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Genetics

Identification of a nonsense mutation in the granulocyte-colony-stimulating factor receptor in severe congenital neutropenia

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ABSTRACT **Severe congenital neutropenia (Kostmann syndrome) is characterized by profound absolute neutropenia and a maturation arrest of marrow progenitor cells at the promyelocyte–myelocyte stage. Marrow cells from such patients frequently display a reduced responsiveness to granulocyte-colony-stimulating factor (G-CSF). G-CSF binds to and activates a specific receptor which transduces signals critical for the proliferation and maturation of granulocytic progenitor cells. Here we report the identification of a somatic point mutation in one allele of the G-CSF receptor gene in a patient with severe congenital neutropenia. The mutation results in a cytoplasmic truncation of the receptor. When expressed in murine myeloid cells, the mutant receptor transduced a strong growth signal but, in contrast to the wild-type G-CSF receptor, was defective in maturation induction. The mutant receptor chain may act in a dominant negative manner to block granulocytic maturation.**

Severe congenital neutropenia (SCN, or Kostmann syndrome) was first described in 1956 (1). This disorder of granulopoiesis is characterized by severe absolute neutropenia (peripheral blood neutrophil count of $\leq 0.2 \times 10^9$/L) and a maturation arrest of marrow myeloid progenitor cells at the promyelocyte–myelocyte stage. Erythropoiesis and thrombopoiesis are generally normal. Patients with SCN have frequent episodes of severe bacterial infections, usually starting in the first month of life. Affected infants have a poor prognosis and often succumb in the first or second decade despite improvements in supportive care. Patients also have an increased risk for developing acute leukemia (2). Although an autosomal recessive pattern of inheritance was suggested for SCN, sporadic cases have been reported as well (3–6).

The etiology of SCN remains elusive. No serum inhibitors of marrow cell growth have been detected in patients with SCN (5). The mononuclear cells from these patients can produce biologically active granulocyte-colony-stimulating factor (G-CSF) and the serum levels of G-CSF are generally increased (7–9). However, cultured marrow cells from patients with SCN frequently display a markedly suppressed responsiveness to G-CSF stimulation (4, 6, 9, 10). With the clinical availability of recombinant hematopoietic growth factors, it has been observed that most patients respond favorably to *in vivo* administration of G-CSF, as evidenced by a significant increase in circulating neutrophils and a dramatic clinical improvement (3, 4, 9). In contrast, therapy with granulocyte/macrophage-colony-stimulating factor (GM-CSF) is generally ineffective in SCN. These data suggest that a specific defect in G-CSF signal transduction may exist in SCN that can be overcome by providing the patients with pharmacologic dosages of G-CSF.

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G-CSF acts primarily in the granulocytic lineage and appears to be the major hematopoietic growth factor involved in the regulation of *in vivo* production of mature neutrophils (11). G-CSF induces the proliferation, differentiation, and survival of granulocytic progenitor cells. The diverse effects of G-CSF are mediated through the interaction of G-CSF and a specific cell surface receptor (G-CSF-R). The human G-CSF-R cDNA predicts an 813-aa peptide with a single membrane-spanning domain (12, 13). Expression of the human G-CSF-R in murine hematopoietic cells resulted in high-affinity binding sites on the cell surface and rendered the cells responsive to G-CSF (14).

The exclusive abnormality of granulopoiesis in SCN prompted us to investigate the G-CSF-R in these patients. We used reverse transcription (RT)–PCR to amplify the G-CSF-R cDNA of patients with congenital neutropenia and screened for mutations by single-strand conformation polymorphism (SSCP) analysis. In one patient, we identified a somatic point mutation which results in the cytoplasmic truncation of the G-CSF-R. Further functional characterization revealed that the truncated G-CSF-R was unable to transduce a maturation signal.

MATERIALS AND METHODS

Patients and Samples. Patient D, a 12-year-old boy now, suffered from severe recurrent infections from 3 days of age onwards. The diagnosis of SCN was established on clinical and laboratory grounds. There was no family history of similar disease. From age 10, the patient started treatment with recombinant human G-CSF and showed a favorable response with an increase of neutrophils from below detectable to $>10^9$/L.

Bone marrow or blood samples were obtained from patient D after informed consent from the parents. Marrow progenitor cells were enriched by Ficoll-Isopaque centrifugation and complement-mediated cytolysis of mature T cells and monocytes following 30 min of incubation with optimal concentrations of monoclonal antibodies against CD3, CD14, and CD15 (15). $CD34^+$ cells were then sorted following CD34 labeling (BI3C5, Sera-Lab, Crawley Down, Sussex, U.K.). T lymphocytes were isolated from peripheral blood by erythrocyte rosetting of cells from the Ficoll-Isopaque interface. Monocytes were recovered from the latter cell population after plastic adherence in 6-cm Petri dishes (1 hr at 37°C), and B lymphocytes were collected as nonadherent erythrocyte-rosette-negative mononuclear cells. Granulocytes were obtained from blood as the sedimented cell fraction after

Abbreviations: Epo, erythropoietin; G-CSF, granulocyte-colony-stimulating factor; G-CSF-R, G-CSF receptor; GM-CSF, granulocyte/macrophage-colony-stimulating factor; IL-3, interleukin 3; SCN, severe congenital neutropenia; SSCP, single-strand conformation polymorphism.

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Ficoll-Isopaque centrifugation and further depleted of erythrocytes by hypotonic lysis. Erythroid colony cells were picked from *in vitro* cultures supported by erythropoietin (Epo) and interleukin 3 (IL-3).

Clonogenic Assays and Recombinant Human Growth Factors. Colony cultures were set up essentially as described (15). Marrow cells were plated in duplicate at 1×10^4 per ml ($CD34^+$ cells) or 4×10^4 per ml (Ficoll interface) in semisolid medium containing Iscove's modified Dulbecco's medium (IMDM), 1.1% methylcellulose, 30% fetal bovine serum, transferrin, lecithin, sodium selenite, and 2-mercaptoethanol with or without growth factors. Colonies were scored after 14 days of incubation at 37°C in humidified 5% CO_2 atmosphere. IL-3 (Gist Brocades, Delft, The Netherlands) was used at 3 ng/ml. GM-CSF and macrophage colony stimulating factor (M-CSF) (Genetics Institute, Cambridge, MA) were used at 25 and 50 ng/ml. G-CSF and Epo (Amgen) were used at 10 ng/ml and 1 unit/ml, respectively.

Amplification of Genomic DNA and cDNA by PCR. Genomic DNA was isolated as described (16). Total granulocyte RNA was extracted (17) and first-strand cDNA was synthesized from 1 μg of RNA by using avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) and oligo(dT) or reverse primers. Three sets of primers (Table 1) were designed to amplify the entire coding region of the G-CSF-R cDNA. Part of exon 17 of the G-CSF-R gene was amplified with primers FR4 and RV3. Amplification was performed with Vent DNA polymerase (New England Biolabs) on 1/10th of the cDNA reaction mixture or 0.5 μg of genomic DNA for 35 cycles of 1 min at 94°C, 1.5 min at 51°C, and 2 min at 72°C in a thermal cycler (Perkin–Elmer/Cetus).

SSCP Analysis. Amplified PCR fragments were purified (Geneclean, Bio 101) after agarose gel separation and 1/100th of the products was subjected to second-round amplification in the presence of [α-^{32}P]ATP. To increase the sensitivity of SSCP analysis, the labeled products were cleaved into fragments of 50–250 bp by digestion with appropriate restriction enzymes (three or four enzymes in each case). Samples were then denatured by boiling for 5 min in loading buffer [95% (vol/vol) formamide/20 mM EDTA/0.05% (wt/vol) each bromophenol blue and xylene cyanol] and loaded on a nondenaturing 8% polyacrylamide gel (acrylamide/methylenebisacrylamide weight ratio, 49:1) containing 10% (vol/vol) glycerol. Electrophoresis at 30 W for 5–8 hr was followed by autoradiography.

Subcloning of PCR Fragments and DNA Sequencing. After agarose gel purification, PCR fragments were ligated to the *Hinc*II site of pBluescript (Stratagene). Nucleotide sequences were determined on both strands by the dideoxy method using the T7 sequencing kit (Pharmacia).

G-CSF-R Expression Constructs. The wild-type human G-CSF-R cDNA (13) derived from clone pHQ3 was excised from pBluescript and inserted into the *Hpa* I site of the retroviral expression vector pLNCX (18). To reconstitute a full-length mutant cDNA, the cloned PCR fragment obtained from granulocytes of patient D with primers FR3 and RV3 was cut with *Bsr*FI and *Cla* I, and the resultant fragment of 499 bp was used to replace the corresponding part of the wild type G-CSF-R cloned in pLNCX.

Table 1. Primers used for PCR amplification

Primer	Sequence (5′ → 3′)	Position
FR1	TCGGAAAGGTGAAGTAACTTGTCC	111–134
RV1	TCCATGGGATCAAGACACAG	818–837
FR2	TGCAGGCAGAGAATGCGCTG	777–796
RV2	GAAGATCTCATAGAGCTGAAAG	1657–1678
FR3	TGTGATCATCGTGACTCCCTT	1669–1689
RV3	GTAGATCTTAGTCATGGGCTTATGG	2750–2774
FR4	CCATCACCAAGCTCACAGTG	2244–2263

Underlined nucleotides indicate introduced mismatches. FR, forward; RV, reverse.

Cell Line and Gene Transfection. A murine myeloid cell line, L-GM (19), was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 10% WEHI-3B cell conditioned medium (WEHI-CM). G-CSF-R expression constructs were linearized by *Pvu* I digestion and then introduced into L-GM cells by electroporation. After transfection, L-GM cells were selected in culture medium containing G418 at 0.8 mg/ml. Single G418-resistant clones were expanded and tested for the ability to respond to human G-CSF. For maturation induction, L-GM transfectants were transferred to RPMI 1640 containing 10% fetal bovine serum and recombinant human G-CSF at 10 ng/ml. Cell morphology was examined every 2–3 days.

RESULTS

***In Vitro* Response to Hematopoietic Growth Factors.** Colony cultures of highly enriched progenitor cells from patient D were used to evaluate the responsiveness to several hematopoietic growth factors. The numbers of erythroid, macrophage, and eosinophilic colonies generated from marrow cells of patient D in response to Epo, IL-3, and GM-CSF were comparable to normal controls (Table 2). However, G-CSF-induced granulocytic colony formation was significantly impaired. Dose titration experiments with marrow cells recovered from Ficoll-Isopaque interface without further enrichment revealed that the patient's marrow cells not only generated lower numbers of granulocytic colonies but also required higher concentration of G-CSF for maximal colony formation than normal marrow cells (Fig. 1). These results showed that the response to G-CSF was exclusively impaired in the patient.

SSCP Analysis of G-CSF-R cDNA. The entire coding region of the G-CSF-R cDNA was amplified from total RNA extracted from granulocytes of patient D by using three sets of primers. The resultant PCR fragments were initially separated in agarose gel, but no differences in size were detected between the patient's and wild-type G-CSF-R. To search for small alterations, the PCR fragments were subjected to SSCP analysis following digestion with various restriction enzymes. An abnormally migrating band, in addition to the normally migrating one, was consistently detected when the PCR fragment extending from nt 1669 to nt 2774 (pHQ3) was digested with *Pvu* II (Fig. 2, lane 9). This fragment was then subcloned and the same mobility shift was detected in 9 out of 16 individual clones analyzed. An abnormally migrating band was also detected following *Dde* I digestion, and by comparing the patterns of SSCP obtained after *Pvu* II and *Dde* I digestion, the region containing the mutation was

Table 2. *In vitro* response of marrow progenitor cells from normal individuals and patient D to various hematopoietic growth factors

Subject(s)	Growth factor	Colony number			
		G	M	Eo	E
Normal (n = 4)	IL-3	0	0–17	15–71	0
	GM-CSF	0	0–36	9–75	0
	G-CSF	44–269	0–35	0	0
	Combination*	107–450	18–83	23–61	107–450
Patient D	IL-3	0	12	37	0
	GM-CSF	0	7	22	0
	G-CSF	7	17	0	0
	Combination*	78	35	58	108

Numbers indicate colonies per 10^4 $CD34^+$ cells. G, granulocytic; M, macrophage; Eo, eosinophilic; E, erythroid.
*IL-3, GM-CSF, G-CSF, and Epo.

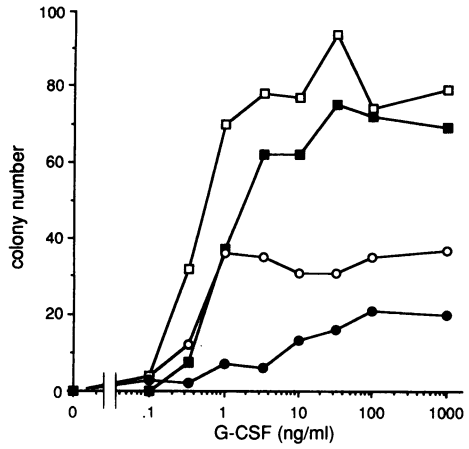

FIG. 1. *In vitro* response to G-CSF of marrow cells from patient D and normal individuals. A reduced sensitivity of marrow cells from patient D (●) to G-CSF stimulation as compared with the marrow cells from 3 healthy donors (○, □, ■) was apparent, as indicated by the reduced colony numbers and the much greater concentration of G-CSF required for maximal colony formation. The results obtained with marrow cells recovered after Ficoll-Isopaque separation are shown. Cells were cultured at 4×10^4 per ml and colonies were counted after 14 days of incubation.

narrowed down to nt 2311–2429. No other mutations in the coding region of the G-CSF-R sequence were identified in patient D or in five other patients with congenital neutropenia and two patients with acquired idiopathic neutropenia (Fig. 2).

Identification of a Point Mutation. Five independent clones were sequenced and a C → T transition at nt 2384 (pHQ3) was identified in three clones displaying the variant SSCP patterns (Fig. 3). The mutation was not present in two other clones displaying the normal SSCP pattern. Direct sequencing of PCR products amplified from genomic DNA of the patient's granulocytes confirmed the presence of both the normal and the mutant allele (data not shown). The mutation changes the CAG glutamine codon to a TAG stop codon at aa 716 (Fig. 3). Thus, one of the patient alleles is predicted to code for a truncated G-CSF-R lacking the C-terminal 98 aa.

Lineage Specificity of the Mutation. To investigate whether the mutation was inherited or represented a *de novo* germline or somatic event, a fragment of 530 bp covering the mutation site was amplified from genomic DNA prepared from various cellular sources of patient D and from blood mononuclear cells of the parents. After *Pvu* II digestion, the amplified

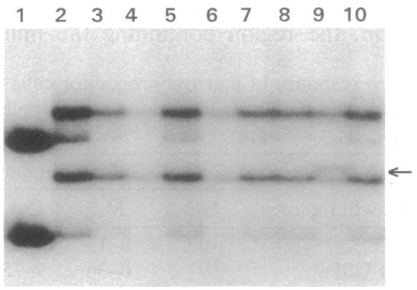

FIG. 2. Screening for mutations in the G-CSF-R gene by PCR–SSCP analysis. The fragment extending from nt 1669 to nt 2774 was amplified from wild-type cDNA (pHQ3, lane 2) and reverse-transcribed cDNA from mRNA extracted from six pediatric patients with congenital neutropenia (lanes 3–6, 8, and 9) and two adult patients with chronic idiopathic neutropenia (lanes 7 and 10). Part of the autoradiograph of the SSCP gel obtained after *Pvu* II digestion is shown. Position of double-strand DNA is shown in lane 1. An abnormally migrating band was seen in lane 9 (arrow).

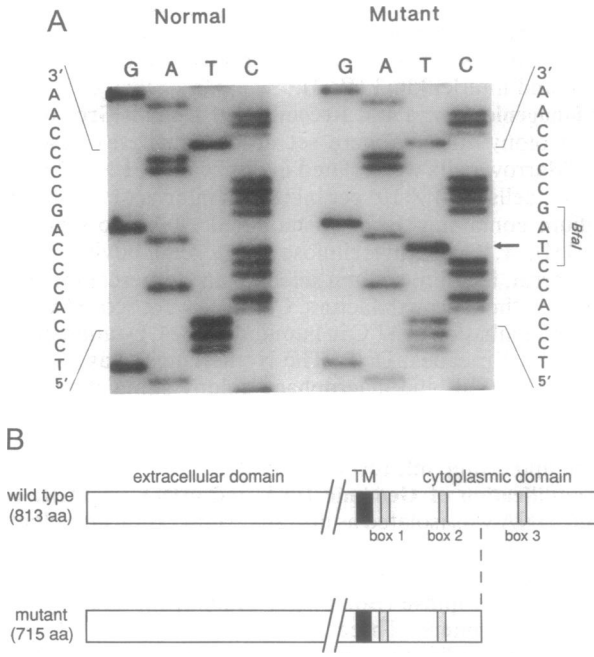

FIG. 3. Identification of a point mutation in G-CSF-R cDNA. (*A*) Partial nucleotide sequences of normal and mutant G-CSF-R cDNA. PCR fragment amplified with primers FR3 and RV3 (nt 1669–2774) was subcloned into the *Hinc*II site of pBluescript SK vector by blunt-end ligation. Nucleotide sequences of five independent clones were determined by DNA sequencing. Shown is a portion of the nucleotide sequences spanning the point mutation (C to T) as indicated by the arrow. The mutation was confirmed in two other clones displaying the variant SSCP. The restriction site for *Bfa* I created by the mutation is also indicated. (*B*) Structure of wild-type and mutant G-CSF-Rs. Boxes 1–3, regions conserved in certain members of the cytokine receptor family; TM, transmembrane domain.

fragment was subjected to SSCP analysis. In two independent experiments the mutation was readily detected in patient's granulocytes (Fig. 4, lane 2), but not in monocytes, erythroid colony cells, T and B lymphocytes, and skin fibroblasts of patient D (lanes 3–7). DNA isolated from mononuclear cells of the parents manifested a normal SSCP pattern (lanes 8 and 9). Since the C → T transition creates an additional *Bfa* I site (CCAG → CTAG; Fig. 3), we performed

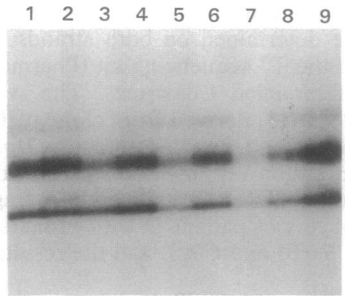

FIG. 4. Detection of the point mutation in genomic DNA from various cell populations by PCR–SSCP analysis. Part of exon 17 of the G-CSF-R gene that contains the mutation was amplified as described in *Materials and Methods*. PCR products were digested with *Pvu* II before SSCP analysis. The same abnormally migrating band as that shown in Fig. 2 was observed in DNA isolated from patient granulocytes (lane 2), but not in DNA from monocytes, erythroid colony cells, T lymphocytes, B lymphocytes, and fibroblasts of the patient (lanes 3–7). DNA isolated from the mononuclear cells of the patient's father and mother exhibits a normal SSCP pattern (lanes 8 and 9). Lane 1 shows the SSCP pattern of wild-type cDNA.

Bfa I digestion of PCR products to confirm the findings and comparable results were obtained (data not shown).

Functional Characterization of the Truncated G-CSF-R. To show that the truncated receptor is functionally defective, the mutant and wild-type G-CSF-R cDNAs were subcloned in the mammalian expression vector pLNCX (18) and then introduced into murine L-GM cells, an IL-3- and GM-CSF-dependent myeloid cell line lacking endogenous G-CSF-R (19). Parental L-GM cells did not show any response to G-CSF and exhibited an immature phenotype when maintained in IL-3-containing medium (Fig. 5*A*). The forced expression of either the wild-type or the mutant receptor rendered these cells responsive to human G-CSF. L-GM cells expressing the wild-type G-CSF-R responded poorly to G-CSF and lost viability gradually in G-CSF-containing medium, a process which was accompanied by terminal maturation of the cells toward neutrophilic granulocytes. Morphological examination of the cells revealed that almost all cells were at the end stage of granulocytic maturation after 9 days of G-CSF treatment (Fig. 5*B*). In contrast, L-GM cells expressing the truncated G-CSF-R continuously proliferated in G-CSF-containing medium and did not show evidence of terminal granulocytic maturation (Fig. 5*C*).

DISCUSSION

SCN is a rare pediatric disorder and to date about 120 cases have been reported (2). On the basis of the reduced *in vitro* response of marrow progenitor cells to G-CSF, increased serum levels of G-CSF, and differential therapeutic effects of G-CSF and GM-CSF in these patients, a perturbed G-CSF signal transduction has been suggested. Normal or increased numbers of G-CSF-Rs are expressed on the neutrophils of SCN patients, with affinities comparable to those on normal neutrophils, suggesting that the aberrant response to G-CSF in SCN is not due to alterations in G-CSF-R expression (20). However, mutations, particularly in the cytoplasmic domain of G-CSF-R, that affect only G-CSF signal transduction cannot be excluded. Rauprich *et al.* (21) have demonstrated that an intracellular protein of ≈160 kDa is spontaneously phosphorylated in neutrophils from patients with SCN, but not in normal neutrophils. Neutrophils from patients with SCN display an abnormal response to fMet-Leu-Phe, with decreased superoxide anion production and impaired mobilization of cytosolic calcium (22–24). Nevertheless, the exact nature of molecular defects in SCN has remained unknown.

We describe here the identification of a point mutation in one allele of the G-CSF-R gene in a patient with SCN which results in the deletion of 98 aa from the C terminus of the receptor. The mutation was predominantly present in the granulocytic lineage, suggesting that patient D is mosaic for the mutation. Functional analysis of the mutant receptor revealed that the underlying defect of the truncated G-CSF-R was its inability to transduce signals for terminal granulocytic maturation. These observations are consistent with the clinical finding that granulocytic maturation is disturbed in patient D and with the fact that marrow cells from the patient displayed an exclusively abnormal response to G-CSF stimulation *in vitro*. Our data strongly imply that the expression of the truncated receptor is the direct cause of neutropenia in the patient.

Two lines of evidence suggest that the truncated receptor was expressed in patient D, although direct demonstration of the presence of the mutant protein was hampered by the fact that anti-human G-CSF-R antibodies are not yet available. According to SSCP analysis the transcripts of the normal and the mutant G-CSF-R gene were present in the patient's granulocytes (Fig. 2). Flow cytometry with biotinylated G-CSF demonstrated that the surface levels of both the wild-type and the truncated G-CSF-R were low but comparable in L-GM cells (data not shown). These data indicate that the mutant G-CSF-R protein is stable and can be expressed on the cell surface. This notion may have important implications for understanding the mechanism whereby the truncated receptor interferes with the normal granulopoiesis. Oligomerization of receptor molecules is required for high-affinity binding and subsequent signal transduction (25–29). Thus, the truncated G-CSF-R could form a heterodimer with

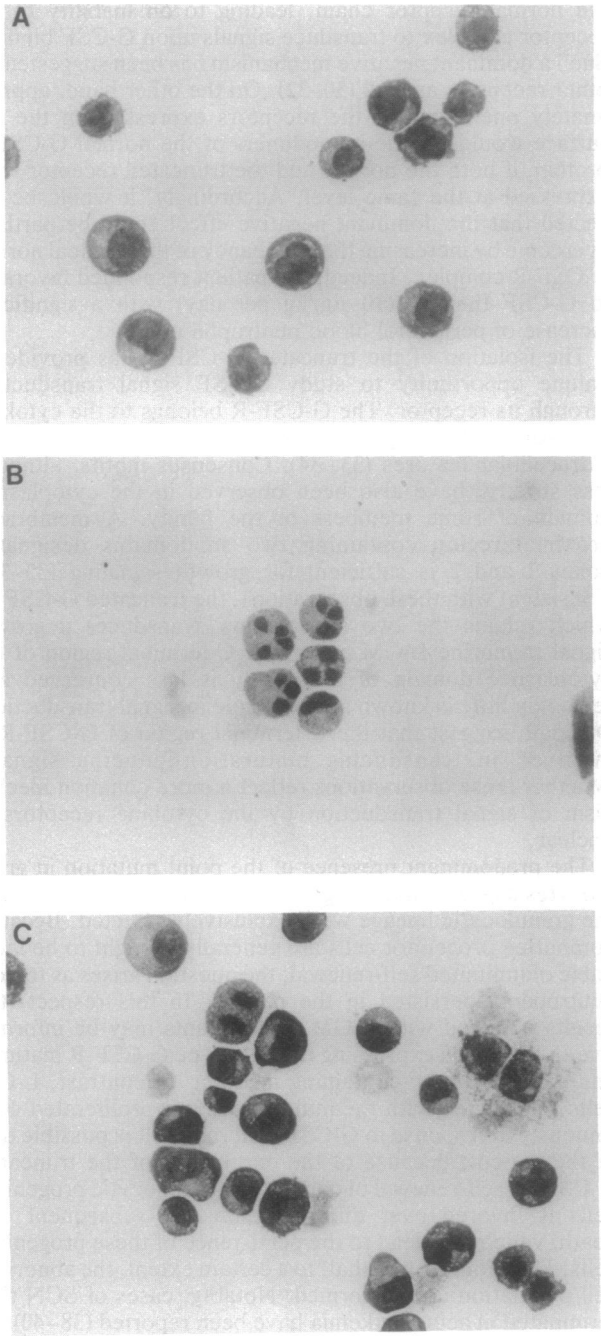

FIG. 5. Morphological analysis of L-GM cells transfected with the wild-type and the mutant G-CSF-R. (*A*) L-GM cells grown in 10% WEHI cell conditioned medium (as a crude source of murine IL-3) display a phenotype of immature myeloid cells. (*B*) L-GM transfectants of the wild-type human G-CSF-R consist of terminally mature neutrophilic granulocytes after incubation with G-CSF for 9 days. (*C*) L-GM cells expressing the truncated G-CSF-R remain morphologically immature following stimulation with G-CSF. Expression of the G-CSF-R was confirmed by Northern blot and flow cytometric analysis using biotinylated G-CSF. Slides were stained with May–Grünwald–Giemsa stain.

the normal receptor chain, leading to an inability of the receptor complex to transduce signals upon G-CSF binding. Such a dominant negative mechanism has been suggested for other receptors as well (30–32). On the other hand, approximately one-fourth of the receptors expressed on the cell surface would still be homodimers of the normal G-CSF-R protein, if both the normal and the truncated receptor were expressed at the same level. Accordingly, it would be expected that the dominant negative effect could be partially overcome by increasing the occupancy of the residual normal G-CSF-R complex. Indeed, the patient responded favorably to G-CSF therapy (10 μg/kg per day) with a significant increase of peripheral blood neutrophil count.

The isolation of the truncated G-CSF-R has provided a unique opportunity to study G-CSF signal transduction through its receptor. The G-CSF-R belongs to the cytokine receptor superfamily, which is characterized by common extracellular features (33, 34). Consensus motifs, although less strictly, have also been observed in the cytoplasmic domain of some members of the family. A membrane-proximal region containing two subdomains designated boxes 1 and 2 is sufficient for growth signaling (35–37). Consistent with these observations, the truncated G-CSF-R, which retains the two subdomains, transduces a growth signal in murine L-GM cells. The C-terminal region of the cytoplasmic domain of the family is less conserved and relatively little is known about its role in signal transduction. Our data suggest that the C-terminal region of G-CSF-R is involved in transducing maturation-inducing signals. Whether these observations reflect a more common mechanism of signal transduction by the cytokine receptors is unclear.

The predominant presence of the point mutation in granulocytes suggests that progenitor cells mainly committed to the granulocytic lineage were exclusively affected. Because committed progenitor cells are generally thought to be incapable of unlimited self-renewal, the question arises as to how neutropenia persisted in the patient. In this respect, the results obtained with L-GM transfectants may be informative. L-GM cells expressing the wild-type G-CSF-R matured and died in G-CSF-containing medium. In contrast, L-GM cells transfected with the mutant receptor proliferated continuously in response to G-CSF. Therefore, it is possible that in the patient, because of the expression of the truncated G-CSF-R, self-renewal of committed granulocytic progenitor cells is favored over differentiation and subsequent cell death, which may lead to the persistence of these progenitor cells. This would imply that, to a certain extent, the abnormal cell population is transformed. Notably, cases of SCN that terminated in acute leukemia have been reported (38–40). In addition, our data indicate that G-CSF therapy in certain cases of SCN should be carefully evaluated, because such treatment may lead to an overstimulation of the abnormal cell population. At least three SCN patients have developed leukemia or myelodysplastic syndrome following G-CSF treatment (2).

It is conceivable that SCN consists of a heterogeneous group of disorders with variable underlying etiology. In fact, six patients with congenital neutropenia were initially screened with SSCP analysis but an abnormal G-CSF-R was identified in only one patient (Fig. 2), suggesting that abnormalities of molecules involved in G-CSF signal transduction downstream of the receptor could also play a role in SCN pathogenesis. However, with more patients being examined, mutations in other important regions of G-CSF-R may be identified in SCN. In addition, our results also validate the investigation of the involvement of an abnormal G-CSF-R in other neutropenias as well as in leukemia. Studies of this kind may help to elucidate the pathogenesis of some hematopoietic disorders and may improve our understanding of G-CSF signal transduction.

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