The Glycosylation and *in Vivo* Stability of Human Granulocyte-Macrophage Colony-Stimulating Factor Produced in Rice Cells

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Recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) has been produced in several different cell types, and has different properties depending on the production process used. There has been no report of glycosylation ratio or of the role of glycans for plant cells-derived rhGM-CSF. We investigated the terminal sialylation of rhGM-CSF produced in rice cells (rrhGM-CSF) using lectins. Glycosylation ratios of rrhGM-CSF and yeast-derived rhGM-CSF (yrhGM-CSF) were evaluated by chemical deglycosylation. After intravenous administration to rats, the clearance rates of rrhGM-CSF and yrhGM-CSF as well as deglycosylated forms of them were evaluated. *In vitro* bioactivities of rrhGM-CSF and yrhGM-CSF were measured in dose- and time-dependent manners. Although rrhGM-CSF does not have terminal sialic acids, the glycosylation ratio was two-fold higher than that of yrhGM-CSF, and rrhGM-CSF sustained longer than yrhGM-CSF in the blood circulation. Moreover, yrhGM-CSF and rrhGM-CSF have almost same bioactivity *via* dose- and time-dependent manners. Deglycosylated rrhGM-CSF was cleared faster than native forms, while deglycosylated yrhGM-CSF and yrhGM-CSF were similarly cleared in blood circulation. These results suggest that the glycans on rrhGM-CSF are superior to the glycans on yrhGM-CSF in terms of *in vivo* stability.

Key words granulocyte-macrophage colony-stimulating factor; lectin; sialic acid; chemical deglycosylation

Human granulocyte-macrophage colony-stimulating factor (hGM-CSF) is a glycoprotein hemopoietic growth factors that stimulates the proliferation of myeloid precursor cells and activates mature granulocytes and macrophages.¹⁾ Recombinant hGM-CSF (rhGM-CSF) is produced by a number of cell types including lymphocytes, endothelial cells, monocytes, fibroblasts, and some malignant cells.²⁾ cDNA of hGM-CSF has been cloned and used to produce rhGM-CSF in cultured animal cells,^{3,4)} yeast,⁵⁾ *Escherichia coli*,^{6,7)} and plant cells.^{8–11)} hGM-CSF has two potential *N*-glycosylation sites (Asn 27, Asn 37) and three potential *O*-glycosylation sites (Ser 7, Ser 9, Thr 10).^{4,12,13)} *Escherichia coli*-derived rhGM-CSF has a molecular weight of 14.5 kDa and is nonglycosylated.⁷⁾ Yeast-derived rhGM-CSF has a molecular weight of 16–18 kDa and is *O*-glycosylated, which contains leucine instead of proline at position 23 of mature form of wild-type hGM-CSF.^{14,15)} Human lymphoblastoid Namalwa cell-derived rhGM-CSF molecules are *O*-glycosylated and produced in 0N, 1N and 2N forms, according to the degree of *N*-glycosylation.¹⁴⁾ *N*-Glycosylation of hGM-CSF has an important role in prolonging its half-life in the blood stream.^{13,14)} Therefore, the *in vivo* stability of heavily glycosylated animal cell-derived rhGM-CSF is superior to that of the yeast and *Escherichia coli*-derived forms.¹⁴⁾ Transgenic plant-derived rhGM-CSF has been produced in tobacco cells,⁸⁾ tomato cells,¹⁰⁾ sugarcane¹¹⁾ and rice cells.⁹⁾ The most important features of production in transgenic plants are its very low investment and operating costs.^{16,17)} Downstream processing has obvious economic advantages over traditional fermentation methods because recombinant proteins can be produced in plants at 2–10% of the cost of microbial fermentation systems and at 0.1% of the cost of mammalian cell culture.^{18,19)} *N*-Linked glycans in plant have a core substituted by two *N*-acetylglucosamine (GlcNAc) residues, as observed in mammals. However the plant *N*-glycans lacks ter-

минаl sialic acids, and have plant-specific α 1,3-fucose and β 1,6-xylose residues.²⁰⁾ The absence of terminal sialic acids in glycoproteins markedly decreases their *in vivo* stability.^{21,22)} However, recent reports of Shah *et al.*²³⁾ and Takashima *et al.*²⁴⁾ suggest a possible genetic and enzymatic basis for sialylation in plants and thus the potential for terminal sialylation of glycoproteins produced in plant cells. However there has been no report of terminal sialylation or of the role of glycans in glycoproteins produced in plant cells.

In this study, we analyzed the terminal sialylation of rice cell-derived rhGM-CSF (rrhGM-CSF). We measured the *in vitro* bioactivities of rrhGM-CSF and commercial yeast-derived rhGM-CSF (yrhGM-CSF) and evaluated the dependence of their *in vivo* stabilities on glycosylation by chemically deglycosylating them.

MATERIALS AND METHODS

Proteins For obtaining the rrhGM-CSF, mRNA of hGM-CSF was isolated from peripheral blood mononuclear cells and cloned as described in Shin *et al.*⁹⁾ The hGM-CSF expression vector pMYN44 containing the hGM-CSF gene under the control of the Ramy3D promoter and with a signal peptide coding sequence was transfected into rice cells and transgenic derivatives were selected with hygromycin B. mRNA and proteins were obtained from suspension cell cultures and were identified by Northern and Western blotting, respectively.⁹⁾ Proteins were purified by DEAE anion-exchange chromatography. Leucogen (Sargramostim, LG Life Science, Seoul, Korea) was used for yrhGM-CSF. The yrhGM-CSF was used for chemical deglycosylation, pharmacokinetic experiment and *in vitro* bioactivity assay, as a control.

Preparation of Asialo Chinese Hamster Ovary Cell-Derived Recombinant Human Erythropoietin (CHO-EPO)

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Terminal sialic acids were removed with $\alpha(2\rightarrow3,6,8,9)$ neuraminidase, proteomics grade, from *Arthrobacter ureafaciens* with 5 $\times$ reaction buffer (Sigma, St. Louis, MO, U.S.A.). 170 μ g of samples and neuraminidase mixture were incubated at 37 °C for 3 h and the reactions were stopped by heating for 5 min at 100 °C.^{25,26} The solutions were dialyzed against distilled water to remove remaining sialic acids. Removal of terminal sialic acids was confirmed by examining terminal sialylation using lectins, as described below.

Analysis of Terminal Sialylation $\alpha2,3$ and $\alpha2,6$ -Terminal sialylation of rrhGM-CSF was determined using biotinylated *Maackia amurensis* lectin II (MAA II) (Vector Laboratories, Burlingame, CA, U.S.A.) and biotinylated *Sambucus nigra* lectin (SNA) (Vector Laboratories), respectively.²⁷ 96-well microtitre plates were coated overnight at 4 °C with 30 ng of sample per well. Unbound proteins were removed by washing three times with Tris buffered saline (TBS) containing 0.1% Tween 20 (TBST) and the plates were incubated with TBST for 1 h at 37 °C. After washing twice with 1 mM Tris pH 7.5 containing 0.1% Tween 20 and 1 mM CaCl_2 , 1 mM MgCl_2 , 1 mM MnCl_2 and biotin-labeled lectins were added and left at 37 °C for 1 h. The plates were washed four times with TBST, streptavidin-HRP (PIERCE, Rockford, IL, U.S.A.) was added and incubation continued at room temperature for 30 min.²⁷ Color reactions were developed using TMB substrate and measured at 450 nm. Values are shown as averages and standard deviations of duplicate measurements.

Chemical Deglycosylation Deglycosylation of rrhGM-CSF and yrhGM-CSF was performed with trifluoromethanesulfonic acid (TFMS, Sigma). Pre-cooled 10% anisole in TFMS solution was added to 1 mg of lyophilized rhGM-CSF sample and the mixture was intermittently shaken for 3 h at 4 °C. 0.2% bromophenol blue solution (indicator) was added followed by pre-cooled 60% pyridine solution until the color changed to light purple or blue.^{28–30} The solution was dialyzed against distilled water to remove remaining glycans and the deglycosylated rhGM-CSF was determined by SDS-PAGE analysis. Concentrations were measured by sandwich ELISA using an OptEIA™ set (BD Bioscience, San Jose, CA, U.S.A.).

SDS-PAGE All samples were subjected to 12% PAGE in the presence of sodium dodecyl sulfate according to the method of Laemmli.³¹ Separated proteins were visualized by silver staining (Amersham Pharmacia Biotech, Uppsala, Sweden).

Clearance Experiment in the Rat Three independent pharmacokinetics experiments were performed, using six weeks old male Sprague-Dawley rats (155–165 g) (Orient-bio, Kyonggi, Korea). Animals were acclimated for 7 d under controlled environmental conditions with free access to solid food and water. The animals were maintained at a constant temperature of 21 ± 1 °C, 12 h light/dark cycle. The protocol of this study was approved by the Animal Care and Use Committee of the College of Pharmacy, Chung-Ang University. Groups of three male rats received single intravenous injections of rrhGM-CSF, yrhGM-CSF, deglycosylated rrhGM-CSF or deglycosylated yrhGM-CSF, each at a dose of 180–187 μ g/kg. Blood samples were withdrawn at 0.25, 1, 2, 4, 8, 10, 24 and 48 h post injection.³² These were centrifuged and the sera were stored at -80 °C.^{14,32} The serum protein levels of rhGM-CSFs were determined by sandwich ELISA

using an OptEIA™ set (BD Bioscience).

In Vitro Bioactivity Assay Bioactivities of rrhGM-CSF and yrhGM-CSF were determined using the human erythroleukemic cell line (TF-1 cell line). The TF-1 cells were maintained in RPMI-1640 (GIBCO BRL, Grand Island, NY, U.S.A.) containing 10% FBS. To investigate dose-dependent effects of GM-CSFs, aliquots (100 μ l; 5×10^4 cells) of a cell suspension were incubated in wells of flat-bottomed 96-well tissue culture plate for 24 h. rhGM-CSF samples were serially diluted to 100–10000 pg/ml with medium and added to the plates. To investigate time-dependent effects of rhGM-CSFs, aliquots (1 ml; 1×10^5 cells) of a cell suspension were incubated in wells of flat-bottomed 6-well tissue culture plate for 24 h. 10000 pg/ml of rhGM-CSF samples added to the plates. After shaking for 1 min, 1 Ci/ml of [^3H] thymidine was added and the plates were incubated for 24 h and 48 h at 37 °C in a humidified atmosphere of 5% CO_2 . The cells were washed three times and lysed with 10% Triton X-100. The lysates were centrifuged for 10 min at 10000 g , and the radioactivity of the supernatants was measured with a β -counter.³³

RESULTS

Terminal Sialylation of rrhGM-CSF $\alpha2,3$ -Terminal sialic acids of rrhGM-CSF were detected using MAA II (Fig. 1A). The positive control, CHO-EPO has only $\alpha2,3$ -linked sialic acids due to the absence of a $\alpha2,6$ -sialyltransferase in Chinese hamster ovary (CHO) cells.^{34,35} As shown in Fig. 1A, the optical density of CHO-EPO was over 0.8, whereas that of non-glycosylated BSA (negative control) was less

Fig. 1. Analysis of $\alpha2,3$ - and $\alpha2,6$ -Terminal Sialylation

$\alpha2,3$ -terminal sialic acids of rrhGM-CSF were detected using biotinylated *Maackia amurensis* lectin II (MAA II) (A) and $\alpha2,6$ -terminal sialic acids of rrhGM-CSF were detected using biotinylated *Sambucus nigra* Lectin (SNA) (B). MAA II and SNA were detected using poly-HRP streptavidin. The values were measured at OD 450 nm. $\alpha2,3$ -terminal sialylation of rrhGM-CSF was compared with BSA (negative control) and EPO produced in CHO cells (CHO-EPO, positive control). $\alpha2,6$ -terminal sialylation of rrhGM-CSF was compared with apo-human transferrin (positive control) and asialo CHO-EPO (negative control). Values are means $\pm$ S.D. of duplicate measurements. Asialo refers to the desialylated form.

than 0.2. Asialo CHO-EPO (terminal sialic acids removed by neuraminidase) had the same optical density as BSA, confirming removal of α 2,3-linked sialic acids (Fig. 1A). rrrhGM-CSF had the same optical density as BSA and asialo CHO-EPO. Therefore, α 2,3-linked sialic acid was not detected in rrrhGM-CSF (Fig. 1A).

The α 2,6-terminal sialic acids of rrrhGM-CSF were detected using SNA (Fig. 1B). SNA primarily binds to α 2,6-linked sialic acids and secondarily to α 2,3-linked sialic acids.³⁶⁾ The positive control, apo-human transferrin is known to have only α 2,6-linked sialic acids.³⁷⁾ As shown Fig. 1B, apo-human transferrin had the highest value followed by CHO-EPO. This result agrees with the properties of SNA. The value for asialo CHO-EPO was about 0.2 and for rrrhGM-CSF less than 0.2 (Fig. 1B). Therefore, α 2,6-linked sialic acid was not detected in rrrhGM-CSF (Fig. 1B). These results indicate that mammals like α 2,3- and α 2,6-linked terminal sialic acid do not exist enough to be detected or are not contained in rrrhGM-CSF.

Deglycosylation of rrrhGM-CSF and yrrhGM-CSF
TFMS removes *O*-glycans and *N*-glycans while not affecting other post-translational modifications or amino acids of glycoprotein^{29,38,39)} This property of TFMS is useful for determining the glycosylation ratio of uncharacterized glycoproteins.^{40–42)} TFMS is particularly useful for removing plant glycans that are not removed by enzymatic deglycosylation.²⁹⁾ After TFMS treatment, the molecular weight changes of rrrhGM-CSF and yrrhGM-CSF were determined by SDS-PAGE. rrrhGM-CSF has a molecular weight of 24–28 kDa, similar to the 1N-type of human lymphoblastoid Namalwa cell-derived rhGM-CSF (Fig. 2).^{13,14)} After TFMS treatment, its molecular weight was shifted to 15 kDa,¹⁴⁾ showing that 9–13 kDa of glycans had been removed (Fig. 2). yrrhGM-CSF has a molecular weight of 16–18 kDa and is known to be *O*-glycosylated.⁴³⁾ Its molecular weight was shifted to 14.5 kDa, the same as nonglycosylated hGM-CSF, after TFMS treatment (Fig. 2).^{7,14)} Thus the glycans of rrrhGM-CSF represent 37–47% of its initial molecular weight and the glycans of yrrhGM-CSF represent 10–20% of its initial molecular weight. *N*-Glycosylation sites of yrrhGM-CSF were removed by site specific mutation. Therefore, yrrhGM-CSF has three potential *O*-glycosylation sites⁴⁴⁾ whereas rrrhGM-CSF has two potential *N*-glycosylation sites and three potential *O*-glycosylation sites.⁹⁾ Glycoproteins containing *N*-gly-

cosylation site has a molecular weight greater than the expected value due to the remaining innermost *N*-acetylglucosamine after the TFMS treatment.²⁹⁾ The 15 kDa molecular weight of the deglycosylated rrrhGM-CSF was larger than expected value, suggesting that the innermost *N*-acetylglucosamine of *N*-glycan was not removed by the TFMS treatment.²⁹⁾

Clearance Rate of rhGM-CSF in the Rat We determined the circulating serum protein levels of rrrhGM-CSF, yrrhGM-CSF, deglycosylated rrrhGM-CSF and deglycosylated yrrhGM-CSF by sandwich ELISA following intravenous administration to rats. Serum protein levels were measured from 0.25 to 48 h post injection.

Although yrrhGM-CSF had the highest serum protein level up to 4 h, it was cleared rapidly after that and was undetectable 10 h post injection (Fig. 3). Although the level of rrrhGM-CSF was lower than that of yrrhGM-CSF up to 4 h, it was cleared more slowly after 10 h and was 180 pg/ml at 24 h and 30 pg/ml at 48 h (Fig. 3). The deglycosylated forms of

Fig. 2. SDS-PAGE Analysis of Glycosylated rhGM-CSF and Deglycosylated rhGM-CSF

rrhGM-CSF and yrrhGM-CSF were deglycosylated by TFMS. Changes of molecular weight were identified by SDS-PAGE. Lane 1, rrrhGM-CSF; lane 2, yrrhGM-CSF; lane 3, deglycosylated rrrhGM-CSF; lane 4, deglycosylated yrrhGM-CSF.

Fig. 3. Circulating Plasma Levels of rhGM-CSF

rhGM-CSF was administrated to 6-week-old male SD rats. rhGM-CSF levels in serum samples were measured by sandwich ELISAs. Data are means $\pm$ S.D. of three rats per group. Data are representative of three independent experiments in which similar results were obtained.

rrhGM-CSF and yrhGM-CSF were undetectable 10 h post injection. Therefore, the two deglycosylated rhGM-CSFs have similar clearance rates (Fig. 3). Because rrhGM-CSF persisted for longer than deglycosylated rrhGM-CSF, clearance rate clearly depends on the presence or absence of glycans. However, although yrhGM-CSF had higher serum protein levels than deglycosylated yrhGM-CSF, it was also undetectable 10 h post injection (Fig. 3). These results indicate that the removed glycans of rrhGM-CSF are very important for *in vivo* stability and that the different clearance rates of rrhGM-CSF and yrhGM-CSF are due to differences between their individual glycans. Therefore, the glycans of rrhGM-CSF is more effective in maintaining *in vivo* stability than yrhGM-CSF.

In Vitro Bioactivity of rhGM-CSF We measured bioactivities of rhGM-CSFs *via* dose- and time-dependent manners. Bioactivities of rrhGM-CSF and yrhGM-CSF are similar to each other at 100, 500, 1000 and 10000 pg/ml (Fig. 4A). In addition, 100 pg/ml of rrhGM-CSF had bioactivity. These findings indicate that the serum protein levels of rrhGM-CSF from 10 h onwards are valid reflections of their bioactivity (Fig. 3). Proliferation of TF-1 cells was little more increased by rrhGM-CSF than yrhGM-CSF at 24 h and 48 h, respectively (Fig. 4B). These results indicate that bioactivities of rrhGM-CSF and yrhGM-CSF are similar to each other.

Fig. 4. *In Vitro* Bioactivities of rrhGM-CSF and yrhGM-CSF

The data represent proliferation of TF-1 cells in response to rrhGM-CSF and yrhGM-CSF. Proliferation of TF-1 cells was determined by ^3H -thymidine uptake as described in Materials and Methods. (A) Dose-dependent effects of rrhGM-CSF and yrhGM-CSF. (B) Time-dependent effects of rrhGM-CSF and yrhGM-CSF. Controls were incubated with culture medium only. Data are means $\pm$ S.D. of duplicate measurements.

DISCUSSION

hGM-CSF has two potential *N*-glycosylation sites (Asn27, Asn37) and three potential *O*-glycosylation sites (Ser7, Ser9, Thr10).^{4,12,13} It is known that degrees of glycosylation differ according to cell type and that *in vivo* stability increases with *N*-glycan content.¹⁴ Terminal sialic acids are important for the *in vivo* stability of glycoproteins. There is no report concerning terminal sialylation of plant cell-derived glycoproteins, although Shah *et al.*²³ and Takashima *et al.*²⁴ reported that plants have the genetic and enzymatic potential for sialylation. Therefore, the lack of terminal sialic acids in plant cell-derived glycoproteins has limited their use as therapeutic agents.²⁰ In this study, we analyzed the terminal sialylation of rrhGM-CSF using lectins. As shown in Fig. 1, mammals like α 2,3- and α 2,6- linked terminal sialic acids were not detected in rrhGM-CSF (Fig. 1A, B). These lectin methods are limited to detect other forms of sialylation.⁴⁵ Therefore, existence of other form of terminal sialylation can not be excluded.

The glycosylation ratio of rrhGM-CSF was two-fold higher than that of commercial rhGM-CSF (yrhGM-CSF) as shown by experimental deglycosylation of rrhGM-CSF and yrhGM-CSF using TFMS (Fig. 2). Although rrhGM-CSF had lower serum protein levels than yrhGM-CSF up to 4 h after injection, its level was sustained up to 48 h, whereas yrhGM-CSF was undetectable after 10 h. This result parallels the findings of a pharmacokinetic study by Denzlinger *et al.* in which rhGM-CSF was administered to humans and serum protein and leukocytes levels were determined⁴⁶: heavily glycosylated CHO cell-derived rhGM-CSF gave rise to lower serum protein levels than non-glycosylated *Escherichia coli*-derived rhGM-CSF up to 8 h but thereafter the opposite was the case. In addition, although the serum protein levels of CHO cell-derived rhGM-CSF were lower than those of *Escherichia coli*-derived rhGM-CSF up to 10 h, leukocytes numbers were stimulated to the same extent by the two preparation up to 16 h, and from 24 h onwards the CHO cell-derived rhGM-CSF was more effective in increasing leukocyte numbers. In a report of Okamoto *et al.*, colony formation by human marrow mononuclear cells was no longer increased by rhGM-CSF when its concentration exceeded 100000 pg/ml.¹⁴ This indicates that stimulation of leukocyte multiplication is limited to a certain range of rhGM-CSF concentrations. Therefore, the serum protein level of rhGM-CSF from 10 h is more important for sustaining leukocyte levels than its early level. In our study similarly, although early serum protein levels of rrhGM-CSF were lower than yrhGM-CSF, the level of rrhGM-CSF was better maintained than that of yrhGM-CSF. Hence rrhGM-CSF was more effective than yrhGM-CSF in maintaining leukocyte levels (Fig. 3). The stability of rrhGM-CSF in rats depends on the presence of glycans since rrhGM-CSF was sustained until 48 h whereas deglycosylated rrhGM-CSF was undetectable 10 h post injection. Although yrhGM-CSF was maintained at a higher level than deglycosylated yrhGM-CSF, the latter, like the former, was undetectable 10 h post injection. Therefore the stability of yrhGM-CSF depends on only a limited extent on the presence of glycans.

Thus, although the glycans on rrhGM-CSF lack terminal sialic acids, rrhGM-CSF is more stable than commercial

yrhGM-CSF *in vivo*. Although amino acid sequences of rrhGM-CSF and yrhGM-CSF are different, deglycosylated forms of them have similar clearance rates. Therefore, the glycans on rrhGM-CSF promote *in vivo* stability more effectively than the glycans on yrhGM-CSF. This property of rrhGM-CSF suggests new possibilities for using transgenic plants in glycoprotein production systems. We are currently performing a detailed study of the *in vivo* bioactivity and glycosylation profiles of rrhGM-CSF.

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