Serum Granulocyte Colony-Stimulating Factor Levels in Healthy Volunteers and Patients With Various Disorders as Estimated by Enzyme Immunoassay

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In order to better understand the patho-physiologic role of granulocyte colony-stimulating factor (G-CSF), we estimated its serum levels in healthy persons and patients with various disorders, using a newly developed enzyme immunoassay (Motojima et al.). In 49 of 56 normal healthy persons (88%), the levels were beneath the sensitivity of the assay (<30 pg/mL), while in the remaining seven healthy persons, the levels ranged from 33 to 163 pg/mL. On the other hand, nine of 11 patients (82%) with chronic lymphoid leukemia (CLL), one patient with Fanconi’s anemia, six of 12 patients (50%) with myelodysplastic syndrome (MDS), five of 12 patients (42%) with acute lymphoid leukemia (ALL) (L1: three, L2: three; in the FAB classification), four with chronic myeloid leukemia (CML), one of two patients with chronic lymphoid leukemia (CLL), two of four patients with lung cancer, one patient with cyclic neutropenia, two of seven patients with malignant lymphoma, and four patients with acute infection had G-CSF levels ranging from 46 pg/mL to >2,000 pg/mL. Interestingly, a reverse correlation between blood neutrophil count and serum G-CSF level was clearly demonstrated for aplastic anemia (r = –0.8169, P < .01). Moreover, it was found that the G-CSF level rose during the neutropenic phase of cyclic neutropenia and after chemotherapy or bone marrow transplantation (BMT) in three patients with leukemia; also high G-CSF levels were positively correlated to blood neutrophil counts in some cases of infectious disorders and lung cancer. The cellular sources and the mechanisms for production and secretion of circulating G-CSF were not investigated in this study, but the data presented here strongly indicate that G-CSF plays an important role as a circulating neutrophilopoietin.

G-Granulocyte colony-stimulating factor (G-CSF) is a glycoprotein that stimulates the production and functional activation of neutrophilic granulocytes in vivo, as well as in vitro. However, the kinetics and patho-physiologic roles of G-CSF in the body have not yet been clarified. Therefore, it is of great interest to examine serum G-CSF levels in normal persons and in patients with various disorders whose blood neutrophil number or function is altered. To assay G-CSF, the in vitro clonal culture of granulocyte/macrophage colony-forming cells or (3H)-thymidine uptake by CSF-responsive immature leukemia cells have so far been used. However, both methods have inherent defects in their specificity and simplicity.

Recently, Motojima et al. developed an enzyme immunoassay (EIA) specific for human G-CSF. This method makes it possible to estimate serum G-CSF levels higher than 30 pg/mL. Here we report the EIA results for serum G-CSF in normal healthy volunteers and in patients with various hematologic and nonhematologic disorders. The observed relationships between serum G-CSF levels and blood neutrophil count provide strong evidence that G-CSF acts as a circulating neutrophilopoietin.

MATERIALS AND METHODS

Sera. Serum samples were obtained from 56 healthy volunteers (20 to 86 years old; 46 men and 10 women) and from 89 patients with various disorders after obtaining informed consent, including 11 with idiopathic aplastic anemia (AA), one with Fanconi’s anemia, two with pure red cell aplasia, 12 with myelodysplastic syndrome (MDS), six with acute myeloid leukemia (AML) (M2: 1; M4: 4; M5: 1; in the French American British [FAB] classification), 18 with chronic myeloid leukemia (CML), six with acute lymphoid leukemia (ALL) (L1: three; L2: three; in the FAB classification), two with chronic lymphoid leukemia (CLL), two with Hodgkin’s lymphoma, five with non-Hodgkin’s lymphoma, three with multiple myeloma, three with polycythemia vera, four with acquired immunodeficiency syndrome (AIDS), one with cyclic neutropenia, 11 with solid tumors (breast cancer: six; colon cancer: one; lung cancer: four), and two with bacterial infection (sepsis: one; pneumonia: one).

Serum was separated by centrifugation shortly after collection, and all samples were stored frozen until use. Before the G-CSF assay, each sample was serially diluted 3:4, 1:2, and 1:4 with EIA buffer (50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 0.25% bovine serum albumin, 0.05% Tween-20, and 0.1% NaN3).

G-CSF and anti-G-CSF serum. DNA encoding G-CSFb (general form of G-CSF) was introduced into a Chinese hamster ovary (CHO) cell expression system, and the secreted recombinant G-CSF was purified to homogeneity (specific activity: 1 × 107 U/mg protein) as described previously. For production of rabbit anti-G-CSF serum, 0.5 mg of the purified recombinant G-CSF was dissolved in 0.5 mL of saline, emulsified with complete Freund’s adjuvant, and injected subcutaneously three times at 2-week intervals. Serum collected from the immunized rabbit seven days after the last injection was subjected to fractional precipitation with ammonium sulfate (50% saturation) and then to Ultrogel AcA44 gel filtration (LKB) for isolation of the IgG fraction. The IgG was adjusted to 20 µg/mL in 150 mmol/L NaCl containing 50 mmol/L borate buffer, pH 8.0 (BBS). The antibody was proved to specifically bind to recombinant G-CSF by the Western blotting method and was also shown to completely inhibit in vitro granulocyte colony formation by normal human bone marrow at a dilution of 1:100. From the purified anti-G-CSF IgG, the Fab’ fragment was prepared by pepsin digestion and 2-aminothi ethanol reduction and was conjugated to protein A, allowing specific binding to G-CSF.

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gated to horseradish peroxidase (HRP) by the method of Hashida et al. The HRP-Fab' conjugate was isolated by Ultrogel AcA44 gel filtration, lyophilized, and then redissolved to a concentration of 20 µg/mL in BBS for use as the secondary antibody in the EIA assay. EIA technique. The EIA technique used here has been described elsewhere. In brief, 200 µL of each sample and its three dilutions (3:4, 1:2, 1:4), or serial dilutions of recombinant G-CSF, together with 500 µL of EIA buffer containing 2% polyethylene glycol (molecular weight [mol wt]: 6,000), were added to a polystyrene tube coated with rabbit anti-G-CSF IgG. After two hours incubation at room temperature, 100 µL of the HRP-conjugated anti-G-CSF Fab' solution was added and further incubated for two hours at room temperature. After washing three times with 20 mmol/L Tris-HCl, pH 8.0, containing 0.005% benzalkonium chloride, 1 mL of reaction mixture (3 mg/mL o-phenylenediamine dihydrochloride, 2.3% [wt/vol] disodium hydrogenphosphate, 0.38% citric acid, 0.1% salicylic acid, and 0.015% H₂O₂) was added for color reaction. After incubation for one hour at room temperature in the dark, the reaction was stopped by adding 1 mL of 4N sulfuric acid, and the resulting optical density was measured at 492 nm using a Hitachi spectrophotometer (Hitachi Co., Ltd, Tokyo). From the standard curve obtained with serial 2:1 dilutions of recombinant G-CSF, the within-assay and between-assay coefficients of variation were 4.3% to 6.2% and 6.2% to 9.0%, respectively, for the range of 30 to 2,000 pg G-CSF. Furthermore, it was shown that a highly significant correlation (y = 0.84x + 1.45, r = 94) was present between the G-CSF value determined by the EIA method and the bioactivity assessed by the NFS-60 cell assay, and that addition of macrophage (M) CSF (up to 2,000 ng/mL), granulocyte/macrophage CSF (up to 2,000 ng/mL), or interleukin-3 (up to 16 ng/mL) to the assay system has no effect on the G-CSF standard curve. Curves obtained from the three dilutions of positive serum samples always paralleled the standard curve, and G-CSF values of the test samples could be reproducibly determined by superimposing them on the standard curve.

Blood neutrophil count. Blood samples for neutrophil count and G-CSF assay were drawn on the same day. The absolute neutrophil count (ANC) was obtained by multiplying the WBC count by the percentage of neutrophils in the conventional way.

RESULTS

G-CSF levels in healthy volunteers. G-CSF values for all 56 normal healthy persons, whose ANC were within normal range (1.5 × 10⁹ to 6.0 × 10⁹/L), are plotted according to age and sex in Fig 1. In 49 of the 56 persons (88%) tested, the G-CSF levels were under the sensitivity of the assay (<30 pg/mL). In the remaining seven persons, the levels ranged between 33 and 163 pg/mL; the ages of these persons were all between 30 and 50 years: the highest value was seen in a 47-year-old woman. Any difference in G-CSF levels due to gender could not be determined in this study, as too few women were included.

G-CSF levels in patients with various disorders. Figure 2A shows all the G-CSF values estimated by EIA for patients having various disorders without any evidence of infection, in relation to ANC. In AA, nine of the 11 patients (82%) had G-CSF levels of much more than 30 pg/mL: the mean value was approximately 260 pg/mL, with the highest at 855 pg/mL. In a patient with Fanconi's anemia, the value exceeded 2,000 pg/mL with an ANC of 0.18 × 10⁹/L. Interestingly, in AA there was a reverse correlation between the G-CSF levels and the ANC (r = −.8169, P < .01), as shown in Fig 2B. Similarly, a patient with cyclic neutropenia showed an elevation of G-CSF levels (up to 165 pg/mL) during a neutrophilic phase (ANC: 0.05 × 10⁹/L), although the levels were low during a neutropenic phase (Table 1). On the other hand, six of the 12 patients with MDS whose ANC were between 0.75 × 10⁹/L and 1.6 × 10⁹/L had G-CSF values of <30 pg/mL. In MDS patients, we do not know whether a reverse correlation between the G-CSF levels and ANC may be present because the range of ANC was too narrow.

The G-CSF levels in AML and ALL are shown in Fig 2A, determined long after chemotherapy when the leukemia blast cells had disappeared in blood but having various ANC. Four patients with AML and three patients with ALL had G-CSF levels of <30 pg/mL, but the remaining one patient with AML (M4), one with AML (M5), and three with ALL (L1: one and L2: two) had relatively high G-CSF levels (68 to 270 pg/mL). In these cases, there was no relationship between the G-CSF level and ANC. In CML under the treatment (ANC ranging from 0.04 × 10⁹/L to 49.5 × 10⁹/L), 12 of the 18 patients (67%) had G-CSF levels of <30 pg/mL, while the remaining six patients had levels of 47 to 499 pg/mL. The relationship between the G-CSF levels and ANC was not statistically significant (r = 0.4175, P = 0.8). The CML patients with high G-CSF levels had clinical features no different from those with low G-CSF levels.

One of the two patients with CLL, one of the two with Hodgkin's lymphoma, four of the five with non-Hodgkin's lymphoma, three with multiple myeloma, three with polycythemia vera, one of the two with pure red cell aplasia, six with breast cancer, two of the four with lung cancer, and one with colon cancer had G-CSF levels of <30 pg/mL, with almost normal ANC. In the remaining one patient with CLL, one with Hodgkin's lymphoma, one with non-Hodgkin's lymphoma, and one with pure red cell aplasia, ANC were all around 2 × 10⁹/L, and the G-CSF levels were between 55 and 642 pg/mL. On the other hand, the remaining two patients with lung cancer showed the high G-CSF
the two patients with AIDS who had suffered from bacterial infection. Unlike the AIDS patients, these cases were associated with increased ANC: 468 pg/mL with 9.3 x 10^9/L and 122 pg/mL with 11.9 x 10^9/L. Interestingly, the G-CSF levels in all these patients fell corresponding with the elimination of infection and/or neutrophilia (data not shown).

Effects of cytoreductive therapy on G-CSF levels. Serial changes in G-CSF levels were examined during the course of cytoreductive therapy in some of the patients with hematologic malignancies. Figure 3A shows a representative result for a case of AML that received intensive postremission chemotherapy. The G-CSF levels began to rise in opposition to a decrease in ANC, with a peak G-CSF value of 450 pg/mL at day 14. This elevation was followed by neutrophilic recovery. A similar pattern was also found for a case of bone marrow transplantation (BMT) (Fig 3B). Thus, the G-CSF levels were undetectable before, during, and shortly after the conditioning, but thereafter began to rise rapidly, with a peak G-CSF value of 634 pg/mL at day 13, followed by a gradual rise in ANC.

Serial changes in the G-CSF levels of the two patients with CML undergoing treatment with interferon (IFN)-α are shown in Fig 4. In the patient whose G-CSF levels were high before the treatment, the G-CSF levels gradually decreased with a concomitant fall in ANC (Fig 4A), while in the other patient, who initially had a G-CSF level of 30 pg/mL, the levels remained low during the course of treatment (Fig 4B).

DISCUSSION

Although the newly developed EIA was not so sensitive as to determine serum G-CSF levels in most normal persons, we did demonstrate with the use of this method that the G-CSF levels are often higher than normal in most patients with abnormal ANC. Most typically, high G-CSF levels were found in severely neutropenic patients. It is interesting to note that a clear reverse correlation between the G-CSF level and ANC was observed for cases of AA and for a case of cyclic neutropenia. Moreover, a transient G-CSF elevation (up to 450 or 634 pg/mL) was observed to precede neutrophilic recovery after cytoreductive therapy or after BMT in leukemia. In addition, elevation of the G-CSF level was obviously accompanied by an increased ANC in some cases of bacterial infection. The degree of G-CSF elevation varied among the disorders, presumably reflecting different abilities to produce G-CSF. These findings strongly indicate, however, that serum G-CSF levels fluctuate according to the requirements for stimulation of neutrophil production, supporting the view that G-CSF is one of the circulating hormones specific for neutrophilopoiesis.

On the other hand, most of the patients with the other disorders (if they had no obvious evidence of bacterial or fungal infection) had levels of <30 pg/mL, as seen for most of the normal persons. There were some exceptional cases. For example, some patients with CML and AML (M4 and M5) had relatively high G-CSF levels. We have reported

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**Table 1. Serum G-CSF Level and Blood Neutrophil Count in a Patient With Cyclic Neutropenia**

<table>
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<th>Day</th>
<th>ANC (x 10^9/L)</th>
<th>G-CSF (pg/mL)</th>
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<tr>
<td>1</td>
<td>1.0</td>
<td>&lt;30</td>
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<tr>
<td>3</td>
<td>0.3</td>
<td>48</td>
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<tr>
<td>7</td>
<td>0.05</td>
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<td>10</td>
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elsewhere that myelomonocytic leukemic cells from the other two patients with AML (M4 and M5) in relapse produced G-CSF. These data might indicate that the elevation of G-CSF levels observed in the present AML (M4 and M5) patients reflect an excess production of G-CSF by the residual leukemia cells; however, we could not explain why the high G-CSF levels did not lead to increases in ANC in these patients. In contrast, a parallel relation was observed between ANC and G-CSF levels in six of 18 patients with CML and two of four patients with lung cancer. In this context, the one case of CML undergoing IFN-α therapy may be interesting. Further analysis of G-CSF expression at the mRNA level will be necessary to ascertain aberrant production of G-CSF by these malignant cells.

Another interesting point to be covered is the cellular source for the circulating G-CSF in AA. Production of G-CSF by fibroblasts under the stimulation of interleukin-1 was reported to occur at the mRNA level. We also recently
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observed G-CSF mRNA expression in bone marrow stromal cells under the stimulation of M-CSF (unpublished data). On the other hand, Gascon et al recently reported that production of interleukin-1 by monocytes in AA decreased. Investigation of the capacity of these cells to produce G-CSF and the kinetics of interleukin-1 or M-CSF in AA will provide much information about the regulatory mechanisms for physiologic neutrophilopoiesis.

It should be kept in mind, however, that we do not know whether the antigenic factor being bound in the EIA is biologically active or not. Indeed, there were some cases in which the relationship between the ANC and the G-CSF level was not easily understandable. In such cases, it would be necessary to estimate the G-CSF levels by other methods that guarantee biologic activity. Nevertheless, it is apparent that the EIA method is simple, specific, and sensitive for assaying serum G-CSF in most cases. Therefore, the EIA for serum G-CSF will be of much help in the differential diagnosis of several disorders and in understanding the pathogenesis of them. In addition, it will be useful for determining the proper dosing schedule for recombinant G-CSF, which has recently become available for clinical use.

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Fig 4. (A) Serial changes in serum G-CSF levels and in blood neutrophil counts in a 50-year-old man with CML who initially showed high G-CSF levels and subsequently was treated with IFN-α (6 x 10⁶ U/d). (B) Serial changes in serum G-CSF levels and in blood neutrophil counts in a 22-year-old woman with CML who initially showed low G-CSF levels and subsequently was treated with IFN-α (1 x 10⁶ U/d).

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