Abstract  Background. In severe congenital neutropenia the maturation of myeloid progenitor cells is arrested. The myelodysplastic syndrome and acute myeloid leukemia develop in some patients with severe congenital neutropenia. Abnormalities in the signal-transduction pathways for granulocyte colony-stimulating factor (G-CSF) may play a part in the progression to acute myeloid leukemia.

Methods. We isolated genomic DNA and RNA from hematopoietic cells obtained from two patients with acute myeloid leukemia and histories of severe congenital neutropenia. The nucleotide sequences encoding the cytoplasmic domain of the G-CSF receptor were amplified by means of the polymerase chain reaction and sequenced. Murine myeloid 32D.C10 cells were transfected with complementary DNA encoding the wild-type or mutant G-CSF receptors and tested for their responses to G-CSF.

Results. Point mutations in the gene for the G-CSF receptor were identified in both patients. The mutations, a substitution of thymine for cytosine at the codon for glutamine at position 718 (Gln718) in one patient and at the codon for glutamine at position 731 (Gln731) in the other, caused a truncation of the C-terminal cytoplasmic region of the receptor. Both mutant and wild-type genes for the G-CSF receptor were present in leukemic cells from the two patients. In one patient, the mutation was also found in the neutrophilic stage, before the progression to acute myeloid leukemia. The 32D.C10 cells expressing mutant receptors had abnormally high proliferative responses but failed to mature when cultured in G-CSF. The mutant G-CSF receptors also interfered with terminal maturation mediated by the wild-type G-CSF receptor in the 32D.C10 cells that coexpressed the wild-type and mutant receptors.

Conclusions. Mutations in the gene for the G-CSF receptor that interrupt signals required for the maturation of myeloid cells are involved in the pathogenesis of severe congenital neutropenia and associated with the progression to acute myeloid leukemia. (N Engl J Med 1995;333: 487-93.)
Expression Vectors for G-CSF Receptor

A cDNA encoding a truncated G-CSF receptor (mutant DA\textsuperscript{18}) was cloned at the EcoRI restriction site of the pBabe-puro retroviral expression vector that contains a puromycin-resistance gene,\textsuperscript{27} giving rise to the pBabe-DA construct. To constitute the full-length G-CSF receptor in the two patients, PCR fragments obtained from Patients 1 and 2 with primer sets FWI16–RV1 and FW2–RV1, respectively, were inserted into the HindII restriction site of the pBluescript vector (Stratford, Leduc, Alberta, Canada). Amplification with the polymerase chain reaction (PCR) was performed as previously described.\textsuperscript{21} The following primers were used: FW2, 5′TGATGTCATGGGCTTATGG3′ (forward); FW3, 5′TGATGTCATGGGCTTATGG3′ (forward); FW4, 5′CGACAGGAGTTCCACCCAGGCCC3′ (forward); FW16, 5′ACCTTTTGTTTCCAACAGATG3′ (forward); RV1, 5′AAAGGTCTGTTTACAXACTCTGAAAG3′ (reverse); RV2, 5′GTATGTCATGGGCTTATGG3′ (reverse); and RV3, 5′TGATGTCATGGGCTTATGG3′ (reverse). The underlined nucleotides indicate the introduced mismatches.

Nucleotide Sequencing

After electrophoresis on agarose gels, PCR fragments were purified with the Geneclean kit (Bio 101, La Jolla, Calif.) and sequenced directly or after subcloning in the pBluescript vector (Stratford with the Geneclean II kit (Bio 101, La Jolla, Calif.) and sequencing data and to examine the ratio of mutant-to-normal genes for the G-CSF receptor in bone marrow cells from Patient 1. A single mismatch was introduced in primer FW4; it created a HindII restriction site of the pBluescript vector (Stratagene, La Jolla, Calif.), with the use of the T7 Sequencing Kit (Pharmacia P-L Biochemicals, Milwaukee).

Cell-Line and Gene Transfection

A subline of the murine myeloid cell line 32D, called 32DC10, was fully dependent on murine interleukin-3 for proliferation and was unresponsive to G-CSF.\textsuperscript{25} The 32DC10 cells were maintained in RPMI medium supplemented with 10 percent fetal-calf serum and 10 ng of interleukin-3 per milliliter. The expression constructs pBabe-1, pBabe-2, and pLNCX-WT were linearized with PvuI and introduced into the 32DC10 cells by electroporation. After 48 hours of incubation, cells were selected with puromycin (1 μg per milliliter) or G418 (0.8 mg per milliliter) in semisolid culture medium containing 0.9 percent methylcellulose. Single colonies were subsequently expanded in liquid culture for further analyses.

Antibodies to the G-CSF Receptor

Antiserum was raised by immunizing rabbits with a fusion protein consisting of a hexahistidine–residue tag and G-CSF receptor containing the extracellular domain of the receptor from amino acid 17 to amino acid 345. A corresponding BamHI fragment of the G-CSF receptor cDNA was inserted into the BamHI restriction site of the bacteria expressing vector pQE-10 (Qiagen, Düsseldorf, Germany). A purified immunoglobulin fraction was obtained by protein A Sepharose affinity chromatography.

Western Blotting and Assays of Cell Proliferation

Cell lysates were prepared as described elsewhere\textsuperscript{28} and analyzed by a standard method of Western blotting. Tritium-labeled–thymidine uptake and long-term cell proliferation in response to G-CSF were measured as described elsewhere.\textsuperscript{29}

**Methods**

**Polymerase-Chain-Reaction Amplification**

Genomic DNA was isolated from different cellular sources, as described elsewhere.\textsuperscript{22} Total RNA was isolated from leukemic cells from Patient 2 by the method of Chomczynski and Sacchi.\textsuperscript{23} RNA was reverse-transcribed into complementary DNA (cDNA) with the use of the reverse transcriptase of Moloney murine leukemia virus (GIBCO-BRL, Breda, the Netherlands). Amplification with the gene, La Jolla, Calif.), with the use of the T7 Sequencing Kit (Pharmacia P-L Biochemicals, Milwaukee), the myelodysplastic syndrome (the subtype characterized by refractory anemia and excess of blasts) and thrombocytopenia developed. After an additional eight months, the patient presented with acute myeloid leukemia (subtype M1 according to the French–American–British classification) and subsequently died.

**Results**

**Mutations in the Gene for the G-CSF Receptor**

The entire exon 17,\textsuperscript{21,26} which encodes the 156 amino acids of the G-terminal cytoplasmic region, and part of intron 16 of the G-CSF–receptor gene were amplified by PCR from genomic DNA isolated from bone marrow cells obtained from Patient 1 with primers FWI16 and RV1. The PCR product was subcloned, and a pool of 18 clones was sequenced. This sequence contained a cytosine-to-thymine (C-to-T) transition at nucleotide 2390 of the G-CSF receptor cDNA\textsuperscript{26} (Fig. 1A). Direct sequencing of PCR products confirmed the presence of the point mutation (data not shown). This mutation changes the CAA codon for glutamine at position 718 (Gln718) to the TAA stop codon, thus truncating a C-terminal region of 96 amino acids, including the conserved box-3 segment of the receptor’s cytoplasmic domain\textsuperscript{20} (Fig. 1B).

Enzyme-restriction analysis was used to confirm the sequencing data and to examine the ratio of mutant-to-normal genes for the G-CSF receptor in bone marrow cells from Patient 1. A single mismatch was introduced in primer FW4; it created a StuI restriction site in the PCR product if the point mutation was present in the DNA (Fig. 2A). Analysis of eight individual clones showed that five contained the mutation (Fig. 2B). StuI digestion of the PCR product obtained from DNA of bone marrow cells collected at various times during the course of leukemia showed that the mutated gene made up a minor proportion of the DNA. The mutation was not detected in the liver or the spleen by StuI digestion and nucleotide sequencing (data not shown). These results indicate that the mutation did not occur in the germ line.

Only RNA samples were available from leukemic cells in peripheral blood obtained from Patient 2. Reverse-transcriptase PCR with primers FW2 and RV1 (Filarstatin) in Hannover, Germany. At that time, bone marrow studies revealed an arrest of myelopoiesis at the promyelocytic or myelocytic stage, with an absence of bands and segmented neutrophils. There were no signs of dysplasia, and the cellularity of the bone marrow was normal. Treatment with G-CSF (3 μg per kilogram of body weight per day) increased the neutrophil count to a level above 2000 per cubic millimeter within two weeks. During the next two years, the neutrophil count was maintained at this level with the same dose of G-CSF, and the patient had no severe infections.

Two years after the start of G-CSF treatment, a routine bone marrow examination demonstrated monosomy 7 in the myeloid lineage, but there was no sign of dysplasia or leukemia. G-CSF treatment was immediately discontinued, but it was restarted two months later, at the patient’s request, because of severe stomatitis. Eleven months later, the myelodysplastic syndrome (the subtype characterized by refractory anemia and excess of blasts) and thrombocytopenia developed. After an additional eight months, the patient presented with acute myeloid leukemia (subtype M1 according to the French–American–British classification) and subsequently died.
was used to amplify the entire transmembrane and cytoplasmic domains, as well as part of the extracellular domain, of the G-CSF–receptor cDNA. After subcloning of the PCR product, nucleotide sequencing was performed with a pool of 16 clones. A C-to-T point mutation was identified at nucleotide 2429 (Fig. 1A). This mutation, which changes the CAG codon for glutamine at position 731 (Gln731) to the TAG stop codon, deleted the 83 C-terminal amino acids of the G-CSF receptor (Fig. 1B). The mutation destroys a *Pvu*II restriction site in the G-CSF receptor cDNA. *Pvu*II digestion of PCR products obtained with primers FW3 and RV2 revealed transcripts of both the normal and mutated G-CSF receptor alleles (Fig. 3). To determine whether the point mutation was present before acute myeloid leukemia developed in Patient 2, DNA was isolated from a bone marrow smear prepared when the patient was in the neutropenic phase, before the acquisition of monosomy 7. A minor proportion of the DNA contained the mutation (Fig. 3), indicating that it had arisen from a somatic event.

### Transduction of Proliferative and Maturation Signals by Wild-Type and Mutant G-CSF Receptors

The function of the mutant G-CSF receptors from the two patients was tested in murine myeloid 32D.C10 cells transduced with retroviruses containing either wild-type G-CSF receptor cDNA or the mutated G-CSF receptor cDNA from Patients 1 and 2.

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**Figure 1.** Mutations in the Gene for the G-CSF Receptor in Patients 1 and 2.

Panel A shows the sequences flanking the point mutations and the wild-type sequence. The numbers indicate the nucleotide positions (upper numbers) and amino acid positions (lower numbers). The point mutations are underlined. Panel B shows the structures of the wild-type and truncated G-CSF receptors. Boxes 1, 2, and 3 represent cytoplasmic subdomains conserved in several members of the hematopoietin-receptor superfamily. The horizontal lines in the cytoplasmic domains for Patients 1 and 2 indicate the C-terminal regions that have been deleted because of the point mutations. The numbers denote amino acid positions.

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**Figure 2.** *Stu*I Restriction-Enzyme Analysis of the PCR Product.

In Panel A, the point mutation in the template DNA (in the antisense orientation) is boxed. A mismatch (underlined) introduced in the forward primer, together with the point mutation in the template DNA, creates a *Stu*I restriction site in the PCR product. The arrows indicate the direction of the primers. Panel B shows *Stu*I digestion of individual clones of the PCR product obtained from leukemic cells from Patient 1. The nondigested product (116 base pairs [bp]) was derived from normal G-CSF–receptor sequences; the digested product (94 bp and 22 bp) was derived from the mutated allele. PCR products were separated on a 3 percent NuSieve agarose gel. M denotes the molecular-size markers.
cells that were transfected with cDNA encoding the wild-type or mutated G-CSF receptors. The expression of the G-CSF–receptor proteins in the transfected 32D.C10 cells was examined by Western blot analysis. The wild-type G-CSF–receptor protein had an apparent molecular weight of 140,000 to 150,000, whereas the mutant proteins from Patients 1 and 2 had a molecular weight of 115,000 to 135,000 and 120,000 to 140,000, respectively (data not shown). These variations in molecular weight were probably due to differences in protein glycosylation.\textsuperscript{15}

The capacities of the G-CSF receptors to transduce proliferative signals were analyzed in assays with tritium-labeled thymidine. The 32D.C10 cells expressing the wild-type receptor (32D.WT) had a dose-dependent response to G-CSF and proliferated most efficiently at the level of 3 ng of G-CSF per milliliter, which is about 75 percent of the response to interleukin-3 (Fig. 4A). The 32D.C10 cells that expressed the mutant receptors from Patient 1 (32D.1) or Patient 2 (32D.2) had a considerably increased sensitivity to G-CSF, requiring concentrations of the factor that were approximately 10 times lower than the concentrations required by the 32D.WT cells for maximal proliferation. Unlike the cells transfected with the wild-type cDNA, the 32D.1 and 32D.2 cells had maximal responses to G-CSF that were similar to the responses to interleukin-3 (Fig. 4A).

In long-term cultures, the 32D.WT cells proliferated transiently in medium containing G-CSF. The cells gradually lost viability after 4 to 6 days in the medium (Fig. 4B) and died after 12 to 14 days. In contrast, the 32D.1 and 32D.2 cells proliferated continuously and could be maintained in G-CSF–containing culture medium for at least one month.

When cultured in medium containing interleukin-3, the 32D.WT cells had morphologic features that were typical of those of immature myeloid cells, and 50 to 60 percent of the cells displayed weak myeloperoxidase staining. Despite the death of substantial numbers of cells after 8 to 12 days of culture in G-CSF–containing medium, the surviving 32D.WT cells exhibited morphologic features characteristic of terminal granulocytic maturation (Fig. 5A), and nearly all the cells showed strong myeloperoxidase staining. In striking contrast, G-CSF treatment of 32D.1 and 32D.2 cells induced neither morphologic changes indicative of granulocytic maturation nor an increase in the expression of myeloperoxidase protein as indicated by myeloperoxidase staining (data not shown).

**Effect of Mutant Receptors on Granulocytic Maturation Mediated by the Wild-Type G-CSF Receptor**

Because the leukemic cells from both patients expressed not only the mutated genes for the G-CSF receptor but also the normal alleles of the gene, we performed studies to determine whether the mutant receptors interfere with the function of the wild-type receptor. Two 32D.WT clones were transfected with pBabe-puro expression vector carrying the cDNAs of the mutant receptors from Patient 1 and Patient 2 or only empty pBabe-puro vector (the negative control). Expression of the wild-type and mutant G-CSF–receptor proteins in single clones was verified by Western blot analysis, and those that expressed approximately equal levels of the wild-type and mutant G-CSF–receptor proteins were examined. Transfection of 32D.WT cells with the empty vector did not alter G-CSF–induced proliferation and maturation (data not shown). The 32D.WT cells coexpressing the mu-
tant receptor from Patient 1 (32D.WT-1) or Patient 2 (32D.WT-2) had increased proliferative responses to G-CSF (Fig. 4A) and proliferated continuously in culture medium containing G-CSF alone (Fig. 4B). Both the 32D.WT-1 cells and the 32D.WT-2 cells were unable to mature terminally in response to G-CSF (Fig. 5B and 5C). In fact, the responses of the 32D.WT-1 cells and the 32D.WT-2 cells to G-CSF were indistinguishable from the response of the 32D.1 and 32D.2 cells.

**DISCUSSION**

In this study, we have detected point mutations in the gene for the G-CSF receptor in two patients with acute myeloid leukemia and histories of severe congenital neutropenia. The mutations truncate the C-terminal cytoplasmic region of the receptor that participates in the transduction of maturation signals. In one patient the mutation was already present in the neutropenic phase, before the progression to acute myeloid leukemia. When expressed in murine 32D.C10 cells, the truncated G-CSF receptors from the two patients transduced stronger proliferative signals than the wild-type receptor but were defective in inducing maturation. Moreover, the mutant receptors blocked granulocytic maturation even in the presence of wild-type G-CSF receptors, presumably by forming heterodimers with the wild-type receptors. Taken together, our data suggest that disruption of the maturation-signaling function of the G-CSF receptor contributes to leukemogenesis.

Not all patients with severe congenital neutropenia have mutations in the G-CSF receptor. No such mutations corresponding to the cytoplasmic domain were found in three patients with severe congenital neutropenia who were members of the Swedish families in which the disease was originally described (unpublished data). However, a point mutation in the gene for the G-CSF receptor, causing truncation of the C-terminal region, has been identified in a patient with severe congenital neutropenia and monosomy 7 but no signs of the myelodysplastic syndrome or acute myeloid leukemia (unpublished data). The mutation was detected in myeloid cells from this patient but not in B lymphocytes, indicating its acquisition by a committed progenitor cell. Thus far, we have found mutations in the gene for the G-CSF receptor in 4 of 14 patients with severe congenital neutropenia; in all 4, the mutation truncated the C-terminal region. Patients with severe congenital neutropenia and such truncated receptors may represent a subgroup of patients in whom the neutropenia is a preleukemic disorder.

Recombinant human G-CSF, now used frequently in the treatment of severe congenital neutropenia, can have favorable results. However, acute myeloid leukemia or the myelodysplastic syndrome has developed after the administration of G-CSF in patients with severe congenital neutropenia; in all 4, the mutation truncated the C-terminal region. Patients with severe congenital neutropenia and such truncated receptors may represent a subgroup of patients in whom the neutropenia is a preleukemic disorder.

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![Figure 4. G-CSF–Induced Proliferative Responses of 32D.C10 Clones Expressing Various G-CSF Receptors.](https://example.com/gcsf.png)

Panel A shows the response to G-CSF in comparison with the response to interleukin-3. DNA synthesis was determined by the uptake of tritium-labeled thymidine. Data are presented as the percentage of the maximal response to 10 ng of murine interleukin-3 per milliliter for each clone. Analogous results were obtained with at least three independent clones for each form of receptor. Panel B shows the level of cell proliferation induced by G-CSF, according to the numbers of days in culture. Cells were cultured in medium containing 10 ng of G-CSF per milliliter. The number of viable cells was determined on the basis of trypan-blue exclusion. 32D.WT denotes cells expressing the wild-type receptor, 32D.1 cells expressing the mutant receptor from Patient 1, 32D.2 cells expressing the mutant receptor from Patient 2, 32D.WT-1 cells expressing the wild-type receptor and mutant receptor from Patient 1, and 32D.WT-2 cells expressing the wild-type receptor and mutant receptor from Patient 2.
Figure 5. Morphologic Features of 32D.C10 Clones Expressing 492 THE NEW ENGLAND JOURNAL OF MEDICINE Aug. 24, 1995

The cells were cultured for 10 days in medium containing G-CSF (10 ng per milliliter). Panel A shows cells expressing the wild-type receptor (32D.WT), Panel B cells expressing the mutant receptor from Patient 1 (32D.WT-1), and Panel C cells expressing the wild-type receptor and the mutant receptor from Patient 2 (32D.WT-2). The 32D.WT cells matured into neutrophilic granulocytes in response to G-CSF, whereas the 32D.WT-1 and 32D.WT-2 cells retained a myeloblastic appearance.

and the contribution of G-CSF therapy to leukemogenesis.

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REFERENCES


