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Pharmacokinetics of Recombinant Human Granulocyte Colony-stimulating Factor Conjugated to Polyethylene Glycol in Rats

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ABSTRACT

The pharmacokinetics of recombinant human granulocyte colony-stimulating factor conjugated to polyethylene glycol (PEG-rhG-CSF) and rhG-CSF were studied in male Sprague-Dawley rats. The serum concentration after i.v. administration at a dose of 100 μg protein/kg was investigated by a bioassay. The serum rhG-CSF concentration decreased steadily after injection with a terminal half-life of 1.79 h. The PEG-rhG-CSF concentration after injection decreased much more slowly with a half-life of 7.05 h. The slower disappearance of PEG-rhG-CSF resulted in a greater area under the concentration-time curve. The neutrophil count after 100 μg of protein/kg of rhG-CSF administration reached a peak 12 h after injection and returned to the control level 48 h after injection. The neutrophil count after 100 μg of protein/kg of PEG-rhG-CSF administration was identical to that of rhG-CSF after 12 h but the highest level was maintained for 24 to 72 h after injection and returned to the control level after 168 h. These data indicated that PEG-rhG-CSF administration exerted a sustained biological effect on peripheral blood neutrophils. It is expected that PEG-rhG-CSF may contribute greatly to human G-CSF treatment because it has a prolonged neutrophil-proliferating activity enabling fewer administrations.

INTRODUCTION

G-CSF[2] was identified because it induced differentiation in a murine myelomonocytic leukemic cell line and stimulated granulocyte colony formation by normal progenitor cells (1). The hematopoietic actions of this regulator are exclusive to cells of the neutrophilic granulocyte lineage (2–4). Murine and human G-CSF have been purified from various sources (1–3) and the genes encoding human and murine G-CSF have been isolated (5–7). Characterization of the gene encoding G-CSF has led to the production of the protein by recombinant DNA techniques. rhG-CSF is also capable of supporting the formation of granulocytic colonies from committed precursor cells. The broad species cross-reactivity of hG-CSF has allowed its *in vivo* action to be studied in mice (8, 9), hamsters (10), rats (11), monkeys (12), and humans (13). The predominant response in all species was a rapid dose-dependent neutrophilia. The availability of large quantities of molecularly homogeneous, biologically active hG-CSF by recombinant DNA technology has made it possible to explore the use of hG-CSF as a therapeutic agent. Based on the preclinical findings, rhG-CSF has recently been applied clinically to patients to accelerate the hematological recovery after high-dose chemotherapy with or without autologous bone marrow rescue, and it has been found to markedly shorten the duration of neutropenia (14–18). Hence, rhG-CSF may be very useful in the treatment of cancer patients with drug- or irradiation-induced myelosuppression.

We have previously reported the pharmacokinetics of rhG-CSF in male Sprague-Dawley rats (11, 19, 20) where rhG-CSF is rapidly cleared from the circulation resulting in short-duration pharmacological effects. The same phenomena were observed in humans and most neutropenic patients received rhG-CSF via daily injection (13–18). Increasing the half-life of rhG-CSF may increase its potency, thus enhancing the use of this protein as a drug. Covalent attachment of PEG to a variety of proteins resulted in lowering the chances of anaphylactic reactions to the protein and extension of half-life (21–25). Modification of protein drugs, such as L-asparaginase, interleukin 2, and superoxide dismutase, with PEG increased its potency in therapeutic use (26–30).

This paper describes the comparison studies in the pharmacokinetics and *in vitro* and *in vivo* biological activities in male Sprague-Dawley rats, using rhG-CSF and PEG-rhG-CSF. The PEG-rhG-CSF exhibits an increased circulatory half-life *in vivo* and it also has a longer duration of action than rhG-CSF *in vivo*.

MATERIALS AND METHODS

Animals. All mice and rats were obtained from Japan SLC Co., Ltd. (Shizuoka, Japan). Female mice (C3H/He strain) were used when they were between 6 and 14 weeks old; male Sprague-Dawley rats were used when 7 weeks old. They were kept in specific-pathogen-free animal rooms and allowed food and water *ad libitum*.

Reagents. McCoy's 5a medium was purchased from Gibco (Grand Island, NY). FCS was obtained from Bocknek Laboratories, Inc. (lot SF70318; Toronto, Ontario, Canada). Activated PEG_2 (average molecular weight of the whole PEG_2, 10,000) was obtained from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan). All chemicals were of analytical grade and were obtained commercially.

Preparation of rhG-CSF and PEG-rhG-CSF. rhG-CSF was produced in *Escherichia coli* and purified to homogeneity as described previously (5). rhG-CSF was chemically modified with activated PEG_2 at the amino groups of the four lysine residues and NH_2-terminal methionine residue. Preparation of PEG-rhG-CSF is described elsewhere.[3] In brief, activated PEG_2 was added in stoichiometric molar ratio of 5 to amino groups of rhG-CSF in 0.25 M sodium borate (pH 10.0). The solution was stirred for 1 h at room temperature; then the pH of the solution was adjusted to 4.0. The PEG-rhG-CSF was purified from the reaction mixture by Sephadex G-25 chromatography to desalt, followed by ion-exchange chromatography on a Waters Protein Pak DEAE-5PW column to remove unmodified rhG-CSF and unreacted PEG. The proteins were eluted with an increasing salt gradient (NH_4HCO_3; 0.01–0.5 M). The protein concentration of PEG-rhG-CSF was measured by the method of Bradford (31).

SDS-PAGE. SDS-PAGE was carried out as described by Laemmli (32) using 15% polyacrylamide gels. Coomassie Brilliant Blue R-250 was used to stain protein bands.

Collection of Serum Samples. Rats were randomly assigned to groups corresponding to the serum sampling times. This design provided three animals per sampling time. rhG-CSF or PEG-rhG-CSF (100 μg of

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[2] The abbreviations used are: G-CSF, granulocyte colony-stimulating factor; rhG-CSF, recombinant human granulocyte colony-stimulating factor; PEG, polyethylene glycol; FCS, fetal calf serum; activated PEG_2, 2,4-bis(*O*-methoxypolyethylene glycol)-6-chloro-*s*-triazine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

[3] R. Satake-Ishikawa, Y. Okada, M. Kakitani, M. Kawagishi, M. Ishikawa, S. Matsuki, and K. Asano, submitted for publication.

protein/kg) was administered i.v. via the tail vein. Between 6 and 7 ml of arterial blood were collected in an ice-cold polypropylene tube from the abdominal aorta of animals under light ether anesthesia at indicated time points (10 min and 2, 4, 8, 24, and 48 h) after administration. Blood was centrifuged at 18,000 × *g* for 5 min to separate the serum. The obtained serum was filtered (0.22 μm; Japan Millipore, Tokyo, Japan) into a polypropylene microtube. The filtered serum was then frozen in liquid nitrogen and stored at −80°C until use.

Bioassay for rhG-CSF. Bioassay was performed using previously published procedures (11). Briefly, 4 × 10^4 freshly obtained nonadherent light (<1.077 g/cm^3) bone marrow cells from female C3H/He mouse femur were plated in 150 μl McCoy's 5a medium containing 10% FCS into a flat-bottomed 96-well plate. Serial 2-fold dilutions of rat serum or standard containing rhG-CSF or PEG-rhG-CSF were performed with untreated pooled rat serum for pharmacokinetic studies. For comparison of *in vitro* activities between PEG-rhG-CSF and rhG-CSF, these samples were diluted with McCoy's 5a medium containing 10% FCS. Each sample was plated at 50 μl into the well plate and each assay was performed in triplicate. After 3 days of incubation, 1 μCi [^{3}H]thymidine (20 Ci/mmol; New England Nuclear, Boston, MA) in 50 μl McCoy's 5a medium was added to each well. After an additional 5 h of incubation, cells were harvested. Radioactivity of incorporated [^{3}H]thymidine was measured using a liquid scintillation counter.

For comparison of *in vitro* activities between PEG-rhG-CSF and rhG-CSF, the dose-response curves were linearized using probit transformation and the potency ratio of PEG-rhG-CSF to rhG-CSF was calculated. For pharmacokinetic studies, the dose-response curves were determined by plotting decreasing cpm against log dilution. Parallelism was checked between the linear portions of sample curves and that of the standard curve of a known amount of rhG-CSF or PEG-rhG-CSF. The amount of rhG-CSF or PEG-rhG-CSF in the samples was then calculated as

$$\text{Sample conc.} = \text{Standard conc.} \times \text{relative potency of sample}$$

WBC, RBC, Platelet, and Neutrophil Counts. Blood was collected from the tail vein of rats using 70-μl micropipets at the indicated time points (0, 6, 12, 24, 48, 72, 96, 120, 144, and 168 h) after rhG-CSF or PEG-rhG-CSF administration. WBC, RBC, and platelets were counted by a microcell counter CC-180A (Toa Medical Electronics, Kobe, Japan) and neutrophil counts were measured on blood smears stained with May-Grünwald-Giemsa.

Pharmacokinetic Data Analysis. Serum concentration-time data were analyzed by compartmental and noncompartmental methods. The area under the serum concentration-time curve (AUC) and area under the moment curve (AUMC) were calculated using the trapezoidal rule and extrapolating to infinity (33). The mean residence time (MRT) was calculated as

$$\text{MRT} = \frac{\text{AUMC}}{\text{AUC}}$$

The systemic clearance (CL) was calculated as

$$\text{CL} = \frac{\text{Dose}}{\text{AUC}}$$

Serum half-life was estimated using a nonlinear least squares program, NONLIN 84 (Statistical Consultants, Inc., Lexington, KY), operated on a VAX 8350 computer.

RESULTS

Preparation of PEG-rhG-CSF. Purified rhG-CSF (M_r 18,800) was modified by activated PEG_2 (average molecular weight, 10,000) as described in "Materials and Methods." The molecular weight estimation for PEG-rhG-CSF by SDS-PAGE revealed that its average molecular weight was about 45,000 distributed among 30,000 (10%), 40,000 (70%), and 66,000 (20%). This PEG-rhG-CSF preparation was used in following studies.

Activities of rhG-CSF and PEG-rhG-CSF *in Vitro*. The effect of PEG-rhG-CSF on [^{3}H]thymidine uptake in cultured mice bone marrow cells was compared to that of rhG-CSF. Typical dose-response curves are shown in Fig. 1. The relative potency of PEG-rhG-CSF to rhG-CSF obtained from three separate experiments was 41 ± 13% (SD).

Pharmacokinetics of rhG-CSF and PEG-rhG-CSF. The pharmacokinetics of rhG-CSF and PEG-rhG-CSF after i.v. administration to male Sprague-Dawley rats was studied at a dose of 100 μg protein/kg. At selected time points after injection rats were arteriotomized and sera were tested *in vitro* to assay the activity. Typical experiments are shown in Figs. 2 (rhG-CSF) and 3 (PEG-rhG-CSF). Both figures show the decrease in activity with time. After rhG-CSF was injected, the serum activity declined rapidly and sera obtained 24 and 48 h after injection showed no detectable activities. After PEG-rhG-CSF was administered a protracted elimination profile was observed. The average concentrations in sera after i.v. administration are shown in Fig. 4 and the pharmacokinetic parameters are presented in Table 1. The half-life, the area under the PEG-rhG-CSF serum concentration-time curve, and mean residence time were much greater than those of rhG-CSF.

Activities of rhG-CSF and PEG-rhG-CSF *in Vivo*. The effects of rhG-CSF and PEG-rhG-CSF administered i.v. to male Sprague-Dawley rats were determined at a dose of 100 μg protein/kg. As shown in Fig. 5, a marked increase in total WBC and absolute neutrophil count in rats receiving rhG-CSF and PEG-

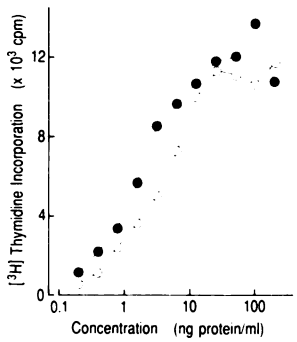

Fig. 1. Comparison of *in vitro* biological activities of rhG-CSF and PEG-rhG-CSF. [^{3}H]Thymidine uptake over 5 h by murine bone marrow cells was measured after a 67-h incubation in the presence of rhG-CSF (●) or PEG-rhG-CSF (○). *Points*, mean values of triplicates.

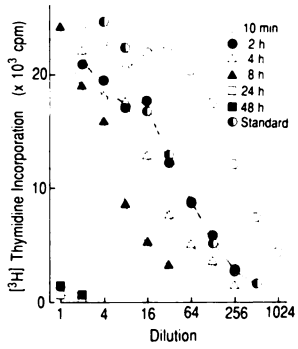

Fig. 2. Serum activity after rhG-CSF injection; 100 μg protein/kg were injected i.v. and blood samples were collected. Serum samples were serially 2-fold diluted and [^{3}H]thymidine uptake over 5 h by murine bone marrow cells was measured after a 67-h incubation. The standard comprised 200 ng protein/ml rhG-CSF in serum.

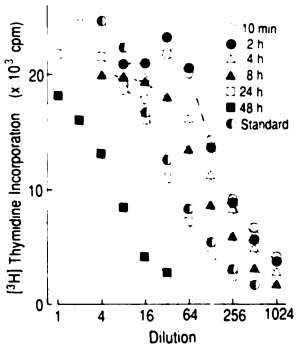

Fig. 3. Serum activity after PEG-rhG-CSF injection; 100 μg protein/kg were injected i.v. and blood samples were collected. Serum samples were serially 2-fold diluted and [^{3}H]thymidine uptake over 5 h by murine bone marrow cells was measured after a 67-h incubation. The standard comprised 200 ng protein/ml PEG-rhG-CSF in serum.

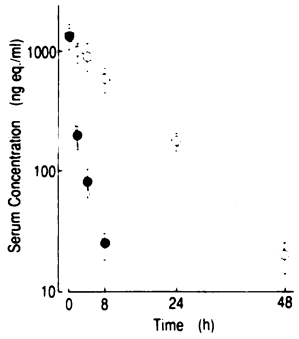

Fig. 4. Serum rhG-CSF and PEG-rhG-CSF concentration after i.v. administration to rats. Male Sprague-Dawley rats (7 weeks old) were given injections of 100 μg protein/kg, *i.e.*, rhG-CSF (●) or PEG-rhG-CSF (○), and serum concentration was assessed at subsequent time points. *Points*, mean of three animals; *bars*, SD.

Table 1 *Pharmacokinetic parameters of rhG-CSF and PEG-rhG-CSF obtained after i.v. administration of 100 μg protein/kg to rats*

Materials	AUC[a] (ng•h/ml)	CL (ml/h/kg)	$t_{1/2}$ (h)	MRT (h)
rhG-CSF	2,000	50.0	1.79[b]	1.65
PEG-rhG-CSF	16,195	6.2	7.05	10.8

[a] AUC, area under the serum concentration-time curve; *CL*, total body clearance; $t_{1/2}$, half-life; MRT, mean residence time.
[b] $t_{1/2}$ of rhG-CSF was determined from the points between 2 and 8 h.

rhG-CSF was observed. The neutrophil count in peripheral blood reached a peak 12 h after rhG-CSF injection and returned to the control level after 48 h. The neutrophil count after PEG-rhG-CSF administration was identical to that 12 h after rhG-CSF was given but the neutrophil count was maintained at a high level for 24 to 72 h and returned to the control level 168 h after injection. There was no marked change in the control animals receiving only vehicle during the same time period. These data indicated that PEG-rhG-CSF administration caused a sustained biological effect on neutrophilic hematopoiesis *in vivo*. Changes in RBC and platelet counts were not obvious (Fig. 6).

DISCUSSION

Human peptide hormones have been produced on a large scale by using recombinant DNA expression vectors in cultured cell. These techniques have enabled the therapeutic application of human peptide hormones. Souza *et al.* (5) have described a method for large-scale production of rhG-CSF by recombinant DNA techniques and rhG-CSF is now being clinically studied with Phase I/II/III data showing efficacy in neutropenia. We prepared PEG-rhG-CSF and *in vitro* and *in vivo* studies were performed using PEG-rhG-CSF. We used mouse bone marrow cells for *in vitro* studies of hematopoietic activities, because the response of rat bone marrow cells to G-CSF is very low in [^{3}H] thymidine uptake assay (data not shown). For *in vivo* studies we used rats, because we are able to obtain a sufficient volume of serum sample for pharmacokinetic studies.

A comparative study of the *in vitro* actions on hematopoietic cells of rhG-CSF and PEG-rhG-CSF was performed in murine bone marrow cell culture. The response to PEG-rhG-CSF was smaller than that to rhG-CSF, suggesting that PEG molecule(s) may sterically inhibit rhG-CSF/G-CSF receptor binding or that some of lysine residues may play important role in biological activities. To determine the exact extent and mechanisms of the loss of *in vitro* rhG-CSF activity after chemical modification, more detailed studies, such as a receptor binding assay, are necessary.

The pharmacokinetics of rhG-CSF in animals and humans has been reported using various assay techniques for rhG-CSF determination in plasma and serum (10, 11, 14, 16, 20). A good agreement between the [^{3}H]thymidine uptake assay and the sandwich enzyme-linked immunosorbent assay was observed with rat serum samples after administration of rhG-CSF (20). Thus, there are a few factors that influence rhG-CSF activity

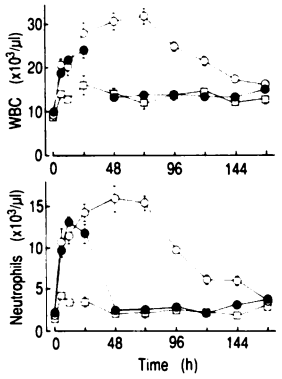

Fig. 5. Time course of peripheral WBC and neutrophil counts of male Sprague-Dawley rats (7 weeks old) given injections of rhG-CSF or PEG-rhG-CSF. Rats were treated with 100 μg protein/kg, *i.e.*, rhG-CSF (●) or PEG-rhG-CSF (○) via i.v. injection. Control rats (□) received only vehicle via i.v. injection. *Points*, mean of 5 animals; *bars*, SE.

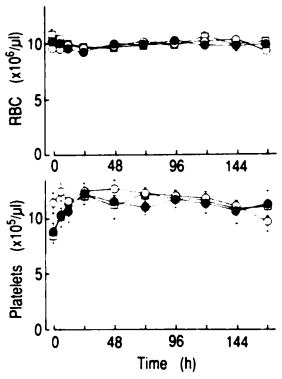

Fig. 6. Time course of peripheral RBC and platelet counts of male Sprague-Dawley rats (7 weeks old) given injections rhG-CSF or PEG-rhG-CSF. Rats were treated with 100 μg protein/kg, *i.e.*, rhG-CSF (●) or PEG-rhG-CSF (○) via i.v. injection. Control rats (□) received only vehicle via i.v. injection. *Points*, mean of 5 animals; *bars*, SE.

in rat serum and it is possible to measure the functionally active, intact rhG-CSF concentrations by using this [^{3}H]thymidine uptake assay. An immunological assay for rhG-CSF cannot be used for PEG-rhG-CSF because PEG modification inhibits the association between rhG-CSF and anti-rhG-CSF antibody (data not shown). Therefore we used a [^{3}H]thymidine uptake assay for this rhG-CSF and PEG-rhG-CSF pharmacokinetic study in rats.

The serum concentration after i.v. administration of a dose of 100 μg protein/kg was investigated in male Sprague-Dawley rats. The serum concentration after rhG-CSF administration rapidly decreased (Fig 4). The rapid clearance of rhG-CSF in rats was due to the combination of a small volume of distribution and moderate systemic clearance (11, 20). Injection of PEG-rhG-CSF resulted in relatively prolonged presence in serum. Pharmacokinetic analysis of the serum concentration-time data gave the following disposition characteristics for rhG-CSF and PEG-rhG-CSF. As presented in Table 1, the total body clearance of rhG-CSF decreased from 50.0 ml/h/kg to 6.2 ml/h/kg after PEG modification. Previously, pharmacokinetic studies of rhG-CSF in rats have indicated that the serum concentration profile of rhG-CSF after i.v. dosing show biexponential elimination (11, 20). The half-life of the α phase could not be determined in this experiment because of the few data points in the early period. The terminal half-life obtained from the data points between 2–8 h was 1.79 h and this value was in good agreement with the previously reported value (11, 20). In contrast, the concentration of PEG-rhG-CSF decreased much more slowly following an apparently monoexponential pattern of elimination with a half-life of 7.05 h. The slower disappearance of PEG-rhG-CSF resulted in greater values for the area under the serum concentration-time curve.

The number of exponentials needed to describe adequately such a plasma concentration *versus* time curve determines the number of kinetically "homogeneous" compartment that a drug confers on the body (33). The change from biexponential elimination profile for rhG-CSF to monoexponential for PEG-rhG-CSF indicated a fundamental difference(s) in clearance and distribution processes. Further studies are needed to clear the detailed mechanism of the clearance and distribution that restrict elimination profiles of rhG-CSF and PEG-rhG-CSF.

In general, small proteins are eliminated from circulatory blood by glomerular filtration and proteolysis (34, 35). Conjugation of PEG to proteins increases their size; the larger the protein, the slower is the clearance by the kidney. This is an explanation for the decrease in clearance by PEG-modified protein. Another explanation for the decrease in clearance may be the modification or shielding of the proteolytic sites in the protein molecule by PEG inasmuch as PEG-modified proteins have been shown to be more resistant to proteolysis than the corresponding unmodified proteins (21–25). Urinary excretion of rhG-CSF injected into rats is negligible (19); therefore the decrease in clearance of rhG-CSF by PEG modification was due to reduction in metabolism.

The effect of rhG-CSF administration in animals is a specific activity on the neutrophil lineage with increase of neutrophils in peripheral blood (12, 13). The effects of rhG-CSF and PEG-rhG-CSF on hematopoiesis *in vivo* were determined in male Sprague-Dawley rats receiving an i.v. dose of 100 μg protein/kg (Fig. 5). A marked increase in total WBC and absolute neutrophil count in rats receiving rhG-CSF or PEG-rhG-CSF was observed and the increased WBC was based on the increase of neutrophils. PEG modification of rhG-CSF yielded a sustained biological effect on the peripheral blood neutrophil count. It is suggested that the effects of G-CSF on hematopoiesis are more positively influenced by the duration of G-CSF presence in serum than by the peak G-CSF concentration.

The current studies show that covalent linking of PEG to rhG-CSF decrease its total body clearance and provide a sustained biological effect on peripheral blood neutrophil count. It is expected that PEG-rhG-CSF may make a pronounced contribution to treatment with rhG-CSF because it has prolonged neutrophil-proliferating activity, enabling fewer administrations. If these observations of PEG-rhG-CSF potency in rats can be extended, covalent modification of rhG-CSF with PEG may facilitate the potential therapeutic use of this protein.

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