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Long-acting Recombinant Human Granulocyte Colony Stimulating Factor (rhG-CSF) with a Trimer–Structured Polyethylene Glycol

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ABSTRACT − Mono PEGylated rhG-CSF (PEG-G-CSF) prepared by utilizing unique PEG was purified and characterized by cation-exchange chromatography. A unique, trimer-structured PEG was chosen for PEGylation of rhG-CSF among various PEG moieties. The *in-vitro* bioactivity, stability, and pharmacokinetics of mono-PEG-G-CSF were examined and compared to those of native rhG-CSF. Mono PEG-G-CSF exhibited reduced in-vitro bioactivity to native rhG-CSF but showed an excellent *in-vivo* bioactivity and stability. Furthermore, it showed markedly reduced clearance in rats, thereby increasing the biological half-life by about 4.5-fold compared to that of native rhG-CSF. The results suggest that this unique, trimer-structured 23 kDa PEG can provide advantages to improve the bioactivity of therapeutic proteins in clinical use.

Key words − G-CSF, PEG, Structure, Characteristics

Granulocyte colony-stimulating factor (G-CSF) is a growth factor that serves as a major regulator of the proliferation and differentiation of neutrophilic granulocytes. Recombinant human G-CSF (rhG-CSF) produced in E. coli is a 175-residue protein that folds into a 4 helical bundle, typical of a cytokine (Hill et al., 1993; Manavalan et al., 1992; Souza et al., 1986).

It has been generally known that the proteins such as rhG-CSF have short life in the body due to their rapid clearance (Bronchud et al., 1988). The short half-life of a recombinant protein such as rhG-CSF can be increased by covalent modification with the polyethylene glycol (PEG), in a procedure termed 'PEGylation' (Molineux, 2002; Yowell and Blackwell, 2002). Covalent modification of therapeutic proteins with PEG can result in increased serum half life due to decreased renal clearance (Crawford, 2002). PEGylation is known to improve the physicochemical properties of protein stability, increased solubility and decreased immunogenicity compared to their parent molecule by increasing protein molecular size and shielding the metabolic sites (Rajan et al., 2006; Lee et al., 2005; Yang et al., 2004; Tsuji et al., 1985). Due to these advantages, PEGylated protein therapeutics can enhance therapeutic efficacy and reduce undesirable effects.

The increasing size of the attached PEG, however, may result in loss of bioactivity (Bowen et al., 1999). Therefore, the successful development of a PEGylated therapeutic protein requires an optimized balance between enhanced pharmacokinetics and reduced bioactivity by the judicious selection of PEG size. In addition, PEG structure also plays a crucial role in bioactivity and pharmacokinetic behavior reported that linear PEG was distributed with a larger distribution volume, whereas branched PEG was distributed with a smaller distribution volume (Caliceti, 2004). These reports showed the advantages of branched PEG in the therapeutic use of PEGylated proteins by minimizing the loss of in-vitro bioactivity and maximizing the blood residual time. Therefore, the proper PEGylation, the selection of size and shape for PEG is one of the most important points.

In this study, a unique, trimer-structured, 23 kDa PEG was conjugated to rhG-CSF by forming an amide bond to improve the pharmacokinetic properties and minimize the loss of native protein bioactivity. in-vitro bioactivity, in-vivo stability, and pharmacokinetics of 23 kDa mono PEG-G-CSF prepared with this PEG molecule were assessed and compared to those of native rhG-CSF.

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Material and Method

Materials

rhG-CSF was obtained from the Research Laboratories of Dong-A Pharm. Co., Ltd. (Yong In, Korea). Purified rhG-CSF gave a single peak (>99%) on reverse-phase high performance liquid phase chromatography (RP-HPLC) and SDS-PAGE analysis (data not shown). Trimer PEG with N-hydroxy succinimidyl group (mPEG2CONHPEG-NHS, 23 kDa), branched PEG with N-hydroxy succinimidyl group (mPEG2-NHS, 40 kDa and 20 kDa), linear PEG with N-hydroxy succinimidyl group (mPEG-NHS, 10 kDa) were obtained from NOF corporation (Tokyo, Japan). 20 kDa linear PEG with aldehyde group was obtained from Nektar (San Carlos, USA).

Preparation and purification of mono-PEGylated rhG-CSFs

PEGylation was carried out by mixing of rhG-CSF(final concentration of 4 mg/mL) and PEG reagent at 1:1.5 molar ratio in 50 mM sodium borate buffer (pH 8.5) at 4°C. After 1.5 h, the reaction was stopped by adjusting the pH of the mixture to 4.0 with acetic acid. The reaction mixture was subjected to size-exclusion high performance liquid chromatography (SE-HPLC) on a Shodex KW803 column (ShowaDenko K.K., Tokyo, Japan) eluted with 50 mM phosphate buffer with 1 M sodium chloride (pH 6.0), and the degree of PEGylation was monitored with SE-HPLC and SDS-PAGE to optimize the condition for the fabrication of the mono PEG-G-CSF. For isolation of the mono PEG-G-CSF species, the reaction mixture was diluted 10-fold with distilled deionized water and applied into a column (4 cm × 15 cm) packed with SP-Sepharose resin (Amersham Biosciences, Sweden). The column was washed with the equilibration buffer to remove excess PEG reagent. The desired mono PEG-G-CSF was then eluted with a NaCl gradient, and concentrated to approximately 10 mg/mL for further experiments.

MALDI-TOF mass spectrometry

MALDI-TOF MS analysis was performed using a Voyager-RP Biospectrometry Workstation (PerSeptive Biosystems, Cambridge, MA) with a slight modification of a method described elsewhere (Lee et al., 2005). Samples were prepared by mixing 5 μL of an aliquot with 30 μL of the matrix solution, which was a saturated solution of sinapinic acid in 50% of water/acetonitrile with 0.1% trifluoroacetic acid. One microliter of the sample mixture was spotted into a well of the sample plate and dried by vacuum evaporation prior to mass spectrometry. Data for 2 ns pulses of the 337 nm nitrogen lasers were averaged for each spectrum in a linear mode, and positive ion TOF detection was performed by using an accelerating voltage of 25 kV. A mixture of cytochrome C and bovine serum albumin was adapted for the external calibration.

Circular dichroism

Circular dichroism (CD) analysis was used to examine the secondary or tertiary structural conformation of PEG-G-CSF. The concentration was adjusted to 0.5 mg/mL with distilled deionized water. The spectrum was analyzed and compared to native rhG-CSF using a Jasco J600 CD spectropolarimeter (Jasco, Tokyo, Japan).

Size exclusion high performance liquid chromatography (SE-HPLC)

HPLC was performed on a Waters Liquid Chromatography (Millford, MA) equipped with WISP 717 plus autosampler refrigerated at 4°C, 1525 Binary HPLC Pump and a 2487 Dual λ absorbance UV/Vis detector. Data was recorded by Waters Empower software. Chromatographic separations were performed on a Shodex Toso Hass G3000 Silica based GFC column KW-803 (8.0 × 300 mm, 5 μm) with an isocratic mobile phase of 20 mM sodium acetate, pH 4.0 containing 200 mM sodium chloride. The absorbance at 214 nm is reported as mAU.

Ion-exchange high performance liquid chromatography (IE-HPLC)

Analytical ion exchange samples were analyzed on a TSK gel SP-5PW column (4.6 × 75 mm), initially equilibrated in 25 mM sodium acetate, pH 4.0 (buffer A), and eluted with a linear gradient of 100 mM sodium acetate, pH 7.8 (buffer B) at linear gradient of 50-100% over 100 min. The absorbance at 280 nm is reported as mAU.

Reversed-phase high performance liquid chromatography (RP-HPLC)

Chromatographic separations were performed on a reversed phase HPLC column, Vydac C4 214TP54 (4.6 mm × 250mm, 5 μm) with the column temperature of 35°C equilibrated in buffer A (10% of HPLC grade acetonitrile with 0.1% TFA), using a linear gradient starting at 45% buffer B (80% of HPLC grade acetonitrile with 0.1% TFA), increasing to 75% over 30 min. The absorbance at 214 nm is reported as mAU.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were diluted with either reducing or non-reducing

buffer and 5.0 μg of protein was loaded into each well. The gels were run on a discontinuous buffer system and stained with Coomassie staining.

***In-vitro* biological assay**

NFS-60 cells (ATCC CRL-1838) were grown in RPMI 1640 medium (Gibco BRL, 22400-089) supplemented with 5% FBS and 1 ng/mL of IL-3. Cells were collected at a density of 2×10^5 cells/mL and 100 μL of cell culture was added to each well of 96-well microplate. Mono PEGylated rhG-CSF or rhG-CSF was diluted in the medium with varying concentrations and added to the wells in a volume of 100 μL. After 2-day incubation, cell proliferation was quantitated by MTS assay kit (Promega, Madison, WI). The 50% effective concentration (EC_{50}), the concentration required to saturate 50% of the maximal response, was calculated from dose response curve (Hara et al., 1988).

Stability of PEGylated G-CSFs

The stabilities of various PEG conjugates were accomplished and compared at the harsh condition of 37ºC for 1 week. Degradation and aggregation were monitored by RP-HPLC and SE-HPLC. The pH associated stability of mono PEGylated rhG-CSF was shown over the pH range of 4.0-8.0 after 1 day incubation at 37ºC. Thermal stability was also observed over the range of 4-100ºC. Stability data was achived by SDS-PAGE, SE-HPLC and *in-vitro* assay which have been described previously.

Pharmacokinetic parameters

The pharmacokinetics of rhG-CSF and 23 kDa PEG-G-CSF were evaluated after a single subcutaneous injection in SD male rats (7-8 weeks old, 230-250 g). Two rats in each group received 400 μg/rat of rhG-CSF and 23 kDa PEG-G-CSF. Blood samples were withdrawn at properly designed time points, and the serum samples were analyzed by ELISA.

Pharmacodynamics

The Pharmacodynamics of 23 kDa PEG-G-CSF was evaluated after a single subcutaneous injection in female neutropenic mice (7-8 weeks old) or in male neutropenic rats (7-8 weeks old, 230-250 g). The neutropenic condition was induced by cyclophosphamide in 3 mice or 5-fluorouracil in 7 rats. In cyclophosphamide–induced neutropenia group, each animal received 0.125 mg/kg of rhG-CSF twice a day for 4 days and 1 mg/kg of 23 kDa PEG-G-CSF once via s.c. administration route. In 5-fluorouracil–induced neutropenia group, each animal received 10, 100 and 1000 μg/kg of 23 kDa PEG-G-CSF via s.c. administration route. The ANC counting was accomplished using ADVIA-120 (Bayer).

Results and discussion

It has been generally known that the covalent attachment of PEG is currently being employed to extent the circulation time of proteins in the body. For the successful introduction of PEG modified proteins, optimization of PEG is need for appropriate physicochemical properties because slightly different PEG can offer thoroughly different effects on the pharmaceutical properties of PEG-protein conjugate. We have found conditions (see materials and methods) which the PEGylation reaction can take place. For this study, we used various PEG moieties that have different molecule size and structure (Figure 1).

Preparation and stability study of PEGylated G-CSFs

rhG-CSF is known to aggregate under physiological conditions (e.g., pH 6.9 and 37ºC) (Ribarska et al., 2008; Rajan et al., 2006; Krishnan et al., 2002). Several studies have been reported that PEG conjugation resulted in the increased physical stability of rhG-CSF, thereby affording enhanced physical stability and increased solubility because of the hydrophilicity of the PEG molecule. PEGylation has also been shown to decrease aggregation and prevented the formation of insoluble precipitate (Rajan et al., 2006) thereby increasing the formulation stability of the protein (Jain and Jain et al., 2008; Ryan et al., 2008; Yamasaki et al., 1994).

We studied various PEG-G-CSF conjugates that modified

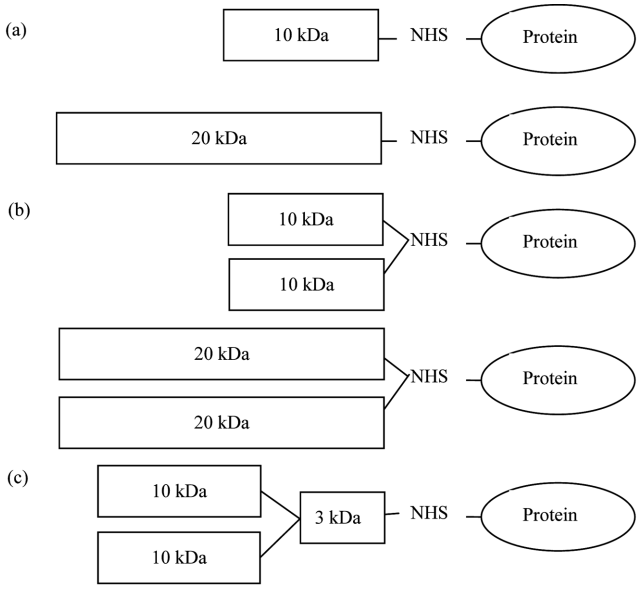

Figure 1. Various PEG moieties with different size and structure. (a): linear PEG; (b): branched PEG; (c): trimer PEG.

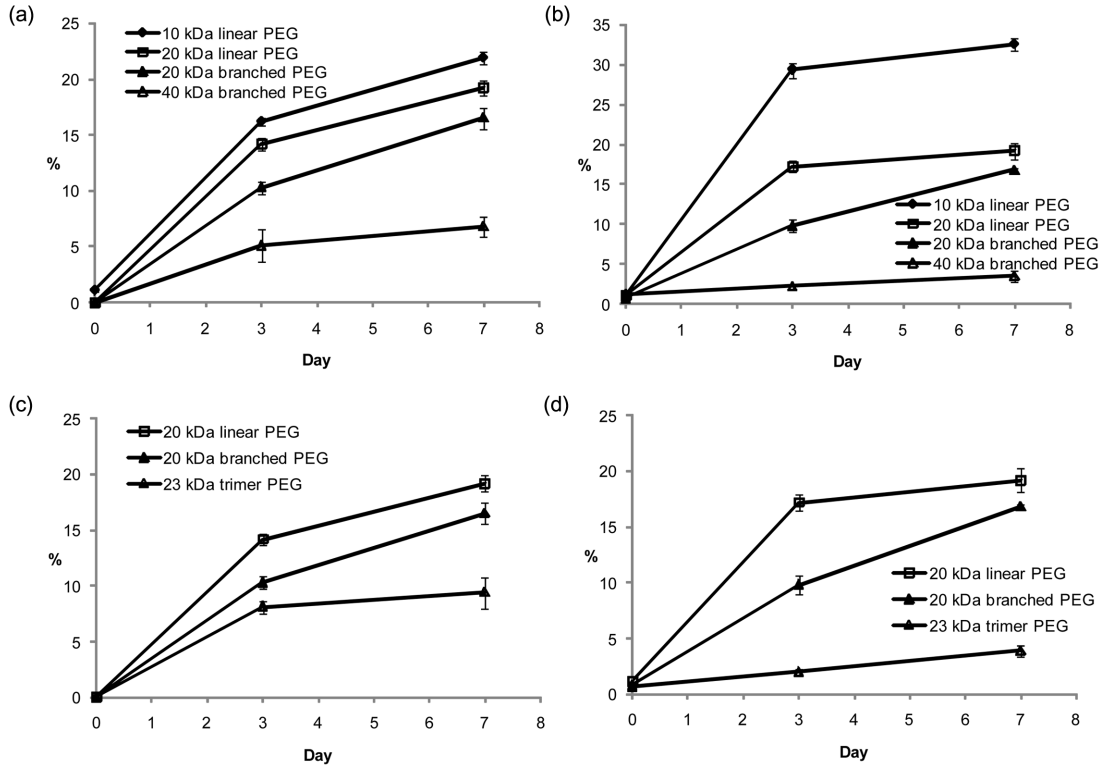

Figure 2. Stability of different PEG-G-CSF conjugates stored at 37°C for 7 days (RP-HPLC and SE-HPLC). (a) and (c); the percentages of dePEGylated rhG-CSF in the PEG-G-CSF conjugates from the results of RP-HPLC; (b) and (d); the percentages of soluble aggregate in the various PEG-G-CSF conjugates from the results of SE-HPLC. (n=3)

with PEG moieties of different size and structure as illustrated above to select most effective PEG moiety for rhG-CSF modification. The mono PEG-G-CSF conjugates were prepared via an amide bond as a result of the reaction between an N-hydroxysuccinimide ester derivative of a PEG molecule and the free amino group of rhG-CSF. After conjugation of 23 kDa trimer, 40 kDa branch, 20 kDa branch, 20 kDa linear or 10 kDa linear PEG moieties to rhG-CSF, all conjugates were dialysed with 50 mM sodium phosphate buffer (pH 7.0), then stored at 37°C for 7 days. Samples were analyzed with RP-HPLC, SE-HPLC and SDS-PAGE to compare the stability of each conjugate.

Among the various PEG-G-CSF conjugates with different size and structure PEG, the conjugate that modified with bigger sized and more hindered-structured PEG molecule has the most effective stability. In example, 20 kDa linear PEG-G-CSF produced less dePEGylated rhG-CSF and soluble aggregate than 10 kDa linear PEG-G-CSF, and 40 kDa branched PEG-G-CSF also did than 20 kDa linear PEG-G-CSF in the harsh condition as results from RP-HPLC and SE-HPLC. And the PEG-G-CSF conjugates modified with more hindered-structured PEG moiety showed more effective stability (23 kDa trimer PEG-G-CSF > 20 kDa branched PEG-G-CSF > 20 kDa linear PEG-G-CSF) (Figure 2). These data showed that the conjugate modified with 23 kDa trimer-structured PEG moiety showed the most effective stability at the phygiological condition.

With the comparison of the 23 kDa trimer-structured PEG-G-CSF conjugate (23 kDa PEG-G-CSF) and 20 kDa linear-structured PEG-G-CSF conjugate (20 kDa PEG-G-CSF), the former showed more dePEGylated rhG-CSF due to the degradation procedure in the phygiological condition, but less soluble aggregate formation than the latter. PEG conjugated at the Lys residue was shown to have less stability than at the N-terminus residue as previously (Kinstler et al., 1996), but the steric hindrance of the trimer structured PEG molecule may reduce the formation of the soluble aggregate (Figure 3).

Preparation, purification and characterization of 23kDa-PEG-G-CSFs

The 23 kDa trimer-structured PEG-G-CSF conjugate (23 kDa PEG-G-CSF) was prepared as described above. Reaction mixture was analyzed by SE-HPLC to consider the ratio of mono-PEGylated conjugate, di-PEGylated conjugate and unconjugated form (Figure 4). The size-exclusion chromatogram showed that the reaction mixture contained di-PEGylated rhG-CSF species, mono-PEGylated rhG-CSF species, and

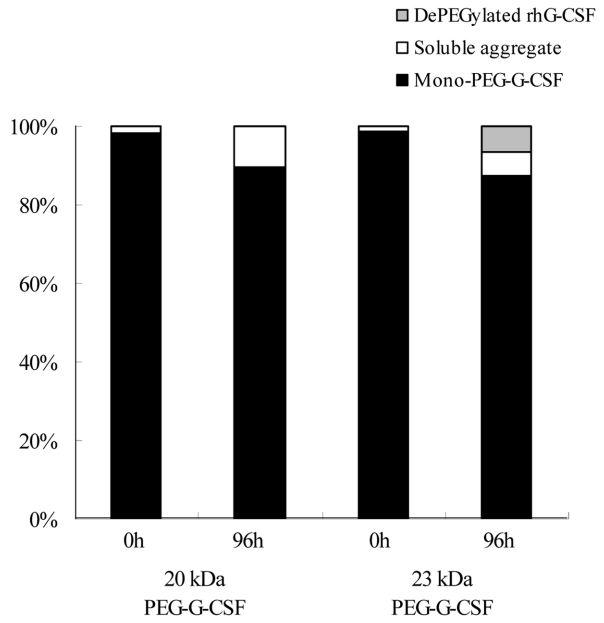

Figure 3. Formation of soluble aggregate and degraded rhG-CSF in the different PEG-G-CSF conjugates by SE-HPLC results. Left bar; 0 h, right bar; after 96 h.

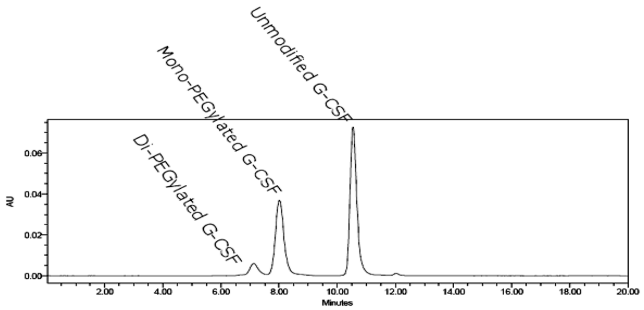

Figure 4. Ion-exchange chromatogram of 23 kDa PEG-G-CSF reaction mixture.

unmodified rhG-CSF for the conjugate PEGylated with 23 kDa trimer PEG. Then, the mono PEGylated rhG-CSF was separated from di-PEG-G-CSF and the unconjugated rhG-CSF molecules by SP-Sepharose cation-exchange chromatography, and the purity was approximately 99% as determined by SE-HPLC and RP-HPLC (Figure 5a and 5b). The purified 23 kDa PEG-G-CSF isolated by cation exchange chromatography was assessed by SDS-PAGE (Figure 6). SDS-PAGE analysis with Coomassie staining revealed an intense broad band corresponding to 23 kDa PEG-G-CSF. The purified 23 kDa PEG-G-CSF appeared as a single band. The estimated molecular weight from SDS-PAGE suggests that 23 kDa PEG-G-CSF contains only a single PEG molecule and a small portion of unmodified rhG-CSF. The retarded mobility of PEGylated proteins on SDS-PAGE gels has been previously discussed (Kurfürst, 1992) therefore the actual molecular weight of the 23 kDa PEG-G-CSF is best determined by MALDI-TOF/MS.

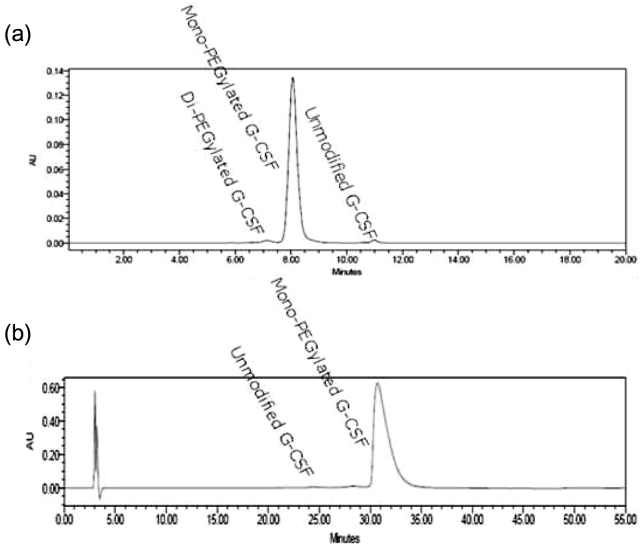

Figure 5. Chromatograms of purified 23 kDa PEG-G-CSF by (a) size-exclusion chromatogram and (b) reverse-phase high pressure liquid chromatographies.

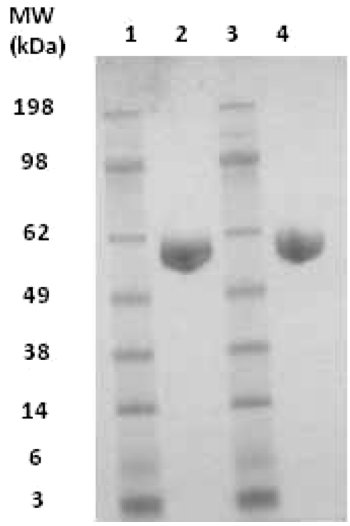

Figure 6. SDS-PAGE analysis of 23 kDa PEG-G-CSF with Coomassie staining. Lane 1 and 3; molecular weight marker, lane 2; 23 kDa PEG-G-CSF under reducing condition, lane 3; 23 kDa PEG-G-CSF under non-reducing condition.

The MALDI-TOF mass spectrometry (MALDI-TOF/MS) was used to determine the molecular weight of the purified 23 kDa PEG-G-CSF. The molecular mass of the purified 23 kDa PEG-G-CSF was determined to be 42368.75 Da by MALDI-TOF/MS as shown in Figure 7. Although the molecular weight determination of PEG modified proteins is complicated due to the inherent polydispersity which is typical of a polymer like PEG, these molecular weights are in good agreement with the

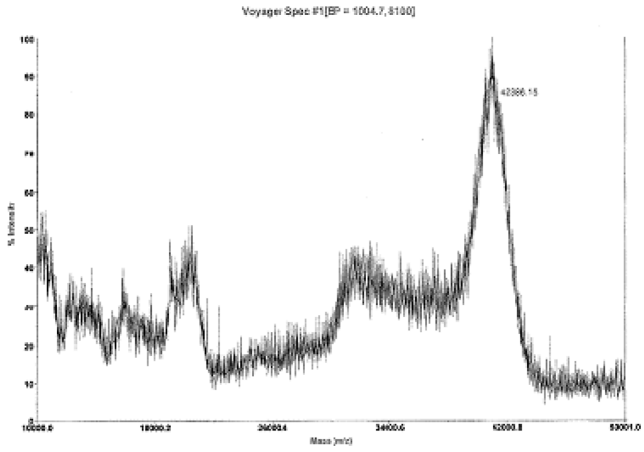

Figure 7. MALDI-TOF mass spectrum of 23 kDa PEG-G-CSF.

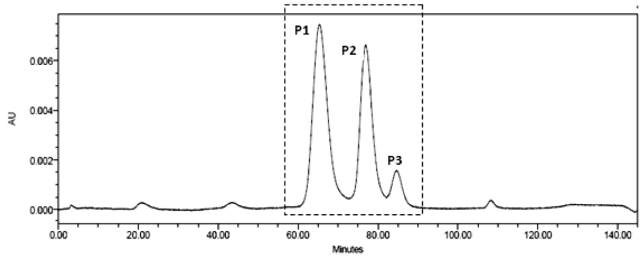

Figure 8. Positional isomers of 23 kDa PEG-G-CSF separated with cation exchange chromatography.

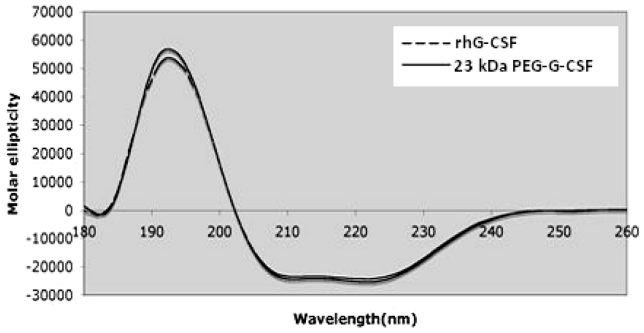

Figure 9. Circular dichroism spectra of 23 kDa PEG-G-CSF and rhG-CSF.

rhG-CSF conjugates containing a single 23,000 dalton PEG derivative.

Cation exchange chromatography was used to monitor the potential positional isomers in the PEGylated rhG-CSF (Figure 8). As rhG-CSF has 4 Lys residues, PEGylation can occur at the Lys and N-terminus residue. Therefore, the number of potential positional isomers of 23 kDa PEG-G-CSF is theoretically 5. In the ion-exchange chromatogram, 23 kDa PEG-G-CSF was separated to 3 positional isomers which could be attributed to the reactivity difference of PEG with each Lys residue which is under slight different pKa and steric hindrance.

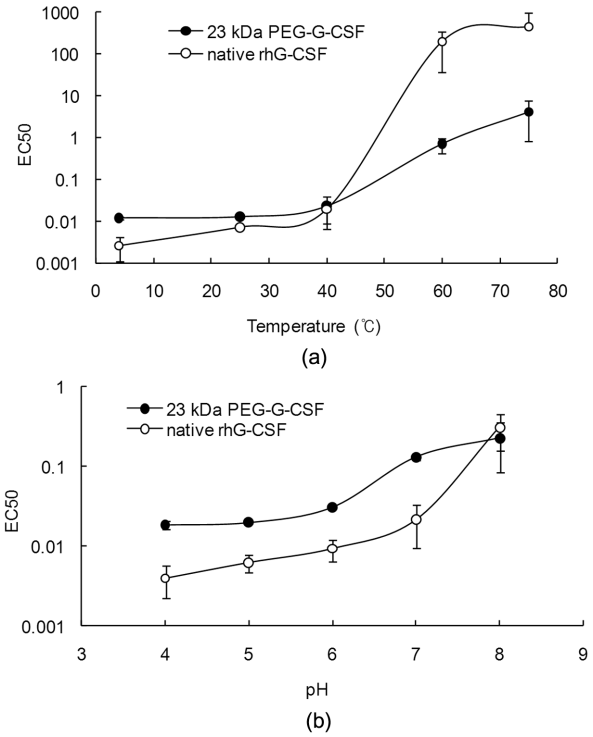

Figure 10. The stability of 23 kDa PEG-G-CSF in various temperature (a) or pH (b) judged by *in-vitro* assay.

The CD spectra for rhG-CSF and 23 kDa PEG-G-CSF were nearly superimposed across the near-to-far UV spectrum with minimum at 222 nm, suggesting that PEGylation had no significant effect on the secondary or tertiary structure of the rhG-CSF (Figure 10).

The 23 kDa PEG-G-CSF showed approximately 20% of *in-vitro* activity of native rhG-CSF (2.0×10^7 I.U./mg, data not shown)

Stability

23 kDa PEG-G-CSF conjugate was stored at various temperature within 4°C to 100°C for 15 min to examine the effect of temperature on the stability of conjugate. The effect of pH on the stability was examined over a pH range of 4-8. SDS-PAGE, SE-HPLC and In-vitro activity assay were used for the studies. SDS-PAGE and SE-HPLC results showed the reduced formation of soluble aggregate and dePEGylated rhG-CSF (data not shown). A representative in-vitro assay results (Figure 11) shown that 23 kDa PEG-G-CSF has lower value of EC_{50} compared to rhG-CSF (it corresponds with an inverse of in-vitro activity) as temperature and pH increase therefore it can be mentioned that 23 kDa PEG-G-CSF is more stable and can preserve *in-vitro* activity than native rhG-CSF. Reduced SDS-PAGE suggested that the high molecular weight species were produced as increasing temperature and pH (data not

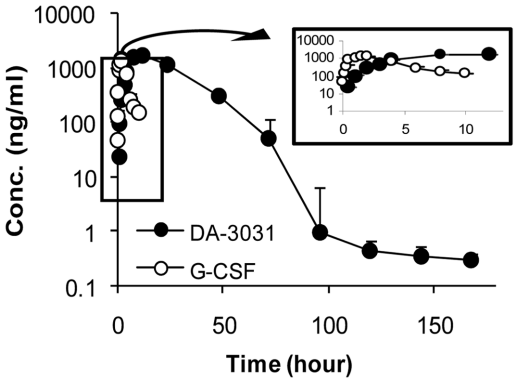

Figure 11. Serum concentration versus time curves following subcutaneous injection (400 ng/rat) of rhG-CSF and 23 kDa PEG-G-CSF in rats.

shown). As expected, pH 4.0 was determined to afford optimal stability (e.g., minimal aggregation and dePEGylation) for 23 kDa PEG-G-CSF.

Pharmacokinetics and pharmacodynamics

It is known that PEGylation leads to the prolonged elevation of the plasma levels of rhG-CSF as compared to unmodified rhG-CSF by lowering renal clearance and prolonging drug circulation in the blood (Werle and Bernkop-Schnürch, 2006). As expected, 23 kDa trimer PEG–G-CSF showed a dramatic improvement in various pharmacokinetic parameters (Table I). The serum concentration versus time curves obtained after subcutaneous injection of native rhG-CSF and 23 kDa PEG-G-CSF in rats are shown in Figure 12. The serum concentration of 23 kDa PEG-G-CSF remained over 0.3 ng/mL for 7 days, and the half-life of 23 kDa PEG-G-CSF was increased 4.5-fold compared to that of native rhG-CSF.

Pharmacodynamics of rhG-CSF and 23 kDa PEG-G-CSF were evaluated by increases of neutrophil counts after s.c. administration in rats. The concentration versus time (data not shown) and ANC (absolute neutrophil count) time profiles

Table I. *Summary of Pharmacokinetic Parameters of rhG-CSF and 23 kDa PEG-G-CSF (n = 2). $T_{1/2}$; Terminal Half-life, AUC; Area Under the Concentration-time Curve, MRT; Mean Residual Time, CL; Serum Clearance after s.c dosing, C_{max}; Observed Maximal Serum Concentration, T_{max}; Time for C_{max}*

PK parameter	rhG-CSF	23 kDa PEG-G-CSF
$T_{1/2}$ (hr)	2.395±0.134	10.75±0.212
AUC (ng·hr/mL)	6142.5±77.1	47421±2511.6
MRT (hr)	4.09±0.156	24.1±0.141
CL (L/kg hr)	0.261±0.0003	0.034±0.002
C_{max} (ng/mL)	1383.2±97.7	1676.75±49.6
T_{max} (hr)	1.5±0.0	12±0.0

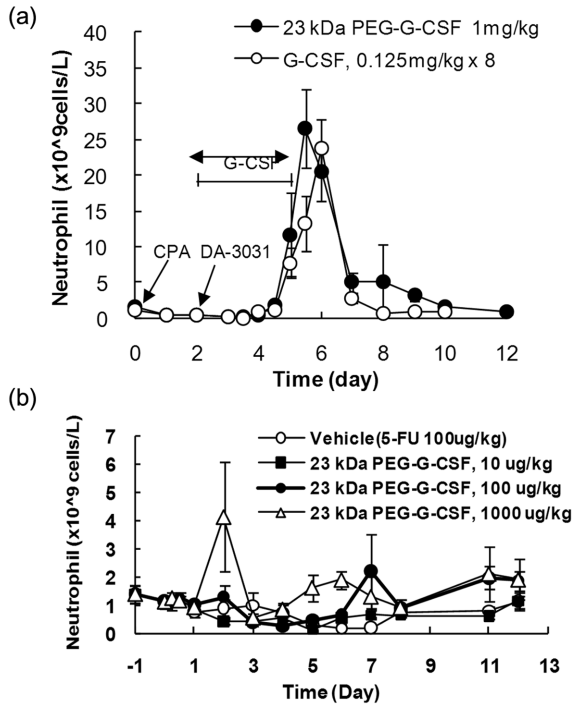

Figure 12. The induction of peripheral ANC recovery by the s.c. injection of either rhG-CSF or 23 kDa PEG-G-CSF in neutropenic condition induced by (a) cyclophosphamide (n=3) or (b) 5-fluorouracil (n=7)

from the cyclophosphamide-induced neutropenic mice were compared (Figure 13a). The single s.c. administration of 23 kDa PEG-G-CSF significantly improved the time for recovery of ANC when comparing daily administration of rhG-CSF. The ANC induced by 23 kDa PEG-G-CSF peaked 5 days after the s.c. injection, whereas the maximal ANC induced by rhG-CSF was reached at 6 days after the daily injection. A single s.c. injection of the 23 kDa PEG-G-CSF also leads to prolonged elevation of ANC in neutropenic rats. The concentration versus time (data not shown) and ANC versus time profiles from the 5-fluorouracil-induced neutropenic rats (n=7) were compared (Figure 13b). 23 kDa PEG-G-CSF showed improvement of neutropenic condition induced by anticancer drug, and the efficacy was dose-dependent.

Conclusions

This study was focused on the unique, trimer-structured PEG and its use for bioactive proteins. We selected most proper PEG among various PEG moieties with different size and structure for the most effective physiological stability and efficacy. The long-acting rhG-CSF prepared by utilizing this unique PEG showed dramatically enhanced properties compared to those of rhG-CSF. 23 kDa PEG-G-CSF exhibited

superior performance to native rhG-CSF and linear 20 kDa linear PEG-conjugated rhG-CSF. These results appeared to be attributable to the differences of the size and trimer structure of PEG moiety. Consequently, the 23 kDa trimer-structured PEG has the potential to endow promising therapeutic proteins with clinically useful properties.

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