An intestinal bacterial metabolite (M1) of ginseng protopanaxadiol saponins inhibits tumor-induced neovascularization

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Abstract

The present study demonstrated that an intestinal bacterial metabolite (M1) of protopanaxadiol-type ginsenosides significantly inhibited the growth of implanted tumor and the intrahepatic metastasis by the implantation of a small fragment of colon 26-L5 tumor into the liver when it was administered orally. These findings indicates that M1 was effective for the inhibition of the growth and metastasis of colon26-L5 cells in addition to lung metastasis of B16-BL6 melanoma cells as have been reported previously. The conditioned medium of colon 26-L5 cells (CM-L5) induced *in vitro* tube formation of hepatic sinusoidal endothelial (HSE) cells on Matrigel-coated substrates, which is considered to be an important step in the processes of tumor angiogenesis. This activity of CM-L5 was abrogated by noncytotoxic concentrations of M1 in a concentration-dependent manner. Similarly, M1 eliminated the ability of CM-L5 to promote the migration of HSE cells concentration-dependently. These findings indicate that M1-induced inhibition of tumor growth and intrahepatic metastasis may be partly related to the suppression of tumor angiogenic responses including capillary tube formation and migration of HSE cells.

Key words *Panax ginseng,* ginsenosides, intestinal bacterial metabolite, metastasis, angiogenesis, hepatic sinusoidal endothelial cell.

Introduction

Ginseng (the root of *Panax ginseng* C. A. MEYER, Araliaceae) has been used in traditional medicine in China, Korea, Japan and other Asian countries for the treatment of various diseases, including psychiatric and neurologic diseases as well as diabetes mellitus. So far, ginseng saponins (ginsenosides) have been regarded as the principal components responsible for the pharmacological activities of ginseng. Ginsenosides are glycosides containing an aglycone (protopanaxadiol or protopanaxatriol) with a dammarane skeleton and have been shown to possess various biological activites, including the enhancement of cholesterol biosynthesis, stimulation of serum protein synthesis, immunomodulatory effects, and anti-inflammatory activity.[1-7] There are many reports on antitumor effects of various ginsenosides, particularly the inhibition of tumor invasion and metastasis.[8-10]

Previously, it has been reported that protopanaxadiol-type ginsenosides such as Rb_1, Rb_2 and Rc are metabolized by intestinal bacteria after oral administration to their derivative 20-O-β-D-glucopyranosyl-20 (*S*)-protopanaxadiol, which is referred to as M1[11] or compound K,[12,13] as shown in Fig. 1. When Rb_1 was

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administered orally into C57BL6 mice, M1 was found in serum for 24 h, but Rb_1 was not detectable.[14] Thus, our pharmacokinetic and antimetastatic studies demonstrated that expression of the *in vivo* antimetastatic effect of the protopanaxadiol-type ginsenosides was primarily based on their metabolite M1 resulted from the orally administered ginsenosides.[14,15] We also found that anti-proliferative activity of M1 against tumor cells was partly associated with the induction of apoptosis.[16]

Tumor angiogenesis is one of the most important events concerning tumor growth and metastasis.[17] The angiogenic response is considered to be composed of a series of sequential steps that endothelial cells degrade the surrounding basement membrane, migrate into the stroma, proliferate and finally differentiate to give rise to new capillary vessels.[18] The induction of tumor angiogenesis is believed to reflect a balance between positive and negative regulatory factors.[19]

In the present study, we investigated the effect of M1 on the solitary tumor growth at the implanted site and intrahepatic metastasis by the implantation of colon 26-L5 tumor fragment into the liver.[20] We also examined whether M1 inhibited tumor-induced neovascularization and migration of hepatic sinusoidal endothelial (HSE) cells *in vitro*.

Materials and Methods

Chemicals: Ginsenoside-Rb_1 was isolated from the ginseng extract according to the reported procedures,[21] and their major intestinal bacterial metabolite M1 was the same as that described previously.[11] For *in vitro* experiments, M1 was dissolved in dimethyl sulfoxide at a concentration of 100 mM for a stock solution and kept at -20°C until use. The chemical structures of ginsenoside-Rb_1 and its metabolic compound M1 are shown in Fig. 1.

Cell line: The liver metastatic cell line of the colon26 carcinoma (colon26-L5) was obtained by the *in vivo* selection method.[22] Colon26-L5 cells were maintained as monolayer cultures in RPMI-1640 supplemented with 10 % fetal bovine serum (FBS) and L-gulutamine at 37°C in a humidified atmosphere of 5 % CO_2/95 % air. Hepatic sinusoidal endothelial (HSE) cells were kindly provided by Dr. G. L. Nicolson (M.D. Anderson Cancer Center, USA) and were maintained in Dulbecco's Modified Eagle Medium (DMEM)/F12 supplemented with 5 % FBS, L-glutamine, and 0.1 mg/ml endothelial mitogen in a humidified atmosphere of 5 % CO_2 at 37°C.

Mice: Specific pathogen-free female BALB/c (5-6 weeks old) mice were purchased from Japan SLC Inc. (Hamamatsu). They were maintained in the Laboratory for Animal Experiments, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, under laminar air-flow conditions. This study was conducted in accordance with the standards established by the Guideline for the Care and Use of Laboratory Animals of Toyama Medical and Pharmaceutical University.

In vivo anti-tumor activity against intrahepatic growth of colon 26-L5 tumor: A fragment of colon

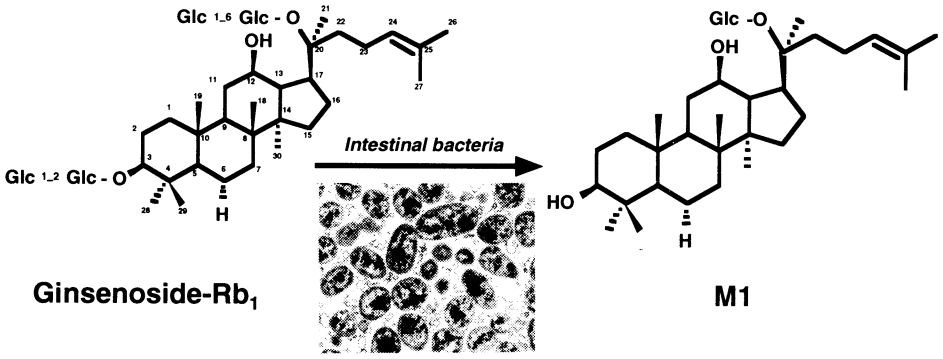

Fig. 1 Structure of ginsenoside-Rb_1 and its metabolic compound M1. Ginsenoside Rb_1 is hydrolyzed to M1 by intestinal bacteria, particularly *Prevotella and Eubacterium*. Electron micrograph, a thin section of an isolate (*Prevotella oriss*12) (×60000).

26-L5 tumor (~1 mm^3) was implanted in the left lobe of the liver of BALB/c mice to form a single tumor nodule in the liver as described previously.[20] M1 was administered *p.o.* for 14 days, beginning on day 1 after tumor implantation. The mice implanted with colon 26-L5 tumor were sacrificed on day 15. The tumor in the liver was removed and weighted. Intrahepatic metastasis was evaluated by counting the number of liver tumor colonies. The results represent mean ± S.D. of 10 mice per group.

Preparation of conditioned medium of colon 26-L5 cells (CM-L5) : Colon 26-L5 cells (1×10^5/well) were seeded into 6-well plates and incubated for 24 h. The medium was replaced with fresh RPMI 1640 with 5 % FBS in the absence or presence of M1. After 24 h of incubation, the medium was harvested and filtered through a 0.2-μm pore membrane.

Assay for in vitro tube formation by HSE cells induced by CM-L5 : HSE cells (1×10^3/well) were incubated for 4 h on 48-well plates coated with Matrigel (Collaborative Biochemical Products, Inc.) with 50 % CM-L5, which was prepared in the absence or presence of M1 (2.5, 5, or 10 μM), as described previously.[20] The cultures were fixed with 2.5 % glutaraldehyde and stained with hematoxylin to observe morphological changes.

Assay for the migration of HSE cells induced by CM-L5 : The migration of HSE cells was assayed in Chemotaxicell chamber (Fig. 4, Kurabo Co. Ltd., Osaka, Japan) as described previously.[23] The filters (8.0 μm pore size) of the chamber were coated with 10 μg/50 μl of gelatin on the lower surface, before being dried at room temperature. HSE cells (1×10^5/chamber) were added to the upper compartment of the chamber and incubated with CM-L5 treated with various concentrations of M1 in the lower compartments. After 2-h incubation, the cells that had migrated to the lower surface were determined by crystal violet staining. Each assay was performed in triplicate cultures.

Statistical analysis : The statistical significance of differences between the groups was determined by applying Student's two-tailed *t*-test. Statistical significance was defined as $p<0.05$.

Results

Effect of M1 on the growth of the inoculated tumor and intrahepatic metastasis after the implantation of colon 26-L5 tumor fragment into the liver

We first examined the effect of M1 on the tumor growth and metastasis caused by intrahepatic implantation of a small fragment of colon 26-L5 tumor. Oral administration of M1 (1 or 10 mg/kg) for 14 days

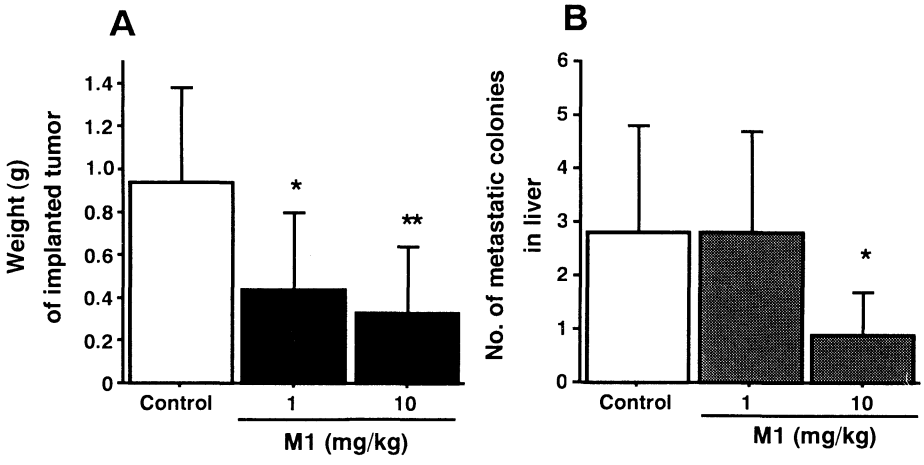

Fig. 2 Effect of oral administration of M1 on the growth of the inoculated tumor and intrahepatic metastasis by the implantation of a fragment of colon 26-L5 tumor. A small fragment of colon26-L5 tumor was implanted in the left lobe of the liver in female BALB/c mice. M1 were given orally for 14 days. Mice were sacrificed on day 15 after the implantation, and then the weight of implanted tumor was measured and the number of tumor colonies in the liver were manually counted. *, $p<0.05$; **, $p<0.01$ compared with untreated control by Student's *t*-test.

resulted in a significant inhibition of the growth of implanted tumors as compared with untreated control (Fig. 2). M1 at the dose of 10 mg/kg markedly inhibited the intrahepatic metastasis. These results indicate that oral administration of M1 (or ginsenosides) was effective at inhibiting intrahepatic metastasis of colon 26-L5 tumor as well as lung metastasis of B16-BL6 melanoma as had been reported previously.[14]

Effect of M1 on tube formation by HSE cells induced by CM-L5 in vitro

We examined the effect of M1 on the formation of capillary-like structures by endothelial cells *in vitro*. The incubation of HSE cells with 50 % CM-L5 in Matrigel-coated wells caused tube-like formations by HSE cells within 4 h (Fig. 3). Addition of CM-L5 obtained from the culture of colon 26-L5 cells with

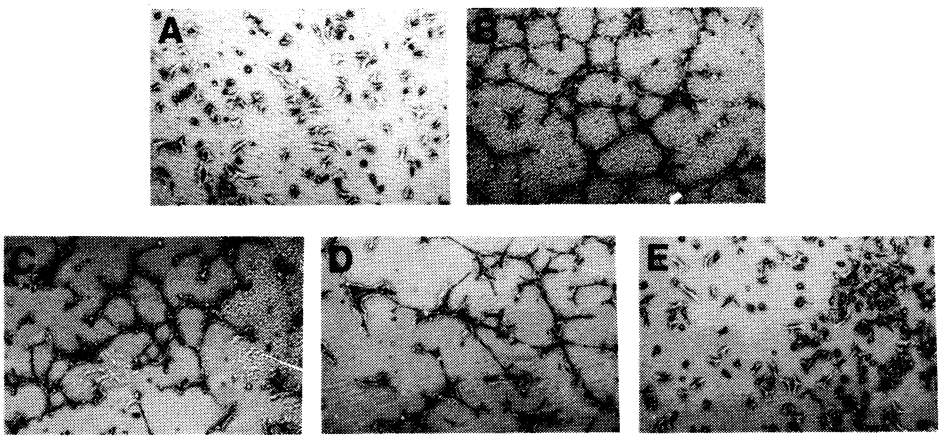

Fig. 3 Effect of M1 on tumor-induced morphological change of HSE cells. HSE cells (1×10^3/well) were seeded into Matrigel-coated 48-well plates in the absence (A) or presence (B to E) of 50 % CM-L5. B, control ; C, 2.5 μM of M1 ; D, 5 μM of M1 ; and E, 10 μM of M1. After 4 h of incubation, the plates were fixed with glutaraldehyde and stained with hematoxylin. Magnification, ×100.

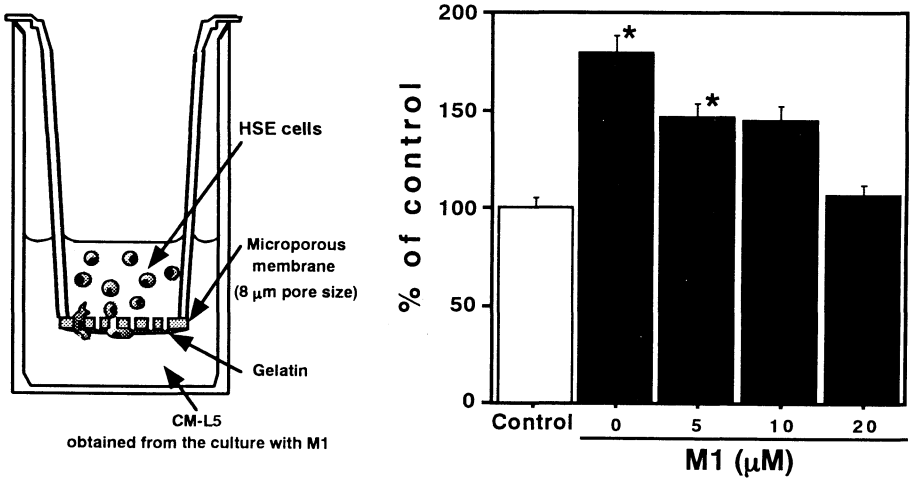

Fig. 4 Effect of M1 on the migration of HSE cells induced by CM-L5 *in vitro*.
Filters of Chemotaxicell chambers (left panel) were pre-coated with 10 μg gelatin on their lower surfaces. HSE cells (1×10^5/well) in 0.1 % BSA medium were seeded into the upper compartment and incubated with or without CM-L5 obtained from the culture with appropriate concentrations of M1 in the lower compartment. After a 2-h incubation, crystal violet staining was performed. *, $p<0.05$ compared with untreated control by Student's t-test.

various concentrations of M1 (2.5, 5, or 10 μM) for 24 h resulted in a dose-dependent inhibition of the tube formation by HSE cells. The incubation of HSE cells with M1 at the concentrations used in this study for 24 h did not affect the direct cytotoxicity against HSE cells, nor the formation of tube-like structures (data not shown).

Effect of M1 on the migration of HSE cells induced by CM-L5 in vitro

We next investigated the effect of M1 on the migration of HSE cells *in vitro*. HSE cells in the upper compartment of the chamber was incubated with CM-L5 obtained from the culture with M1 (5, 10 or 20 μM) in the lower compartment (Fig. 4). The incubation with CM-L5 markedly enhanced the migration of HSE cells as compared with untreated control. CM-L5 obtained from the culture of colon 26-L5 cells with M1 resulted in a concentration-dependent decrease of CM-L5-enhanced migration (Fig. 4). However, M1 at the concentrations used in this study did not directly affect the inhibition of HSE cell migration (date not shown).

Discussion

We have previously reported that protopanaxadiol-type ginsenosides and their major metabolite M1 markedly inhibited the lung metastasis of B16-BL6 melanoma cells when they were administered *p.o.* into mice. In addition, the ginsenosides was not found in serum after the oral administration, but only M1 was detected in serum and showed the inhibitory effects on the proliferation, migration and invasion of tumor cells *in vitro*.[14] These findings clearly indicated that M1, a major metabolite resulted from orally administered ginsenosides, is primarily responsible for the induction of the *in vivo* anti-metastatic effect.

In the present study, a small fragment of colon 26-L5 tumor was directly implanted into the left lobe of the liver to form a single tumor nodule in the liver for evaluation of antitumor and antiangiogenic activities. Oral administration of M1 inhibited the growth of implanted tumor and the intrahepatic metastasis after the implantation of a small fragment of colon 26-L5 tumor (Fig. 2). This indicates that M1 was effective at inhibiting tumor growth and metastasis of colon 26-L5 cells as well as melanoma cells. However, the detailed mechanism underlying the antitumor and antimetastatic effects of M1 has not been fully understood yet.

Neovascularization toward and into tumor is a crucial step for nutrient delivery to tumor and for hematogenous metastasis of tumor cells, and it consists of proliferation, migration, and capillary tube formation of endothelial cells.[17-19] It has been reported that some angiogenesis-related molecules, such as hepatocyte growth factor (HGF), VEGF and basic fibroblast growth factor (bFGF) play an important role in positively regulating the formation of tumor neovascularization.[19,24] Recently, hepatic sinusoidal endothelial (HSE) cells have been reported to be associated with tumor-induced angiogenesis in the liver.[25] We have also shown that vascular endothelial growth factor (VEGF) in CM-L5 is related to the stimulation of the proliferation and tube formation of HSE cells.[20] The coculture with noncytotoxic concentrations of M1 eliminated the ability of CM-L5 to induce the capillary tube formation and the migration of HSE cells in a concentration-dependent manner (Fig. 3 and 4). Previously, we reported that M1 transfers immediately from the blood to the liver after the intravenous administration.[26] Our preliminary study observes that orally administered M1 (10 mg/kg) reached the maximum level in the liver (7.1 μg/g) 2 h after the administration, and that the M1-level (more than 10 μM) required for inhibition of the tube formation of HSE cells *in vitro* (Fig. 3) was sustained for 6 h (data not shown). These results indicate that the suppression of tumor-induced neovascularization by M1 may be partly associated with the down-regulation of tube formation and migration of HSE cells, and consequently lead to the inhibition of intrahepatic tumor growth and metastasis. Further study will be needed to examine how M1 regulates the formation of capillary tube-like structures induced by VEGF in CM-L5 or directly influences the function of HSE cells.

On the other hand, our previous study has shown that the up-regulation of the $p27^{Kip1}$ and down-regulation of c-Myc and cyclin D1 by M1 treatment are responsible for the induction of apoptosis in B16-BL6 melanoma cells, suggesting the importance of cell cycle-related proteins for M1-induced apoptosis.[16]

Therefore, *in vivo* antitumor effect of M1 may be associated with the induction of apoptosis in tumor as well as angiogenesis.

In conclusion, the present study demonstrated that M1 inhibited the intrahepatic growth and metastasis of colon 26-L5 cells partially through the inhibition of angiogenic responses such as capillary tube formation and migration of HSE cells.

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和文抄録

本研究では，薬用人参（*Panax ginseng* C.A. MEYER）の protopanaxadiol-type ginsenosides の主要な腸内細菌代謝物 M1 の経口投与は，マウス結腸癌 colon 26-L5 細胞の腫瘍小片を肝へ直接移植した後の移植部位での増殖と肝内移転に対して有意に抑制効果を示すことを明らかにした。この結果は，B16-BL6 メラノーマ細胞による肺転移を抑制した以前の報告と同様に，colon 26-L5 細胞に対しても有効であることが示された。肝類洞内皮細胞（HSE 細胞）をマトリゲルをコートした基質上で，colon 26-L5 細胞の培養上清（CM-L5）とともに培養すると，腫瘍血管新生の過程における重要なステップのひとつである，内皮細胞の管腔形成を誘導した。CM-L5 による管腔形成能は，細胞傷害性を示さない濃度範囲の M1 により，濃度依存的に抑制された。同様に，CM-L5 による HSE 細胞の移動能の亢進を，M1 は濃度に依存して抑制した。

以上，M1 による結腸癌の肝における増殖及び肝内転移の抑制は，内皮細胞の管腔形成及び移動能を含む血管新生反応の抑制と部分的に関係していることが示唆された。

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