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A Novel Recombinant Human Granulocyte Colony-Stimulating Factor (G-CSFa) Enhances Peripheral Platelet Recovery in Mice Exposed to Radiotherapy and Has No Immunogenicity in Rats

Keyong Shi[1,3#], Zhenwang Jie[1,2#], Chang Chu[2], Qing-Yu Zhang[2], Xinxin Ding[2] and Yongping Jiang[1,2*]

[1]*Biopharmagen Corporation, Suzhou, 215126, China*
[2]*Biopharmaceutical R&D Center, Chinese Academy of Medical Sciences & Peking Union Medical College, Suzhou, 215126, China*
[3]*State Key Laboratory of Experimental Hematology, Institute of Hematology, Chinese Academy of Medical Sciences & Peking Union Medical College, Tianjin, 300020, China*
[#]*These authors contributed equally to this study*

Abstract

Using site-direct mutagenesis and recombinant DNA technology, we had previously obtained a structurally modified derivative of human G-CSF termed G-CSFa. G-CSFa contains alanine 17 (instead of cysteine 17 as in wild-type G-CSF) as well as four additional amino acids (methionine, arginine, glycine and serine) at the amino terminus. Previous studies showed that G-CSFa is more potent than the wild-type counterpart in stimulating proliferation and differentiation of myeloid cells of the granulocytic lineage, both *in vitro* and *in vivo*. Here, we show that G-CSFa can significantly accelerate peripheral platelet recovery in C57BL/6 mice exposed to radiotherapy. We further demonstrate that G-CSFa is not immunogenic in rats, by confirming the absence of any binding antibodies, analyzed using ELISA, or neutralizing antibodies, determined using the NFS-60 cell proliferation bioassay, to G-CSFa in the sera of Sprague-Dawley rats following repeated G-CSFa administration. Taken together, these findings further support the benefits of G-CSFa for clinical therapy.

Keywords: Human granulocyte colony-stimulating factor; Radiotherapy; Peripheral platelet recovery; Site-direct mutagenesis

Introduction

Granulocyte colony-stimulating factor (G-CSF), a glycoprotein, stimulates the proliferation, differentiation, and maturation of neutrophilic granulocyte precursors [1,2]. More importantly, G-CSF can mobilize bone marrow to produce granulocytesand stem/progenitor cells, and release them into the peripheral blood circulation [3]. Since 1991, recombinant G-CSF has been widely used for neutropenia treatment and marrow hematopoietic stem cell mobilization in clinical applications. However, due to its intrinsic instability, recombinant G-CSF needs to be administered to patients frequently and/or in excessive amounts, in order to maintain a plasma concentration high enough to achieve therapeutic effects [4]. Therefore, there is a need for the development of G-CSF derivatives that are more stable and have greater active *in vivo*.

Using site-directed mutagenesis and recombinant DNA technology, this laboratory has developed a structurally modified derivative of human G-CSF, termed G-CSFa [5], in which the cysteine 17 (Cys 17) residue of the G-CSF molecule was replaced by an alanine (Ala), in order to diminish the possibility of intermolecular disulfide bond formation. In addition, four amino acids (Met-Arg-Gly-Ser) were added to its N-terminus, to enhance its binding affinity to the membrane receptors on target cells. G-CSFa, expressed in *E. coli* using a pQE vector expression system, was found to have greater biological activities in previous studies; it was more potent in stimulating proliferation and differentiation of myeloid cells of the granulocytic lineage than the wild-type counterpart, both *in vitro* and *in vivo* [6]. Pharmacokinetics study in rats showed that the half-life of G-CSFa was longer than that of wild-type G-CSF [7]. Additionally, G-CSFa showed no vascular stimulation when added to rabbit vascular endothelial cell, and no hemolyzation or agglutination when added torabbit red blood cells, *in vitro*.

The present work was designed to further characterize the safety and therapeutic profiles of G-CSFa. We determined whether G-CSFa can accelerate peripheral platelet recovery in a mouse model of radiotherapy. More than half of all cancer patients receive radiation as a part of their cancer treatment. Radiation therapy, which causes DNA damage in cancer cells as well as normal cells, can lower blood cell counts, especially white blood cells (which can increase the risks of infection) and platelets (which can raise the risk of bruising or bleeding) [8]. We also evaluated the immunogenicity (antibody formation) of G-CSFa in rats, which, if present, can lead to adverse drug response.

Materials and Methods

Animals and treatments

Male C57BL/6 mice (20 ± 2 g, specific pathogen-free) purchased from the Shanghai SLAC laboratory Animal Co., Ltd (Shanghai, China) were used in this study. The mice were exposed to a single dose of 5 Gy whole-body radiation from a Cesium-137 source, and then were randomly separated into 2 groups (n=12 each). On day 5 through day 19 after the irradiation, mice received a once daily subcutaneously injection of either vehicle or G-CSFa at 25 μg/kg. Peripheral white

***Corresponding author:** Yongping Jiang, Professor & Director of Biopharmaceutical R&D Center, Chinese Academy of Medical Sciences & Peking Union Medical College, Suzhou, 215126, China, Tel: 86-512-63851269; E-mail: yjiang@biopharmagen.com

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blood cell counts, platelets counts and hemoglobin concentration were measured at day 0, day 5, day 9, day 12, day 16, and day 19 after irradiation.

Sprague-Dawley rats (female and male, 200 ± 20 g, specific pathogen-free), purchased from Shanghai SLAC laboratory Animal Co., Ltd (Shanghai, China), were injected with G-CSFa subcutaneously at the dose of 0 (placebo), 20, 100, or 500 μg/kg (once daily for 4 weeks). Peripheral blood was collected, and serum was obtained for analysis, either at the end of the 4-week treatment period or after a further 4-week recovery period.

All animals were housed in controlled conditions (temperature 21 ± 2°C and lighting 8:00 to 20:00), and received a standard rodent chow and tap water *ad libitum* throughout the study. All animal experiments were conducted in compliance with the Guidelines for Animal Experimentation issued by the Chinese Association for Laboratory Animal Science and the Standards related to the Care and Management of Experimental Animals.

ELISA for the determination of serum G-CSFa binding antibody levels

A biotin-avidin amplified enzyme-linked immunosorbent assay (BA-ELISA) method was employed to detect serum antibody levels. Briefly, G-CSFa antigen (GMP standard, Beihai Fangzhou Biopharmagen Co., China), dissolved in bicarbonate/carbonate coating buffer (pH 9.6), was used to coat the plate (1 μg/100 μl to each well) overnight at 4°C. On day 2, the plate was rinsed, and non-specific protein binding sites were blocked with 2% bovine serum albumin in PBS at 37°C for 1 h. After a further washing step, 100 μl of serum or diluted serum samples (diluted at 1:1, 1:5, 1:25, 1:125, 1:625 and 1:1250, respectively) were added. Normal rat serum was used as the negative control, and a commercially available anti-G-CSF antibody was included as a positive control. Biotin conjugated goat anti-rat IgG antibody (BioLegend; diluted 1:1000) was applied and incubated for 45 min at 37°C, and followed by incubation with streptavidin-peroxidase (Sigma-Aldrich; diluted 1:1000) for another 15 min. o-Phenylenediamine (Sigma-Aldrich) was used as the substrate to developed color. The plates were read on an ELISA plate reader (Bio-Rad) at 490 nm wavelength. Positive detection of binding antibody was defined by an OD value in the treatment group that is at least 2.1 fold greater than the value for the vehicle control group [9].

Detection of G-CSFa neutralizing antibody using cell-based bioassay

Neutralizing antibody was detected by measuring the capability of serum samples to inhibit proliferation of NFS-60 cells [10,11], which grow only in the presence of G-CSFa or other known growth factors.

The NFS-60 cells suspensions (2×10^5/mL, 50 μL in each well) were cultured with RPMI 1640 medium, supplemented with 10%FBS and 20 ng/mL G-CSFa, as well as 50 μL rat serum. After incubation of the cells at 37°C and 5% CO_2, for 48 h, 20 μL of a MTT solution was applied to each well. Following further incubation for 5 h, 100 μL lysis buffer (2.8% HCl and 10% Triton X-100 in isopropanol) were added, and the OD was read at 570 nm wavelength.

Data analysis

Data are expressed as the mean ± SD. Two-way ANOVA followed by Bonferroni posttest was employed for statistical analysis. A valve of $P<0.05$ was considered statistically significant.

Results

Irradiated C57BL/6 mice were injected with G-CSFa(at 25 μg/kg) or vehicle, and platelet counts and hemoglobin levels were determined at various times after the cytokine injection. The same animal model had been used previously to demonstrate the superior ability of G-CSFa over wild-type G-CSF in stimulating proliferation and differentiation of myeloid cells of the granulocytic lineage [6], a finding further confirmed in this study (data not shown).

As shown in Table 1, peripheral platelet counts decreased drastically after the irradiation, to ~40% of the pre-irradiation level by day 9 and gradually increased thereafter, in both vehicle control and G-CSFa treated mice. On day 19, whereas the platelet counts in vehicle control group were still significantly below the pre-irradiation level, the counts in the G-CSFa treated group have fully recovered. The difference in counts between the vehicle and G-CSFa groups was statistically significant at day 19. This ability of G-CSFa to accelerate recovery of peripheral platelet counts in the irradiated mice was also confirmed in additional experiments (not shown), where a higher dose of G-CSFa (50 or 100 μg/kg) was used.

The exposure to radiation also caused large decreases in hemoglobin levels. As shown in Table 2, the level of hemoglobin decreased by 20-30% at all times tested following the irradiation, and the level did not fully recover during the entire experiment period. However, hemoglobin levels were not significantly different between the vehicle and G-CSFa treated groups ($P>0.05$), either before or at any time tested after the irradiation. The same results were obtained when a higher G-CSFa dose (50 or 100 μg/kg) was used (data not shown). Thus, G-CSFa had no stimulatory effect on hemoglobin levels in the radiotherapy mouse model.

Considering the adverse effects of immunogenicity (antibody formation), we further evaluated the potential immunogenicity of G-CSFa in rats. Forty rats received daily subcutaneous injection of G-CSFa for 4 weeks, at various doses (0-500 μg/kg). Serum was obtained for analysis either at the end of the 4-week treatment period or after a further 4-week recovery period. A biotin-avidin amplified enzyme-linked immunosorbent assay (BA-ELISA) method was developed for the determination of serum antibody levels, whereas a cell-based G-CSFa-responsive bioassay was used to detect G-CSFa-neutralizing antibody activity.

As shown in Table 3, none of the G-CSFa treated rats was positive for binding antibody in the ELISA test, during either the treatment or the recovery period. Similarly, none of the serum samples was positive for the neutralizing antibody activity when tested in the NFS-60 cell-based bioassay. Briefly, the OD values of three G-CSFa treatment groups at the end of the 4-week treatment period ranged from 0.317 to 0.425, at the same time the OD value of the vehicle control group was 0.363 ± 0.046. The similar bioassay results were obtained at the end of 4-week recovery period. There was no significant difference between G-CSFa treatment groups and vehicle control group ($P>0.05$). The above-mentioned results clearly revealed that G-CSFa is not immunogenic in rats under the conditions used, which further supported the validity of the biological activity of G-CSFa in accelerating peripheral platelet recovery in the radiotherapy mouse model.

Discussion

G-CSF has been widely used in the clinic for over 20 years, primarily for accelerating neutrophil recovery in cancer patients

Groups	Platelet counts (10^9/L)					
	Before Irradiation (d0)	Days after irradiation				
		d5	d9	d12	d16	d19
Control	111 ± 13	100 ± 19	44 ± 6[b]	42 ± 13[b]	83 ± 11	81 ± 4[b]
G-CSFa	103 ± 9	91 ± 14	35 ± 11[b]	45 ± 11[b]	77 ± 12	100 ± 16[c]

Table 1: Effect of G-CSFa on peripheral platelet counts in irradiated C57BL/6 mice[a].

Groups	Hemoglobin concentration (g/L)					
	Before Irradiation (d0)	Days after irradiation				
		d5	d9	d12	d16	d19
Control	156 ± 8	116 ± 8[b]	116 ± 13[b]	120 ± 13[b]	130 ± 7[b]	129 ± 8[b]
G-CSFa	162 ± 16	126 ± 9[b]	107 ± 13[b]	112 ± 13[b]	128 ± 10[b]	125 ± 11[b]

[a]Mice were treated as described in Table 1. Peripheral hemoglobin concentration were determined at selected days after irradiation, and shown as means ± SD, n=12. Statistical analysis was conducted using two-way ANOVA, with Bonferronipost test. There was no significant difference between control and G-CSFa groups (P>0.05).
[b]P<0.001, compared to d0

Table 2: Effect of G-CSFa on whole blood hemoglobin concentration in irradiated C57BL/6 mice[a].

Dosage(μg/kg)	Sample	OD value		Number of antibody-positive animals	
		Week 4	Week 8	Week 4	Week 8
0	Serum	0.068 ± 0.006	0.068 ± 0.008	0/5	0/5
20	Serum	0.064 ± 0.004	0.073 ± 0.009	0/5	0/5
100	Serum	0.064 ± 0.007	0.073 ± 0.003	0/5	0/5
500	Serum	0.068 ± 0.019	0.072 ± 0.013	0/5	0/5
—	Positive antibody (anti-G-CSF)	1.109 ± 0.090		—	—

[a]Adult Sprague-Dawley rats were injected daily with G-CSFa subcutaneously at the dose of 0 (vehicle control), 20, 100, or 500 μg/kg, for up to 4 weeks. Peripheral blood serum was obtained for ELISA analysis either at the end of the 4-week treatment period (week 4) or after a further 4-week recovery period (week 8)

Table 3: Incidence of binding antibody detection after repeated G-CSFa administration in rats[a].

with myelo-suppressive radiotherapy or chemotherapy. During the past decade or so, great efforts have been directed at finding a more stable and thus more effective G-CSF, given the instability of the wild-type G-CSF *in vivo*. Amino acid locus mutation as well as chemical modifications of G-CSF have been reported [12-15], and PEG-rhG-CSF injection has already been marketed [16,17]. Our previous studies showed that G-CSFa is more potent in stimulating proliferation and differentiation of myeloid cells of the granulocytic lineage than the wild-type counterpart, both *in vitro* and *in vivo* [6]. The present work further documented that G-CSFa can significantly accelerate peripheral platelet recovery in the radiotherapy mouse model, which adds to the benefits of its clinical use. The exact mechanism of platelet recovery induced by G-CSFa in mice exposed to radiotherapy is not known. Although G-CSF-mediated thrombopoietin release triggers neutrophil motility and mobilization from bone marrow [18], G-CSF did not affect nadir platelet counts in Hodgkin's disease patients treated with radiation therapy [19], and G-CSF displayed little effect on platelet in cancer patients with neutropenia caused by chemotherapy and radiotherapy [20]. Future studies to determine whether G-CSFa has greater ability to induce increased production of thrombopoietin in the bone marrow are warranted.

Potential immunogenicity is a major disadvantage of protein drugs. Most therapeutic proteins could induce immunological response *in vivo* [21]. Antibody formation against the protein drug may lead to a loss of efficacy and/or general immune system effects (i.e., infusion reactions, anaphylaxis and anaphylactoid reactions) [22,23]. Therefore, it is important to ascertain whether G-CSFa has immunogenicity *in vivo*. Our results from studies in rats proved the absence of anti-G-CSFa antibodies in the serum either at the end of the 4-week treatment period or after a further 4-week recovery period. We further confirmed, using a bioassay, the absence of any G-CSFa neutralizing antibodies in the serum. These evidences support the conclusion that G-CSFa is not immunogenic in rats.

Given potential species differences between rodents and primates, the relevance of the present study using rodent models to G-CSFa therapy in humans remains to be established. In that regard, the non-human primates have been considered as better animal models for evaluating drug efficacy and immunogenicity. Therefore, further testing of the safety and efficacy of G-CSFa in a primate model, such as the Macaca, is warranted.

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