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Journal of Nutritional Biochemistry 16 (2005) 121–128

Journal of Nutritional Biochemistry

Effects of DNA polymerase inhibitory and antitumor activities of lipase-hydrolyzed glycolipid fractions from spinach☆

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Received 10 June 2004; accepted 9 August 2004

Abstract

We succeeded in purifying the major glycolipid fraction in the class of sulfoquinovosyl diacylglycerol, monogalactosyl diacylglycerol and digalactosyl diacylglycerol (DGDG) from a green vegetable, spinach (*Spinacia oleracea* L.). This glycolipid fraction was an inhibitor of DNA polymerases and a growth inhibitor of NUGC-3 human gastric cancer cells, and, interestingly, the activities were much stronger when the fraction was hydrolyzed by lipase. Glycolipids in the hydrolyzed fraction consisted of sulfoquinovosyl monoacylglycerol (SQMG), monogalactosyl monoacylglycerol (MGMG) and DGDG. In the in vivo antitumor assay using Greene's melanoma, the fraction containing SQMG, MGMG and DGDG showed to be a promising suppressor of solid tumors. Spinach glycolipid fraction might be a potent antitumor compound if directly injected into a tumor-carrying body, and this fraction may be a healthy food material that has antitumor activity.

Keywords: Spinach; Glycolipids; DNA polymerase; Enzyme inhibitor; Antitumor effect; Cytotoxicity

1. Introduction

Multiple organisms are known to contain at least 14 types of DNA polymerase [1]. DNA polymerases catalyze both DNA replication and repair [2]. Such inhibitors could be employed as anticancer chemotherapy agents because they inhibit cell proliferation. Based on this idea, we have searched for and found many new DNA polymerase inhibitors over the past 9 years (e.g., long-chain fatty acids and their derivatives [3–7], bile acids such as lithocholic acid [8], terpenoids [9–11], flavonoids [12,13], sulfate-containing glycolipids [14–22], vitamin A-related compounds [23] and nucleotide analogs [24,25] from natural resources). Of these, in particular, sulfoglycolipids in the class of sulfoquinovosyl diacylglycerol (SQDG; Fig. 1A) and sulfoquinovosyl monoacylglycerol (SQMG; Fig. 1B)

Abbreviations: SQDG, sulfoquinovosyl diacylglycerol; SQMG, sulfoquinovosyl monoacylglycerol; MGDG, monogalactosyl diacylglycerol; MGMG, monogalactosyl monoacylglycerol; DGDG, digalactosyl diacylglycerol; pol, DNA-directed DNA polymerase (EC 2.7.7.7); dTTP, 2′-deoxythymidine 5′-triphosphate; PBS, phosphate-buffered saline; TLC, thin-layer chromatography.

☆ This work was partly supported by a Grant-in-Aid for Kobe Gakuin University Joint Research (B) (Y.M., C.M., H.I., Y.F. and H.Y.). Y.M. acknowledges Grant-in-Aid from Hyogo Science and Technology Association, The Japan Food Chemical Research Foundation and Grant-in-Aid 16710161 for Scientific Research, The Ministry of Education, Culture, Sports, Science and Technology, Japan.

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doi:10.1016/j.jnutbio.2004.08.005

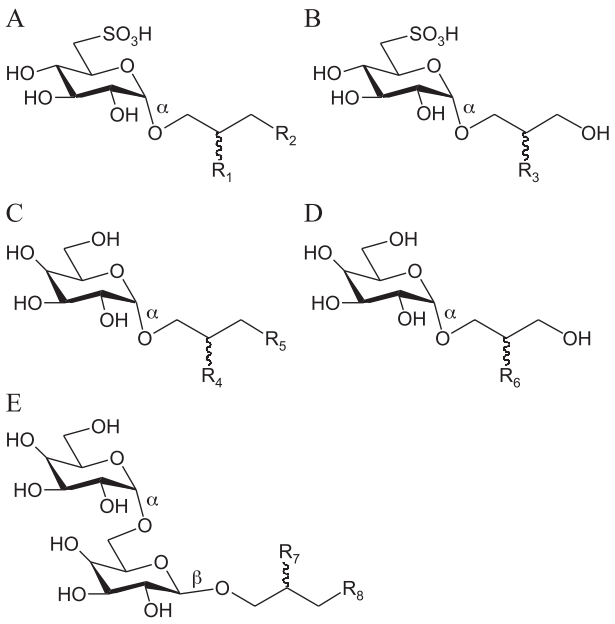

Fig. 1. Chemical structures of the glycolipids from spinach. (A) SQDG. (B) SQMG. (C) MGDG. (D) MGMG. (E) DGDG. R_1 to R_7 in these structures are fatty acids.

from a fern [14] and an alga [15,16] were potent DNA polymerase inhibitors. SQDG and SQMG were not only potent inhibitors of the DNA polymerases in vitro but were also growth inhibitors of human lung cancer in vivo [26]. Given this connection, therefore, we have widely screened for other types of natural glycolipids that show such inhibitory activities and consequently found and reported previously that some of the glycolipids from a green vegetable, spinach (*Spinacia oleracea* L.), showed these activities [27]. The glycolipid fraction from spinach was the strongest inhibitor of mammalian DNA polymerase of other vegetables' fractions of glycolipids (Mizushina et al., personal communication). In this report, we report that some of this fraction extracted from spinach leaves can inhibit mammalian cultured cell growth and in vivo solid tumor proliferation and, when hydrolyzed by pancreatic lipase, can be much more potent DNA polymerase inhibitors than the original spinach extract, possibly inhibiting mammalian cultured cell growth and in vivo solid tumor proliferation.

2. Materials and methods

2.1. Materials

Dried spinach (*S. oleracea* L.) was obtained from Shinyu Co. Ltd. (Hiroshima, Japan). Diaion HP-20 was obtained from Mitsubishi Chemical Inc. (Tokyo, Japan). Nucleotides and chemically synthesized DNA template primers such as [^{3}H]-2′ -deoxythymidine 5′ -triphosphate (dTTP; 43 Ci/mmol) and poly(dA)/oligo(dT)$_{12–18}$ were purchased from Amersham Biosciences Inc. (Buckinghamshire, UK). Porcine pancreatic lipase was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Precoated Silica-Gel 60 plates (10×20 cm, 0.25 mm layer thickness) for thin-layer chromatography (TLC) were purchased from Merck (Darmstadt, Germany). All other reagents were of analytical grade and were purchased from Nacalai Tesque Inc. (Kyoto, Japan).

2.2. Extraction and purification of glycolipid fraction and water-soluble and fat-soluble fractions from spinach

The effective purification methods of the glycolipid fraction from spinach (*S. oleracea* L.) are shown in Fig. 2. The substances containing glycolipids were extracted from dried spinach (100 g) with 90% ethanol. The extract was applied to a first Diaion HP-20 column chromatography, a hydrophobic type of chromatography, washed with 90% ethanol and then eluted using chloroform. This chloroform solution was a fat-soluble fraction (Fraction III, 1 g). The washed 90% ethanol solution was concentrated and

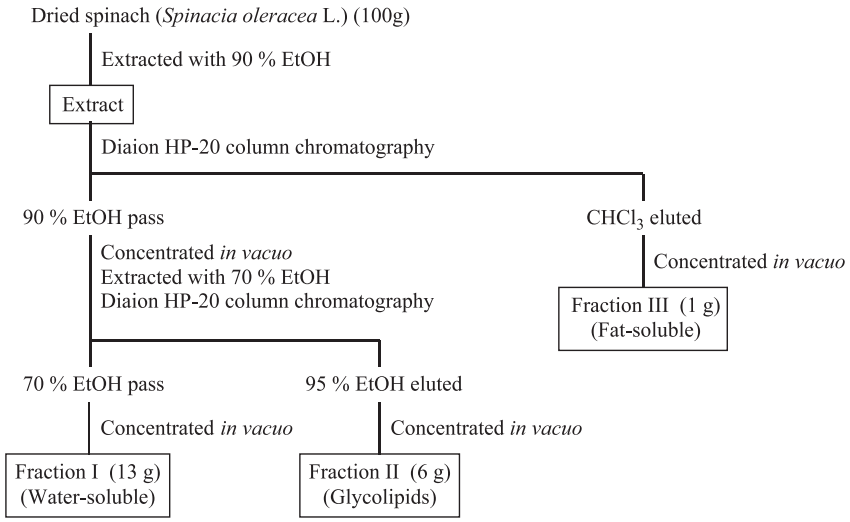

Fig. 2. The method of purification of glycolipid fraction (Fraction II) from dried spinach (*S. oleracea* L.).

dissolved in 70% ethanol. The dissolved materials were subjected to a second Diaion HP-20 column chromatography, and the column was washed with 70% ethanol. The washed solution was a water-soluble fraction (Fraction I, 13 g). The second column was then eluted using 95% ethanol. The 95% ethanol solution was a glycolipid fraction (Fraction II, 6 g).

2.3. Hydrolysis of glycolipid fraction by pancreatic lipase

Enzymatic hydrolysis of glycolipid fraction (Fraction II) from spinach was mediated by pancreatic lipase [28]. One gram of glycolipid fraction was suspended in 20 ml of 0.2 M Tris–HCl buffer (pH 7.6) containing 1 g of pancreatic lipase and 0.25 M of $CaCl_2$. The reaction mixture was incubated at 37°C for 20 min, and then 1 ml of 6 N HCl was added to the reaction mixture. Thereafter, the hydrolysates were extracted with *n*-butanol.

2.4. DNA polymerase assays

DNA-directed DNA polymerase (EC 2.7.7.7) [pol] α was purified from a calf thymus by immunoaffinity column chromatography as described previously [29]. Pol β was purified from a recombinant plasmid expressing rat pol β [30]. The activities of the DNA polymerases were measured by methods described previously [3,4]. For the DNA polymerases, poly(dA)/oligo$(dT)_{12–18}$ (A/T=2:1) and dTTP were used as the DNA template primer and the nucleotide substrate, respectively. The spinach fractions were dissolved in dimethyl sulfoxide at various concentrations and sonicated for 30 s. Four microliters of each sonicated sample was mixed with 16 μl of each enzyme (final 0.05 units) in 50 mM Tris–HCl (pH 7.5) containing 1 mM dithiothreitol, 50% glycerol and 0.1 mM EDTA and kept at 0°C for 10 min. These inhibitor-enzyme mixtures (8 μl) were added to 16 μl of each of the enzyme standard reaction mixtures, and incubation was carried out at 37°C for 60 min. One unit of each DNA polymerase activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of deoxyribonucleoside triphosphates into synthetic DNA template primers at 37°C for 60 min.

2.5. Cell culture and measurement of cell viability

A human gastric cancer cell line, NUGC-3, was obtained from the Health Science Research Bank (Osaka, Japan). The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37°C in a humid atmosphere of 5% CO_2/95% air. For the cell viability assay, NUGC-3 cells were plated at 3×10^5 cells into each well of 96-well microplates with various concentrations of the spinach fractions. These compounds were dissolved in phosphate-buffered saline (PBS) at a concentration of 10 mM as a stock solution. The stock solutions were diluted to the appropriate final concentrations with growth medium just before use. The cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay [31].

2.6. In vivo assessment of antitumor assay

Female Syrian (golden) hamsters, 5 weeks old, were purchased from Japan SLC, Inc. (Shizuoka, Japan). Hamsters receiving standard laboratory chow and water ad libitum were acclimatized for 1 week before the tumor implantation. Greene's melanoma (melanotic No. 179 cell, D_1-179) [32], considered a human melanoma counterpart biologically and pathologically, was maintained by subcutaneous transplantation of a tumor cell fragment every 2 weeks. For in vivo experiments, a D_1-179 fragment (2×2×2 mm) was subcutaneously inoculated into the right back of a Syrian hamster. The in vivo antitumor experiments were performed 8 days after the inoculation, when the diameter of the tumor mass reached about 10 mm (0.81±0.06 g) and the body weight was 99.7±10.6 g. The hamsters were divided randomly into 3 groups (n=5/group). One of the 3 groups was a control group, the subjects of which were injected with 0.25 ml of PBS alone; of the other 2 groups, one group's subjects were injected with a glycolipid fraction (Fraction II) from spinach and the other group's subjects were injected with a lipase-hydrolyzed Fraction II dissolved in PBS at a dose of 10 mg/kg. The above administrations all took place between Day 8 and Day 24 subsequent to implantation. All hamsters were injected subcutaneously nine times at 1-day intervals with these compounds and PBS alone (control). Tumor growth was measured at 1-day intervals for 26 days after implantation, and the statistics were analyzed by using Student's t test. At the end of in vivo antitumor assay, some hamsters treated with glycolipid fraction and PBS were separately examined to observe pathohistological features of the tumors and major organs such as lung, heart, spleen, stomach, liver, pancreas, kidney, intestine and brain.

3. Results and discussion

3.1. Purification of glycolipid fraction from spinach

As briefly described in the Introduction, we screened for and found many DNA polymerase inhibitors from natural resources [3–16,21–25]. Some of the natural glycolipids such as SQDG and monogalactosyl diacylglycerol (MGDG) [Fig. 1A and C] were found to inhibit the activities of DNA polymerases, whereas digalactosyl diacylglycerol (DGDG) [Fig. 1E] was found not to [14–22,27]. This is the reason we investigated the effects of the glycolipids and other compounds including dried spinach on calf pol α activity.

The components of the extract of 90% ethanol and three purified fractions from dried spinach (*S. oleracea* L.) by hydrophobic column chromatography such as Diaion HP-20 were analyzed by TLC (Fig. 3). Fractions I and III consisted

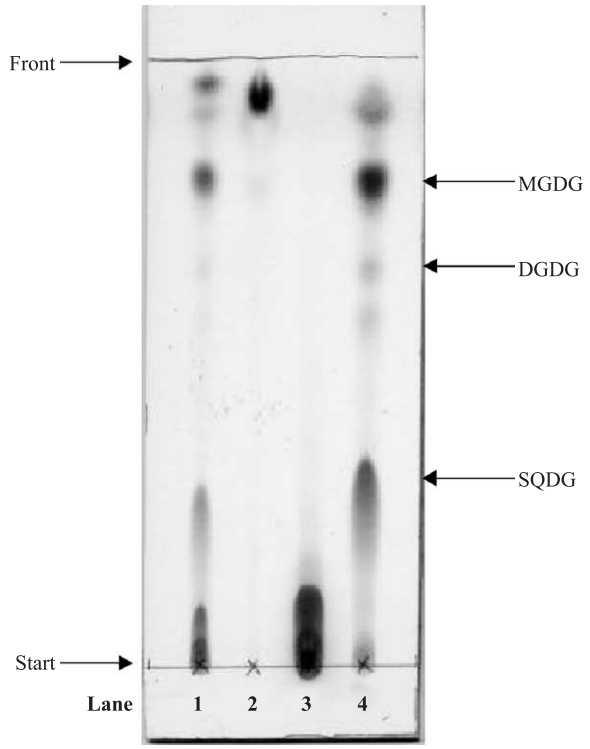

Fig. 3. Purified spinach fractions. Lanes 1, 2, 3, and 4 are extracts with 90% ethanol, Fraction III (fat-soluble fraction), Fraction I (water-soluble fraction) and Fraction II (glycolipid fraction), respectively. A photograph of TLC (chloroform/methanol=3: 1) detected by 50% sulfuric acid spray is shown.

of water-soluble and fat-soluble compounds, respectively (Lanes 3 and 2 in Fig. 3, respectively). The color of the compounds consisting of Fraction III was dark green; therefore, the major material was chlorophyll. Fraction II did not consist of water-soluble and fat-soluble materials, but had three major compounds as detected by TLC (lane 4 in Fig. 3). Each of these compounds was completely purified by silica gel column chromatography, and then the chemical structures were determined by ^{1}H-, ^{13}C- and distortionless enhancement by polarization transfer (DEPT) NMR spectroscopic analyses. These compounds were glycolipids such as MGDG (Fig. 1C), DGDG (Fig. 1E) and SQDG (Fig. 1A). The weight-percents of MGDG, DGDG and SQDG in Fraction II from dried spinach were 5.89%, 0.23%, and 2.06%, respectively, and no other glycolipid was detected. From the fatty acid analysis by gas chromatography, the major fatty acid in DGDG and SQDG was palmitic acid (16:0), and MGDG mostly consisted of stearic acid (18:0), oleic acid (18:1) and linolenic acid (18:3). The extract of 90% ethanol from the dried spinach consisted of all compounds containing Fractions I to III (Lane 1 in Fig. 3). The extract of 90% ethanol and three purified fractions such as Fraction I (i.e., water-soluble compounds), Fraction II (i.e., glycolipids) and Fraction III (i.e., fat-soluble compounds such as chlorophyll) from spinach were used in this study.

3.2. Effects of spinach fractions on the activities of pol α and human cancer cell growth

Fraction II dose-dependently inhibited calf thymus pol α activity with an IC_{50} value of 43 μg/ml and Fraction III slightly inhibited the activity of pol α, although Fraction I did not show such effects (Fig. 4). Interestingly, spinach extract had no effect on pol α, although the extract contained DNA polymerase inhibitory glycolipids such as SQDG and MGDG. There may be compounds (especially water-soluble compounds) that prevent pol α inhibitory activity by glycolipids in spinach.

To clarify the cytological effects of Fractions I, II and III, we tested their influence on human stomach cancer cell (NUGC-3) survival. The cells were incubated with spinach fractions for 48 h. As shown in Fig. 5, neither Fractions I and III nor spinach extract inhibited cell growth. On the other hand, Fraction II inhibited cell growth, and the LD_{50} value was approximately 80 μg/ml. The glycolipids in Fraction II must be able to penetrate into cells. Since the value is approximately twice the IC_{50} value on the pol α activity, the inhibition must be mostly led by the function of pol α. Fraction II also suppressed the growth of other human cancer cell lines such as HeLa (cervix) and HL-60 (peripheral blood) [data not shown].

3.3. Enzymatic hydrolysis of spinach glycolipids by pancreatic lipase

As described previously [14–22], some of glycolipids could be very strong DNA polymerase inhibitors and antitumor agents. Fraction II mainly consists of three glycolipids — MGDG, DGDG and SQDG (Fig. 3, and Lane 1 in Fig. 6). The glycolipids in Fraction II, however, are not known to be antitumor agents, although SQDG with two C18-saturated fatty acids is known as an immunosup-

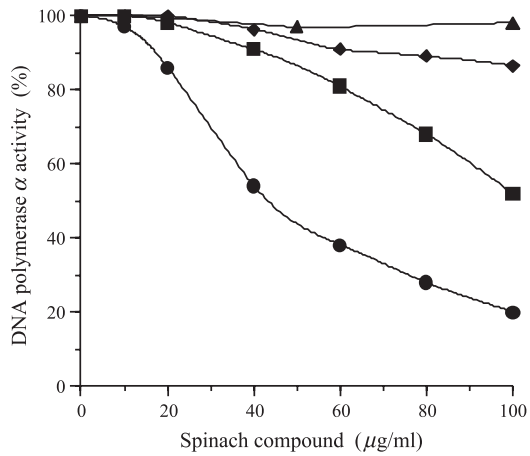

Fig. 4. Inhibition of activity of calf pol α by spinach fractions. Pol α activity (0.05 units; 5000 cpm) in the absence of the compounds was taken as 100%. The symbols and spinach compounds tested used are as follows: triangle, extract with 90% ethanol; diamond, Fraction I (water-soluble fraction); circle, Fraction II (glycolipid fraction); square, Fraction III (fat-soluble fraction).

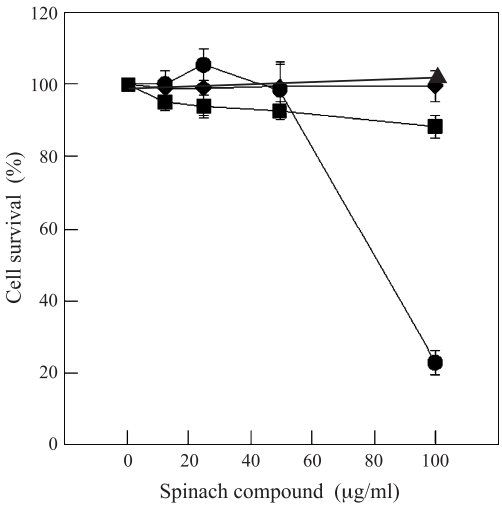

Fig. 5. Effect of spinach fractions on the proliferation of human cancer cells. Dose–responsive curves of growth inhibition of human stomach cancer cells (NUGC-3) incubated with extract with 90% ethanol [triangle], Fraction I (water-soluble fraction) [diamond], Fraction II (glycolipid fraction) [circle] and Fraction III (fat-soluble fraction) [square] for 48 h. Cell proliferation was determined by MTT assay [31]. Data are shown as means±S.E.M. of four independent experiments.

pressant [33,34]. On the other hand, SQMG, which has a molecule of fatty acid located at the 2 position in the glycerol moiety, was a potent antitumor agent [22]. Therefore, if the glycolipids in Fraction II were digested to monoglyceride forms, the activity would occur. Based on this idea, we attempted enzymatic digestion of these glycolipids including Fraction II using pancreatic lipase as described in Materials and methods. After a 20-min incubation with the pancreatic lipase, MGDG and SQDG were hydrolyzed to monogalactosyl monoacylglycerol (MGMG) and SQMG, respectively, and were almost reduced (Lane 2 in Fig. 6). The structures of MGMG and SQMG are shown in Fig. 1D and B, respectively. The produced MGMG and SQMG were purified through silica gel column chromatography, the structures were determined by ^{1}H-, ^{13}C- and DEPT NMR spectroscopic analyses and these results indicated that MGMG and SQMG had one molecule of fatty acid at the 2 position in the glycerol moiety. DGDG was not digested by the lipase (Fig. 6). The enzymatically treated Fraction II was routinely used in subsequent experiments.

3.4. Effects of the lipase-treated spinach glycolipids on the activities of mammalian DNA polymerases and the proliferation of human cancer cells

Fig. 7 shows the dose–response curves of the inhibition of Fraction II before or after pancreatic lipase treatment against calf thymus pol α and rat pol β. Pol α and pol β were used as representatives of replicative and repair polymerases, respectively, because so many species of mammalian DNA polymerase have been reported. Fraction II inhibited pol α activity but did not suppress pol β activity. However, the lipase-hydrolyzed Fraction II inhibited not only the activity of pol α but also that of pol β in a dose-dependent manner (Fig. 7). The IC_{50} values of pol α and rat pol β were 21 and 91 μg/ml, respectively. The inhibitory effect was obviously enhanced after lipase treatment. The inhibitory effect was strengthened about twofold. The dose–response curves in Fig. 7 did not change when activated DNA was used as the DNA template primer instead of a chemically synthesized DNA such as poly(dA)/oligo(dT)$_{12–18}$ (data not shown). The monoglyceride forms such as MGMG and SQMG were suggested to be better polymerase inhibitors than the diglycerides (MGDG and SQDG). The same tendency was observed in the cytotoxicity test described as follows.

Fraction II could also suppress NUGC-3 cell growth in a dose-dependent manner, and the LD_{50} value was 57 μg/ml (Fig. 8). The cell growth effect was clearly enhanced. As described previously, some of the monoglycerides from glycolipids showed antitumor activity [22]. We next focused on the effects of the lipase-hydrolyzed Fraction II in in vivo antitumor experiments.

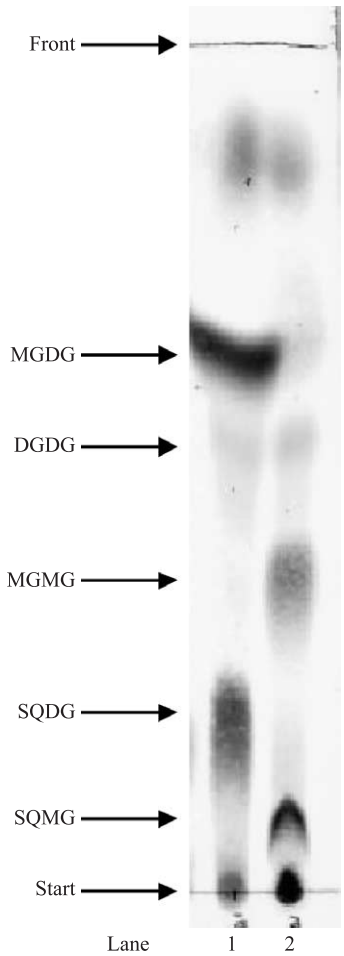

Fig. 6. Enzymatic hydrolysis of glycolipid fraction (Fraction II) from spinach by pancreatic lipase. Lanes 1 and 2 are purified Fraction II and lipase-hydrolyzed Fraction II, respectively. The enzyme reactions were described in the Materials and methods section. A photograph of TLC (chloroform/methanol=3:1) detected by 50% sulfuric acid spray is shown.

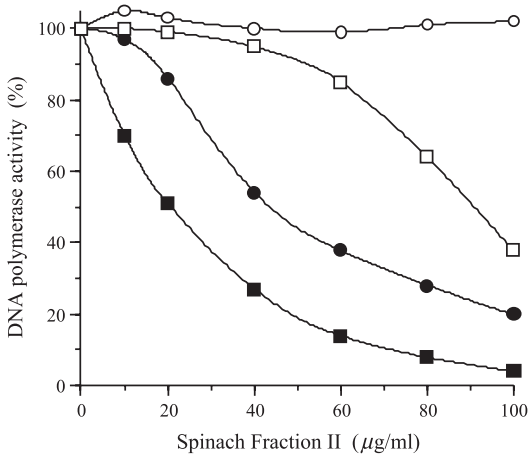

Fig. 7. Effects of the lipase-treated glycolipid fraction (Fraction II) from spinach on the activities of mammalian DNA polymerases. Pol α (black symbols; 0.05 units) and pol β (white symbols; 0.05 units) were incubated with lipase-treated Fraction II (square) and nontreated Fraction II (circle). Enzyme activity (5000 cpm each) in the absence of the compounds was taken as 100%.

3.5. Effect of the lipase-treated spinach glycolipids on in vivo antitumor assay

At 8 days after the implantation of D_1-179 cells, the hamsters bearing a solid tumor were injected with Fraction II from spinach at 1-day intervals until the 24th days. As shown in Fig. 9, both lipase-treated and nontreated Fraction II groups showed suppression of tumor growth at 26 days as compared with the control group. None of the hamsters showed any significant loss of body weight throughout the experimental period (data not shown). It was also noted that the main visceral organs such as lung, heart, spleen, stomach, liver, pancreas, kidney, intestine and brain of all the groups showed no toxic or degenerative histological appearance (data not shown).

The in vivo antitumor effect of the pancreatic lipase-hydrolyzed Fraction II was significantly stronger than that of the untreated Fraction II (Fig. 9A). To understand the mechanism of the suppressive effects at the tumor sites, we assessed the pathological findings of tumors treated with the lipase-digested glycolipid fraction in comparison with the control. The tumors treated with the lipase-hydrolyzed Fraction II showed much more extensive hemorrhagic necrosis compared as with the controls (Fig. 9B and C). This might indicate that DNA polymerase inhibition leads to the death of rapidly proliferating cells in the body and, subsequently, to the induction of necrosis.

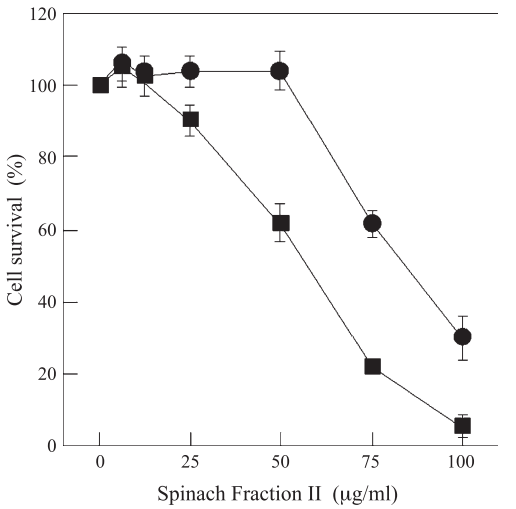

Fig. 8. Effect of the lipase-treated glycolipid fraction (Fraction II) from spinach on NUGC-3 cancer cell growth. Dose–responsive curves of growth inhibition of NUGC-3 cells incubated with lipase-treated Fraction II (square) and nontreated Fraction II (circle) for 48 h. Cell proliferation was determined by MTT assay [31]. Data are shown as means±S.E.M. of four independent experiments.

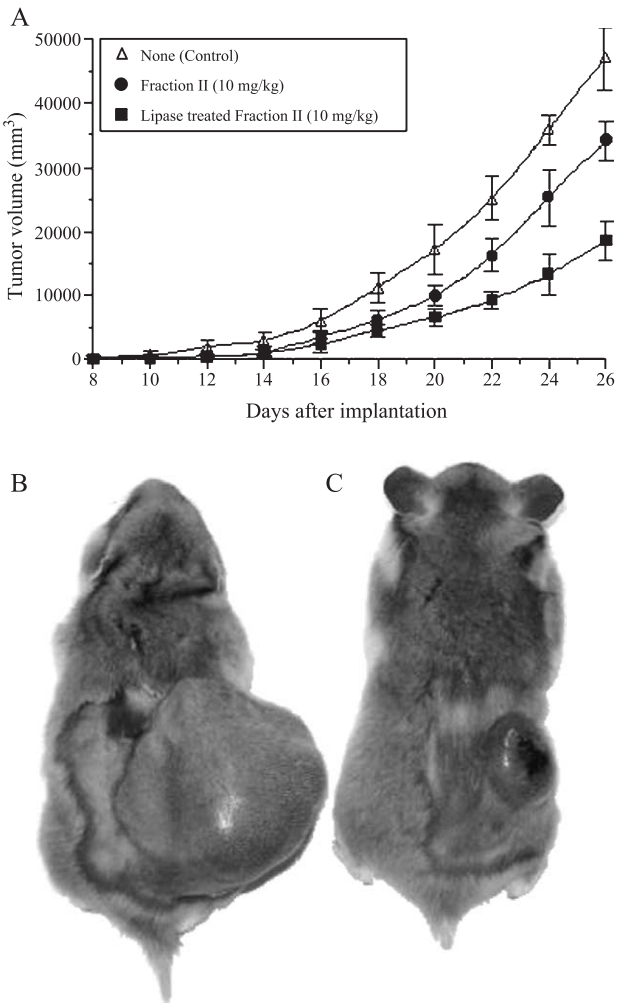

Fig. 9. In vivo study of antitumor effects of glycolipid fractions (Fraction II) from spinach. (A) The hamsters bearing D_1-179 solid tumors were injected with PBS only as a control group (white triangle), lipase-treated Fraction II (black square) and nontreated Fraction II (black circle) at a dose of 10 mg/kg. The assay method was described in the Materials and methods section. Data are shown as means±S.E.M. of five independent animals. (B, C) A photograph of hamsters bearing D_1-179 solid tumors at 26 days after implantation that were injected with PBS only (B) and 10 mg/kg of lipase-treated Fraction II (C) is shown.

To our knowledge, there is no information indicating that the ingestion of plenty of spinach as a vegetable is either cancer preventive or oncogenic. Probably, the reason must be that any of the glycolipids are rapidly converted to glucose and fatty acids in the stomach and intestine. Therefore, when these glycolipids are injected intravenously, they could be antineoplastic and perhaps oncogenic. In summary, the results of the present study indicate that the glycolipids extracted from dried spinach are useful as mammalian DNA polymerase inhibitors and anticancer materials both in vivo and in vitro.

Acknowledgments

We are grateful to Dr. Masaharu Takemura of the Nagoya University and Dr. Akio Matsukage of the Japan Women's University for preparing calf pol α and rat pol β, respectively, and for their valuable discussions concerning inhibitors. We thank Dr. Fumio Sugawara, Mr. Shinji Kamisuki, Mr. Satoshi Nakazawa and Ms. Akitsu Ogawa of the Tokyo University of Science for analyses of NMR spectroscopy.

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