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# Enhanced circulating half-life and hematopoietic properties of a human granulocyte colony-stimulating factor/immunoglobulin fusion protein

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***Objective.*** **The aim of this study was to determine whether fusion proteins comprising human granulocyte colony-stimulating factor (G-CSF) joined to human immunoglobulin G1 and G4 (IgG1 and IgG4) Fc and $C_H$ domains are biologically active and have improved pharmacokinetic and hematopoietic properties in vivo.**

***Material and Methods.*** **Chimeric genes encoding human G-CSF fused to the N-termini of the Fc and $C_H$ domains of human IgG1 and IgG4 were constructed and used to transfect monkey COS cells. The fusion proteins were purified from the conditioned media by protein A affinity chromatography. Bioactivities of the proteins were measured in a G-CSF–dependent in vitro bioassay. Pharmacokinetic and granulopoietic properties of the G-CSF/IgG1-Fc fusion protein were measured in normal rats.**

***Results.*** **The G-CSF/IgG-Fc and G-CSF/IgG-$C_H$ fusion proteins were secreted from transfected COS cells primarily as disulfide-linked homodimers. On a molar basis, the purified G-CSF/IgG-Fc fusion proteins were as active as G-CSF in in vitro bioassays, whereas bioactivities of the purified G-CSF/IgG-$C_H$ fusion proteins were decreased 3- to 4-fold. The G-CSF/IgG1-Fc fusion protein displayed a slower plasma clearance rate and stimulated greater and longer lasting increases in circulating neutrophils and white blood cells than G-CSF following intravenous and subcutaneous administration to rats.**

***Conclusion.*** **Fusion of G-CSF to human IgG domains results in homodimeric fusion proteins possessing high in vitro bioactivities, long circulating half-lives, and enhanced hematopoietic properties in vivo.** **© 2004 International Society for Experimental Hematology. Published by Elsevier Inc.**

Granulocyte colony-stimulating factor (G-CSF) is a 19-kDa cytokine that stimulates the proliferation, differentiation, and functional activation of cells of the granulocyte lineage. Recombinant methionyl–G-CSF (met–G-CSF) is widely used to ameliorate neutropenia following myelosuppressive chemotherapy and bone marrow transplantation and to mobilize peripheral blood progenitor cells for transplantation and blood banking [1,2]. Recombinant met–G-CSF is cleared rapidly from the body and is administered to patients by daily subcutaneous injection. Development of methods to prolong the circulating half-life, and potentially the therapeutic effectiveness, of G-CSF is of significant interest to patients and healthcare providers.

Previous studies showed that the circulating half-life and in vivo hematopoietic properties of G-CSF can be improved by modification of the protein with polyethylene glycol (PEG), by covalent attachment of G-CSF, via a small PEG linker, to human serum albumin, or by covalent fusion of the amino-terminus of G-CSF to the carboxy-terminus of human serum albumin [3–9]. However, modification of G-CSF with PEG reagents, which attach primarily to lysine residues and the N-terminal amino acid, results in a heterogeneous product mixture with significantly reduced (3- to 50-fold) in vitro biologic activity [3,7]. Loss of in vitro bioactivity is greatest with large PEGs, which are the most useful PEGs for extending a protein's half-life. Less significant (2- to 3-fold) loss of in vitro bioactivity can be achieved by preferential attachment of PEG to the N-terminus of met–G-CSF [5,6]. In vitro bioactivities of the G-CSF/PEG albumin complex and G-CSF/albumin fusion protein were not reported [4,8]. Despite their reduced in vitro biologic activities, the in vivo hematopoietic properties of these

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longer-acting G-CSF conjugates are improved, although potentially not optimized.

Human IgG1 and IgG4 immunoglobulins, which have circulating half-lives of 21 days in humans [10], have been used to create a number of long-acting fusion proteins [11]. Therapeutic proteins typically have been joined via their carboxy-termini to the amino-termini of Fc (Hinge-CH2-CH3) and $C_H$ (CH1-Hinge-CH2-CH3) domains of human IgGs. When expressed in mammalian cells IgG fusion proteins often are secreted as disulfide-linked homodimers due to interchain disulfides that form between cysteine residues located in the IgG Hinge region. The dimeric structure of IgG fusion proteins further increases their effective size and circulating half-lives.

Biologically active IgG fusion proteins have been created most often with the extracellular domains of cell surface receptors [11]. There are fewer reports of proteins that are normally soluble, e.g., growth factors and cytokines, which have been fused to IgG domains and retained significant biologic activity [12,13]. More often, in vitro biologic activity of the fusion protein is significantly reduced relative to the nonfused protein [14,15]. Whether or not a protein will be biologically active when fused to an IgG domain will depend upon whether the C-terminus of the protein is required for biologic activity and whether the protein can bind properly to its receptor as a homodimer. Little is known about the role of the carboxy-terminus in G-CSF biologic activity. In this report, we describe the construction, purification, and in vitro characterization of four G-CSF/IgG fusion proteins. We also compare the pharmacokinetic and hematopoietic properties of one of the fusion proteins to met–G-CSF in normal rats.

## Materials and methods

*Reagents*

RPMI 1640 and Dulbecco's modified Eagle medium (DMEM) media, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Gibco (Grand Island, NY, USA). Restriction endonucleases were purchased from New England BioLabs (Beverly, MA, USA). Oligonucleotides were obtained from Macromolecular Resources (Colorado State University, Fort Collins, CO, USA). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and nitrocellulose filters were obtained from Invitrogen Corporation (Carlsbad, CA, USA).

*Construction of G-CSF/IgG-Fc and G-CSF/IgG-$C_H$ genes*

A cDNA encoding G-CSF [16,17] was amplified by the reverse transcriptase-polymerase chain reaction (RT-PCR) method [18] from total RNA isolated from the human bladder carcinoma cell line 5637 (American Type Culture Collection, Rockville, MD, USA) using forward primer BB91 (5′-CGCAAGCTTGCCACCATGGCTGGACCTGCCACCCAG-3′) and reverse primer BB92 (5′-CGCGGATCCTCCGGAGGGCTGGGCAAGGTGGCGTAG-3′). The PCR product was digested with *Hind*III and *Bam*HI and cloned into similarly cut plasmid pCDNA3.1(+) (Invitrogen), creating pBBT165.

cDNAs encoding IgG1-Fc and IgG4-Fc were amplified by PCR from human leukocyte single-stranded cDNA (CLONTECH, Inc., Palo Alto, CA, USA). IgG1-Fc was amplified using forward primer BB83 (5′-CGCGGATCCGGTGGCTCAGAGCCCAAATCTTGTGACAAAACT-3′) and reverse primer BB82 (5′-CGCTCTAG AGGTACGTGCCAAGCATCCTCG-3′). IgG4-Fc was amplified using forward primer BB84 (5′-CGCGGATCCGGTGGCTCAGAGTCCAAATATGGTCCCCCATGC-3′) and reverse primer BB82. The IgG1-Fc and IgG4-Fc PCR products were digested with *Bam*HI and *Xba*I and cloned into similarly cut pCDNA3.1(+), creating pBBT167 and pBBT158, respectively.

A cDNA encoding IgG1-$C_H$ was amplified from human leukocyte single-stranded cDNA (CLONTECH, Inc.) by PCR using forward primer BB81 (5′-CGCGGATCCGGTGGCTCAGCCTCCACCAAGGGCCCATC-3′) and BB82. The PCR product was digested with *Bam*HI and *Xba*I and cloned into similarly cut pCDNA3.1, creating pBBT166. A cDNA encoding IgG4-$C_H$ was created by changing the DNA sequence of the IgG1 CH1 domain in pBBT166 to match the IgG4 CH1 coding sequence and then joining the IgG4 CH1 domain to the IgG4-Fc domain in a PCR splicing reaction [19]. To construct the IgG4 CH1 sequence, mutagenic primers BB119 (5′-TCCACCAAG GGCCCATCCGTCTTCCCCCTG GCGCCCTGCTCCAGGAGCACCTCCGAGAGCACAGC-3′) and BB120 (5′-TCTCTTGTCCACCTTGGTGTTGCTGGGCTTGTGATCTACGTTGCAGGTGTAGGTCTTCGTGCCCAA-3′) were used in a PCR reaction with pBBT166. The purified PCR product was used as template in a second PCR reaction with primers BB81 and BB121 (5′-TGGGGGACCATATTTGGACTCAACTCTCTTGTCCACCTT-3′). The product of this PCR reaction was used as one of the template molecules in the PCR splicing reaction. The other template for the splicing reaction was generated by PCR of the cloned IgG4-Fc sequence in pBBT158 with primers BB85 (5′-TCCAAATATGGTCCCCCATGCCCATCA-3′) and BB82. The PCR splicing reaction used primers BB81 and BB82 and generated a full-length IgG4-$C_H$ spliced product. This PCR fragment was digested with *Bam*HI and *Sac*II and the 530-bp fragment containing the CH1 and Hinge domains and a portion of the CH2 domain was cloned into pBC-SK+ (Stratagene, Inc., La Jolla, CA, USA), creating plasmid pBBT182.

DNA sequences encoding the IgG1-Fc, IgG4-Fc, and IgG1-$C_H$ domains were excised from appropriate pCDNA3.1(+) plasmids as *Bam*HI–*Xba*I fragments and cloned into similarly cut plasmid pBBT165. The G-CSF/IgG4-$C_H$ fusion was constructed by replacing the 240-bp *Bam*HI–*Sac*II fragments of the G-CSF/IgG4-Fc clone with the 530-bp *Bam*HI–*Sac*II fragment of pBBT182. DNA sequences of all clones were confirmed [20,21].

*Expression and purification of G-CSF/IgG fusion proteins*

COS-1 cells (American Type Culture Collection) were transfected with plasmid DNAs encoding the fusion proteins in T-75 flasks using LipofectAMINE reagent (Invitrogen). After washing, the transfected cells were grown in DMEM media supplemented with 50 units/mL penicillin, 50 μg/mL streptomycin, and 2mM glutamine at 37°C, 5% $CO_2$ in a humidified tissue culture incubator. Conditioned media were harvested on days 3, 6, 9, and 12 and stored at −80°C.

Approximately 300 mL of conditioned media for each fusion protein was pooled and concentrated using an Ultrafiltration cell and either a YM3 or YM30 DIAFLO Ultrafiltration membrane (Amicon, Beverly, MA, USA). Concentrated pools were loaded

onto a 1-mL HiTrap recombinant protein A column (Amersham-Pharmacia, Piscataway, NJ, USA) that previously had been equilibrated with 20 mM sodium phosphate at pH 7.0. Bound proteins were eluted with 100 mM sodium citrate at pH 3.0 and collected into sufficient volume of 1M TRIS at pH 9.0 to achieve a final pH of approximately 7.0. Protein samples were prepared in SDS-PAGE sample buffer with (reducing) or without (nonreducing) the addition of 1–5% (V/V) β-mercaptoethanol, electrophoresed on precast 14% or 8–16% TRIS-glycine polyacrylamide gels (Invitrogen), and stained with Coomassie blue (Bio-Rad Laboratories, Richmond, CA, USA). Western blots used polyclonal goat anti-human G-CSF antisera (R&D Systems, Inc., Minneapolis, MN, USA) and an alkaline phosphatase-conjugated rabbit anti-goat IgG secondary antibody (Pierce Chemical Company, Rockford, IL, USA).

*In vitro bioassay*

The murine NFS-60 cell line [22] was maintained in RPMI 1640 media supplemented with 10% FBS, 50 units/mL penicillin, 50 μg/mL streptomycin, 2 mM glutamine, and 17 to 170 units/mL mouse interleukin-3 (IL-3; R&D Systems, Inc.). For bioassays, NFS-60 cells were washed and resuspended at a concentration of $1 \times 10^5$/mL in cell maintenance media minus IL-3. A total of 50 μL ($5 \times 10^3$ cells) of the cell suspension was aliquoted per test well of a flat-bottom 96-well tissue culture plate. Serial 3-fold dilutions of the protein samples were prepared in maintenance media minus IL-3. Serial dilutions of recombinant human met–G-CSF (*Escherichia coli*-expressed; R&D Systems) were analyzed in parallel. At total of 50 μL of the diluted protein samples was added to the test wells and the plates incubated at 37°C in a humidified 5% $CO_2$ tissue culture incubator. Protein samples were assayed in triplicate wells. After 3 days, 20 μL of CellTiter 96 AQueous One Solution (Promega Corporation, Madison, WI, USA) was added to each well and the plates incubated at 37°C in the tissue culture incubator for 1 to 4 hours. Absorbance of the wells was read at 490 nm using a microplate reader. Control wells contained media but no cells. Mean absorbance values for the triplicate control wells were subtracted from mean values obtained for the test wells.

*Pharmacokinetic and efficacy experiments*

Experiments were performed with the approval of BolderPATH's Institutional Animal Care and Use Committee. Male Sprague Dawley rats (weight 310–365g) were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA). Groups of three rats received a single intravenous (lateral tail vein) or subcutaneous (lateral side) injection of recombinant met–G-CSF (Neupogen, Amgen, Inc., Thousand Oaks, CA, USA) or G-CSF/IgG1-Fc, each at a dose of 100 μg/kg total protein. Protein concentrations were determined using a Bradford dye binding assay kit (Bio-Rad Laboratories) and bovine serum albumin as the standard. At selected time points, blood samples (0.4 mL) were drawn from the rats into EDTA anticoagulant tubes. A portion of the blood sample was used for a complete blood cell count analysis (performed by IDEXX Laboratories, Inc., Irvine, CA) for the intravenous study and by Antech Diagnostics, Inc. (Irvine, CA, USA) for the subcutaneous study. The remainder of the blood sample was centrifuged and the plasma frozen at −80°C. Blood samples were drawn at 0.25, 1.5, 4, 8, 12, 16, 24, 48, 72, 96, 120, and 144 hours postinjection. A predose blood sample was drawn 1 day prior to injection of test compounds. Rats were anesthetized with isoflurane prior to blood sampling and intravenous injections. Plasma levels of the test proteins were quantitated using Quantikine human G-CSF ELISA kits (R&D Systems), which do not detect rat G-CSF. Serial dilutions of plasma samples from one rat in each test group were analyzed initially in the in vitro bioassay to identify dilutions expected to fall within the linear range of the ELISA kits (39–2,500 pg/mL). Duplicate samples of appropriate dilutions of plasma samples from all rats then were analyzed in the enzyme-linked immunosorbent assays (ELISAs). ELISA values were adjusted based upon standard curves prepared for each test protein. Rats were euthanized by cervical dislocation while under isoflurane anesthesia. At necropsy, the spleen, kidney, and sternum from each animal were removed and collected in 10% neutral buffered formalin. The sternums were decalcified in 5% formic acid and processed with the spleens and kidneys for histopathologic analysis. Paraffin sections were stained with hematoxylin and eosin. Statistical analyses between samples were compared using a Student's *t*-test, with significance set at $p < 0.05$.

## Results

Fusion proteins comprising human G-CSF joined via a 7-amino-acid flexible linker (SerGlyGlySerGlyGlySer) to the N-termini of the Fc and $C_H$ domains of human IgG1 and IgG4 were constructed (Fig. 1). The proteins were expressed by transfection of COS cells and purified from the conditioned media by protein A affinity chromatography, yielding a single peak in the elution step. Fractions across the major protein peak were analyzed by SDS-PAGE and

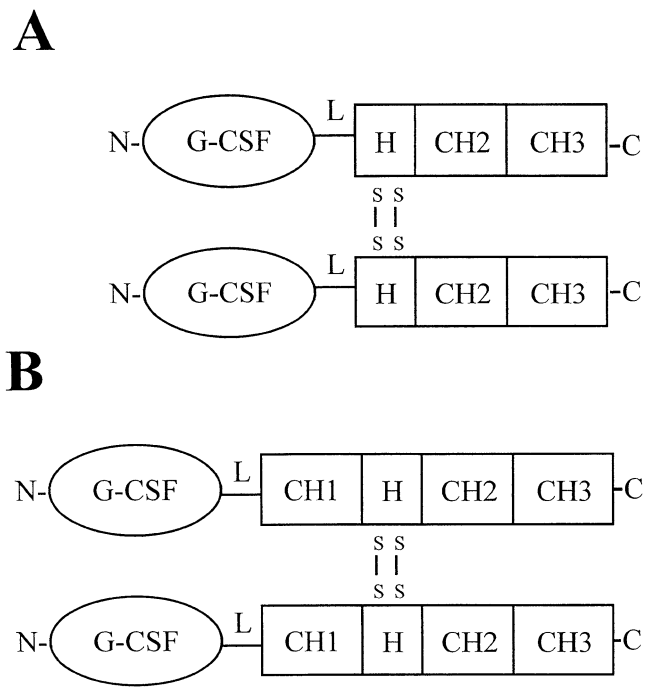


**Figure 1.** Schematic diagram of **(A)** G-CSF/IgG-Fc and **(B)** G-CSF/IgG-$C_H$ fusion proteins. The carboxy-terminus of G-CSF is joined via a seven amino acid linker (L) to the amino termini of the IgG-Fc and IgG-$C_H$ domains. The Hinge (H) and CH1, CH2, and CH3 regions of the IgG domains are indicated. The fusion proteins are dimeric due to disulfide bonds (S-S) that form between cysteine residues located in the IgG Hinge region.

column fractions enriched for the IgG fusion proteins were pooled. Figure 2 shows reducing and nonreducing SDS-PAGE analysis of the four purified G-CSF/IgG fusion proteins. The G-CSF/IgG fusion proteins were recovered principally as disulfide-linked dimers, presumably joined through disulfide bond(s) between cysteine residues in the Hinge regions of the IgG domains. The apparent molecular weights of the major G-CSF/IgG-Fc and G-CSF/IgG-$C_H$ fusion protein bands were approximately 50,000 and 60,000, respectively, under reducing SDS-PAGE conditions and 120,000 and 190,000, respectively, under nonreducing conditions. The apparent molecular weights of the reduced IgG-Fc and IgG-$C_H$ fusion proteins were consistent with their predicted molecular weights (Table 1), whereas the apparent molecular weights of the nonreduced IgG-Fc and IgG-$C_H$ fusion protein dimers (120,000 and 190,000, respectively) were significantly greater than their predicted molecular weights (90,000 and 110,000, respectively). The major monomeric and dimeric G-CSF/IgG fusion protein bands reacted with polyclonal antisera specific for G-CSF in Western blots of the samples (data not shown). When analyzed by nonreducing SDS-PAGE, minor protein bands with apparent molecular weights expected for monomeric fusion proteins (50,000–60,000) were observed in all of the purified protein samples but were more abundant with the IgG4 fusion proteins (Fig. 2). Western blots of the samples indicated that the minor protein bands reacted with anti–G-CSF antisera (data not shown), suggesting that they represent monomeric fusion protein or nondisulfide-bonded dimeric fusion protein present in the samples.

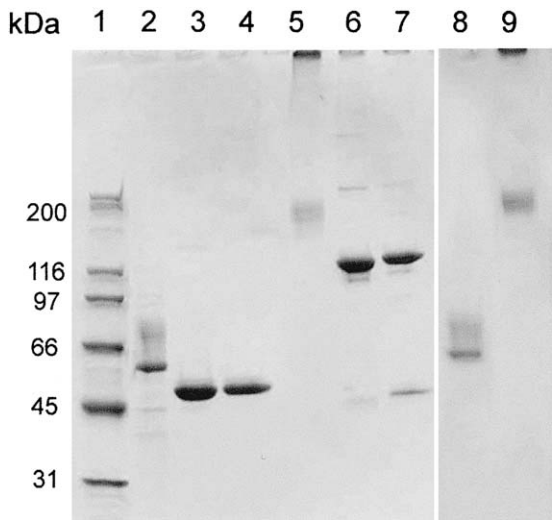


**Figure 2.** SDS-PAGE analysis of purified G-CSF/IgG fusion proteins. Lane 1 = molecular weight standards; lane 2 = G-CSF/IgG1-$C_H$ reduced; lane 3 = G-CSF/IgG1-Fc reduced; lane 4 = G-CSF/IgG4-Fc reduced; lane 5 = G-CSF/IgG1-$C_H$ nonreduced; lane 6 = G-CSF/IgG1-Fc nonreduced; lane 7 = G-CSF/IgG4-Fc nonreduced; lane 8 = G-CSF/IgG4-$C_H$ reduced; lane 9, G-CSF/IgG4-$C_H$ nonreduced. Lanes 2 to 9 contains 2 μg of protein.

**Table 1.** In vitro bioactivities of G-CSF/IgG fusion proteins

| Protein | Predicted MW* | | Mean $EC_{50}$† | |
|---|---|---|---|---|
| | Monomer | Dimer | pg/mL | pM‡ |
| met–G-CSF | 18,987 | — | 17.7 ± 0.6 | 0.93 |
| G-CSF/IgG1-Fc | 45,551 | 91,102 | 38.3 ± 4.0 | 0.84 |
| G-CSF/IgG4-Fc | 45,222 | 90,444 | 56.7 ± 5.9 | 1.25 |
| G-CSF/IgG1-$C_H$ | 55,564 | 111,128 | 182 ± 18.9 | 3.2 |
| G-CSF/IgG4-$C_H$ | 55,399 | 110,798 | 215 ± 21.2 | 3.9 |

*Does not include the molecular weight contribution due to glycosylation.

†Mean ± SD from three assays, except for the G-CSF/IgG4-$C_H$ fusion protein for which only two assays were performed.

‡Calculated using monomer molecular weights for the fusion proteins.

Both G-CSF/IgG-$C_H$ fusion proteins contained a large aggregate that migrated at the top of the gel when the samples were electrophoresed under nonreducing conditions (Fig. 2). The aggregates disappeared, and the amount of protein at the positions of the major G-CSF/IgG-$C_H$ bands increased proportionately, when the samples were electrophoresed under reducing conditions (Fig. 2). The aggregates reacted with anti–G-CSF antisera (data not shown), suggesting that they represent disulfide-linked oligomers of the G-CSF/IgG-$C_H$ fusion proteins.

*In vitro bioactivities of G-CSF/IgG fusion proteins*

In vitro bioactivities of the G-CSF/IgG fusion proteins were measured using the G-CSF–dependent mouse NFS-60 cell line. Representative assay results for the G-CSF/IgG1-Fc and G-CSF/IgG1-$C_H$ fusion proteins are shown in Fig. 3. Results for the G-CSF/IgG4-Fc and G-CSF/IgG4-$C_H$ fusion proteins were similar. The met–G-CSF standard had an $EC_{50}$ of 18 pg/mL (Table 1), which is similar to the $EC_{50}$ reported by the manufacturer (10–30 pg/mL; R&D Systems). The G-CSF/IgG1-Fc and G-CSF/IgG4-Fc fusion proteins had $EC_{50}$s of 38 and 57 pg/mL, respectively (Fig. 3 and Table 1). On a molar basis (using monomer molecular weights for the fusion proteins), the $EC_{50}$ values of met–G-CSF, G-CSF/IgG1-Fc, and G-CSF/IgG4-Fc fusion protein were similar, approximately 1 pM (Table 1). In contrast, the G-CSF/IgG1-$C_H$ and G-CSF/IgG4-$C_H$ fusion proteins had $EC_{50}$s of 182 pg/mL (3.2 pM) and 215 pg/mL (3.9 pM), respectively, which represent a 3- to 4-fold reduction in specific activity relative to the G-CSF/IgG-Fc fusion proteins and the met–G-CSF standard (Fig. 3 and Table 1). $EC_{50}$ values for the G-CSF/IgG-Fc and G-CSF/IgG-$C_H$ fusion proteins would be approximately 0.5 pM and 1.8 pM, respectively, if dimer molecular weights were used for the calculations.

*Pharmacokinetic and efficacy experiments in normal rats*

Circulating levels of the G-CSF/IgG1-Fc fusion protein and recombinant met–G-CSF were determined following a single intravenous or subcutaneous injection into Sprague-Dawley rats, each at a dose of 100 μg/kg total protein. On a molar basis, rats receiving met–G-CSF received approximately 2.5 times as much G-CSF as the rats receiving

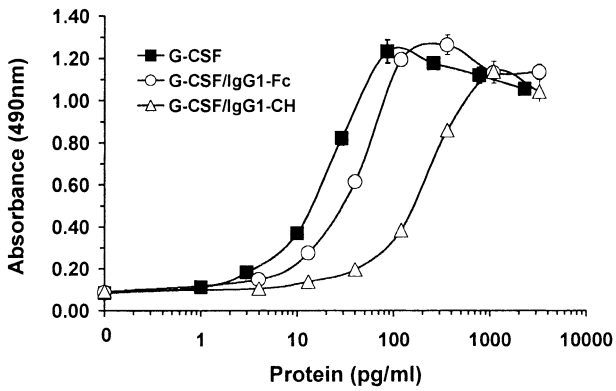


**Figure 3.** Representative dose-response curves for G-CSF/IgG1-Fc, G-CSF/IgG1-$C_H$, and met–G-CSF for stimulating proliferation of NFS-60 cells. Data are given as mean ± SD for triplicate wells.

G-CSF/IgG1-Fc. Blood samples were drawn at various times postinjection and plasma levels of met–G-CSF and the G-CSF/IgG1-Fc protein quantitated by ELISA. Plasma samples from 0.25 to 48 hours postinjection from one rat in each test group were tested initially in the in vitro bioassay to determine whether the circulating proteins were biologically active and to obtain an estimate of their approximate concentrations. There was an excellent correlation between protein concentrations estimated by the in vitro biologic activity and ELISA for all of the samples.

Figure 4, panels A, B, C, and D, show mean plasma G-CSF protein levels, blood neutrophil counts, total white blood cell counts, and red blood cell counts, respectively, for the different test groups given intravenous injections of the test compounds. met–G-CSF cleared rapidly from the rats, with a $t_{1/2}$ of 1.8 hours, which is in good agreement with the value of 1.79 hours previously reported [3]. In contrast, the G-CSF/IgG1-Fc fusion protein was cleared much more slowly. Elimination of the fusion protein was biphasic, with an elimination $t_{1/2}$ of 9.3 hours during the initial 24 hours postinjection, followed by a more rapid elimination phase ($t_{1/2}$ of 4.2 hours) between 24 and 72 hours postinjection. The initial distribution phase (0–1.5 hours) also appeared to be slower for G-CSF/IgG1-Fc than met–G-CSF, but more

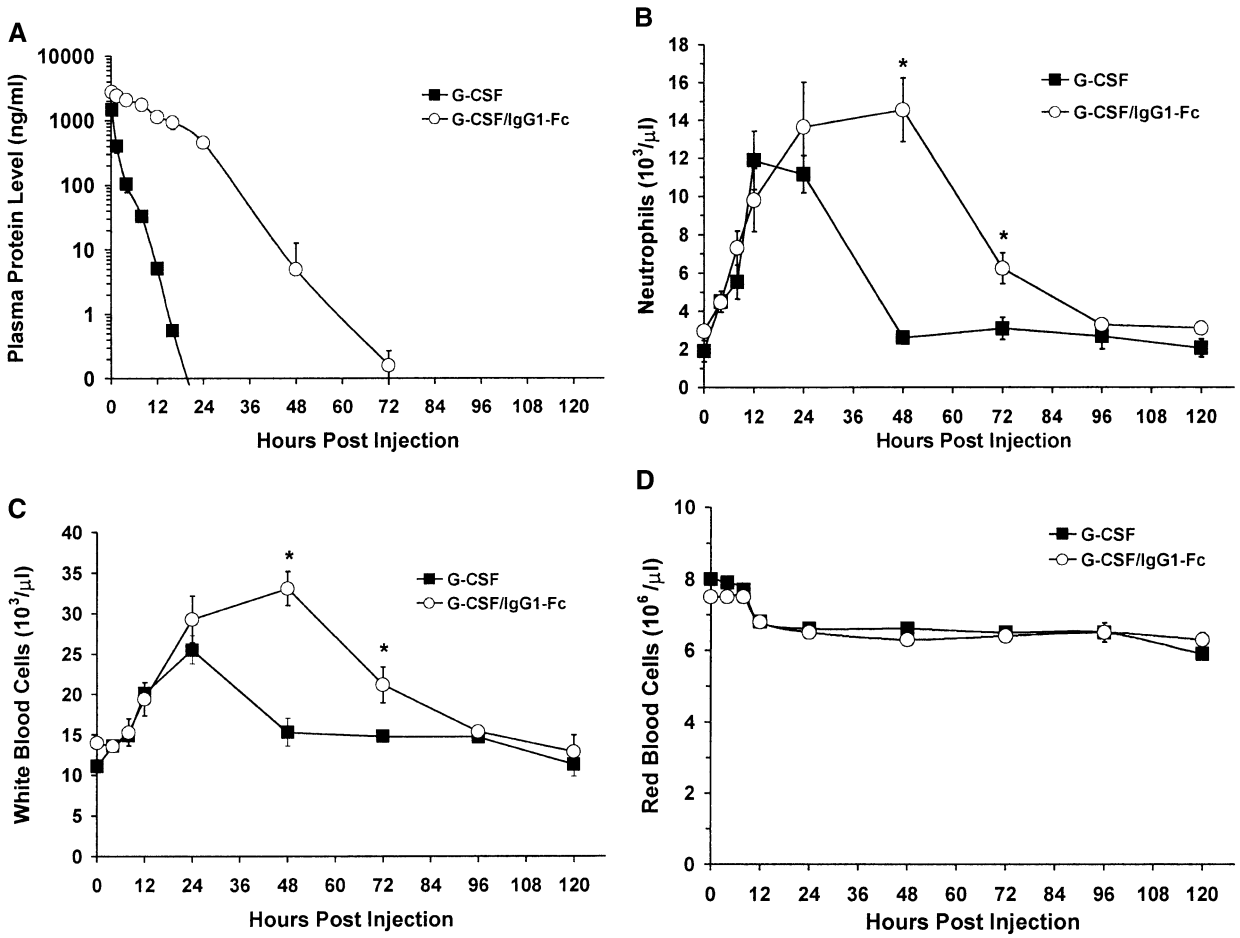


**Figure 4.** Changes in **(A)** plasma protein levels, **(B)** blood neutrophil counts, **(C)** white blood cell counts, and **(D)** red blood cell counts following intravenous administration of G-CSF/IgG1-Fc and met–G-CSF to rats. Data are given as mean ± SD **(A)** or SE **(B, C, D)** for three rats per group. Asterisks denote neutrophil and white blood cell values that are significantly different between animals treated with G-CSF/IgG1-Fc and met–G-CSF ($p < 0.05$).

early sampling time points are needed to quantitate this precisely. Both G-CSF and G-CSF/IgG1-Fc stimulated time-dependent increases in peripheral white blood cells and neutrophils over baseline values. The rates of increases in blood neutrophils were similar for the various test groups in the first 12 hours postinjection. White blood cell and neutrophil counts for the test group receiving met–G-CSF peaked 12 hours postinjection and returned to baseline values by 48 hours. In contrast, white blood cell and neutrophil counts for the rats receiving G-CSF/IgG1-Fc peaked around 48 hours postinjection and did not return to baseline values until 96 hours postinjection. White blood cell counts and neutrophil counts for the animals receiving G-CSF/IgG1-Fc were significantly higher than the corresponding values for animals receiving met–G-CSF at 48 and 72 hours postinjection ($p < 0.05$). Red blood cell counts in both test groups decreased by 14–17% during the first 24 hours postinjection and remained at this reduced level for the remainder of the experiment (Fig. 4D). The reduction in red blood cell numbers may be a result of the frequent blood sampling and amount of blood drawn from the animals during the first 24 hours postinjection. No significant changes in platelet numbers were noted for animals in either test group (data not shown).

Figure 5, panels A, B, C, and D, show the mean plasma protein levels, blood neutrophil counts, white blood cell counts, and red blood cell counts, respectively, for the rats receiving subcutaneous injection of G-CSF/IgG1-Fc and met–G-CSF. Plasma levels of met–G-CSF peaked at 183 ng/mL 2 hours postinjection and dropped to undetectable levels (<39 pg/mL) by 24 hours postinjection. The terminal half-life for met–G-CSF was estimated to be 2.6 hours. In contrast, G-CSF/IgG1-Fc was absorbed more slowly and reached peak plasma levels of 53 ng/mL 24 hours postinjection. G-CSF/IgG1-Fc also was cleared more slowly, with a terminal half-life of 20.4 hours. At 144 hours postinjection, mean plasma levels of G-CSF/IgG1-Fc were 600 pg/mL, well above its in vitro $EC_{50}$ value of 38 pg/mL. Blood

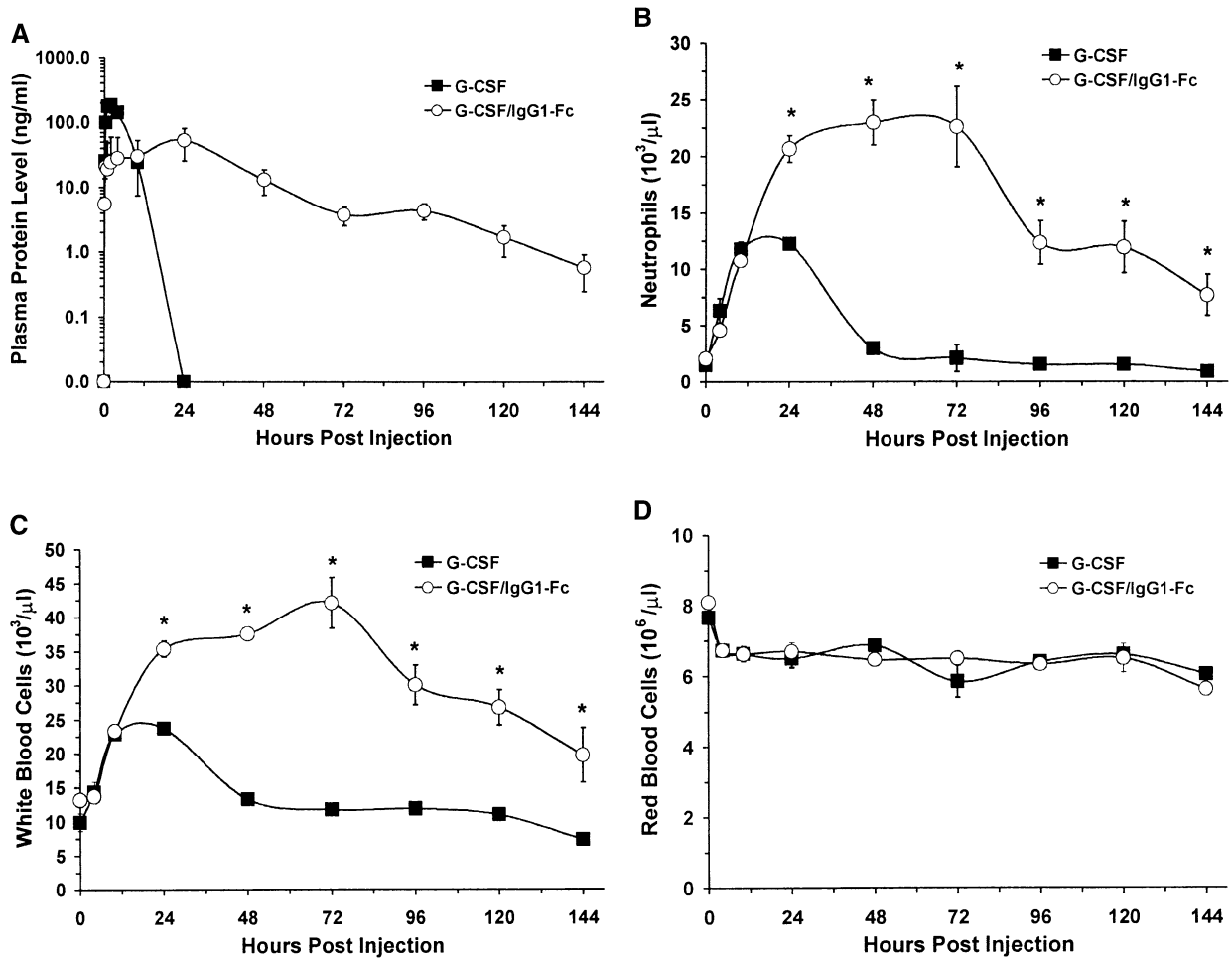


**Figure 5.** Changes in **(A)** plasma protein levels, **(B)** blood neutrophil counts, **(C)** white blood cell counts, and **(D)** red blood cell counts following subcutaneous administration of G-CSF/IgG1-Fc and met–G-CSF to rats. Data are given as mean ± SD **(A)** or SE **(B, C, D)** for three rats per group. Asterisks denote neutrophil and white blood cell values that are significantly different between animals treated with G-CSF/IgG1-Fc and met–G-CSF ($p < 0.05$).

neutrophil and white blood cell counts mirrored these results. In the animals receiving met–G-CSF, blood neutrophil levels and white blood cell counts peaked 24 hours postinjection and returned to baseline values by 48 hours. In contrast, in the animals receiving G-CSF/IgG1-Fc, blood neutrophil and white blood cell counts did not peak until 48–72 hours postinjection and were still significantly above baseline values at the termination of the experiment at 144 hours. Blood neutrophil and white blood cell counts in the animals receiving G-CSF/IgG1-Fc were significantly greater than the corresponding values for animals receiving met–G-CSF from 24 to 144 hours postinjection ($p < 0.05$). Red blood cell numbers decreased by 15–20% in all animals during the first 12 hours postinjection but showed no further changes for the remainder of the study (Fig. 5D). Platelets were not quantitated in this experiment. Analysis of histologic sections of bone marrow taken from the animals at necropsy (144 hours) showed marked granulopoiesis, with large numbers of fully mature, segmented neutrophils in the G-CSF/IgG1-Fc treated animals (myeloid/erythroid ratio of 9:1), whereas normal histology (myeloid/erythroid ratio of about 1:1) was noted for the met–G-CSF treated animals (Fig. 6). No obvious signs of toxicity in the bone marrow, spleen, or kidney were observed in animals from either test group.

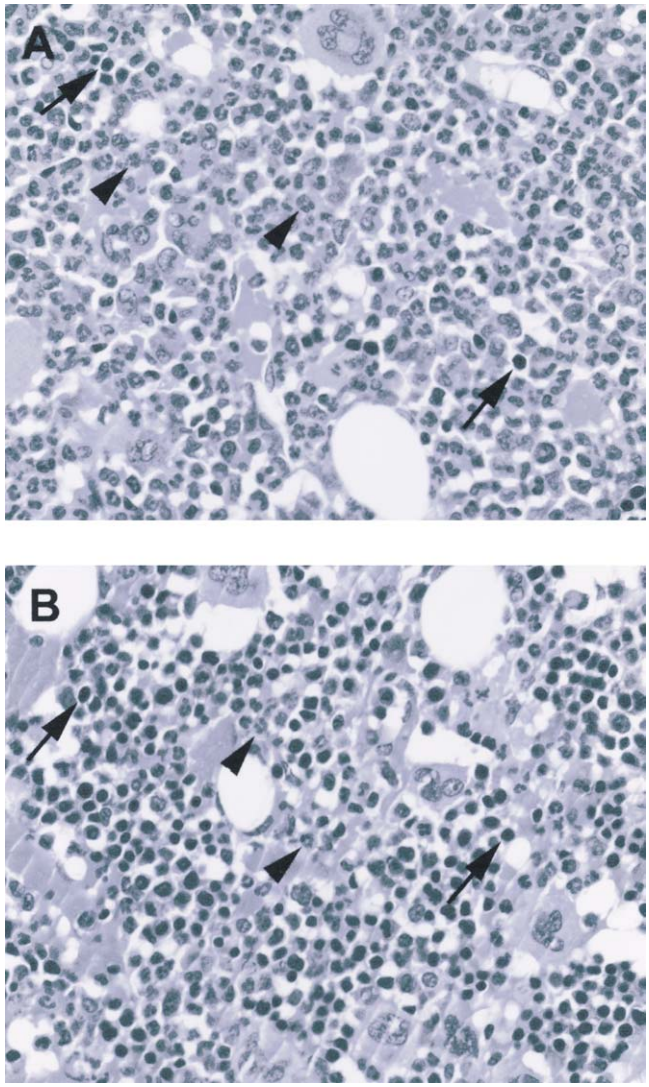


**Figure 6.** Photomicrographs of histologic sections from bone marrow of rats 6 days following a single subcutaneous injection of 100 μg/kg of **(A)** G-CSF/IgG1-Fc or **(B)** met–G-CSF. Animals treated with G-CSF/IgG1-Fc exhibit marked granulopoiesis, with a significantly greater proportion of granulocyte precursors (arrowheads) compared to erythrocyte precursors (arrows). Animals treated with met–G-CSF exhibit a normal bone marrow appearance, with approximately equal numbers of granulocyte and erythrocyte precursors (original magnification ×40). (Photographs courtesy of Dr. Stephen Ruby.)

## Discussion

Our results demonstrate that human G-CSF can be fused to human IgG1-Fc and IgG4-Fc domains without significantly affecting in vitro biologic activity of G-CSF. On an absolute weight basis, in vitro bioactivities ($EC_{50}$ values) of the G-CSF/IgG-Fc fusion proteins are reduced 2- to 3-fold relative to G-CSF, whereas on a molar basis $EC_{50}$ values of the fusion proteins are equal to, or 2-fold lower, i.e., more potent, than that of G-CSF, depending upon whether monomer or dimer molecular weights of the fusion proteins, respectively, were used in the calculations. Because there are two G-CSF molecules per fusion protein dimer, G-CSF/IgG1-Fc and G-CSF appear to be equipotent when compared on a G-CSF molar basis. The high specific activities of the G-CSF/IgG-Fc fusion proteins suggests that both G-CSF proteins in each G-CSF/IgG-Fc dimer may be capable of binding and activating cell surface G-CSF receptors. The fusion proteins are secreted from mammalian cells principally as disulfide-linked homodimers, presumably joined by intermolecular disulfide bonds formed between cysteine residues located in the Hinge regions of the IgG domains. Dimerization of the G-CSF/IgG-Fc fusion proteins increases their effective size without apparently affecting in vitro biologic activities of the proteins. By nonreducing SDS-PAGE, the apparent molecular weight of the G-CSF/IgG1-Fc fusion protein dimer is approximately 6-fold greater than that of G-CSF (120,000 vs 19,000). As expected for a larger protein, the G-CSF/IgG1-Fc fusion protein has a longer circulating half-life (5- to 8-fold) than G-CSF following intravenous and subcutaneous administration to rats. The G-CSF/IgG1-Fc fusion protein stimulates significantly greater and longer-lasting increases in circulating neutrophils and white blood cells than met–G-CSF, even when administered at a lower molar dose. The increases in circulating neutrophils stimulated by G-CSF/IgG1-Fc are reflected by morphologic changes in the bone marrow, which still display markedly enhanced granulopoiesis 6 days following a single subcutaneous injection of G-CSF/IgG1-Fc. Thus, the longer circulating half-life of the G-CSF/IgG1-Fc fusion protein allows

the G-CSF moiety to exert its biologic effects for a longer period of time, enhancing its therapeutic benefits.

The circulating half-life of the G-CSF/IgG1-Fc fusion protein compares favorably to that of PEG-modified G-CSF and G-CSF coupled via a PEG linker to albumin, which have 4- to 6-fold longer half-lives than G-CSF in rodents [3,4,7]. Half-life of the G-CSF/IgG1-Fc fusion protein appears to be longer than that of the G-CSF-albumin fusion protein, which has a 2-fold longer half-life than G-CSF in mice [8]. These conclusions must be considered tentative, however, because half-lives of the various G-CSF conjugates have not been measured directly in the same experiment. Clearance of the G-CSF/IgG1-Fc fusion protein accelerates when neutrophil levels become elevated, similar to what has been reported for G-CSF [23]. This effect is less pronounced when G-CSF/IgG1-Fc is administered by subcutaneous injection. The more rapid clearance of the fusion protein when neutrophil levels are elevated likely is due to receptor-mediated endocytosis by neutrophils and bone marrow cells, which are known to express G-CSF receptors [23]. Receptor-mediated endocytosis has been identified as a major mechanism regulating G-CSF metabolism and neutrophil homeostasis in vivo [23].

We constructed both IgG1-Fc and IgG4-Fc fusion proteins because the Hinge regions of these two IgG isotypes differ in size and flexibility [24] and because the IgG1-Fc fusion protein potentially could activate complement and be toxic in vivo (IgG4 has a reduced ability to activate complement compared to IgG1 [10]). However, we found that the IgG1-Fc and IgG4-Fc fusion proteins had comparable in vitro biologic activities, suggesting that the size and flexibility of the IgG1 and IgG4 Hinge regions do not play significant roles in determining bioactivity of the attached G-CSF domain. We also observed no signs of systemic or tissue toxicity with the G-CSF/IgG1-Fc fusion protein in the rat efficacy studies.

In contrast to the results obtained with the G-CSF/IgG-Fc fusion proteins, the G-CSF/IgG-$C_H$ fusion proteins displayed 3- to 4-fold reduced in vitro biologic activities relative to G-CSF, apparently due to significant amounts of disulfide-linked aggregates/oligomers in the samples. We have encountered this problem with several other cytokine/IgG-$C_H$ fusion proteins (unpublished results). Other researchers have not reported significant aggregation of IgG-$C_H$ fusion proteins [12,25]. The CH1 domain present in the IgG-$C_H$ constructs contains a hydrophobic interface that normally associates with the immunoglobulin light chain [9]. It is possible this hydrophobic surface is responsible for aggregation of the proteins. G-CSF also is attached to the CH1 domain, rather than to the Hinge region, in the IgG-$C_H$ constructs, which may contribute to the lower activity of these fusion proteins.

G-CSF has a four-helix bundle structure that is shared by a number of other cytokines and growth factors [26–28]. Critical receptor binding regions in G-CSF have been localized to the first and fourth α-helical regions and the loop region joining the first and second α-helices [17,29–31]. G-CSF contains only two amino acids, Q173 and P174, following the final α-helix. These two amino acids appear to have an unordered structure [26]. Our finding that G-CSF/IgG-Fc fusion proteins possess essentially complete in vitro biologic activity suggests that the extreme carboxy-terminus of G-CSF is largely nonessential for G-CSF bioactivity. The long circulating half-life and enhanced hematopoietic properties of G-CSF/IgG-Fc make it a promising candidate for future testing in humans.

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## References

1. Anderlini P, Przepiorka D, Champlin R, Korbling M. Biologic and clinical effects of granulocyte colony-stimulating factor in normal individuals. Blood. 1996;88:2819–2825.
2. Welte K, Gabrilova J, Bronchud MH, Platzer E, Morstyn G. Filgrastim (r-metHuG-CSF): the first 10 years. Blood. 1996;88:1907–1929.
3. Tanaka H, Satake-Ishikawa R, Ishikawa M, Matsuki S, Asano K. Pharmacokinetics of recombinant human granulocyte colony-stimulating factor conjugated to polyethylene glycol in rats. Cancer Res. 1991;51:3710–3714.
4. Page AG, Whitcomb KL, Liu J, Kinstler O. Prolonged circulation of recombinant human granulocyte-colony-stimulating factor by covalent linkage to albumin through a heterobifunctional polyethylene glycol. Pharm Res. 1995;12:1883–1888.
5. Kinstler OB, Brems DN, Lauren SL, Paige AG, Hamburger JB, Treuheit MJ. Characterization and stability of N-terminally PEGylated rhG-CSF. Pharm Res. 1996;13:996–1002.
6. Gaertner HF, Offord RE. Site-specific attachment of functionalized poly(ethylene glycol) to the amino terminus of proteins. Bioconjug Chem. 1996;7:38–44.
7. Bowen S, Tare N, Inoue T, et al. Relationship between molecular mass and duration of activity of polyethylene glycol conjugated granulocyte colony-stimulating factor mutein. Exp Hematol. 1999;27:425–432.
8. Halpern W, Riccobene TA, Agostini H, et al. Albugranin™, a recombinant human granulocyte colony stimulating factor (G-CSF) genetically fused to recombinant human albumin induces prolonged myelopoietic effects in mice and monkeys. Pharm Res. 2002;19:1720–1729.
9. Molineux G, Kinstler O, Briddell B, et al. A new form of filgrastim with sustained duration in vivo and enhanced ability to mobilize PBPC in both mice and humans. Exp Hematol. 1999;27:1724–1734.
10. Roitt I, Brostoff J, Male D. Immunology. London: Gower Medical Publishing; 1989.
11. Chamow SM, Ashkenazi A. Immunoadhesins: principles and applications. Trends Biotech. 1996;14:52–60.
12. Landolfi NF. A chimeric IL-2/Ig molecule possesses the functional activity of both proteins. J Immunol. 1991;146:915–919.
13. Zeng XX, Steele AW, Nickerson PW, Steurer W, Steiger J, Strom TB. Administration of noncytolytic IL-10/fc in murine models of

lipopolysaccharide-induced septic shock and allogeneic islet cell transplantation. J Immunol. 1995;154:5590–5600.
14. LaRochelle WJ, Dirsch OR, Finch PW, et al. Specific receptor detection by a functional keratinocyte growth factor-immunoglobulin chimera. J Cell Biol. 1995;129:357–366.
15. Dikov MM, Reich MB, Dworkin L, Thomas JW, Miller GG. A functional fibroblast growth factor-1 immunoglobulin fusion protein. J Biol Chem. 1998;273:15811–15817.
16. Souza LM, Boone TC, Gabrilove J, et al. Recombinant human granulocyte colony-stimulating factor: effects on normal and leukemic myeloid cells. Science. 1986;232:61–65.
17. Nagata S, Tsuchiya M, Asano S, et al. The chromosomal gene structure and two mRNAs for human granulocyte colony-stimulating factor. EMBO J. 1986;5:575–581.
18. Kawasaki ES. Amplification of RNA. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. PCR Protocols. San Diego: Academic Press; 1990. p. 21.
19. Horton RM. In vitro recombination and mutagenesis of DNA. In: White BA, ed. Methods in Molecular Biology. Totawa, NJ: Humana Press; 1993;15:251–266.
20. Ellison J, Buxbaum J, Hood L. The nucleotide sequence of a human immunoglobulin C gamma 4 gene. DNA. 1981;1:11–18.
21. Ellison JW, Berson BJ, Hood LE. The nucleotide sequence of a human immunoglobulin C gamma 1 gene. Nucleic Acids Res. 1982;10:4071–4079.
22. Shirafuji N, Asano S, Matsuda S, Watari K, Takaku F, Nagata S. A new bioassay for human granulocyte colony-stimulating factor (hG-CSF) using murine myeloblastic NFS-60 cells as targets and estimation of its levels in sera from normal healthy persons and patients with infectious and hematological disorders. Exp Hematol. 1989;17:116–119.
23. Kuwabara T, Kato Y, Kobayashi S, Suzuki H, Sugiyama Y. Nonlinear pharmacokinetics of a recombinant human granulocyte colony-stimulating factor derivative (nartograstim): species differences among rats, monkeys and humans. JPET. 1994;271:1535–1543.
24. Dangl JL, Wensel TG, Morrison SL, Stryer L, Herzenberg LA, Oi VT. Segmental flexibility and complement fixation of genetically engineered chimeric human, rabbit and mouse antibodies. EMBO J. 1988;7:1989–1994.
25. Capon DJ, Chamow SC, Mordenti J, et al. Designing CD4 immunoadhesins for AIDS therapy. Nature. 1989;337:525–531.
26. Hill CP, Osslund TD, Eisenberg D. The structure of granulocyte-colony-stimulating factor and its relationship to other growth factors. Proc Natl Acad Sci USA. 1993;90:5167–5171.
27. Bazan F. Haemopoietic receptors and helical cytokines. Immunol Today. 1990;11:350–354.
28. Mott RM, Campbell ID. Four-helix bundle growth factors and their receptors: protein-protein interactions. Curr Opin Struct Biol. 1995;5:114–121.
29. Kuga T, Komatsu Y, Yamasaki M, et al. Mutagenesis of human granulocyte colony stimulating factor. Biochem Biophys Res Commun. 1989;159:103–111.
30. Kubota N, Orita T, Hattori K, Oh-eda M, Ochi N, Yamazaki T. Structural characterization of natural and recombinant human granulocyte colony-stimulating factors. J Biochem. 1990;107:486–492.
31. Layton JE, Morstyn G, Fabri LJ, et al. Identification of a functional domain of human granulocyte colony-stimulating factor using neutralizing monoclonal antibodies. J Biol Chem. 1991;266:23815–23823.