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Gc Protein-derived Macrophage Activating Factor (GcMAF): Isoelectric Focusing Pattern and Tumoricidal Activity

SAHARUDDIN BIN MOHAMAD[1], HIDEKO NAGASAWA[1], HIDEYUKI SASAKI[1], YOSHIHIRO UTO[1], YOSHINORI NAKAGAWA[2], KEN KAWASHIMA[3] and HITOSHI HORI[1]

[1]*Department of Biological Science and Technology, Faculty of Engineering, The University of Tokushima, Minamijyosanjimacho-2, Tokushima, 770-8506;*
[2]*Hayashibara Biochemical Laboratory, Kanko-Shikiso Institute Inc. Okayama, 700-0907;*
[3]*Meneki Bunseki Kenkyu Center Co. (MBK), Tsuyama, Okayama, 708-0806 Japan*

Abstract. *Background: Gc protein is the precursor for Gc protein-derived macrophage activating factor (GcMAF), with three phenotypes: Gc1f, Gc1s and Gc2, based on its elecrophoretic mobility. The difference in elecrophoretic mobility is because of the difference in its posttranslational sugar moiety composition. Materials and Methods: We compared the difference between Gc protein and GcMAF elecrophoretic mobility using the isoelectric focusing (IEF) method. The tumoricidal activity of GcMAF-treated macrophage was evaluated after coculture with L-929 cell. The tumoricidal mechanism was investigated using TNF bioassay and nitric oxide (NO) release. Results: The difference in Gc protein and GcMAF elecrophoretic mobility was detected. The tumoricidal activity of GcMAF-treated macrophage was detected, but no release of TNF and NO was detected. Conclusion: The difference of isoelectric focusing mobility in Gc protein and GcMAF would be useful to develop a GcMAF detection method. GcMAF increased macrophage tumoricidal activity but TNF and NO release were not involved in the mechanism.*

Microbial infection and cancerous tissue cause inflammation that induces chemotaxis and activation of macrophages, which finally leads to immune development (1,2). Thus, macrophages are essential for host defense and play an important role in orchestrating the immune response of the host against threat signals.

Correspondence to: Hitoshi Hori, Department of Biological Science and Technology, Faculty of Engineering, The University of Tokushima, Minamijyosajimacho-2, Tokushima, 770-8506 Japan. Tel: +81-88-656-7514, Fax: +81-88-656-9164, e-mail: hori@bio.tokushima-u.ac.jp

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Gc protein has been reported to be the precursor for a potent macrophage activating factor (GcMAF; Gc protein-derived macrophage activating factor) (3). Gc protein is a highly polymorphic serum glycoprotein predominantly synthesized in the liver as a single-chain glycoprotein with molecular weight of approximately 58 kDa (4). Lysophosphatidylcholine released from inflammation tissue induces beta-galactosidase in B cells and sialidase in T cells, and converts Gc protein into GcMAF through a stepwise pathway (3, 5).

Yamamoto *et al.* reported the possibility of using GcMAF as an immunomodulator for cancer treatment (6). For its pharmacokinetic analysis, it is important to provide an assay system specific for GcMAF in human serum. Gc protein is known with three major polymorphisms (Gc1f, Gc1s and Gc2), with the difference being in their sugar moiety composition (7), as shown by isoelectric focusing (IEF) mobility (8-10). Gc protein and GcMAF only differ in their sugar moiety composition as shown in Figure 1. We have reported a method to analyze GcMAF using *Helix pomatia* lectin blotting. The differences in Gc protein and GcMAF sugar moiety composition would cause the difference in their isoeletric focusing mobility. We tried the isoelectric focusing method as another candidate in developing an analysis method for GcMAF. We also evaluated the tumoricidal activity of GcMAF-treated macrophages and investigated the mechanism of action using TNF and NO assay.

Materials and Methods

Materials. Female ICR mice (7 to 8 weeks old) were purchased from Japan SLC, Inc.. Other chemicals (biochemical grade) were purchased from the Wako Pure Chemical Industries Co., Japan.

Serum samples. Blood samples from 15 healthy human (age: 22 ~ 88 years old) were taken and centrifuged 2,500 x g, 4°C for 10 minutes, and serum samples were collected and stored at -30°C until used.

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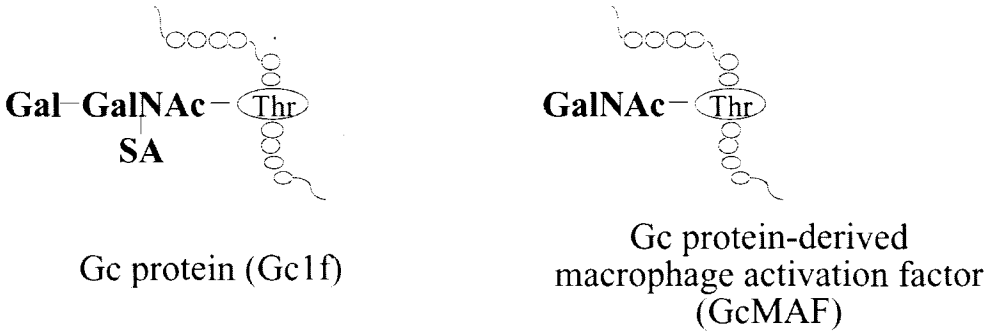


Figure 1. *Sugar moiety composition of Gc protein (1f) and GcMAF. Gal: beta-galactose, GalNAc: alpha-N-acetylgalactosamine, SA: sialic acid.*

Macrophage culture. Resident mouse peritoneal macrophages (female ICR mice, 7 weeks of age) were collected and, after centrifugation at 1,000 x g, 4°C for 10 minutes, the collected macrophages were cultured in RPMI 1640 medium with the cell number as indicated in each biological assay system. J774.1 mouse macrophage-like cell line, NR8383 mouse alveolar macrophage cell line and L-929 mouse fibroblast cell line were maintained in 10% heat-inactivated fetal bovine serum (FBS)-RPMI 1640, 15% FBS-HAM F-12K and 10% FBS- EMEM medium.

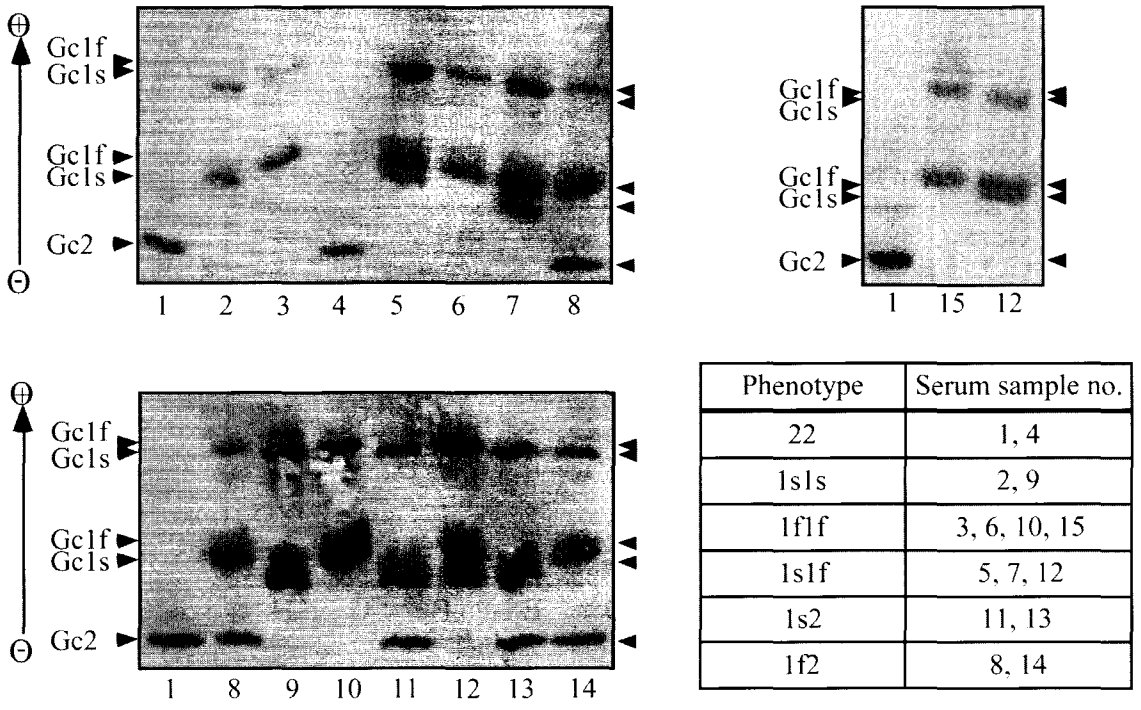
GcMAF preparation. (1) Isoelectric focusing. Serum sample number 6 was diluted 10 times with 100 mM, pH 5.0 sodium phosphate buffer and treated with 0.05 Unit beta-galactosidase and sialidase for 1 hour. The reaction was stopped by means of an ice bath and the sample applied to PD-10 column to desalt it. The sample was then stored at 4°C until used. (2) *Biological activity*: GcMAF was prepared as reported by Mohamad *et al.* (11). Briefly, 400 μg of Gc protein purified from human serum was 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 using 1M NaH_2PO_4. The supernatant in 100 mM sodium phosphate buffer, pH 6.0 was incubated with immobilized sialidase (1 Unit) at 37°C by rotation movement for 1 hour. The immobilized enzyme was removed and the supernatant was made sterile by filtration and the protein concentration was determined using the BCA method.

Isoelectric focusing method. Gc typing analysis was done using PhastSystem polyacrylamide gel isoelectric focusing (IEF) electrophoresis. PhastGel Dry IEF (5% T, 3% C) was re-swelled in 2.5% Pharmalyte 4.5-5.4, 1.68% (w/w) MOPS [3-(*N*-morpholino)propanesulfonic acid], 1.12% (w/w) HEPES [N-(2-hydroxyethyl) piperazine-*N'*-2-ethanesulfonic acid] for 2 hours. One μl of sample (diluted serum sample or GcMAF) was applied to the sample applicator and electrophoresis was performed as reported by Takaki and Ujie (9). Detection was done using Western blot with anti-human Gc globulin and horseradish peroxidase-labeled anti-rabbit IgG.

Tumoricidal assay. The tumoricidal activity of the macrophages was assayed by measuring lactate dehydrogenase (LDH) released from the target cells using a cytotoxicity assay kit (TAKARA BIO Inc., Ostu, Japan). Macrophages (2.1 x 10^4 cells/well) were plated in a 96-well plate. After a 1-hour culture to allow macrophage to adhere to the well, the macrophages were treated as indicated for 3 hours. Then the macrophages were cocultured with L-929 (target) cells at an E:T ratio of 40:1. After 4 hours of culture, the supernatants were evaluated for LDH activity released by the damaged cells and the results expressed as percent cytotoxicity. Macrophage incubated without target cells, target cells lysed with Triton X-100 and cell-free assay medium served as controls for calculating the percent cytotoxicity. All experiments were performed in triplicate.

TNF bioassay. The TNF bioactivity of the cell-free culture media of macrophages was determined by measuring the death of actinomycin D-primed, TNF-sensitive L-929 cells induced by the culture media (12). One x 10^5 cells/well macrophages were plated in a 96-well plate with culture condition as mentioned in the Results section. Macrophages were treated for 3 hours and the conditioned medium was collected and diluted to a range of concentration for the cytotoxicity assay. Eight x 10^4 L-929 cells were plated in another 96-well plate and allowed to adhere for 6 hours. L-929 cells were added to a range of concentration of recombinant mouse TNF-alpha or conditioned medium of macrophages and incubated for 18 hours. After staining with 0.5% crystal violet, colorimetric intensity at 520 nm was measured. The cell viability of L-929 cells was calculated and the TNF bioactivity of each sample was based on the cell viability of L-929 cells towards 24 Unit/ml recombinant mouse TNF-alpha.

NO assay. The amount of NO accumulated in the culture supernatant of the J774.1 cell line cultured in (10% FBS) RPMI1640 medium after a 24-hour treatment was determined by a colorimetric assay using Griess reagent (13). Cell-free supernatant was incubated with an equal volume of Griess reagent (1% sulfanilamide, 0.1% *N*-naphthylethylenediamine dichloride and 2.5% orthophosphoric acid) at room temperature for 10 minutes. OD was measured at 550 nm. The concentration of NO released was determined from the standard curve made with varying concentrations of sodium nitrate (0.01-1 mM).



Phenotype	Serum sample no.
22	1, 4
1s1s	2, 9
1f1f	3, 6, 10, 15
1s1f	5, 7, 12
1s2	11, 13
1f2	8, 14

Figure 2. *Electrophoretic mobility of Gc protein in human serum samples using isoelectric focusing (IEF) method.*

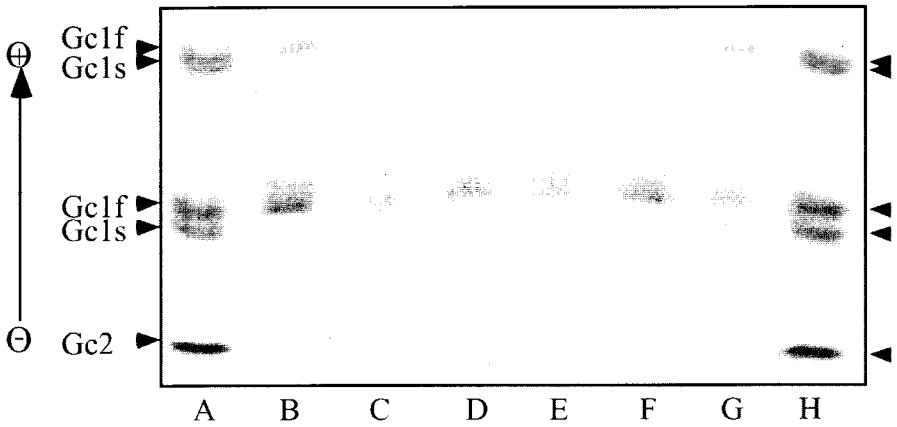
Results

Isoelectric focusing. The results of human serum Gc typing by IEF is shown in Figure 2. Gc1f1f, where the sugar moiety is thought to be *N*-acetylgalactosamine (GalNAc) branched with galactose (Gal) and sialic acid (SA) as shown in Figure 1 (14), is detected in sample nos. 3, 6, 10 and 15. In order to detect any difference in IEF mobility pattern of GcMAF and Gc protein, we treated Gc1f1f type human serum (sample no. 6) with glycosidases as shown in Figure 3a. The results showed that, after beta-galactosidase and sialidase co-treatment, the upper band (close to anode) of Gc protein disappears and probably a new band was detected at the lower (close to cathode) band. Serum treated with beta-galactosidase did not cause any changes in the mobility of Gc, but sialidase treatment caused the same changes that were observed with beta-galactosidase and sialidase co-treatment. Co-treatment of beta-galactosidase, sialidase and alpha-NaGalase, which is thought to hydrolyse the entire sugar moiety also, gave the same observation.

Serum sample no. 15 was used in the purification of Gc protein and GcMAF preparation by immobilized-glycosidases, as we previously reported (11). Figure 3b shows the comparison of the IEF pattern of Gc protein and GcMAF. Gc1f band was detected in both Gc protein and GcMAF, but no difference was detected between them.

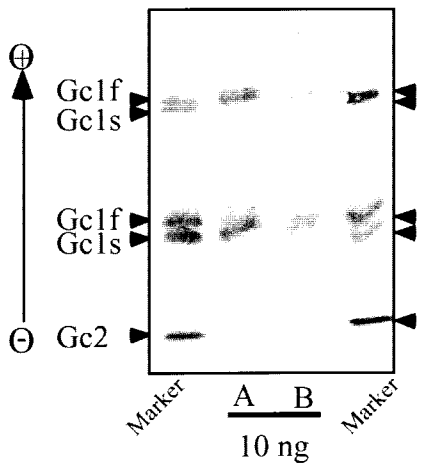
Tumoricidal activity of GcMAF-treated macrophage and its mechanism. The results of coculture of GcMAF-treated mouse peritoneal macrophages with L-929 cells show that GcMAF increases the cytotoxicity activity of mouse peritoneal macrophages more than the non-treatment control (Figure 4). Then, we examined which factor(s) would cause the cytotoxicity of macrophage after GcMAF treatment. First, we evaluated the biological activity of TNF released from macrophages by assaying the cytotoxicity of macrophages conditioned medium in actinomycin D-primed, TNF-sensitive L-929 cells (Figure 5a). The results of mouse peritoneal macrophages and NR8383 cells show that GcMAF treatment did not induce TNF secretion in both cells. LPS, which was used as a positive control for the experiment, was found not to release TNF in mouse peritoneal macrophages. In order to avoid any effect of serum factor in the assay system, FBS-free medium was used in mouse peritoneal macrophages culture condition. The lack of LPS-binding protein (LBP) in the culture media and a short period of treatment would be the factor that causes LPS not to be able to induce a satisfactory activation in macrophage to release TNF. In NR8383 cells, 5% FBS medium was used in the culture condition and a high release of TNF was detected, but GcMAF was found not to release TNF in NR8383 cells. Another macrophage cytotoxicity factor, NO, was assayed

(a). Human serum treated with soluble glycosidase.



A: Marker (Gc2 + Gc1f1s)
B: Serum (Gc1f1f); sample no. 6
C: 0.05 U beta-galactosidase*
D: 0.05 U sialidase*
E: 0.05 U beta-galactosidase* + 0.05 U sialidase*
F: 0.05 U beta-galactosidase* + 0.05 U sialidase* + 0.05 alpha-NaGalase**
G: Serum
H: Marker (Gc2 + Gc1f1s)
* Soluble glycosidase
** alpha-NaGalase from chicken liver

(b). GcMAF prepared by immobilized-glycosidases.



Marker: Gc2 + Gc1f1s
A: Gc protein (purified from Gc1f1f serum)
B: GcMAF (immobilized glycosidases)

Figure 3. *Electrophoretic mobility of Gc protein and GcMAF. (a). Samples were prepared by treatment of human serum with soluble glycosidase. (b). GcMAF was prepared by treatment of purified Gc protein with immobilized-glycosidases.*

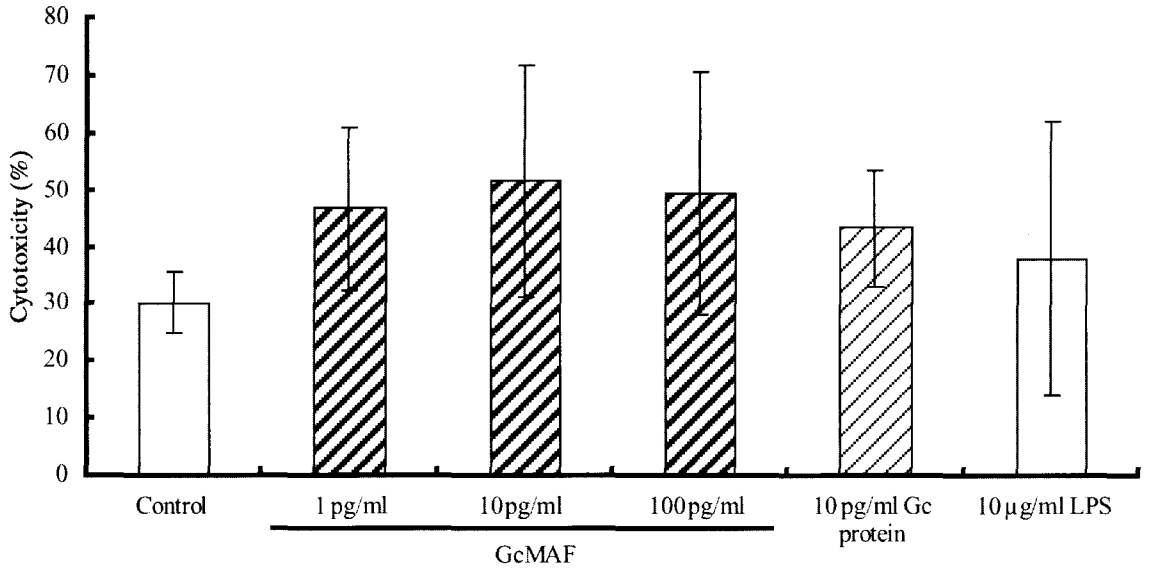


Figure 4. *Tumoricidal activity of GcMAF-treated mouse peritoneal macrophage. All data are shown as the mean±standard deviation of n=2, triplicate experiments.*

in the J774.1 mouse macrophage cell line. After 24 hours treatment in 10% FBS medium culture condition, LPS (positive control) was detected to release NO in J774.1 cells, but no release of NO was detected in GcMAF treatment (Figure 5b).

Discussion

The difference in sugar moiety composition in Gc protein was reported to cause the difference in its IEF mobility (8). Gc protein and GcMAF are different only in their sugar moiety composition, so we expected that they would show a difference in IEF mobility. The results of Gc1f1f treated with glycosidases showed that the difference in IEF mobility occurred after sialidase treatment. The observation indicates that only the sialic acid moiety is responsible for the difference in IEF mobility and that other sugar moieties may not directly influence IEF mobility. The comparison of Gc protein and GcMAF (Figure 3b), however, showed no difference in their IEF mobility. It is thought that the glycosylation activity was not enough for 100% preparation of GcMAF. However, both the lectin blot and the biological activity studies supported the generation of GcMAF prepared using the method of immobilized-enzymes (11, 15). Yet, the percentage of GcMAF formation is still unknown because of the lack of a standard method to quantitatively analyze GcMAF and the detection method is still under study. We are testing other methods to increase the efficiency of GcMAF production.

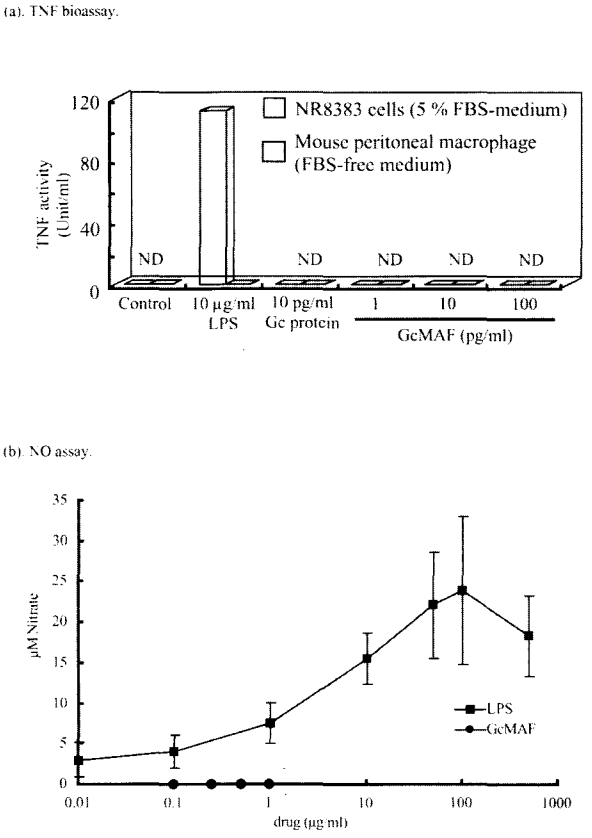


Figure 5. *Mechanism of tumoricidal activity in GcMAF-treated mouse peritoneal macrophage. (a). TNF bioassay by measuring the TNF-sensitive L-929 cell killing activity. All data are shown as the mean±standard deviation of triplicate experiments. (b). NO release measured by Griess reagent. All data are shown as the mean±standard deviation of n=3.*

Three-hour treatment of GcMAF increased the cytotoxicity of macrophages, but no release of TNF and NO were detected. So, we examined factors which increase the cytotoxicity of GcMAF-treated macrophages in the *in vitro* culture. Superoxide (O_2^-), NO and TNF constitute the major cytotoxic effector molecules in macrophage-mediated killing of microbes and tumor cells (16). Previously, we have reported the increase of O_2^- generation activity in GcMAF-treated resident mouse peritoneal macrophage (11, 15). Probably the cytotoxicity of GcMAF-treated macrophages is exerted by reactive oxygen species (ROS). ROS are formed after activation of a membrane-associated reduced nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase. The initial product is the O_2^-, from which other cytotoxic oxygen species, including hydrogen peroxide, hydroxyl radical and hypochlorous acid, are derived. *In vitro* experiments have shown that the lysis of different tumor cells by monocytes was dependent on the production of O_2^- and the hydroxyl radical; this proves that monocytes or macrophages perform their cytotoxicity *via* ROS (17).

Another macrophage activation property that GcMAF enhanced was the phagocytosis activity (11, 15). GcMAF has been reported to increase Fc receptor-mediated phagocytosis in murine peritoneal macrophages by inducing translocation of FcγRI and FcγRII from the intracellular compartment to the cell surface of macrophage (18). FcγRI on monocytes, polymorphonuclear leukocytes (PMN) and macrophages has been reported to be a potent trigger molecule for ADCC (Ab-dependent cellular cytotoxicity) and phagocytosis of tumor cells. So, we suggest that ROS and phagocytosis would be the mediator for GcMAF-mediated macrophage tumoricidal activity instead of TNF and reactive nitrogen species.

GcMAF was reported to be involved in inflammation-primed macrophage activation, which finally leads to immune development. Swamy *et al.* reported data to support the essential role of the GalNAc sugar moiety of GcMAF in the macrophage activation cascade using baculovirus-expressed form of Gc protein, which is glycosylated (19). We also reported supporting evidence for the role of GalNAc in GcMAF-mediated macrophage activation, where tumor-derived alpha-*N*-acetylgalactosaminidase was found to reduce GcMAF bioactivity (20). Vitamin D_3 derivatives are known as macrophage activators. The GcMAF precursor, Gc protein, was purified by the use of 25-hydroxyvitamin D_3 [25(OH)D_3] affinity chromatography (which specifically binds vitamin D binding sites of the protein), which should eliminate any of the vitamin D_3 derivatives that contaminate the GcMAF preparation. Furthermore, the endogenous ligand of Gc protein 25(OH)D_3 does not influence the activity of GcMAF to activate macrophage (19). Recently, GcMAF has been reported to have antiangiogenics activity by a direct effect on endothelial cells (21).

GcMAF has shown to be an excellent candidate as immunomodulator for cancer treatment and, for this reason, it is important to provide an assay system to prepare and detect GcMAF. We were able to detect the difference between Gc protein and GcMAF using the IEF method. Our studies showed that, other than lectin blotting by *Helix pomatia* agglutinin that we have reported previously, the IEF method is another candidate for the development of a GcMAF detection method.

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