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Preparation of Gc Protein-derived Macrophage Activating Factor (GcMAF) and its Structural Characterization and Biological Activities

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Abstract. *Background: Gc protein has been reported to be a precursor of Gc protein-derived macrophage activation factor (GcMAF) in the inflammation- primed macrophage activation cascade. An inducible beta-galactosidase of B cells and neuraminidase of T cells convert Gc protein to GcMAF. Materials and Methods: Gc protein from human serum was purified using 25(OH)D_3 affinity column chromatography and modified to GcMAF using immobilized glycosidases (beta-galactosidase and neuraminidase) The sugar moiety structure of GcMAF was characterized by lectin blotting by Helix pomatia agglutinin. The biological activities of GcMAF were evaluated by a superoxide generation assay and a phagocytosis assay. Results: We successfully purified Gc protein from human serum. GcMAF was detected by lectin blotting and showed a high biological activity. Conclusion: Our results support the importance of the terminal N-acetylgalactosamine moiety in the GcMAF-mediated macrophage activation cascade, and the existence of constitutive GcMAF in human serum. These preliminary data are important for designing small molecular GcMAF mimics.*

Inflammation induces chemotaxis and activation of macrophages, which thus leads to immune development (1). Macrophages are known to play a critical role in antitumor immunity (2). They can also infiltrate tumor tissues and are found at most tumor sites (2, 3). Meanwhile, Group specific component (Gc protein), also known as Gc globulin or vitamin D_3-binding protein, is a serum protein which has been reported as a precursor for macrophage activating factor (4). The role of Gc protein in inflammation-mediated macrophage activation was established by Yamamoto *et al.* (4, 5). Gc protein can be converted by an inducible beta-galactosidase of B cells and neuraminidase of T cells to a potent macrophage-activating factor (GcMAF), a protein with *N*-acetylgalactosamine (GalNAc) as the remaining sugar moiety (5).

Gc protein has been reported to have multifunctional properties as a transporter of serum vitamin D_3 and its metabolites, an actin scavenger during cellular injury, a binder fatty acid, and a chemotaxin for phagocytic cells (6, 7) and also to play a role in macrophage activation as a precursor for macrophage activating factor (4). The structure of Gc protein is highly homologous to serum albumin and it has a triple-domained modular structure termed as domains I, II and III (8). Domain III of Gc protein (C-terminal end) harbors a single glycosylation site. The terminal GalNAc moiety in domain III is the region involved in the GcMAF-mediated macrophage activation cascade (4, 9).

Gc protein has a high affinity for the 25-hydroxyvitamin D_3 [25(OH)D_3], $Ka = 5 \times 10^8\ M^{-1}$ (10), a major circulating forms of vitamin D_3 metabolites that is generated in the liver. In order to study the sugar-moiety character of GcMAF, we purified Gc protein using 25(OH)D_3 affinity column chromatography as reported by Link *et al.* (11). The sugar moiety of the Gc protein before and after immobilized glycosidases (beta-galactosidase and neuramindase) treatment was characterized by lectin blotting using *Helix pomatia* agglutinin.

In addition, the importance of the terminal GalNAc moiety of GcMAF was also determined by evaluating the biological activities of GcMAF in resident mouse peritoneal macrophages.

Materials and Methods

Materials. 25(OH)D_3 was a gift from Dr. Nobuto Yamamoto from the Socrates Institute for Therapeutic Immunology, Philadelphia, USA. Other chemicals (biochemical grade) were purchased from the Wako Pure Chemical Industries Co., Japan.

Purification of human serum Gc protein. This procedure was adapted

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from the methods reported by Link *et al.* (11). Briefly, human serum was diluted 1:1 with column buffer (50 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, 150 mM NaCl, 0.1% Triton X-100) and applied to the 25(OH)D_3-Sepharose column that we prepared as reported by Link *et al.*(11). 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 with the protein peak of the guanidine eluted fraction were pooled and dialyzed with 10 mM sodium phosphate, pH 7. Then, a hydroxyappatite column 5 ml (Econo- Pac HTP Cartridge 1, Bio-Rad) was equilibrated in 10 mM sodium phosphate, pH 7. The dialyzed sample from 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 applied to the column chromatography. Fractions were collected and the protein concentration was determined using the bichinconinic acid (BCA) method (Pierce Chemical Co., Rockford, IL, USA). BSA was used as a standard.

GcMAF preparation. Four hundred μg of Gc protein purified from human serum as above were incubated with immobilized beta-galactosidase (4 Unit in 100 mM sodium phosphate buffer, pH 7.0) in a microcentrifuge tube at 37°C by rotation movement for 1 hour. The immobilized enzyme was removed by centrifugation and the pH of the supernatant was adjusted to pH 6.0, by 1 M NaH_2PO_4. The supernatant in 100 mM sodium phosphate buffer, pH 6.0, was incubated with immobilized neuraminidase (1 Unit) at 37°C by rotation movement for 1 hour. The immobilized enzyme was removed and the supernatant was made sterile by filtration; the protein concentration was determined using the BCA method.

Isolation and culture of mouse peritoneal macrophages. Resident mouse peritoneal macrophages (female ICR mice, 7 weeks of age) were collected and after centrifugation at 1,000 × g, 4°C for 10 minutes, the collected macrophages were cultured in 6-well plates with a density of $1\sim2 \times 10^6$ cells/ well in RPMI 1640 (serum-free) for 1 hour. The cultured cells were then washed 3 times with Hank's solution to separate adherent macrophages from non-adherent cells (T and B cells). The cultured macrophages were then treated as indicated for 3 hours and the biological assay (superoxide generation and phagocytosis) was performed as described below.

Superoxide generation assay. The method was modified from that reported by Johnston *et al.* (12). Briefly, after sample treatment for three hours, the plates were washed twice with PBS(-) and once with Krebs-Ringer phosphate buffer, pH 7.4 and 1.5 ml of 50 μM cytochrome *c* in Krebs-Ringer phosphate buffer was added and phorbol myristate acetate (PMA) was added to a final concentration of 5 μg/ml in each well and cultured for 90 minutes (37°C/5% CO_2) in a humidified incubator. The reaction was stopped by means of an ice-bath. The cultured medium was placed in an Eppendorf tube, and centrifuged at 8,000 × g. The optical density of the supernatant was determined spectrometrically at 550 nm with reference at 540 nm (U-2000, HITACHI) using mixtures from plates without cells as blanks. The concentration of reduced cytochrome *c* was determined by the equation $\Delta E_{550nm} = 2.1 \times 10^4\ M^{-1}\ cm^{-1}$.

Phagocytosis assay. Mouse peritoneal cells were layered onto coverslips in a 24-well plate. After three hours of drug treatment, the cultures were assayed for phagocytic activity. Sheep red blood cells (SRBC) were opsonized by rabbit haemolytic serum (anti-sheep red blood C12HSB cells, Serotec Ltd. England). Opsonized-SRBC (0.5%) in RPMI 1640 (serum-free) was overlaid on each macrophage-coated coverslip and was cultured for 90 minutes. The noninternalized erythrocytes were lysed by immersing the coverslip in a hypotonic solution (1/5 PBS). The macrophages were fixed with methanol, air-dried and stained with Giemza stain. The number of phagocytosed erythrocytes per cell was determined microscopically; 250 macrophages were counted for each data point. The data are expressed as the phagocytosis index, which is defined as the percentage of macrophages with ingested erythrocytes multiplied by the average number of erythrocytes ingested per macrophage (13).

Western bloting and lectin bloting. Gc protein before and after immobilized glycosidases treatment was subjected to SDS-PAGE under reducing conditions and electroblotting onto a PVDF membrane. Non-specific binding was blocked by incubation in Tris-buffer saline, pH 7.4 containing 0.1% Tween 20 and 3% bovine serum albumin (BSA) overnight at 4°C. For Western blotting, the blots were probed with anti-human Gc globulin (Code No. A 0021, DAKO Japan Co. Ltd.) and, after extensive washing, the blots were incubated with the secondary antibody (Horseradish Peroxidase-labeled anti-rabbit IgG, Amersharn Pharmacia Biotech UK Ltd.). For lectin blotting, the blots were incubated with Horseradish Peroxidase-labeled *Helix pomatia* agglutinin (Sigma- Aldrich Japan Co.). The blots were developed using an ECL Western blotting detection system (Amersham Pharmacia Biotech UK Ltd.).

Results

Gc protein was purified with a good yield (1.96 mg protein/10 ml serum). Figure 1 shows the Western blot analysis of Gc protein isolated from human serum using a 25(OH)D_3 affinity column. A single band was detected by anti-human Gc globulin antibody. Lectins are good tools for exploring the interaction of sugar moieties because each lectin recognizes specific sugars. In order to examine the existence of terminal GalNAc, a lectin blot was done using *Helix pomatia* agglutinin. *Helix pomatia* agglutinin is a lectin possessing particularly high specificity for terminal GalNAc residue (13). We revised the method of lectin blot from what we had reported recently (14) by adding 3% BSA during incubation of the PVDF membrane with HRP-labeled *Helix pomatia* agglutinin, to avoid any non-specific binding of the lectin to the protein. Human serum and BSA was used as a positive and negative control in both Western and lectin blot analysis. We observed a band of Gc protein before and after immobilized glycosidases treatment at the same position (about 52 kDa) as detected in Western blot. A more strongly stained band was detected in Gc protein after immobilized glycosidases treatment (GcMAF) compared to that before the treatment (Gc protein), which shows the increase of terminal GalNAc residue in Gc protein after immobilized glycosidases treatment.

We evaluated the *in vitro* biological activities of GcMAF in resident mouse peritoneal macrophage after a short time treatment (3 hours). Figure 2 shows the results of the superoxide generation assay. We found that the treatment with 10 pg/ml GcMAF increased the macrophage superoxide generation more than the control (non-treatment) and with 10 pg/ml Gc protein (control: 2.24±1.5 nmol $O_2^-/10^6$ cells/90 minutes, 10 pg/ml GcMAF: 3.45± 1.9, 10 pg/ml Gc protein: 2.56±1.6). Lipopolysaccharide (10 μg/ml LPS: 3.32 ± 1.8 nmol $O_2^-/10^6$ cells/90 minutes) showed almost the same activity to GcMAF, at a concentration 10^6 times higher. Another marker to evaluate macrophage activity is the phagocytosis activity of macrophage, determined as the

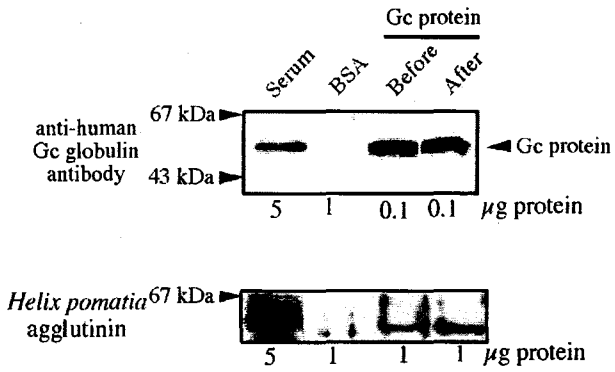

Figure 1. *Western blot and lectin blot analysis of Gc protein before and after the treatment with immobilized glycosidases.*

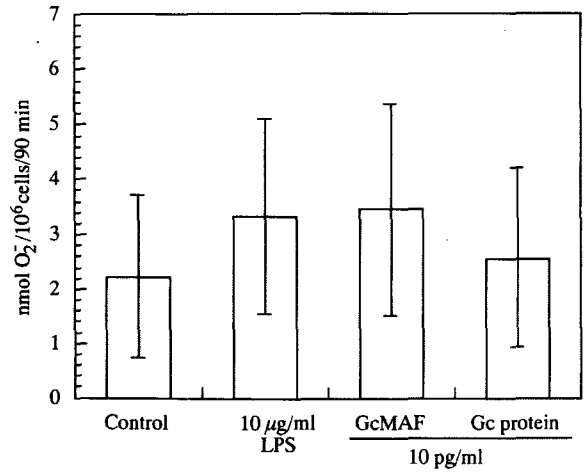

Figure 2. *Superoxide generation assay after 3 hours treatment in resident mouse peritoneal macrophages. All data are shown as the mean ± standard deviation of n = 5, duplicate experiments.*

phagocytosis index. Figure 3 shows a significantly higher macrophage activity in 10 pg/ml GcMAF treatment than in either control, 10 μg/ml LPS or 10 pg/ml Gc protein (control: 11.2±5.6, 10 μg/ml LPS: 72.6±2.8, 10 pg/ml GcMAR 107.1 ±22.1, 10 pg/ml Gc protein: 29.1±11.3).

Discussion

We herein report our findings regarding a qualitative study of GcMAF sugar moiety using *Helix pomatia* agglutinin, which is known to specifically bind to the terminal GalNAc residue. We could detect the difference between Gc protein and GcMAF by lectin blot. As shown in Figure 1, in lectin blot analysis using *Helix pomatia* agglutinin, both GcMAF and Gc protein gave a band at the same position. However, the GcMAF band was stronger than the Gc protein band. The result showed that by immobilized glycosidases, we were able to prepare GcMAF. This observation also showed the existence of constitutive GcMAF in human serum and also corresponding the lect lot results of non-inducible GcMAF in human serum, reported by Kannan *et al.* (15). We were successful in preparing GcMAF using immobilized glycosidases, but we are not certain whether all Gc protein was converted to GcMAF because a quantitative analysis of GcMAF has yet to be performed. Furthermore, the binding ratio of the terminal GalNAc residue of GcMAF to *Helix pomatia* agglutinin is unknown.

GcMAF is a potent macrophage activation factor which increases the macrophage activity after three hours of incubation as demonstrated by superoxide generation and phagocytosis assay. The superoxide generation activities do not seem to be statistically significant. However, we found the same tendency of macrophage activity in each separate experiment, where 10 pg/ml GcMAF treatment demonstrated the highest activity comparetd to control, 10 μg/ml LPS and 10 pg/ml Gc protein. The phagocytosis activity of macrophage treated with GcMAF was 9.5 times higher than the non-

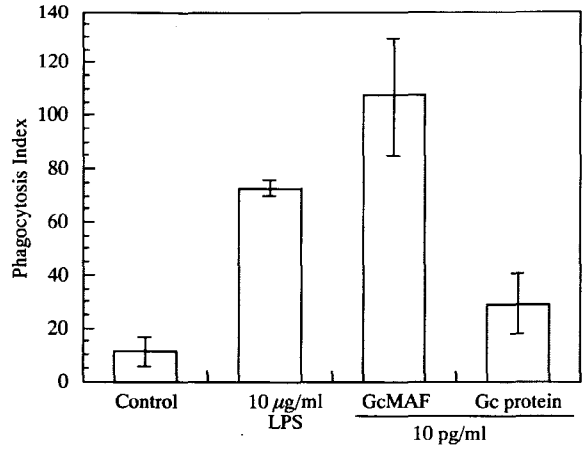

Figure 3. *Phagocytosis assay after 3 hours treatment in resident mouse peritoneal macrophages. All data are shown as the mean ± standard deviation of n = 3.*

treatment. GcMAF increases macrophage phagocytosis activity *via* Fc-gamma receptor (4), but the detailed mechanism of this phenomenon has yet to be studied. Meanwhile, alpha-NaGalase has been reported to accumulate in cancer patients and is responsible for the deglycosylation of Gc protein, which thus finally leads to immunosuppression in advanced cancer patients (16, 17). Recently, we showed *in vitro* evidence that tumor-derived alpha-NaGalase reduced GcMAF activity to release superoxide in thioglycolate-elucidated mouse peritoneal macrophage (18).

Gc protein has been reported to contribute to innate

immunity. A Gc protein-null mouse line was found to reduce cell recruitment to the peritoneum in response to thioglycollate. These observations, however, require further reconsideration, since immune functions can be affected by a complex genetic interaction, such as the difference of strain of mouse used in the study (19). Swamy *et al.* reported that the endogenous ligand of Gc protein [25(OH)D_3] does not influence the activity of GcMAF to activate macrophages (20). Recently, the three dimensional structure of the Gc protein complex with vitamin D_3 analogs ligand has been studied in order to elucidate the unique vitamin D_3- binding properties of Gc protein (21). This study, however, did not reveal the sugar- moiety structure of Gc protein.

Our results show the importance of the terminal GalNAc moiety in the GcMAF-mediated macrophage activation cascade. The above findings provide valuable information for designing small molecular GcMAF mimics which can thus be used for the development a new immunotherapeutic regimens.

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