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Inhibitory effects of preventive and curative orally administered spinach glycoglycerolipid fraction on the tumor growth of sarcoma and colon in mouse graft models

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ABSTRACT

The glycoglycerolipid fraction from spinach was purified, and this fraction was used to clarify bioactive functions, such as inhibition of DNA polymerase activity and cancer cell growth. The effects of the spinach glycoglycerolipid fraction on preventive and curative antitumor activities, and acute toxicity were investigated. After oral administration of 20 mg/kg of the glycoglycerolipid fraction for 2 weeks as preliminary medication, colon tumor growth was delayed, and the protein expression level of proliferating cell nuclear antigen (PCNA) was decreased in tumor tissue. Five days after tumor cell implantation, oral administration of, not only 70 mg/kg of the glycoglycerolipid fraction, but also the γ-cyclodextrin (CD) glycoglycerolipid fraction complex, for 4 weeks, suppressed sarcoma formation with no adverse reactions in mice. In the acute toxicity test, 2000 mg/kg of orally administered glycoglycerolipid fraction did not show evident toxicity. Hence, these results suggest that the spinach glycoglycerolipid fraction is a safe and effective anticancer bioactive agent and/or food material.

1. Introduction

Epidemiological and prospective studies indicate that the intake of vegetables and fruits is associated with a reduced risk of cancer (Michels et al., 2000; Terry et al., 2001). These results suggest that some food compounds, such as carotene and vitamins, have bioactivity as anticancer agents. Furthermore, we have searched for new bioactive compounds, and found some glycoglycerolipids from green plants to be potent anticancer agents (Ohta et al., 1998). In vegetables, fruits and grains, most of the lipid composition of thylakoid membranes is glycoglycerolipids, which consist of mainly monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG) and sulfoquinovosyl diacylglycerol (SQDG) (Roughan & Batt, 1969).

The glycoglycerolipids inhibit DNA polymerase activity (Mizushina et al., 1998), cancer cell proliferation (Hossain, Kurihara, Hosokawa, & Takahashi, 2005; Ohta et al., 2000), inflammation (Bruno et al., 2005), tumor promotion (Colombo et al., 2005; Morimoto et al., 1995) and tumor growth (Sahara et al., 2002). Therefore, to clarify the glycoglycerolipid properties, we succeeded in purifying a fraction including three glycoglycerolipids from spinach (Maeda et al., 2005). Spinach was the best source for obtaining the glycoglycerolipid fraction of the vegetables tested (Kuriyama et al., 2005). In an *in vitro* study and parenteral treatment *in vivo*, the spinach glycoglycerolipid fraction potently affected the decrease of replicative DNA polymerase activity, cancer cell proliferation and solid tumor size (Maeda, Hada, Yoshida, & Mizushina, 2007a; Maeda et al., 2007b).

Although vegetables, fruits and grains, which contain glycoglycerolipids (MGDG, DGDG, SQDG) are ingested orally every day, the bioactive function of these glycoglycerolipids is unknown. Thus, in this report, we evaluated the bioactivity, especially antitumor activity, of the orally administered spinach glycoglycerolipid fraction. The glycoglycerolipid fraction has characteristic high viscosity and low solubility; therefore, γ-cyclodextrin (CD) was used to solve this problem, and then compare the CD glycoglycerolipid fraction complex with the normal glycoglycerolipid fraction for antitumor efficacy.

Abbreviations: MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol; SQDG, sulfoquinovosyl diacylglycerol; CD, γ-cyclodextrin; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; H&E, hematoxylin and eosin.

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In this report, we investigate colon prevention and sarcoma treatment with the orally administered glycoglycerolipid fraction in mouse solid tumors. Moreover, we attempted to evaluate its safety by oral administration of a high concentration of the spinach glycoglycerolipid fraction in mice to develop an anticancer food compound.

2. Materials and methods

2.1. Materials

The purification method of the glycoglycerolipid fraction from spinach was established as described previously (Maeda et al., 2005). Briefly, water-soluble substances were extracted from dried spinach (*Spinacia oleracea* L., 20 g) with 1000 ml of warm water (60 °C). The tissue cake was added to 1000 ml of warm 100% ethanol (60 °C), and substances containing glycoglycerolipids were extracted. The 100% ethanol extract was diluted with water to a 70% ethanol solution. This solution was subjected to Diaion HP-20 (Mitsubishi Chemical Inc., Tokyo, Japan) column chromatography (200 ml), a hydrophobic type of chromatography, washed with 1000 ml of 70% ethanol, and then eluted using 500 ml of 95% ethanol. The 95% ethanol eluted solution was the "spinach glycoglycerolipid fraction" (Fig. 1).

The preparation of the CD glycoglycerolipid fraction complex is also shown in Fig. 1. Briefly, 12.5% CD (Bizen Chemical Co. Ltd., Okayama, Japan) was dissolved in distilled water, and the spinach glycoglycerolipid fraction was added to achieve an equal weight of CD. The mixture was homogenised at 2500 rpm for 30 min at room temperature. After standing overnight at room temperature in a dark place, the CD glycoglycerolipid fraction complex mixture was freeze-dried in a vacuum at −50 °C overnight. The glycoglycerolipid fraction and the CD glycoglycerolipid fraction complex contained mainly three glycoglycerolipids, MGDG, DGDG and SQDG (Fig. 2).

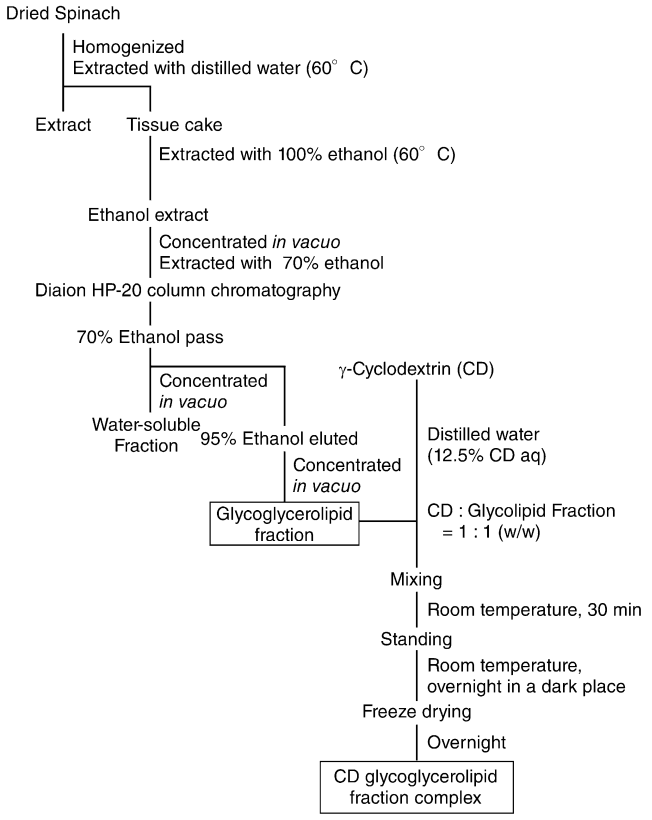

Fig. 1. The method of purifying the glycoglycerolipid fraction from spinach, and preparation of CD glycoglycerolipid fraction complex powder.

A mouse anti-proliferating cell nuclear antigen (PCNA) monoclonal antibody (sc-56) was purchased from Santa Cruz Biotechnology, Inc. (CA, USA). Peroxidase-conjugated forms of goat anti-rabbit or mouse IgGs (i.e., secondary antibody) were obtained from Nichirei Biosciences Inc. (Tokyo, Japan). All reagents were of analytical grade and purchased from Nacalai Tesque Ltd. (Kyoto, Japan).

2.2. Cell line and cell culture

Mouse colon adenocarcinoma cell line, colon-26, and mouse sarcoma cell line, CCRF S-180 II (S-180), were provided by the Cell Resource Center for Biomedical Research (Tohoku University, Sendai, Japan), and the Health Science Research Bank (Osaka, Japan), respectively. The colon-26 cells were cultured in RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum (Equitech-Bio, Inc., Texas, USA), penicillin (100 units/ml, Nacalai Tesque Ltd., Kyoto, Japan) and streptomycin (100 μg/ml, Nacalai Tesque Ltd., Kyoto, Japan). S-180 cells were cultured in Eagle's minimal essential medium (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) with non-essential amino acids (Invitrogen Corporation, CA, USA), 5% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). All cells were cultured in an atmosphere of 95% air and 5% CO_2 at 37 °C.

2.3. In vitro assay of cancer cell growth inhibition

Colon-26 and S-180 cells were trypsinized and plated in 96-well plates at 5000 cells per well ($n = 5$) in complete medium and allowed to adhere overnight. The culture was then washed and referred with medium containing the spinach glycoglycerolipid fraction from 0 μg/ml to 100 μg/ml. After 24 h, the inhibitory activity of cell growth was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT, Sigma, St. Louis, MO, USA) assay (Mosmann, 1983).

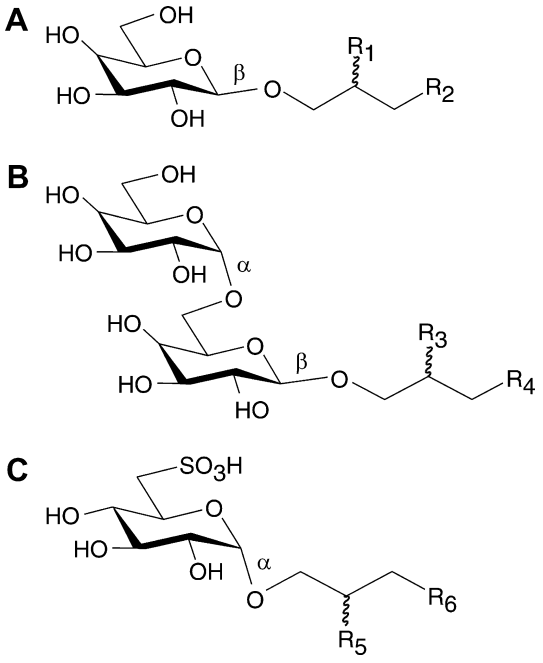

Fig. 2. Chemical structures of monogalactosyl diacylglycerol (A), digalactosyl diacylglycerol (B) and sulfoquinovosyl diacylglycerol (C), which are the main constituents of the spinach glycoglycerolipid fraction. R_1–R_6 are fatty acids.

2.4. Animals

ICR mice and BALB/c mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). Mice were housed and acclimatised for at least one week in the animal research facilities before being used in the present study. These mice were provided with free access to laboratory standard diet (MF; Oriental Yeasts Co., Ltd., Osaka, Japan) and water. All animal studies were approved by the Kobe-Gakuin University Animal Committee, and were performed according to the guidelines for the "Care and Use of Laboratory Animals" of Kobe-Gakuin University.

2.5. Safety measurement of the spinach glycoglycerolipid fraction in mice

Acute oral toxicity of the spinach glycoglycerolipid fraction was evaluated using female ICR mice (6 weeks of age) in compliance with OECD guidelines for the testing of chemicals, No. 420-acute oral toxicity-fixed dose method, adapted in December, 2001. First, one female mouse was used to assess whether oral administration of the spinach glycoglycerolipid fraction was fatal or toxic. In the first sighting study, we selected and orally administered the glycoglycerolipid fraction at a dose of 300 mg/kg in distilled water, using a gastric feeding needle. Since the mouse did not die or show signs of toxicity, we next administered the spinach glycoglycerolipid fraction to another mouse at 2000 mg/kg, and conducted the same observation. Mouse administration of 2000 mg/kg of the glycoglycerolipid fraction showed no mortality or toxicity; therefore, the main study was commenced. In the main study, the mice were divided randomly into a control group ($n = 5$) or orally administered at a dose of 2000 mg/kg in the spinach glycoglycerolipid fraction group ($n = 5$), and we administered distilled water or the glycoglycerolipid fraction in distilled water to these groups, respectively. The changes of all mouse conditions, such as skin, fur, eyes, respiratory pattern, and autonomic and behaviour patterns were observed for 14 days, and then the mice were killed humanely. The histopathological features of major organs were assessed, and the body, brain, heart, lungs, liver, spleen and kidneys were weighed.

2.6. Effect of pre-orally administered spinach glycoglycerolipid fraction on colon-26 tumor growth

First, female BALB/c mice (6–7 weeks of ages) were randomly distributed into a control group or orally administered glycoglycerolipid fraction group ($n = 10$ per each group). Once a day, mice were treated by oral administration of phosphate-buffered saline (PBS) alone (control group) or the spinach glycoglycerolipid fraction with PBS at 20 mg/kg/day, using a gastric feeding needle for 2 weeks (total of 14 administrations). Eight hours after the last administration of PBS or the glycoglycerolipid fraction, colon-26 cells (1×10^6 cells) were subcutaneously injected into these BALB/c mice. Five days after cancer cell implantation, the tumor volume was measured [tumor volume = length $\times$ (width)$^2 \times 0.5$]. Mice that did not develop less than 50 mm^3 of tumor volume during the study period were excluded from the analyses.

Twenty-eight days after tumor implantation, all mice were killed and histopathological features of the tumors and major organs, such as the lung, heart, spleen, stomach, liver, pancreas, kidney, intestine and brain were observed.

The tumor tissues were fixed with 10% formalin in PBS (pH 7.2) and processed for paraffin embedding. The 3 μm-thick sections were deparaffinized in xylene and alcohol, and transferred to PBS. The deparaffinized sections were stained with hematoxylin and eosin (H&E), and immunohistochemically stained to evaluate tumor cell proliferation. The mitotic index [number of mitoses/high power field (HPF)] of five to six random non-necrotic fields by H&E stain at 400$\times$ magnification was used. For immunohistochemical staining, the deparaffinized sections were incubated overnight with an anti-PCNA primary antibody (1:500 dilution) at 4 °C. Section samples were then rinsed three times with PBS and incubated with the peroxidase-conjugated secondary antibody for 10 min at room temperature. The sections were rinsed three times in PBS, and the positive reaction was visualised by incubating the sections with 3,3′-diaminobenzidine (DAB; Nichirei Biosciences Inc., Tokyo, Japan) for 5 min. Then, the sections were washed with water and counterstained with hematoxylin. The slides were analysed for the percentage of positive cells (i.e., three to six PCNA high expression spots at 400$\times$ magnification).

2.7. Assessment of antitumor activity of the spinach glycoglycerolipid fraction on S-180 tumor growth in mice

S-180 cells (1×10^6 cells) were subcutaneously inoculated into female ICR mice (6–7 weeks of ages). After mice formed approximately 100 mm^3 of solid tumor, they were divided randomly into three groups: a control group with orally administered PBS alone ($n = 5$), orally administered glycoglycerolipid fraction dissolved in PBS at a dose of 70 mg/kg group ($n = 4$) or CD glycoglycerolipid fraction complex dissolved in PBS at a dose of 70 mg/kg group ($n = 5$). Each day, all mice were orally administered with PBS or the glycoglycerolipid fractions using a gastric feeding needle, and the tumor volume was measured. At the end of the *in vivo* antitumor assay, the mice were killed and observed with gross diagnosis of major organs.

2.8. Statistical analysis

All measurements are expressed as the means ± SEM. Significant differences were determined using the Mann-Whitney *U*-test for colon-26 tumor volume and pathological analysis measurements, and the Steel–Dwass test for *in vitro* cancer cell growth inhibition study and *in vivo* S-180 solid tumor volume using the KyPlot 5.0 software package (KyensLab, Inc., Tokyo, Japan). Statistical significance was defined as $P < 0.05$.

3. Results

3.1. In vitro growth inhibition of colon and sarcoma cells by the spinach glycoglycerolipid fraction

The murine colon and sarcoma cells were cultured on each plate for 24 h in media containing various concentrations of the glycoglycerolipid fraction from spinach, and the growth of these cancer cells was inhibited in a dose-dependent manner (Fig. 3). At 100 μg/ml, the survival of colon-26 and S-180 cells, as determined by the MTT assay, decreased to 75.4% and 91.5%, respectively, compared with the control ($P < 0.05$). The IC_{50} values of the glycoglycerolipid fraction were 74.5 μg/ml and 51.7 μg/ml for colon and sarcoma cells, respectively, and these results were almost the same values as in our previous study of stomach cancer cells and cervical cancer cells from human (Maeda et al., 2005, 2007b).

3.2. Acute oral safety test of the spinach glycoglycerolipid fraction

Analyses of the acute oral safety of the spinach glycoglycerolipid fraction were performed in mice according to the standard protocols outlined by OECD 420. We performed a sighting study at 300 mg/kg and another sighting study at 2000 mg/kg. After checking for adverse effects, we used five female mice and assessed the acute safety of orally administered glycoglycerolipid fraction at 2000 mg/kg for 14 days as the main study. At 2000 mg/kg of the

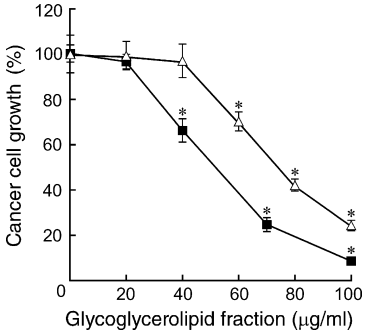

Fig. 3. Dose-responsive curves of the effects of spinach glycoglycerolipid fraction on the growth inhibition of the cultured cancer cells. Each cell line (5000 cells/well), S-180 sarcoma cells (closed square) and colon-26 colorectal cancer cells (open triangle), was cultured for 24 h in the media containing various concentrations of the glycoglycerolipid fraction. Cell proliferation was determined by MTT assay (Mosmann, 1983). All values are shown as the means ± SEM of five independent wells. *Different from the control, $P < 0.05$.

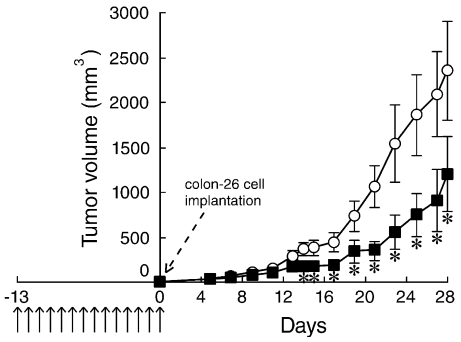

Fig. 4. Effect of the spinach glycoglycerolipid fraction on the established colon cancer graft model for preliminary medication. Vertical arrows show the injection timings of the glycoglycerolipid fraction (i.e., for 2 weeks continuous intake before implantation). ○, control PBS; ■, the spinach glycoglycerolipid fraction (20 mg/kg/day for 2 weeks). After the implantation of subcutaneous colon-26 cells (1×10^6 cells), all mice (BALB/c) were measured for tumor volume for one month with no drug administration. All values are shown as the means ± SEM of five independent experiments. *Different from the control, $P < 0.05$.

spinach glycoglycerolipid fraction, no toxicity was noted at necropsy, indicated by a total lack of gross pathological alterations (Table 1).

3.3. Preliminary medication of tumor growth inhibition by the spinach glycoglycerolipid fraction

The mice were randomized into two groups, and we started orally administering the glycoglycerolipid fraction from spinach or PBS (i.e., control) for 2 weeks. Then, 1×10^6 cells of colon-26 cell line were implanted, and we measured the tumor volume of mouse solid tumor for 28 days after the implantation of cancer cells ($n = 9$ of the each group). The spinach glycoglycerolipid fraction inhibited the tumor growth volume to 48.9% of the control tumor ($P < 0.05$, Fig. 4, Table 2). None of the mice showed any significant loss of body weight (control: 24.3 ± 0.4 g and the glycoglycerolipid fraction: 22.8 ± 0.4 g) or organopathy throughout the experimental period. We next analysed all mouse tumors for histopathological examination. PCNA, which is a marker protein for proliferating cells, is a reported biomarker for cancer (Kubben et al., 1994). The PCNA express level in tumor tissue was significantly decreased by orally administered spinach glycoglycerolipid fraction treatment ($P < 0.05$, Table 2). Moreover, the tumor tissue of these mice was stained with H&E and mitoses were counted. There was a significant decrease of the mitosis index in the administered glycoglycerolipid fraction group compared with the control group ($P < 0.05$, Table 2).

3.4. Antitumor activity of the spinach glycoglycerolipid fraction in mouse model

1×10^6 S-180 cells were implanted into the subcutaneous tissue of ICR mice. Four days after the implantation of sarcoma cells, the first group received daily PBS, the second group received daily oral administration of the glycoglycerolipid fraction from spinach at 70 mg/kg, and the third group received daily oral administration of the CD glycoglycerolipid fraction complex at 70 mg/kg (i.e., the spinach glycoglycerolipid fraction of the third group was the same amount as that of the second group). For 27 days after oral administration, the tumor volumes and weights of all mice were measured once a day, and then the mice were killed humanely. In the present study, we investigated whether oral administration of the glycoglycerolipid fraction or the CD glycoglycerolipid fraction complex had a superior antitumor effect. Oral administration of both the glycoglycerolipid fraction and the CD glycoglycerolipid fraction complex significantly decreased sarcoma tumor growth compared with the control mouse tumor (90% and 92%, respectively; $P < 0.05$, Fig. 5), with tumor tissue almost lacking in mice (data not shown). The *in vivo* antitumor effect of the glycoglycerolipid fraction was not different from that of the CD glycoglycerolipid fraction complex. These antitumor effects induced no adverse drug reaction (i.e., no decrease of body weight, no damage to major organs or drug-related animal death) (data not shown).

4. Discussion

The orally administered glycoglycerolipid fraction of spinach inhibited solid tumor growth with no side effects in our animal studies. The antitumor efficacy is on colon tumor prevention after preliminary medication with the spinach glycoglycerolipid fraction. Second, the glycoglycerolipid fraction inhibited tumor vegetation of growing sarcoma. These dual antitumor actions suggest that continuous oral administration of the spinach glycoglycerolipid fraction strongly reduces cancer risk.

In a preliminary medication study on colon-26 tumor prevention, the glycoglycerolipid fraction, at a dose of 20 mg/kg, induced the reduction of both the PCNA expression level and mitotic index of tumor tissue, and delayed the tumor proliferation rate (Fig. 4, Table 2). This animal model indicated, not only tumor prevention, but also some of the steps of metastasis. Major processes are involved in metastasis: migration, intravasation, transport, extrava-

Table 1
Acute oral safety of the spinach glycoglycerolipid fraction in ICR mice

Weight (g)	Body	Brain	Heart	Lungs	Liver	Spleen	Kidneys
Control	30.7 ± 1.6	0.475 ± 0.010	0.128 ± 0.001	0.193 ± 0.006	1.538 ± 0.136	0.132 ± 0.014	0.435 ± 0.025
Glycoglycerolipid fraction	31.5 ± 1.1	0.465 ± 0.016	0.139 ± 0.004	0.199 ± 0.006	1.643 ± 0.101	0.128 ± 0.012	0.460 ± 0.028

Mice treated with water or the spinach glycoglycerolipid fraction at 2000 mg/kg by oral administration in accordance with OECD 420. Fourteen days after administration, the body and major organ weights of mice were measured as the means ± SEM of five independent animals.

Table 2
Effect of the spinach glycoglycerolipid fraction on tumor growth in mice for preliminary medication

	Tumor volume (mm^3)	PCNA (%)	Mitosis (/HPF)
Control	2356.7 ± 550.1	64.0 ± 1.7	15.3 ± 1.7
Glycoglycerolipid fraction	1203.5 ± 416.0*	55.0 ± 1.7*	11.9 ± 0.4*

The spinach glycoglycerolipid fraction at 20 mg/kg or PBS (i.e., control) was orally administered for 14 days, before colon-26 cells (1×10^6 cells) were implanted. The tumor volume of mice on day 28 after cancer cell implantation was measured, and tumor tissue underwent histopathological analysis. Percentage of PCNA-positive cells and mitosis count in tumor tissue were used as an index of cell proliferation. Measurements are shown as the means ± SEM of nine independent experiments. *Different from the control, $P < 0.05$.

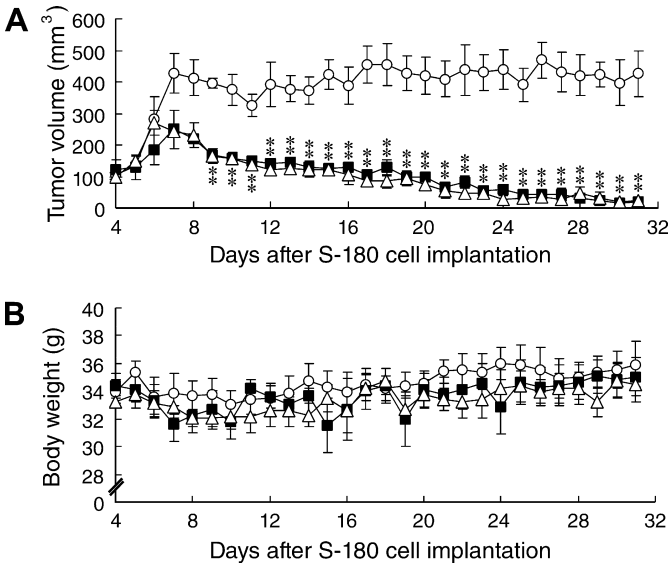

Fig. 5. *In vivo* antitumor effect of the glycoglycerolipid fraction from spinach and the CD glycoglycerolipid fraction complex. Four days after implantation of S-180 cells (1×106 cells), we started orally administered PBS (control; open circle), the glycoglycerolipid fraction (closed square) or the CD glycoglycerolipid fraction complex (open triangle) at 70 mg/kg or 70 mg/kg equivalent, respectively, for 27 days. All data are shown as the means ± SEM of four to five independent animals. *Different from the control, $P < 0.05$.

sation, and metastatic colonisation (Fidler, 1990). Metastatic colonization, (adherence, growth and proliferation process of cancer cells) was shown in this study; therefore, the spinach glycoglycerolipid fraction must inhibit metastatic focus growth in tumor tissue.

In the next antitumor study of treatment by the glycoglycerolipid fraction of spinach after cancer cell implantation, there were two important findings: oral administration of 70 mg/kg of the spinach glycoglycerolipid fraction had potent antitumor activity for mouse sarcoma (Fig. 5A). In our previous study, some glycoglycerolipids, such as MGDG and SQDG, showed angiogenesis in an *ex vivo* rat model (Matsubara et al., 2005) and subcutaneous injection of the glycoglycerolipid fraction inhibited the growth of solid tumor *in vivo* and cell proliferation in tumor tissue (Maeda et al., 2007b). These reports indicate that oral administration of the glycoglycerolipid fraction from spinach influences both tumor angiogenesis and proliferation. Second, although CD had no effective antitumor activity, the CD glycoglycerolipid fraction complex has the same antitumor efficiency as the normal (i.e., no complex) glycoglycerolipid fraction (Fig. 5A). CD can change the solubility of materials from fat-soluble to water-soluble; therefore, the CD glycoglycerolipid fraction complex must be useful as an anticancer functional food and/or drug.

Regarding the safety of the spinach glycoglycerolipid fraction, we conducted three studies in our present investigations: an acute oral study at a dose of 2000 mg/kg (Table 1) and continuous oral studies at doses of 20 and 70 mg/kg for 2–4 weeks. These results showed that the glycoglycerolipid fraction did not have side effects, such as animal death or evident toxicity, loss of body weight and/or major organ damage. In addition, CD in itself is digested in the body, and it is a safe agent (Fukuda et al., 1992). In fact, CD, in our previous investigation of antitumor tests for one month, did not induce damage to mice (Fig. 5B).

In conclusion, this study showed that the glycoglycerolipid fraction from spinach inhibited the tumor growth of sarcoma and colon cells in mouse models with no adverse reactions. Preventive oral administration of the glycoglycerolipid fraction at 20 mg/kg for 2 weeks delayed tumor growth. Furthermore, therapeutic administration of both the glycoglycerolipid fraction and the CD glycoglycerolipid fraction complex at doses of 70 mg/kg for 4 weeks suppressed tumor growth. We conclude that the spinach glycoglycerolipid fraction is a bioactive and safe agent and/or a food substance with anticancer effects.

Acknowledgments

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