Monosomy 7 and Activating RAS Mutations Accompany Malignant Transformation in Patients With Congenital Neutropenia

By Ruby Kalra, David Dale, Melvin Freedman, Mary Ann Bonilla, Mark Weinblatt, Arnold Ganser, Paul Bowman, Sharon Abish, Jack Priest, Ronald S. Oseas, Kristin Olson, Dorothy Paderanga, and Kevin Shannon

While receiving rhG-CSF. The bone marrows of 10 patients showed monosomy 7 and 5 had activating RAS mutations. These abnormalities were not detected in pretreatment bone marrows and cessation of rhG-CSF was not associated with either clinical improvement or cytogenetic remission. We conclude that patients with severe forms of congenital neutropenia are at relatively high risk of developing MDS and AML. The occurrence of monosomy 7 and RAS mutations in these cases suggests that the myeloid progenitors of some patients are genetically predisposed to malignant transformation. The relationship between therapeutic rhG-CSF and leukemogenesis in patients with severe chronic neutropenia is unclear.

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Severe chronic neutropenia and recurrent serious infections are features of a heterogeneous group of disorders of myelopoiesis including congenital agranulocytosis (Kostmann's syndrome [KS]), cyclic neutropenia, and idiopathic congenital neutropenia. The bone marrow of affected patients typically show a paucity of mature myeloid cells. KS is a subtype of congenital neutropenia with onset in early childhood, profound neutropenia (absolute neutrophil count <200/μL), recurrent life-threatening infections, and a maturation arrest of myeloid precursors at the promyelocyte-myelocyte stage of differentiation. Until recently, most patients with KS died early in life from infections. Severe chronic neutropenia is also seen in the context of inherited bone marrow failure syndromes, including Fanconi's anemia, dyskeratosis congenita, and Shwachman-Diamond syndrome. Finally, a subset of patients with congenital neutropenia (CN) who cannot be classified as having KS show heterogeneous laboratory findings and an unpredictable clinical course.

The proliferation and terminal differentiation of hematopoietic cells is regulated by soluble proteins called hematopoietic growth factors. The genes that encode many of these cytokines have been cloned over the past decade and therapeutic quantities have been used in a variety of clinical settings. Recombinant human granulocyte colony-stimulating factor (rhG-CSF) enhances the production and function of mature neutrophils in both normal subjects and in patients with neutropenia. Administration of pharmacologic doses of rhG-CSF to patients with various forms of chronic neutropenia induces a marked increase in circulating neutrophil counts and is associated with both a significant reduction in serious infections and improved quality of life. Although the molecular basis of most cases of chronic neutropenia and the reasons that affected individuals show a therapeutic response to rhG-CSF are largely unknown, the available data are consistent with an intrinsic defect of immature myeloid cells rather than impaired G-CSF production.

Patients with a variety of inherited and acquired conditions, including occupational or medical exposure to mutagens, aplastic anemia, paroxysmal nocturnal hemoglobinuria, and Fanconi's anemia, are predisposed to preleukemic myelodysplastic syndromes (MDS) and acute myelogenous leukemia (AML). Molecular analysis has shown acquired mutations at codons 12, 13, or 61 of the KRAS or NRAS proto-oncogenes in 20% to 30% of adults and children with preleukemia and AML. In addition, the bone marrows of many patients with MDS and AML acquire nonrandom cytogenetic abnormalities, including specific chromosomal translocations, duplications, and deletions. Loss of all or part of chromosomes 5 and 7 are particularly common in cases of chronic neutropenia, while receiving rhG-CSF. The bone marrows of 10 patients showed monosomy 7 and 5 had activating RAS mutations. These abnormalities were not detected in pretreatment bone marrows and cessation of rhG-CSF was not associated with either clinical improvement or cytogenetic remission. We conclude that patients with severe forms of congenital neutropenia are at relatively high risk of developing MDS and AML. The occurrence of monosomy 7 and RAS mutations in these cases suggests that the myeloid progenitors of some patients are genetically predisposed to malignant transformation. The relationship between therapeutic rhG-CSF and leukemogenesis in patients with severe chronic neutropenia is unclear.

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of MDS and AML that develop in patients with known genetic or occupational predispositions. Although AML has been reported in 3 patients with KS who never received rhG-CSF, many affected children die during the first few years of life, and the association between KS and myeloid leukemia is incompletely defined.

We report on 13 patients with inherited disorders of myelopoiesis who developed MDS or AML while receiving rhG-CSF. Another patient with KS developed a clonal cytogenetic abnormality during rh-G-CSF therapy without clinical evidence of MDS or AML. The bone marrows of 10 patients showed complete or partial monosomy 7. We hypothesized that activating RAS mutations might contribute to leukemogenesis in this clinical setting and detected activating RAS mutations in 5 cases. We also compared bone marrow samples obtained before and after the start of rhG-CSF therapy to ask if monosity 7 and RAS mutations were acquired during treatment and present data that support this hypothesis.

MATERIALS AND METHODS

Patients and treatment. Patients were diagnosed with KS, CN, or Shwachman-Diamond syndrome on the basis of clinical criteria and bone marrow examination. All patients with chronic neutropenia had a history of recurrent infections, many of which were life-threatening. Thirteen patients participated in clinical trials of rhG-CSF. Another patient with KS developed a clonal cytogenetic abnormality during rh-G-CSF therapy without clinical evidence of MDS or AML. The bone marrows of 10 patients showed complete or partial monosomy 7. We hypothesized that activating RAS mutations might contribute to leukemogenesis in this clinical setting and detected activating RAS mutations in 5 cases. We also compared bone marrow samples obtained before and after the start of rhG-CSF therapy to ask if monosity 7 and RAS mutations were acquired during treatment and present data that support this hypothesis.

DNA extraction and polymerase chain reaction (PCR). The laboratory procedures were approved by the Committee for the Protection of Human Subjects at the University of California, San Francisco. DNA was extracted from bone marrow and blood cells, specimen smears on glass slides, and paraffin-embedded tissues, as previously described. DNA was suspended at a final concentration of 50 ng/mL in 10 mmol/L Tris-1 mM MgCl2. EDTA buffer for use in PCR experiments. DNA samples were amplified in a DNA Thermocycle Machine (Perkin-Elmer, Norwalk, CT) using reaction mixtures that include 10 pmol/L of respective 3′ and 5′ primers, 100 ng of target genomic DNA, 1 U of Taq polymerase (AmpliTaq: Cetus, Emeryville, CA), and 100 μmol/L final concentrations of deoxynucleotides in a final reaction volume of 25 μL. For SSPC, 1 μL of 32P-dATP or 32P-dCTP was incorporated into the PCR reaction mixture to label the fragments. Oligonucleotide primers described by Suzukin were used to amplify both exons of the NRAS (located on chromosome 12) and KRAS (on chromosome 3) fragments for single-strand conformational polymorphism (SSCP) and primers designed by van Munsfeld and Bos for allele-specific restriction enzyme digests. PCR conditions were as described previously.

SSCP. We performed SSCP as recommended by Suzukin with minor modifications. Briefly, gels are prepared from an 80 mL solution that includes 25 mL of an enhanced acrylamide solution (MDE; Hydronil Inc, Malvern, PA) mixed in 0.6X Tris:Borate: EDTA (TBE). We do not include glycerol in the gel mixture. Amplified RAS fragments are diluted in a solution of 0.5 mol/L NaOH, 10 mmol/L EDTA, and 90% deionized formamide and then denatured at 95°C for 5 minutes before loading. We pour 1-mm-thick gels in 20 × 40 cm plates. Gels are electrophoresed at 8 W of constant power at both room temperature and at 4°C for 16 hours followed by autoradiography.

Allele-specific restriction enzyme digests. This screening method is based on assays described by Jakobsen and Mills and van Munsfeld and Bos that take advantage of the fact that all known RAS mutations occur at one of three sites (codons 12, 13, and 61). The 5′ PCR primer is designed to introduce a single nucleotide substitution into the codon immediately preceding the codon of interest. This nucleotide mismatch creates a restriction site if the normal sequence is present at the codon of interest. Normal RAS fragments generated by PCR are cleaved with the appropriate restriction enzyme, whereas mutant products remain undigested. Mutant and wild-type fragments are then separated by electrophoresis for 45 minutes at 180 V of constant power in 10 cm × 11 cm gels containing 8% acrylamide and can be visualized by ethidium bromide staining.

Molecular cloning and sequencing of mutant RAS fragments. Fragments that appeared abnormal by either screening procedure were cloned and sequenced using a vector system that is available commercially (Clon A, Gibco BRL, Gaithersburg, MD). As described elsewhere, the sequence 5′ CUACUACUAUCA 3′ is added to 3′ end of the sense primers and 5′ CAUCAUCAUCAU 3′ is added to the 5′ end of the antisense primer. We enrich for mutant fragments by amplifying with 5′ primers that introduce the same restriction sites used in the allele-specific screening procedure. The PCR products are digested and then annealed to the proper vector in the presence of uracil DNA glycosylase. The annealed products are then transformed into DH5α competent cells, streaked, and grown overnight on ampicillin plates. Colonies are picked and grown up in 5 mL of L broth, and plasmid DNA is extracted using standard methods. A polyethylene glycol precipitation is then performed to further purify the DNA and double-stranded sequencing is performed using Sequenase, version 2.0 (US Biochemicals, Pittsburgh, PA).

PCR analysis of chromosome 7 sequence polymorphisms. Polymorphisms at the GCK, COL1A2, MET, and D7S23 (XVIIc) loci were detected by PCR, as previously described. Amplifications were performed in a final volume of 25 or 50 μL. The GCK1 and COL1A2 polymorphisms consist of short tandem nucleotide repeats that are cleaved by electrophoresis on 5% acrylamide DNA sequencing type gels followed by autoradiography. The primers used to type MET, CFTR, and D7S23 loci amplify short regions of DNA that surround known polymorphic restriction sites. The PCR products are cleaved with the appropriate restriction enzyme and the products are separated on 10 × 10 cm acrylamide gels, stained with ethidium bromide, and photographed under UV light.

RESULTS

Patient characteristics. Table 1 summarizes clinical and laboratory data from the 14 cases. The patients included 9 with KS, 3 with CN, and 2 with Shwachman-Diamond syndrome. Patient no. 3 showed a constellation of anomalies, including cyanotic congenital heart disease, hypospadias, microcephaly, growth failure, and mild mental retardation, and had episodes of neutropenia with irregular oscillations. All 13 patients with MDS or AML were receiving rhG-CSF when they developed a myeloid disorder. The mean duration of rhG-CSF treatment before the onset of MDS or AML was 47.7 ± 24 months and the mean of the most frequently used rhG-CSF dose for each patient during the entire course of...
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We detected the amino acid substitution in the mutant protein (ASP, aspartic acid).

Five patients who developed monosomy 7 had cytogenetic abnormalities or in association with other findings (Table 1). In patients no. 6, 7, and 10, monosomy 7 was identified.

**Table 1. Clinical and Laboratory Characteristics of Patients**

| Patient No./Sex | Primary Diagnosis | Secondary Diagnosis | Age When rhG-CSF Started | Months of rhG-CSF Therapy* | rhG-CSF Dose (μg/kg/d) | Cytogenetic Findings | RAS Mutation
<table>
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<tr>
<td>1/M</td>
<td>CN</td>
<td>MDS</td>
<td>44 yr</td>
<td>48</td>
<td>2.4</td>
<td>46,XY/45,XY,−7,del(6)(q21)</td>
<td>None</td>
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<tr>
<td>2/M</td>
<td>KS</td>
<td>AML</td>
<td>18 mo</td>
<td>18</td>
<td>30</td>
<td>46,XY/45,XY,−7,+11</td>
<td>K12ASP</td>
</tr>
<tr>
<td>3/M</td>
<td>CN</td>
<td>MDS</td>
<td>10 yr</td>
<td>24</td>
<td>5</td>
<td>46,XY/45,XY,−7,+21</td>
<td>K12ASP</td>
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<tr>
<td>4/M</td>
<td>KS</td>
<td>MDS</td>
<td>4 mo</td>
<td>11</td>
<td>10-15</td>
<td>45,XY,−7</td>
<td>None</td>
</tr>
<tr>
<td>5/M</td>
<td>CN</td>
<td>MDS, AML</td>
<td>20 yr</td>
<td>24</td>
<td>2.5</td>
<td>46,XY/45,XY,−7,+21/45,XY,−7,iso(17q)</td>
<td>None</td>
</tr>
<tr>
<td>6/F</td>
<td>KS</td>
<td>AML</td>
<td>11 mo</td>
<td>45</td>
<td>20</td>
<td>46,XX/45,XX,−7</td>
<td>N12ASP</td>
</tr>
<tr>
<td>7/F</td>
<td>KS</td>
<td>AML</td>
<td>7 yr</td>
<td>39</td>
<td>40</td>
<td>46,XX,−7,+t(12q20q)</td>
<td>N12ASP</td>
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<tr>
<td>8/F</td>
<td>KS</td>
<td>AML</td>
<td>11 mo</td>
<td>73</td>
<td>20</td>
<td>46,XX/47,XX,+mar</td>
<td>N12ASP</td>
</tr>
<tr>
<td>9/F</td>
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<td>AML</td>
<td>16 mo</td>
<td>44</td>
<td>12.5</td>
<td>48,XX,inv(16)(p13;q22)/46,XXt(5;6)(q31;q27)</td>
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<tr>
<td>10/M</td>
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<td>AML</td>
<td>6.2 yr</td>
<td>84</td>
<td>6.5</td>
<td>46,XX,der(11);t(16;9)(q15;q16);q34/47,XYadd(3q28)</td>
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<td>11/M</td>
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<td>MDS</td>
<td>12.9 yr</td>
<td>30</td>
<td>2.6-10</td>
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<td>5 yr</td>
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<td>MDS</td>
<td>7 yr</td>
<td>59+</td>
<td>5</td>
<td>46,XX/46,XX,t(7)(q22-q34)</td>
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</tr>
<tr>
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<td>MDS</td>
<td>17 mo</td>
<td>83+</td>
<td>15</td>
<td>46,XX/46,XX,t(16;13)(q21;q32),−7</td>
<td>ND</td>
</tr>
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**Abbreviations:** CYN, cyclic neutropenia; SDS, Shwachman-Diamond syndrome; ND, not done.

* Refers to the duration of treatment before the onset of MDS or AML.
† Refers to the dose of rhG-CSF required to maintain adequate neutrophil counts over time.
‡ Mutations are presented according to the RAS gene involved (NRAS, N; KRAS, K), the number of the codon affected (12, 13, or 81), and the amino acid substitution in the mutant protein (ASP, aspartic acid).

Treatment was 16.4 ± 14.6 μg/kg/d. Patients no. 13 and 14 are siblings with Shwachman-Diamond syndrome who developed monosomy 7 after 59 and 83 months of rhG-CSF therapy (Table 1). Patient no. 13 had normal bone marrow morphology when del(7)(q22-34) was detected in 5 of 20 cells. He progressed to MDS within a few months. His sister’s marrow showed mild dysplasia at the time monosomy 7 was identified.

**Cytogenetics.** Analysis of unstimulated bone marrow mononuclear cells from 11 patients (no. 1 through 7, 11, 13, and 14) showed monosomy 7 or del(7q) as either the only cytogenetic abnormality or in association with other findings (Table 1). In patients no. 1, 2, 3, and 13, monosomy 7 was detected on routine yearly aspirates before they had evidence of MDS or AML. In these cases, the number of cytogenetically abnormal cells initially seen was 25% to 100%. Patient no. 12 developed a clone with a t(X;5)(q13;q35) in 4 of 18 cells in March 1994, with normal peripheral blood counts and bone marrow morphology. This abnormality was not detected in a follow-up bone marrow study performed 6 months later (25 metaphases analyzed) and she remains well located on chromosome 7 to show that loss of constitutional heterozygosity occurred in the bone marrows of patients no. 4 and 7 during treatment with rhG-CSF (Fig 1).

**Acquisition of monosomy 7 during rhG-CSF therapy.** Five patients who developed monosomy 7 had cytogenetic studies performed before receiving rhG-CSF. The pretreatment karyotype was normal in unstimulated bone marrow obtained from patients no. 1, 9, 11, and 14, and monosomy 7 was not detected by fluorescent in situ hybridization in patient no. 3. In addition, we used polymorphic markers located on chromosome 7 to show that loss of constitutional heterozygosity occurred in the bone marrows of patients no. 4 and 7 during treatment with rhG-CSF (Fig 1).

**Activating RAS mutations.** As summarized in Table 1, we detected RAS mutations in the bone marrows of 5 patients. Four also had monosomy 7 (Table 1). In patients no. 2 and 3, DNA fragments amplified from exon 1 of KRAS showed abnormal migration on SSCP gels and were not cleaved in the allele-specific restriction enzyme assay for codon 12 (Fig 2). SSCP of NRAS exon 1 and the allele-specific assay for codon 12 mutations gave abnormal results in patients no. 6, 7, and 8. These 5 fragments were cloned and sequence analysis showed GGT (glycine) to GAT (aspartic acid) substitutions at codon 12 in all of them. DNA was available from bone marrow specimens obtained before the onset of MDS or AML in the patients with RAS mutations. We amplified these samples and examined them on SSCP gels. In every case, abnormal migration was only seen in the specimens collected after the onset of a myeloid disorder (Fig 3). These data provide strong evidence that RAS mutations arise de novo during the evolution from severe chronic neutropenia to myeloid leukemia.

**Clinical course of MDS and AML.** The fact that malignant myeloid disorders emerged during rhG-CSF therapy raised the possibility that the proliferation of the abnormal clones might be growth factor-dependent. However, cessation of rhG-CSF was not associated with either durable clinical improvements or cytogenetic remissions. All patients who were asymptomatic when monosomy 7 was first detected progressed to MDS or AML within a few months. In addition, the MDS evolved rapidly toward frank leukemia in patients no. 1, 4, and 5 (over 2 to 8.5 months).

**DISCUSSION**

Our data are disturbing for the families of patients with KS and CN and their physicians because they suggest that these disorders are associated with a significant risk of MDS and AML. Most of the patients in our series participated in clinical trials to test the safety and efficacy of rhG-CSF in
severe neutropenia. A preliminary analysis performed in mid-1994 showed 9 cases of MDS or AML among 185 patients with KS and CN (4.8%). Eight of these 9 patients are included in our series. In addition, we studied 5 patients from this cohort who recently developed MDS or AML; these new cases raise the incidence to 7.5%. No cases of MDS or AML have developed in 140 patients who received long-term treatment with rhG-CSF for other indications who were observed concurrently. The latter group included 74 patients classified as having idiopathic neutropenia, 48 with cyclic neutropenia, and 18 with other diagnoses. Continued observation of this large cohort will be essential to determine if the incidence of clonal disorders of myelopoiesis will continue to increase over time or will stabilize. The answer to this question will likely depend, in large part, on the underlying genetic and biochemical basis of these disorders and, in particular, on how the causative mutations affect the genetic stability of immature myeloid cells.

Laboratory studies of stromal and bone marrow mononuclear cells in patients with KS and CN suggest that the primary defect is at the level of the myeloid progenitor. In particular, these experiments have shown normal G-CSF levels, normal G-CSF production by marrow stromal cells ex vivo, a normal or increased number of G-CSF receptors on hematopoietic cells, and defective myeloid colony growth. The G-CSF receptor contains discrete cytoplasmic domains that transduce proliferative and differentiating signals. Dong et al found a mutation that removed the carboxy tail of the G-CSF receptor in a patient after he developed MDS. This truncated receptor retained the ability to stimulate cell growth, but deleted the differentiation domain. The same investigators recently reported carboxy terminal truncations of the G-CSF receptor gene in patients no. 5 and 11. In all cases, the G-CSF receptor mutation was restricted to the myeloid lineage and was not detected in DNA extracted from other tissues. These data indicate that G-CSF receptor mutations may be acquired during treatment with rhG-CSF and raise the possibility that these alter-

Fig 1. Loss of constitutional heterozygosity in malignant bone marrows at the COL1A2 locus on the long arm of chromosome 7. (A) Amplification of DNA from the parents of patient no. 4 (lanes 1 and 2) and from 2 bone marrow samples obtained from patient no. 4. The specimen studied in lane 3 was obtained before rhG-CSF treatment and the sample is lane 4 is from after he developed MDS. (B) Amplified bone marrow DNA samples from before (lane 5) and after (lane 6) the onset of AML in patient no. 7. The labels A, B, and C refer to alleles detected by PCR amplification.

Fig 2. SSCP analysis of bone marrow samples for abnormalities in exon 1 of KRAS. The samples in lanes 2 through 4 show abnormal migration. The fragment in lane 2 was amplified from the bone marrow of patient no. 2. The fragment in lane 3 was from patient no. 3. The fragment in lane 4 was amplified from a child with congenital amegakaryocytic thrombocytopenia who developed MDS. Arrows show the positions of normal fragments. The same abnormal band is visible in lanes 2 through 4 above the lower normal fragment and (faintly) below the upper normal fragment. Sequence analysis of cloned PCR products showed a G-to-A transition in the second position of codon 12 that changed glycine (GGT) to aspartic acid (GAT) in all 3 cases.
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Monosomy 7 was a specific recurring cytogenetic alteration in the leukemias of our patients. The high frequency of monosomy 7 in the malignant myeloid disorders that arise in children with Fanconi’s anemia or neurofibromatosis type 1 and its strong association with secondary MDS and AML implicate chromosome 7 loss as a secondary event in leukemogenesis. The idea is also consistent with data showing that the genetic predisposition to leukemia is not linked to chromosome 7 in siblings with the rare familial form of bone marrow monosomy 7,32,51. The strong association of monosomy 7 with the leukemias that arise in patients with inherited or acquired predispositions suggests a similar intrinsic susceptibility in some patients with KS and other forms of CN. Fanconi’s anemia is especially provocative in this regard because it is characterized by defective hematopoiesis, an increased risk of MDS and AML, and an excessive rate of DNA damage after exposure to bipolar alkylating agents.20

Deregulated signaling through Ras proteins plays a central role in the pathogenesis of MDS and AML and may occur by a number of different genetic mechanisms.23,24,52-55. The frequent observation of RAS alterations in sporadic MDS and AML supports the idea that the predisposition to myeloid leukemia seen in patients with KS and CN does not involve a novel oncogenic mechanism, but is due to an increased risk of acquiring common secondary mutations. This, in turn, suggests that the underlying defect(s) in some patients with inherited disorders of granulocyte production is not restricted to myeloid differentiation but affects the intrinsic genetic stability of immature myeloid cells. The finding of a transient clonal cytogenetic abnormality in the bone marrows of patient no. 12 without clinical evidence of MDS is also consistent with this idea. Although it is intriguing that all of the RAS mutations detected in our patients involved G-to-A transitions at the second position of codon 12, these alterations are not specific to patients with chronic neutropenia but represent the most frequent type of RAS mutation seen in de novo MDS and AML.46. It is of interest that we did not detect an RAS mutation in the bone marrows of the 2 patients with an acquired G-CSF receptor mutation. Exposure to G-CSF increases the percentage of Ras in the active, GTP-bound conformation and induces mitogen-activated protein (MAP) kinase activation in myeloid cell lines.44 If acquired G-CSF receptor mutations deregulate myeloid growth though a Ras-dependent mechanism, activating RAS mutations would be functionally redundant if they did not confer any further proliferative advantage. Additional studies are required to establish the relative roles of G-CSF receptor and RAS mutations in leukemogenesis and to address the biochemical consequences of these alterations.

The relationship between rhG-CSF treatment and the development of malignant myeloid disorders is unclear. Alter and Young9 performed a literature review that included more than 100 cases of KS. The overall prognosis was poor, with a median survival of 3 years.7 These investigators identified 2 cases of AML,28,29 and another patient with KS who developed AML before receiving rhG-CSF was reported re-

Fig 3. SSCP of fragments amplified from bone marrows obtained from 2 patients before and after the development of MDS or AML. SSCP of NRAS exon 1 in patients no. 7 (lanes 1 and 2) and 8 (lanes 3 and 4). Arrows show the positions of normal fragments. The pretreatment sample from patient no. 7 (lane 1) shows a normal pattern of migration, whereas amplification of DNA prepared from bone marrow obtained after the onset of AML (lane 2) shows an aberrant fragment (asterisk). Similarly, a fragment showing abnormal migration is present in the leukemic bone marrow of patient no. 8 (lane 4), but not in a previous sample obtained when the patient had neutropenia (lane 3). DNA sequence analysis confirmed GGT-to-GAT substitutions at NRAS codon 12 in the samples amplified in lanes 2 and 4.
cently. 30 Interestingly, all 3 patients developed leukemia during the second decade of life. 28-30 By preventing early deaths from infection, rhG-CSF may allow time for later expression of the leukemic potential in patients with CN and KS. The absence of malignant myeloid disorders in patients with either cyclic neutropenia or idiopathic neutropenia maintained on chronic rhG-CSF 31 also supports the hypothesis that the risk of leukemic transformation is a function of the underlying myelopoeitic defect rather than a direct effect of growth factor treatment. We recently investigated an unusual child with bone marrow failure who never received rhG-CSF yet developed MDS with both monosomy 7 and an activating RAS mutation (Fig 2). She presented with congenital amegakaryocytic thrombocytopenia during infancy, progressed to marrow aplasia with peripheral pancytopenia by 2 years of age, and then evolved to MDS 1 year later.

Although these data suggest that rhG-CSF therapy does not play a direct role in leukemogenesis, they do not exclude the possibility that treatment accelerates the onset of leukemia. It is worrisome that 2 patients in our series who survived recurrent infections for more than 20 years developed MDS or AML 23 and 48 months after starting rhG-CSF treatment (Table 1). An important role of apoptosis (programmed cell death) in regulating the growth of normal hematopoietic cells has recently become apparent. Erythropoietin stimulates erythropoiesis, in part, by suppressing apoptosis in erythroid progenitors, 37,58 and interleukin-6 and G-CSF inhibit apoptosis in some murine myeloid leukemia cell lines. 39,60 By analogy, it is possible that pharmacologic doses of rhG-CSF might contribute to the development of leukemia by rescuing genetically defective myeloid progenitors from apoptosis.

Our findings have significant implications for both clinical practice and laboratory research. Patients affected with severe neutropenia have benefited greatly from rhG-CSF. For example, patient no. 7 had more than 40 hospital admissions and many documented severe infections before starting rhG-CSF at 7 years of age. We continue to recommend that patients with a well-documented history of severe chronic neutropenia and recurrent serious infections receive rhG-CSF. We suggest cytogenetic analysis of unstimulated bone marrow and cryopreservation of an aliquot of cells when treatment begins. Patients receiving rhG-CSF should be maintained on the lowest possible dose and should be observed closely for clinical evidence of MDS or AML. Careful follow-up of a large cohort of patients with severe chronic neutropenia is planned through the establishment of an International Registry that aims to enroll patients regardless of treatment. This will provide essential information about the risk of leukemic transformation in specific disorders of granulocyte production over time. Elucidating the cellular and molecular basis of KS and CN may identify a subset of mutant alleles that strongly predispose to myeloid leukemia and could provide a rational basis for assessing the risks and benefits of rhG-CSF in individual patients.

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REFERENCES

17. Hestdal K, Welte K, Lie SO, Keller JR, Russett FW, Abrahamsen TG: Severe congenital neutropenia: Abnormal growth and differentiation of myeloid progenitors to granulocyte colony-stimu-
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lating factor (G-CSF) but normal responses to G-CSF plus stem cell factor. Blood 82:2991, 1993
37. Van Mansfeld AD, Bos JL: PCR-based approaches for detection of mutated ras genes. PCR Methods Applications 1:211, 1992


60. Lotem J, Sachs L: Hematopoietic cytokines inhibit apoptosis induced by transforming growth factor β1 and cancer chemotherapy compounds in myeloid leukemic cells. Blood 80:1750, 1992
Monosomy 7 and activating RAS mutations accompany malignant transformation in patients with congenital neutropenia

R Kalra, D Dale, M Freedman, MA Bonilla, M Weinblatt, A Ganser, P Bowman, S Abish, J Priest and RS Oseas