

Degalactosylated/Desialylated Bovine Colostrum Induces Macrophage Phagocytic Activity Independently of Inflammatory Cytokine Production

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Abstract. *Background/Aim:* Colostrum contains antibodies, such as immunoglobulin G (IgG), immunoglobulin A (IgA) and immunoglobulin M (IgM), and, therefore, has potent immunomodulating activity. In particular, IgA has an O-linked sugar chain similar to that in the group-specific component (Gc) protein, a precursor of the Gc protein-derived macrophage-activating factor (GcMAF). In the present study, we investigated the macrophage-activating effects of degalactosylated/desialylated bovine colostrum. *Results:* We detected the positive band in degalactosylated/desialylated bovine colostrum by western blotting using *Helix pomatia* agglutinin lectin. We also found that degalactosylated/desialylated bovine colostrum could significantly enhance the phagocytic activity of mouse peritoneal macrophages *in vitro* and of intestinal macrophages *in vivo*. Besides, degalactosylated/desialylated bovine colostrum did not mediate the production of inflammatory cytokines such as tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β). *Conclusion:* Similar to the use of GcMAF, degalactosylated/desialylated bovine colostrum can be used as a potential macrophage activator for various immunotherapies.

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Colostrum is a type of milk produced by the mammary glands of mammals just prior to giving birth. It contains serum proteins and antibodies, such as albumin, insulin-like growth factor (IGF), epidermal growth factor (EGF), nerve growth factor (NGF), lactoferrin, immunoglobulin G (IgG), immunoglobulin A (IgA) and immunoglobulin M (IgM) to protect the newborn against various infectious diseases; however, it contains lower amounts of carbohydrates and lipids than does mature milk (1). IgA, in particular, is known to protect against various kinds of infections and interact with the Fc receptor called Fc α RI (or CD89) to initiate inflammatory reactions (2). In addition, IgA has an O-linked sugar chain and the binding property for the Fc receptor decreases if there are many sialic acid residues (3). It has been reported that the number of GalNAc moieties attached to IgA O-linked glycans was significantly decreased in patients with Crohn's disease and strongly correlated with clinical activity (4).

The group-specific component (Gc) protein, also known as vitamin D-binding protein (DBP), has an O-linked sugar chain. Inflammation results in the hydrolysis of the terminal galactose and sialic acid of the Gc protein to produce Gc protein-derived macrophage-activating factor (GcMAF) with an N-acetylgalactosamine (GalNAc) moiety (5). GcMAF has been shown to possess several biological activities, such as macrophage activation, anti-angiogenic activity and antitumor activity (6-10). Furthermore, GcMAF-containing human serum demonstrates macrophage phagocytic activation, antitumor activity and remarkable clinical effects in cancer patients (11, 12).

We hypothesized that colostrum could be a macrophage-activator if enzymatically modified IgA and Gc protein had activity similar to that of GcMAF. Therefore, we propose a novel macrophage-activator function for degalactosylated/desialylated bovine colostrum and report the potential role of this modified bovine colostrum in stimulating phagocytosis in macrophages *in vitro* and *in vivo*.

Materials and Methods

Preparation of degalactosylated/desialylated bovine colostrum and GcMAF-containing human serum. Bovine colostrum was obtained from Jun Sei Co. Ltd. (Tokyo, Japan). One milligram of bovine colostrum powder was dissolved in 1 ml of 50 mM sodium phosphate buffer (pH 7.0) and incubated with 65 mU of β -D-galactosidase (from *Escherichia coli*; WAKO Pure Chemical Industries, Ltd., Osaka, Japan) either with or without 65 mU of neuraminidase (sialidase from *Clostridium perfringens*; Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 1 h. The reaction mixture was then heated at 60°C for 10 min to deactivate the enzymes. The protein concentrations were determined using a Pierce® BCA protein assay kit (Thermo Scientific, Waltham, MA, USA). GcMAF-containing human serum was prepared as previously reported by Kuchiike *et al.* (11).

SDS-PAGE and western blotting. Degalactosylated/desialylated bovine colostrum was subjected to sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE; XV PANTERA GEL MP, 7.5-15%; DRC Co., Ltd., Tokyo, Japan) 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% BSA at 4°C. The membranes were then probed with biotin-conjugated *Helix pomatia* agglutinin (HPA) lectin (Sigma-Aldrich) specific for GalNAc moiety. After membrane washing, the blots were incubated with horseradish peroxidase (HRP)-labeled streptavidin (GE Healthcare Life Sciences, Uppsala, Sweden) as a secondary antibody. The blots were developed using an ECL western blotting detection system (GE Healthcare). The visualization and quantification of the Western blot bands were achieved using an ECL Western blotting detection system (GE Healthcare), a LumiCube chemiluminescence analyzer and JustTLC image analysis software (Liponics, Tokyo, Japan).

In vitro phagocytosis assay. 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.* (13), 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. Mouse peritoneal macrophages were layered onto coverslips in a 24-well plate and cultured for 15 h. After 3 h of degalactosylated/desialylated bovine colostrum 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). The macrophages were fixed with methanol, air-dried and stained with Giemsa stain. The number of phagocytosed erythrocytes per cell was determined microscopically; in total, 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.

In vivo phagocytosis assay. Seven-week-old female C57BL/6 mice under anesthesia were injected with 300 μ l of degalactosylated/desialylated bovine colostrum directly into the small intestine. After 1 h, 300 μ l of AF488-labeled ovalbumin (OVA) protein (Invitrogen, Tokyo, Japan) was injected into the small intestine. After 1 h, mice were sacrificed and the small intestine was extracted. Fat and a Peyer's patch were removed, washed by PBS and, then, stirred at 37°C in 20 ml of FACS buffer for 20 min. Collagenase (Roche Diagnostics K.K., Tokyo, Japan) was also added and then cut the small intestine finely. After stirring at 37°C for 1 h, EDTA was added and stirred for an additional 5-min period. The supernatant was filtered with a cell strainer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and the residue was suspended in 10 ml of FACS buffer. The supernatant was removed by centrifugation. The cell pellet was suspended in 10 ml of 40% percoll (GE Healthcare), followed by addition of 5 ml of 75% percoll to the bottom and centrifugation. After the supernatant was removed, the residue was suspended in 9 ml of FACS buffer and then centrifuged again. After the supernatant was removed, anti-mouse F4/80, anti-mouse/human CD11b and anti-mouse/human CD16/32 (BioLegend, Inc., San Diego, CA, USA) antibodies were added and reacted for 15 min. After the supernatant was removed, wash buffer was added and the mixture subjected to a final centrifugation. 7-Aminoactinomycin D (Sigma-Aldrich) was added and analyzed by FACSCantoII (Becton, Dickinson and Company).

Flow cytometry assay. Mouse peritoneal macrophages were cultured in 24-well plates at a density of 5×10^5 cells/well in serum-free RPMI 1640 for 15 h. The cultured cells were washed two times with serum-free RPMI 1640 and then treated with degalactosylated/desialylated bovine colostrum for 24 h. The supernatant (50 μ l) was added to a mixture of capture bead diluent (48 μ l), mouse IL-1 β capture bead E5 (1 μ l) and mouse TNF- α capture bead C8 (1 μ l) (Becton, Dickinson and Company). After the solution containing beads was incubated at room temperature for 1 h, the mixture of capture bead diluent (48 μ l), mouse IL-1 β PE detection reagent (1 μ l) and mouse TNF- α PE detection reagent (1 μ l) were added. The solution containing beads was incubated at room temperature for 1 h, the beads were washed with 1 ml of wash buffer (Becton, Dickinson and Company) and centrifuged at $200 \times g$ for 5 min. The supernatant was removed and then 300 μ l of wash buffer was added and analyzed using FACSVerse.

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

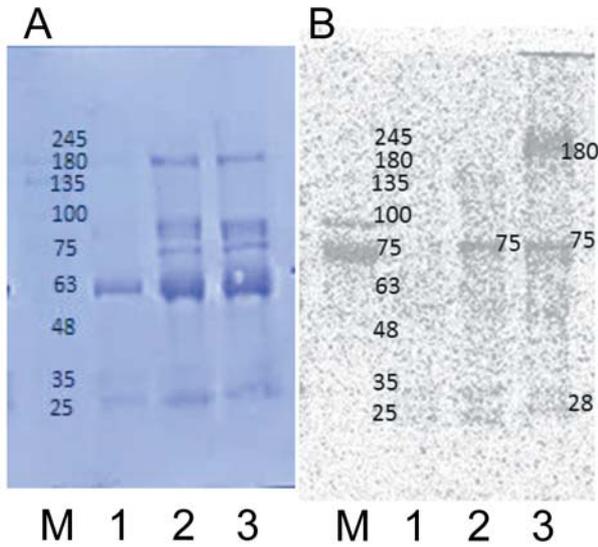


Figure 1. SDS-PAGE of degalactosylated and degalactosylated/desialylated bovine colostrum. (A) CBB-stain and (B) western blots probed with anti-human Gc globulin and *Helix pomatia* agglutinin (HPA) lectin. M, Marker; lane 1, bovine colostrum; lane 2, degalactosylated bovine colostrum; lane 3, degalactosylated/desialylated bovine colostrum.

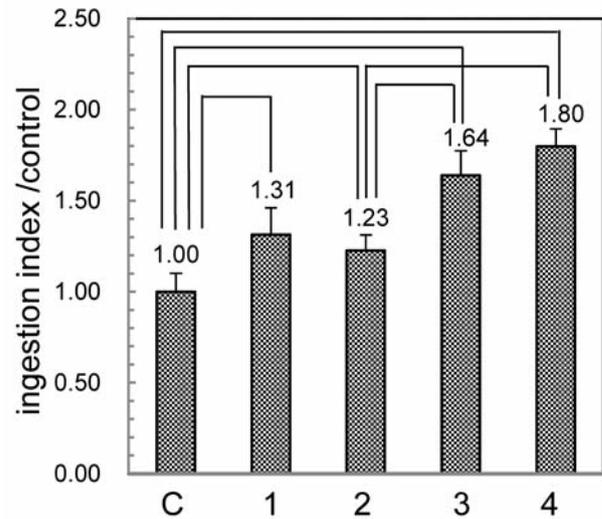


Figure 2. *In vitro* phagocytic activity of mouse peritoneal macrophages observed using degalactosylated/desialylated bovine colostrum and GcMAF-containing human serum. C, Control; 1, 1 µg of LPS; 2, 10 ng of non-treated bovine colostrum; 3, 10 ng of degalactosylated bovine colostrum; 4, 10 ng of degalactosylated/desialylated bovine colostrum. All experiments were performed in triplicate. Each error bar represents the standard deviation. The number on each bar indicates the mean value. * $p < 0.05$.

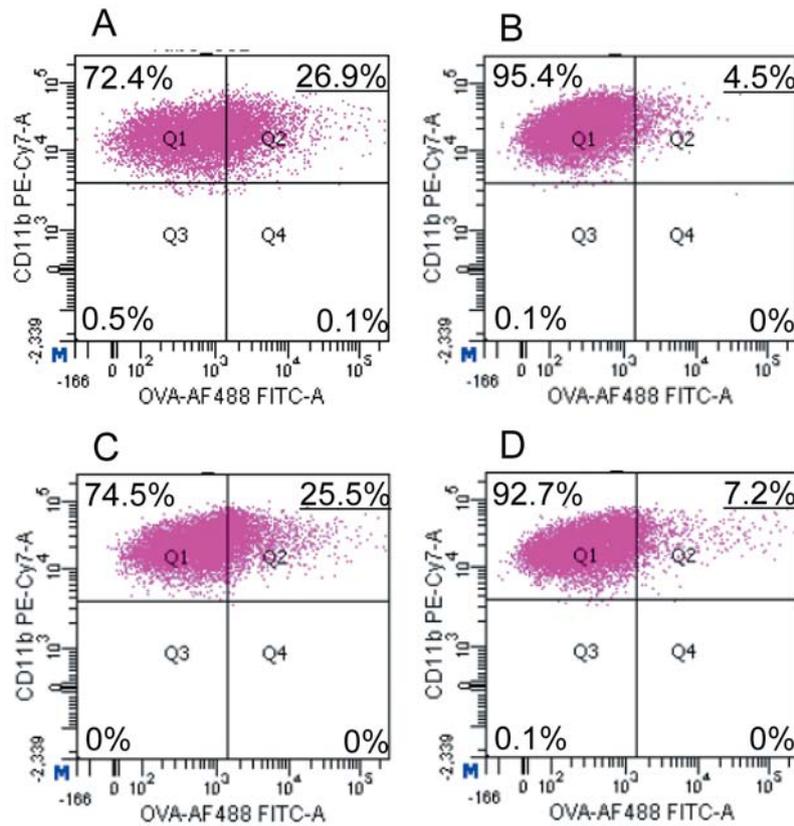


Figure 3. *In vivo* phagocytic activity of mouse intestinal macrophages observed using degalactosylated/desialylated bovine colostrum and GcMAF-containing human serum. The macrophage cells gated by expression of F4/80 and CD11b. Q2 area express the phagocytic macrophage. (A) 1 mg/kg of LPS; (B) 1 mg/kg of bovine colostrum; (C) 1 mg/kg of degalactosylated/desialylated bovine colostrum; (D) 1 mg/kg of GcMAF-containing human serum.

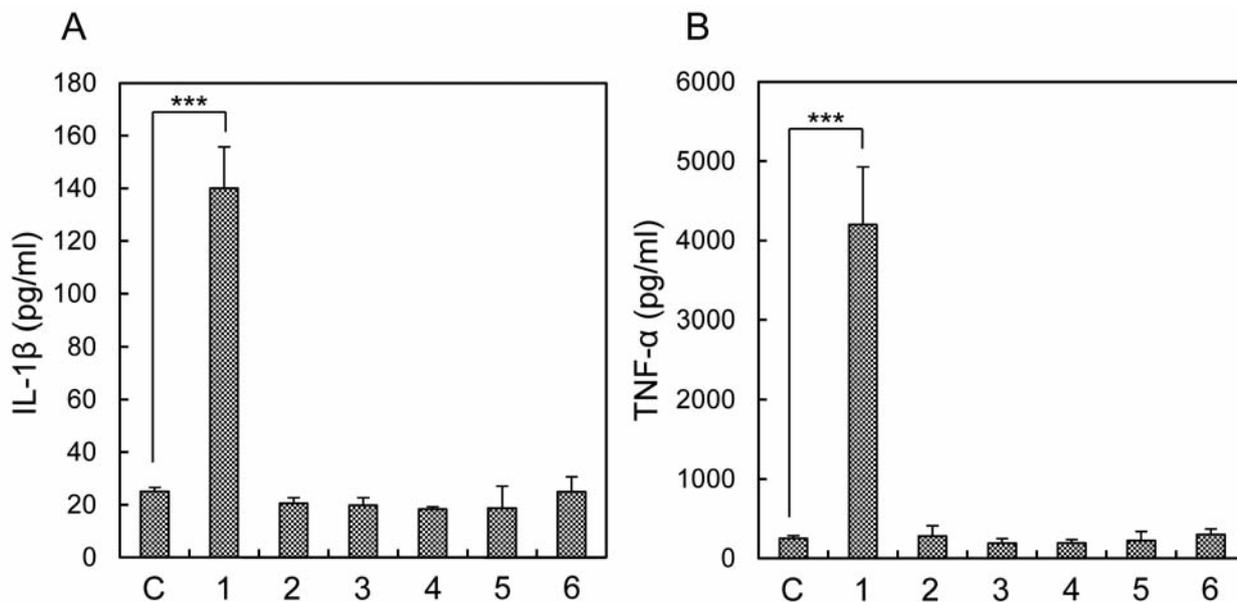


Figure 4. Induction of inflammatory cytokines by mouse peritoneal macrophage treated with degalactosylated/desialylated bovine colostrum or GcMAF-containing human serum. (A) IL-1β and (B) TNF-α. C, Control; 1, 1 μg of LPS and 10 ng of interferon-γ; 2, 10 ng of non-treated bovine colostrum; 3, 10 ng of degalactosylated bovine colostrum; 4, 10 ng of degalactosylated/desialylated bovine colostrum; 5, 10 ng of non-treated human serum; 6, 10 ng of degalactosylated/desialylated human serum. All experiments were performed in triplicate. Each error bar represents the standard deviation. The number on each bar indicates mean value. ***p<0.005.

Results

Preparation and identification of degalactosylated/desialylated bovine colostrum. We first checked the digestion activity of the O-linked sugar chain of the glycoprotein included in the bovine colostrum. Figure 1A shows the Coomassie Brilliant Blue (CBB) stain and Figure 1B shows the western blot of the bovine colostrum (lane 1), degalactosylated bovine colostrum (lane 2) and degalactosylated/desialylated bovine colostrum (lane 3). Five bands (180, 90, 75, 63, 28 kDa) were detected on the CBB stain, but only three bands (180, 75, 28 kDa) were detected by using an HPA lectin, which recognizes the GalNAc moiety.

Stimulating activity of degalactosylated/desialylated bovine colostrum on phagocytic activity of mouse peritoneal macrophages. We examined phagocytic activation by using degalactosylated and degalactosylated/desialylated bovine colostrum against mouse peritoneal macrophages. Figure 2 shows significant phagocytic activation with 10 ng of degalactosylated and degalactosylated/desialylated bovine colostrum, compared to that observed with the control. Degalactosylated/desialylated bovine colostrum (PI=1.80) showed significantly more potent phagocytic activation than did degalactosylated bovine colostrum (PI=1.64). Ten nanograms of non-treated bovine colostrum showed

significant phagocytic activation compared to that observed with the control; however, its activity was relatively weak (PI=1.23).

Stimulating activity of degalactosylated/desialylated bovine colostrum on the phagocytic activity of mouse intestinal macrophages. We examined the *in vivo* phagocytic activation of degalactosylated/desialylated bovine colostrum against mouse intestinal macrophages. In Figure 3, 1 mg/kg of degalactosylated/desialylated bovine colostrum exhibited a proportion of phagocytic macrophage number (C; 25.5%) that was higher than those exhibited by non-treated bovine colostrum (B; 4.5%) and GcMAF-containing human serum (D; 7.2%); its value was equal to the lipopolysaccharide (LPS) (A; 26.9%) of the positive control.

Stimulating activity of degalactosylated/desialylated bovine colostrum on induction of inflammatory cytokines. We checked whether inflammatory cytokines were stimulated in macrophages activated by treatment with degalactosylated/desialylated bovine colostrum. As seen in Figure 4, neither IL-1β (A) nor TNF-α (B) was produced as a major inflammatory cytokine from mouse peritoneal macrophages activated by degalactosylated/desialylated bovine colostrum, similar to that observed with GcMAF-containing human serum.

Discussion

In the present study, we evaluated degalactosylated/desialylated bovine colostrum for its ability to activate mouse peritoneal and intestinal macrophage phagocytosis *in vitro* and *in vivo*. Three HPA-positive bands of the degalactosylated/desialylated bovine colostrum are shown in Figure 1B suggesting that the 75-kDa band corresponds to glycoprotein- α constituting IgA and the 28-kDa band corresponds to DBP (14, 15). IgA and DBP has an *O*-linked sugar chain corresponding to the higher HPA-positive band, present when treated with β -galactosidase and sialidase, compared to when treated with β -galactosidase alone. This result correlated with the higher macrophage phagocytic activity of degalactosylated or degalactosylated/desialylated bovine colostrum in Figure 2. Degalactosylated/desialylated bovine colostrum also activated intestinal macrophages *in vivo* (Figure 3); however, it is generally believed that materials over a molecular weight of 500 Da do not undergo intestinal absorption. In contrast, it has been reported that some peptides of relatively high molecular weight (~15,000 Da) could be absorbed in the mouse intestinal tract (16). Therefore, it is suggested that even a glycoprotein in the bovine colostrum with a high molecular weight can be absorbed. Unlike LPS and interferon γ (IFN- γ), the degalactosylated/desialylated bovine colostrum does not induce production of inflammatory cytokines, such as TNF- α and IL-1 β as shown in Figure 4. We believe that this is a positive result due to the association between inflammatory cytokines and autoimmune disease. Degalactosylated/desialylated bovine colostrum can become an effective therapeutic agent for autoimmune diseases if it can suppress the production of inflammatory cytokines. In particular, it has been recently reported that the etiology of the autoimmune condition inflammatory bowel disease (IBD) involves Th17 cells that mediate the production of inflammatory cytokines (17). We will continue to investigate the relationships of various disease conditions with the macrophage activation mechanism of degalactosylated/desialylated bovine colostrum in the future.

In conclusion, we propose that degalactosylated/desialylated bovine colostrum can be used in various immunotherapies as an effective macrophage activator that does not induce inflammatory cytokines.

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References

- 1 Korhonen H, Marnila P and Gill HS: Milk immunoglobulins and complement factors. *Br J Nutr* 84: S75-S80, 2000.
- 2 Snoeck V, Peters I and Cox E: The IgA system: a comparison of structure and function in different species. *Vet Res* 37: 455-467, 2006.
- 3 Basset C, Devauchelle V, Durand V, Jamin C, Penneç YL, Youinou P and Dueymes M: Glycosylation of immunoglobulin A influences its receptor binding. *Scand J Immunol* 50: 572-579, 1999.
- 4 Inoue T, Iijima H, Tajiri M, Shinzaki S, Shiraishi E, Hiyama S, Mukai A, Nakajima S, Iwatani H, Nishida T, Mizushima T, Yasui T, Isaka Y, Kanto T, Tsujii M, Miyoshi E, Wada Y and Takehara T: Deficiency of *N*-acetylgalactosamine in *O*-linked oligosaccharides of IgA is a novel biologic marker for Crohn's disease. *Inflamm Bowel Dis* 18: 1723-1734, 2012.
- 5 Yamamoto N and Kumashiro R: Conversion of vitamin D3 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 Mohamad SB, Nagasawa H, Uto Y and Hori H: Preparation of Gc protein-derived macrophage activating factor (GcMAF) and its structural characterization and biological activities. *Anticancer Res* 22: 4297-4300, 2002.
- 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 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.
- 9 Mohamad SB, Nagasawa H, Sasaki H, Uto Y, Nakagawa Y, Kawashima K and Hori H: Gc protein-derived macrophage activating factor (GcMAF): Isoelectric focusing pattern and tumoricidal activity. *Anticancer Res* 23: 4451-4457, 2003.
- 10 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* 172: 116-122, 2012.
- 11 Kuchiike D, Uto Y, Mukai H, Ishiyama N, Abe C, Tanaka D, Kawai T, Kubo K, Mette M, Inui T, Endo Y and Hori H: Degalactosylated/desialylated human serum containing GcMAF induces macrophage phagocytic activity and *in vivo* antitumor activity. *Anticancer Res* 33: 2881-2885, 2013.
- 12 Inui T, Kuchiike D, Kubo K, Mette M, Uto Y, Hori H and Sakamoto N: Clinical experience of integrative cancer immuno-therapy with GcMAF. *Anticancer Res* 33: 2917-2919, 2013.
- 13 Uto Y, Yamamoto S, Mukai H, Ishiyama N, Takeuchi R, Nakagawa Y, Hirota K, Terada H, Onizuka S and Hori H: β -Galactosidase treatment is a common first-stage modification of the three major subtypes of Gc protein to GcMAF. *Anticancer Res* 32: 2359-2364, 2012.
- 14 Butler JE: Physicochemical and immunochemical studies on bovine IgA and glycoprotein- α . *Biochim Biophys Acta* 251: 435-449, 1971.

- 15 Maruyama K, Ebisawa K and Nonomura Y: Purification of vitamin D-dependent 28,000-Mr calcium-binding protein from bovine cerebellum and kidney by calcium-dependent elution from DEAE-cellulose DE-52 column chromatography. *Anal Biochem* 151: 1-6, 1985.
- 16 Oesser S, Adam M, Babe W and Seifert J: Oral administration of ¹⁴C labeled gelatin hydrolysate leads to an accumulation of radioactivity in cartilage of mice (C57/BL). *J Nutr* 129: 1891-1895, 1999.
- 17 Ogino T, Nishimura J, Barman S, Kayama H, Uematsu S, Okuzaki D, Osawa H, Haraguchi N, Uemura M, Hata T, Takemasa I, Mizushima T, Yamamoto H, Takeda K, Doki Y and Mori M: Increased Th17-inducing activity of CD14⁺ CD163^{low} myeloid cells in intestinal lamina propria of patients with Crohn's disease. *Gastroenterology* 145: 1380-1391, 2013.

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