Anti-cancer Effect of Spinach Glycoglycerolipids as Angiogenesis Inhibitors Based on the Selective Inhibition of DNA Polymerase Activity

N. Maeda[1,2], K. Matsubara[2,3], H. Yoshida[1,2] and Y. Mizushina*[,1,2]

[1]*Laboratory of Food and Nutritional Sciences, Department of Nutritional Science, Kobe-Gakuin University, Nishi-ku, Kobe, Hyogo 651-2180, Japan*

[2]*Cooperative Research Center of Life Sciences, Kobe-Gakuin University, Chuo-ku, Kobe, Hyogo, 650-8586, Japan*

[3]*Department of Human Life Science Education, Graduate School of Education, Hiroshima University, Higashi-Hiroshima, Hiroshima 739-8524, Japan*

Abstract: Plants contain major glycoglycerolipids, such as monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG) and sulfoquinovosyl diacylglycerol (SQDG), in the chloroplast membrane. The bioactivities of purified MGDG, DGDG and SQDG from spinach have been investigated extensively. MGDG and SQDG have been shown to inhibit the activities of mammalian DNA polymerases, but DGDG has no such inhibitory effect. The effect of these glycoglycerolipids on cancer cells, angiogenesis and solid tumor growth might be mediated *via* their inhibition of replicative DNA polymerase activities. On the basis of these findings, we discuss the mode of action of plant chloroplast glycoglycerolipids as anti-cancer therapeutic agents.

Keywords: Monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), sulfoquinovosyl diacylglycerol (SQDG), DNA polymerase (E.C.2.7.7.7), enzyme inhibitor, cytotoxicity, anti-angiogenesis, anti-tumor effect.

INTRODUCTION

Diets rich in vegetables are known to reduce the risk of cancer, suggesting that edible plants might be potential sources of anti-cancer agents. Many organisms are known to contain at least 15 types of DNA polymerase (pol) [1], which catalyzes DNA replication, repair and recombination [1, 2]. Pol inhibitors could therefore be employed as anti-cancer chemotherapy agents because they inhibit cell proliferation. On the basis of this idea, we have found many new pol inhibitors from natural compounds, in particular plant food materials, over the past 15 years. Of these, glycoglycerolipids in the class sulfoquinovosyl diacylglycerol (SQDG, 1,2-di-*O*-acyl-3-*O*-(6-deoxy-6-sulfo-α-D-glucopyranosyl-sn-glycerol) from a fern [3] and an alga [4] are particularly potent inhibitors of eukaryotic pols.

The lipid composition of thylakoid membranes in chloroplasts is highly conserved among higher plants such as spinach, algae and cyanobacteria, and comprises mainly of the following three glycoglycerolipids: monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), and SQDG [4, 5]. Here, we review results showing how these major glycoglycerolipids purified from spinach might be promising agents for cancer chemotherapy.

*Address correspondence to these authors at the Laboratory of Food and Nutritional Sciences, Department of Nutritional Science, Kobe-Gakuin University, Nishi-ku, Kobe, Hyogo 651-2180, Japan; Tel: +81-78-974-1551 (Ext. 3232); Fax: +81-78-974-5689;
E-mail: mizushin@nutr.kobegakuin.ac.jp

EFFECT OF EACH GLYCOGLYCEROLIPID FROM SPINACH ON THE ACTIVITIES OF DNA METABOLIC ENZYMES

The three major glycoglycerolipids, MGDG, DGDG and SQDG, from spinach were purified using silica gel column chromatography [5], and the purification grade of each compound was more than 98% pure. The structures of these glycoglycerolipids are shown in Fig. (**1**). The inhibitory activities of these glycoglycerolipids on pols and other DNA

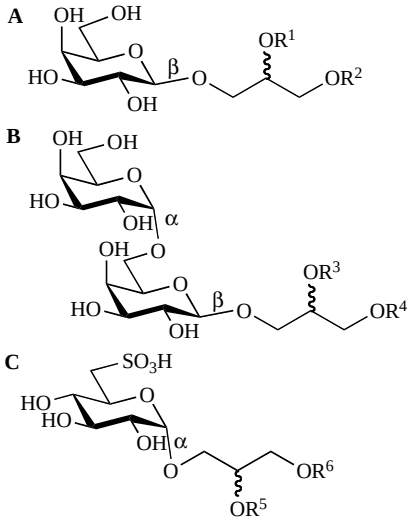

Fig. (1). Chemical structures of major glycoglycerolipids from spinach. (A) monogalactosyl diacylglycerol (MGDG), (B) digalactosyl diacylglycerol (DGDG), (C) sulfoquinovosyl diacylglycerol (SQDG). R^1 to R^6 in these structures are acyl chains.

1389-5575/11 $58.00+.00

metabolic enzymes have been investigated extensively. Pols conduct cellular DNA synthesis [1], and eukaryotic cells reportedly contain three replicative types, namely pols α, δ, and ε; mitochondrial pol γ; and thirteen repair types, namely pols β, δ, ε, ζ, η, θ, ι, κ, λ, μ, ν, REV1 and terminal deoxynucleotidyl transferase (TdT) [2]. Because pols are essential for DNA replication, repair and recombination, and subsequently for cell division, inhibition of these enzymes will lead to cell death, especially under proliferation conditions. As a result, inhibitors of eukaryotic pols should be considered potential agents for cancer chemotherapy. In the assay for pol activity, poly(dA)/oligo(dT)$_{18}$ and 2'-deoxythymidine 5'-triphosphate (dTTP) are used as the DNA template-primer and nucleotide substrate [i.e., 2'-deoxynucleoside 5'-triphosphate (dNTP)], respectively [6-8].

As shown in Fig. (**2A**), 100 μg/mL of MGDG and SQDG has been found to inhibit the activities of mammalian pols, but DGDG has no such inhibitory effect. Interestingly, MGDG significantly affects pols α, γ, δ and ε activities, but has no effect on pol β, pol λ and TdT, which are repair-related and/or recombination pols. The inhibitory effect of SQDG is more than 10-fold stronger than that of MGDG, because IC_{50} values of MGDG and SQDG for pol α were 24.0 and 1.6 μg/mL, respectively.

To elucidate the mechanism by which MGDG and SQDG inhibited pols α and β, the extent of inhibition as a function of the DNA template-primer or dNTP substrate concentration was studied (Table **1**). Lineweaver-Burk plots of the obtained data showed that the MGDG-induced inhibition of pol α activity was non-competitive with respect to

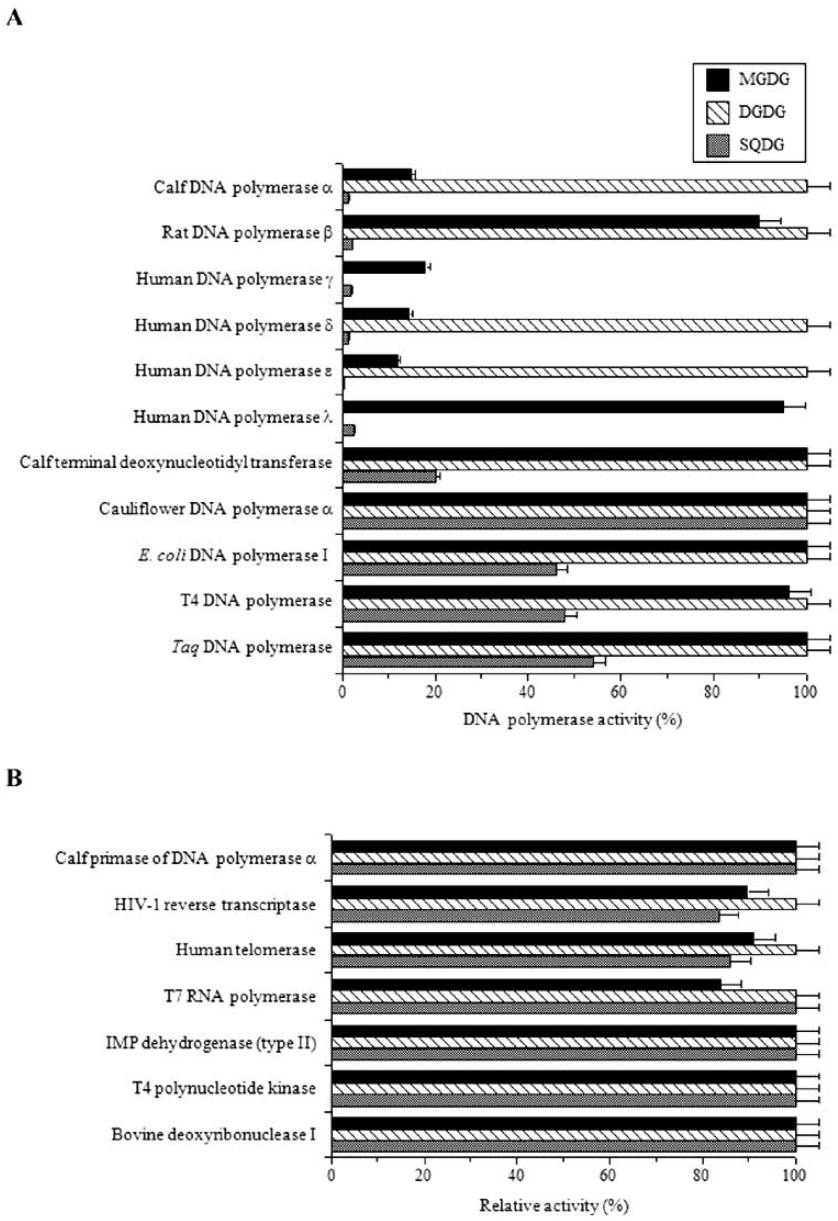

Fig. (2). Effects of the purified spinach glycoglycerolipids on the activities of various DNA polymerases and other DNA metabolic enzymes. (**A**) Pols from mammals and various species, (**B**) other DNA metabolic enzymes. MGDG, DGDG and SQDG (100 mg/mL each) were incubated with each enzyme [6-8]. % of relative activity. Enzyme activity in the absence of the compounds was taken as 100%. Data are shown as the means ± SD (n=3).

Table 1. Kinetic Analysis of the Inhibitory Effects of Spinach Glycoglycerolipids on the Activities of Calf Pol α and Rat Pol β as a Function of the DNA Template-primer Dose and the Nucleotide Substrate Concentration

Spinach glycoglycerolipid	Pol	IC_{50} (μg/ml)	DNA template-primer [a]		dNTP substrate [b]	
			Ki [c] (μg/ml)	Inhibitory mode [d]	Ki [c] (μg/ml)	Inhibitory mode [d]
MGDG	α	24.0	8.5	Non-competitive	12.4	Non-competitive
SQDG	α	1.6	0.47	Non-competitive	0.76	Non-competitive
	β	38.7	4.1	Competitive	13.7	Competitive

[a] That is, poly(dA)/oligo$(dT)_{18}$.
[b] That is, dTTP.
[c] These data were obtained from Dixon plot.
[d] These data were obtained from Lineweaver–Burk plot.

both the DNA template-primer and the dNTP substrate (Michaelis constant (K_m) was unchanged at 13.0 and 1.65 μM, respectively). Inhibition constant (Ki) values, obtained from Dixon plots, were found to be 8.5 μg/ml and 12.4 μg/ml for the DNA template-primer and dNTP substrate, respectively. The mode of inhibition of pol α by SQDG was the same as by MGDG. The inhibition of pol β by SQDG was competitive with both the DNA template-primer and dNTP substrate, since the maximum velocity (V_{max}) values were constant (111 and 62.5 pmol/h, respectively). Ki values of SQDG against pol β were found to be 4.1 μg/ml and 13.7 μg/ml for the DNA template-primer and dNTP substrate, respectively. Because the Ki value for the DNA template-primer was significantly smaller than that for the dNTP substrate, the affinity of MGDG and SQDG was greater for the enzyme-DNA template-primer binary complex than for the enzyme-nucleotide substrate complex.

These three glycoglycerolipids have no inhibitory effect on pol α from plant (cauliflower). SQDG moderately inhibits the activities of prokaryotic pols, such as *E. coli* pol I (Klenow fragment), T4 pol and *Taq* pol. On the other hand, these glycoglycerolipids do not influence the activities of other DNA-metabolic enzymes, such as calf DNA primase of pol α, human immunodeficiency virus type-1 (HIV-1) reverse transcriptase, human telomerase, T7 RNA polymerase, inosine 5'-monophosphate (IMP) dehydrogenase (type II), T4 polynucleotide kinase and bovine deoxyribonuclease I (Fig. (**2B**)). These results suggest that MGDG and SQDG are selective pol inhibitors.

EFFECTS OF SPINACH GLYCOGLYCEROLIPIDS ON HUMAN CANCER AND NORMAL CELL GROWTH

To clarify the cytological effects of purified MGDG, DGDG and SQDG from spinach, their influence on human cultured cell growth was tested by an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay [9] in which the cells were incubated with these glycoglycerolipids for 48 h. As shown in Table **2**, MGDG was found to suppress the proliferation of six cancer cell lines, and promyelocytic leukemia (HL60) cells were suppressed the most with an IC_{50} value of 39.2 μg/mL. The value is approximately 1.6 and 4-folds the IC_{50} and Ki values for pol α, respectively, of MGDG for the activities of mammalian replicative pols, such as pol α, δ and ε. On the other hand, neither DGDG nor SQDG influences human cell growth, although SQDG strongly inhibits pol activity (Fig. (**2**)). These

Table 2. IC_{50} Values of the Purified Spinach Glycoglycerolipids (i.e., MGDG, DGDG and SQDG) on the Growth of Human Cancer and Normal Cell Lines

Species of human cells		IC_{50} values of compound (μg/mL)		
		MGDG	DGDG	SQDG
Cancer cell line	A549	52.2 ± 4.3	>200	>200
	BALL-1	41.3 ± 3.4	>200	>200
	HCT116	45.9 ± 3.8	>200	>200
	HeLa	50.4 ± 4.1	>200	>200
	HL60	39.2 ± 3.2	>200	>200
	NUGC-3	50.9 ± 4.2	>200	>200
Normal cell line	HDF	>200	>200	>200

Human cancer cells, such as A549 (lung cancer cell line), BALL-1 (acute lymphoblastoid leukemia cell line), HCT116 (colon carcinoma cancer cell line), HeLa (cervix cancer cell line), HL60 (promyelocytic leukemia cell line) and NUGC-3 (stomach cancer cell line), and normal human cells, such as HDF (human dermal fibroblast), were incubated with these compounds for 48 h. Cell viability was determined by MTT assay [27]. The results were calculated as a percentage of the value obtained with untreated cells, and data are shown as the means ± SD (n=5).

three glycoglycerolipids were found to have no effect on the proliferation of human normal cell lines, such as human dermal fibroblasts (HDFs) (Table **2**).

A significant correlation has been found between MGDG content and the inhibition of replicative pols [10]; therefore, MGDG may be able to penetrate cancer cells and reach the nucleus, therein inhibiting the activities of pols α, δ and ε. As a result, the inhibition of these pol activities by MGDG may lead to selective cancer cell growth suppression.

HL-60 CELL GROWTH INHIBITORY PROPERTIES OF SPINACH MGDG

After MGDG was found to selectively inhibit the activities of mammalian replicative pols and human cancer cell growth, the effect of this compound on cell cycle regulation was investigated. When HL60 cells, which showed the most inhibition of proliferation by MGDG among the human cancer cell lines tested, were treated with 39.2 μg/mL (= IC_{50} value) of the compound for 48 h, the percentage of cells in G1-phase increased (35.3% to 61.0%) and the percentage of cells in G2/M-phase decreased (29.6% to 9.8%). The number of S-phase cells was moderately decreased through incubation (35.1% to 29.2%). This effect of MGDG on the cell cycle was investigated by determining the expression of cyclin proteins using Western blotting. Cyclin A and cyclin E proteins, which are regulated in G1/S-phase [11-14], increased with MGDG treatment, whereas cyclin B, which is regulated in G2/M-phase [15], decreased significantly. These results indicate that MGDG induces G1- and S-phase arrest in human cancer cells, suggesting that HL60 cells treated with MGDG, which inhibits the activity of replicative pols, overcome the S-phase block, divide, enter a new G1-phase and then stop cycling, being unable to replicate DNA and pass the G1/S checkpoint; thus, it is probable that MGDG may act not so much as a DNA synthesis inhibitor (like, e.g., aphidicolin) but as a mild anti-proliferative agent. Furthermore, DNA ladder formation was observed in HL60 cells treated with MGDG at its IC_{50} value (39.2 μg/mL) for 24 h. These results suggest that MGDG from spinach causes both *in vivo* cell cycle arrest and the apoptosis in HL60 cells, and that the inhibition of mammalian replicative pol activity by this compound has a strong apoptotic effect on human cancer cells.

INHIBITORY EFFECTS OF SPINACH GLYCOGLYCEROLIPIDS ON *IN VITRO* AND *EX VIVO* ANGIOGENESIS

Angiogenesis, the formation of new blood vessels from preexisting blood vessels, is deeply related to tumor growth [16], and thus the effect of purified MGDG, DGDG and SQDG from spinach on a rat model of angiogenesis has been investigated. In this model, a rat aortic ring is cultured in collagen gel, and this *ex vivo* angiogenesis model is recognized as a very useful tool for investigating angiogenesis and anti-angiogenic agents [17-20]. In the absence of glycoglycerolipids, fibroblastic fusiform cells migrate from the ends of the aortic rings after 2 to 3 days, and then spread in the collagen gel. Microvessels appear from the ends of aortic rings after 5 to 6 days, and then elongate (the control of Fig. (**3A**)). As shown in Fig. (**3A**), MGDG and SQDG were found to strongly inhibit the outgrowth of microvessels at 200 μg/mL, although the inhibitory effect of DGDG was very weak at this concentration.

To clarify the anti-angiogenic activity of spinach glycoglycerolipids, their effects have also been examined in human umbilical vein endothelial cells (HUVECs) in an *in vitro* angiogenesis model based on a HUVEC tube formation assay. Such *in vitro* angiogenesis models are widely used, and many anti-angiogenic agents exert a suppressive effect in at least one model. HUVECs form blood vessel-like structures (tube formation) on a reconstituted basement membrane (Matrigel™) as shown in Fig. (**3B**). MGDG was found to completely inhibit HUVEC tube formation at 100 μg/mL, and this inhibitory effect on HUVEC tube formation was dose-dependent (Fig. (**3C**)). On the other hand, SQDG showed a weak inhibitory effect in the assay (Fig. (**3B**) and Fig. (**3C**)). Overall, MGDG was the most effective glycoglycerolipid in this assay. SQDG shows no inhibitory effect on cell growth, although SQDG inhibited pols more potently than MGDG. (Fig. (**2**) and Table **2**). Thus, it seems that MGDG can exert a suppressive effect on HUVEC proliferation by inhibiting mammalian replicative pols. However, pols are not direct targets in the HUVEC tube formation assay; therefore, MGDG might be able to affect signal transduction pathways in endothelial cells.

ANTI-TUMOR ACTIVITY OF SPINACH MGDG, IN A MOUSE MODEL

Lastly, MGDG has also been shown to suppress the cell growth of mouse cancer cell lines, such as colon-26, with almost the same cell growth inhibitory results as cancer cell lines from humans. In this experiment, 1 x 10^6 colon-26 cells were implanted into the subcutaneous tissue of BALB/c mice. Starting five days after implantation, one group of mice received daily γ-cyclodextrin (CD) at 200 mg/kg and the second group received daily oral administration of the complex of purified MGDG from spinach and CD at 20 and 200 mg/kg, respectively. At 5, 12 and 19 days after implantation, the tumor volume was measured in all mice. As shown in Fig. (**4**), oral administration of MGDG significantly decreased colon tumor growth as compared with the control mouse tumor (49% and 51% at 12 and 19 days after implantation, respectively; $p<0.05$). None of the mice showed any significant loss of body weight throughout the experimental period, and the *in vivo* anti-tumor effect of MGDG induced no adverse drug reaction (i.e., no damage to major organs or drug-related animal death). These results suggest that *in vitro* inhibition of cancer cell proliferation and *in vivo* induction of anti-tumor activity by MGDG may be mediated through the inhibition of replicative pols.

DISCUSSION AND CONCLUSION

Thylakoid membranes in plant chloroplasts and cyanobacterial cells are unique in possessing photosynthetic electron transport and photophosphorylation systems for the conversion of light to chemical energy. The chloroplast lipid mainly comprises the following three glycoglycerolipids, MGDG, DGDG, and SQDG [4]. Several studies have reported that these glycoglycerolipids have specific biological activities, including anti-tumor-promoting, anti-inflammatory, anti-algal, hemolytic and anti-viral properties. MGDG and

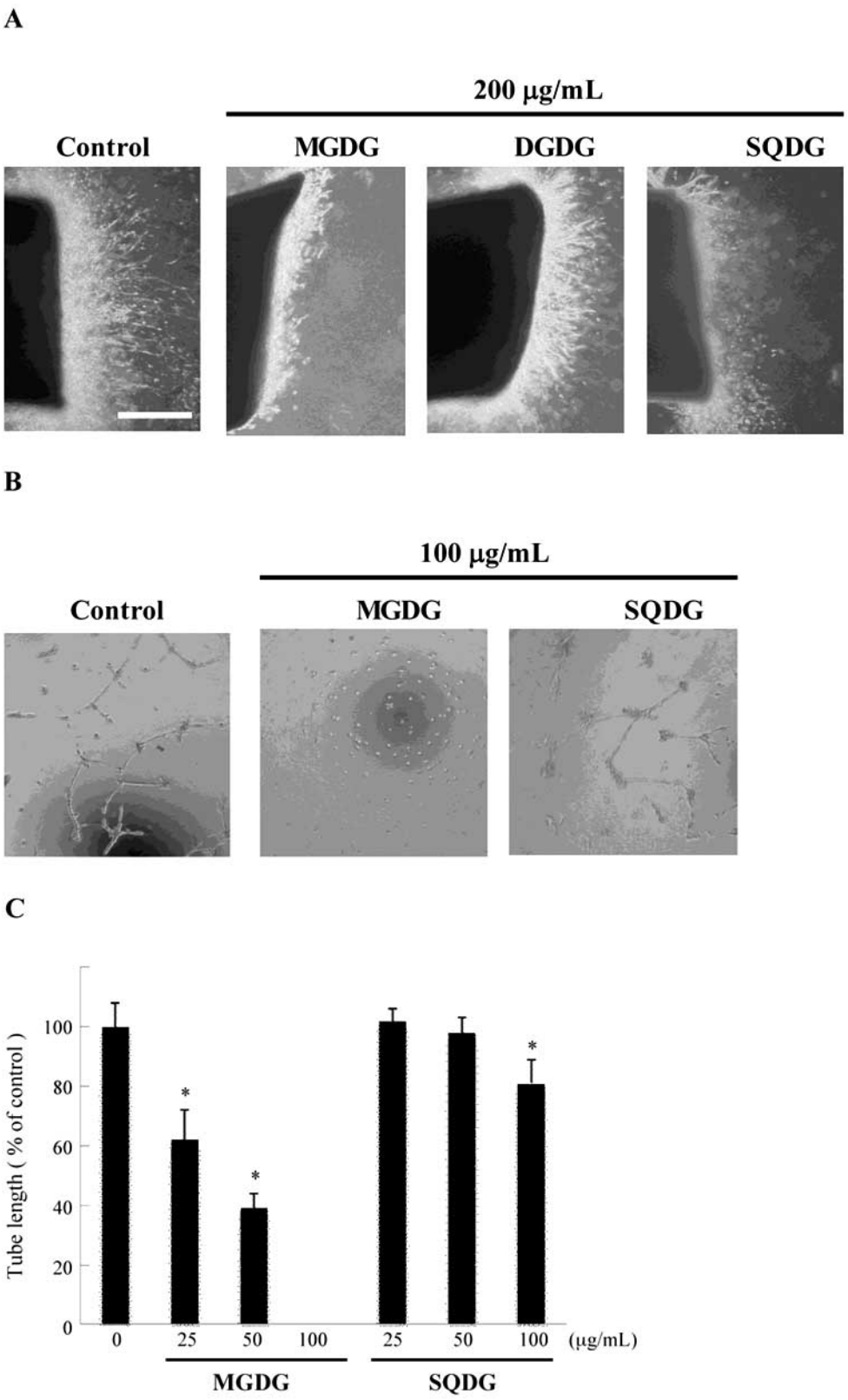

Fig. (3). Effects of the purified spinach glycoglycerolipids on angiogenesis. (**A**) Representative result of the inhibitory effect of MGDG, DGDG and SQDG (200 μg/mL each) on *ex vivo* angiogenesis. Bar equals 500 μm. (B and C) Inhibition of HUVEC tube formation by MGDG and SQDG on a reconstituted basement membrane gel. (**B**) Cells were plated on the reconstituted gel and observed 12 h later. (**C**) Capillary length was measured, and values are means ± SD (n=3). Means with an asterisk (*$p<0.01$) are significantly different from control.

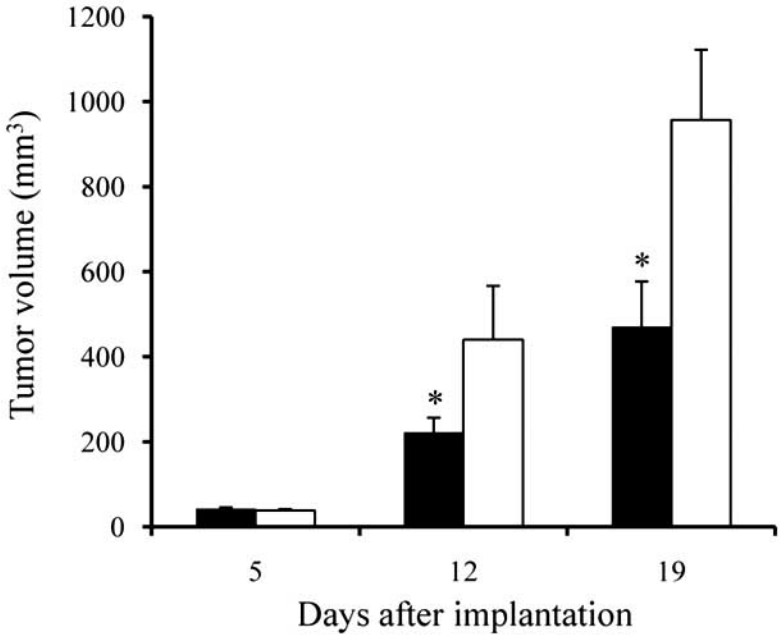

Fig. (4). *In vivo* anti-tumor effect of purified MGDG from spinach. Five days after implantation of colon-26 cells (1 x 10^6 cells) into 6 female BALB/c mice, mice were given orally administered CD (200 mg/kg [this concentration is 50 mg/mL]) (control; white bars) and MGDG + CD complex (20 mg/kg [5 mg/mL] + 200 mg/kg [50 mg/mL], respectively) (black bars) for 5, 12 and 19 days. Data are shown as the means ± SE (n=6). Means with an asterisk (*$p<0.05$) are significantly different from control.

DGDG are noncharged lipids, whereas SQDG possesses a negatively charged head group. SQDG and MGDG are potent mammalian pol inhibitors (Fig. (**2**)). SQDG consist of a sulfoquinovose, a glycerol and two fatty acids (Fig. (**1**)). Both SQDG and MGDG are glycoglycerolipids, and the two difference between MGDG and SQDG are (1) the presence of an acidic group (i.e., a $–SO_3H$ group) on the sugar and (2) the stereochemical differences at C-1 and C-4; therefore, both this acid side and the stereochemistry may be required for inhibition of pols β and λ activities.

In this review, we have described studies showing that a chloroplast glycoglycerolipid, MGDG, can selective inhibit mammalian replicative pol activity, human cultured cancer cell growth, *ex vivo* angiogenesis and *in vivo* solid tumor proliferation after oral administration. Moreover, MGDG shows anti-tumor-promoting [21, 22] and anti-inflammatory [23] actions. Thus, MGDG could have potential use as an anti-cancer therapeutic agent.

ACKNOWLEDGEMENTS

We are grateful for the donations of calf pol α, rat pol β, human pol γ, human pols δ, ε, and human pol λ by Dr. M. Takemura of Tokyo University of Science (Tokyo, Japan), Dr. A. Matsukage of Japan Women's University (Tokyo, Japan), Dr. M. Suzuki of Nagoya University (Nagoya, Japan), Dr. K. Sakaguchi of Tokyo University of Science (Chiba, Japan), and Dr. O. Koiwai of Tokyo University of Science (Chiba, Japan), respectively. This work was supported in part by a Grant-in-aid for Kobe-Gakuin University Joint Research (A), and "Academic Frontier" Project for Private Universities: matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology), 2006-2010, (H. Y. and Y. M.). Y. M. acknowledges a Grant-in-Aid for Young Scientists (A) (No. 19680031) from MEXT.

ABBREVIATIONS

CD	=	γ-cyclodextrin
DGDG	=	digalactosyl diacylglycerol
dNTP	=	2'-deoxynucleoside 5'-triphosphate
dTTP	=	2'-deoxythymidine 5'-triphosphate
HDF	=	human dermal fibroblast
HIV-1	=	human immunodeficiency virus type-1
HUVEC	=	human umbilical vein endothelial cell
IMP	=	inosine 5'-monophosphate
Ki	=	inhibition constant
MGDG	=	monogalactosyl diacylglycerol
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
Pol	=	DNA polymerase (EC.2.7.7.7)
SQDG	=	sulfoquinovosyl diacylglycerol
TdT	=	terminal deoxynucleotidyl transferase

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Received: July 22, 2010 Revised: September 20, 2010 Accepted: September 24, 2010