Prevention of Growth and Metastasis of Murine Melanoma through Enhanced Natural-Killer Cytotoxicity by Fatty Acid-Conjugate of Protopanaxatriol

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Ginsenosides, the glycosides of Panax ginseng, are metabolized (deglycosylated) by intestinal bacteria after oral administration. 20(S)-Protopanaxatriol (M4) is the main bacterial metabolite of propanaxatriol-type ginsenosides and mediates their antitumor effects. To clarify the mechanism of the M4-mediated antitumor effect, the antitumor activity and metabolism of M4 was examined, using the C57BL/6 mice implanted with B16-BL6 melanoma. The chronic oral administration of M4 inhibited the growth of B16-BL6 melanoma at the implanted site. Analyses using TLC, HPLC, MS and NMR suggest that orally administered M4 was absorbed from the small intestine into the mesenteric lymphatics followed by the rapid esterification of M4 with fatty acids and its accumulation in the tissues including the liver and lung. The administration of M4 prior to the intravenous injection of B16-BL6 cells abrogated the enhanced lung metastasis in the mice pretreated with 2-chloroadenosine more effectively than in those pretreated with anti-asialo GM1. The esterified M4 (EM4) did not directly affect tumor growth in vitro, whereas it stimulated splenic NK cells to become cytotoxic to tumor cells. These results indicate that the antitumor activity of M4 is based on the NK cell-mediated tumor lysis enhanced by EM4.

Key words ginsenoside; 20(S)-protopanaxatriol (M4); fatty acid; esterification; immunostimulation; NK cell

Ginseng (the roots of Panax ginseng C. A. MEYER, Alaricaceae) has been used as one of the most valuable traditional medicines in the Orient for over 2000 years. It contains a number of active constituents including saponins, essential oil, phytosterol, carbohydrates and sugars, organic acids, nitrogenous substances, amino acids and peptides, plus vitamins and minerals.1,2) Pharmacological and clinical studies conducted over the past 40 years have focused on radioprotective, antitumor, antiviral and metabolic effects; antioxidant activities; nervous system and reproductive performance; effects on cholesterol and lipid metabolism, and endocrinological activity.3,4) More recently, epidemiological studies have identified an association between ginseng intake and a decreased incidence and growth of cancers.5–9) The main ingredients of ginseng are ginsenosides, glycosides containing an aglycone (protopanaxadiol or protopanaxatriol) with a dammarane skeleton. Numerous researchers have contributed to the accumulation of evidence that ginsenosides are responsible for the pharmacological effects of ginseng; however, some have obtained results from direct addition of ginsenoside into cell cultures in vitro or from intraperitoneal (i.p.) or intravenous (i.v.) injection to experimental animals, though ginseng is generally taken orally. Pharmacokinetic studies have demonstrated that orally administered ginsenosides pass through the stomach and small intestine without decomposition by either gastric juice or liver enzymes into the large intestine, where ginsenosides are metabolized by colonic bacteria. Protopanaxadiol monogluco- side (M1) and protopanaxatriol (M4) are major bacterial metabolites of ginsenosides,10) and we have shown that these metabolites mediate the anticancer actions of ginsenosides.11,12) We recently have found that M1 is further biotransformed to its fatty acid-conjugates,13) which potentiate the activity of M1 through effective accumulation in the body.14) This allows us to hypothesize that M4 is also esterified with fatty acids, though Kasai et al. have reported that M4 is biotransformed to its 20,24-epoxide (protopanaxatriol oxide II, PO) by liver microsomes in vitro.15) Thus, we investigated the real active principle of M4 in the body, using C57BL/6 mice bearing B16-BL6 melanoma.

MATERIALS AND METHODS

Instruments Mass spectra were obtained on a JEOL JMS-700T spectrometer by the FAB method. NMR spectra were recorded on a JEOL JNM-LA400 spectrometer [solvent, dimethyl sulfoxide-d6 (DMSO-d6); internal standard, tetramethylsilane (TMS); chemical shifts, δ (ppm); abbreviations, d (doublet), t (triplet) and m (multiplet)].

Chemicals M4 was prepared from intestinal bacterial fermentation of propanaxatriol-type ginsenosides16) and PO from majonoside R2 of Vietnamese ginseng (Panax vietnamensis).17) Streptomycin, kanamycin and anti-asialo GM1 serum were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), penicillin from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and 2-chloroadenosine from Research Biochemicals Inc. (Natick, MA). Tween 20 (Tw) was kindly provided by NOF Co. (Tokyo). All other chemicals were of reagent grade or better.

Animals and Housing C57BL/6 mice, colonized with the intestinal anaerobes Bacteroides/Prevotella and Lactobacillus to an extent similar to that seen in humans,18) 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. The animals were fed a low-fat diet for long-term breeding.

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(MM-3, Funabashi Farm, Co., Ltd., Funabashi, Japan) and had free access to drinking water. Mice that were 12—16 weeks of age at the start of the experiments were used.

**Cell Line** A highly metastatic subline of murine B16 melanoma, B16-BL6, was kindly provided by Dr. K. Komiya, Research Center for Clinical Pharmacology, The Kitasato Institute (Tokyo). YAC-1 was generously provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging, and Cancer, Tohoku University (Sendai). These 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, and 100 U/ml penicillin (growth medium).

**Evaluation of Antitumor Activity** C57BL/6 mice were implanted subcutaneously (s.c.) with B16-BL6 cells in the right hind footpad. Diet dosed with or without M4 was administered from day −5 to day 30 or day −21 to day −1 of tumor implantation. Dosage was calculated based on the measurements of diet intake and animal body weight. Tumor growth was measured every 2—7 d and expressed as volume, which was calculated using the following formula: tumor volume (mm³) = 0.4 × (large diameter) × (small diameter)². For another lung metastasis model, mice were or were not administered orally with M4 for 5 d (5 mg/kg/d) before the i.v. injection of B16-BL6 cells (3 × 10⁶ cells/mouse). Anti-asialo GM1 serum (20 μg/mouse) or 2-chloroadenosine (50 μg/mouse) was administered i.v. at 24 h before tumor implantation. After 14 d of tumor implantation, the animals were sacrificed and the number of tumor colonies in the lung was counted manually.

**Assays for Biodistribution and Metabolism of M4** The tumor-bearing animals were sacrificed 30 d after tumor implantation. Immediately following sacrifice, contents of the gastrointestinal tract and the digestive organs were collected from 4 animals of the control or M4-treated group. The biological samples were homogenized in distilled water (10 ml) and extracted twice with 40 ml of ethyl acetate (AcOEt), with which M4 and its monoglycosides were extracted. The weights of tissues (g wet) and their AcOEt extract (mg) are summarized in the table below Fig. 2. To make sure, the residue was further extracted twice with MeOH (40 ml). Each organic solvent was evaporated to dryness and then dissolved in 0.5 ml of the same solvent for TLC analysis [TLC plates, silica gel 70 F₂₅₄, Wako Pure Chemical Industries, Ltd.; sample amount, 2 μl; developing solvent, CHCl₃–EtOH (10:1 or 50:1 v/v) or AcOEt; detection, spraying with 8% vanillin–MeOH/72% H₂SO₄ (1:5 v/v) followed by heating at 140 °C for 3 min].

**Alkaline Hydrolysis** A part (50 mg) of AcOEt extract from the contents of the small intestine of M4-treated mice was reacted with 0.1 ml of 10% NaOH–MeOH overnight. Water (0.4 ml) was added to the reaction mixture and the whole was extracted with 0.1 ml of AcOEt. An aliquot of the AcOEt layer (2 μl) was then analyzed by TLC.

**Synthesis of Oleoyl M4** M4 was chemically or enzymically esterified with oleic acid. Chemical synthesis of oleoyl M4 (10) was performed using the Schotten–Baumann method. Briefly, 100 mg (210 μmol) of M4 was dissolved in 30 ml of AcOEt and mixed with 30 ml of saturated aqueous NaHCO₃. Oleoyl chloride (12.5 mmol) was added under ice-cooling and the mixture was stirred at room temperature overnight. The reaction mixture was extracted with 10 ml of AcOEt 3 times, and the AcOEt layer was evaporated to dryness. The residue (3.26 g) was chromatographed twice over Kieselgel (70—230 mesh, Merck) 60 column (30 g and 15 g, column i.d., 2 cm) with CHCl₃ to afford oily oleoyl M4 (10, 48.0 mg, 64.8 μmol). Meanwhile, enzymic synthesis of oleoyl M4 (11) was done using a microbial lipase of Candida cylindracea (MY30, Meito Sangyo Co., Ltd., Tokyo), which can esterify various 3-hydroxy-sterols including cholesterol with fatty acids.19) Briefly, 100 mg (210 μmol) of M4 was dissolved in oleic acid (3.85 g, 13.6 mmol) and isooctane (27 ml), and mixed with phosphate buffered saline (PBS, 54 ml) with MY30 (1 g). The mixture was shaken at 37 °C overnight. The reaction mixture was subjected to the same isolation procedure as 10 to afford oily oleoyl M4 (11, 59.9 mg, 80.8 μmol). These esters showed different Rf values (10, 0.11; 11, 0.15) (TLC plate, silica gel 70 F₂₅₄, Wako; solvent, CHCl₃–EtOH, 50:1 v/v) but almost the same t₀ value of 24.5 min (HPLC conditions: column, IPG-ODS, 8—10 μm, 4.6 i.d.×150 mm, Wako Pure Chemical Industries, Ltd.; solvent, 100% MeOH; detection, UV 215 nm; column temperature, 18 °C; flow rate, 0.1 ml/min).

**Ex Vivo Incubation of M4 with Digestive Enzymes** The contents of small intestine (0.587 g) from a normal mouse were homogenized in PBS (2 ml) and 0.5 ml of M4 (5 mg/ml) dissolved in 8% Tw-PBS was added to the homogenized suspension. The mixture was shaken at 37 °C for 18 h. The reaction mixture was extracted with AcOEt (1 ml), and an aliquot of the AcOEt layer (2 μl) was then analyzed by TLC.

**Cytotoxicity Assay** Because the amount of EM4 was insufficient to be assayed in vitro, EM4 was substituted by oleoyl M4 (11). Test drugs (M4 and 11) were dissolved in DMSO and diluted by growth media so that the final concentration of DMSO in cell culture could be less than 1%. Splenocytes were obtained by pressing the spleen through a stainless steel mesh. After lysing erythrocytes with a hypotonic solution [0.17 M NH₄Cl, 0.01 mM EDTA, and 0.1 M Tris (pH 7.3)], the splenocytes (1×10⁹/well) were seeded into 96-well culture plates (Falcon 3072, Becton Dickinson Labware, Bedford, MA, U.S.A.) and cultured in growth medium (200 μl/well) with or without serial 1:2 dilutions of test drugs at 37 °C in a humidified atmosphere of 5% CO₂. After 40 h of culture, the viable cell number was counted using the trypan blue dye exclusion method.

For splenic NK activity assay, splenocytes (1—3×10⁶/well) were cultured in 96-well culture plates with round bottoms (Asahi Techno Glass, Co., Tokyo) with YAC-1 target cells (1×10⁵/well) at various effector/target (E/T) ratios in the presence or absence of test drugs (0.1—10 μM). After 40 h of culture, the number of viable tumor cells was visually counted under a microscope. The ability of splenocytes to lyse tumor cells was calculated using the following formula: specific lysis (%) = (1—tested group/control group)×100, where tested group = the number of viable tumor cells in the culture with splenocytes in the presence or absence of test compounds; control group = the number of viable tumor cells in the culture without splenocytes.

**Statistical Analysis** Student’s two-sided t-test was used
to examine the significance of difference among groups.

RESULTS AND DISCUSSION

We first examined the antitumor activity and metabolism of M4 in C57BL/6 mice implanted s.c. with B16-BL6 melanoma. M4 was administered orally to the mice from day ~5 of tumor implantation onwards. As shown in Fig. 1, the chronic oral administration of M4 (73 mg/kg/d) significantly inhibited the growth of B16-BL6 melanoma compared to the untreated control (Fig. 1). The M4-treatment caused no significant changes in animal weight, indicating that the dose was not excessively toxic.

The tumor-bearing mice were sacrificed 30 d after tumor implantation, and the contents of gastrointestinal tract and the digestive organs (biological samples) were analyzed in detail by TLC and HPLC. The typical TLC profiles are summarized in Fig. 2. As shown in Fig. 2B, M4 was detected in the diet dosed with M4 (lane 1+), the stomach (lane 2+), the large intestine (lane 4+), the feces (lane 5+) and the mesenteric lymph tissues (lane 7+), whereas it was not detected in either the small intestine (lane 3+), the blood (lane 6+), the liver (lane 8+) or the lung (lane 9+). Based on the comparison of biological samples between the M4-treated and untreated animals using various solvent systems, the new band at the Rf value of 0.44 (termed EM4) was found to exist in the small intestine (lane 3+), the mesenteric lymph tissues (lane 7+, though the band was twisted), the liver (lane 8+) and the lung (lane 9+), but not in the blood (lane 6+). Although the band of EM4 appears to exist in the diet dosed with M4 (lane 1+), precise TLC analyses have denied such possibility (data not shown). Furthermore, EM4 did not result from ex vivo incubation of M4 in the contents of small intestine from untreated mice (data not shown). These results suggest that orally administered M4 is completely absorbed from the small intestine and transferred mainly into the mesenteric lymphatics followed by formation of EM4 and its spread to other organs in the body and excretion as bile. When M4 was injected into the tail vein of mice, it was undetectable in the blood or liver even 3 min after administration (data not shown), so the metabolic reaction seems to progress quickly in the body. M4 appeared again in the large intestine (Fig. 2B, lane 4+), suggesting the deacylation of EM4 by colonic bacteria. Some might enter the enterohepatic circulation and potenti ate the antitumor activity of M4. Further studies on the pharmacokinetics of M4 are warranted.

Alkaline treatment of the contents of small intestine from M4-administered mice resulted in M4 (Fig. 2D, lane 3#), indicating that EM4 is ester. Because oleic acid (OA) was rich in the diet and all the tissues tested (Fig. 2B), the oleic acid esters of M4 were synthesized and compared with EM4. On a TLC plate, the Rf value of EM4 was consistent with that of the enzymically synthesized M4 oleate (11) (Fig. 2E, lanes 11 and 3+). HPLC analysis showed the same tR value of 24.5 min between EM4 and 11. Therefore, EM4 was considered identical to 11, however, it was not isolated in sufficient amounts to be analyzed for MS and NMR. The linkage posi-

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**Fig. 1.** Effect of Oral Administration of M4 on the Growth of B16-BL6 Cells Implanted in the Footpad

Female C57BL/6 mice were inoculated s.c. with B16-BL6 cells (1×10⁶ cells/mouse) in the hind footpad. The diet dosed with or without 0.05% M4 was administered from day −5 to day 30 of tumor implantation. Tumor growth was measured every 4−7 d and expressed as volume (mm³). The results represent the mean±S.E.M. of 4 mice per group. *p<0.05; **p<0.01 vs. control by Student’s two-sided t-test.

**Fig. 2.** TLC Profiles of Distribution and Metabolism of M4 in the Gastrointestinal Tract and Digestive Organs after Its Oral Administration

The tumor-bearing animals were sacrificed on day 30 after tumor implantation, and the contents of gastrointestinal tract and the digestive organs were extracted with AcOEt. The weights of tissues (g wet) and their AcOEt extract (mg) are summarized in the table below the figure. The AcOEt extracts (tissue samples) were analyzed by TLC (TLC plate, silica gel 70 F254 developing solvent, CHCl₃–EtOH (10 : 1 v/v) (A, B and D), AcOEt (C) or CHCl₃–EtOH (50 : 1 v/v) (E); plate A, TLC data from control animals (lane 1, diet; lane 2, stomach; lane 3, small intestine; lane 4, large intestine; lane 5, feces; lane 6, blood; lane 7, mesenteric lymph tissues; lane 8, liver; lane 9, lung; lane M4, M4); plate B, TLC data from the small intestine and transferred mainly into the mesenteric lymphatics followed by formation of EM4 and its spread to other organs in the body and excretion as bile. When M4 was injected into the tail vein of mice, it was undetectable in the blood or liver even 3 min after administration (data not shown), so the metabolic reaction seems to progress quickly in the body. M4 appeared again in the large intestine (Fig. 2B, lane 4+), suggesting the deacylation of EM4 by colonic bacteria. Some might enter the enterohepatic circulation and potenti ate the antitumor activity of M4. Further studies on the pharmacokinetics of M4 are warranted.

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tion and number of oleic acid(s) to M4 in EM4 were determined using 11. Low resolution FAB-MS of 10 and 11 in the presence of added NaCl produced the same strong ion at m/z 763 corresponding to the pseudomolecular ion [C₄₈H₈₄O₅Na⁺]. Furthermore, high resolution FAB-MS gave ions at m/z 763.6248 (C₄₈H₈₄O₅Na⁺; theoretical m/z 763.6217) for 10 and 763.6202 (C₄₈H₈₄O₅Na⁺; theoretical m/z 763.6216) for 11. These data indicate that both esters are mono-esters of M4 with oleic acid. In the 13C- and 1H-NMR spectra of 10 and 11, carbon and proton signals were assigned by 1H–1H shift correlation spectroscopy (COSY), heteronuclear multiple-quantum correlation (HMQC) and heteronuclear multiple-bond correlation (HMBC) measurements. Although the C-6 and C-20 carbon signals remain almost unchanged, the C-3 and C-12 carbon ones differ between the two (Table 1). Such differences are also observed in the 1H-NMR spectrum. The downfield shifts of C-12 car–bon and H-12 proton signals in 10 or C-3 carbon and H-3 proton signals in 11 show that 10 is 12-O-oleoyl M4 and 11 is 3-O-oleoyl M4. Therefore, the metabolic reaction of M4 in the body may be catalyzed by an enzyme with similar mechanical properties to the microbial lipase MY30 produced by Candida cylindracea.

Although Kasai et al. have reported that the oxidation of M4 by a rat liver microsomal fraction affords its 24,25-epox-
iderivative of the microbial lipase MY30 produced in the body may be catalyzed by an enzyme with similar mechanical properties to the microbial lipase MY30 produced by Candida cylindracea. Although Kasai et al. have reported that the oxidation of M4 by a rat liver microsomal fraction affords its 24,25-epoxide (PO),15) PO was undetectable in the liver from M4-treated rats by a rat liver microsomal fraction affords its 24,25-epoxide. It is possible that PO reacts with cytochrome P450 and is oxidized to a metabolite similar to cholesterol. This concept is partly supported by the inhibitory activity of M4 against acyl CoA: cholesterol acyltransferase (ACAT),23) In addition, Tabas et al. found the triterpene esters containing 80% stearate and 20% palmitate in the liver of rabbits and humans,22) and hypothesized that the origin of fatty acid triterpene esters may be via dietary absorption of plant triterpenes followed by fatty acid esterification of the triterpene in animal tissues that is catalyzed by acyl CoA: triterpene acyltransferase which differs from ACAT.

The oral administration of M4 at low doses (3—13 mg/kg/d) prior to tumor implantation also prevented the growth of B16-BL6 melanoma at the implanted site in both male and female mice (Fig. 3). This suggested that M4 induced host-mediated responses required to inhibit tumor growth. Such responses are involved in immune cells. NK cells destroy tumor cells by injecting perforin into target cells,24) and macrophages kill tumors through activation of T cells. Anti-asialo GM1 serum and 2-chloroadenosine can selectively eliminate NK cells and macrophages, respectively.25) In the lung metastasis produced by i.v. implantation of B16-BL6 cells, the treatment with anti-asialo GM1 serum or 2-chloroadenosine before tumor implantation increased the number of tumor metastatic colonies in the lung by 5 fold or more.

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Table 1. 13C-, 1H-NMR Data of Hydroxy Carbons and Protons Involved in the Oleoyl Derivatives of M4

a) Measured in DMSO-d₆ at 400 MHz. Chemical shifts are in δ (ppm) and J values are in Hz.

Fig. 3. Effects of Oral Administration of M4 before Tumor Implantation on the Growth of B16-BL6 Cells Implanted into the Footpad

Male (A) or female (B) C57BL/6 mice were given the diet dosed with or without M4 (3.0, 5.5 or 13.3 mg/kg/d) for 3 weeks and then inoculated s.c. with B16-BL6 cells (4×10⁵ cells/male, 3×10⁵ cells/female) in the hind footpad. Tumor growth was measured every 2—4d and expressed as volume (mm³). The results represent the mean±S.E.M. of 6 (A) or 7—8 (B) mice per group. * p<0.05; ** p<0.01 vs. control by Student’s two-sided t-test.
The animals were sacrificed and the number of tumor colonies in the lung was counted manually. The results represent the mean±S.D. of 3 dishes (106/well) and YAC-1 cells (105/well) were co-cultured with or without M4 or EM4. The variation of concentrations from 0.1 µM to 100 µM of drug at the E/T ratio of 20; B, variation of E/T ratios from 10 to 40 at the drug concentration of 10 µM. *p<0.01; **p<0.001 vs. control by Student’s two-sided t-test.

These results clearly indicate that, if the esterification of M4 against B16-BL6 melanoma through cell-mediated tumor lysis enhanced by EM4. This may offer a clue to the mechanism of other pharmacological actions of ginseng.

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REFERENCES AND NOTES


