Human Granulocyte Colony-Stimulating Factor Specifically Binds to Murine Myeloblastic NFS-60 Cells and Activates Their Guanosine Triphosphate Binding Proteins/Adenylate Cyclase System

By Satoru Matsuda, Naoki Shirafuji, and Shigetaka Asano

The specific binding of human granulocyte colony-stimulating factor (G-CSF) to its receptors on NFS-60 cells acts as a primer for cellular proliferation. There are approximately 400 binding sites per cell, with a binding constant of about 100 pmol/L. Before the proliferative response, the affinity constant of the membrane particulate fraction to \(^{35}\text{S}\)-labeled guanosine triphosphate-gamma-S (\(^{35}\text{S}\text{-GTP}\gamma\text{S})\) and the intracellular cyclic adenylate monophosphate (cAMP) level increased in the presence of G-CSF to about 2.5-fold and about fivefold higher, respectively, than the levels seen in the absence of G-CSF. The increases were time-dependent, with a peak occurring 15 minutes after the addition of G-CSF at 37°C. These findings suggest that, following the binding of the G-CSF to its surface receptors, the activation of the guanosine triphosphate (GTP)-binding protein/adenylate cyclase system may be involved in the proliferation of immature myeloid cells.

G \-RANULOCYTE colony-stimulating factor (G-CSF), a glycoprotein hematopoietic regulator with a molecular weight of approximately 19,000, is capable of specifically stimulating both neutrophil production and functional activation of mature neutrophils in vitro and in vivo. This factor, therefore, can be of great use in analyzing the inherent characteristics of acute myeloid leukemia cells, which is to say their abnormal proliferation and maturation block. However, the receptor-effector systems for the diverse array of cellular responses to G-CSF have not yet been fully investigated in either normal or leukemia cells.

Previously we purified human G-CSF and showed in molecular cloning studies that its amino acid and nucleic acid sequences are very similar to those of murine G-CSF. These findings confirm those of Nicola and Metcalf who used murine WEHI-3B (D\(^+\)) myelomonocytic leukemia cells to indicate a cross-reactivity between human and murine G-CSFs; furthermore, they showed that the basis for the unresponsiveness of the variant (D\(^-\)) cells to G-CSF-induced differentiation appears to be the failure of these cells to express membrane receptors for G-CSF. However, Weinstein et al. reported that another murine myeloblastic cell line, designated as NFS-60, is stimulated by murine G-CSF to proliferate but not differentiate. Since the latter situation seems more representative of human myeloid leukemia cells, NFS-60 cells may serve as a more useful model than WEHI-3 (D\(^+\)) cells in further studying the inherent abnormalities of leukemia cells. We have recently investigated the binding of purified recombinant human G-CSF to NFS-60 cells and the subsequent cellular responses in more detail. Here we describe that \(^{125}\text{I}-\text{G-CSF}\) specifically bound to its receptors on the surface of NFS-60 cells, after which the affinity constant of the membrane particulate fraction to \(^{35}\text{S}\)-labeled guanosine triphosphate-gamma-S (\(^{35}\text{S}\text{-GTP}\gamma\text{S})\), the activity of adenylate cyclase, and the intracellular cyclic adenylate monophosphate (cAMP) level increased before cell proliferation. These data suggest that the GTP-binding proteins/adenylate cyclase system of the membrane may be involved in an early phase of the G-CSF signal transduction for proliferation of immature myeloid cells.

MATERIALS AND METHODS

Cells. NFS-60 cells, kindly provided by Dr J. Ihle (St. Jude Children's Research Hospital, Memphis, TN) were maintained in RPMI 1640 medium containing 10% fetal calf serum and 10% WEHI-3B conditioned medium as a source of mouse interleukin-3 (IL-3), replacing with freshly prepared medium every 3 days.

Reagents. G-CSF was secreted and purified to homogeneity from mouse C127I cells transfected with human G-CSF cDNA encoding a 204-amino acid polypeptide (type b) (specific activity: 1 x 8 U/mg protein), as described previously. Purified recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) and native macrophage colony-stimulating factor (M-CSF) were kindly provided by Drs D.W. Golde (UCLA School of Medicine, CA) and K. Motoyoshi (Jichi Medical School, Tochigi, Japan), respectively. \(^{35}\text{S}\text{-GTP}\gamma\text{S}\) and cAMP kits were purchased from New England Nuclear (NEN, Boston, MA). All other reagents were of analytical grade obtained from Sigma (St. Louis, MO).

\(^{1}\text{H}-\text{labeled thymidine uptake.}\) After being washed three times with RPMI 1640 medium, NFS-60 cells were resuspended in medium to a concentration of 1 x 10\(^6\) cells/mL. Fifty \(\mu\)L of the cell suspension was then mixed with 50 \(\mu\)L of serially diluted (2:1) G-CSF, M-CSF, or GM-CSF solution in each well of a microtiter plate (No. 167008, Nunc, Roskilde, Denmark), followed by incubation at 37°C in a 5% CO\(_2\) incubator. Twenty-four hours later, 0.25 \(\mu\text{Ci}\) of \(^{3}H\)-thymidine (specific activity 2 Ci/mmol, NEN) was added to each well and the mixtures were further incubated for 6 hours at 37°C. The cells were then harvested onto membranes using an automated LABO-MASH cell harvester unit (Labo Science, Natwick, MA) and assayed for \(^{3}H\)-thymidine incorporation.

Membrane fractionation. The membrane fraction of NFS-60 cells for the study of \(^{125}\text{I}-\text{G-CSF}\) and \(^{35}\text{S}\text{-GTP}\gamma\text{S}\) bindings was prepared as follows in the presence of 1 mmol/L phenylmethylsulfo-
nylfluoride. NFS-60 cells were ruptured by N2 gas cavitation at 75 kg/cm² for 1 hour on ice using an IYEDA press (Israel). The homogenate was centrifuged at 500 × g for 5 minutes at 4°C and the opalescent supernatant was collected. This fraction was then placed over a 23% sucrose cushion and ultracentrifuged at 100,000 × g at 4°C for 2.5 hours in a Hitachi ST40 rotor (Tokyo, Japan). The fraction at the interphase was recovered and centrifuged at 200,000 × g for 30 minutes at 4°C. The pellet was resuspended in 20 mmol/L HEPES buffer, pH 7.4, containing 0.1% bovine serum albumin (BSA), and was frozen at −70°C until use.

125I-labeled G-CSF binding. G-CSF was radioiodinated with 125I-NaI by the two-phase chloramin-T method reported previously by Tejedor and Ballesta. This procedure yielded a high specific radioactivity (about 3 × 10⁶ cpm/μg) of G-CSF with little loss of bioactivity. Various concentrations of 125I-G-CSF were incubated with the cells or the cell membrane fractions with and without 100-fold excess of unlabeled G-CSF at 37°C for 1 hour in RPMI 1640 medium with 0.1% BSA for cellular binding or in 20 mmol/L HEPES buffer with 0.1% BSA, pH 7.4, for membrane binding. After being washed three times with ice-cold phosphate buffered saline, the suspensions were filtered through 0.2 μm Millipore EGWP filters for counting the radioactivity.

35S-labeled GTPγS binding. Binding of 35S-GTPγS to NFS-60 cells was assayed as reported by Evans et al. Briefly, NFS-60 cells at a concentration of 1 × 10⁷/mL in ice-cold 25 mmol/L Tris HCl, pH 7.6, containing 5 mmol/L MgCl₂, were sonicated for 30 seconds and centrifuged at 10,000 × g for 5 minutes. The pellet was then resuspended in 50 μL of 10 mmol/L Tris HCl, pH 7.8, containing 10 mmol/L MgCl₂, 1 mmol/L EDTA, 0.2% BSA, 0.5 mmol/L ascorbic acid, 2 mmol/L adenyln-5-yl imidodiphosphate, and 100 mmol/L 35S-GTPγS, with or without 50 ng G-CSF, and was incubated at 30°C for various periods. Next, 450 μL of an ice-cold buffered stop solution composed of 25 mmol/L GTPγS, 100 mmol/L NaCl, 0.1% Lubrol, and 10 mmol/L Tris HCl, pH 7.4, was added and the mixture filtered through Millipore HAWP membranes (0.45 μm). The membranes were washed extensively with 20 mL of 10 mmol/L Tris HCl, pH 7.4, containing 100 mmol/L NaCl and 0.1% Lubrol before counting the radioactivities. Effect of cholera toxin on the 35S-GTPγS binding was also examined using the membrane fraction. In this experiment, the membranes were preincubated in 30 mmol/L Tris HCl, pH 7.5, containing 10 mmol/L MgCl₂, 1 mmol/L EDTA, 0.5 mmol/L ascorbic acid, 1 mmol/L adenyl-5-yl imidodiphosphate, 0.1 mmol/L adenosine triphosphate (ATP), 0.4 mmol/L dithiothreitol (DTT), 10 μmol/L nicotinamide adenine dinucleotide (NAD), and 0.1 mmol/L GTPγS with or without 40 μg/mL of cholera toxin at 30°C for 30 minutes. After being washed three times, the membranes were incubated with or without G-CSF for 15 minutes for the 35S-GTPγS binding study.

Intracellular cAMP. Five million NFS-60 cells were incubated in 1 mL of RPMI 1640 medium with or without 50 ng/mL of G-CSF at 37°C. At various intervals, the cells were harvested and extracted with 1 mL of 6% (wt/vol) trichloracetic acid (TCA). After removal of the TCA, the extracts were lyophilized and then solubilized in 1 mL of deionized water for cAMP assay. Each assay of cAMP levels, using a commercially available cAMP RIA Kit (NEN), required 100 μL of this solution.

Adenylate cyclase activity. A constant amount of the NFS-60 cell membrane (20 μg protein) was incubated in 40 μL of the reaction mixture composed of 30 mmol/L Tris HCl, pH 7.5, 10 mmol/L MgCl₂, 0.12 mmol/L ATP, 2 mmol/L potassium phosphate, 4 μg/mL pyruvate kinase, 8 μg/mL myokinase, 0.4 mmol/L DTT, 0.2 mmol/L isobutylmethylxanthine, 0.1 mmol/L GTP, and 10 mmol/L NAD, in the presence and absence of 50 ng/mL G-CSF at 37°C. At intervals, the reactions were stopped by adding 1 mL of an ice-cold buffered stop solution composed of 25 mmol/L GTPγS, 100 mmol/L NaCl, 0.1% Lubrol, and 10 mmol/L Tris HCl, pH 7.4, and the mixture filtered through Millipore HAWP membranes (0.45 μm). The membranes were washed extensively with 20 mL of 10 mmol/L Tris HCl, pH 7.4, containing 100 mmol/L NaCl and 0.1% Lubrol before counting the radioactivities.
RESULTS

Proliferative response to G-CSF. As shown in Fig 1, the presence of G-CSF in the culture medium increased 3H-thymidine incorporation into NFS-60 cells in a dose-dependent manner. On the other hand, GM-CSF and M-CSF did not affect uptake, even at concentrations of more than 100 ng/mL. Morphological changes in NFS-60 cells were not observed in the presence of any of these CSFs.

Specific binding of 125I-G-CSF. 125I-G-CSF was found to bind specifically to NFS-60 cells. Representative data on the binding kinetics are shown in Fig 2. The specific binding was saturable (Fig 2A), linear with the cell number, and time- and temperature-dependent. Scatchard analysis of the data obtained at 4°C gave a straight line (Fig 2B). From the negative inverse of the slope, a dissociation constant of about 100 pmol/L was calculated for the binding of 125I-G-CSF to NFS-60 cells; from the intercept on the abscissa, it was calculated that the cells contained about 400 receptors per cell. Similar results were obtained repeatedly, and the values were almost the same as those for WEHI-3B(D) cell receptors as reported by Nicola and Metcalf. When either GM-CSF or M-CSF was added to the membrane fractions containing 125I-G-CSF, no competition with G-CSF was observed at 37°C (Fig 3).

Effects of G-CSF on 35S-GTPγS binding to the membranes. To determine whether G-CSF signals are mediated by GTP-binding proteins or not, effects of G-CSF on 35S-GTPγS binding to the NFS-60 cell membranes were examined before and after cholera toxin treatment. Approxi-
mately sevenfold increase in the $^{35}$S-GTPyS binding in response to G-CSF was observed with the cholera toxin-pretreated membranes, while no increase was observed with non-pretreated membranes (Fig 4). Figure 5 gives representative data on the kinetics using the non-pretreated membranes. The presence of G-CSF increased the $^{35}$S-GTPyS binding time-dependently compared with the absence of G-CSF, and the increase in response to G-CSF was almost completely blocked by co-existence of 2 mmol/L guanosine diphosphate (Fig 5A). By Scatchard analysis of the specific binding based on the data obtained at 30°C for 10 minutes, it was calculated that the affinity constants of the G-CSF-treated and the nontreated fractions were about 33 mmol/L and 75 mmol/L, respectively (Fig 5B). On the other hand, no change was seen in the number of binding sites per cell with the G-CSF treatment.

**Activation of membrane adenylate cyclase by G-CSF.** GTP-binding proteins are known to regulate adenylate cyclase activity. To determine whether this is also the case in NFS-60 cells, we examined effects of G-CSF on the enzyme activity of the isolated membranes themselves. As shown in Fig 6, the activity was 48 pmol cAMP/min/mg protein in the presence of 50 ng/mL G-CSF, while only 1.2 pmol cAMP/min/mg protein in the absence of G-CSF.

**Elevation of intracellular cAMP by G-CSF.** In many cell types it has been proposed that cAMP produced by adenylate cyclase plays a major role in cell proliferation. Therefore, effects of G-CSF on intracellular cAMP levels were examined in NFS-60 cells. The levels of intracellular cAMP increased rapidly in the presence of G-CSF, reaching a maximum level (approximately 20 pmol/10$^6$ cells) after 15 minutes of incubation (Fig 7). The increase was dose-dependent, with a plateau at concentrations of more than 50 ng G-CSF/mL (Fig 8).

**DISCUSSION**

Human G-CSF has been shown to stimulate murine NFS-60 cells to proliferate markedly without differentiation, presumably via its binding to surface receptors specific for G-CSF. The dissociation constant for $^{125}$I-G-CSF binding to NFS-60 cells was almost the same as that for WEHI-3B(D$^+$) cells, and the number of binding sites per cell was low as is generally the case within the CSF family.
For many cell types, it has been shown that alpha-subunits of GTP-binding proteins (Gs) are directly coupled to receptors, are capable of activating adenylate cyclase, thereby elevating levels of intracellular cAMP, and also that cholera toxin directly induces ADP-ribosylation of Gs necessary for the adenylate cyclase activation. The above findings, therefore, suggest that the G-CSF signal in NFS-60 cell proliferation may be mediated by the GTP-binding proteins/adenylate cyclase/cAMP system.

It is interesting to note that the effect of G-CSF on cAMP levels reported here was quite different from that of IL-3 on murine IL-3-dependent stem cells reported by Dexter et al. They have described that the deprivation of IL-3 from the cells had no major effect on cAMP levels in the first few hours and also that its addition to IL-3-deprived cells caused a decrease in cAMP. These data suggest that the signal transduction systems for G-CSF and IL-3 are probably different.

However, we have not determined in this report how the elevated intracellular cAMP elevation is related to growth. We have not observed cholera toxin or cAMP stimulating NFS-60 cells to proliferate (data not shown). For better understanding of the biologic significance of cAMP elevation for NFS-60 cell proliferation, further study will be needed to determine whether the same early component of signal transduction system is involved in activation of nonproliferating mature neutrophils as well, what proteins are phosphorylated by cAMP-dependent protein kinases, and how these proteins affect the expression of oncogenes such as the c-myb gene of NFS-60 cells.

ACKNOWLEDGMENT

We are grateful to Dr Shigekazu Nagata for providing purified G-CSF and to Dr Fumimaro Takaku for encouraging us. Keiko Nakano is acknowledged for her secretarial assistance.

REFERENCES

5. Nicola NA, Metcalf D: Binding of the differentiation-inducer, granulocyte colony-stimulating factor, to responsive but not unresponsive leukemic cell lines. Proc Natl Acad Sci USA 81:3765, 1984
gene structure and two mRNAs for human granulocyte colony-stimulating factor. EMBO J 5:575, 1986


Human granulocyte colony-stimulating factor specifically binds to murine myeloblastic NFS-60 cells and activates their guanosine triphosphate binding proteins/adenylate cyclase system

S Matsuda, N Shirafuji and S Asano