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Chemical Modification of Recombinant Human Granulocyte Colony-Stimulating Factor by Polyethylene Glycol Increases its Biological Activity *in vivo*

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ABSTRACT. **Recombinant human granulocyte colony-stimulating factor (rHuG-CSF) produced in *Escherichia coli* was chemically modified by polyethylene glycol (PEG) of molecular weights 4,500 or 10,000. The neutrophils observed at 32 hours after intravenous injection of the rHuG-CSF modified with PEG (4,500) or PEG (10,000) to mice were, respectively, 2.5 times and 5 times more than that observed after the injection of the unmodified rHuG-CSF. These results show that the covalent attachment of PEG to rHuG-CSF enhanced its pharmacological activity *in vivo* and that the modification with the larger PEG molecule is more effective to enhance the *in vivo* activity of rHuG-CSF.**

Human granulocyte colony-stimulating factor (G-CSF) is a hematopoietic growth factor with an approximate molecular weight of 19,600 that regulates the proliferation and differentiation of neutrophilic granulocytes (7, 16). Human G-CSF has been purified to homogeneity from the conditioned medium of the bladder carcinoma cell line 5637 and its cDNA has been cloned (10, 13). Recombinant human G-CSF (rHuG-CSF), successfully produced in *Escherichia coli*, is also capable of supporting the formation of granulocytic colonies from bone marrow cells (13) and is very useful in the treatment of cancer patients with drug- or irradiation-induced myelosuppression.

When rHuG-CSF is injected into rats, it is rapidly cleared from the circulation, resulting in short-term pharmacological effects (14). It is thus conjectured that increasing the half-life of rHuG-CSF will increase its potency. It is generally observed that when physiologically active proteins are administered, their pharmacological activities are short-lived due to their high turnover in the body. Chemical modifications of several proteins, such as asparaginase (1), superoxide dismutase (8), and recombinant human interleukin-2 (3, 4) with polyethylene glycol (PEG), have increased their half-lives *in vivo*.

We previously reported that the rHuG-CSF modified with PEG exhibits an increased circulatory half-life *in vivo* (15). This paper reports that the pharmacological activity of rHuG-CSF *in vivo* was enhanced by the conjugation with PEG, and that the modification with a larger PEG molecule was more effective to enhance it.

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Abbreviations: G-CSF, granulocyte-colony stimulating factor; PEG, polyethylene glycol; IEF, isoelectric focusing.

MATERIALS AND METHODS

Materials. rHuG-CSF was produced in *Escherichia coli* and purified to homogeneity as previously described (13). Methoxypolyethylene glycol succinimydyl succinate (activated PEG (4,500); average molecular weight 4,500) (1) was obtained from Nippon Oil and Fats, Co., Ltd. 2,4-Bis (O-methoxypolyethylene glycol)-6-chloro-s-triazine (activated PEG (10,000); average molecular weight 10,000) (6) was obtained from Seikagaku Kogyo Co., Ltd.

Modification of rHuG-CSF. rHuG-CSF was incubated in 0.25 M sodium borate buffer (pH 8.0) for 1 hour at 4°C with the activated PEG (4,500) at a ratio of 1 to 50 times the molar concentration of free amino groups in rHuG-CSF.

Activated PEG (10,000) was incubated with rHuG-CSF at a ratio of 5 times the molar concentration of free amino groups of rHuG-CSF in 0.25 M sodium borate buffer (pH 10.0) for 1 hour at room temperature.

The reaction mixture was subjected to Sephadex G-25 (Pharmacia, Uppsala, Sweden) which had been equilibrated with 10 mM ammonium bicarbonate for buffer-exchange, and then to Protein Pak DEAE-5PW (0.75 × 75 mm, Japan Millipore Ltd., Tokyo, Japan) so as to remove excess PEG reagent and unmodified rHuG-CSF. The protein concentrations of rHuG-CSF and modified rHuG-CSF were determined by the method of Bradford (2).

In vitro biological assays. Biological activities of rHuG-CSF and modified rHuG-CSF were measured by the uptake of [^{3}H]-thymidine into mouse bone marrow cells following the

procedure of Moore *et al.* (9) and Ralph *et al.* (11) with some modifications. In brief, low-density (< 1.077 g/cm^3) nonadherent bone marrow cells from female Balb/c mice were incubated for 3 days in dilutions of samples in supplemented McCoy's 5a medium (10% fetal calf serum). The cell concentration was 8×10^4 cells per well in a volume of 0.2 ml in 96 well flat-bottom plates. After incubation, the cells were pulse-labelled with ^{3}H-thymidine for 5 hours (1 μCi per well), harvested on glass fiber filters and assayed for radioactivity using a liquid-scintillation counter. Biological activities of samples were calculated by a parallel line assay. A purified preparation of rHuG-CSF, quantified by the mouse bone marrow colony assay, was used as a standard.

In vivo biological assays. Male ICR mice were injected intravenously at a dose of 10 μg protein/kg of rHuG-CSF or modified rHuG-CSF. After the injection, blood was collected from the orbital vein at selected times and neutrophil counts were performed on blood smears stained with May-Grunwald-Giemsa.

Other techniques. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (5). Isoelectric focusing (IEF) analysis was performed using an LKB Ampholine Pagplate gel, pH 3.5–9.5 (12). Both SDS-PAGE and IEF gels were scanned after staining with Coomassie blue by using a chromato-scanner (Shimazu Corporation CS-930).

RESULTS AND DISCUSSION

Effect of chemical modification on in vivo activity. rHuG-CSF was chemically modified at the amino groups of the 4 lysine residues and the N-terminal methionine residue by activated PEG (4,500) as described in METHODS. The modified products eluted from Sephadex-G25 were analyzed by SDS-PAGE and IEF to examine the degree of modification. On SDS-PAGE (Fig. 1-A, a–e), at least three species of modified rHuG-CSF with different molecular weights (26-KDa, 34-KDa, 43-KDa) were observed. When the molar ratio of activated PEG to the number of free amino groups of rHuG-CSF

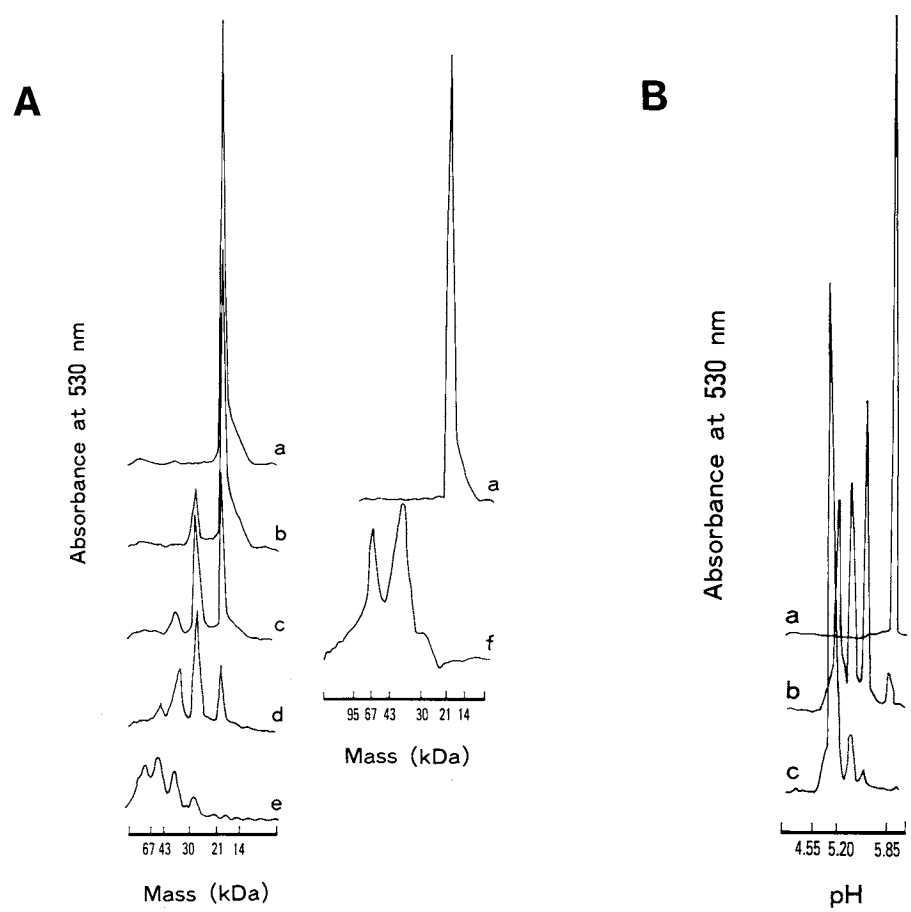

Fig. 1. Densitometric scanning of SDS-PAGE and IEF gel of modified products. The products modified with activated PEG (4,500) or activated PEG (10,000) were analyzed by (A) SDS-PAGE or (B) IEF. (A) (a) rHuG-CSF, (b) products with equimolar PEG (4,500), (c) products with 5-fold excess PEG (4,500), (d) products with 10-fold excess PEG (4,500), (e) products with 50-fold excess PEG (4,500), (f) products with 5-fold excess PEG (10,000); (B) (a) rHuG-CSF, (b) products with 10-fold excess of PEG (4,500), (c) products with 50-fold excess PEG (4,500).

was increased, the population of larger molecular mass species increased. On IEF (Fig. 1-B), modified rHuG-CSF species having different pI values were detected (pI 5.5, 5.3, 5.1). The population of lower pI species increased according to the molecular ratio of the activated PEG.

To estimate the effect of chemical modification on *in vivo* activity of rHuG-CSF, the number of neutrophils was measured at 24 hours after intravenous injection of variously modified rHuG-CSF to mice. The relationship between the average molecular weight of modified rHuG-CSF and the effect on granulopoiesis is shown in Figure 2. As the average molecular weight increased from 19 to 60 kDa, the number of neutrophils increased from 18 to 58×10^2 cells/mm^3. This result shows that the more highly modified rHuG-CSF has a higher activity *in vivo*. The relationship between the modification rate and the increase of *in vivo* activity was about the same as the modification with PEG (10,000) (data not shown).

Comparison of the effects of PEG (4,500) and PEG (10,000). rHuG-CSF was incubated with activated PEG (4,500) or PEG (10,000) in order to examine the effect of the molecular weight of PEG on *in vivo* activity of rHuG-CSF. The rHuG-CSF was incubated with the 50-fold excess activated PEG (4,500) and the highly modified rHuG-CSF was fractioned with Protein Pak DEAE-5PW. It was designated as PEG (4,500)-rHuG-CSF. The average molecular weight on SDS-PAGE of PEG (4,500)-rHuG-CSF was about 60-kDa. Next, rHuG-CSF was modified by activated PEG (10,000). The average molecular weight of this species was about 45-kDa (Fig. 1f). This sample was designated as PEG (10,000)-rHuG-CSF. Peripheral blood neutrophil counts in mice after injection of rHuG-CSF, PEG (4,500)-rHuG-CSF or PEG (10,000)-rHuG-CSF (10 μg protein/kg) were measured (Fig. 3). PEG (4,500)-rHuG-CSF and PEG (10,000)-rHuG-CSF caused a greater increase of neutrophils than unmodified rHuG-CSF and maintained the high neutrophil cell counts even after 32 and 48 hours, respectively. The neutrophils observed at 32 hours after the injection of PEG (4,500)-rHuG-CSF and PEG (10,000)-rHuG-CSF were, respectively, 2.5 times and 5 times more than that observed after the injection of unmodified rHuG-CSF. PEG (10,000)-rHuG-CSF exhibited a higher activity *in vivo* than PEG (4,500)-rHuG-CSF, even though its average molecular weight was smaller. These results indicated that the modification with a larger PEG molecule is more effective on the enhancement of the pharmacological activity of rHuG-CSF. The biological activity *in vitro* of the modified rHuG-CSF was determined by incorporation of [^{3}H]thymidine into bone marrow cells of mice. The *in vitro* activities of PEG (4,500)-rHuG-CSF and PEG (10,000)-rHuG-CSF were 15% and 32% of that of unmodified rHuG-CSF, respectively (Table I). These results suggest that PEG (10,000)-rHuG-CSF is more effective *in vivo* with less decrease *in*

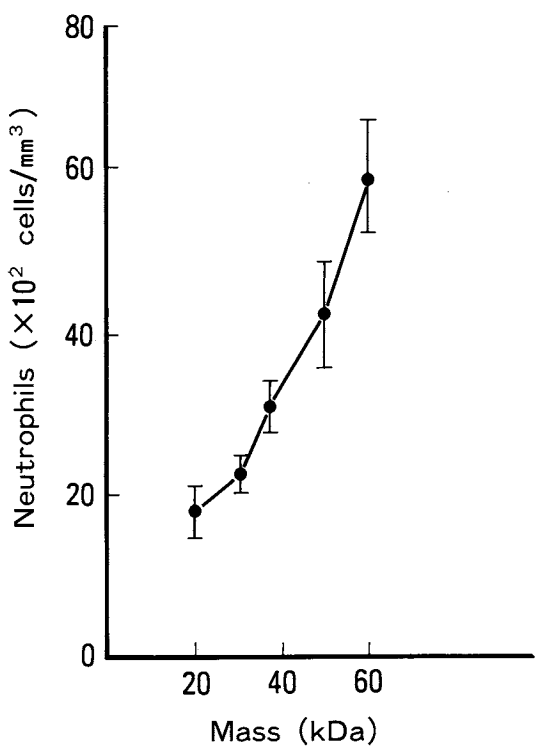

Fig. 2. Neutrophil counts in mice after injection of rHuG-CSF modified with PEG. 10 μg protein/kg of rHuG-CSF modified by PEG (4,500) with different average molecular weights as indicated was injected intravenously to mice. After 24 hours, peripheral blood neutrophils were counted as described in METHODS.

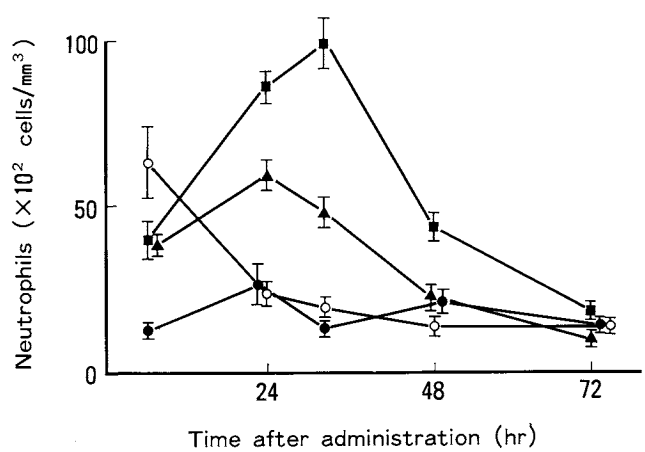

Fig. 3. Time course of neutrophil counts in mice after injection of rHuG-CSF and PEG-rHuG-CSF. rHuG-CSF (○), PEG (4,500)-rHuG-CSF (▲), PEG (10,000)-rHuG-CSF (■) or vehicle (●) was injected intravenously to mice at a dose of 10 μg protein/kg. Each point is the mean of six animals with standard errors indicated by bars.

Table I. Comparison of modified rHuG-CSFs.

sample	MW (kDa)	activity *in vivo* (%)	activity *in vitro* (%)
rHuG-CSF	19	100	100
PEG (4,500)-rHuG-CSF	60	250	15
PEG (10,000)-rHuG-CSF	45	500	32

Activities are expressed using a value of 100% for intact rHuG-CSF.

vitro activity than PEG (4,500)-rHuG-CSF. The partial decrease of the *in vitro* activity of chemically modified protein was also observed in other proteins (3, 8), because PEG covered their active sites or changed their tertiary structures. The fact that the *in vivo* activity of modified rHuG-CSF increased, although the *in vitro* activity decreased, suggests that modified rHuG-CSF has a longer plasma half-life. In fact, when the pharmacokinetics of rHuG-CSF and PEG (10,000)-rHuG-CSF were investigated in male rats, the serum rHuG-CSF concentration after intravenous administration at a dose of 100 μg protein/kg detected by *in vitro* biological assays decreased steadily with a terminal half-life of 1.79 h. On the other hand, the PEG (10,000)-rHuG-CSF concentration after injection decreased much more slowly with a half-life of 7.05 h (15). It is speculated that selective modification at specific residues with the larger PEG, using techniques such as site-directed mutagenesis, might have more positive results.

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