β-Galactosidase Treatment Is a Common First-stage Modification of the Three Major Subtypes of Gc Protein to GcMAF

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Abstract. *Background: The 1f1f subtype of the group-specific component (Gc) protein is converted into Gc protein-derived macrophage-activating factor (GcMAF) by enzymatic processing with β-galactosidase and sialidase. We previously demonstrated that preGc$_{1f1f}$MAF, a full Gc$_{1f1f}$ protein otherwise lacking a galactosyl moiety, can be converted to GcMAF by treatment with mouse peritoneal fluid. Here, we investigated the effects of the β-galactosidase-treated 1s1s and 22 subtypes of Gc protein (preGc$_{1s1s}$MAF and preGc$_{22}$MAF) on the phagocytic activation of mouse peritoneal macrophages. Results: We demonstrated the presence of Gal-GalNAc disaccharide sugar structures in the Gc$_{1s1s}$ protein by western blotting using peanut agglutinin and Helix pomatia agglutinin lectin. We also found that preGc$_{1s1s}$MAF and preGc$_{22}$MAF significantly enhanced the phagocytic activity of mouse peritoneal macrophages in the presence and absence of mouse peritoneal fluid. Conclusion: We demonstrate that preGc$_{1s1s}$MAF and preGc$_{22}$MAF proteins can be used as effective macrophage activators.*

The group-specific component (Gc) protein, a vitamin D-binding protein (DBP), Gc globulin, is a 53-kDa human plasma protein (1), of which there are six major subtypes, the homodimers and heterodimers of Gc1f, Gc1s, and Gc2 (2). The 1f1f subtype of the Gc protein (Gc$_{1f1f}$ protein) contains a branched trisaccharide with a galactose (Gal) and a sialic acid (SA) moiety bound to *N*-acetylgalactosamine (GalNAc) attached to the core protein (3). Furthermore, the 1s1s subtype of the Gc protein (Gc$_{1s1s}$ protein) contains two different sugar structures: a branched trisaccharide with Gal and a mannose (MA) moiety bound to GalNAc (4), or a linear trisaccharide with SA-Gal-GalNAc (5, 6). The 22 subtype of the Gc protein (Gc$_{22}$ protein) contains a linear Gal-GalNAc disaccharide sugar structure (3). In an inflammatory response, the terminal Gal and SA of the Gc$_{1f1f}$ protein were hydrolyzed by membrane-bound β-galactosidase on an activated B-cell and sialidase on a T-cell, respectively, to produce Gc protein-derived macrophage-activating factor (GcMAF) (7). GcMAF has some interesting biological activities, such as promotion of macrophage activation *via* superoxide generation (8, 9), and phagocytic activation (10), in addition to antiangiogenic (11, 12) and antitumor activities (13-15). GcMAF has also been shown to have clinical activity against metastatic colorectal, metastatic breast, and prostate cancer, with additional activities in non-anemic HIV-infected patients (16-19).

Until now, the interesting GcMAF biological activity data, concerned only the 1f1f GcMAF subtype, although the biological or clinical applications for Gc$_{1s1s}$ and Gc$_{22}$ protein-derived GcMAF have not been reported. If GcMAF is an endogenous macrophage activator, all Gc protein subtypes should have the possibility of being converted into GcMAF. We previously demonstrated that preGc$_{1f1f}$MAF, a full Gc$_{1f1f}$ protein lacking only a galactosyl moiety, can be converted to GcMAF by treatment with mouse peritoneal fluid (20). We hypothesized that if β-galactosidase treatment could be applied to all Gc protein subtypes (1f, 1s and 2), the clinical use of GcMAF could be attained from all human serum.

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Key Words: Human serum Gc protein, β-galactosidase-treatment, 1s1s and 22 subtypes, phagocytic activity, mouse peritoneal macrophages, group-specific component protein.

Here, we present the effect of β-galactosidase-treated Gc_{1s1s} and Gc_{22} proteins (preGc_{1s1s}MAF and preGc_{22}MAF) on the phagocytic activation of mouse peritoneal macrophages.

Materials and Methods

Materials. Lipopolysaccharide (LPS; from *Escherichia coli* O128:B12) and Sepharose CL-6B (cross-linked) were purchased from Sigma-Aldrich Japan Co. Ltd. (Tokyo, Japan). 25-Hydroxyvitamin D_3 [25(OH)D_3] and other biochemical-grade chemicals were purchased from WAKO Pure Chemical Industries Co. Ltd. (Osaka, Japan).

Preparation of human serum Gc_{1s1s} and Gc_{22} protein. The Gc_{1s1s} and Gc_{22} proteins were prepared as reported by Link *et al*. (21). Briefly, serum from healthy humans containing Gc_{1s1s} or Gc_{22} proteins (each 7.0 ml) was diluted 1:1 with column buffer (50 mM Tris-HCl, 1.5 mM EDTA, 150 mM NaCl, 0.1% Triton X-100; pH 7.4) and applied to a 25(OH)D_3-Sepharose column which was prepared in our laboratory according to the method reported by Link *et al*. (21). The column was washed with 300 ml of column buffer. The protein remaining on the matrix was eluted with 6 M guanidine-HCl, and a 1-ml fraction was collected. Fractions of the protein peak encompassing the guanidine-eluted fraction were pooled and dialyzed with 10 mM sodium phosphate (pH 7.0). Subsequently, a hydroxyapatite column with a volume of 5 ml (Bio-Scale Mini CHT Type II Cartridge; Bio-Rad Laboratories, Tokyo, Japan) was equilibrated with 10 mM sodium phosphate (pH 7.0). The dialyzed sample obtained following 25(OH)D_3-Sepharose column chromatography was applied to the column. A linear gradient elution from 10 mM sodium phosphate to 200 mM sodium phosphate (pH 7.0) was used for column chromatography. Fractions were collected and concentrated with a centricon-concentrating unit (30,000 MWCO; Nihon Millipore Co. Ltd., Tokyo, Japan), and their protein concentrations were determined using the bicinchoninic acid (BCA) method (Pierce Chemical Co., Rockford, IL, USA). Bovine serum albumin (BSA) was used as a standard. The yields of purified Gc_{1s1s} and Gc_{22} proteins were 2.294 mg and 1.867 mg, respectively.

Preparation of preGc_{1s1s}MAF and preGc_{22}MAF. The purified Gc_{1s1s} and Gc_{22} proteins (200 μg) were incubated with 1000 mU β-galactosidase (grade III from bovine liver; Sigma-Aldrich Japan Co. Ltd., Osaka, Japan) in 100 mM sodium phosphate buffer (pH 7.0) in an Eppendorf tube at 37.5˚C for 3 h. The reaction mixture was mixed with 25(OH)D_3-Sepharose beads and was stirred for 10 min at room temperature using a vortex mixer to separate the β-galactosidase. The solution was concentrated using a microcon-concentration unit (10,000 MWCO; Nihon Millipore Co. Ltd., Tokyo, Japan). The protein concentrations were determined using the BCA method, and the yields of preGc_{1s1s}MAF and preGc_{22}MAF were calculated as being 80.2 μg and 57.8 μg, respectively.

Western blotting. The Gc_{1s1s} and Gc_{22} proteins and the preGc_{1s1s}MAF and preGc_{22}MAF subtypes were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and subsequently electroblotted onto a polyvinylidene fluoride (PVDF) membrane. Non-specific binding was blocked by overnight incubation in Tris-buffered saline (pH 7.4) containing 0.1% Tween 20 and 1% BSA at 4˚C. The membranes were then probed with anti-human Gc globulin (DakoCytomation Co. Ltd., Kyoto, Japan) against the Gc_{1s1s} and Gc_{22} proteins, and with Peanut Agglutinin (PNA) lectin (*Arachis hypogaea*, biotin-conjugated; Sigma-Aldrich Japan Co. Ltd., Osaka, Japan) and Helix pomatia (HPA) lectin (*Helix pomatia*, biotin-conjugated; Sigma-Aldrich Japan Co. Ltd., Osaka, Japan) for the Gc_{1s1s} and Gc_{22} protein subtypes preGc_{1s1s}MAF and preGc_{22}MAF. After extensive washing, the blots were incubated with the secondary antibody [horseradish peroxidase (HRP)-labeled anti-rabbit IgG and electrochemiluminescence (ECL) streptavidin-HRP conjugate; GE Healthcare Japan Co., Tokyo, Japan]. The blots were developed using an ECL western blotting detection system (GE Healthcare Japan Co.). The visualization and quantification of the western blot bands was achieved using a LumiCube chemiluminescence analyzer and the JustTLC image analysis software (Liponics Inc., Tokyo, Japan).

Isolation and culture of mouse peritoneal macrophages. Resident mouse peritoneal macrophages were collected from 8-week-old female mice from the Institute for Cancer Research (ICR). After centrifugation at 1,000 rpm, at 4˚C, for 15 min, macrophages were collected and cultured in 24-well plates at a density of 5×10^5 cells/well in serum-free RPMI-1640 (Life Technologies Japan Ltd., Tokyo, Japan) for 1 h. The cultured cells were then washed three times with serum-free RPMI-1640 to separate adherent macrophages from non-adherent cells, such as T- and B-cells. The macrophages were cultured for 15 h, following which, the phagocytosis assay was performed as described below.

Phagocytosis assay for preGc_{1s1s}MAF and preGc_{22}MAF. Mouse peritoneal cells were layered onto coverslips in a 24-well plate. After 3 h of treatment (treated preGc_{1s1s}MAF and preGc_{22}MAF were pre-incubated with peritoneal fluid at 37˚C for 1 h), the cultures were assayed for phagocytic activity. Sheep red blood cells (SRBCs; Nippon Bio-Supp. Center Co., Tokyo, Japan) were opsonized by rabbit hemolytic serum (anti-sheep red blood cells; Cosmo Bio Co., Tokyo, Japan). Opsonized SRBCs (0.5%) in serum-free RPMI-1640 were overlaid onto each macrophage-coated coverslip and were cultured for 1.5 h. The non-internalized erythrocytes were lysed by immersing the coverslip into a hypotonic solution (1/5 phosphate-buffered saline). The macrophages were fixed with methanol, air-dried, and stained with Giemsa stain. The number of phagocytosed erythrocytes per cell was determined microscopically; 250 macrophages were counted for each data point. The data are expressed in terms of the phagocytotic index (PI), which is defined as the percentage of macrophages with ingested erythrocytes multiplied by the average number of erythrocytes ingested per macrophage.

Statistical analysis. Data are expressed as the mean ±SD. The significance of the differences between the results of the independent experiments was analyzed using the Student's *t*-test. A *p*-value of <0.05 was considered significant.

Results

Preparation and identification of preGc_{1s1s}MAF and preGc_{22}MAF. We obtained 2.294 mg of Gc_{1s1s} protein and 1.867 mg of Gc_{22} protein from 7.0 ml of human serum using a 25(OH)D_3 affinity column. Figure 1A shows the SDS-PAGE gel and the western blot of the purified Gc_{1s1s} and

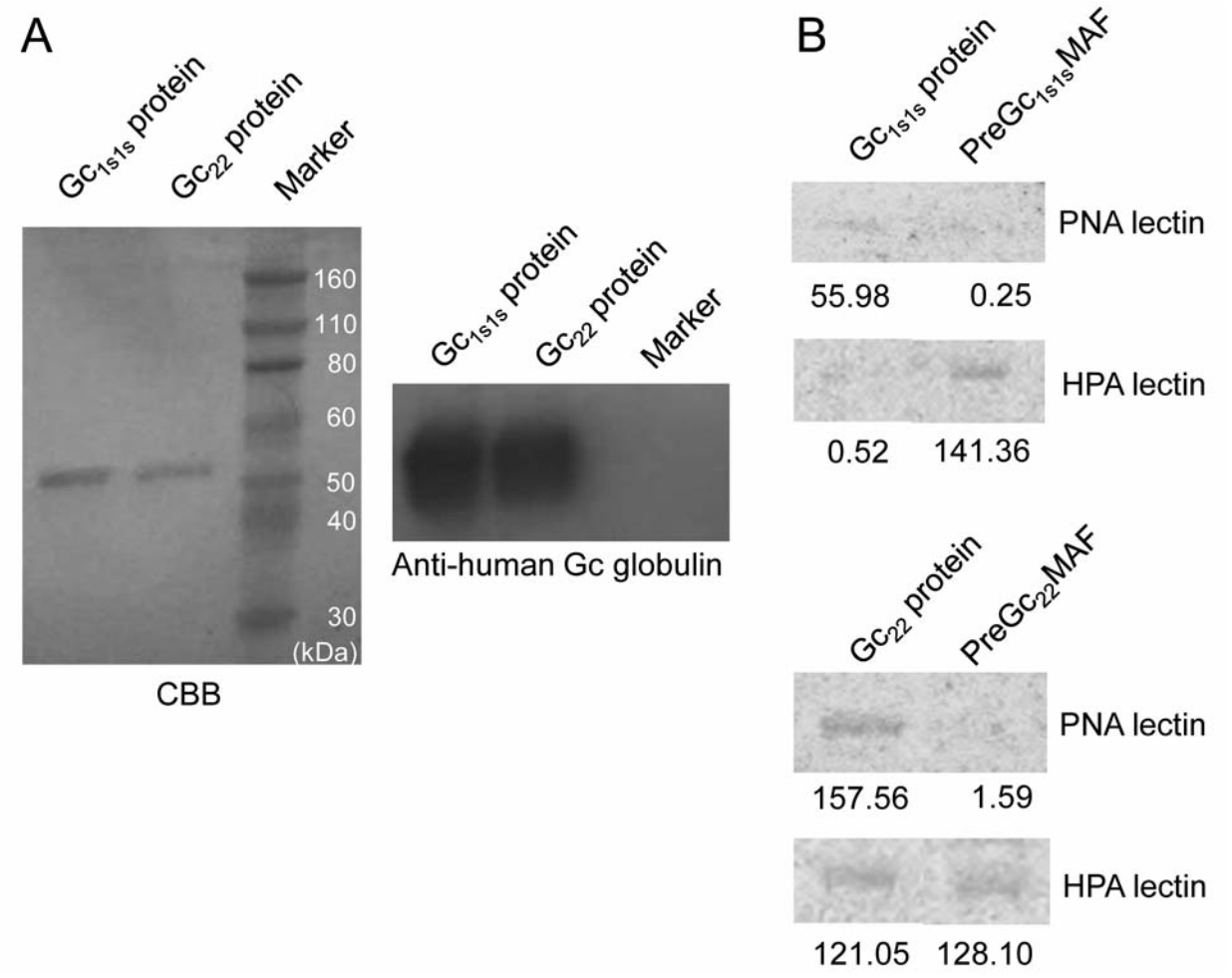
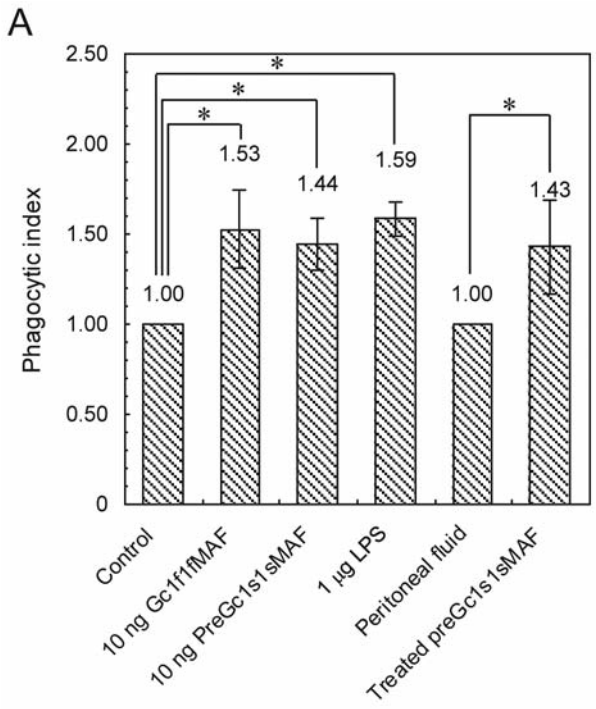


Figure 1. *Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis of preGc$_{1s1s}$MAF and preGc$_{22}$MAF. A: SDS-PAGE gel stained with coomassie brilliant blue (CBB) and a western blot probed with anti-human Gc globulin against purified Gc$_{1s1s}$ and Gc$_{22}$ proteins. B: Western blot of preGc$_{1s1s}$MAF and preGc$_{22}$MAF using Peanut Agglutinin (PNA) lectin (affinity for Gal-GalNAc disaccharide) and Helix pomatia (HPA) lectin (affinity for GalNAc moiety). The values below blots represent the concentrations for each band.*

Gc$_{22}$ proteins. A single band was detected on staining with Coomassie brilliant blue (CBB) and after probing with an anti-human Gc globulin antibody. The purified Gc$_{1s1s}$ and Gc$_{22}$ proteins were treated with β-galactosidase and then checked for purity by western blot using PNA and HPA lectin in order to identify whether Gal had been removed and GalNAc had appeared. We observed the disappearance of the PNA lectin-stained preGc$_{1s1s}$MAF (reduction in the band concentration from 55.98 to 0.25 counts) and the preGc$_{22}$MAF (reduction in the band concentration from 157.56 to 1.59 counts) bands, which had affinity for the Gal-GalNAc moiety (Figure 1B). We also observed the HPA lectin-stained preGc$_{1s1s}$MAF (increase in the band concentration from 0.52 to 141.36 counts) band that had affinity for the GalNAc-end. The reason for this, however, remains unknown, although positive bands were present for the Gc$_{22}$ and preGc$_{22}$MAF proteins (a slight increase in the band concentration from 121.05 to 128.10 counts). These data indicate that both preGc$_{1s1s}$MAF and preGc$_{22}$MAF contained a GalNAc moiety.

Phagocytic activity of preGc$_{1s1s}$MAF and preGc$_{22}$MAF toward mouse peritoneal macrophages. We examined phagocytic activation or macrophage activation by preGc$_{1s1s}$MAF and preGc$_{22}$MAF toward mouse peritoneal macrophages, in order to evaluate their macrophage-activating effects. Figure 2A shows significant phagocytic activation by 10 ng of preGc$_{1s1s}$MAF on treatment with mouse peritoneal fluid, as compared with the control (without mouse peritoneal fluid). The PI of 1.44 for 10 ng of preGc$_{1s1s}$MAF corresponded to that of 10 ng of Gc$_{1f1f}$MAF (PI=1.53) and 1 μg of LPS (PI=1.59). Gc$_{1s1s}$ protein did not



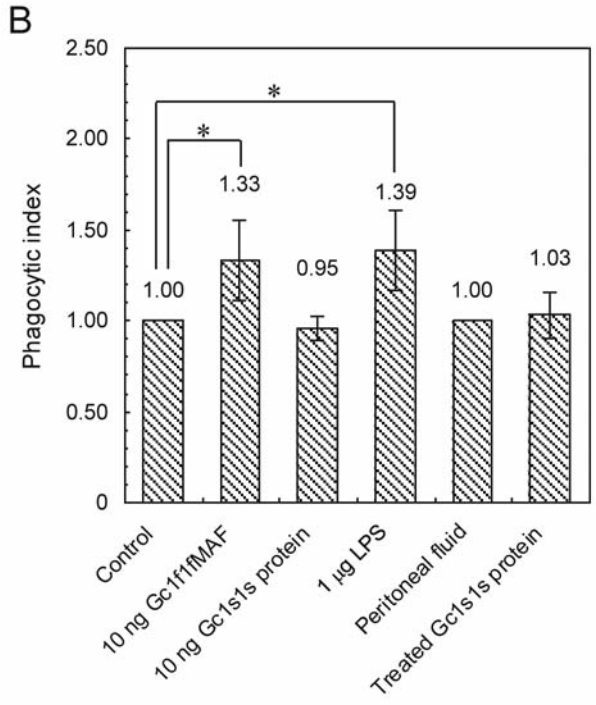
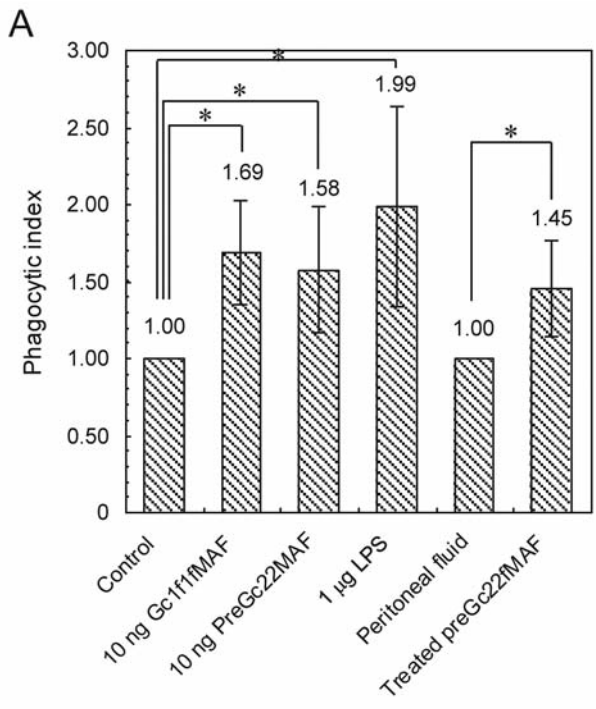


Figure 2. *Phagocytic activity of mouse peritoneal macrophages observed using preGc$_{1s1s}$MAF (A) and Gc$_{1s1s}$ protein (B), treated or not treated with mouse peritoneal fluid. All experiments were performed in triplicate. Each bar represents the mean (±SD). *p<0.05.*



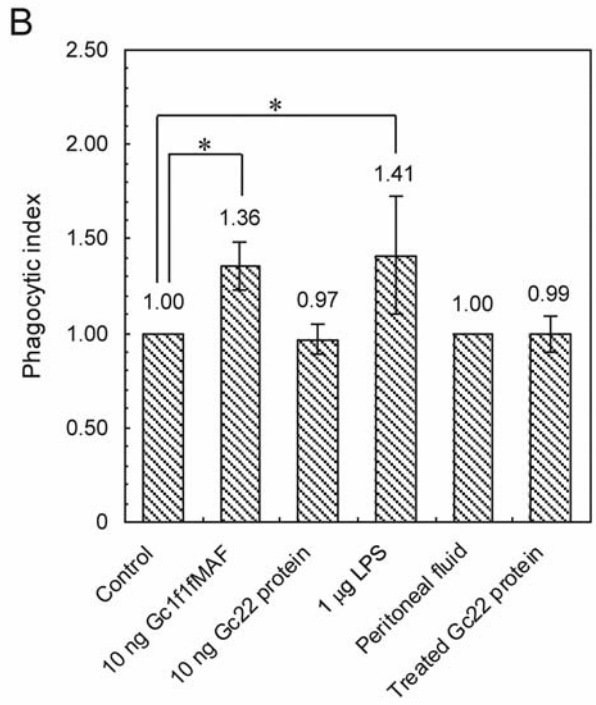


Figure 3. *Phagocytic activity of mouse peritoneal macrophages observed using preGc$_{22}$MAF (A) and Gc$_{22}$ protein (B) treated or not treated with mouse peritoneal fluid. All experiments were performed in triplicate. Each bar represents the mean (±SD). *p<0.05.*

result in significant phagocytic activation with or without mouse peritoneal fluid treatment (Figure 2B). Figure 3A shows significant phagocytic activation by 10 ng of preGc_{22}MAF on treatment with mouse peritoneal fluid compared with the control (without mouse peritoneal fluid). The PI of 1.58 for 10 ng of preGc_{22}MAF corresponded to that of 10 ng of Gc_{1f1f}MAF (PI=1.69) and 1 μg of LPS (PI=1.99). Gc_{22} protein did not result in significant phagocytic activation with or without mouse peritoneal fluid treatment (Figure 3B).

Discussion

We prepared preGc_{1s1s}MAF and preGc_{22}MAF by β-galactosidase treatment of purified Gc_{1s1s} and Gc_{22} proteins and evaluated their effects on macrophage activation. Using western blotting with PNA and HPA lectin, we propose, for the first time, that the Gal-GalNAc disaccharide sugar structure exists as part of the Gc_{1s1s} protein. Dr. Yamamoto suggested that the sugar moiety of the Gc_{1s1s} protein contained a branched trisaccharide with a Gal and an MA moiety bound to GalNAc (4), while Borges and Ravnsborg proposed an alternative sugar structure for the Gc_{1s1s} protein, consisting of a linear SA-Gal-GalNAc trisaccharide, by mass spectrometry (5, 6). We presumed from these results that post-modification of SA or MA might not be a stoichiometric reaction. We have demonstrated that the Gc_{1s1s} protein can be a potential precursor of GcMAF when subjected to β-galactosidase processing, and have supported the previously proposed activation mechanism that Gc_{22} protein containing a linear Gal-GalNAc disaccharide is directly converted into the macrophage activator (GcMAF) by β-galactosidase treatment alone.

In conclusion, we have demonstrated that three major subtypes of β-galactosidase-treated human serum Gc protein can be used as effective macrophage activators *in vivo*.

Acknowledgements

We thank the staff at the Health Service Center of the University of Tokushima for collecting blood samples from healthy volunteers.

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Received April 4, 2012
Revised May 15, 2012
Accepted May 16, 2012