimulating spectrum of human recombinant multi-CSF (IL-3) on human marrow precursors: Importance of accessory cells. Blood 71:1609, 1988