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Production of Soluble Granulocyte Colony-Stimulating Factor Receptors from Myelomonocytic Cells

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It has been speculated that a soluble form of G-CSF might be physiologically present in humans, since G-CSFR mRNA that lacks a transmembrane domain has been identified from a human myelomonocytic cell line. Here, we demonstrate human soluble G-CSFR (sG-CSFR) of two different molecular sizes (80 and 85 kDa) on an immunoblot analysis using Abs generated against the amino-terminal, extracellular domain of the full-length G-CSFR. Both isoforms of sG-CSFR were able to bind recombinant human G-CSF (rhG-CSF). RT-PCR analysis with primers targeted outside of the transmembrane region revealed that membrane-anchored G-CSFR is expressed at all maturation stages of purified myeloid cells, including CD34+CD13+ cells (blasts), CD11b+CD15+ cells (promyelocytes or myelocytes), CD11b+CD15+ cells (metamyelocytes and mature neutrophils), and CD14+ cells (monocytes). On the other hand, sG-CSFR mRNA was detectable in CD11b+CD15+, CD11b+CD15+, and CD14+ cells, but not in the CD34+CD13+ blast population. The serum concentration of both isoforms of sG-CSFR appeared to be correlated with the numbers of neutrophils/monocytes before and after rhG-CSF treatment in normal individuals. Thus, two isoforms of sG-CSFR are physiologically secreted from relatively mature myeloid cells and might play an important role in myelopoiesis through their binding to serum G-CSF. *The Journal of Immunology, 1999, 163: 6907–6911.

Granulocyte CSF possesses biological activity in cells at various stages of hemopoiesis. G-CSF supports the formation of immature myeloid blast colonies that can differentiate into all myeloid lineages, induces proliferation and differentiation of cells that are committed to myelomonocytic lineages (1, 2), and augments the function of mature neutrophils and platelets (3, 4). G-CSF also plays an important role in mobilizing hematopoietic stem cells from bone marrow to circulation in vivo. Consistent with these data, G-CSFR has been shown to be expressed in stem cells, myeloid progenitors, platelets, and mature myelomonocytic cells. The human G-CSFR cDNA has been cloned from the U937 human monocytic leukemia cell line (5). The full-length human G-CSFR is a transmembrane protein containing 813 amino acids and the WXXWS motif, which is highly conserved in the extracellular domain of members of the cytokine receptor superfamily (6). Two additional human G-CSFR cDNAs have been isolated from U937 cells, one of which has an 88-bp deletion in the transmembrane domain and appears to encode a soluble G-CSFR (sG-CSFR) (5).

Soluble form receptors for various cytokines are known to be generated either by alternative splicing of cytokine receptor mRNAs or by proteolytic cleavage of the membrane-anchored proteins (7–17). These include receptors for IL-2, IL-6, leukemia inhibitory factor, TNF, GM-CSF, erythropoietin (Epo), and growth hormone. These soluble receptors are detectable in human serum, and the cells responsible for releasing each soluble receptor have been identified (18–27). However, it remains unclear whether or not the sG-CSFR is physiologically released into serum and whether or not it plays a role in the regulation of hemopoiesis in vivo. In this paper, we show evidence that the two isoforms of sG-CSFR are present in human serum and that both isoforms are released from relatively mature myelomonocytic cells.

Materials and Methods
Collection of blood cells and sera
Bone marrow cells, blood cells, and sera were obtained from healthy volunteers. Bone marrow mononuclear cells (BMMC) and neutrophils were separated by density gradient centrifugation on lymphocyte separation medium (Organon Teknika, Durham, NC) and mono-poly resolving medium (ICN, Tokyo, Japan), respectively. In addition, sera were collected from three healthy male volunteers who received recombinant human G-CSF (rhG-CSF) (Filgrastim; Santkyo/Kirin, Tokyo, Japan) (5 μg/kg, s.c.) for 5 days. In all cases, informed consent was obtained in a written form from each individual as previously reported (28).

Preparation of polyclonal anti-G-CSFR Abs
The peptide CJEQPGGQRQLSDGT, which corresponds to residues 68 to 82 of human full-length G-CSFR, was synthesized, purified, and linked to the KLH carrier protein (Takara, Kyoto, Japan) (29–31). A female New Zealand White rabbit was immunized with this peptide as described by Shih et al. (32).

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4 Abbreviations used in this paper: sG-CSFR, soluble G-CSFR; Epo, erythropoietin; BMMC, bone marrow mononuclear cells; rhG-CSF, recombinant human G-CSF.
**SDS-PAGE and immunoblot analysis**

To characterize the reactivity of the anti-G-CSF-peptide antiserum, we subjected neutrophil membranes to immunoblot analysis. Purified neutrophils were lysed in 1 ml of lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, and 1 mM PMSF) for 15 min on ice. After centrifugation at 10,000 x g for 15 min, the supernatant was subjected to SDS-PAGE on a 4 to 20% gradient gel as described by Laemmli (33). The gel was electroblotted onto a nitrocellulose membrane (Hybond-C super; Amersham, Rainham, U.K.) with a semidis charge transfer cell (Bio-Rad, Richmond, CA). The membrane was incubated with 5% skim milk in TBS (20 mM Tris-HCl (pH 7.6) and 150 mM NaCl) for 1 h and then with the polyclonal antiserum diluted 1:500 in TBS-T (TBS with 0.1% Tween 20) for 1 h. After three washes with TBS-T, the membrane was incubated for 20 min with biotinylated Abs to rabbit Ig (Amersham). Finally, the membrane was incubated with streptavidin conjugated to HRP (Caltag, San Francisco, CA) for 20 min. Chemiluminescence was detected with the ECL system (Amersham).

**Detection of G-CSF-binding proteins**

Sera from healthy volunteers were incubated for 1 h at 37°C with biotinylated rhG-CSF (Fluorokine; R&D Systems, Minneapolis, MN) at a final concentration of 5 µg/ml in the absence or presence of a 100-fold excess of unlabeled rhG-CSF. The sera were then subjected to native PAGE as described by Davis (34). The gel was electroblotted onto a nitrocellulose membrane and incubated sequentially with skim milk and HRP-conjugated streptavidin. Chemiluminescence was detected by the ECL system.

**Purification of myeloid cells**

BMMC were stained with the following mAbs: FITC-conjugated anti-CD15 (Leu M1) and anti-CD34 (HPCA2) Abs (Becton Dickinson, Oxnard, CA), and PE-conjugated anti-CD11b (Leu 15), anti-CD13 (Leu M7) (Becton Dickinson) or anti-CD14 (My4) Abs (Coulter, Hialeah, FL), CD34+, CD13+, CD11b+, CD15+, CD11b-CD15+, and CD14+ populations were purified by a FACStarPlus FACS (Becton Dickinson) (35).

**RT-PCR**

Total RNA was extracted from 10,000 cells of each myeloid population, and cDNA was obtained by reverse transcription as previously described (36). The PCR primers targeted outside the transmembrane region of human full-length G-CSFR were as follows: 5' primer, ACCTGGGCA-CAGCTGGAGTGG; and 3' primer, GCTGCTGTGAGCTGGGTCTGG. These primers have been reported by Fukunaga et al. (5), and the expected sizes of PCR products from membrane-anchored and soluble G-CSFR have been reported by Fukunaga et al. (5), and the expected sizes of PCR products from membrane-anchored and soluble G-CSFR (Fluorokine; R&D Systems, Minneapolis, MN) at a final concentration of 5 µg/ml in the absence or presence of a 100-fold excess of unlabeled rhG-CSF. The sera were then subjected to native PAGE as described by Davis (34). The gel was electroblotted onto a nitrocellulose membrane and incubated sequentially with skim milk and HRP-conjugated streptavidin. Chemiluminescence was detected by the ECL system.

**Results**

**Human sera in normal individuals contain two isoforms of sG-CSFR**

A cell membrane fraction from blood neutrophils was analyzed with polyclonal Abs against an amino-terminal region of the human G-CSFR extracellular domain. The Abs recognized a 150-kDa molecule (Fig. 1A), the size of which was consistent with the molecular mass of human full-length G-CSFR (37). On the other hand, the Abs reacted with 80- and 85-kDa molecules in healthy human sera (Fig. 1B). In both experiments, an addition of antigenic complexes correspond to the molecular mass of biotinylated rhG-CSF with 80- and 85-kDa sG-CSFR, respectively. The excess of unlabeled rhG-CSF competitively inhibited the binding of biotinylated rhG-CSF to sG-CSFR of either isoform, suggesting that each sG-CSFR can bind to one rhG-CSF molecule.

**sG-CSFR mRNA is expressed in relatively mature myelomonocytic cells**

We searched for cells that are responsible for the production of sG-CSFR. Myeloid cells in normal bone marrow were fractionated by FACS into four populations. As shown in Fig. 3, FACS-purified CD34+CD13+ (region A), CD11b-CD15+ (region B), CD11b+CD15+ (region C) and CD14+ (region D) cells are composed of blasts, promyelocytes/myelocytes, metamyelocytes/neutrophils, and monocytes, respectively. The purity of each sorted cell population was >99%. We performed RT-PCR analysis of total RNA extracted from 10,000 cells of each fraction using the rhG-CSF is a 15-kDa molecule, these two biotinylated complexes correspond to the molecular mass of biotinylated rhG-CSF with 80- and 85-kDa sG-CSFR, respectively. The excess of unlabeled rhG-CSF competitively inhibited the binding of biotinylated rhG-CSF to sG-CSFR of either isoform, suggesting that each sG-CSFR can bind to one rhG-CSF molecule.

**Detection of G-CSF-binding proteins in healthy human serum**

Shown is a PAGE analysis of serum incubated with biotinylated rhG-CSF in the absence (lane 1) or presence (lane 2) of a 100-fold excess of unlabeled rhG-CSF. Bands seen under 66 kDa are aggregations of unbound biotinylated rhG-CSF.

**FIGURE 1.** A, Anti-peptide Ab reactivity with cell membranes from peripheral neutrophils. B, Immunoblot analysis of healthy human serum with the anti-peptide Abs. In both panels, lane 2 shows the disappearance of the band in lane 1 by the addition of the antigenic peptide (50 µg/ml) during the primary-Ab incubation.

**FIGURE 2.** Detection of G-CSF-binding proteins in healthy human serum. Shown is a PAGE analysis of serum incubated with biotinylated rhG-CSF in the absence (lane 1) or presence (lane 2) of a 100-fold excess of unlabeled rhG-CSF. Bands seen under 66 kDa are aggregations of unbound biotinylated rhG-CSF.
primers targeted outside the transmembrane region of G-CSFR. In all fractions, 390-bp products, which correspond to membrane-anchored G-CSFR mRNA, were detected. In addition to the 390-bp products, the shorter 302-bp products were found in CD11b<sup>−</sup>CD15<sup>+</sup>, CD11b<sup>+</sup>CD15<sup>−</sup>, and CD14<sup>+</sup> fractions, but not in CD34<sup>−</sup>CD13<sup>−</sup> fraction (Fig. 4). The 302-bp products correspond to sG-CSFR mRNA because the 88 bp of transmembrane domain coding sequences is missing (5). Accordingly, membrane-anchored G-CSFR mRNA is expressed in all maturation stages of myelomonocytic lineage, whereas sG-CSFR mRNA, which might be generated by alternative splicing, is differentially expressed in relatively mature myelomonocytic cells.

Serum concentration of sG-CSFR is correlated with numbers of circulating myelomonocytic cells

We analyzed sera from normal individuals before and after administration of rhG-CSF on an immunoblot assay by using polyclonal anti-G-CSFR Abs. rhG-CSF (5 μg/kg s.c.) was administered from day 0 to day 5. Representative data are shown in Fig. 5. In this case, numbers of mature neutrophils/monocytes were 4,400/μl (day 0), 29,200/μl (day 3), and 36,600/μl (day 5). Both isoforms of sG-CSFR (80 and 85 kDa) were detectable in all samples. However, the concentration of both isoforms of sG-CSFR gradually increased after the rhG-CSF treatment. In contrast, sera taken from aplastic anemia patients, whose blood neutrophils/monocytes numbers were <500/μl, did not contain detectable levels of sG-CSFR. These data strongly suggest that the serum level of both isoforms of sG-CSFR might synchronize with numbers of blood neutrophils/monocytes that can produce sG-CSFR.

Discussion

We have demonstrated the existence of proteins of two different molecular sizes (80 and 85 kDa) that reacted with the anti-G-CSFR

![FIGURE 3](image-url)  
**A.** Purification of myeloid cells. BMMC were stained for CD34 (FITC) and CD13 (PE) (upper left panel), CD15 (FITC) and CD11b (PE) (lower left and upper right panels), or CD14 (PE) (lower right panel). CD34<sup>−</sup>CD13<sup>−</sup> cells (region a), CD11b<sup>−</sup>CD15<sup>+</sup> cells (region b), CD11b<sup>−</sup>CD15<sup>−</sup> cells (region c), and CD14<sup>+</sup> cells (region d) were sorted with a FACStar™. **B.** The morphology of the sorted cells. Cells from regions a to d corresponded to blasts (region a), promyelocytes and myelocytes (region b), mature neutrophils (region c), and monocytes (region d) (May-Giemsa stain, ×600).

![FIGURE 4](image-url)  
Detection of G-CSFR mRNA by RT-PCR. Total RNA from 10,000 cells of the sorted populations corresponding to regions a to d (Fig. 3A) was subjected to RT-PCR. Products of 390 and 302 bp that correspond to membrane-anchored G-CSFR mRNA and sG-CSFR mRNA, respectively, were amplified in regions b (lane 3), c (lane 4), and d (lane 5). On the other hand, the blastic population (region a) expressed only membrane-anchored G-CSFR mRNA, but not sG-CSFR mRNA (lane 2). Lane 1 shows the 100-bp DNA ladder.

![FIGURE 5](image-url)  
Concentration of sG-CSFR in various phases of myelopoiesis. Immunoblot analysis of sG-CSFR in sera from G-CSF-treated healthy volunteers (lanes 1-3) and from the patients with severe aplastic anemia (lanes 4-6) was performed. Representative data of sera from a healthy volunteer at day 0 (lane 1), day 3 (lane 2), and day 5 (lane 3) from the initiation of the consecutive G-CSF administration for 5 days are shown. White blood cell counts were 4400/μl (day 0), 29,200/μl (day 3), and 36,600/μl (day 5). In all three cases of severe aplastic anemia, white blood cell counts were <500/μl.
Abs in human sera, and two corresponding G-CSF-binding proteins in a G-CSF-binding assay. These data strongly suggest that at least two isoforms of sG-CSFR physiologically exist in human sera.

The PCR analysis showed that the G-CSFR mRNA that lacked transmembrane domain was differentially expressed in relatively mature myelomonocytic cells. This indicates that at least one of the two isoforms of sG-CSFR might be generated by an alternative splicing of G-CSFR mRNA. The origin of the other isoform of sG-CSFR in serum remains unclear. It is possible that these two isoforms of sG-CSFR may differ in their patterns of glycosylation. Another possibility is that either one may represent a proteolytic cleavage fragment of the membrane-anchored G-CSFR, as shown in soluble IL-1, IL-2, IL-6, and TNF receptors (15–17, 38–40). However, our data thus far do not support this hypothesis, because we did not see a significant decrease in the expression levels of G-CSFR on neutrophils using flow cytometric analysis (41) after the treatment of either phorbol esters or ionomycin, and because the supernatant of cultured neutrophils after the treatment did not contain detectable levels of sG-CSFR (unpublished data).

The physiological function of sG-CSFR remains unclear. sG-CSFR mRNA is detected only in relatively mature myelomonocytic lineage cells, but not in immature blast cells. Fukunaga et al. (5) reported that sG-CSFR mRNA was undetectable in human placenta by RT-PCR. These data suggest that sG-CSFR production may be limited to relatively late stages of myelopoiesis and that mature myeloid cells may be able to affect hemopoiesis through releasing sG-CSFR. The binding of both isoforms of sG-CSFR with rhG-CSF suggests that the sG-CSFR may be able to compete with membrane-anchored G-CSFR on the target cells and serve as a negative regulator of myelopoiesis. This hypothesis is supported by our data that these sG-CSFR were produced only in relatively mature myelomonocytic cells, whereas membrane-anchored G-CSFR were expressed in immature myeloid blasts as well as mature myelomonocytic cells. The negative feedback from the late stage myeloid cells through secreting sG-CSFR may be important to maintain homeostasis of myelopoiesis. Another possibility is that sG-CSFR may directly stimulate or inhibit the growth of target cells. Ku et al. (42) reported that a recombiant extracellular domain of G-CSFR induced a proliferative signal in primary hemo-poietic progenitors in synergy with steel factor or with the ligand for Flt3/Flik2. In contrast, we have shown that the extracellular domain of G-CSFR can inhibit the G-CSF-dependent leukemic progenitors in acute myelogenous leukemia (43). The mechanisms of these opposing actions of recombiant extracellular domain G-CSFR that have different structures from native sG-CSFR are currently unknown. It is also possible that sG-CSFR may prevent its cognate ligands from degradation as has been shown for soluble growth hormone receptors (23–25).

The serum concentration of various soluble receptors has been shown to correlate with the number or the activity of the cells that express each receptor of the nonsoluble forms. This is probably because both soluble and nonsoluble receptors can be simultaneously produced by the same cells. For example, increased serum concentrations of soluble Epo receptors were observed in individuals with active erythropoiesis after treatment with recombiant Epo (21), and in patients with sickle cell anemia, thalassemia, or megaloblastic anemia. Serum soluble TNF receptors were increased in patients with hairy cell leukemia or chronic lymphocytic leukemia (27). We have also reported that serum concentrations of soluble IL-2 receptors correlate with the severity of graft-vs-host diseases after allogeneic bone marrow transplantation, which reflects the activity of host-derived T cells (44). Consistent with these data, serum concentration of s-CSFR correlated with the numbers of neutrophils/monocytes after G-CSF treatment. Therefore, sG-CSFR may be a useful marker in evaluating the degree of myelopoiesis and the activity of malignant disorders of myeloid lineages. To clarify these issues, the purification of native sG-CSFR is important, to evaluate its neutralizing activity for G-CSF and to generate anti-sG-CSFR mAbs to be used in the ELISA system. This would allow us to assess the clinical relevance of sG-CSFR. It is also possible to generate other mAbs that recognize the amino acid sequences that result from altered reading frame in sG-CSFR mRNA splicing. These studies are ongoing in our laboratory.

Thus, sG-CSFR are physiologically released from mature myelomonocytic cells to the serum. Further studies are necessary to understand the mechanism by which each isoform of sG-CSFR develops, the role of sG-CSFR in myelopoiesis, and the potential usefulness of sG-CSFR measurement in evaluating the activity of various hemopoietic disorders.

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