A comparison of the defective granulopoiesis in childhood cyclic neutropenia and in severe congenital neutropenia

Yasuhiko Sera
Hiroshi Kawaguchi
Kazuhiro Nakamura
Takashi Sato
Masakazu Habara
Satoshi Okada
Nobutsune Ishikawa
Seiji Kojima
Osamu Katoh
Masao Kobayashi

Background and Objectives. Cyclic neutropenia (CyN) in childhood and severe congenital neutropenia (SCN) are congenital disorders that cause chronic neutropenia. Mutations in the neutrophil elastase gene, *ELA2*, have been reported in patients with CyN and in those with SCN. We examined granulopoietic defects in CyN patients with those in SCN patients.

Design and Methods. Three patients with CyN and four with SCN were enrolled in this study. Bone marrow cells were enriched based on the expression of CD34, Kit, and granulocyte colony-stimulating factor receptor (G-CSFR). The purified cells were assayed for colony formation, proliferation, and mRNA expression of granular enzymes.

Results. All patients showed heterozygous mutations of *ELA2*. Flow cytometric analysis demonstrated no differences in the frequency of CD34, Kit, and G-CSFR expression between CyN patients and normal subjects. Significant differences in granulocyte/macrophage (GM)-colony formation of $CD34^+/Kit^+$ cells were observed among CyN patients, SCN patients, and normal subjects in response to hematopoietic factors. Impaired granulopoiesis was found in both $CD34^+/Kit^+/G\text{-}CSFR^+$ and $CD34^+/Kit^+/G\text{-}CSFR^-$ cells in patients with CyN, whereas this impairment was observed only in $CD34^+/Kit^+/G\text{-}CSFR^+$ cells in SCN patients, as previously reported. The mRNA expression of granular enzymes in myeloid precursors and the transcription levels during myeloid cell differentiation in CyN patients were comparable to those in normal subjects, in contrast to the abnormal transcription of granular enzymes in SCN patients.

Interpretation and Conclusions. These results suggest that the underlying granulopoietic abnormalities differ between CyN and SCN, and emphasize the presence of additional genetic pathophysiology specific to each disease.

Keywords: cyclic neutropenia, granulopoiesis, neutrophil elastase, severe congenital neutropenia.

Haematologica 2005; 90:1032-1041

From the Department of Pediatrics, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima, Japan (YS, HK, KN, TS, MH, SO, NI, MK); Department of Developmental Paediatrics, Nagoya University School of Medicine, Nagoya, Japan (SK); Department of Cellular and Molecular Biology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan (OK).

Correspondence:
Masao Kobayashi, M.D. PhD., Department of Pediatrics, Hiroshima University Graduate School of Biomedical Sciences, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan.
E-mail: masak@hiroshima-u.ac.jp

Human cyclic neutropenia (CyN) in children is a rare blood disorder characterized by regular oscillations in the numbers of circulating blood neutrophils, monocytes, eosinophils, lymphocytes, platelets, and reticulocytes.[1-4] A profound decrease in circulating neutrophils is observed at regular 3-week intervals. During phases of neutropenia, patients frequently suffer from symptoms of fever, malaise, aphthous stomatitis, lymphadenopathy and, occasionally, very severe infections. With increasing neutrophil counts, the infections and accompanying symptoms normally disappear. Bone marrow examinations have indicated that the cycling of peripheral blood cells is preceded by oscillations of granulopoietic cells. Severe congenital neutropenia (SCN), also known as Kostmann-type neutropenia, is characterized by an early onset in childhood, recurrent life-threatening infections, and profound neutropenia with an absolute neutrophil count of <200/μL in the peripheral blood.[5-9] The bone marrow usually shows a paucity of mature myeloid cells with an arrest of maturation of neutrophil precursors at the promyelocyte-myelocyte stage of differentiation.

Recently, mutations of the gene encoding neutrophil elastase (NE), *ELA2*, have been identified in both patients with CyN and those with SCN.[10-14] Missense or deletion mutations in the NE gene detected in patients with CyN or SCN are assumed to be involved in the pathogenesis of these disorders. We previously demonstrated that primitive myeloid progenitor cells in patients with SCN show abnormal transcriptional regulation of primary granule

enzymes, including NE, during myeloid proliferation and differentiation.[15] However, neither the mutation of *ELA2* nor the transcriptional abnormality can account for the functional defects in the SCN and CyN phenotypes.[13,14] Although these two disorders characterized by chronic neutropenia have similar genetic molecular abnormalities, the significance of the mutations of *ELA2* in the defective myelopoiesis of patients with CyN or SCN remains unclear.

Bone marrow cells from patients with CyN and those with SCN frequently display abnormal responses to hematopoietic factors in *in vitro* culture.[16-25] A defective response to haematopoietic factors, including granulocyte colony-stimulating factor (G-CSF), may be an important pathophysiologic mechanism underlying both CyN and SCN. We previously reported the presence of qualitative and quantitative abnormalities in the proliferation of primitive myeloid progenitor cells expressing G-CSF receptor (G-CSFR) in patients with SCN in response to hematopoietic factors, including G-CSF.[24,25] In patients with CyN, abnormally increased concentrations of G-CSF and granulocyte/macrophage colony-stimulating factor were required to stimulate maximal granulocyte colony formation of bone marrow cells.[16-18] The requirement of hematopoietic factor for maximal growth could be demonstrated at the $CD34^+$ progenitor cell level.[18] Thus, hematopoietic factors and their receptors may play a pivotal role in furthering our understanding of abnormal granulopoiesis in patients with CyN or SCN. In this study we examined abnormalities in primitive myeloid cells expressing CD34, Kit, and G-CSFR from patients with CyN or SCN. A comparison of CyN and SCN samples revealed a number of differences between the two disorders in terms of the abnormalities of affected myeloid cells.

Design and Methods

Patients

Three patients with CyN and four patients with SCN (characteristics presented in Table 1) were enrolled in this study. The diagnosis of CyN was made based on oscillations in the number of circulating blood neutrophils at regular 3-week intervals. The diagnosis of SCN, also known as Kostmann's syndrome, was made based on the established criteria, including an absolute neutrophil count below 200/μL in the peripheral blood, and maturation arrest at the promyelocyte or myelocyte stage in the bone marrow. No circulating antineutrophil antibodies were detected by granulocyte indirect immunofluorescence tests in either the CyN or SCN patients. The onset of recurrent infections was observed before the age of 24 months. All patients with CyN had received G-CSF in cases of severe infection during the neutropenic phase. All patients with SCN had a history of recurrent life-threatening infections and were receiving G-CSF 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. We have previously reported some of the features of two of the four patients with SCN (SCN 2 and 3).[15,25] The bone marrow cells used in this study were collected during a period in which the patients were not receiving G-CSF, with the exception of one patient with SCN whose bone marrow cells were obtained during G-CSF treatment.

Cytokines

Recombinant human G-CSF, recombinant human interleukin-3 (IL-3), and recombinant human stem cell

Table 1. Characteristics of patients.

	Age (years)/ Sex	WBC (/μL)	Neutrophils (%)	Eosinophils (%)	Monocytes (%)	Lymphocytes (%)	Site of ELA2 mutation[1]	Treatment
Patients with CyN								
CyN 1	4/M	3,300	0	10	5	84	Exon 5, G4943A (Arg191Gln)	G-CSF[2]
CyN 2	5/F	3,400	0	5	6	87	Intron 4, G4716A	G-CSF[2]
CyN 3	12/M	4,400	0	11	14	74	Exon 4, C4534T (Pro110Leu)	G-CSF[2]
Patients with SCN								
SCN 1	0.9/M	4,100	0	9	10	77	Exon 5, C4953A (Cys194stop)	BMT[3]
SCN 2	0.3/F	4,900	2	3	21	69	Exon 4, C4495T (Ser97Leu)	BMT[3]
SCN 3	0.4/M	5,500	0	16	15	68	Exon 5, G4924A (Gly185Arg)	G-CSF[4]
SCN 4	0.2/M	4,000	1	7	10	80	Exon 5, G4981C (Ala204Pro)	BMT[3]

The patients' data reflect the findings at diagnosis. The white blood cell count (WBC) and differentials of patients with CyN represent the nadir in periodic oscillations. One patient with SCN (SCN 3) received G-CSF continuously without developing MDS or AML. [1]Nucleotide position corresponds to GenBank entry AC Y00477. Amino acid number one is the first after the presignal peptide. [2]G-CSF was intermittently administered when severe infections occurred during a neutropenic phase. [3]Allogeneic bone marrow transplantation (BMT) from an HLA-matched donor. [4]Administration of G-CSF four days per week was maintained for nine years without the development of either MDS or AML.

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, USA). Unless otherwise specified, the concentrations of factors used were as follows: G-CSF, 100 ng/mL; SCF, 100 ng/mL; FL, 20 ng/mL; and IL-3, 100 U/mL.

Bone marrow cell separation

Bone marrow samples were obtained with the informed consent of patients and/or their guardians. Normal bone marrow cells for this study were taken from healthy adult volunteers after obtaining informed consent. The study protocol was approved by the Institutional Review Board for Human Research, Hiroshima University Graduate School of Biomedical Sciences. The light density bone marrow cells obtained by centrifugation over Lymphoprep (1.077 g/mL; Nycomed Pharma AS, Oslo, Norway) were washed three times with phosphate-buffered saline (PBS) containing 2% human AB serum (Sigma Chemical Co., St. Louis, MO, USA) and 0.1 mg/mL of DNase I (type II-S; Sigma Chemical Co.), and resuspended in an α-modification of Eagle's medium (αMEM; ICN Biomedicals, Inc., Aurora, OH, USA) containing 10% FBS (ICN Biomedicals, Inc.). Cells were incubated in plastic culture flasks (Becton Dickinson Labware, Lincoln Park, NJ, USA) at 37°C for 1 hour to remove adherent cells. Non-adherent cells were either used in the described purification or were cryopreserved by a standard procedure using 10% DMSO, and then 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. No differences in the results of the experiments were observed between fresh and cryopreserved cells when the cells were purified according to the following procedure.

Purification of bone marrow cells

Cell purification was performed according to previously reported methods with modification.[26,27] In brief, cells (2×10^7/mL) were incubated with fluoroscein isothiocyanate (FITC)-labeled monoclonal anti-CD34 antibody (clone 581, Beckman Coulter, Inc., Fullerton, CA, USA) for 30 minutes at 4°C. FITC-conjugated mouse IgG1a was used as an isotype control. After the addition of propidium iodide (PI, Sigma Chemical Co.) at a concentration of 1 μg/mL, the cells were washed twice and resuspended in PBS-human serum-DNase-sodium azide solution. The initial enrichment of $CD34^+$ cells was carried out by setting a FACS Vantage (Becton Dickinson Immunocytometry Systems, San José, CA, USA), equipped with a 4-W argon laser, to recognize both FITC-positive and negative PI fluorescence, as well as low to medium forward scatter and low side scatter. The enriched $CD34^+$ cells were further stained with a phycoerythrin (PE)-conjugated anti-Kit (clone 95C3, Beckman Coulter, Inc.) for 30 to 40 minutes at 4 °C. After the addition of PI at a concentration of 1 μg/mL, cells were washed twice, then $CD34^+/Kit^+$ cells were sorted by FACS Vantage. When G-$CSFR^{+/-}$ cells on $CD34^+/Kit^+$ cells were purified, enriched $CD34^+$ cells were further stained with Kit-PE, and with biotin-conjugated anti-G-CSFR (clone LMM741, PharMingen, San Diego, CA, USA) for 30 to 40 minutes at 4°C. The cells were then washed twice and stained with streptavidin labeled with allophycocyanin (APC, Molecular Probes, Inc., Eugene, OR) for 15 minutes at 4°C. After the addition of PI at a concentration of 1 μg/mL, cells were sorted by FACS Vantage. The appropriate isotype controls, FITC-, PE-, and biotin-conjugated mouse IgG1a, were used to identify background staining.

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, 300 μg/mL fully iron-saturated human transferrin (98% pure), 10 μg/mL soybean lecithin, 6 μg/mL cholesterol, 1×10^{-7} M sodium selenite, 10 μg/mL insulin, 4.5 mM L-glutamine, 1.5 mM glycine (all from Sigma Chemical Co.), as well as 1.2% 1,500-centipoise methylcellulose (Shinetsu Chemical, Tokyo, Japan), and designated cytokines.[26,27] The cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2/95% air. On day 14 of incubation, granulocyte-macrophage colonies were scored on an inverted microscope using the criteria described previously.[28] Each granulocyte-macrophage colony contained pure granulocyte colonies consisting of primarily neutrophils and their precursors, and mixed granulocyte-macrophage colonies consisting of mainly neutrophils, macrophages/monocytes, and their precursors. The number of colonies reported represents the means of values recorded in triplicate cultures.

Liquid suspension cultures

Ten thousand purified cells were cultured in 24-well microtrays (Corning Coaster Inc., Corning, NY, USA), in serum-deprived liquid suspension media consisting of the same supplements used in the clonal culture described above. Incubation was carried out at 37°C in a humidified atmosphere with 5% CO_2/95% air. The number of cells in each well was scored serially. Aliquots were centrifuged onto slides using Shandon's Cytospin 2 Centrifuge (Shandon Inc., Pittsburgh, PA,

Table 2. Primers for sequencing and real-time PCR.

	Sequence	Nucleotides
Primers for sequencing of the NE gene		
1 Forward primer	CGGAGGGGCAGAGACCCCGGA	1252-1272
Reverse primer	AGACCGGGACGCGGGGTCCGA	1972-1992
Sequencing primer		
Forward primer	CACCCGGTGTGTCCCCAGGCA	1768-1788
2 Forward primer	CTCGAGCACCTTCGCCCTCAG	2065-2085
Reverse primer	TCAACGGCCCATGGCGGGTAT	2497-2517
3 Forward primer	CCTGCCCTGCAGGATCCCAGA	4363-4383
Reverse primer	GGAGAGTGTGGGTGTGGGCAG	5113-5133
Sequencing primer		
Forward primer	AGGAACCGTGGGATCGCCAGC	4599-4619
Reverse primer	TGCAGACGTTGCTGCGACGGC	4660-4680
Primers and probes for real-time PCR		
Neutrophil elastase		
Forward primer	GCTAATCCACGGAATTGCCTC	668-688
Reverse primer	CTCGGAGCGTTGGATGATAGA	768-788
Probe	TTTGCCCCGGTGGCACAGTTTGTA	732-755
Myeloblastin		
Forward primer	TTCTTCTGCCGGCCACATA	586-604
Reverse primer	ATCCCCAGATCACGAAGGAGT	698-718
Probe	CATTTGCACTTTCGTCCCTCGC	606-627
Myeloperoxidase		
Forward primer	TCTTCACCAATGCCTTCC	1641-1658
Reverse primer	GCAAAAAAGACCCTGCTGAGG	1741-1761
Probe	TCATCCAACCCTTCATGTTCCGCC	1674-1697
Lactoferrin		
Forward primer	CCGAGGCCACAAAATGCTT	395-413
Reverse primer	ATGGCCTGGATACACTGGATG	480-500
Probe	TGGCCCTCCTGTCAGCTGCATAAA	444-467
β-actin		
Forward primer	CAGGTCATCACCATTGGCAAT	777-797
Reverse primer	TCTTTGCGGATGTCCACGT	898-916
Probe	TTGAAGGTAGTTTCGTGGATGCCACAGG	853-880

Nucleotide positions correspond to GenBank entries NM_001972 (neutrophil elastase), X00351 (β-actin), NM_002777 (myeloblastin), X04876 (myeloperoxidase), and NM_002343 (lactoferrin).

USA) for morphological examination of Wright-Giemsa staining.

Real-time quantitative polymerase chain reaction

Total cellular RNA extracted from fresh and cultured cells using the guanidinium thiocyanate extraction method was converted into cDNA by reverse transcriptase. The primers and probes of NE, myeloblastin, myeloperoxidase, and lactoferrin, and the β-actin for PCR used in this study are listed in Table 2. The commercial reagents (TaqMan PCR Reagent Kit, Applied Biosystems, Foster City, CA, USA) used in this study, as well as the PCR conditions, were those recommended by the manufacturer.[15] Thus, 10 μL of cDNA and 5 μL of oligonucleotides together with a final concentration of 200 nmol/L of primers and 100 nmol/L of TaqMan hybridization probe were added to a 25 μL reaction mixture. The amplification conditions for quantification were an initial 2 minutes of incubation at 50°C, 10 minutes at 95°C, followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing/extension at 60°C for 1 minute. The reactions were performed with the ABI PRISM 7700 sequence detection system equipped with a 96-well thermal cycler (Applied Biosystems). The data were collected and subsequently analyzed using Sequence Detector v1.6 software (Applied Biosystems). NE, myeloblastin, myeloperoxidase and lactoferrin values were corrected for the values obtained for β-actin from the same cDNA. In brief, the mean of experimentally obtained NE, myeloblastin, myeloperoxidase, or lactoferrin copy numbers obtained from a sample run in triplicate was divided by the expected value based on the amount of cDNA added to a reaction to obtain a β-actin normalizing value. In preliminary experiments we confirmed that the β-actin copy number was linearly dependent on the amount of cDNA that had been extracted, varying relative to the number of bone marrow cells.

Sequencing of PCR products

Mutational analysis was performed by sequencing PCR-amplified genomic DNA extracted from peripheral blood leukocytes or light density bone marrow cells with Applied Biosystems PRISM BigDye terminator chemistry on an ABI PRISM 310 Analyzer (Applied Biosystems). Each exon of neutrophil NE was sequenced from both directions in each individual. The primers used for the sequencing are listed in Table 2. The presence of mutation was confirmed using restriction endonuclease digestion of the relevant PCR fragment according to a method described by Ancliff *et al.*[12]

Statistical analysis

The statistical significance of the data was determined by ANOVA and unpaired two-group t-test using StatView software (version 5.0, SAS Institute, Inc., Cary, NC, USA).

Results

Mutations of ELA2

Several types of mutations in the gene encoding NE have been identified in CyN and SCN patients.[10-14] As shown in Table 1, heterozygous mutations were identified in all patients enrolled in this study. The site of mutation in three patients with CyN was consistent with that reported in the previous study.

The mutation found in one CyN patient (CyN 3) was reported to correspond to that in several patients with SCN. There have been no reports on the loci of mutations in patients with SCN (SCN 1 and SCN 4).

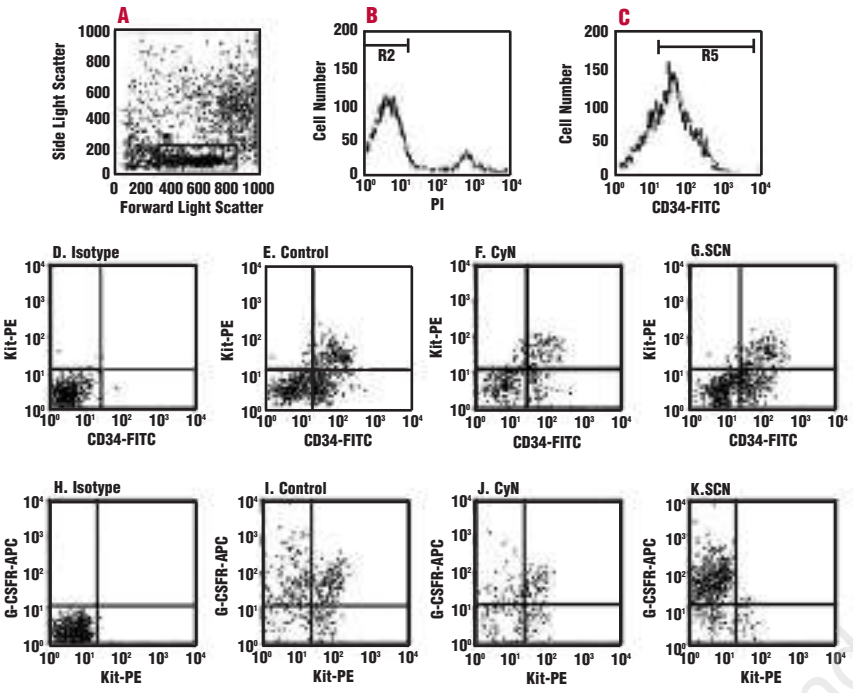

Figure 1. Flow cytometric analysis of bone marrow cells based on the expression of CD34, Kit, and G-CSFR. Low to medium forward scatter, and low side scatter (R1), and negative for PI fluorescence gates (R2) were used in all analyses (A, B). Mouse IgG1-FITC, IgG1-PE, and IgG1-biotin were used as isotype controls. The expression of CD34 and Kit (CD117) within the gated cells described above (R1 and R2) is shown for an isotype control (D), a representative normal subject (E), for a patient with CyN (F), and for a patient with SCN (G). The expression of Kit and G-CSFR (CD114) within R1, R2, and R3 (positive for CD34 in panel C) gates is shown for an isotype control (H), a representative normal subject (I), for a patient with CyN (J), and for a patient with SCN (K).

Flow cytometric analysis of bone marrow cells

We previously reported a remarkably reduced frequency of $CD34^+/Kit^+/G\text{-}CSFR^+$ cells in patients with SCN.[25] The representative expression of CD34, c-Kit, and G-CSFR on bone marrow cells in a patient with CyN, a patient with SCN, and a normal subject is shown in Figure 1. The expression of both CD34 and c-Kit on bone marrow cells was comparable among CyN patients, SCN patients, and normal subjects. Table 3 summarizes the quadrant percentage of $Kit^+/G\text{-}CSFR^+$, $Kit^+/G\text{-}CSFR^-$, $Kit^-/G\text{-}CSFR^+$, and Kit^-/G^-CSFR^- cells in $CD34^+$ cells in three patients with CyN, patients with SCN, and normal subjects. A statistically significant difference in the frequency of $Kit^+/G\text{-}CSFR^+$ expression on CD34 cells was observed among CyN patients, SCN patients, and normal subjects as determined by ANOVA ($p< 0.001$). Post-hoc analysis showed that the frequency of $Kit^+/G\text{-}CSFR^+$ expression on CD34 cells in patients with SCN was clearly lower than that in CyN patients or normal subjects ($p<0.001$). There was no difference between patients with CyN and normal subjects in terms of the expression of CD34, Kit, and G-CSFR.

GM-colony formation of $CD34^+/Kit^+$ cells in response to various hematopoietic factors

Light density bone marrow cells were enriched for primitive myeloid progenitors using CD34 antibody and anti-Kit receptor antibody, because CFU-GM was enriched in the $CD34^+/Kit^+$ fraction. $CD34^+/Kit^+$ cells were cultured under serum-deprived conditions that included various hematopoietic factors. As shown in Table 4, significant differences in the number of GM colonies formed in response to G-CSF alone and to combinations of SCF, FL, and IL-3 with or without G-CSF were found among CyN patients, SCN patients, and normal subjects on analysis by ANOVA ($p<0.001$). Post-hoc analysis (Tukey's method) revealed that $CD34^+/Kit^+$ cells in normal subjects yielded a greater number of GM colonies than did those in patients with CyN or SCN ($p<0.01$), and $CD34^+/Kit^+$ cells in CyN patients yielded a greater number of GM colonies than did those in SCN patients ($p<0.05$). These results imply that patients with SCN have a more severe granulopoietic defect than do patients with CyN. In one patient with CyN (CyN 1), bone

Table 3. Frequency of the expression of Kit and G-CSFR on CD34 cells.

	Quadrant percentages of cells					
Cells	*CyN 1*	*CyN 2*	*CyN 3*	*CyN (Mean ± SD, n=3)*	*SCN (Mean ± SD, n=4)*	*Normal Subjects (Mean±SD, n=12)*
$Kit^+/G\text{-}CSFR^+$	35.3	38.2	29.0	34.2±3.8[a]	5.9±3.5[a]	30.4±9.8[a]
$Kit^+/G\text{-}CSFR^-$	12.5	15.3	23.5	17.1±4.7	15.4±8.9	18.1±5.2
$Kit^-/G\text{-}CSFR^+$	25.3	21.1	28.1	24.8±2.9	45.2±21.0	27.4±8.5
$Kit^-/G\text{-}CSFR^-$	27.0	25.3	19.4	23.9±3.3	33.5±13.6	24.2±13.6

Data demonstrating the quadrant percentage of each fraction in $CD34^+$ cells illustrated in Figure 1-I, J, and K. [a]ANOVA revealed statistical significance ($p< 0.001$). [b]$p< 0.001$, Normal subjects or CyN vs. SCN by post-hoc analysis (Tukey's method).

Table 4. Formation of GM colonies of $CD34^+/c\text{-}Kit^+$ cells supported by various factors.

Factor(s)	CyN 1	CyN 2	CyN 3	Number of GM colonies[a] CyN (Mean±SD, n=3)	SCN (Mean±SD, n=4)	SCN (Range, n=4)	Normal Subjects (Mean±SD, n=10)	Normal Subjects (Range, n=10)
G-CSF	8	9	13	10±3[b]	4±2[b]	2-5	19±5[b]	14-35
SCF, FL, IL-3	31	28	38	32±5[b]	19±5[b]	16-24	53±12[b]	41-68
SCF, FL, IL-3, G-CSF	62	65	70	66±4[b,c]	31±8[b,c]	25-37	95±20[b]	76-128

Cultures were performed under serum-deprived conditions including 500 $CD34^+/c\text{-}Kit^+$ cells and the designated factors. CyN patient data represent the mean values in triplicate cultures. SCN and control data represent 4 and 10 subjects, respectively. [a]ANOVA revealed statistical significance ($p< 0.001$). [b]$p< 0.01$, Normal subjects vs. CyN or SCN by post-hoc analysis (Tukey's procedure). [c]$p< 0.05$, CyN vs. SCN by post-hoc analysis (Tukey's procedure).

Table 5. Formation of GM colonies of purified cells supported by various factors.

Factor(s)	CyN 1	CyN 2	CyN 3	Number of GM colonies[a] CN (Mean±SD, n=3)	SCN (Mean±SD, n=4)	SCN (Range, n=4)	Normal Subjects (Mean±SD, n=10)	Normal Subjects (Range, n=10)
$CD34^+/Kit^+/G\text{-}CSFR^+$ Cells								
G-CSF	8	15	16	13±4[b,c]	3±1[b,c]	1-4	28±8[b]	21-35
SCF, FL, IL-3	21	30	25	25±3[b,c]	15±5[b,c]	9-18	41±8[b]	28-47
SCF, FL, IL-3, G-CSF	39	49	55	48±8[b,c]	29±8[b,c]	21-35	70±5	56-76
$CD34^+/Kit^+/G\text{-}CSFR^-$ Cells								
G-CSF	0	1	1	1±1	3±1	1-4	2±1	0-3
SCF, FL, IL-3	9	20	13	14±6[d]	23±6	16-30	21±6[d]	22-35
SCF, FL, IL-3, G-CSF	25	32	26	28±4[d]	39±9	28-46	38±7[d]	32-51

Cultures were performed under serum-deprived conditions including 250 $CD34^+/Kit^+/G\text{-}CSFR^+$ or $CD34^+/Kit^+/G\text{-}CSFR^-$ cells and the designated factors. CyN patient data represent the mean values in triplicate cultures. SCN and controls data represent 4 and 10 subjects, respectively. [a]ANOVA revealed statistical significance ($p< 0.001$); [b]$p< 0.01$, Normal subjects vs. CyN or SCN by post-hoc analysis (Tukey's procedure). [c]$p< 0.05$, CyN vs. SCN by post-hoc analysis (Tukey procedure). [d]$p< 0.01$, Normal subjects vs. CyN by post-hoc analysis (Tukey's procedure).

marrow aspiration was performed at three different time points over 21 days. No significant differences in the GM-colony formation of $CD34^+/Kit^+$ cells were observed relative to the point of oscillation (data not shown). These findings suggest the possibility that the primitive myeloid progenitor cells of patients with CyN and those with SCN are defective in their response not only to G-CSF, but also to other hematopoietic factors involved in granulopoiesis.[29-31]

GM-colony formation of $CD34^+/Kit^+$ cells expressing G-CSFR

The $CD34^+/Kit^+$ cells were further purified to obtain $CD34^+/Kit^+/G\text{-}CSFR^+$ and $CD34^+/Kit^+/G\text{-}CSFR^-$ cells. No difference in quadrant percentages of $Kit^+/G\text{-}CSFR^+$ and $Kit^+/G\text{-}CSFR^-$ cells on $CD34^+$ cells was noted between patients with CyN and normal subjects (Figures 1-I and J). This result is completely different from that previously reported for SCN patients.[25] Similar to the results seen in $CD34^+/Kit^+$ cells, the number of GM colonies formed was significantly different (ANOVA, $p<0.001$) in $CD34^+/Kit^+/G\text{-}CSFR^+$ cells among CyN patients, SCN patients, and normal subjects (Table 5). The ranking established according to the number of GM colonies formed was similar to that in $CD34^+/Kit^+$ cells. In contrast to the case of $CD34^+/Kit^+/G\text{-}CSFR^+$ cells, no difference in GM colony formation was observed between normal subjects and patients with SCN in the case of $CD34^+/Kit^+/G\text{-}CSFR$ cells. However, GM-colony formation of $CD34^+/Kit^+/G\text{-}CSFR$ cells in patients with CyN was significantly reduced compared with that in normal subjects and in patients with SCN in response to hematopoietic factors ($p< 0.01$). These findings suggest that the affected cell population differs between patients with CyN and those with SCN.

Proliferation of $CD34^+/Kit^+$ cells in liquid suspension culture

The proliferation of $CD34^+/Kit^+/G\text{-}CSFR^+$ and $CD34^+/Kit^+/G\text{-}CSFR^-$ cells in response to various hematopoietic factors, including G-CSF, was examined in a serum-deprived liquid suspension culture. The total number of cells in the wells was recorded

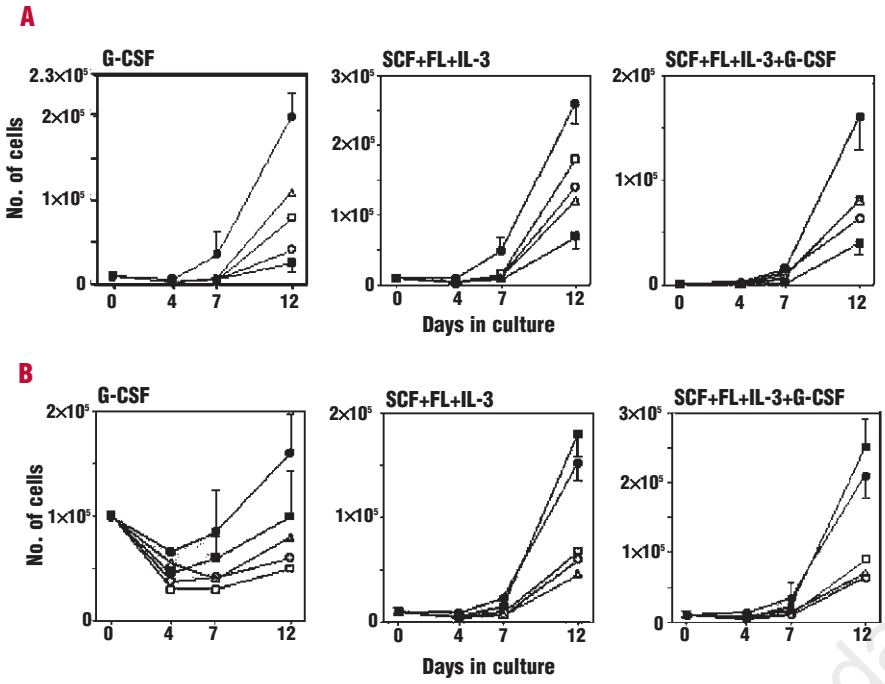

Figure 2. Proliferation of CD34⁺/Kit⁺/G-CSFR⁺ and CD34⁺/Kit⁺/G-CSFR⁻ cells in liquid suspension culture. Ten thousand purified CD34⁺/Kit⁺/G-CSFR⁺ (A) and CD34⁺/Kit⁺/G-CSFR⁻ cells (B) were cultured with the hematopoietic factors indicated under serum-deprived conditions. Data represent the cell number (mean ± SD) in six normal subjects (●), in four patients with SCN (■), and in three patients with CyN (CyN 1, ○; CyN 2, □; CyN 3, △).

serially. As shown in Figure 2, the proliferation of CD34⁺/Kit⁺/G-CSFR⁺ cells in response to G-CSF alone, or to SCF, FL, and IL-3, with and without G-CSF, was less in patients with CyN than in normal subjects. A more substantial decrease in the proliferation of CD34⁺/Kit⁺/G-CSFR⁺ cells in response to hematopoietic factors was found in SCN patients than in CyN patients. Similarly, the CD34⁺/Kit⁺/G-CSFR⁻ cells of patients with CyN proliferated less in response to SCF, FL, and IL-3, with and without G-CSF, than did the corresponding cells from normal subjects or patients with SCN. These results are consistent with the data showing a reduced level of GM-colony formation in CD34⁺/Kit⁺/G-CSFR⁺ cells and a comparably reduced level in CD34⁺/Kit⁺/G-CSFR⁻ cells in semisolid culture. Thus, defective granulopoiesis in patients with CyN was primarily manifested in the level of CD34⁺/Kit⁺ cells, irrespectively of the expression of G-CSFR.

Granular protein mRNA expression in myeloid precursor cells

We recently demonstrated that the primitive myeloid progenitor cells in patients with SCN show an abnormal transcriptional regulation of primary granule enzymes, including NE, during myeloid proliferation and differentiation.[15] In the culture of CD34⁺/Kit⁺ cells with G-CSF, granular protein mRNA expression, including that of NE, was examined using real-time quantitative PCR. As shown in Figure 3, the transcription level of primary granule enzymes in CD34⁺/Kit⁺ cells was enhanced to relatively high levels on days 4 and 7, and then decreased on day 12 in normal subjects, as previously reported.[15] The transcription levels of primary granule enzymes in patients with CyN showed a pattern similar to that of normal subjects. The cell composition reflecting proliferation and differentiation in this culture was almost the same between CyN patients and normal subjects,

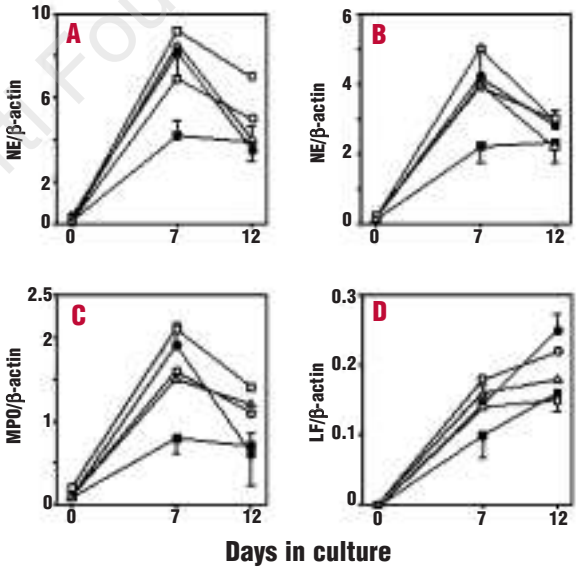

Figure 3. Expression of neutrophil elastase (NE), myeloblastin (MBN), myeloblastin myeloproxidase (MPO) and lactoferrin (LF) genes during the culture of CD34⁺/Kit⁺ cells with G-CSF. Total cellular RNA extracted from cells during culture with G-CSF under serum-deprived conditions was converted into cDNA and submitted to a quantitative real-time PCR analysis. The ratios of the copies of NE (A), MBN (B), MPO (C), and LF (D) to those of β-actin are presented as the mean ± SD of four normal subjects (●), the mean ± SD of four patients with SCN (■), and that of three patients with CyN (CyN 1, ○; CyN 2, □; CyN 3, △).

Table 6. Expression of granular protein genes in myeloid precursor cells of bone marrow.

	CyN 1	CyN 3	SCN (Mean±SD, n = 4)	Normal Subjects (Mean±SD, n = 5)
Neutrophil elastase	12.6	24.4	2.9±1.5	17.1±6.4
Myeloblastin	3.5	7.2	1.1±0.6	4.6±1.8
Myeloperoxidase	3.6	6.5	0.9±0.3	3.9±1.6
Lactoferrin	1.5	2.2	1.7±0.6	3.5±2.9

Fresh bone marrow cells were stained with CD14-FITC, CD16-FITC, and CD13-PE, and then the $CD13^+/CD14^-/CD16^-$ cells were sorted by FACS Vantage. cDNA in $CD13^+/CD14^-/CD16^-$ cells were extracted and then subjected to real-time quantitative PCR analysis. The ratios of the copies of neutrophil elastase, myeloblastin, myeloperoxidase and lactoferrin, respectively, to the copies of β-actin are presented.

as determined by cytospin preparation (*data not shown*). Although abnormal proliferation was observed in CyN patients, the difference in transcription levels between CyN patients and normal subjects was not significant. In contrast, initial up-regulation and subsequent down-regulation of primary granule enzyme transcription were not clearly observed in the SCN patients, as previously reported.[15] In addition, no differences in the levels of lactoferrin expression were observed among normal subjects, CyN patients, and SCN patients.

To confirm the levels of transcription in myeloid cells, bone marrow myeloid precursor cells were isolated from fresh light density bone marrow cells from two patients with CyN. The cells positive for CD13 and negative for both CD14 and CD16 were purified in order to avoid contamination by monocytes and mature neutrophils. Cytospin preparation with Wright-Giemsa staining revealed that more than 90% of cells were myeloblasts, promyelocytes, and myelocytes. The cDNA extracted from these cells was subjected to quantitative real-time PCR analysis. As shown in Table 6, no significant differences in the levels of transcription of primary granule enzymes were noted between patients with CyN and normal subjects. The transcription levels in CyN patients were normal, irrespective of the time point in the course of the CyN cycle (*data not shown*). These observations suggest that the regulation of transcription of primary granule proteins in patients with CyN is normal. Thus, the mutations of the NE gene observed in CyN patients may not be directly involved in the transcription of primary granule proteins.

Discussion

Several reports have noted that the bone marrow cells of CyN and SCN patients exhibit abnormal *in vitro* growth responses to hematopoietic factors.[16-25] Recently, mutations in the gene encoding NE have been identified in all patients with CyN and in the majority of patients with SCN.[10-14] These findings have provided genetic evidence that mutations in *ELA2* play a key role in the pathogenesis of both CyN and SCN. However, the involvement of *ELA2* mutations in myelopoietic defects in CyN and SCN patients remains unclear. In a comparison of granulopoiesis of myeloid progenitor cells and expression of granular proteins in myeloid precursor cells of patients with CyN and those with SCN, both groups exhibiting heterozygous mutations of the NE gene, different defects were observed. In a previous series we found that affected myeloid progenitor cells in SCN patients were predominantly primitive progenitor cells expressing G-CSFR. In contrast, the cells affected in CyN patients were independent of the presence of G-CSFR, and were found in primitive stage hematopoietic progenitor cells expressing CD34 and Kit (CD117). Human bone marrow cells capable of multilineage differentiation and long-term engraftment are known to be present in the $CD34^+/Kit^+$ population.[32] This effect in CyN patients might be attributable to regulatory defects in multilineage haematopoietic progenitor cells and to characteristics of the periodic decrease in circulating neutrophil levels corresponding with the oscillation of platelets, monocytes, and, occasionally, reticulocytes and lymphocytes.[1-4]

Abnormal responsiveness to hematopoietic factors including G-CSF may be an important pathophysiologic mechanism underlying CyN and SCN. Previous studies have shown that myeloid progenitor cells from CyN patients are 5 to 10 times less responsive to G-CSF and GM-CSF than are normal progenitors.[16-18] A requirement for an increased concentration of hematopoietic factors could be demonstrated at the level of $CD34^+$ cells, especially in response to G-CSF, in both serum-supplemented and serum-deprived cultures.[18] The present study confirmed abnormalities among myeloid cells by using highly purified progenitor cells under serum-deprived conditions. We found a reduction in GM-colony formation and decreased proliferation in response to various hematopoietic factors involved in myelopoiesis. The combination of SCF, FL, IL-3, and G-CSF maximally stimulated GM-colony formation in cells from normal subjects in serum-deprived culture.[24] However, an increased concentration of hematopoietic factors did not induce the formation of the same number of GM colonies as that formed in normal subjects (*data not shown*), implying a decreased number of myeloid progenitor cells in the primitive hematopoietic cell compartment of the $CD34^+/Kit^+$ population. Together, these findings provide evidence that myeloid progenitor cells in CyN patients exhibit qualitative and quantitative abnor-

malities. Avalos and co-workers reported that the lower responsiveness was not the result of a defect in the G-CSF signal transduction pathway at a point distal to G-CSF receptor binding in canine cyclic neutropenia.[19] In humans with CyN, the number of G-CSF binding sites on mature neutrophils has been reported to be normal.[33] The present study also showed no difference in the percentage of cells expressing G-CSFR on $CD34^+/Kit^+$ cells between normal subjects and CyN patients. The difference in G-CSFR expression on $CD34^+/Kit^+$ cells between patients with CyN and those with SCN may suggest a completely different pathophysiologic mechanism underlying these respective disorders. The intensive involvement of G-CSF and G-CSFR in SCN patients may underlie a different prognosis for SCN and CyN patients. A long-term follow-up study of patients included in the Severe Chronic Neutropenia International Registry found that MDS and/or AML developed in some SCN patients, but neither of these disorders developed in CyN patients.[8,34]

The roles played by granular proteins and their expression in the regulation of myelopoiesis are currently unknown. The expression profiles of granule protein mRNA during neutrophilic granulocyte differentiation have already been described.[35-42] Cells positive for NE mRNA transcripts were shown to be present during a very limited period of neutrophil differentiation, and were predominantly found in the promyelocyte and late promyelocyte stages, with a much smaller percentage of positive myelocytes.[37,39,40] We recently demonstrated the dysregulation of transcription in primary granule enzymes during myeloid proliferation and differentiation in SCN patients. In patients with CyN, the expression levels of primary granule constituents were comparable to those in normal subjects during *in vitro* myeloid differentiation. Moreover, no differences were observed between normal subjects and CyN patients in terms of the transcriptional levels of primary granule enzymes in fresh bone marrow cells enriched for myeloblasts, promyelocytes, and myelocytes using CD13, CD14, and CD16 antibodies. These results suggest that the transcription pattern of granular proteins in patients with CyN is normal and, therefore, differs from that in patients with SCN. It appears that mutations of *ELA2* may not be directly involved in the regulation of transcription.

Taken together, the above findings provide no direct evidence of a correlation between the two disorders and the presence of *ELA2* mutations. However, an overlap of mutations of *ELA2* was found in CyN and SCN patients. As demonstrated in this study, faulty myelopoiesis occurred in different subpopulations of myeloid progenitor cells. The expression pattern of granular protein mRNA was also different between these two disorders. Furthermore, in mice, the lack of NE as well as the engineered expression of a mutated NE was insufficient to induce an SCN phenotype.[43,44] Further studies are, therefore, necessary to clarify the relationship between *ELA2* mutations and additional underlying molecular defects in the pathophysiology of CyN and SCN.

All authors meet the criteria for being contributing authors. Design of the study, analysis of data, and writing of the manuscript; YS, SK, and MK; cell sorting, TS and MH; ELA2 mutation analysis and real time quantitative PCR data, HK, KN, and OK; cell culture, SO, NI and YS.

All authors were involved in discussing and interpreting the data. The authors declare that they have no potential conflicts of interest.

This study was supported in part by Grants-in-Aid for Scientific Research (C) from the Ministry of Education, Science, Sports and Culture of Japan. We are grateful to Kirin Brewery Company Ltd. (Tokyo, Japan) for supporting this study with the special grant and for providing the cytokines.

Manuscript received January 26, 2005. Accepted June 21, 2005.

References

1. Wright DG, Dale DC, Fauci AS, Wolff SM. Human cyclic neutropenia: clinical review and long-term follow-up of patients. Medicine (Baltimore) 1981; 60:1-13.
2. Dale DC, Hammond WP 4th. Cyclic neutropenia: a clinical review. Blood Rev 1988;2:178-85.
3. Hammond WP 4th, Price TH, Souza LM, Dale DC. Treatment of cyclic neutropenia with granulocyte colony-stimulating factor. N Engl J Med 1989; 320: 1306-11.
4. Dale DC, Bolyard AA, Aprikyan A. Cyclic neutropenia. Semin Hematol 2002;39:89-94.
5. Kostman R. Infantile genetic agranulocytosis. A review with presentation of ten new cases. Acta Paediatr Scand 1975;64:362-8.
6. Welte K, Dale D. Pathophysiology and treatment of severe chronic neutropenia. Ann Hematol 1996;72:158-65.
7. Welte K, Boxer LA. Severe chronic neutropenia: pathophysiology and therapy. Semin Hematol 1997; 34:267-78.
8. Glader BE, Guinan E, Lipton JM, Boxer LA. Congenital bone marrow failure syndromes: Diagnosis and therapeutic strategies. In: McArthur JR, Schechter GP, Schrier SL, eds. American Society of Hematology Education Program Book. Washington DC: American Society of Hematology 1998:384-403.
9. Zeidler C, Welte K. Kostmann syndrome and severe congenital neutropenia. Semin Hematol 2002;39:82-8.
10. Horwitz M, Benson KF, Person RE, Aprikyan AG, Dale DC. Mutations in ELA2, encoding neutrophil elastase, define a 21-day biological clock in cyclic haematopoiesis. Nat Genet 1999; 23:433-6.
11. Dale DC, Person RE, Bolyard AA, Aprikyan AG, Bos C, Bonilla MA, et al. Mutations in the gene encoding neutrophil elastase in congenital and cyclic neutropenia. Blood 2000;96:2317-22.
12. Ancliff PJ, Gale RE, Liesner R, Hann IM, Linch DC. Mutations in the ELA2 gene encoding neutrophil elastase are present in most patients with sporadic severe congenital neutropenia but only in some patients with the familial form of the disease. Blood 2001;98:2645-50.
13. Germeshausen M, Schulze H, Ballmaier M, Zeidler C, Welte K. Mutations in the gene encoding neutrophil elastase (ELA2) are not sufficient to cause the phenotype of congenital neutropenia. Br J Haematol 2001;115:222-4.
14. Ancliff PJ, Gale RE, Watts MJ, Liesner R, Hann IM, Strobel S, et al. Paternal mosaicism proves the pathogenic nature of mutations in neutrophil elastase in severe congenital neutropenia. Blood 2002;100:707-9.

15. Kawaguchi H, Kobayashi M, Nakamura K, Konishi N, Miyagawa S, Sato T, et al. Dysregulation of transcriptions in primary granule constituents during myeloid proliferation and differentiation in patients with severe congenital neutropenia. J Leukoc Biol 2003;73:225-34.
16. 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 1989; 83:1414-8.
17. Migliaccio AR, Migliaccio G, Dale DC, Hammond WP. Hematopoietic progenitors in cyclic neutropenia: effect of granulocyte colony-stimulating factor in vivo. Blood 1990;75:1951-9.
18. Hammond WP, Chatta GS, Andrews RG, Dale DC. Abnormal responsiveness of granulocyte-committed progenitor cells in cyclic neutropenia. Blood 1992;79:2536-9.
19. Avalos BR, Broudy VC, Ceselski SK, Druker BJ, Griffin JD, Hammond WP. Abnormal response to granulocyte colony-stimulating factor (G-CSF) in canine cyclic hematopoiesis is not caused by altered G-CSF receptor expression. Blood 1994;84:789-94.
20. Kawaguchi Y, Kobayashi M, Tanabe A, Hara M, Nishi Y, Usui T, et al. Granulopoiesis in patients with congenital neutropenia. Am J Hematol 1985;20:223-34.
21. Bonilla MA, Gillio AP, Ruggeiro M, Kernan NA, Brochstein JA, Abboud M, et al. Effects of recombinant human granulocyte colony-stimulating factor on neutropenia in patients with congenital agranulocytosis. N Engl J Med 1989;320:1574-80.
22. Kobayashi M, Yumiba C, Kawaguchi Y, Tanaka Y, Ueda K, Komazawa Y, et al. Abnormal responses of myeloid progenitor cells to recombinant human colony-stimulating factors in congenital neutropenia. Blood 1990; 75:2143-9.
23. Hestdal K, Welte K, Lie SO, Keller JR, Ruscetti FW, Abrahamsen TG. 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. Blood 1993;82:2991-7.
24. Konishi N, Kobayashi M, Miyagawa S, Sato T, Katoh O, Ueda K. Defective proliferation of primitive myeloid progenitor cells in patients with severe congenital neutropenia. Blood 1999; 94:4077-83.
25. Nakamura K, Kobayashi M, Konishi N, Kawaguchi H, Miyagawa S, Sato T, et al. Abnormalities of primitive myeloid progenitor cells expressing granulocyte colony-stimulating factor receptor in patients with severe congenital neutropenia. Blood 2000; 96:4366-9.
26. Kobayashi M, Laver JH, Kato T, Miyazaki H, Ogawa M. Thrombopoietin supports proliferation of human primitive hematopoietic cells in synergy with steel factor and/or interleukin-3. Blood 1996; 88:429-36.
27. Kobayashi M, Laver JH, Lyman SD, Kato T, Miyazaki H, Ogawa M. Thrombopoietin, steel factor and the ligand for flt3/flk2 interact to stimulate the proliferation of human hematopoietic progenitors in culture. Int J Hematol 1997; 66:423-34.
28. Leary AG, Yang YC, Clark SC, Gasson JC, Golde DW, Ogawa M. Recombinant gibbon interleukin 3 supports formation of human multilineage colonies and blast cell colonies in culture: comparison with recombinant human granulocyte-macrophage colony-stimulating factor. Blood 1987; 70:1343-8.
29. Ogawa M. Differentiation and proliferation of hematopoietic stem cells. Blood 1993;81:2844-53.
30. Gabbianelli M, Pelosi E, Montesoro E, Valtieri M, Luchetti L, Samoggia P, et al. Multi-level effects of flt3 ligand on human hematopoiesis: expansion of putative stem cells and proliferation of granulomonocytic progenitors/monocytic precursors. Blood 1995;86:1661-70.
31. Rusten LS, Lyman SD, Veiby OP, Jacobsen SE. The FLT3 ligand is a direct and potent stimulator of the growth of primitive and committed human CD34+ bone marrow progenitor cells in vitro. Blood 1996; 87:1317-25.
32. Kawashima I, Zanjani ED, Almaida-Porada G, Flake AW, Zeng H, Ogawa M. $CD34^+$ human marrow cells that express low levels of Kit protein are enriched for long-term marrow-engrafting cells. Blood 1996;87:4136-42.
33. Kyas U, Pietsch T, Welte K. Expression of receptors for granulocyte colony-stimulating factor on neutrophils from patients with severe congenital neutropenia and cyclic neutropenia. Blood 1992;79:1144-7.
34. Freedman MH. Safety of long-term administration of granulocyte colony-stimulating factor for severe chronic neutropenia. Curr Opin Hematol 1997; 4:217-24.
35. Borregaard N, Cowland JB. Granules of the human neutrophilic polymorphonuclear leukocyte. Blood 1997; 89: 3503-21.
36. Cowland JB, Borregaard N. The individual regulation of granule protein mRNA levels during neutrophil maturation explains the heterogeneity of neutrophil granules. J Leukoc Biol 1999; 66:989-95.
37. Borregaard N, Theilgaard-Monch K, Sorensen OE, Cowland JB. Regulation of human neutrophil granule protein expression. Curr Opin Hematol 2001; 8:23-7.
38. Takahashi H, Nukiwa T, Basset P, Crystal RG. Myelomonocytic cell lineage expression of the neutrophil elastase gene. J Biol Chem 1988; 263:2543-7.
39. Fouret P, du Bois RM, Bernaudin JF, Takahashi H, Ferrans VJ, Crystal RG. Expression of the neutrophil elastase gene during human bone marrow cell differentiation. J Exp Med 1989; 169: 833-45.
40. Zimmer M, Medcalf RL, Fink TM, Mattmann C, Lichter P, Jenne DE. Three human elastase-like genes coordinately expressed in the myelomonocyte lineage are organized as a single genetic locus on 19pter. Proc Natl Acad Sci USA 1992;89:8215-9.
41. Bories D, Raynal MC, Solomon DH, Darzynkiewicz Z, Cayre YE. Down-regulation of a serine protease, myeloblastin, causes growth arrest and differentiation of promyelocytic leukaemia cells. Cell 1989;59:959-68.
42. Lutz PG, Moog-Lutz C, Coumau-Gatbois E, Kobari L, Di Gioia Y, Cayre YE. Myeloblastin is a granulocyte colony-stimulating factor-responsive gene conferring factor-independent growth to hematopoietic cells. Proc Natl Acad Sci USA 2000;97:1601-6.
43. Belaaouaj A, McCarthy R, Baumann M, Gao Z, Ley TJ, Abraham SN, et al. Mice lacking neutrophil elastase reveal impaired host defense against gram negative bacterial sepsis. Nat Med 1998;4:615-8.
44. Grenda DS, Johnson SE, Mayer JR, McLemore ML, Benson KF, Horwitz M, et al. Mice expressing a neutrophil elastase mutation derived from patients with severe congenital neutropenia have normal granulopoiesis. Blood 2002;100:3221-8.