Granulocyte Colony-Stimulating Factor (G-CSF) Production and G-CSF Receptor Structure in Patients With Congenital Neutropenia

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Congenital neutropenia (Kostmann's syndrome [KS]) is an autosomal recessive syndrome that is characterized by profound neutropenia, resulting in major clinical infections and death. Since the neutropenia and symptoms in KS improve in response to exogenous administration of granulocyte colony-stimulating factor (G-CSF), we studied bone marrow cytokine (G-CSF, granulocyte-macrophage CSF [GM-CSF], and interleukin-6) production under both basal and stimulated conditions. No differences in G-CSF, GM-CSF, or IL-6 gene expression were found in bone marrow stromal cells between normal controls and KS patients, and all three cytokines were detected by enzyme-linked immunosorbent assay (ELISA) in medium conditioned by bone marrow stromal cells from normal donors and patients with KS. Each KS patient tested had detectable, functional G-CSF in their own serum before exogenous G-CSF administration. Since G-CSF production appeared normal in KS patients, we then asked whether we could detect structural defects in the signaling portion of G-CSF receptor genes. Polymerase chain reaction (PCR) amplification of the G-CSF receptor transmembrane region alone, and of the transmembrane plus cytosolic portions of the receptor, yielded the size products predicted from the sequences of the normal G-CSF receptor. Single-strand conformational polymorphism (SSCP) analysis of G-CSF receptor PCR products demonstrated no variance in structural conformation between KS patients and normal subjects. These results demonstrate that bone marrow stromal cells in patients with KS secrete normal concentrations of functional G-CSF and suggest that the neutropenia in KS patients is caused by an inability of neutrophil progenitor and precursor cells to respond to normal, physiologic levels of G-CSF. Such a defect, clinically responsive to pharmacologic doses of G-CSF, might be caused by defects in the post-G-CSF receptor signal transduction pathway.

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Blood and bone marrow samples. Blood or bone marrow samples were obtained from patients who met the diagnostic criteria for KS from diagnostic or therapeutic procedures or from paid, normal volunteers after informed consent under a protocol approved by the University of Michigan Human Investigations Committee. Serum was separated from blood cells by centrifugation in a plug tube. Adherent bone marrow stromal cell preparation. Adherent bone marrow mononuclear cells were separated by density centrifugation over Ficoll/Hypaque, then grown to confluence on plastic in a modified Dulbecco's medium (MGM; Gibco-BRL Laboratories, Grand Island, NY) with 10% fetal calf serum (FCS).

MATERIALS AND METHODS

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Hyclone, Logan, UT) and 10% equine serum (Hyclone) at 37°C. Adherent stromal cell monolayers were maintained by biweekly exchange of 50% of the culture medium, and then trypsinized and passaged at approximately 80% confluence. In some experiments, 10 x 10^6 nonadherent bone marrow mononuclear cells from KS patients were added either to an autologous adherent bone marrow stromal monolayer or to an adherent bone marrow stromal monolayer from a normal allogeneic marrow donor. In selected cultures, either GM-CSF (20 ng/mL; Genzyme, Boston, MA) or G-CSF (200 U/mL; AMGEN Biologicals, Thousand Oaks, CA) were added as well. Nonadherent cells removed during feeding were either used in cytosin preparations or for methylycellulose culture.

Cytospin preparation. A maximum of 2 x 10^5 cells were resuspended in FCS and cytospres prepared by centrifuging the cells onto glass slides for 15 minutes at 600 rpm using a cytcentrifuge (Shandon, Pittsburgh, PA).

Methylycellulose cultures. A maximum of 2 x 10^5 cells/mL were cultured in methylcellulose in the presence of 2 U erythropoietin and either 20 ng/mL GM-CSF or 200 U/mL GM-CSF. All cytokines were obtained from R & D Systems, Minneapolis, MN.

Preparation of conditioned media and ELISA. Primary adherent confluent stromal layers were cultured in IMDM alone for 96 hours. At that time, aliquots were stimulated with 10 ng/mL IL-1α for 72 hours and conditioned. Conditioned media (CM) were assayed by ELISA using the double-antibody sandwich method using commercially available kits: G-CSF (AMGEN), GM-CSF (Genzyme), IL-6 (R & D Systems). Based on parallel assays of known diluted standards, the sensitivities of the assays were as follows: G-CSF, 40 to 5,000 pg/mL; GM-CSF, 2 to 500 pg/mL; IL-6, 30 to 2,000 pg/mL. Similarly, G-CSF levels in patient and control serum samples were determined by ELISA, and in the case of the patients, both before and after administration of recombinant human (rh)G-CSF (AMGEN).

Hematopoietic cell proliferation assay. To test whether the G-CSF produced by the Kostmann stromal cells could induce cellular proliferation, the murine IL-3- or human G-CSF-dependent myeloblastic cell line NFS-60 (a generous gift of Dr. James Ihle, St. Jude's, Memphis, TN) was grown in RPMI-1640 (GIBCO-BRL, Gaithersburg, MD) with 10% FCS and 25% WEHI-3 CM (a source of IL-3). When the cells were in exponential growth, they were washed three times to remove all WEHI-3 CM. The cells were plated in a 96-well plate at a concentration of 4 x 10^3 cells in a final volume of 100 μL RPMI with 10% FCS. To quadruplicate wells, one of the following was added: rh-G-CSF, 25% WEHI-3 CM, or CM from IL-1α-stimulated normal or Kostmann stromal cells. The cells were incubated for 37°C under 5% CO₂, and then 1 μCi 3H-dTr was added to each microwell. Following 6 more hours, the wells were harvested on a cell harvester (PHYD, Cambridge, MA), and thymidine incorporation into newly synthesized DNA was assayed by scintillation counting.

RNA preparation. Total cellular RNA was isolated from normal or Kostmann bone marrow mononuclear cells, separated from whole bone marrow by density centrifugation, using the guanidinium isothiocyanate method. Briefly, the cells were injected into guanidinium isothiocyanate, vortexed, and the RNA separated over a cesium density gradient. The RNA pellet was then subjected to a phenol/chloroform extraction, and precipitated in ethanol. The purified RNA was resuspended in water. Alternatively, after appropriate purification, total cellular RNA was isolated by direct precipitation from GITC solution. Cells were suspended in GITC solution (final concentrations are 4 mol/L guanidine isothiocyanate, 0.5% sarcosyl, 25 mmol/L citric acid, pH 7.0, and 0.007% β-mercaptoethanol), then sequentially mixed with 0.2 mol/L sodium acetate, pH 4.0, 1 vol phenol, 1/10 vol 49:1 chloroform:isoamyl alcohol, then cooled on ice for 15 minutes. The aqueous layer was extracted and precipitated at -20°C in isopropanol. The RNA was precipitated in equal volumes of GITC solution and isopropanol, dried, and resuspended in RNase-free water.

Oligonucleotide primers. Sense and antisense primers were prepared in the oligonucleotide synthesis core at the University of Michigan or at the University of Arkansas for Medical Sciences. The primers used were as follows: GM-CSF, (nucleotides 100 to 124, sense) GAGCATGTGAATGCCATCCAGGAG and (nucleotides 415 to 442, antisense) CTCTCGAGATTGCCTCCACGA-GTCAAA22, abl, (nucleotides -150 to -120, sense) CGAGCC-GGGCCTGAGCCGGCCCCAGCA and (nucleotides 63 to 100, antisense) TCACGTGTCACAGGAAAGTTTCC-TTGGAGTT24, G-CSF, (nucleotides 95 to 115, sense) CAGACT- GCACTCTGAGACGTGCAGGA and (nucleotides 480 to 510, antisense) CACTCCCCGTTTCTTCCATGCTGCAGAT25, IL-6, (nucleotides 26 to 52, sense) GGATGTTGCTCCCTCACAGCGCTTCGGTCCA and (nucleotides 421 to 450, antisense) AAGCCTGGTCTCCACTCTCATCCTAAGTCGCT. The primers used for the transmembrane and intracytosolic portions of the G-CSF receptor were as follows: transmembrane, (nucleotides 1965 to 1989, sense) ATGGCTGCCAGGCGGCTTGGGCC and (nucleotides 2165 to 2189, antisense) GGCCACCCAGGAGGGCCAGGCTGCT; intracytosolic, (nucleotides 2310 to 2331, sense) GAGACCTGTGCTCCCTCCACGTC, (nucleotides 2312 to 2333, antisense) CAGAGTTGGGAGGCAACAGTGC, and (nucleotides 2642 to 2675, antisense) CTAAGGCTTCCCCAGCGCTTC-CATC.27,28

Reverse-transcriptase PCR. DNA 1.0 μg 10x reverse transcriptase (RT) buffer (1X RT buffer contains 50 mmol/L Tris, pH 8.3, 50 mmol/L KCl, 8.0 mmol/L MgCl₂ and 10 mmol/L dithiothreitol), 25 mmol/L dXTP mix (25 mmol/L of each dXTP [ACGT]), 3.0 μg oligo dT), and 2.5 μRT (AMV Reverse Transcriptase; Gibco-BRL) were mixed in a 50-μL reaction and incubated at 41°C for 1 hour. One-fifth of the double-stranded product was mixed with 10X Taq/RT buffer (1X Taq/RT buffer consists of 10 mmol/L Tris, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.01% gelatin, and 2.0 mmol/L dithiothreitol), 1 mmol/L dXTP mix, 500 ng each of the sense and antisense oligonucleotides of interest, and 2.5 U Taq polymerase (AmpliTaq DNA polymerase; PerkiElmer Cetus, Norwalk, CT). The reaction mixture underwent thermal cycling at 94°C for 1 minute and 72°C for 3 minutes for 35 cycles, then finally a 10-minute extension at 72°C (Perkin Elmer Cetus DNA Thermal Cycler). Analysis of PCR products was performed by agarose gel electrophoresis.

SSCP assay. From the cDNA solution, prepared by reverse transcription of RNA as described above, 4 μL was mixed with 2 mL each of the four deoxyxynucleotides, 10X Taq/RT buffer, 1 μL of 100P-dCTP (3,000 Ci/mm, Dupont, Boston, MA), 10 pmol of each sense and antisense primer of interest, and 2.5 U Taq polymerase (Perkin Elmer Cetus). The reaction mixture underwent thermal cycling as described above, using sets of PCR primers that amplified either a 369-bp region spanning the transmembrane region and the submembranous cytosolic receptor domain or a 366-bp region spanning the terminal portion of the receptor. To ensure that conformational changes resulting from a single base pair mutation could be detected, restriction enzyme digestion of the PCR products was performed to produce products less than 300 bp. The digestion consisted of 2 μL of PCR product, 1 μL 10% bovine serum albumin, 1 μL restriction buffer D (Promega, Madison, WI), 6 mmol/L Tris/HCl, 6 mmol/L MgCl₂, 150 mmol/L NaCl, 1 mmol/L DTT, pH 7.0, 0.5 mL BSTX 1 (Promega), and incubated at 50°C overnight. One microliter of the undigested PCR mixture was mixed with 100 μL of 0.1% sodium dodecyl sulfate (SDS) and 10 mmol/L EDTA.
Alternatively, the 10-μL digest was mixed with 90 mL of the SDS/EDTA diluent. Two microliters of diluted product was mixed with 2 μL of 95% formamide, 20 mmol/L EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol and denatured at 80°C for 7 minutes. Two microliters of the denatured product was applied to a 5% polyacrylamide gel containing 90 mmol/L Tris-borate, pH 8.3, 4 mmol/L EDTA, and, in some cases, 10% glycerol. The gels were run for 3 hours at 30 watts at room temperature (with glycerol) or at 4°C (without glycerol). The gel was dried on filter paper and exposed to x-ray film at −80°C.

RESULTS

To evaluate the possibility that bone marrow stromal cells were defective in their ability to produce HGFs regulating neutrophoiesis, multiply passaged bone marrow stromal fibroblasts were evaluated for cytokine gene induction and cytokine production. In the absence of stimulation with IL-1α, KS stromal cells constitutively expressed message for IL-6, but did not transcribe message for G-CSF, and expressed little or no message for GM-CSF. The gene for GM-CSF was expressed either at very low levels or not at all. In the presence of IL-1α, all three cytokine genes were expressed (Fig 1). While these studies were not strictly quantitative, the results obtained were qualitatively indistinguishable from those obtained with passaged bone marrow fibroblasts from normal donors, as reported previously. We next asked whether we could detect normal levels of G-CSF, GM-CSF, and IL-6 secreted by primary cultured stromal monolayers. In these studies, ELISAs were performed on CM collected at 24 hours from stromal cells cultured in the presence and absence of IL-1α. While production of G-CSF under unstimulated conditions varied among five KS patients' monolayers, in each case IL-1α-stimulated stromal cells produced G-CSF at concentrations comparable to concentrations produced by normal bone marrow stromal monolayers (Fig 2). Similarly, no differences were found in the levels of constitutive or IL-1α-induced IL-6 or GM-CSF secretion between KS and normal bone marrow stromal monolayers (data not shown).

Although these data demonstrated that bone marrow stromal cells from KS patients clearly were capable of secreting G-CSF in vitro, it was theoretically possible that for unknown reasons G-CSF was not produced in these patients in vivo. We therefore performed G-CSF ELISAs on patient serum samples obtained before and after exogenous G-CSF therapy. In all pretreatment samples, KS patients demonstrated normal to slightly elevated G-CSF levels as compared with normal controls (0 to 100 pg/mL) (Fig 3). Not surprisingly, following in vivo treatment with G-CSF, patients' serum G-CSF levels were found to be in the nanomolar range.

To determine whether the G-CSF protein detected immunologically by ELISA in KS bone marrow stromal cell CM induced cellular proliferation, the murine myeloblastic cell line NFS-60 was grown in the presence of CM from IL-1α-stimulated Kostmann stromal cells. The NFS-60 cell line was chosen because it is absolutely dependent on mu-
rinue IL-3 or human G-CSF for proliferation. In addition, it does not cross-react or proliferate in response to GM-CSF. In the present study, IL-1α-stimulated KS stromal cell CM stimulated NFS-60 cell proliferation in the same manner as native human rhG-CSF (Fig 4). Therefore, the G-CSF produced by stromal cells from KS patients induces normal cellular proliferation.

Taken together, these studies suggested that the production of secreted G-CSF in KS patients was normal. We wondered whether KS bone marrow might display a defect in the local presentation of the G-CSF molecule to myeloid cells in the bone marrow microenvironment. To address this question, we asked whether the failure of neutrophilic maturation could be corrected by the presence of normal bone marrow stromal cells in vitro. Specifically we evaluated the degree of neutrophilic maturation achieved by KS bone marrow mononuclear cells when cultured on autologous or allogeneic stromal cells. For both autologous and allogeneic stroma, the bone marrow mononuclear cells were grown on stroma alone, or in the presence of either stroma and GM-CSF or stroma and G-CSF, for an interval of 4 to 6 weeks. Each week, methylcellulose cultures and cytospins were prepared from the nonadherent cells on the stromal layers. No differences in either survival or degree of differentiation of the KS bone marrow mononuclear cells were found between autologous and allogeneic stromal layers. Importantly, neutrophilic differentiation remained left-shifted, with a predominance of early myeloid forms, even on the allogeneic layer (Fig 5). In addition, we found that adding GM-CSF or G-CSF to the culture flasks did not increase the percentage of nonadherent cells that developed into mature neutrophils (data not shown). These data confirm that the KS maturation defect could not be corrected by normal stromal cells, and therefore appeared to be localized to the differentiating myeloid cells themselves.

If KS myeloid cells were defective in their response to physiologic doses of G-CSF, the molecular locus of the defect could be at either the G-CSF binding or signal transduction domains of the G-CSF receptor, or at downstream sites in the G-CSF signal transduction pathway. Since studies by Kyas et al.19 have shown that neutrophils from KS patients display normal to increased numbers of G-CSF receptor numbers, with apparently normal CSF affinities, we asked whether the nonbinding domains of the G-CSF receptor might be structurally abnormal. RNA was prepared from unstimulated whole bone marrow cells, and segments of the transmembrane and cytosolic portions of the G-CSF receptor were amplified by PCR. Message of the expected size was easily detected in both normal and KS samples for both the transmembrane and cytosolic portions of the G-CSF receptor (Fig 6).

Since a point or nonsense mutation could conserve the correct length of the transcript while altering the function of the G-CSF receptor, the structure of the G-CSF receptor cDNA in KS patients was analyzed for conformational polymorphisms by SSCP. In these assays, changes in three-dimensional conformation induced by point mutations would be detected by altered mobility in the gel. However, this analysis displayed indistinguishable patterns of mobility of the amplified products between normal and KS G-CSF receptor transmembrane and cytoplasmic domains (Fig 7).

DISCUSSION

KS is characterized by profound neutropenia and a neutrophilic maturation defect in the bone marrow, both of

![Fig 4. G-CSF produced by KS stromal cells induces proliferation of the G-CSF-dependent myeloblastic cell line NFS-60. NFS-60 cells in exponential growth were plated in the presence of either rhG-CSF or IL-1α-stimulated KS stromal cells. The cells were incubated for 18 hours, then pulsed for 6 hours with 1 μCi tritiated thymidine. (*) CPM derived from tritiated thymidine incorporation into NFS-60 cells stimulated by known rhG-CSF standards; (f) CPM for IL-1α-stimulated KS stromal cells. Data represent the mean of quadruplicate samples of tritiated thymidine incorporation of proliferating cells.](www.bloodjournal.org by guest on October 23, 2017. For personal use only.)
which are ameliorated in response to pharmacologic doses of exogenous G-CSF. To understand the basis for the disease and its response to pharmacologic therapy, we analyzed G-CSF bone marrow physiology at several levels. First, we found that KS bone marrow stromal cells produce normal to slightly elevated levels of G-CSF, and that this G-CSF appears to stimulate G-CSF-responsive cells to proliferate. In addition, GM-CSF and IL-6 all are detectable as both the message and protein at levels comparable to those found in normal control stromal cells and supernatants. Furthermore, support of KS progenitor cells on both autologous and allogeneic normal stromal cells leads to no difference in differentiation or survival. These data suggest that the neutropenia of KS is not the result of a stromal cell-derived growth factor deficiency. The present data therefore confirm and extend the observations of Mempel et al, who found detectable levels of biologically active G-CSF protein in the serum of children with KS as well.

Taken together, the accumulated data suggested that the deficiency in KS was more likely due to a defect in the ability of the myeloid target cells to respond to cytokine signals. Kyas et al had demonstrated that the number and affinity of G-CSF receptors was normal in KS patients, suggesting that both the number and structure of G-CSF receptor extracellular domains is normal in these patients. We therefore wondered whether we could detect transmembrane or cytosolic G-CSF receptor cDNAs of abnormal size or conformation in KS patients. However, our evaluation of the transmembrane and cytosolic portions of the G-CSF receptor by PCR and SSCP demonstrated no abnormalities in the expected size or conformation in any of the patients studied. In these experiments, the transmembrane product amplified was 225 bp and the transmembrane plus cytosolic product was 711 bp. Since this latter product is too large to analyze by SSCP, which optimally requires products of less than 300 bp, new primers were prepared that divided the entire transmembrane-cytosolic sequence into two products of approximately 350 bp each. This sequence was scanned and a restriction site for BSTXI was found midway in each product. Polyacrylamide gel electrophoresis of the digested PCR products demonstrated no conformational changes in the receptor. Since products of this size should be expected to demonstrate conformational variation in 97% of cases, we believe that it is unlikely that a mutation of the transmembrane or cytosolic portions of the G-CSF receptor exists in KS cells, at least in the majority of KS patients.

These present data are of interest in light of the findings of both the clinical response of most KS patients to pharmacologic doses of G-CSF and the data of Kyas et al finding normal to increased numbers and normal affinities of G-CSF receptors. Although the KS patients most often do respond to G-CSF in vivo, the doses required are often much higher than needed to stimulate myelopoiesis in normal individuals. Furthermore, neutrophils from some patients with KS following treatment with G-CSF have a reduced ability to generate superoxide following exposure to phorbol myristate acetate. Thus, the G-CSF target cells appear to be relatively insensitive to G-CSF stimulation. Al-

Fig 5. In vitro differentiation of KS nonadherent bone marrow cells on autologous and normal, allogeneic adherent monolayers. Cytospins of nonadherent KS bone marrow mononuclear cells supported either by autologous or normal, allogeneic Dexter layers were prepared weekly. Percentages represent the average of two differential cytospin counts, prepared from two different Dexter layers. Note that lack of maturation of KS myeloid elements was not corrected by a normal allogeneic stromal layer.

Fig 6. RT PCR from RNA prepared from KS bone marrow mononuclear cells demonstrates that the transmembrane (225 bp, A) and cytosolic (711 bp, B) domains of the G-CSF receptor are of the expected sizes.
though the molecular basis for this phenomenon is not clear, either a defect in the G-CSF signal transduction pathway itself, or in a costimulatory pathway required for CFU-GM maturation on stimulation with G-CSF, would seem to be most likely. Further studies will therefore need to focus on such pathways, eg, the class of GTP-binding proteins associated with ligand receptor complexes that have been found in neutrophils,\textsuperscript{34,36} phosphotyrosine alterations, or pathways of phospholipase C and D metabolism that have been associated with neutrophil activation.\textsuperscript{37,38} On the other hand, rare patients with KS fail to increase their circulating neutrophil counts in response to pharmacologic doses of G-CSF. Unlike the patients analyzed in the present study, bone marrow cells from truly G-CSF-resistant KS patients might indeed have a structural abnormality in their G-CSF receptors.

In summary, we have found that patients with congenital neutropenia appear to produce normal amounts of G-CSF both in vitro and in vivo. However, bone marrow mononuclear cells in KS patients appear unable to differentiate normally in response to these normal levels of produced G-CSF. Analyses of the size of the G-CSF receptor and the conformation of the receptor cDNA in patient bone marrow mononuclear cells indicate no obvious structural abnormality in the receptor per se. Therefore, we hypothesize that the defect in most patients with KS resides downstream from the G-CSF receptor, in its signal transduction pathway or in the pathway of a requisite costimulatory molecule required for G-CSF-induced neutrophil differentiation.

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