Degalactosylated/Desialylated Human Serum Containing GcMAF Induces Macrophage Phagocytic Activity and *In Vivo* Antitumor Activity

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Abstract. *Background: The group-specific component protein-derived macrophage-activating factor (GcMAF) has various biological activities, such as macrophage activation and antitumor activity. Clinical trials of GcMAF have been carried out for metastatic breast cancer, prostate cancer, and metastatic colorectal cancer. In this study, despite the complicated purification process of GcMAF, we used enzymatically-treated human serum containing GcMAF with a considerable macrophage-stimulating activity and antitumor activity. Results: We detected GcMAF in degalactosylated/desialylated human serum by western blotting using an anti-human Gc globulin antibody, and Helix pomatia agglutinin lectin. We also found that GcMAF-containing human serum significantly enhanced the phagocytic activity of mouse peritoneal macrophages and extended the survival time of mice bearing Ehrlich ascites tumors. Conclusion: We demonstrated that GcMAF-containing human serum can be used as a potential macrophage activator for cancer immunotherapy.*

The group-specific component (Gc) protein, also known as vitamin D-binding protein (DBP) or Gc globulin, is a 53-kDa human plasma glycoprotein (1). Inflammation results in the hydrolysis of terminal galactose and sialic acid of the Gc protein and this is mediated by membrane-bound β-galactosidase present on activated B-cells and sialidase on T-cells to produce Gc protein-derived macrophage-activating factor (GcMAF) (2). GcMAF has been shown to possess several biological activities, such as macrophage activation *via* superoxide generation (3, 4) and phagocytic activation (5), anti-angiogenesis effect (6, 7), and antitumor activities (8-10). Moreover, it has been demonstrated that GcMAF administration has clinical benefits in patients with metastatic colorectal, metastatic breast, and prostate cancer, and non-anemic HIV-infected patients, (11-14).

However, a major problem is associated with the purification of GcMAF for clinical use. In previous studies, GcMAF was purified from human serum using an affinity column modified with 25-hydroxy-vitamin D3 (15). However, it is difficult to prevent contamination when a column is repeatedly used. Additionally, purified GcMAF is unstable in the presence of oxygen at room temperature and in the absence of antioxidants such as albumin and uric acid that are abundantly present in blood (16). To overcome problems associated with GcMAF purification, we prepared non-purified GcMAF by using autologous serum as a source. Thus, we propose a novel method for preparation of autologous serum containing GcMAF by degalactosylation and desialylation and report the potential role of autologous GcMAF in stimulating phagocytosis in macrophages and *in vivo* antitumor activity.

Materials and Methods

Preparation of GcMAF-containing human serum. Gc protein and GcMAF were prepared as previously reported by Uto *et al.* (17, 18). Human serum containing 1f1f-subtype of Gc protein (100 μl, 100

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Key Words: Degalactosylated/desialylated human serum, GcMAF, macrophage phagocytic activity, *in vivo* antitumor activity.

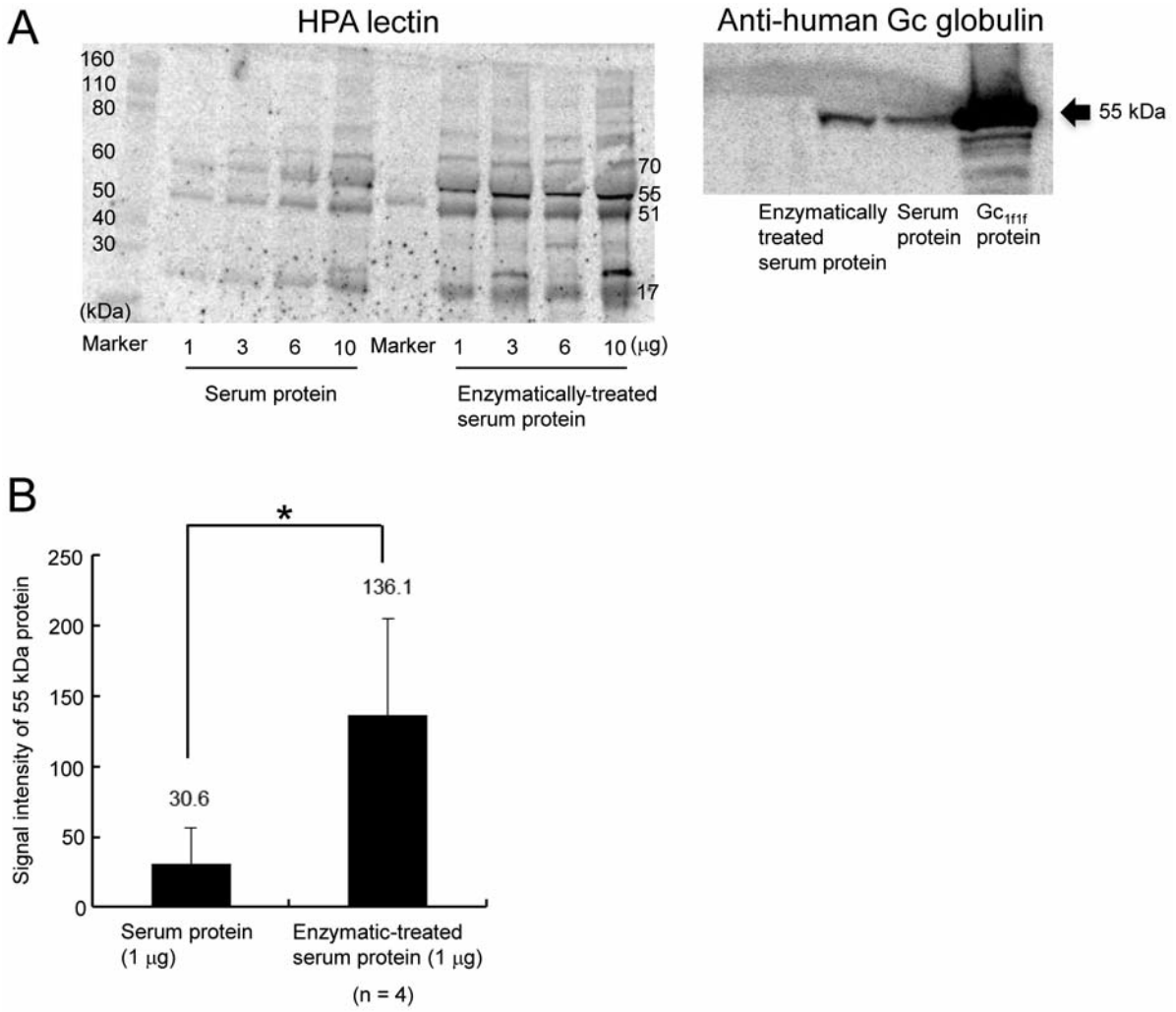

Figure 1. *Analysis of group-specific component protein-derived macrophage-activating factor (GcMAF) generated by the enzymatic treatment of human serum. A: Western blots probed with anti-human Gc globulin and Helix pomatia agglutinin (HPA) lectin. B: Quantification of the signal intensity of the detected protein bands was performed using JustTLC image analysis software.*

μg/μl) was obtained from human volunteers and each serum sample was incubated with 65 mU of β-D-galactosidase (from *Escherichia coli*; Wako, Osaka, Japan) and neuraminidase (sialidase, from *Clostridium perfringens*; Sigma-Aldrich Japan Co. Ltd., Tokyo, Japan) in 100 mM sodium phosphate buffer (pH 7.0) at 37˚C for 3 h. The reaction mixture was heated at 60˚C for 10 min and the solution was concentrated using a microcon concentration unit (10,000 MWCO; Nihon Millipore Co. Ltd., Tokyo, Japan). The protein concentrations were determined using a BCA protein assay kit (Pierce Chemical Co., Rockford, IL, USA).

Western blotting of GcMAF-containing human serum. The GcMAF-containing human serum was subjected to sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and subsequently electroblotted onto a nitrocellulose membrane. Non-specific binding was blocked by overnight incubation in Tris-buffered saline (pH 7.4) containing 0.1% Tween 20 and 1% bovine serum albumin (BSA) at 4˚C. The membranes were then probed with anti-human Gc globulin (DakoCytomation Co. Ltd., Kyoto, Japan) and with biotin-conjugated *Helix pomatia* agglutinin (HPA) lectin (Sigma-Aldrich, St. Louis, MO, USA) specific for GcMAF with *N*-acetylgalactosamine (GalNAc) moiety. After membrane washing, the blots were incubated with horseradish peroxidase (HRP)-labeled anti-rabbit IgG (GE Healthcare Life Sciences, Uppsala, Sweden) as a secondary antibody. The blots were developed using an ECL western blotting detection system (GE Healthcare Life Sciences, Uppsala, Sweden) and visualization and quantification of the western blot bands was carried out with a LumiCube chemiluminescence analyzer and JustTLC image analysis software (Liponics, Tokyo, Japan).

Isolation and culture of mouse peritoneal macrophages. Mouse peritoneal adherent cells containing macrophages were collected from 8-week-old female ICR mice (Japan SLC, Hamamatsu, Japan), as previously reported by Uto *et al*. (17, 18) and cultured in 24-well plates at a density of 5×10^5 cells/well in serum-free RPMI-1640 (Life Technologies, Carlsbad, CA, USA) 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 cultured macrophages were treated with different concentrations of serum proteins for 15 h and used for phagocytosis assay as described below.

Phagocytosis assay. Mouse peritoneal macrophages were layered onto coverslips in a 24-well plate. After 3 h of human serum treatment, the cultures were assayed for phagocytic activity. Sheep red blood cells (SRBCs; Nippon Bio-Supp. Center, 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 cultured for 1.5 h. The non-internalized erythrocytes were lysed by immersing the coverslip into a hypotonic solution [1/5-diluted phosphate-buffered saline (PBS)]. 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 were expressed in terms of the phagocytosis index (PI), which was defined as the percentage of macrophages with ingested erythrocytes multiplied by the mean number of erythrocytes ingested per macrophage.

Animals. ICR female mice weighing 20-25 g were housed in polycarbonate cages under standard laboratory conditions (24±1˚C, 12 h light/dark cycle) with food and water *ad libitum*. All procedures used for animal experimentation were approved by the Animal Research Committee of the University of Tokushima (TokuDobutsu12025). Ehrlich ascites carcinoma (EAC) cells were obtained from the Cancer Research Institute of Kanazawa University and were maintained in the peritoneal cavity of mice by injecting 0.1 ml of ascitic fluid every seven days. Ascitic tumor cell counts were carried out in a Neubauer hemocytometer using the trypan blue dye exclusion method. Tumor cell suspensions were prepared in PBS.

In vivo antitumor activity assay. Ten-week-old female ICR mice were inoculated with 1×10^7 EAC cells (0.2 ml/mouse) intraperitoneally (*i.p.*). The day of tumor implantation was assigned as day '0'. On day 1, the animals were randomized and divided into five groups (n=5) and administered *i.p.* with PBS, GcMAF (40 ng/kg/d), or GcMAF-containing human serum (172, 517, and 1,552 μg protein/kg/d) for seven days. The animals with ascitic tumor were weighed every day. Kaplan-Meier survival curves were analyzed by the log-rank test.

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

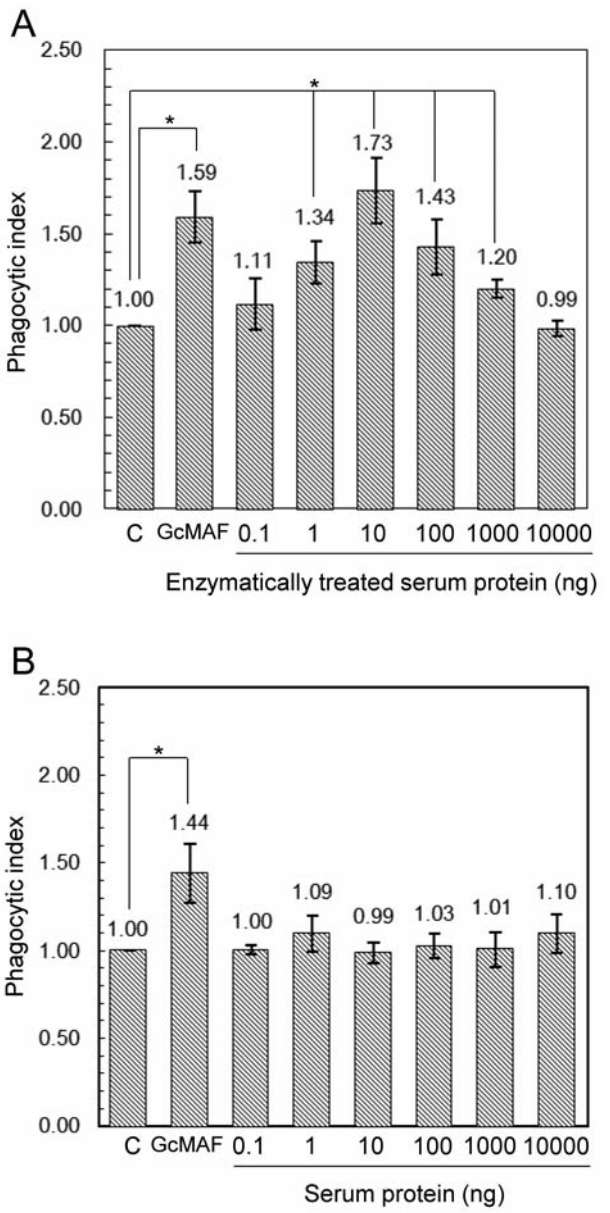

Figure 2. *Phagocytic activity of mouse peritoneal macrophages observed using enzymatically-treated human serum protein (A) or serum protein (B). All experiments were performed in triplicates. Each error bar represents the standard deviation. The number on each bar indicates the mean value. *p<0.05.*

Results

Preparation and characterization of GcMAF-containing human serum. We obtained 8.8 mg of serum protein from 100 μl of healthy human serum by degalactosylation/ desialylation and concentration using a microcon centrifugal filter. Figure 1A shows the western blot of the degalactosylated/ desialylated human serum. Four bands (70 kDa, 55 kDa, 51 kDa and 17 kDa) were detected on the blot membranes by using an HPA lectin, which recognizes the GalNAc moiety. Since the 55-kDa protein band was specifically detected by anti-human Gc globulin, it was confirmed that GcMAF was present in the degalactosylated/desialylated human serum. The signal intensity of the 55-kDa protein was significantly higher than that of the control serum, as shown in Figure 1B. Therefore, it was clearly demonstrated that degalactosylation/ desialylation of human serum is a useful method to produce GcMAF.

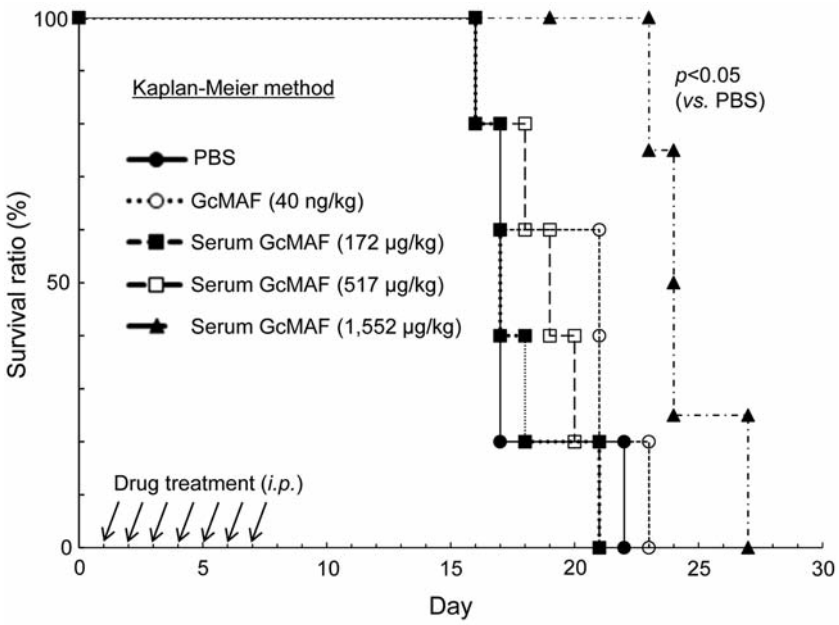

Figure 3. *In vivo antitumor activity assessed using enzymatically-treated human serum protein and Gc protein-derived macrophage-activating factor (GcMAF). All experiments were performed in triplicates. Each error bar represents the standard deviation. The number on each bar indicates the mean value. *p<0.05.*

Stimulating activity of GcMAF-containing human serum on phagocytic activity of mouse peritoneal macrophages. We examined the phagocytic activation by using GcMAF-containing human serum against mouse peritoneal adherent cells containing macrophages. Figure 2A shows significant phagocytic activation with 1 to 1000 ng of GcMAF-containing human serum as compared to that of the control. The maximum PI of 1.73 for 10 ng of GcMAF-containing human serum corresponded to that of 10 ng of GcMAF (PI=1.59). No phagocytic activation was shown in peritoneal macrophages incubated with non-treated human serum (Figure 2B).

In vivo antitumor activity of GcMAF-containing human serum in the EAC model. Figure 3 shows the antitumor activity of GcMAF-containing human serum against EAC inoculated *i.p.* into mice. Treatment of EAC-bearing mice with 1,552 μg protein/kg dosage of GcMAF-containing human serum resulted in a significant prolongation of life span, and the [treated/control (T/C) (%)] value was 138% compared to that of the EAC-bearing control group.

Discussion

We prepared GcMAF-containing human serum by treating human serum with β-galactosidase and sialidase and then evaluated its ability to activate peritoneal macrophage phagocytosis and its *in vivo* antitumor activity. The western blot analysis with HPA lectin showed four positive bands, including a GcMAF-positive band. On the basis of the molecular weights of the other three bands (70 kDa, 51 kDa and 17 kDa), we speculated that the bands may be serum glycoproteins, α1T-glycoprotein (75 kDa), immunoglobulin (Ig) heavy chain (50-70 kDa) and α1-antitrypsin (51 kDa). However, it has been reported that α1T-glycoprotein has no GalNAc moiety (19). Therefore, the 70-kDa band may be IgA or IgD heavy chain with O-linked oligosaccharides that consist of GalNAc, galactose, and sialic acid with the same sugar chain composition as Gc protein. IgA is the most common immunoglobulin in the blood, and the plasma of a healthy adult contains about 90-400 mg/dl of immunoglobulins. Additionally, plasma contains about 3-5 mg/dl of Gc protein.

FcαRI (or CD89), the Fc receptor for IgA, is expressed on immune effector cells and it initiates inflammatory reactions (20, 21). Additionally, it has been demonstrated that the number of GalNAc attached to IgA *O*-linked glycans was significantly reduced in patients with Crohn's disease and was strongly correlated with clinical activity (22). These data suggest that GcMAF, as well as IgA, is activated by GalNAc that is presented by β-galactosidase and sialidase treatment. If this hypothesis is correct, it may explain the mechanisms by which 10 ng of GcMAF-containing serum protein induced phagocytic activation comparable to that of 10 ng of purified GcMAF.

IgA deficiency is also of interest to us; it is the most common immune deficiency disorder (23). People with this disorder have low levels of or a lack of IgA which affects

proper functioning of the immune system and increases episodes of bronchitis, conjunctivitis (eye infection), gastrointestinal inflammation including ulcerative colitis, Crohn's disease and upper respiratory tract infections (23, 24). Whether or not IgA deficiency has implications for endogenous GcMAF levels has yet to be elucidated. In addition, exogenous GcMAF treatment for people with selective IgA deficiency disorder is also of interest. Further investigation is required to elucidate whether active glycoproteins, other than GcMAF, are present in human serum.

In conclusion, we propose that GcMAF-containing human serum can be used as an effective phagocytosis activator for macrophages and antitumor agent for cancer immunotherapy.

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