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 neutrophilic 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.

Congenital neutropenia is characterized by profound neutropenia and recurrent severe infections. When inherited in an autosomal recessive fashion, the syndrome has been termed Kostmann’s syndrome (KS). Bone marrow aspirates in KS patients demonstrate normal erythroid and megakaryocytic maturation. However, myeloid maturation is markedly deficient, with a majority of cells arrested at the promyelocytic stage of development. Although the cause of KS is not known, the nature of the defect suggests that the key defect could lie at the level of a hematopoietic growth factor (HGF) or HGF receptor. Interleukin (IL)-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte CSF (G-CSF), and IL-6 all have the ability to regulate neutrophil differentiation. IL-3 and/or GM-CSF are required for multilineage hematopoietic cell differentiation, and G-CSF exhibits in vitro synergy with IL-3, IL-6, and c-kit ligand. In addition, G-CSF and GM-CSF stimulate the differentiation and activation of committed neutrophilic precursors. Thus, deficiencies or abnormalities in the function of any of these growth factors could be expected to be manifested by decreased neutrophil production or function. Given the severe lack of neutrophil differentiation in the bone marrow in KS, and given the lack of involvement of other hematopoietic lineages, a role for G-CSF seems likely and has been hypothesized.

Both GM-CSF and G-CSF have been used in the clinical management of KS patients. GM-CSF has had variable effects on neutrophils in these patients, and has also resulted in a profound concomitant eosinophilia not observed in non-KS patients receiving GM-CSF. G-CSF, on the other hand, has been found to correct nearly all KS patients’ neutropenia with few side effects, resulting in fewer clinical infections. Given these data, we investigated the molecular basis of the clinical phenotype seen in KS patients, and in particular we hypothesized a deficiency in G-CSF or its receptor.

To test for a deficiency in cytokine production, production of message and protein for G-CSF, GM-CSF, and IL-6 by bone marrow stromal cells was assessed by polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA). The ability of G-CSF produced by bone marrow stromal cells from KS patients to induce cellular proliferation was also measured. In addition, since binding studies of G-CSF to the G-CSF receptor have been reported to be normal, we analyzed the transmembrane and cytosolic portions of the G-CSF receptor by PCR and single-strand conformational polymorphism (SSCP) analysis.

MATERIALS AND METHODS

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 Iscove’s modified Dulbecco’s medium (IMDM; GIBCO-BRL Laboratories, Grand Island, NY) with 10% fetal calf serum (FCS;...
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 cyto centrifuge preparations or for methylenumelose culture.

**Cyto centrifugation.** A maximum of 2 x 10^6 cells were suspended in FCS and cytopreps prepared by centrifuging the cells onto glass slides for 15 minutes at 600 rpm using a cyto centrifuge (Shandon, Pittsburgh, PA).

**Methylcellulose cultures.** A maximum of 2 x 10^5 cells/mL were cultured in methylcellulose culture in the presence of 2 U erythropoietin and either 20 ng/mL GM-CSF or 200 U/mL G-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 could be detected, restriction enzyme digestion of PCR products could be detected, and the RNA was synthesized as described above, 4 µL was mixed with 2 µL of each of the four deoxynucleotides, 10X Taq/RT buffer, 1 µL of 32P-dCTP (3,000 Ci/mm, Dupont, Boston, MA), 10 pmol of each sense and antisense primer of interest, and 2.5 U Taq polymerase (Promega). 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. 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 µL each of the four deoxynucleotides, 10X Taq/RT buffer, 1 µL of 32P-dCTP (3,000 Ci/mm, Dupont, Boston, MA), 10 pmol of each sense and antisense primer of interest, and 2.5 U Taq polymerase (Promega Elmer 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. The reaction mixture was then added to a single base pair mixture 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 of 10% bovine serum albumin, 1 µL restriction buffer D (Promega, Madison, WI), 6 µmol/L Tris (pH 8.3), 6 µmol/L MgCl2, 150 µmol/L NaCl, 1 µmol/L DTT, pH 7.0, 0.5 µL 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 µmol/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% bromophenol 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).

![Figure 1](image1.png)

**Fig 1.** Expression of G-CSF, GM-CSF, and IL-6 mRNA in passaged adherent bone marrow stromal cells from patients with KS. Stromal cell monolayers were cultured in the presence or absence of IL-1α for 6 hours. The layers were killed, RNA prepared, and the message for specific cytokines analyzed by RT PCR. RNA from stromal cell monolayers from normal bone marrow donors cultured in the presence of IL-1α for 6 hours are shown as positive controls for the G-CSF, GM-CSF, and IL-6 RT PCR signals (+C), and samples without added RNA amplified in parallel with the experimental samples are shown as negative controls (−C).

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-
rime 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 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
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 BSTX I 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-
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, \(^{34,35}\) phosphotyrosine alterations, or pathways of phospholipase C and D metabolism that have been associated with neutrophil activation. \(^{37,38}\) 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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REFERENCES


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