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

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Abstract. Background: The 1f1f subtype of the Gc protein (Gc1f1f 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 preGc1f1fMAF, the only Gc1f1f protein lacking galactose, can be converted to GcMAF in vivo because sialic acid is cleaved by residual sialidase. Hence, we investigated the effect of preGc1f1fMAF on the phagocytic activation of mouse peritoneal macrophages. Results: We examined the sugar moiety of preGc1f1fMAF with a Western blot using peanut agglutinin (PNA) and Helix pomatia agglutinin (HPA) lectin. We also found that preGc1f1fMAF 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: PreGc1f1fMAF can be used as an effective macrophage activator in vivo.

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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 (Gc1f1f 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 Gc1f1f-derived macrophage-activating factor precursor (preGc1f1fMAF), the Gc1f1f 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 preGc1f1fMAF 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 D3 (25(OH)D3) and other chemicals of biochemical grade were purchased from Wako Pure Chemical Industries Co. Ltd. (Osaka, Japan). 25-Hydroxyvitamin D3 (25(OH)D3) and other chemicals of biochemical grade were purchased from Wako Pure Chemical Industries Co. Ltd. (Osaka, Japan). 25-Hydroxyvitamin D3 (25(OH)D3) and other chemicals of biochemical grade were purchased from Wako Pure Chemical Industries Co. Ltd. (Osaka, Japan).

Preparation of human serum Gc1f1f protein. The Gc1f1f protein was prepared as reported by Link et al. (20). Briefly, healthy human serum (containing Gc1f1f, 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)D3-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)D3-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 MWCO; Nihon Millipore Co.). The protein concentrations were determined using the BCA method, and the amount of preGc1f1fMAF was 17 μg.

Western blotting. The Gc1f1f protein and preGc1f1fMAF 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 Gc1f1f 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 preGc1f1fMAF. 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 preGc1f1fMAF. The purified Gc1f1f protein (25 μg) was incubated with 250 μl 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)D3-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 MWCO; Nihon Millipore Co.). The protein concentrations were determined using the BCA method, and the amount of preGc1f1fMAF was 17 μg.

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 1,000xg rpm at 4°C for 15 minutes, the collected macrophages were cultured in 24-well plates at a density of 5x10^4 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 Gc1f1fMAF, preGc1f1fMAF, Gc1f1f protein, LSP, peritoneal fluid, or peritoneal fluid treated preGc1f1fMAF (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 washed with methanol, air-dried, and stained with Giemsa stain. The number of phagocytosed erythrocytes per cell was determined using a microscope under a ×200 magnification (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.
Results

Preparation and identification of preGc1f1fMAF. We obtained 743 μg of Gc1f1f protein from 3.5 ml of healthy human serum using the 25(OH)D3 affinity column. Figure 1A shows the SDS-PAGE gel and Western blot of the purified Gc1f1f protein. A single band was detected using Coomassie brilliant blue (CBB) stain and anti-human Gc globulin antibody stain. The purified Gc1f1f 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 preGc1f1fMAF band that had affinity for the Gal-GalNAc moiety (Figure 1B upper panel). We also observed the disappearance of the HPA lectin-stained preGc1f1fMAF band that had affinity for the GalNAc-end (Figure 1B lower panel). These data indicated that preGc1f1fMAF containing disaccharide resembles the SA-GalNAc structure.

Phagocytic activity of peritoneal fluid-treated preGc1f1fMAF against mouse peritoneal macrophages. We examined phagocytic activation or macrophage activation of preGc1f1fMAF 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 preGc1f1fMAF as compared with the control on treatment with mouse peritoneal fluid. The phagocytic index (PI) of 10 ng of preGc1f1fMAF (PI=1.63) treated with mouse peritoneal fluid corresponds to that of 10 ng Gc1f1fMAF (PI=1.67) and 1 μg LPS (PI=1.65). Gc1f1f 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 preGc1f1fMAF by the enzymatic conversion of the purified Gc1f1f protein and evaluated the macrophage activation effect of preGc1f1fMAF. We were able to determine whether preGc1f1fMAF 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 preGc1f1fMAF can be used for all Gc subtypes. Yamamoto suggested that the sugar moiety of the Gc1s1s protein contains GalNAc, which is branched with Gal and mannose (MA), and that the Gc22 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 Gc1f1f and Gc1s1s proteins, consisting of a linear SA-Gal-GalNAc trisaccharide (21, 22). We already reported that only the Gc1f1f protein shows phagocytic activation of mouse peritoneal macrophage through β-galactosidase and sialidase processing (7). In the present study, the band of the Gc1f1f protein observed on performing Western blot using PNA lectin disappeared after treatment with β-galactosidase; this suggested that the sugar end of the Gc1f1f protein was Gal and not SA. We propose that the Gc1f1f 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 Gc1f1f protein is yet to be elucidated. Our aim to convert the Gc1f1f protein to GcMAF in vivo only in the presence of β-galactosidase was successfully achieved as determined by demonstration of macrophage activation by preGc1f1fMAF treated with mouse peritoneal fluid.
In conclusion, we demonstrated that preGcMAF, as well as GcMAF, can be used as an effective and precursor- or generator-type macrophage activator in vivo.

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References


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