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Low concentrations of recombinant granulocyte macrophage-colony stimulating factor derived from Chinese hamster ovary cells augments long-term bioactivity with delayed clearance *in vitro*

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ABSTRACT

To date, the biological activity of granulocyte macrophage-colony stimulating factor (GM-CSF) has been investigated by using mostly *Escherichia coli*- or yeast cell-derived recombinant human GM-CSF (erhGM-CSF and yrhGM-CSF, respectively). However, Chinese hamster ovary cell-derived recombinant human GM-CSF (crhGM-CSF), as well as natural human GM-CSF, is a distinct molecule that includes modifications by complicated oligosaccharide moieties. In the present study, we reevaluated the bioactivity of crhGM-CSF by comparing it with those of erhGM-CSF and yrhGM-CSF. The effect of short-term stimulation (0.5 h) on the activation of neutrophils/monocytes or peripheral blood mononuclear cells (PBMCs) by crhGM-CSF was lower than those with erhGM-CSF or yrhGM-CSF at low concentrations (under 60 pM). Intermediate-term stimulation (24 h) among the different rhGM-CSFs with respect to its effect on the activation of TF-1 cells, a GM-CSF-dependent cell line, or PBMCs was not significantly different. In contrast, the proliferation/survival of TF-1 cells or PBMCs after long-term stimulation (72–168 h) was higher at low concentrations of crhGM-CSF (15–30 pM) than that of cells treated with other GM-CSFs. The proportion of apoptotic TF-1 cells after incubation with crhGM-CSF for 72 h was lower than that of cells incubated with other rhGM-CSFs. These effects were attenuated by desialylation of crhGM-CSF. Clearance of crhGM-CSF but not desialylated-crhGM-CSF by both TF-1 cells and PBMCs was delayed compared with that of erhGM-CSF or yrhGM-CSF. These results suggest that sialylation of oligosaccharide moieties delayed the clearance of GM-CSF, thus eliciting increased long-term bioactivity *in vitro*.

1. Introduction

Granulocyte macrophage-colony stimulating factor (GM-CSF) is a hematopoietic growth factor that regulates the growth, differentiation, and maturation of myeloid precursor cells and promotes the function of mature neutrophils, eosinophils, and monocytes [1–4]. It elicits these diverse effects through interaction with a unique dodecameric receptor complex on cells, which consists of α and common β chains [5–7]. GM-CSF signaling induces phosphorylation of Janus kinase 2 (JAK2) and the common β chains, followed by activation of signal transducers and activators of transcription (STATs) [5,7,8]. Upon immune stimulation, it is produced by a variety of cell types, including T cells, macrophages, endothelial cells, and fibroblasts. Although GM-CSF is produced locally [3], it can

Abbreviations: ACN, acetonitrile; ANOVA, analysis of variance; CHO, Chinese hamster ovary; crhGM-CSF, CHO-derived recombinant human GM-CSF; erhGM-CSF, *Escherichia coli*-derived recombinant human GM-CSF; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte macrophage-colony stimulating factor; JAK2, Janus kinase 2; MIP-1α, macrophage inflammatory protein; NaN_3, sodium azide; PBMCs, peripheral blood mononuclear cells; SDS–PAGE, sodium dodecyl sulfate–polyacrylamidegel electrophoresis; STAT5, signal transduction and activator of transcription; TFA, trifluoroacetic acid; TOF mass spectrometer, time-of-flight mass spectrometer; yrhGM-CSF, yeast cell-derived recombinant human GM-CSF.

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act in a paracrine fashion to recruit circulating neutrophils, monocytes, and lymphocytes to enhance their function in host defense [9,10]. GM-CSF is used clinically to prevent neutropenia and associated infections by promoting the proliferation of hematopoietic progenitor cells after chemotherapy, by promoting the differentiation of myeloid cells, and by enhancing the antibacterial activities of neutrophils and macrophages [10–14].

Natural human GM-CSF (hGM-CSF) has been purified from several sources, including medium conditioned with placenta cells or activated blood lymphocytes [15–19]. It is a glycoprotein that consists of 127 amino acid residues, with four cysteines involved in two disulfide bonds, forming a compact globular structure that comprises four α-helices joined by loops. It is found extracellularly as a homodimer [6,7] with two N-glycosylation sites at Asn27 and Asn37 and three O-glycosylation sites at Ser7, Ser9, and Thr10 [15]. The most heavily glycosylated hGM-CSF, with a molecular weight of 28–32 kDa, has two N-linked carbohydrate moieties, whereas the partially glycosylated hGM-CSF, with a molecular weight of 23–25 kDa, contains one N-linked carbohydrate moiety. A minimally glycosylated hGM-CSF with molecular weight of 16–18 kDa consists of only one O-linked carbohydrate [15,20].

Cells from various species can produce recombinant hGM-CSF (rhGM-CSF) [21,22]. However, only commercial preparations produced from *Escherichia coli* and *Saccharomyces cerevisiae* are available for clinical use. Commercial *E. coli*-derived recombinant hGM-CSF (erhGM-CSF), Molgramostim, is non-glycosylated, consists of 127 amino acid residues, has a molecular weight of 14.5 kDa, and is methylated at the N-terminal end [23]. Commercial *Saccharomyces*-derived recombinant hGM-CSF (yrhGM-CSF), Sargramostim, is a glycoprotein of 127 amino acids composed of three primary molecular species having molecular weights of 19.5, 16.8, and 15.5 kDa [23]. Its amino acid sequence differs from hGM-CSF by a substitution of leucine at position 23 [23]. On the other hand, rhGM-CSF derived from Chinese hamster ovary (CHO) cells (crhGM-CSF) has a molecular weight of 15–32 kDa with the same N-glycosylation and O-glycosylation sites as those of hGM-CSF, although the carbohydrate moieties added are probably different. Forno et al. demonstrated that the N-glycan terminal contains mono- and disialic acid residues, but has predominantly tri- or tetrasialic acid residues with and without N-acetyllactosamine repeat units. N-glycans contain more than 90% α-1,6-linked fucose at the proximal end [20].

The pattern of glycosylation on GM-CSF is known to affect its biological activity. Proliferation of a human monocytic leukemia cell line incubated with the heavily glycosylated hGM-CSF (28–32 kDa) was reduced six fold compared with proliferation after treatment with non-glycosylated erhGM-CSF, while neutrophil superoxide anion production was reduced by up to 10-fold. Partially glycosylated hGM-CSF (23–25 kDa) and minimally glycosylated hGM-CSF (16–18 kDa) have biological activity similar to that of erhGM-CSF. The binding capacity of these hGM-CSFs for the rhGM-CSF receptor on neutrophils decreases with increasing molecular weight [15]. Similarly, most studies on mammalian cell-derived, glycosylated GM-CSF (including crhGM-CSF) demonstrate that glycosylation of GM-CSF prolongs the *in vivo* half life by stabilizing the protein, but reduces its binding avidity to the GM-CSF receptor and decreases its biological activities such as colony-forming activity of bone marrow cells and neutrophil superoxide anion production [15,24].

In contrast to previous studies [15,24], we showed in the present study that glycosylated rhGM-CSF produced by CHO cells exhibited increased proliferation/survival of TF-1 cells, PBMCs and monocytes at low GM-CSF concentrations compared with that of erhGM-CSF and yrhGM-CSF *in vitro*. Desialylation of crhGM-CSF attenuated this effect, indicating that the sialyl residue is crucial for augmenting the long-term activity of GM-CSF. Moreover, we examined the mechanism of this effect by measuring the clearance of rhGM-CSF by cells.

2. Materials and methods

2.1. Material

2.1.1. Cells

TF-1, a GM-CSF-dependent cell line, was kindly provided by Kitamura et al. [22].

Peripheral blood mononuclear cells (PBMCs) and monocytes were isolated from the peripheral blood of healthy donors as described previously [8]. Written informed consent was obtained under protocols approved by the institutional review boards of the Niigata University Medical Dental Hospital.

2.1.2. rhGM-CSF

Molgramostim and Sargramostim were purchased from Amoytop Biotech Co., Ltd. (Xiamen, Fujian, PRC) and Genzyme Corporation (Cambridge, MA, USA), respectively. crhGM-CSF was kindly provided by JCR Pharmaceuticals Co., Ltd. (Ashiya, Hyogo, Japan).

2.1.3. Desialylation of crhGM-CSF

crhGM-CSF (1 mg/ml) was incubated with neuraminidase agarose from *Clostridium perfringens* (0.05 U/ml, Sigma–Aldrich, MO, USA) in 100 mM sodium acetate buffer with $CaCl_2$ (pH 5.0) for 60 min at 37 °C. After the agarose was removed, the solution was dialyzed against PBS overnight at 4 °C.

2.2. Mass spectrometry

Protein (10 μl) was mixed with 90 μl of 0.1% trifluoroacetic acid (TFA) and 0.5 μl of MB-HIC8 magnetic C8 beads (Bruker Daltonics, Hercules, MA, USA) in a PCR tube and then incubated for 5 min at room temperature. The tube was subsequently placed in a magnetic beads separator and the supernatant was removed by using a pipette. The magnetic beads were then washed three times with 100 μl of 0.1% TFA. The bound proteins were eluted from the magnetic beads by using 4.5 μl of 60% acetonitrile (ACN) in 0.1% TFA. Two microliters of the eluate was mixed with 1 μl of matrix solution (10 g/l sinapinic acid in 70% ACN, 0.1% TFA) and was spotted on a polished steel plate. The mass spectra were obtained on an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics, Hercules, MA, USA) operated in positive-ion linear mode.

2.3. Phosphorylated STAT5 detection assay

Heparinized fresh whole blood was incubated with 15, 30, 60, or 500 pM rhGM-CSF, for 30 min at 37 °C and fixed, and then red blood cells were lysed in Fix/Lyse buffer (BD Biosciences, Franklin Lakes, New Jersey, USA) for 20 min at 37 °C. White blood cells were collected by centrifugation and fixed in ice-cold methanol at −20 °C for 1 h. After centrifugation, the cells were resuspended in 3% FCS/0.01% NaN_3/PBS solution and incubated with Alexa Fluor 647-labeled anti-pSTAT5 (BD Biosciences, San Jose, CA, New Jersey, USA). Cells with phosphorylated STAT5 in granulocytes/monocytes detected by flow cytometry (Cell Analyzer, Sony, Tokyo, Japan).

2.4. Neutrophil CD11b stimulation index assay

The neutrophil CD11b assay was performed as described previously [25]. Aliquots of heparinized fresh whole blood were incubated with rhGM-CSF, and cell-surface CD11b levels were quantified by flow cytometry (Sony, Tokyo, Japan). The CD11b