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CHARACTERIZATION OF HUMAN Gc PROTEIN-DERIVED MACROPHAGE ACTIVATION FACTOR (GcMAF) AND ITS FUNCTIONAL ROLE IN MACROPHAGE TUMORICIDAL ACTIVITY

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1. INTRODUCTION

Macrophages are essential for host defense and play an important role in orchestrating immune response of the host against threat signals. Macrophages are also known to have a critical role in antitumor immunity,[1] can infiltrate into tumor, and are found in most tumor sites.[2,3] Meanwhile, Gc protein (also known as vitamin D_3-binding protein) is a serum protein with multifunctional properties[4] and has been reported as a precursor for macrophage activation factor.[5] Gc protein can be converted by an inducible ß-galactosidase of B cells and neuraminidase of T cells to a potent macrophage activating factor (GcMAF), a protein with *N*-acetylgalactosamine (GalNAc) as the remaining sugar moiety.[6,7] Activated macrophages express tumoricidal activity by ingestion of tumor cells and release of reactive oxygen species (ROS) and reactive nitrogen species (RNS), or both.[5] We reported that *in situ* modification of Gc protein with ß-galactosidase and neuraminidase increased the release of superoxide in thioglycolate-elucidated mouse peritoneal macrophage.[8] Yamamoto et al. reported the possibility of using GcMAF as an immunomodulator for cancer treatment,[9] so it is important to provide an assay for GcMAF. Kanan et al. reported the quantitative analysis of GcMAF from human serum.[10] However, the sugar moiety of GcMAF has never been qualitatively studied. Here we present the study of qualitative analysis of GcMAF from purified human serum as well as its influence on the macrophage activity.

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2. MATERIALS AND METHODS

2.1. Materials

25-OH-Vitamin D_3 was a gift from Dr. Nobuto Yamamoto from Socrates Institute for Therapeutic Immunology and Albert Einstein Cancer Center, Philadelphia, USA. Other chemicals (biochemical grade) were purchased from Wako Pure Chemical Industries Co., Japan.

2.2. Purification of human serum Gc protein

The procedure was adapted from methods reported by Link et al.[11]

Human serum was diluted 1:1 with column buffer and applied to the 25-OH-D_3-Sepharose column at a rate of 0.4 mL/min. Absorbance at 280 nm was used to monitor protein elution from the column. After the sample was applied, the column was washed with 300 mL of column buffer at 2 mL/min. The protein remaining on the matrix was eluted with 6 M guanidine-HCl at 1 mL/min and 1 mL/fraction was collected. Fractions with protein peak of the guanidine eluted fraction were pooled and dialyzed with 10 mM sodium phosphate, pH 7.0 for the hydroxyappatite chromatography.

2.2.2. Hydroxyappatite chromatography

A 5-mL hydroxyappatite column (Econo-Pac HTP Cartridge 1, Bio-Rad) was equilibrated in 10 mM sodium phosphate, pH 7.0. The dialyzed sample from 25-OH-D_3-Sepharose chromatography was applied to the column (0.5 mL/min) and the column was washed with 50 mL 10 mM sodium phosphate, pH 7.0 (2 mL/min). A linear gradient from 10 mM sodium phosphate to 200 mM sodium phosphate, pH 7.0 was applied to the column. Fractions with protein peak were collected and the protein concentration was determined using the BCA method. Collected fractions were stored at -80 °C.

2.3. GcMAF preparation

Purified Gc protein (100 μg) was incubated with immobilized ß-galactosidase (1 Unit, in 0.5 mL 100 mM sodium phosphate buffer, pH 7.0) in a microcentrifuge tube at 37 °C by rotation movement for 1 hr. The immobilized enzyme was removed by centrifugation and pH of the supernatant was adjusted to pH 6.0 using 1M NaH_2PO_4. The supernatant (1.5~2.0 mL in 100 mM sodium phosphate buffer, pH 6.0) was incubated with immobilized neuraminidase (0.5 Unit) in a microcentrifuge tube at 37 °C by rotation movement for 1 hr. The immobilized enzyme was removed by centrifugation and the supernatant was made sterile by filtration and protein concentration was determined using the BCA method and then stored at -80 °C.

2.4. Isolation and culture of mouse peritoneal macrophages

Resident mouse peritoneal macrophages (female ICR mice, 7 weeks of age) were collected and after centrifugation at 1000 G at 4 °C for 10 min, the collected macrophages were cultured in 6-well plates with a concentration of 1~2 x 10^6 cells/ well in RPMI 1640 (serum free). Cultured cells were then washed 3 times with Hank's solution to separate adherent macrophages from non-adherent cells (T and B cells). The cultured macrophages were then treated as indicated for 3 hr, and superoxide generation assay was done as described below.

2.5. Superoxide generation assay

The method was modified from that reported by Johnston et al.[12] Briefly, after drug treatment for the times indicated, the plates were washed twice with PBS(-) and once with Krebs-Ringer phosphate buffer, and 1.5 mL of 50 μM cytochrome *c* in Krebs-Ringer phosphate buffer was added to the plates. Phorbol myristate acetate (PMA) was added to the final concentration 5 μg/mL in each well and cultured for 90 min at 37 °C/5% CO_2 in a humidified incubator. The reaction was stopped by means of an ice-bath, and the cultured medium was placed in an Eppendorf tube, and promptly cleared by centrifugation at 8000 G. The optical density of the supernatant was determined spectrometrically at 550 nm with reference at 540 nm (U-2000, HITACHI) using mixtures from plates without cells as blanks. The concentration of reduced cytochrome *c* was determined using the equation $\Delta E_{550\,nm} = 2.1 \times 10^4\ M^{-1}\ cm^{-1}$.

2.6. Phagocytosis assay

For phagocytosis assay mouse peritoneal cells were layered onto coverslips in a 24-well plate and incubated for 30 min to allow macrophage adherence. GcMAF was added to the culture to the final concentration of 10 pg/mL GcMAF and cultured for 3 hr and then assayed for phagocytic activity. Sheep red blood cells (SRBC) were opsonized by rabbit haemolytic serum (anti-sheep red blood C12HSB cells) purchased from Serotec Ltd. 0.5% opsonized-SRBC in RPMI 1640 (serum free) was overlaid on each macrophage-coated (monolayer) coverslip and cultured at 37 °C in a humidified 5% CO_2 incubator for 90 min. Noninternalized erythrocytes were lysed by immersing the coverslips in a hypotonic solution (1/5 PBS) for 5 to 10 sec. The macrophages were fixed with methanol, air dried and stained with Giemza stain. The morphological photograph of phagocytotic macrophage was taken using a phase contrast microscope (x 400).

2.7. Western blot and Lectin blot

0.1 and 0.2 μg protein for western blot and 1 μg protein for lectin blot were subjected to SDS-PAGE under reducing conditions followed by electroblotting onto a PVDF membrane. Nonspecific binding was blocked by incubation in Tris-buffer phosphate containing 0.1% Tween 20 and 3% BSA for overnight at 4 °C. For western blot, the blots were probed with anti-human Gc globulin (Code No. A 0021, DAKO) and after extensive washing, the blots were incubated with the secondary Ab (HRP-labeled

anti-rabbit IgG). For lectin blot, the blots were incubated with HRP-labeled *Helix pomatia* lectin (Sigma-Aldrich Japan Co). The blots were developed using ECL Western blotting detection system (Amersham).

3. RESULTS AND DISCUSSION

Human serum was applied to 25-OH-D_3-Sepharose affinity and hydroxyappatite chromatography as described under Materials and Methods. Gc protein fraction was pooled and 0.1 and 0.2 μg of Gc protein was subjected to western blot. Fig. 1a shows the

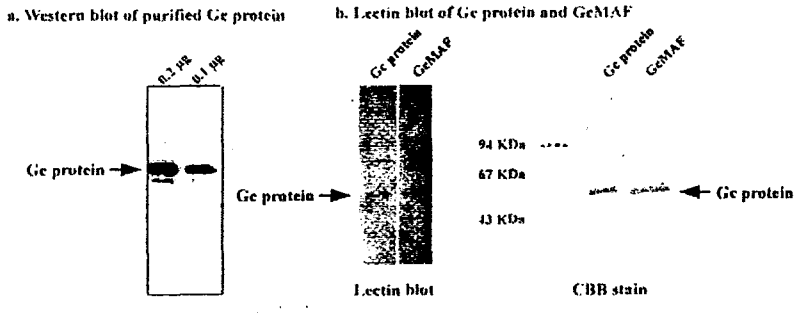

Fig. 1. Western blot and lectin blot analysis of Gc protein and Gc MAF. (1 μg protein was applied in each lane for lectin blot).

high degree of purity of Gc protein that was achieved after the two-step chromatography procedure. Lectins are good tools for exploring the interaction of carbohydrate because each lectin recognizes specific carbohydrates. *Helix pomatia* agglutinin, a lectin possesses particularly high specificity for terminal *N*-acetylgalactosaminyl residue.[13] 1 μg of purified Gc protein and prepared GcMAF was subjected to lectin blot (Fig. 1b). CBB stain of both Gc protein and GcMAF shows the detected band was about 52 KDa. Lectin blot analysis of both Gc protein and GcMAF gave a band at the same position as shown in CBB stain. As *Helix pomatia* lectin would be expected to specifically bind to terminal *N*-acetylgalactosaminyl residue, it would be possible that the GcMAF formation was not successful, or the band in Gc protein was an unspecific bound of lectin with the protein. We evaluated the influence of prepared GcMAF in macrophage activity using superoxide generation assay as described under Materials and Methods. Resident mouse peritoneal macrophages show higher superoxide generation activity at 10 ng/mL GcMAF treatment compared to the control (Fig.2). Increasing the GcMAF treatment was found to be inversely proportional to the macrophage activity. We also checked the phagocytosis activity of macrophages after 3 hr treatment of 10 pg/mL GcMAF (Fig.2). Fig. 2 shows the morphology of opsonized-SRBC phagocyted macrophages. 10 pg/mL of GcMAF enhanced the phagocytic activity of mouse peritoneal macrophage. Therefore, we consider that GcMAF was successfully prepared using the immobilized enzymes. The reason why an unexpected band was detected in Gc protein, is probably because of the

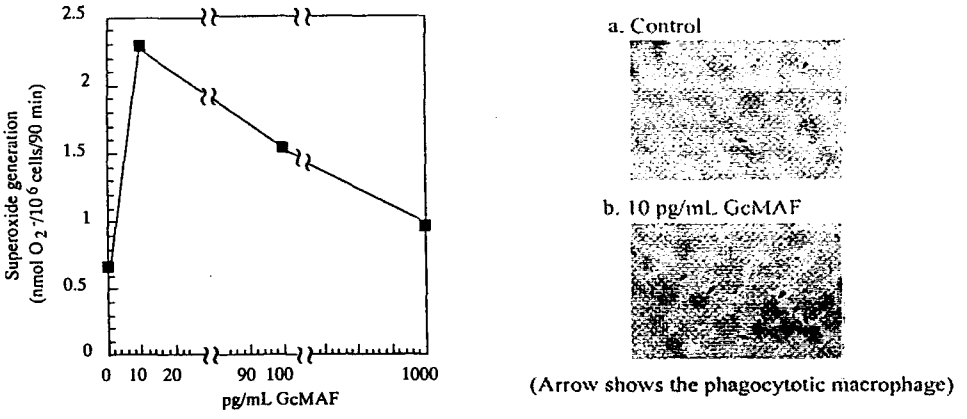

Fig.2. Superoxide generation assay of mouse peritoneal macrophage and morphology of opsonized-SRBC phagocytosed macrophage (Arrow shows phagocytotic macrophage).

unspecific bound of Gc protein to *Helix pomatia* lectin at the volume applied to the SDS-PAGE. We are revising the experimental methods of lectin blot to improve the quality of the results. GcMAF was reported to be involved in inflammation-primed macrophage activation, which finally leads to immune development. A lack of vitamin D_3-binding protein derived macrophage activation factor (DBP-MAF) was suggested to be involved in osteoperotic mutations because, DBP-MAF mediates bone resorption by activating osteoclast, which are responsible for bone resorption.[14] However, Odgren et al. reported that DBP-MAF production is not a universal characteristic of osteopetrotic mutations.[15] Swamy et al. reported data to support the essential role of GalNAc sugar moiety of GcMAF in Macrophage activation cascade using baculovirus expressed form of Gc protein, which is Glycosylated.[16] Furthermore, Swamy et al. also reported that the endogenous ligand of Gc protein (25-OH vitamin D_3) does not influence the activity of GcMAF to activate macrophage.[16] Because GcMAF has shown to be an excellent candidate immunomodulator for cancer treatment,[9] it is important to provide an assay system to characterize GcMAF formation. Here we provided important data and views to characterize GcMAF.

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