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# Association of the Macrophage Activating Factor (MAF) Precursor Activity with Polymorphism in Vitamin D-binding Protein

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**Abstract.** *Background: Serum vitamin D-binding protein (Gc protein or DBP) is a highly expressed polymorphic protein, which is a precursor of the inflammation-primed macrophage activating factor, GcMAF, by a cascade of carbohydrate processing reactions. In order to elucidate the relationship between Gc polymorphism and GcMAF precursor activity, we estimated the phagocytic ability of three homotypes of Gc protein, Gc1F-1F, Gc1S-1S and Gc2-2, through processing of their carbohydrate moiety. Materials and Methods: We performed Gc typing of human serum samples by isoelectric focusing (IEF). Gc protein from human serum was purified by affinity chromatography with 25-hydroxyvitamin $D_3$-sepharose. A phagocytosis assay of Gc proteins, modified using β-glycosidase and sialidase, was carried out. Results: The Gc1F-1F phenotype was revealed to possess Galβ1-4GalNAc linkage by the analysis of GcMAF precursor activity using β1-4 linkage-specific galactosidase from jack bean. The GcMAF precursor activity of the Gc1F-1F phenotype was highest among three Gc homotypes. Conclusion: The Gc polymorphism and carbohydrate diversity of Gc protein are significant for its pleiotropic effects.*

Serum vitamin D-binding protein (Gc ptotein or DBP) is a highly-expressed polymorphic protein, which was also known as the group-specific component (Gc-globulin). The Gc protein exhibits multiple functions including possible roles in the immune system and host defense, besides the transport of vitamin D metabolites (1). The Gc protein is the precursor of the inflammation-primed macrophage activating factor (MAF), GcMAF, by a cascade of reactions including an inducible β-galactosidase of B cells and sialidase of T cells to a protein with N-acetylgalactosamine (GalNAc) as the remaining sugar moiety (2, 3). It was shown that the processed carbohydrate moiety of Gc protein is essential for the osteoclast activating property of GcMAF (4). We also reported that the terminal GalNAc moiety of GcMAF is necessary for its macrophage activating effect, to increase phagocytic ability and tumoricidal activity through the superoxide generation (5-7). Furthermore, it is interesting to note that GcMAF was shown to inhibit angiogenesis and tumor growth recently (8).

There are three common alleles (Gc*2, Gc*1F and Gc*1S) and more than 120 variants of the Gc system in the human population (9). Gc polymorphism was originally analyzed by immunoelectrophoresis (10) and afterwards by isoelectric focusing (IEF) (11, 12). The relationship between Gc polymorphism and genetic disease remained unclear, but it was recently shown to be one of the candidates for the susceptibility to chronic obstructive pulmonary disease (COPD) (13, 14). There are major phenotypes such as Gc1, including subtypes of Gc1F and Gc1S, and Gc2, which differ in only four amino acids (152, 311, 416 and 420) (15, 16). The C-terminal end of the Gc protein (domain III) harbors a single glycosylation site in the vicinity of amino acids 418-420. The carbohydrate structures were elucidated by the analysis of the products treated with some glycolytic enzymes (2, 17, 18). Gc1 contains a branched trisaccharide with GalNAc attached to the core protein, a galactose moiety, and a sialic acid (in Gc1F) or a mannose moiety (in Gc1S). Gc2 has a simple glycosylation pattern with a core GalNAc linked to a terminal galactose moiety. Note, however, that in human more than 90 % of Gc2 is likely to be in the non-glycosylated form (2).

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Gc proteins are converted to GcMAF through the processing of their carbohydrate moiety by certain glycolytic enzymes. The macrophage activation by the modified Gc proteins should depend on the phenotypes and/or their carbohydrate structures. Therefore, the ability of MAF derived from various Gc phenotypes is defined herein as GcMAF precursor activity. In order to elucidate the relationship between the Gc polymorphism including carbohydrate structure diversity and the GcMAF precursor activity, we purified three phenotypes of Gc protein, Gc1F-1F, Gc1S-1S and Gc2-2, each from human serum of the corresponding homozygote genotype and estimated their phagocytic ability by treatment with glycolytic enzymes. Furthermore, the carbohydrate structures of the homotypes of the Gc protein have been considered.

## Materials and Methods

*Animals.* Female ICR mice (8 to 10 weeks old) were purchased from Japan SLC, Inc. (Shizuoka, Japan). The animals were maintained and used according to the guidelines for the Care and Use of Animals approved by the Council of the Physiological Society of Japan.

*Materials.* Rabbit polyclonal anti-human Gc-globulin antibody (Code No. A 0021) was purchased from DAKO Co., Ltd. (Kyoto, Japan). Rabbit hemolytic serum (Code No. C12HSB; anti-sheep red blood cells) was bought from Serotec Ltd. (England). β-galactosidase from bovine testes (Code No. G 4142) and neuraminidase (Code No. N 2876) were from Sigma-Aldrich Japan Co. (Tokyo, Japan) and β-galactosidase from jack bean was from Seikagaku Co. (Tokyo, Japan). Other chemicals (biochemical grade) were obtained from the Wako Pure Chemical Industries Co. (Osaka, Japan). PhastGel Dry IEF and Pharmalyte pH 4.5-5.4 were from Amersham Biosciences (New Jersey, USA).

*Serum samples.* Serum samples from 24 healthy human (Age: 22 ~ 57 years old) were taken were separated by centrifugation at 3,000 g for 10 min at 4˚C and then stored at –30˚C until use.

*Purification of human serum Gc protein.* Gc protein was purified by affinity chromatography with 25-hydroxyvitamin $D_3$ [25(OH)$D_3$]-sepharose from a 60% ammonium sulphate precipitation of the serum samples according to the method of Link *et al.* (19). In brief, the precipitates were diluted 1:5 with column buffer (50 mM Tris-HCl, pH 7.4) and applied to the 25(OH)$D_3$-sepharose column. The column was washed with 100 ml of column buffer. The protein remaining on the matrix was eluted with 6 M guanidine-HCl. The peak fractions were pooled and dialyzed in 10 mM sodium phosphate buffer (pH 7.0) and then applied to a hydroxylapatite column (Econo- Pac HTP Cartridge 1, Bio-Rad, California, USA) equilibrated in 10 mM sodium phosphate buffer (pH 6.0). A linear gradient elution was done from 10 mM to 200 mM sodium phosphate buffer (pH 6.0). Fractions of the protein peak were collected and buffer-exchanged into 10 mM sodium phosphate buffer (pH 7.0), and then concentrated by Centricon or Microcon (Millipore, Massachusetts, USA). The protein concentrations were measured by BCA assay kit (Pierce Chemical Co., Illinois, USA) using BSA standard.

*GcMAF preparation.* One μl of Gc protein (5-10 μg protein/μl) was diluted 1:50 with 10 mM sodium phosphate buffer (pH 6.0) and treated with 1 μl of sialidase (10 U/ml) and 1 μl of β-galactosidase from jack bean (10 U/ml) or 5 μl of β-galactosidase from bovine testes (1 U/ml) at 37˚C overnight. The reaction was stopped on ice and subjected to a biological assay directly.

*Macrophages.* Resident mouse peritoneal macrophages were collected and centrifuged at 1,000 rpm for 10 min at 4˚C. The collected macrophages (5 X $10^5$ cells/well) were suspended in RPMI-1640 medium and laid on a coverslip placed in 24-well plates. They were incubated at 37˚C in a humidified 5% $CO_2$ incubator for 1 h. After washing with the medium to remove nonadherent cells, the macrophages were treated with GcMAF, as indicated in the Results section, for 3h and then used for the phagocytosis assay.

*Phagocytosis assay.* Sheep red blood cells (SRBC), opsonized with rabbit hemolytic serum in RPMI-1640 medium, were overlaid on each macrophages-coated coverslip and were incubated at 37˚C in a humidified 5% $CO_2$ incubator for 90 min. Non-internalized erythrocytes were lysed by immersing the coverslip in a hypotonic solution (1/5 PBS) for 3 sec. The macrophages were fixed with methanol, air-dried and stained with Giemza stain. The number of phagocytized erythrocytes per cell was determined microscopically, when 400 to 1000 macrophages were counted for each data point. We calculated the ingested index from the data, which was defined as the percentage of macrophages with phagocytized erythrocytes multiplied by the average number of erythrocytes phagocytized per ingested macrophage (2). The data are expressed as a ratio to the control data of non-treated macrophage and the values are the means of at least three independent experiments.

*Isoelectric focusing (IEF) method.* Gc typing analysis was done using PhastSystem (Pharmacia Biotech, Uppsala, Sweden) according to the protocol by Takagi and Ujiie (20). In brief, PhastGel Dry IEF (5% T, 3% C) was re-swelled in 2.5% Pharmalyte pH 4.5-5.4, 1.68% MOPS [3-(N-morpholino)propanesulfonic acid] and 1.12% HEPES [N-(2-hydroxyehyl) piperazine-N'-2-ethanesulfonic acid] for 2 h. One μl of samples (serum sample diluted 1:75 with water) was applied to the sample applicator and electrophoresis was performed as reported by Takagi and Ujiie (20). Gc subtypes were detected by immunoblot with anti-human Gc globulin antibody and horseradish peroxidase (HRP)-labeled anti-rabbit IgG. The peroxidase/3,3-diaminobenzidine tetrahydrochloride (DAB) reaction was used for staining.

## Results

*Gc typing.* Electrophoretic patterns corresponding to different phenotypes with reference to the typical pattern (20) obtained from human serum Gc typing by IEF are shown in Figure 1. In the Gc1F-1F and Gc1S-1S phenotypes, the anodic band presents lower intensity than the corresponding cathodic one, which may be desialylated form of the anodic band. A set of bands of the Gc1F-1F phenotype confers a higher mobility than the one of Gc1S-1S. Gc 1A variant has the highest mobility.

*Phagocytic ability and carbohydrate structure of GcMAF from Gc1F-1F.* We isolated three phenotypes of Gc protein, Gc1F-1F, Gc1S-1S and Gc2-2, each from human serum of

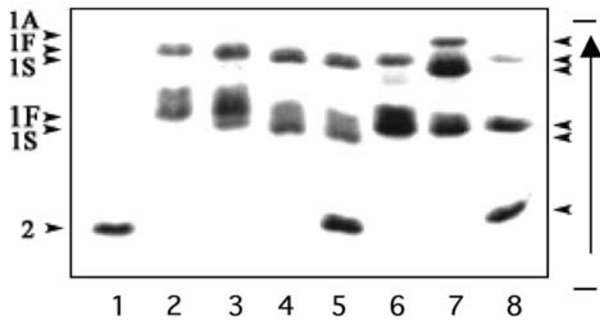


Figure 1. *Electrophoretic pattern of common and variant Gc subtypes after isoelectric focusing (IEF) separation of healthy human serum samples and immunoblot with anti-human Gc globulin antibody. Lane 1, Gc2-2; lanes 2 and 6, Gc1F-1F; lane 3, Gc1F-1S; lane 4, Gc1S-1S; lane 5, Gc2-1S; lane 7, Gc1F-1A; lane 8, Gc2-1F.*

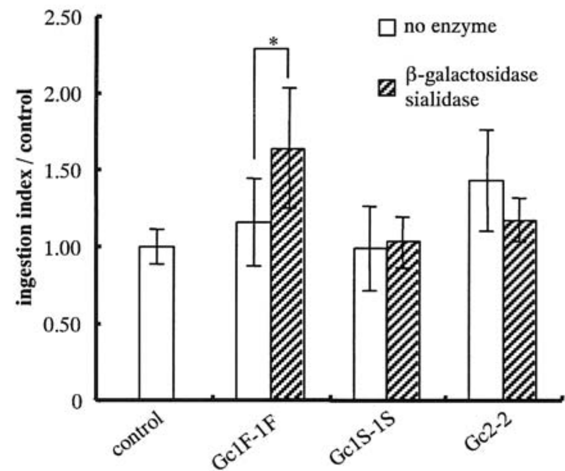


Figure 3. *GcMAF precursor activities of purified Gc homotypes, Gc1F-1F, Gc1S-1S and Gc2-2 evaluated by in vitro activation of mouse peritoneal macrophages for ingestion. Mouse macrophages were treated with Gc proteins of each homotype or the Gc proteins modified by β-galactosidase from jack bean and sialidase at 10 pg/ml for 3 h. All experiments were performed in triplicate. Each bar represents the mean±SD. P<0.01 (*).*

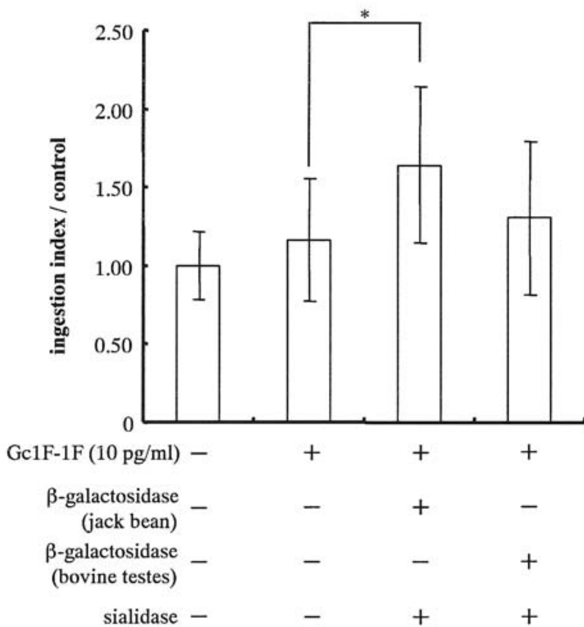


Figure 2. *In vitro activation of mouse peritoneal macrophages by treatment with Gc1F-1F or modified Gc1F-1F at 10 pg/ml for 3 h. Gc1F-1F protein was modified by β-galactosidase from jack bean or bovine testes and /or sialidase. All experiments were performed in triplicate. Each bar represents the mean±SD. P<0.01 (*).*

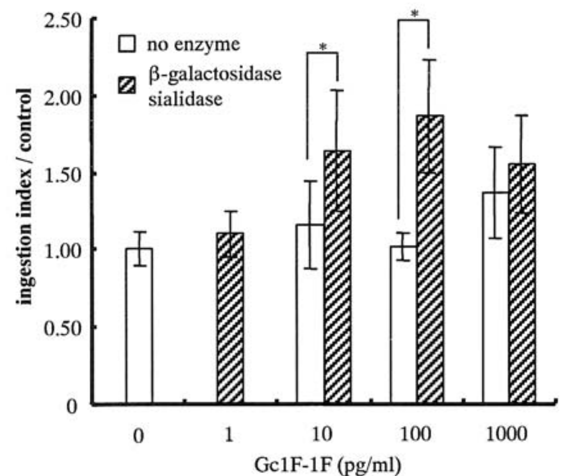


Figure 4. *Dose-response of GcMAF derived from Gc1F-1F phenotype on in vitro activation of mouse peritoneal macrophages. Mouse macrophages were treated with Gc1F-1F protein or GcMAF from Gc1F-1F, which was modified by β-galactosidase from jack bean and sialidase, at concentrations ranging from 1-1000 pg/ml for 3 h and used for phagocytosis assay. All experiments were performed in triplicate or more. Each bar represents the mean±SD. P<0.01 (*).*

the corresponding homozygote genotype determined by Gc typing using the 25(OH)$D_3$-sepharose column as described in Materials and Methods. They were purified in good yield (Gc1F-1F, 6.8 mg protein; Gc1S-1S, 6.3 mg protein; Gc2-2, 4.6 mg protein per 10 ml of serum). We confirmed their purities and Gc types by SDS-PAGE, IEF and Western blot analysis with anti-human Gc globulin antibody.

In order to distinguish between β1-4, β1-6 and β1-3-linked galactose, β-galactosidase from jack bean, which hydrolyzes the Gal β1-3 linkage much slower than other linkages (21), and one from bovine testes, which hydrolyses both the β1-4 and β1-3 linkages(22), were used to modify the Gc protein. As shown in Figure 2, the GcMAF obtained from the Gc1F-1F phenotype treated

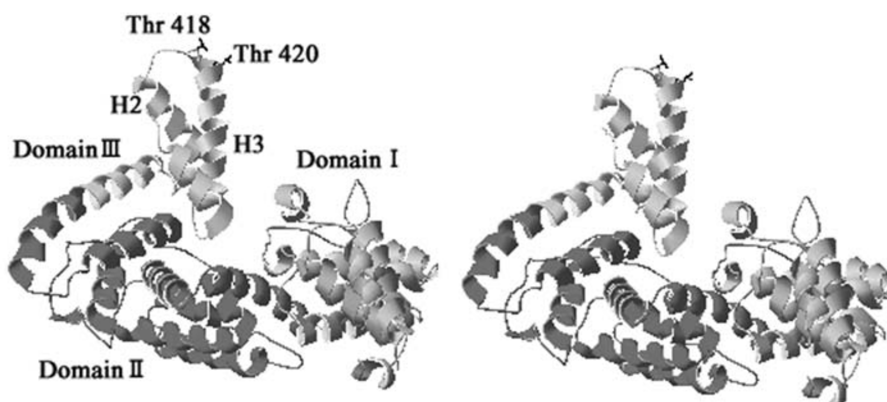


Figure 5. *Stereo view of the overall fold structure of the human vitamin D-binding protein (PDB 1J78)(30). Three domains are shown in different colors and the helices in domain III, H2 and H3 are indicated (26). Possible glycosylation sites, Thr 418 or Thr 420 located at loop-helix (H3) structure are shown. This figure was made using the Swiss-PdbViewer program (v3.7) (31).*

with β-galactosidase from jack bean and sialidase had a significantly higher ingestion index than before the modification of the carbohydrate moiety. There were no significant differences in ingestion index between the two β-galactosidase treatments. These results show that the Gc1F-1F phenotype has an oligosaccharide with a Gal β1-4 linkage.

*GcMAF precursor activities depending on Gc phenotype.* We evaluated the GcMAF precursor activities of three Gc homotypes based on the phagocytic activity of the macrophage induced by the corresponding modified Gc protein, which was converted by β-galactosidase from jack bean and sialidase. As indicated in Figure 3, 3 h-treatment with 10 pg/ml of GcMAF derived from the Gc1F-1F phenotype increased the ratio of ingestion index significantly to the control(Gc1F-1F, 1.16±0.28; modified Gc1F-1F, 1.64±0.39). In Gc1S-1S and Gc2-2 homotypes, there were no significant differences in ingestion index between the precursor and corresponding modified Gc protein (Gc1S-1S, 0.99±0.27; modified Gc1S-1S, 1.03±0.17; Gc2-2, 1.43±0.33; modified Gc2-2, 1.17±0.14). These data suggest that the Gc1F-1F phenotype exhibits the highest GcMAF precursor activity through β-galactosidase and sialidase processing. Dose-response effect of GcMAF from Gc1F-1F on the phagocytic activity of macrophage showed a tendency of bell shape as seen in Figure 4. The macrophage activating ability of GcMAF from Gc1F-1F increased significantly at a concentration of 10 and 100 pg/ml. The highest ingestion activity was observed after treatment with GcMAF at a concentration of 100 pg/ml but a tendency of decreasing activity appeared at 1000 pg/ml.

## Discussion

The role of the Gc protein in the inflammation-mediated activation of macrophages was established by Yamamoto and Homma (3). It is remarkable that the Gc protein is converted to a potent MAF by a cascade of processing reactions involving a simple small trisaccharide attached to threonine located at the C-terminus, domain III. The bell-shaped dose-response curve for the macrophage activating effect of GcMAF as well as its low (*e.g.* 100 pg/ml) , as shown in Figure 4, suggested that the activating effect was mediated by certain receptors. These data bear out the report by Ono *et al.*, which showed GcMAF induced the enhancement of Fcγ receptor-mediated phagocytosis (23).

The carbohydrate structure seems to be involved in the interaction with the receptor. However, the carbohydrate moiety was not shown in the crystal structures of the Gc protein and its complexes with actin or $25(OH)D_3$ as reported recently (24-27). The vicinity of amino acids 418-420 including a glycosylation site form a loop-helix (H 3) structure and the sugar moiety seems to be located on the exterior of a globular conformation (Figure 5) (26). Thus the carbohydrate chain of Gc protein is probably significant for molecular recognition in exerting not only an immunomodulating effect but also antiangiogenic and antitumor effects (8, 28).

In this report, the GcMAF precursor activity of the Gc1F-1F phenotype was the highest among the three Gc homotypes through processing by β-galactosidase and sialidase. This cascade of sugar processing has been suggested that occurs *in vivo* (3). Carbohydrate structures of all phenotypes have not yet been completely elucidated. We showed that the Gc1F-1F phenotype possesses a Galβ1-4GalNAc linkage by analysis of

GcMAF precursor activity through processing by β1-4 linkage-specific galactosidase from jack bean (Figure 2). The sugar structure of all phenotypes should be revealed by direct carbohydrate sequence analysis. This information will be useful for the design of new antitumor agents acting *via* macrophage activation. The glycopeptide of Gc protein is one of candidate for the lead compound of dramatype-based anticancer drug development. In fact, a novel small glycopeptide which was designed and synthesized based on the amino acid sequence at the site of glycosylation in the domain III of Gc protein, exhibited anabolic effects on bone increasing the total bone density and enhancing the strength of the bone (29).

The involvement of the Gc polymorphism and carbohydrate diversity in the pleiotropic effects of the Gc protein is still obscure. However, the structural variation of domain III is a remarkable characteristic of Gc protein and should play a primary role in molecular recognition in the immune system and in cellular signal transduction. We are investigating the structure-activity relationship of carbohydrate moiety of Gc protein for its macrophage activating effect for design of small molecule GcMAF.

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