Effect of the Gc-derived Macrophage-activating Factor Precursor (preGcMAF) on Phagocytic Activation of Mouse Peritoneal Macrophages

YOSHIHIRO UTO[1], SYOTA YAMAMOTO[1], RYOTA TAKEUCHI[1,2], YOSHINORI NAKAGAWA[2], KEIJI HIROTA[3,4], HIROSHI TERADA[3,4], SHINYA ONIZUKA[5], EIJI NAKATA[6] and HITOSHI HORI[1]

[1]*Department of Life System, Institute of Technology and Science, Graduate School, The University of Tokushima, 2-1 Minamijosanjimacho, Tokushima 770-8506, Japan;*
[2]*Morita Pharmaceutical Ind. Ltd., 2-1-3 Nihonbashi, Chuo-ku, Tokyo 103-0027, Japan;*
[3]*Faculty of Pharmaceutical Sciences and* [4]*Center for Drug Delivery Research, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan;*
[5]*Dejima Hospital, 12-23 Dejimacho, Nagasaki 850-0862, Japan;*
[6]*Institute of Advanced Energy, Kyoto University, Uji, Kyoto 011-0011, Japan*

Abstract. *Background: The 1f1f subtype of the Gc protein (Gc_{1f1f} protein) was converted into Gc-derived macrophage-activating factor (GcMAF) by enzymatic processing in the presence of β-galactosidase of an activated B-cell and sialidase of a T-cell. We hypothesized that $preGc_{1f1f}MAF$, the only Gc_{1f1f} protein lacking galactose, can be converted to GcMAF in vivo because sialic acid is cleaved by residual sialidase. Hence, we investigated the effect of $preGc_{1f1f}MAF$ on the phagocytic activation of mouse peritoneal macrophages. Results: We examined the sugar moiety of $preGc_{1f1f}MAF$ with a Western blot using peanut agglutinin (PNA) and Helix pomatia agglutinin (HPA) lectin. We also found that $preGc_{1f1f}MAF$ significantly enhanced phagocytic activity in mouse peritoneal macrophages but only in the presence of the mouse peritoneal fluid; the level of phagocytic activity was the same as that observed for GcMAF. Conclusion: $PreGc_{1f1f}MAF$ can be used as an effective macrophage activator in vivo.*

Correspondence to: Yoshihiro Uto, Department of Life System, Institute of Technology and Science, Graduate School, The University of Tokushima, 2-1 Minamijosanjimacho, Tokushima, 770-8506 Japan. Tel/Fax: +81 886567522, e-mail: uto@bio.tokushima-u.ac.jp; Hitoshi Hori, Department of Life System, Institute of Technology and Science, Graduate School, The University of Tokushima, 2-1 Minamijosanjimacho, Tokushima, 770-8506 Japan. Tel: +81 886567514, Fax: +81 886569164, e-mail: hori@bio.tokushima-u.ac.jp

Key Words: Gc-Derived macrophage-activating factor precursor, preGcMAF, 1f1f subtype of Gc protein, Gc_{1f1f} protein, phagocytic activity, mouse peritoneal macrophages.

The Gc protein, a vitamin D-binding protein (DBP) or Gc globulin, is a 53-kDa serum protein belonging to the albumin superfamily (1, 2). Its physiological functions include vitamin D transport and storage, and scavenging of extracellular G-actin; the Gc protein is also a precursor of the macrophage activator (3, 4). The Gc protein is of six major subtypes; these include the homodimers and heterodimers of Gc1f, Gc1s, and Gc2. The 1f1f subtype of the Gc protein (Gc_{1f1f} protein) contains galactose (Gal) and a sialic acid (SA) moiety because of the binding of an *N*-acetylgalactosamine (GalNAc) moiety to a threonine residue (4). In an inflammatory response, the terminal Gal and SA were hydrolyzed by membrane-bound β-galactosidase of an activated B-cell and sialidase of a T-cell, respectively, to produce a macrophage-activating factor (GcMAF) (5). It is reported that GcMAF showed interesting biological activity, such as macrophage activation *via* superoxide generation (6) and phagocytic activation (7), antiangiogenic activity (8, 9), and antitumor activity (9-11). GcMAF has also shown clinical activity against colorectal, breast, and prostate cancer and HIV infection (12-15). The following are some problems associated with the clinical use of GcMAF prepared from human serum. (i) Enzymatic conversion of the Gc protein to GcMAF is operationally complex because the former has six major subtypes, each with a different sugar moiety. (ii) The pharmacokinetic property of GcMAF may be affected by deglycosidation of SA because the protein structure and surface charge of the Gc protein and GcMAF are different. We hypothesized that the Gc_{1f1f}-derived macrophage-activating factor precursor ($preGc_{1f1f}MAF$), the Gc_{1f1f} protein lacking Gal, could be converted to GcMAF *in vivo* by the cleaving of residual sialidase. In this manner, all of the Gc protein could then be converted to GcMAF, probably because

Gal is a common Gc sugar moiety (1, 16-19). We present here the effect of preGc$_{1f1f}$MAF on the phagocytic activation of mouse peritoneal macrophages.

Materials and Methods

Materials. Lipopolysaccharide (LPS; from *Escherichia coli* 0128:B12) was purchased from Sigma-Aldrich Japan Co. (Tokyo, Japan). 25-Hydroxyvitamin D_3 (25(OH)D_3) and other chemicals of biochemical grade were purchased from WAKO Pure Chemical Industries Co. Ltd. (Osaka, Japan).

Preparation of human serum Gc$_{1f1f}$ protein. The Gc$_{1f1f}$ protein was prepared as reported by Link *et al*. (20). Briefly, healthy human serum (containing Gc$_{1f1f}$, 3.5 ml) was diluted 1:1 with a 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 according to the method reported by Link *et al*. (20). The column was washed with 300 ml of column buffer. The protein remaining on the matrix was eluted with 6 M guanidine-HCl, and 1 ml fractions were collected. Fractions with the protein peak of the guanidine-eluted fraction were pooled and dialyzed with 10 mM sodium phosphate (pH 7.0). A hydroxyapatite column with a volume of 5 ml (Bio-Scale Mini CHT Type II Cartridge; Bio-Rad Laboratories, Tokyo, Japan) was equilibrated in 10 mM sodium phosphate (pH 7.0). The dialyzed sample obtained from 25(OH)D_3-Sepharose column chromatography was then 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 centricon (30,000 MVCO; Nihon Millipore Co., 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 amount of purified Gc$_{1f1f}$ protein was 743 μg.

Western blotting. The Gc$_{1f1f}$ protein and preGc$_{1f1f}$MAF were subjected to sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and subsequently to electroblotting 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. In Western blotting, the blots were probed with anti-human Gc globulin (DakoCytomation, Glostrup, Denmark) for the Gc$_{1f1f}$ protein, and with PNA lectin (*Arachis hypogaea*, biotin-conjugated; Sigma-Aldrich Japan Co.) and HPA lectin (*Helix pomatia*, biotin-conjugated; Sigma-Aldrich Japan Co.) for preGc$_{1f1f}$MAF. After extensive washing, the blots were incubated with 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.).

Preparation of preGc$_{1f1f}$MAF. The purified Gc$_{1f1f}$ protein (25 μg) was incubated with 250 mU of β-galactosidase (Grade III from bovine liver; Sigma-Aldrich) in 100 mM sodium phosphate buffer (pH 7.0) in an Eppendorf tube at 37.5˚C for 1 hour. The reaction mixture was mixed with 25(OH)D_3-Sepharose beads and was subjected to stirring at 4°C using a vortex mixer for 60 minutes to separate the β-galactosidase. The solution was concentrated by microcon (10,000 MVCO; Nihon Millipore Co.). The protein concentrations were determined using the BCA method, and the amount of preGc$_{1f1f}$MAF was 17 μg.

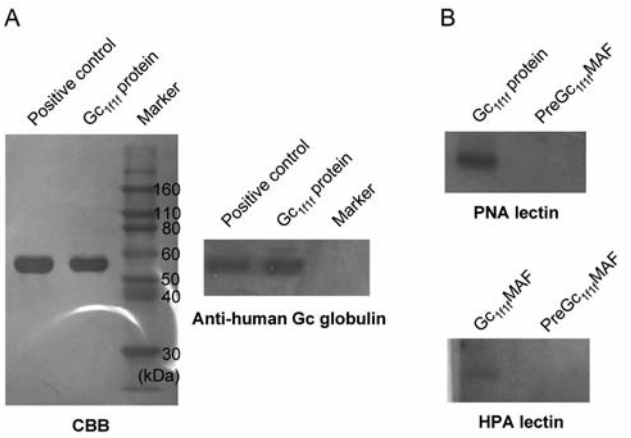

Figure 1. *SDS-PAGE and Western blot analysis of preGc$_{1f1f}$MAF. A: SDS-PAGE with CBB staining and a Western blot with anti-human Gc globulin of purified Gc$_{1f1f}$ protein. B: Western blot of preGc$_{1f1f}$MAF using PNA lectin (affinity for Gal-GalNAc disaccharide) and HPA lectin (affinity for GalNAc moiety).*

Isolation and culture of mouse peritoneal macrophages. Resident mouse peritoneal macrophages were collected from 8-week-old female Institute for Cancer Research (ICR) mice. After centrifugation at 1,000×rpm at 4˚C for 15 minutes, the collected macrophages were cultured in 24-well plates at a density of 5×10^5 cells/well in serum-free RPMI-1640 for 1 hour. The cultured cells were then washed 3 times with serum-free RPMI-1640 to separate adherent macrophages from non-adherent cells such as T- and B-cells. The macrophages were cultured in serum-free RPMI-1640 for 15 hours, and then the phagocytosis assay was performed as described below.

Phagocytosis assay. The macrophages were layered onto coverslips in a 24-well plate, and then treated with Gc$_{1f1f}$MAF, preGc$_{1f1f}$MAF, Gc$_{1f1f}$protein, LSP, peritoneal fluid, or peritoneal fluid treated preGc$_{1f1f}$MAF (pre-incubated with peritoneal fluid at 37˚C for 1 hour) for 3 hours. Sheep red blood cells (SRBCs) 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 on each macrophage-coated coverslip and were cultured for 1.5 hours. The non-internalized erythrocytes were lysed by immersing the coverslip in 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 phagocytic index, 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 in the differences between the results of the independent experiments was analyzed using Student's *t*-test. A *p*-value of <0.05 was considered to be significant.

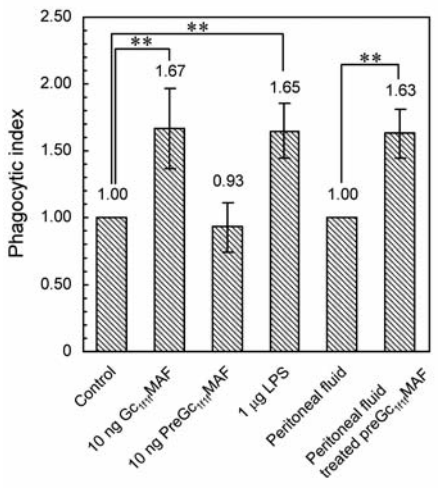

Figure 2. *Phagocytic activity of mouse peritoneal macrophages observed using preGc$_{1f1f}$MAF treated or not treated with mouse peritoneal fluid. All experiments were performed in triplicate. Each bar represents the mean (±SD). **p<0.01.*

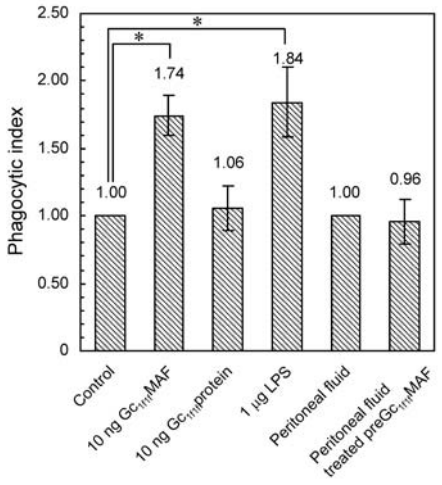

Figure 3. *Phagocytic activity of mouse peritoneal macrophages observed using the Gc$_{1f1f}$ protein treated or not treated with mouse peritoneal fluid. All experiments were performed in triplicate. Each bar represents the mean (±SD). *p<0.05.*

Results

Preparation and identification of preGc$_{1f1f}$MAF. We obtained 743 μg of Gc$_{1f1f}$ protein from 3.5 ml of healthy human serum using the 25(OH)D$_3$ affinity column. Figure 1A shows the SDS-PAGE gel and Western blot of the purified Gc$_{1f1f}$ protein. A single band was detected using Coomassie brilliant blue (CBB) stain and anti-human Gc globulin antibody stain. The purified Gc$_{1f1f}$ protein was treated with β-galactosidase and then checked with Western blot using PNA and HPA lectin in order to identify whether Gal was removed. We observed the disappearance of the PNA lectin-stained preGc$_{1f1f}$MAF band that had affinity for the Gal-GalNAc moiety (Figure 1B upper panel). We also observed the disappearance of the HPA lectin-stained preGc$_{1f1f}$MAF band that had affinity for the GalNAc-end (Figure 1B lower panel). These data indicated that preGc$_{1f1f}$MAF containing disaccharide resembles the SA-GalNAc structure.

Phagocytic activity of peritoneal fluid-treated preGc$_{1f1f}$MAF against mouse peritoneal macrophages. We examined phagocytic activation or macrophage activation of preGc$_{1f1f}$MAF and GcMAF against mouse peritoneal macrophages in order to evaluate their macrophage-activating effects. Figure 2 shows significant phagocytic activation caused by 10 ng of preGc$_{1f1f}$MAF as compared with the control on treatment with mouse peritoneal fluid. The phagocytic index (PI) of 10 ng of preGc$_{1f1f}$MAF (PI=1.63) treated with mouse peritoneal fluid corresponds to that of 10 ng Gc$_{1f1f}$MAF (PI=1.67) and 1 μg LPS (PI=1.65). Gc$_{1f1f}$ protein did not show significant phagocytic activation whether pretreated or not with mouse peritoneal fluid (Figure 3).

Discussion

In this paper, we described the preparation of preGc$_{1f1f}$MAF by the enzymatic conversion of the purified Gc$_{1f1f}$ protein and evaluated the macrophage activation effect of preGc$_{1f1f}$MAF. We were able to determine whether preGc$_{1f1f}$MAF harbors the SA-GalNAc disaccharide sugar moiety by carrying out Western blotting using PNA and HPA lectin. One advantage is that this method of preparing preGc$_{1f1f}$MAF can be used for all Gc subtypes. Yamamoto suggested that the sugar moiety of the Gc$_{1s1s}$ protein contains GalNAc, which is branched with Gal and mannose (MA), and that the Gc$_{22}$ protein is bound to GalNAc *via* Gal (19). Recently, Borges *et al*. and Ravnsborg *et al*. used mass spectrometry and proposed another sugar structure for Gc$_{1f1f}$ and Gc$_{1s1s}$ proteins, consisting of a linear SA-Gal-GalNAc trisaccharide (21, 22). We already reported that only the Gc$_{1f1f}$ protein shows phagocytic activation of mouse peritoneal macrophage through β-galactosidase and sialidase processing (7). In the present study, the band of the Gc$_{1f1f}$ protein observed on performing Western blot using PNA lectin disappeared after treatment with β-galactosidase; this suggested that the sugar end of the Gc$_{1f1f}$ protein was Gal and not SA. We propose that the Gc$_{1f1f}$ protein can be a potential precursor of or an *in vivo* generator of GcMAF after it is subjected to β-galactosidase processing; however, the actual and complete sugar structure of the Gc$_{1f1f}$ protein is yet to be elucidated. Our aim to convert the Gc$_{1f1f}$ protein to GcMAF *in vivo* only in the presence of β-galactosidase was successfully achieved as determined by demonstration of macrophage activation by preGc$_{1f1f}$MAF treated with mouse peritoneal fluid.

In conclusion, we demonstrated that preGc_{1f1f}MAF, as well as GcMAF, can be used as an effective and precursor- or generator-type macrophage activator *in vivo*.

Acknowledgements

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

References

1 Svasti J: Molecular basis for the three major forms of human serum vitamin D binding protein (group-specific component). Biochem *18*: 1611-1617, 1979.

2 Daiger SP: Group-specific component (Gc) proteins bind vitamin D and 25-hydroxyvitamin D. Proc Natl Acad Sci USA *72*: 2076-2080, 1975.

3 White P and Cooke N: The multifunctional properties and characteristics of vitamin D-binding protein. Trends Endocrinol Metab *11*: 320-327, 2000.

4 Yamamoto N and Homma S: Vitamin D_3 binding protein (group-specific component) is a precursor for the macrophage-activating signal factor from lysophosphatidylcholine-treated lymphocytes. Proc Natl Acad Sci USA *88*: 8539-8543, 1991.

5 Yamamoto N and Kumashiro R: Conversion of vitamin D_3 binding protein (group-specific component) to a macrophage-activating factor by the stepwise action of beta-galactosidase of B-cells and sialidase of T-cells. J Immunol *151*: 2794-2802, 1993.

6 Yamamoto N, Lindsay DD, Naraparaju VR, Ireland RA and Popoff SN: A defect in the inflammation-primed macrophage-activation cascade in osteopetrotic rats. J Immunol *15*: 5100-5107, 1994.

7 Nagasawa H, Sasaki H, Uto Y, Kubo S and Hori H: Association of the macrophage activating factor (MAF) precursor activity with polymorphism in vitamin D-binding protein. Anticancer Res *24*: 3361-3366, 2004.

8 Kanda S, Mochizuki Y, Miyata Y, Kanetake H and Yamamoto N: Effects of vitamin D_3-binding protein-derived macrophage activating factor (GcMAF) on angiogenesis. J Natl Cancer Inst *94*: 1311-1319, 2002.

9 Kisker O, Onizuka S, Becker CM, Fannon M, Flynn E, D'Amato R, Zetter B, Folkman J, Ray R, Swamy N and Pirie-Shepherd S: Vitamin D binding protein-macrophage-activating factor (DBP-*maf*) inhibits angiogenesis and tumor growth in mice. Neoplasia *5*: 32-40, 2003.

10 Koga Y, Naraparaju VR and Yamamoto N: Antitumor effect of vitamin D-binding protein-derived macrophage-activating factor on Ehrlich ascites tumor-bearing mice. Proc Soc Exp Biol Med *220*: 20-26, 1999.

11 Nonaka K, Onizuka S, Ishibashi H, Uto Y, Hori H, Nakayama T, Matsuura N, Kanematsu T and Fujioka H: Vitamin D binding protein-macrophage activating factor inhibits HCC in SCID mice. J Surg Res, 2010. [Epub ahead of print]

12 Yamamoto N, Suyama H, Nakazato H, Yamamoto N and Koga Y: Immunotherapy of metastatic colorectal cancer with vitamin D-binding protein-derived macrophage-activating factor, GcMAF. Cancer Immunol Immunother *57*: 1007-1016, 2008.

13 Yamamoto N, Suyama H, Yamamoto N and Ushijima N: Immunotherapy of metastatic breast cancer patients with vitamin D-binding protein-derived macrophage-activating factor (GcMAF). Int J Cancer *122*: 461-467, 2008.

14 Yamamoto N, Suyama H and Yamamoto N: Immunotherapy for prostate cancer with Gc protein-derived macrophage-activating factor, GcMAF. Transl Oncol *1*: 65-72, 2008.

15 Yamamoto N, Ushijima N and Koga Y: Immunotherapy of HIV-infected patients with Gc protein-derived macrophage-activating factor (GcMAF). J Med Virol *81*: 16-21, 2009.

16 Svasti J and Bowman BH: Human group-specific component. Changes in electrophoretic mobility resulting from vitamin D binding and from neuraminidase digestion. J Biol Chem *253*: 4188-4194, 1978.

17 Takaki T and Ujiie K: Gc typing using PhastGel® Dry IEF containing separators by PhastSystem™ . Res Pract Forens Med *36*: 59-62, 1993.

18 Kubo S, Kitamura O, Tsuda R, Hirose W, Mastumoto H and Nakano I: Hereditary recombined three-allele variant of the Gc system. Hum Genet *91*: 71-72, 1993.

19 Yamamoto N: Macrophage-activating factor from vitamin D-binding protein. US Patent 5,326,749, July 5, 1994.

20 Link RP, Perlman KL, Pierce EA, Schnoes HK and DeLuca HF: Purification of human serum vitamin D-binding protein by 25-hydroxyvitamin D_3-Sepharose chromatography. Anal Biochem *157*: 262-269, 1986.

21 Borges CR, Jarvis JW, Oran PE and Nelson RW: Population studies of vitamin D binding protein microheterogeneity by mass spectrometry lead to characterization of its genotype-dependent *O*-glycosylation patterns. J Proteome Res *7*: 4143-4153, 2008.

22 Ravnsborg T, Olsen DT, Thysen AH, Christiansen M, Houen G and Højrup P: The glycosylation and characterization of the candidate Gc macrophage-activating factor. Biochim Biophys Acta *1804*: 909-917, 2010.

Received April 7, 2011
Revised June 1, 2011
Accepted June 2, 2011