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Characterisation of the site-specific monoPEGylated rhG-CSF analogue pegteograstim

Jeungwoon Hong[a,b], Byoungju Lee[b], Kwanyub Kang[c], Seung-Hoon Lee[c], Jaehwan Ryu[c], Gangsoo Jung[b], Jaetaek Oh[b], Eui-Cheol Jo[c], Chan-Wha Kim[a,*]

[a] Department of Biotechnology, College of Life Sciences and Biotechnology, Korea University, 136-701, Anam-dong, Seoungbuk-gu, Seoul, Republic of Korea
[b] Green Cross Corp., Ihyeon-ro 30 Beon-gil 107, Giheung-gu, Yongin-si, Gyeonggi-do, 16924, Republic of Korea
[c] MOGAM Institute for Biomedical Research, Ihyeon-ro 30 Beon-gil 107, Giheung-gu, Yongin-si, Kyonggi-Do, 16924, Republic of Korea

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ABSTRACT

We describe the characterisation of a novel monoPEGylated recombinant human granulocyte colony-stimulating factor analogue, pegteograstim (Neulapeg), prepared by site-specific 20 kDa maleimide-PEG conjugation. An additional cysteine was inserted between Gly136 and Ala137 of filgrastim (methionyl human granulocyte colony-stimulating factor) for site-specific PEGylation, and Cys18 of filgrastim was replaced with Ser18 to prevent unwanted PEGylation. Pegteograstim was produced by *Escherichia coli* and purified by cation exchange chromatography, and its structural, physicochemical, biological and immunological properties were investigated. Male Sprague-Dawley rats were administered pegteograstim (100 μg/kg) and the pharmacokinetics and pharmacodynamics compared with those of filgrastim.

The results of long-term stability testing of pegteograstim revealed no significant change in its quality attributes at 2–8 °C for 36 months. In addition, pegteograstim was stable under the accelerated conditions (25 ± 2 °C, RH of 60 ± 5%) for 6 months. The site-specific monoPEGylated pegteograstim is a highly pure, stable and novel drug for long-lasting treatment of chemotherapy-induced neutropenia.

1. Introduction

Human granulocyte colony-stimulating factor (hG-CSF) is a haematopoietic growth factor that regulates the proliferation and differentiation of neutrophilic granulocytes and stimulates the release of mature neutrophils from bone marrow [1,2]. Recombinant human G-CSF (rhG-CSF) expressed in *Escherichia coli* is non-glycosylated and has been widely used as a therapeutic protein to treat myelosuppression associated with cancer chemotherapy treatments and aplastic anaemia, and for the mobilisation of peripheral blood progenitor cells for transplantation [3].

However, rhG-CSF has a short half-life *in vivo* that necessitates a daily dosing schedule because of rapid renal clearance and specific degradation mediated by G-CSF receptors or by neutrophil elastase [4–11]. To improve the short half-life of protein drugs in the blood, polyethylene glycol (PEG), a neutral, highly hydrophilic and nontoxic polymer, can be covalently conjugated to extend their circulation time [12,13]. Various methodological approaches for conjugating PEG to proteins have been investigated, and some PEGylated proteins have been approved by the US Food and Drug Administration (FDA) [14–17].

PEGylation can improve solubility, stability, resistance to proteolytic inactivation and pharmacokinetic profile. Furthermore, PEGylation is able to decrease renal clearance and immunogenicity of many therapeutic proteins such as cytokines, hormones, enzymes and Fab' antibody fragments [18–21]. However, it has been reported that PEGylation may result in lowing biological activity *in vitro* since binding affinity of PEGylated proteins to their receptors can be decreased due to PEG-induced steric hindrance. In spite of the lower bioactivity of PEGylated proteins, prolonged circulating half-life induced by PEGylation can create plenty of chances for the receptor-ligand interaction *in vivo*. Consequently, although PEGylated protein gives rise to lowering bioactivity *in vitro*, the decreased activity of the PEGylated protein can be compensated *in vivo* with the prolonged circulating half-life [22,23].

The potential PEGylation sites in rhG-CSF are the N-terminus and amino groups of four lysine residues. Therefore, this conjugation produces hetero-rather than mono-PEGylated rhG-CSF, with molecules

* Corresponding author.
E-mail addresses: jwhong@greencross.com (J. Hong), bj0416@greencross.com (B. Lee), gykang@mogam.re.kr (K. Kang), leeshyy@mogam.re.kr (S.-H. Lee), ryukino@mogam.re.kr (J. Ryu), gsjung0402@greencross.com (G. Jung), jtoh@greencross.com (J. Oh), ecjo@mogam.re.kr (E.-C. Jo), cwkim@korea.ac.kr (C.-W. Kim).

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having different bioactivities. Different multimeric and isomeric states can reduce the biological activity of the PEGylated product, making accurate pharmacokinetic measurement and purification difficult [24]. Even though the same overall sizes of conjugated products are obtained, position-specific isomers are produced, which increases the time and cost of the procedure because removal of unwanted conjugates is essential [25].

The rhG-CSF molecule has one free cysteine at position 18, and two disulphide bridges between C36 and C42 and between C64 and C74 that are critical for protein folding of native G-CSF and maintenance of its biological activity. However, because the cysteine residue at position 18 is not involved in biological activity, it can be substituted with serine by site-specific mutation [26–28].

In our previous study, we produced the rhG-CSF Cys18Ser mutant and prepared a rhG-CSF analogue of this mutant with a free cysteine introduced at a position that does not interfere with the biological activity and where it can be easily PEGylated. Specifically, we introduced a free cysteine into the structurally flexible CD loop ($Gly^{126}-Ser^{143}$) of rhG-CSF by substitution or insertion, and conjugated PEG to the free cysteine. We anticipated that the CD loop region would not affect the overall structure of rhG-CSF based on its flexibility in the crystal structure. However, the resultant cysteine-introduced mutants showed considerable differences in stability; while some mutants were expressed at high levels, were highly stable and easily purified, others were poorly expressed, unstable and precipitated during purification [29]. We subsequently screened mutants of PEGylated G-CSF conjugates that maintained their biological activity and structural stability following PEGylation.

Pegteograstim (Neulapeg) is a novel recombinant human G-CSF conjugated with methoxy-maleimide-polyethylene glycol, which was developed by Green Cross Corporation in Korea. Pegteograstim contains an additional cysteine inserted between Gly136 and Ala137 of filgrastim for site-specific PEGylation, Cys18 is substituted with Ser to prevent unwanted PEGylation and the molecule is conjugated in a site-specific manner to 20 kDa maleimide-PEG. Since pegteograstim contains a PEG anchored to a unique site in the structurally flexible CD loop region located far from the receptor binding site, it displays higher biological activity based on its higher affinity for the G-CSF receptor [30].

The results of the pegteograstim phase I study revealed increased activity of pegteograstim toward ANC and $CD34^{+}$ cells compared with pegfilgrastim at the same dose [10,31]. The results of the pegteograstim phase II/III study demonstrated that it was just as effective as pegfilgrastim for preventing severe neutropenia after high-risk chemotherapy in breast cancer patients. The durations of grade 4 neutropenia and the incidence of febrile neutropenia in patients who received prophylactic pegteograstim were comparable to those in patients who received pegfilgrastim. The duration of grade 4 neutropenia observed in phase III studies (1.64 d) substantiated the results in phase II studies lasting 1.54 days for the 6.0 mg group. Among the secondary efficacy endpoints, the time to recovery to ANC $\geq 2000/\mu L$ in the pegteograstim group was significantly shortened by 1 day compared with that in the pegfilgrastim group (8.85 vs. 9.83 days, $p < 0.0001$) [30].

In the present study, we investigated the structural and physicochemical properties of pegteograstim using mass spectrometry and circular dichroism to investigate the primary structure, molecular mass, PEGylation site and secondary structure. PEGylation at Cys137 was confirmed by size exclusion high-pressure liquid chromatography (HPLC) and N-terminal sequencing. Furthermore, ion-exchange (IEX)-HPLC, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), western blotting and isoelectric focussing revealed physicochemical properties. For the biological property, *In vitro* bioassay with M-NFS60 cells was performed, and pharmacokinetic and pharmacodynamic studies were carried out using Sprague-Dawley (SD) rats.

2. Material and methods

2.1. Materials

The rhG-CSF analogue was expressed in *E. coli* and accumulated in inclusion bodies that were subjected to cell disruption and centrifugation, and the resultant pellet was dissolved in urea solution and activated by refolding. Refolded rhG-CSF was purified by cation exchange chromatography.

2.2. PEGylation

PEGylation formed a covalent bond between the 20 kDa maleimide-PEG (SUNBRIGHT ME-200MAOB, NOF, Japan) and Cys137 of the rhG-CSF analogue. The pH of the rhG-CSF analogue sample eluted from the cation exchange chromatography was adjusted to 6.8, and 20 kDa maleimide-PEG was added to a final ratio of maleimide-PEG (20 kDa):rhG-CSF analogue of between 1.5:1 and 3:1. The average molecular weight of 20 kDa maleimide-PEG and a bovine serum albumin (BSA) standard curve were used to calculate the molar ratio of maleimide-PEG to rhG-CSF analogue. The 20 kDa maleimide-PEG was dissolved in dimethyl sulfoxide (DMSO), mixed with rhG-CSF analogue and PEGylated at 2–8 °C for 12–18 h. PEGylation was stopped by adjusting the pH to 3–4 using 10% (v/v) HCl.

2.3. Amino acid sequence

Pegteograstim was reduced with dithiothreitol (65 °C, 30 min) at a final concentration of 10 mM and alkylated with 2-iodacetamide (RT, 30 min) at final concentration of 15 mM in 100 mM ammonium bicarbonate, pH 7.2. Protein digestion was performed by trypsin and trypsin/Glu-C protease:protein ratio of 1:20 (w/w). The protein concentration should be at least 0.1 mg/ml for mass spectrometric analysis. Protein-protease mixtures were incubated for 16 h at 37 °C. Digested peptides were separated by UPLC (Waters) equipped with a BEH C18 column (2.1 × 50 mm, 1.7 μm, Waters, CA, USA). The gradient was as follows: A = 0.1% formic acid in water, B = 0.1% formic acid in ACN; 3–50% B from 0 to 40 min, 100% B from 40 to 45 min and 3% B from 50 to 60 min. A Synapt G2 (ESI-Q-TOF) mass spectrometer (Waters, London, GBR) was used to record peptide spectra over a mass range (m/z) of 100–1990 and MS/MS spectra in information-dependent data acquisition over a mass range of 100–1990. Analytical parameters were as follows: capillary voltage = 3 kV, source temperature = 100 °C, desolvation temperature = 450 °C and desolvation flow rate = 800 L/h. Searching parameters for Biopharmalynx (Waters) were set as follows: enzyme = trypsin and trypsin/Glu-C with one allowed missing cleavage site, peptide mass tolerance = 0.05 Da, MS/MS tolerance = 0.05 Da and carbamidomethyl (C) as a fixed modification.

2.4. PEGylation site

Proteolytic digestion to determine the PEGylation site was performed for 18 h per enzyme using endoproteinase Glu-C. Reaction products were separated by a Superdex peptide HR 10/300 GL column (10 × 300 mm, 13 μm; GE Healthcare, UK) and analysed by MALDI-TOF MS (Bruker Daltonik, CA, USA) and Edman N-terminal sequencing (Applied Biosystems, CA, USA).

2.5. Disulphide linkage

After digestion of pegteograstim using endo-proteases without reduction and alkylation, the resulting peptides were analysed using LC-ESI-MS and MS/MS as described above in 2.3. Masses of coupling peptides linked by disulphide bonds were extracted from total ion chromatography using MassLynx software (Waters) and evaluated by matching MS/MS fragments to amino acid sequences using BioLynx