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Oleoyl triterpene glycoside biotransformed from ginseng suppresses growth and metastasis of murine B16-F10 melanoma *via* immunostimulation

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Abstract

The effects of 20(*S*)-protopanaxadiol 20-*O*-*β*-D-glucopyranoside (M1) and 3-*O*-oleoyl M1 (OM1) on the growth and metastasis of murine B16-F10 melanoma cells were examined in C57BL/6 mice. A single co-injection of M1 (5 mg/kg) with B16-F10 cells into the liver inhibited tumor growth at the inoculation site by 23 % (not significant compared to untreated control). In contrast, the same dosage of OM1 caused a 2.6-fold suppression of tumor growth, compared with M1 treatment ($p<0.02$). Concerning the pharmacokinetics, both M1 and OM1 were selectively taken up into the liver soon after i.v. administration (30 mg/kg). Thereafter, M1 was cleared immediately from the liver ; however, OM1 was retained in the liver at a level of more than 25 % of the administered dose for 24 h after administration. Thus, the antitumor activity paralleled the pharmacokinetic behavior. Moreover, three consecutive i.v. administrations of OM1 (30 mg/kg) inhibited the liver metastasis produced by intrasplenic inoculation of B16-F10 cells by 95 %. OM1 did not directly affect tumor growth *in vitro,* whereas it promoted tumor cell lysis by lymphocytes, particularly non-adherent splenocytes, in a concentration-dependent manner. These results suggest that fatty acid esterification of M1 potentiates the antitumor activity of the parental M1 through delay of the clearance and through immunostimulation.

Key words ginsenoside, intestinal bacterial metabolite, fatty acid triterpene ester, antitumor effect, immunostimulation.

Abbreviations M1, 20(*S*)-protopanaxadiol 20-*O*-*β*-D-glucopyranoside ; OM1, 3-*O*-oleoyl-20(*S*)-protopanaxadiol 20-*O*-*β*-D-glucopyranoside.

Introduction

Ginseng (the root of *Panax ginseng* C. A. MEYER, Araliaceae) is frequently used as an orally taken drug in the traditional medicine of China, Korea, Japan, and other Asian countries for the treatment of diseases including psychiatric and neurologic diseases and diabetes mellitus. Recent epidemiological studies have identified an association between Ginseng intake and decreased incidence and growth of tumors.[1,2] The major active components of ginseng are ginsenosides, glycosides containing an aglycone (protopanaxadiol or protopanaxatriol) with a dammarane skeleton. We have found the antitumor activities of ginsenosides, namely inhibition of tumor-induced angiogenesis and prevention of tumor invasion and metastasis.[3,4]

Orally administered ginsenosides pass through the stomach and small intestine without decomposition by either gastric juice or liver enzymes into the large intestine, where intact ginsenosides are hydrolyzed to active forms by colonic bacteria (*e.g. Prevotella oris*) and subsequently absorbed into the blood (Chart 1).[5-10] 20(*S*)-Protopanaxadiol 20-*O*-*β*-D-glucopyranoside,

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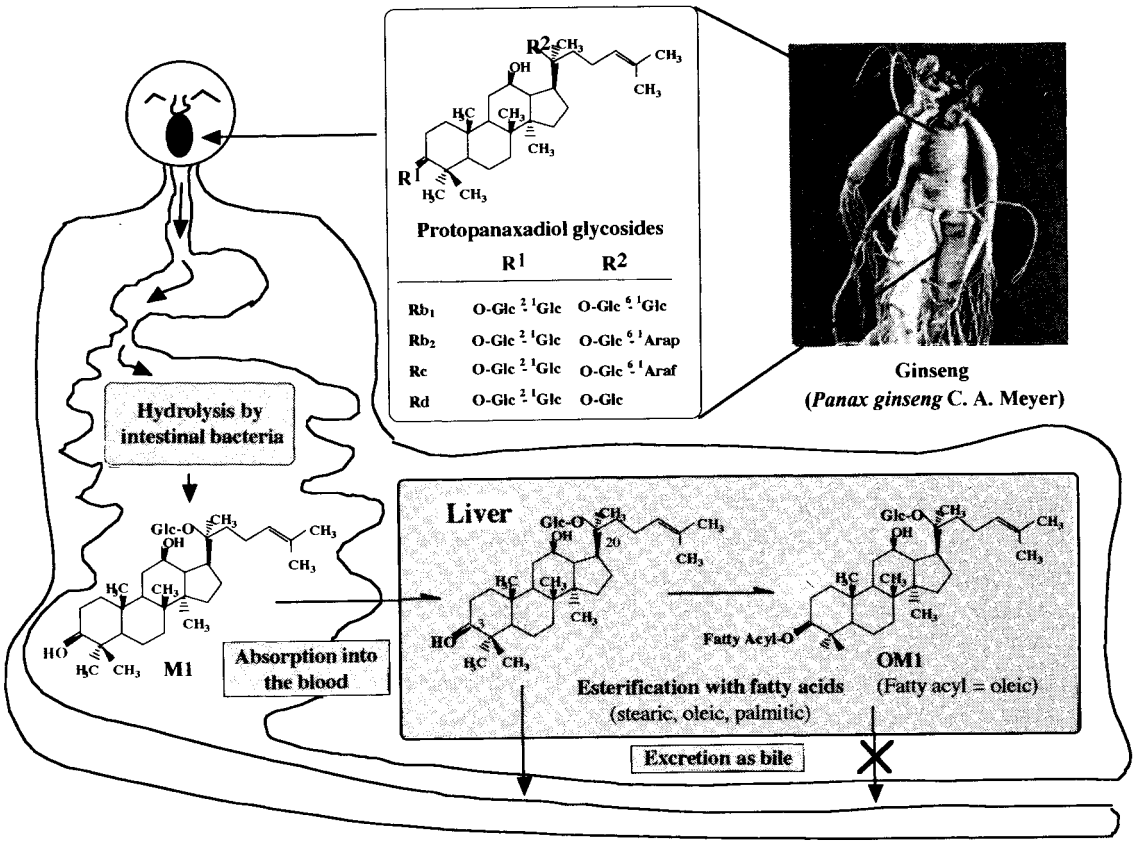

Chart 1 Metabolism of protopanaxadiol-type ginsenosides after oral administration.

referred to as M1[9,14] or compound K[5,8,15-17] is a major intestinal bacterial metabolite of protopanaxadiol-type. We showed that the antitumor effects resulting from oral administration of ginsenosides are caused by their intestinal bacterial metabolites, including M1.[11,18] In addition, incubation of tumor cells with more than 30 μM M1 induces cell cycle arrest and apoptosis within 24 h.[12]

Recently, we found that M1 is further biotransformed to the fatty acid esters, including 3-*O*-oleoyl M1 (OM1), in the liver after the oral or i.v. administration of M1 (Chart 1).[14] These esters are not excreted as M1 is, and consequently sustained in the liver longer than M1. These findings necessarily raise the question whether the fatty acyl M1 is related to the antitumor activity of ginseng. In the present study, we examined the antitumor activity of OM1 in comparison with M1, and found that OM1 possesses the antitumor activity that functions *via* the activation of immune responses.

Materials and Methods

Mice: C57BL/6 mice, colonized with intestinal anaerobes such as *Bacteroides/Prevotella* and *Lactobacillus* to an extent similar to that seen in humans,[10] were maintained and inbred in the Animal Experimental Laboratory, Itto Institute of Life Science Research in accordance with the institute's animal care guidelines. The animals were housed in plastic cages with wire tops and sawdust bedding with a 12-h light-dark cycle under conventional conditions. Food and water were supplied *ad libitum*. Mice that were 12-16 weeks of age at the start of the experiments were used.

Chemicals: M1 was prepared by fermentation of protopanaxadiol glycosides by human flora as described.[9] OM1 was synthesized by the esterification of M1 with oleic acid as reported previously.[14] Dimyristoylphoshatidylcholine (DMPC) was purchased from

NOF Co., Tokyo, Japan. M1 or OM1 was encapsulated into DMPC liposomes (drug : DMPC, 1 : 9 mol%) using the reverse-phase evaporation method as reported.[19] Liposomes were used as drug matrices in subsequent *in vivo* and *in vitro* experiments because of hematological safety.[14]

Detection of drugs in the liver : Mice were given a single i.v. injection of M1 or OM1 at a dose of 30 mg/kg (0.1 ml/20 g body weight). At the indicated times after administration, the dose recovery of M1 and OM1 in the liver was determined by the reported method.[14]

Tumor cells : A highly metastatic subline of murine B16 melanoma, B16-F10, was kindly provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging, and Cancer, Tohoku University (Sendai, Japan). The cells were maintained as monolayer cultures in RPMI 1640 medium (Nissui Pharmaceutical Co. Ltd., Tokyo) supplemented with 10 % fetal bovine serum (CSL Ltd., Parkville, VIC, Australia), 100 μg/ml streptomycin (Wako Pure Chemical Industries Ltd., Osaka, Japan), 60 μg/ml kanamycin (Wako) and 100 U/ml penicillin (Sigma, Chemical Co., St. Louis, MO) (referred to as complete medium).

Tumor growth and metastasis by intrahepatic or intrasplenic implantation of B16-F10 cells : Log-phase cultures of B16-F10 cells were harvested by trypsinization, washed, and resuspended at 1.5×10^7 cells/ml in Hank's balanced salt solution containing 1 mg/ml Matrigel (Becton Dickinson Labware, Bedford, MA). Matrigel was used to prevent the cell suspension from leaking out of the liver or spleen.[20] Intrahepatic implantation of tumor cells was performed as described by Cameron *et al.*[21] Briefly, mice were anesthetized with an i.p. injection of pentobarbital (50 mg/kg) and given a single injection of B16-F10 cells (2×10^5 cells) with or without M1 and OM1 (5 mg/kg drug/mouse) into the liver. Ten days later, the mice were sacrificed, and the tumor in the liver was removed and weighed. In other experiments, mice were given an intrasplenic injection of B16-F10 cells (2×10^5/mouse) to form multiple metastases in the liver. OM1 (30 mg/kg) was administered intravenously on days 0, 2, and 4 after tumor inoculation. The spleen with the primary tumor was removed on day 7 after inoculation and the weights of tumors and the spleen without tumors were measured. The mice were sacrificed 17 days after implantation and the number of tumor colonies in the liver was counted manually.

Preparation of lymphocytes : Lymphocytes from mice were prepared essentially as described by Goossens *et al.*[22] Briefly, the liver or spleen was passed through a stainless steel mesh, suspended in red blood cell lysis solution [0.17 M NH_4Cl, 0.01 mM EDTA, and 0.1 M Tris (pH 7.3)], and centrifuged at $100\times g$ for 6 min at room temperature. The cell pellets were washed twice with complete medium. Lymphocytes were resuspended at 5×10^7 viable cells/ml in complete medium. For culture of hepatic lymphocytes, 20 μM 2-mercaptoethanol was added to complete medium.

Cell-mediated tumor cytotoxicity assay : Log-phase cultures of B16-F10 cells were harvested by trypsinization, washed, and resuspended at 2×10^6 viable cells/ml in complete medium. Tumor cells (1×10^5/well) were cultured with hepatic or splenic lymphocytes ($1\text{-}3\times10^6$/well) in complete medium in the presence or absence of OM1 (0.1-10 μM) at 37°C in a humidified atmosphere of 5 % CO_2. Three days later, the adherent cells were harvested with trypsin and the number of viable tumor cells was visually counted under a microscope. In the other series of experiments, splenic lymphocytes (1.5×10^6) were pretreated with OM1 (0-240 μM). Two days later, the lymphocytes were fractionated into adherent and non-adherent cells and individually cultured with B16-F10 cells (2×10^4). In parallel, tumor cells were cultured with OM1 alone. One day later, the number of viable tumor cells was counted. The ability of lymphocytes to lyse tumor cells was calculated using the following formula: specific lysis (%) = (1- tested group/control group) $\times$ 100, where tested group=the number of viable tumor cells in the culture with lymphocytes in the presence or absence of OM1 ; control group=the number of viable tumor cells in the culture without lymphocytes.

Statistical analysis : The statistical significance of differences between the groups was determined by applying Student's two-tailed t-test.

Results

Antitumor activities of M1 and OM1

We investigated the antitumor effect of M1 or OM1 co-injected with B16-F10 cells in the liver of mice. The weight of tumors grown in the liver was measured on day 10 after the implantation. As shown in Fig. 1, the co-injection of M1 (5 mg/kg) with tumor cells tended to inhibit tumor growth at the inoculation site by 23 % (not significant compared to untreated control). In contrast, the treatment of OM1 at the same dosage as M1 caused a significant inhibition of tumor growth compared with either the untreated control ($p<0.002$) or M1-treated group ($p<0.02$).

Hepatic levels of M1 and OM1 after the i.v. administration

We next investigated the relationship between the antitumor efficacy of 30 mg/kg of M1 or OM1 and their levels in the liver after the i.v. administration. Consistent with our previous study,[14] M1 was selectively taken up into the liver soon after its administration, peaking within 10 min (C_{max}, 65 % recovery), and was thereafter cleared rapidly from the liver (Fig. 2). On the other hand, OM1 was also rapidly accumulated in the liver (T_{max} 40 min; C_{max}, 43 % recovery), but

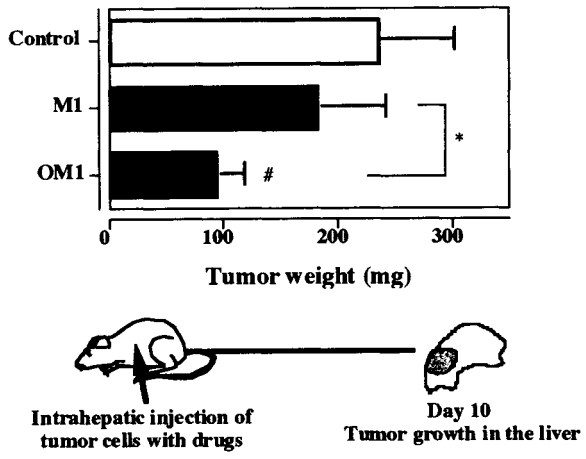

Fig. 1 Effect of M1 and OM1 on the growth of B16-F10 melanoma after intrahepatic implantation. Mice were given a single injection of B16-F10 cells with or without M1 or OM1 (5 mg/kg) into the liver. Ten days later, the mice were sacrificed and the livers were removed to measure tumor weight. Each column represents the mean±S.D. of 6 animals. #, $p<0.002$ *vs.* untreated control; *, $p<0.02$ *vs.* M1.

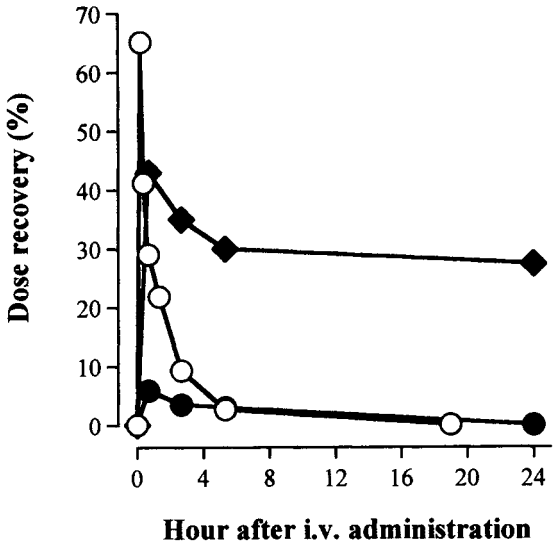

Fig. 2 Time course of the hepatic levels of M1 and OM1 after i.v. administration. Mice were given a single i.v. administration of M1 or OM1 (30 mg/kg). At the indicated times after administration, the dose recovery of M1 and OM1 in the liver was determined. Each point represents the mean value of % dose recovery from 2 to 3 animals (◆, OM1; ○, M1; ●, M1 deacylated from OM1).

the OM1 level decreased gradually with time, and over 25 % of the dose (120 μg/g wet tissue) was sustained for 24 h after administration. These results indicate that the antitumor efficacy of the drug was dependent on the hepatic accumulation. Since only a small amount of M1 resulting from deacylation of OM1 in the liver was detected, the rate of deacylation of OM1 in the liver appears to be very low. In addition, because OM1 is not excreted as bile as M1 is,[14] it must be cleared very slowly from the liver and accumulated in the liver for a longer period than M1.

Antimetastatic activity of OM1

To evaluate the antimetastatic efficacy of OM1, mice were given an intrasplenic injection of B16-F10 cells to form multiple metastases in the liver, followed by three consecutive i.v. administrations of OM1 (30 mg/kg/day). The treatment of OM1 did not show any significant changes in body weight, indicating that the dose was not excessively toxic (data not shown). OM1 treatment did not inhibit tumor growth at the inoculation site (spleen) (Fig. 3A), but markedly suppressed hepatic metastasis (Fig. 3B and 3C). In addition, the weight of spleen without tumors was significantly increased in OM1-treated mice (Fig. 3A). This

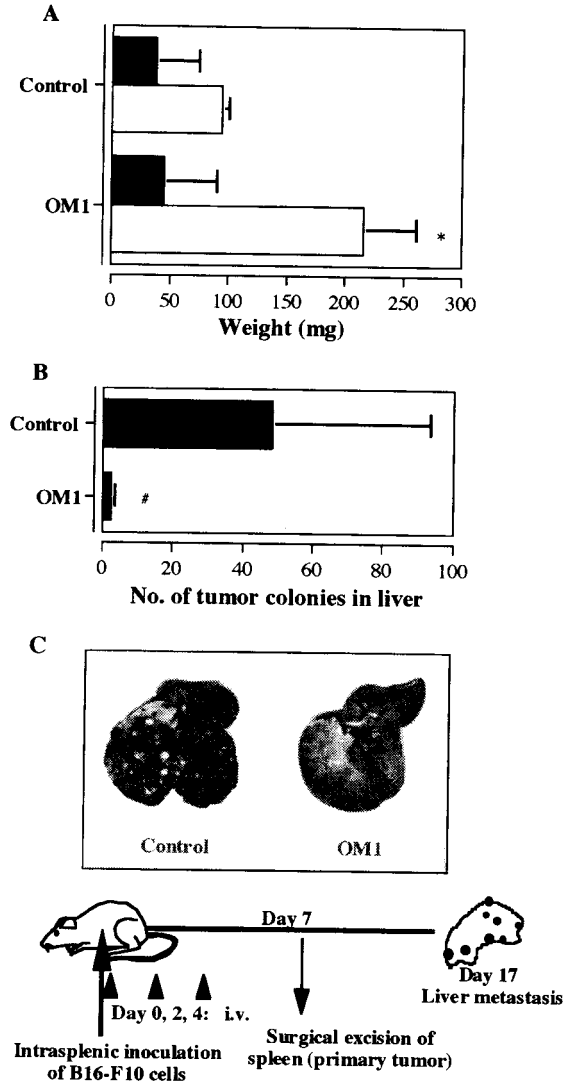

Fig. 3 Effect of OM1 on the liver metastasis produced by intrasplenic injection of B16-F10 cells. Mice were given an intrasplenic injection of B16-F10 cells. OM1 was administered intravenously (30 mg/kg) on days 0, 2, and 4 after tumor inoculation. The spleen with primary tumor was removed on day 7 of inoculation. The mice were sacrificed 17 days after implantation and the number of tumor metastasis in the liver was visually counted. Each column represents the mean±S.D. of 5 animals. *, $p<0.02$ and #, $p<0.05$ vs. untreated control.
A : Weights of tumors (closed column) and the spleen without tumors (open column) on day 7.
B : Number of tumor colonies in the liver on day 17.
C : Photographic view of hepatic metastasis.

finding implies that the suppression of metastasis may involve the stimulation of the immune system by OM1.

Activation of immune responses by OM1

To investigate whether OM1 acts as an immunostimulator, the effect of OM1 on the lymphocyte-mediated tumor cytotoxicity was examined *in vitro*. When B16-F10 cells were cultured for 3 days with hepatic or splenic lymphocytes in the presence or absence of OM1, lymphocyte-mediated tumor cell lysis increased in concentration- and E/T ratio-dependent manners (Fig. 4). To analyze the lymphocyte populations with respect to the lysis, OM1-pretreated splenocytes were fractionated into adherent and non-adherent fractions and then individually incubated with B16-F10 cells. As shown in Fig. 5, the incubation of tumor cells with the non-adherent fraction of OM1-pretreated splenocytes caused concentration-dependent inhibition of tumor growth *in vitro*, whereas the adherent fraction of the treated splenocytes did not affect the tumor cell lysis. OM1 did not show any direct cytotoxicity against tumor cells (Fig. 5), and also M1 was not detected in the conditioned medium (data not shown). These results indicate that OM1-induced inhibition of tumor growth

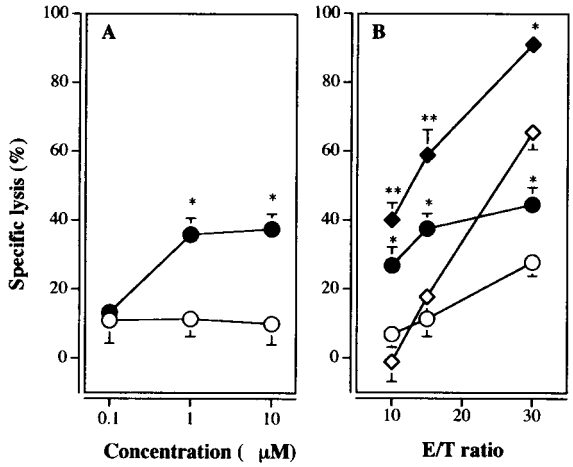

Fig. 4 Effects of OM1 on the lysis of B16-F10 cells by lymphocytes. Hepatic or splenic lymphocytes were co-cultured with B16-F10 cells in the presence or absence of OM1 (0.1-10 μM). Three days later, the adherent cells were harvested with trypsin and the number of viable tumor cells was determined by visual inspection. Each point represents the mean±S.D. of 3 dishes (○, untreated hepatic lymphocytes; ●, OM1-treated hepatic lymphocytes; ◇, untreated splenocytes; ◆, OM1-treated splenocytes). *, $p<0.01$ and **, $p<0.001$ *vs.* untreated control.
A : Tumor cell lysis by OM1-treated lymphocytes at the E/T ratio of 15.
B : Effect of E/T ratio on tumor cell lysis by lymphocytes treated or not treated with 10 μM OM1.

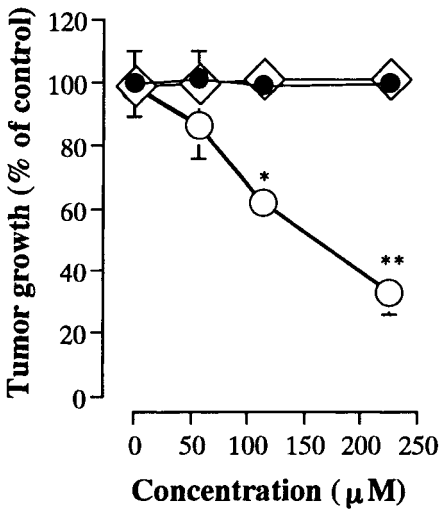

Fig. 5 Cytotoxic activity of fractionated OM1-treated lymphocytes against B16-F10 cells. Splenic lymphocytes were pretreated with OM1 (0-240 μM) for 2 days, fractionated into adherent and non-adherent cells, and individually cultured with B16-F10 cells. In parallel, tumor cells were cultured with OM1 alone. One day later, the adherent cells were harvested with trypsin and the number of viable tumor cells was determined by visual inspection. Each point represents the mean±S.D. of 3 dishes (◇, adherent cells; ○, non-adherent cells; ●, OM1 alone). *, $p < 0.01$ and **, $p < 0.002$ *vs.* untreated control.

and metastasis may be partly related to the stimulation/activation of non-adherent lymphocytes to the tumoricidal state.

Discussion

We demonstrated that fatty acid esterification of M1 resulted in a marked increase of the antitumor potential of M1 (Fig. 1), and that the antitumor activity paralleled the pharmacokinetic behavior (Fig. 2). So far, improvement of antitumor drugs by fatty acid esterification has been experimentally attempted with the aim of increasing the cellular uptake of the drugs and delaying their metabolic deamination and clearance. Breistφl *et al.*[23] reported that a fatty acid ester derivative of cytarabine is more effective for the treatment of hematological malignancies than cytarabine. Thus, the enhanced antitumor effect of OM1 may be closely associated with its persistent retention in the liver.

OM1 consists of a lipophilic moiety containing dammaranediol and oleic acid, and a hydrophilic

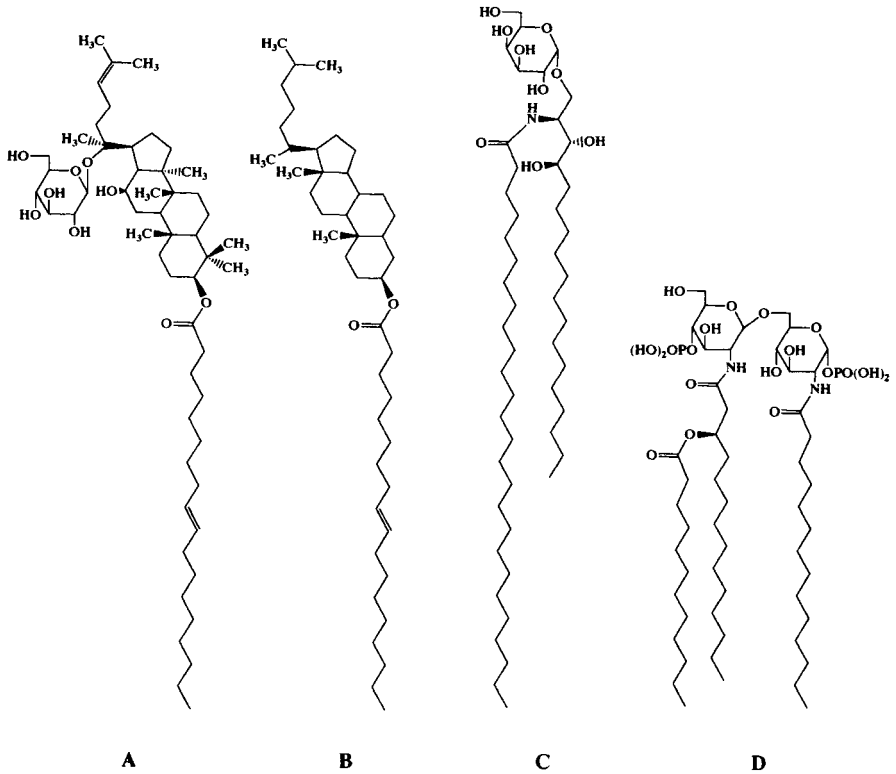

Fig. 6 Chemical structures of OM1 (A), cholesterol oleate (B), an α-galactosylceramide[25,26] (C), and a lipid A analogue[27] (D).

glucose moiety (Chart 1 and Fig. 6). The chemical structure of the dammaranediol in OM1 is very similar to that of cholesterol. Although M1 shows relatively selective cytotoxicity against tumor cells compared with normal cells *in vitro,*[11,12] excessive intracellular M1 may become toxic to normal cells. Since the esterified M1 exhibits less cytotoxicity against tumor cells than M1 *in vitro,*[14] the fatty acid esterification of M1 seems to attenuate the cytotoxicity of M1. Thus, this may indicate a detoxification reaction, just as cholesterol esterification has been shown to prevent the cytotoxicity of excessive intracellular cholesterol.[24] However, neither M1 nor cholesterol oleate (Fig. 6B) stimulated splenic lymphocytes to become cytotoxic to tumor cells (data not shown), while OM1 promoted the tumor cell lysis by lymphocytes, particularly non-adherent splenocytes, in a concentration-dependent manner (Figs. 4 and 5). Some glycolipids, such as α-galactosylceramide and a lipid A analogue (Figs. 6C and 6D), have been reported to stimulate host immune responses *via* surface molecules, including CD1, for T cell recognition of glycolipids.[25] Thus, fatty acid esterification of M1 may induce such host immune responses.

In conclusion, the present and previous studies support the following possible scenario concerning the expression of the antitumor actions of ginsenosides (ginseng) in the body. Ingested ginsenosides are hydrolyzed to active forms (*e.g.* M1) by intestinal bacteria and then absorbed from the intestine.[5,10] M1 directly exerts antitumor effects such as induction of apoptosis and cell cycle arrest;[11,12] however, much higher doses of M1 may be toxic to the host. Thus, most of the absorbed M1 is excreted rapidly, and some M1 (approximately 25 % of the dose) is esterified with fatty acids in tissues.[14] The resulting fatty acyl form, *i.e.* OM1, however, may also act as an active molecule to induce the expression of the antitumor properties of ginseng *via* its immunostimulation.

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和文抄録

薬用人参の主要成分である ginsenoside は，腸内細菌によって活性型に加水分解される。20(*S*)-Protopanaxadiol 20-*O*-β-D-glucopyranoside (M1) は，protopanaxadiol 系サポニンの主要な代謝物である。すでに，我々は M1 が脂肪酸とエステル化されることを示してきた。本研究では，C57BL/6 マウスを用いて M1 及び 3-*O*-oleoyl M1 (OM1) の薬物動態と抗腫瘍活性を調べた。これらの薬物は，静脈内投与 (30 mg/kg) 後直ちに肝臓に選択的に取り込まれた。その後，M1 は肝臓から速やかに除去されたが，OM1 は投与後 24 時間，投与量の 25 % 以上のレベルで肝臓に残留した。OM1 の脱アシル化によって生じる M1 は，投与後 6 時間まで僅かに検出され，脱アシル化率が非常に低いことが示された。薬物動態は，抗腫瘍活性に反映した。M1 (5 mg/kg) をマウスメラノーマ B16-F10 細胞と共に肝臓に注入すると，移植部位での腫瘍増殖は 23 % 抑制されたが (非投与対照との有意差なし)，OM1 処置は M1 処置に比べ 2.6 倍の抑制を示した ($p<0.02$)。さらに，癌細胞の脾内移植後 OM1 を 3 回連続して静脈内投与 (30 mg/kg) した結果，肝転移を 95 % 抑制した。OM1 は *in vitro* の腫瘍増殖に直接影響を及ぼさなかったが，リンパ球，特に非接着性細胞による腫瘍傷害活性を濃度依存的に増強した。以上の結果から，OM1 は生体内クリアランスの遅延及び抗腫瘍免疫の誘導を通して M1 の抗腫瘍効果を増強することが示唆された。

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