Antitumor Effect of Degalactosylated Gc-Globulin on Orthotopic Grafted Lung Cancer in Mice

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Abstract. *Background: Group-specific component (Gc)-globulin-derived macrophage-activating factor (GcMAF) generated by a cascade of catalytic reactions with deglycosidase enzymes exerts antitumor activity. We hypothesized that degalactosyl Gc-globulin (DG3), a precursor of GcMAF, also plays a role in recovery from cancer as well as GcMAF due to progression of deglycosylation by generally resident sialidases and mannosidases. Materials and Methods: We prepared the subtypes of DG3, such as 1f1f and 1s1s and its 22 homodimers, by using vitamin D3-binding Sepharose CL-6B and examined their antitumor activity in mice bearing Lewis lung carcinoma cells, by counting the number of nodules formed in their lungs. Results: Antitumor activity of DG3 was observed regardless of its subtype, being equivalent to that of GcMAF. The injection route of DG3 affected its antitumor activity, with subcutaneous and intramuscular administration being more favorable than the intraperitoneal or intravenous route. In order to obtain significant antitumor activity, more than 160 ng/kg of DG3 were required. Conclusion: DG3 proved to be promising as an antitumor agent, similarly to GcMAF.*

Group-specific component (Gc)-globulin is a protein naturally forming a dimer with a molecular weight of approximately 52 kDa and is predominantly synthesized in the liver (1-3). This protein is classified into three subtypes according to the sugar moiety attached to a threonine residue of the protein, as shown in Figure 1. Humans have Gc-globulin in their blood at a concentration of 200-600 μg/ml, namely, equivalent to 20-50 mg/kg (4,5). The role of Gc-globulin is multiple such as i) delivery of vitamin D (6) and ii) scavenging of actin debris derived from damaged cells (7). In addition, Gc-globulin acquires additional functions due to removal of its sugar moiety galactose by β-galactosidase and of its sialic acid by sialidase; this deglycosylated protein is called Gc-derived macrophage-activating factor (GcMAF). This factor is known to increase the phagocytic activity of macrophage cells (8) and to inhibit tumor growth (9-11). Hence, GcMAF is a promising candidate for cancer therapy and has been subjected to several clinical studies (12-14).

The process of dissociation of sugar moieties of Gc-globulin occurs through a cascade of sequential catalytic reactions involving β-galactosidase followed by sialidase, as shown in Figure 1 (15-17). Degalactosylated Gc-globulin (DG3), the terminal sugar moiety of which is sialic acid (SA) for subtype 1f and α-mannose for subtype 1s, is generated as a precursor of GcMAF. Subtype 2 of DG3 corresponds to GcMAF. We hypothesized that DG3 would exert antitumor activity in the human body following removal of its SA or α-mannose moiety by generally resident sialidases or mannosidases, respectively (18, 19).

In this study, we prepared all homo-subtypes of DG3, namely 1f-1f, 1s-1s, and 2-2 homodimers, from serum of healthy volunteers and investigated their antitumor activity in mice bearing Lewis lung carcinomas (LLC). We found that DG3 reduced the number of nodules on the lungs regardless of the subtype used. In addition, subcutaneous (*s.c.*) or intramuscular (*i.m.*) injection was more effective

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Key Words: Degalactosylated Gc-globulin, a precursor of Gc-derived macrophage activating factor, subtypes of Gc-globulin, antitumor activity, injection route.

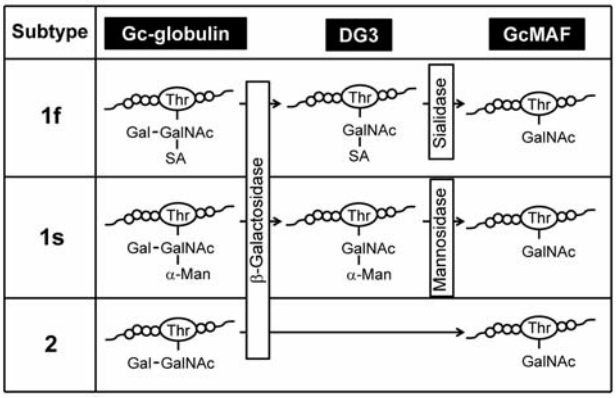

Figure 1. *Structures of three subtypes of group-specific component (Gc)-globulin and the process of removal of sugar moieties associated with the threonine residue of the globulin by a serial cascade reaction mediated by glycosidases. Gal, Galactose; GalNAc, N-acetylgalactosamine; SA, sialic acid; α-Man, α-mannose.*

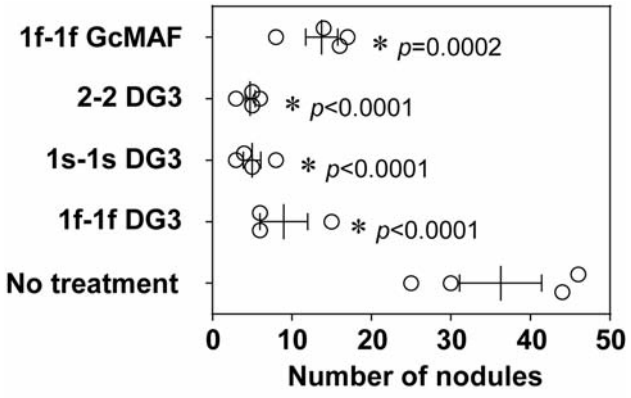

Figure 2. *Antitumor effect of degalactosyl Gc-globulin (DG3) dependent on its subtype. DG3 homodimers 1f-1f, 1s-1s, and 2-2 were intraperitoneally injected into Lewis lung carcinomas (LLC)-tumor-bearing mice at a dose 400 ng/kg; and 1f-1f Gc-globulin-derived macrophage-activating factor (GcMAF) was given by the same route at 4 ng/kg. The number of nodules formed in the lungs was counted after treatment. The results are expressed as a plot of each datum point with vertical lines indicating the mean and horizontal ones, ±S.E.M. Significant difference from the reference (no treatment) at the indicated p-value, as assessed by Bonferroni's multiple comparison test.*

than the intraperitoneal (*i.p.*) or intravenous (*i.v.*) ones; the dose of DG3 required for antitumor activity exertion was more than 160 ng/kg.

Materials and Methods

Materials. β-Galactosidase from bovine liver and Sepharose CL-6B were purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA), and 25-hydroxyvitamin D_3 came from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other chemicals used were of the highest grade commercially available.

Animals. C57BL/6 mice (Japan SLC Inc., Shizuoka, Japan) weighing approx. 20 g were employed in these studies. The animals were housed in an animal room with a 12-h light/12-h dark cycle and constant temperature of 23±1˚C and humidity of 55±5%. A standard diet and water were supplied *ad libitum*. All animal procedures were approved by the Institutional Animal Care and Use Committee at Tokyo University of Science (Y12026).

Preparation of DG3. DG3 samples were prepared as reported by Link *et al*. (20) with some modifications. Briefly, serum from healthy humans containing 1f-1f, 1s-1s, and 2-2 homodimers of Gc-globulin subtypes was obtained according to in-house volunteer rule of Morita Pharmaceutical Ind. Ltd. regarding human material study, diluted 1:1 with column buffer (50 mM Tris-HCl, 1.5 mM EDTA, 150 mM NaCl, 0.1% Triton X-100; pH 7.4), and incubated with 1.0 U/ml of β-galactosidases for 2 h at 37.5˚C. The reaction mixture was then mixed with 25-hydroxyvitamin D3-binding Sepharose CL-6B, which was manufactured in house, and shaken for 30 min. The 25-hydroxyvitamin D3-binding Sepharose CL-6B was washed with column buffer several times to remove unnecessary proteins, and then the bound DG3 was eluted with 1 M acetate buffer at pH 5.0. DG3 was concentrated by using a Microcon concentration unit (10,000 MWCO; Millipore, Billerica, MA, USA), after which the column buffer was replaced with saline solution. The molecular weight of the thus prepared DG3 was determined to be approx. 56 kDa; it appeared as a single band after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation under reducing conditions and subsequent staining with Coomassie brilliant blue. Its concentration was 0.26-0.76 μg/μl, as determined by the bicinchoninic acid (BCA) assay with bovine serum albumin as the standard.

Determination of antitumor effect. LLC cells, derived from a C57BL mouse, were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 60 μg/ml ampicillin. C57BL/6 mice were inoculated with 2×10^5 LLC cells by *i.v.* injection into a tail vein under anesthesia with ether. Injection of DG3, GcMAF or Gc-globulin was initiated on day 7 post inoculation and performed daily for 10 days. On day 17, after sacrifice, the lungs were excised, after which the number of nodules on the lung surface was counted.

Statistics. Data were analyzed by one-way analysis of variance (ANOVA) and post-hoc Bonferroni's multiple comparison tests. A probability level of 5% ($p<0.05$) was considered to be statistically significant. *p*-Values, shown on plots, are given as adjusted *p*-values by use of GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Subtype-dependent antitumor effect of DG3. Gc-globulin is present in human blood as a dimer formed by a combination of its subtypes 1f, 1s, and 2. As it is possible that the antitumor effect would be different dependent on the subtypes

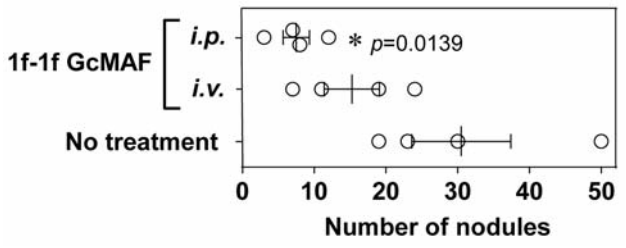

Figure 3. *Comparison between i.p. and i.v. administration in the antitumor effect of Gc-globulin-derived macrophage-activating factor (GcMAF). GcMAF at 4 ng/kg was administered to Lewis lung carcinomas (LLC)-tumor-bearing mice via i.p. or i.v. route. The number of nodules formed in the lungs was counted after treatment. The results are expressed and were evaluated as indicated in the Materials and Methods. p-Values for comparison with reference (no treatment) are shown.*

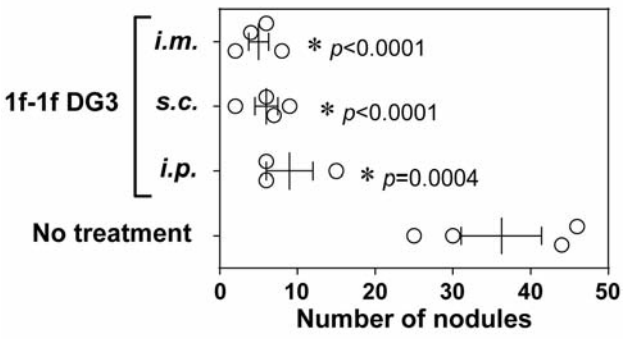

Figure 4. *Antitumor effect of degalactosyl Gc-globulin (DG3) dependent on the type of nonvascular administration route. Lewis lung carcinomas (LLC)-tumor-bearing mice were injected with 1f-1f DG3 at a dose 400 ng/kg by i.p., s.c. or i.m. injections. The results are expressed and were evaluated as indicated in the Materials and Methods. p-Values for comparison with reference (no treatment) are shown.*

due to differences in their terminal sugar moiety, we examined the antitumor effect of all three homodimers, namely 1f-1f, 1s-1s and 2-2, of DG3 on tumor-bearing mice. As shown in Figure 2, all homodimers of DG3 administered at 400 ng/kg exerted a significant antitumor effect, with the number of nodules decreasing to less than a quarter of that without any treatment. These antitumor effects were approximately twice as high as the effect by GcMAF at 4 ng/kg, although the dose of GcMAF used was one hundredth lower than that of DG3. In addition, the antitumor effect was significantly greater for 1s-1s and 2-2 dimers than for the 1f-1f one. These results suggest that the terminal sugar moiety would affect the antitumor activity of DG3.

Administration route-dependent antitumor effect. As DG3 and GcMAF play a role in delivering vitamin D and scavenging actin debris in the bloodstream, these proteins might be expected to work most effectively as antitumor substances when given by a nonvascular route. As shown in Figure 3, we compared the antitumor effect of GcMAF administered *via i.p.* and *i.v.* routes. *I.p.* administration showed a significant antitumor effect, reducing the number of nodules to a quarter of that without treatment, whereas by *i.v.* administration, the decrease was only to one-half. Hence, we regarded the nonvascular administration route as being optimum for Gc-protein derivatives to exert their antitumor activity.

We then investigated three nonvascular administration routes, *i.p.*, *i.m.* and *s.c.*, for their effect on the antitumor activity of DG3. As shown in Figure 4, DG3 administered *via* any of these three routes exerted a significant antitumor effect, with the number of nodules decreasing to a quarter of that without any treatment. Among the administration routes, *s.c.* and *i.m.* injections afforded better antitumor activity than the *i.p.* one. Deglycosylation of DG3 into the active substance GcMAF would be expected to proceed more efficiently in subcutaneous and intramuscular tissues, where the activity of deglycosylating enzymes is higher than that in intraperitoneal tissues (21).

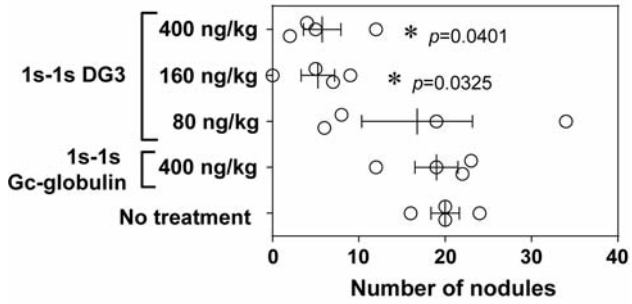

Figure 5. *Dose-dependent antitumor activity of degalactosyl Gc-globulin (DG3). DG3 of the 1s-1s type was subcutaneously administered to Lewis lung carcinomas (LLC)-tumor-bearing mice at a dose 80, 160 or 400 ng/kg. The same homodimer of Gc-globulin was employed as the reference. The results are expressed and were evaluated as stated in the Materials and Methods. p-Values for comparison with reference (no treatment) are shown.*

Dose-dependent antitumor effect. Finally, we investigated the dose dependency of DG3 administered *via* the subcutaneous route for exerting the antitumor effect. As shown in Figure 5, the number of nodules decreased little by treatment with 80 ng/kg of DG3, whereas a dose of 160 ng/kg or higher showed significant antitumor activity. At these doses, the number of nodules was less than a quarter of that without any treatment. However, there was no difference in effect between 160 ng/kg and 400 ng/kg. Gc-globulin at 400 ng/kg had no antitumor effect on the tumor-bearing mice. Hence,

we found that DG3 required a dose of 160 ng/kg or higher, which was, however, 40-times higher than that for GcMAF (4 ng/kg) to exert comparable antitumor activity.

Discussion

DG3 is a promising substance leading to antitumor effects as a precursor of GcMAF. We recently established a method for manufacturing DG3 from human blood serum and found that it potentiated the phagocytic activity of macrophage cells in the presence of peritoneal fluid (22, 23). We assumed that DG3 should play a role in antitumor activity in the human body because DG3 with a terminal sugar moiety of SA for the 1f subtype and α-mannose for the 1s subtype turns into GcMAF by deglycosylation, due to sialidases and mannosidases, respectively.

We found that DG3 exerted its antitumor activity in LLC-tumor-bearing mice regardless of its subtype. This finding means that we all have the potential in our blood to combat malignant neoplasms. However, in people who develop cancer, α-*N*-acetylgalactosaminidase is synthesized by cancer cells and affects Gc-globulin due to deglycosylation of the entire sugar moiety attached to the threonine residue (24, 25). As treatment with DG3 led to a significant reduction in tumor growth in this study, DG3 proved to be useful as an antitumor agent as good as GcMAF.

The effect of DG3 on LLC tumors was dependent on the administration route, with *s.c.* and *i.m.* injections being preferable to *i.p.* and *i.v.* ones. Gc-globulin has multiple functions such as elimination of actin debris and transport of vitamin D. The major role of Gc-globulin is to scavenge actin debris derived from damaged cells, and half of the Gc-globulin in the serum forms a complex with actin (5). In addition, the concentration of vitamin D and its hydroxyl derivative is much higher in the serum than in muscle and liver (26). Hence, DG3 injected into the circulatory system would have unintended functions. For the optimal antitumor activity of DG3, nonvascular administration into tissue where glycosidase activity is abundant, such as hypodermis and muscle, would be favorable.

Several researchers report on elucidating the antitumor mechanisms of GcMAF mentioned that interaction of GcMAF with endothelial and cancer cells inhibited angiogenesis and cell growth, respectively (11, 27). The dose for attaining antitumor activity by GcMAF required is of the microgram order. In this study, it is interesting to note that DG3 exerted antitumor activity towards lung cancer at *s.c.* injections of 160 ng/kg, even though the local concentration of DG3 would be expected to be low. Immunological reactions through macrophage networks may be involved in the antitumor action of DG3 (28). Further studies regarding the mechanism of antitumor activity by Gc-globulin-derived proteins *in vivo* are needed.

In conclusion, we found that DG3 had significant antitumor activity regardless of the subtype used and that *s.c.* injections of DG3 at a dose of 160 ng/kg or higher were preferable for obtaining the desired action.

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