Induction of apoptosis in Lewis lung carcinoma cells by an intestinal bacterial metabolite produced from orally administered ginseng protopanaxadiol saponins

Kazuhito SUDA, Koji MURAKAMI, Hideo HASEGAWA, Ikuo SAIKI*

Department of Pathogenic Biochemistry, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University

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Abstract

The present study demonstrated that oral administration of an intestinal bacterial metabolite (M1) of protopanaxadiol-type saponin significantly inhibited the tumor growth at the implantation site after intrapulmonary implantation of Lewis lung carcinoma (LLC) cells, and suppressed the metastasis to mediastinal lymph nodes. We also investigated the inhibitory mechanism of M1 on the growth of LLC cells. M1 inhibited the proliferation of LLC cells in a concentration-dependent manner, with characteristic morphological changes at the concentration of 30 μM. Treatment of LLC cells with M1 resulted in marked elevation of the caspase-3 activity, peaking at 2 h, and a subsequent time-dependent induction of apoptosis during the period from 3 to 24 h, as evidenced by DNA fragmentation analysis. Since M1-induced growth inhibition of LLC cells was completely abrogated by the pretreatment with a specific inhibitor of caspase-3, Z-DEVD-FMK, M1 functions via the activation of caspase-3 in the process of apoptosis in LLC cells. Thus, the anti-proliferative activity of M1 against LLC cells is primarily due to the induction of apoptosis via promotion of caspase-3 activity, and this induction may lead to the anti-tumor activity *in vivo*.

Key words *Panax ginseng*, ginsenoside, intestinal bacterial metabolite, lymph node metastasis, apoptosis, caspase-3.

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 activities, including the enhancement of cholesterol biosynthesis, stimulation of serum protein synthesis, immunomodulatory effects, anti-inflammatory activity and antitumor effects.[1-10]

Previously, it was reported that after oral administration, protopanaxadiol-type ginsenosides such as Rb_1, Rb_2 and Rc are metabolized by intestinal bacteria 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 administered orally, M1 was found in the serum for 24 h, but Rb_1 was not detectable.[14] Our previous pharmacokinetic and antimetastatic studies demonstrated that expression of the *in vivo* antimetastatic effect of the protopanaxadiol-type ginsenosides was primarily based on the metabolite M1 resulting from the oral administration.[14,15] We also found that M1 caused the cell cycle arrest at G1 phase of tumor cells partly

*〒930-0194 富山市杉谷2630
富山医科薬科大学和漢薬研究所病態生化学部門　済木　育夫
2630 Sugitani, Toyama 930-0194, Japan

through the up/down-regulation of cell cycle-related molecules such as cyclin D and cyclin-dependent kinase (CDK) inhibitors, and consequently induced apoptosis.[16] However, the mechanism of M1-induced apoptosis is not fully understood yet.

In the present study, we investigated the effect of oral administration of M1 on tumor growth and lymph node metastasis in the orthotopic implantation model using murine Lewis lung carcinoma (LLC) cells as described previously.[17] We also examined whether or not M1 can induce apoptosis in LLC cells, and the mechanism of its action.

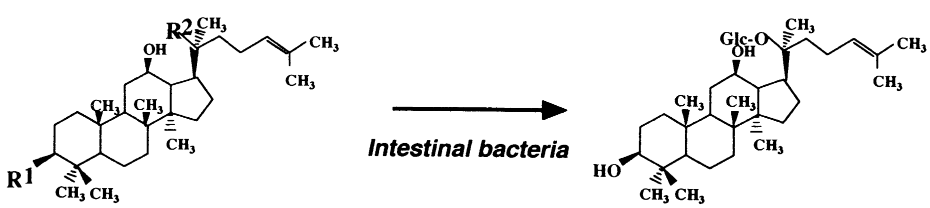
Materials and Methods

Chemicals : M1 was prepared from fermentation of protopanaxadiol-type ginsenosides by human intestinal bacteria as described.[11,18] For *in vitro* experiments, M1 was dissolved in dimethyl sulfoxide at a concentration of 100 mM as a stock solution, and kept at -20°C until use. The chemical structures of protopanaxadiol glycosides and their metabolic compound M1 are shown in Figure 1.

Cell line : Murine Lewis lung carcinoma (LLC) cells were maintained as monolayer cultures in Dulbecco's modified MEM (DMEM) supplemented with 10 % fetal bovine serum (FBS) and L-glutamine (M. A.Bioproducts, Walkersville, MD, USA) in a humidified atmosphere of 5 % CO_2 at 37°C.

Mice: Specific pathogen-free female C57BL/6 mice (5-6 weeks old) were purchased from Japan SLC Inc. (Hamamatsu, Japan). 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.

Intrapulmonary implantation of LLC and evaluation of anti-tumor activity : Orthotopic implantation of LLC cells into the lung was performed as described previously with some modifications.[17] Briefly, the left chests of anesthetized mice were incised (approximately 5 mm in length) and 20-μl aliquots of LLC cell suspension (2×10^3 cells) admixed with 20 μg of Matrigel® (Collaborative Biomedical Products, MA) were injected into the left lung parenchyma through the intercostal space (approximately 3 mm depth). The skin incision was closed with Autoclip® (Becton Dickinson Co., USA). M1 was orally administered daily for 14 days, starting on day 1 after tumor implantation. The antitumor effect was evaluated by measuring the volume of the tumor implanted orthotopically in the lung and the weight of the metastasized tumor at the mediastinal lymph nodes on day 17 after the implantation. The volume of primary tumors was calculated by the following formula : tumor volume (mm^3) $=1/2\times$ (long diameter) $\times$ (short diameter)^2. Tumor growth was indicated as T/C (%), using the following formula : T/C(%) = (mean tumor growth in the M1-treated group/mean tumor growth



Protopanaxadiol glycosides

	R1	R2
Rb_1	O-Glc $^{2\text{-}1}$Glc	O-Glc $^{6\text{-}1}$Glc
Rb_2	O-Glc $^{2\text{-}1}$Glc	O-Glc $^{6\text{-}1}$Arap
Rc	O-Glc $^{2\text{-}1}$Glc	O-Glc $^{6\text{-}1}$Araf
Rd	O-Glc $^{2\text{-}1}$Glc	O-Glc

M1

Fig. 1 Structure of protopanaxadiol glycosides and their metabolic compound M1.

in untreated control group) × 100.

In vitro growth inhibition assay: LLC cells (2×10^3/well) suspended in DMEM supplemented with 5 % FBS were seeded into 96-well culture plates. After a 24-h incubation, various concentrations of M1 in 5 % FCS-DMEM were added to the plates, and the cultures were incubated for a further 24 h. Crystal violet staining was performed to evaluate the activity: Cells were fixed by glutaraldehyde, stained by 0.5 % crystal violet, and then extracted with 30 % acetic acid. The absorbance of cell lysate was colorimetrically measured at 590 nm. In other experiments using caspase-3 inhibitor (Z-DEVD-FMK; MBL, Nagoya, Japan), LLC cells were pre-incubated with various concentrations of Z-DEVD-FMK for 30 min, and then incubated with 30 μM of M1 for 24 h.

Observation of morphological changes: LLC cells (1×10^5/well) suspended in DMEM supplemented with 5 % FBS were seeded into 6-well culture plates (Becton Dickinson, USA). After a 24-h incubation, the medium was replaced with 5 % FBS-EMEM containing M1 (40 μM), and the cultures were incubated for a further 24 h. Morphological changes of cells were photographed using phase contrast microscopy (Olympus, Japan).

DNA extraction and detection of DNA fragmentation: LLC cells (1×10^6 cells) were collected by centrifugation at 1,500 rpm for 5 min. The cell pellet was suspended in 600 μl of cell lysis buffer (10 mM Tris-HCl buffer, pH 7.5, 10 mM EDTA, and 0.2 % Triton X-100) and kept on ice for 10 min. The lysate was centrifuged at 14,000 rpm for 10 min and the supernatant collected. After TE-saturated phenol (Wako Pure Chemical Industries, Co., Ltd., Japan) was added to the supernatant, the mixture was vortexed, and then centrifuged at 14,000 rpm for 10 min. The supernatant was mixed with an equal volume of CIAA solution (chloroform: isoamylalcohol=24:1). DNA in the upper aqueous phase was precipitated in 3 M NaCl and cold ethanol by an overnight incubation at -20°C. After drying, DNA was dissolved in TE buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA, pH 7.5) and incubated with 10 μg/ml RNase (Nippon Gene, Japan) at 37°C for 30 min. Following the addition of

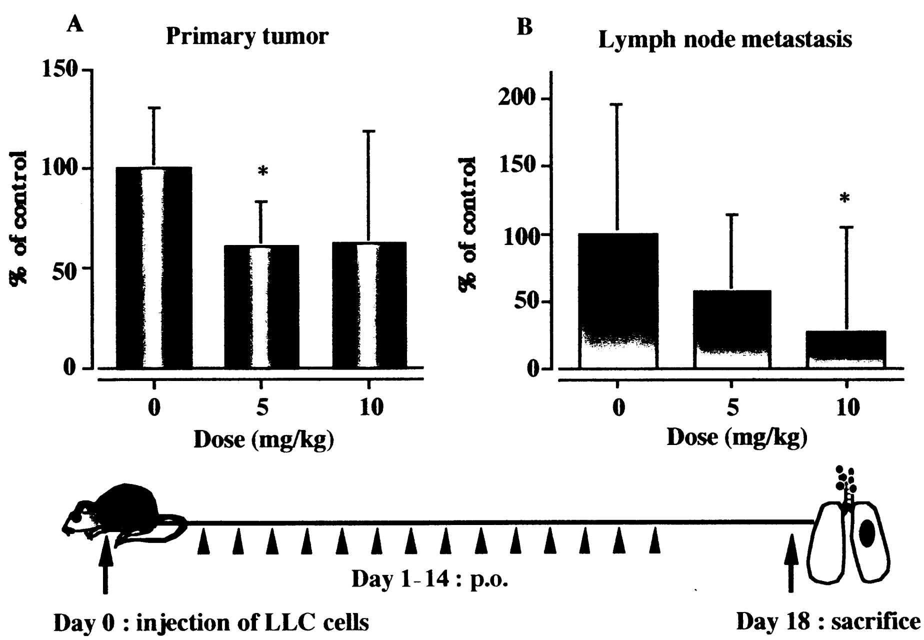


Fig. 2 Effect of oral administration of M1 on the growth of the inoculated tumor and lymph node metastasis after orthotopic implantation of LLC cells. LLC cells (2×10^3 cells/mouse) admixed with Matrigel (20 mg) were orthotopically implanted to the left lungs of female C57BL/6 mice. The intestinal bacterial metabolite of protopanaxadiol-type saponin, M1 (5-10 mg/kg/day), was given orally for 14 days. Mice were sacrificed on day 18 after the implantation, and the primary tumor volume (A) and the weight of metastasis-containing lymph nodes (B) were measured. Each column represents the mean ± S.D. of 7 animals. *, $p < 0.05$ as compared with untreated control (89 ± 27 mm^3 for primary tumor; 0.27 ± 0.26 g for lymph node metastasis) by Student's two-tailed t-test.

loading buffer (Nippon Gene, Japan), fragmented DNA was separated by 2 % agarose gel electrophoresis at 50 V for 1 h, and visualized by staining with 100 ng/ml ethidium bromide. The morphology of apoptotic cells was photographed using phase contrast microscopy (Carl Zeiss, Co., Ltd., Germany).

Caspase-3 activity : LLC cells (1×10^6) were treated with 30 μM of M1 for the appropriate time periods and then lysed with lysis buffer. After centrifugation at 14,000 rpm for 1 min, the supernatant was adjusted to the concentration of 100 μg protein/ml by using a BCA protein assay kit (Pierce Inc. USA). Caspase-3 activity in the supernatant was determined using a caspase-3 assay kit (MBL, Nagoya, Japan) by spectrophotometrically measuring the chromophore *p*-nitroanilide (*p*-NA) resulting from the proteolytic cleavage of the labeled substrate (DVED-*p*NA) at 405 nm.

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

Results

Effect of M1 on the growth of the inoculated tumor and lymph node metastasis after orthotopic implantation of LLC cells

We first examined the effect of M1 on the growth of the inoculated tumor and on lymph node metastasis after orthotopic implantation of LLC cells. Oral administration of M1 (5 mg/kg/day) for 14 days significantly inhibited the growth of LLC tumors at the implantation site as compared with the untreated control (Fig. 2A). Also, M1 administration suppressed

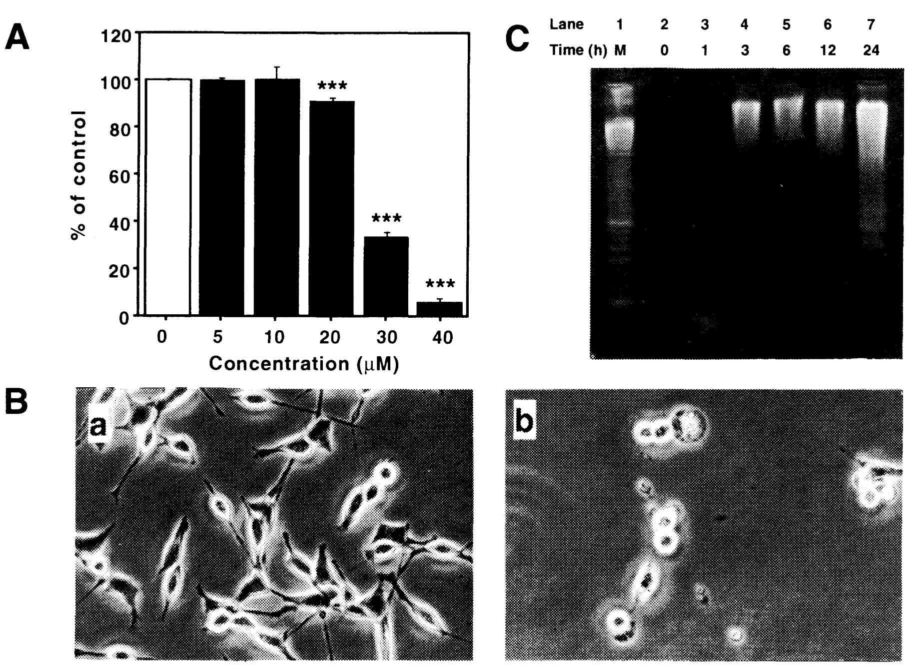


Fig. 3 M1-induced growth inhibition and apoptosis in LLC cells. (A) LLC cells (1×10^4/well) were seeded into each well of 96-well plates, and incubated for 24 h with the indicated concentrations of M1. The cytotoxic activity was determined by the crystal violet staining method, and the absorbance of the cell lysate was colorimetrically measured at 590 nm. Each column represents the mean $\pm$ S.D. of 3 dishes. ***, $p<0.001$ as compared with untreated control by Student's two-tailed *t*-test. (B) Morphological changes of LLC cells treated with M1. LLC cells were seeded into each well of 6-well plates, and incubated for 24 h in the absence (a) or presence (b) of 40 μM M1. Apoptotic bodies and chromatin condensation were seen. Magnification : $\times100$. (C) M1-induced DNA fragmentation in LLC cells. LLC cells (1×10^6) were treated with 30 μM M1 for the indicated time periods and DNA was isolated. The fragmented DNA was electrophoresed on a 1.5 % agarose gel and detected by ethidium bromide staining. Molecular weight markers (lane 1, M) ; 30 μM M1 (lanes 2-7).

the metastasis to the mediastinal lymph nodes in a dose-dependent manner (Fig. 2B).

M1-induced apoptosis in LLC cells

We next investigated whether or not the metabolite M1 could influence the growth of LLC cells *in vitro*. Fig. 3A shows that incubation with various concentrations of M1 for 24 h exhibited the cytotoxic activity against LLC cells in a concentration-dependent manner (in the range from 20 to 40 μM). M1 at the concentration of 40 μM caused dramatic morphological changes, with swollen-round, shrunken and multi-blebbing-shaped LLC cells *in vitro* (Fig. 3B). Since the swollen-shaped morphology of tumor cells is considered to be an apoptotic character, we investigated whether or not treatment with M1 resulted in the induction of apoptosis. As shown in Fig. 3C, fragmentation ladders of extracted DNA were observed in LLC cells treated with 30 μM M1 in a time-dependent manner.

Activity of caspase-3 is required for the process of M1-induced apoptosis

Caspase-3 is a key enzyme for the induction of apoptosis.[19-21] To assess whether activation of caspase-3 is involved in the process of M1-induced apoptosis, we examined the caspase-3 activity in M1-treated LLC cells by measuring the cleavage of caspase-3 substrate. Fig. 4 shows that incubation of LLC cells with 30 μM M1 for various times markedly enhanced the caspase-3 activity. The level of the activity peaked at 2 h of treatment with M1, and thereafter decreased with time. Thus, the anti-proliferative activity of M1 for LLC cells may be related to the induction of apoptosis via the activation of caspase-3.

Therefore, we investigated the effect of the tetrapeptide protease inhibitor (Z-DEVD-FMK), which is known to specifically inhibit caspase-3,[21] on the growth of LLC cells. As shown in Fig. 5, pretreatment of LLC cells with Z-DEVD-FMK for 30 min abrogated the M1-induced inhibition of tumor growth in a concentration-dependent fashion. RT-PCR analysis revealed that the mRNA for caspase-3 was expressed in LLC cells, and that treatment with 30 μM M1

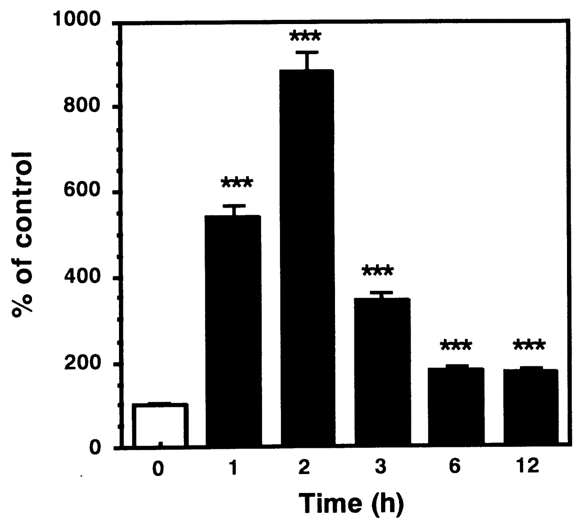


Fig. 4 Caspase-3 activity in LLC cells treated with M1 for various time periods. LLC cells (1×10^6/flask) were seeded into 75 cm^2 culture flasks, and incubated with 30 μM M1 for various time periods. Caspase-3 activity was determined by spectrophotometrically measuring the chromophore *p*-nitroanilide (*p*-NA) after cleavage from the labeled substrate DVED-*p*NA at 405 nm. Each column represents the mean±S.D. of 3 dishes. ***, $p<0.001$ as compared with untreated control by Student's two-tailed *t*-test.

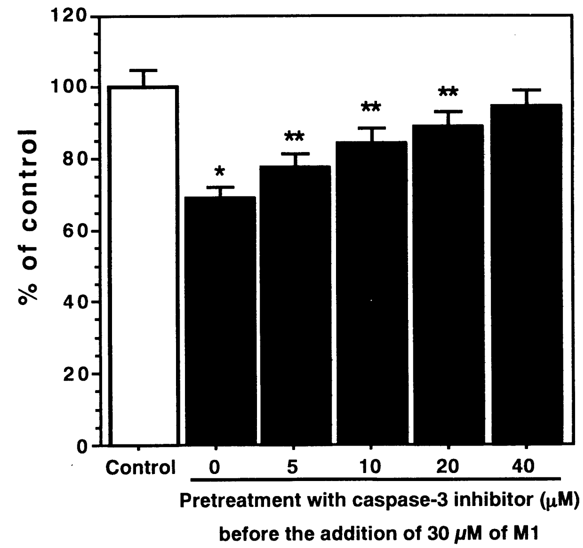


Fig. 5 Effect of a caspase-3 inhibitor on the M1-induced growth inhibition of LLC cells. LLC cells (5×10^3/well) were seeded to each well in the 96-well plate. After 24-h preincubation, medium was replaced with fresh medium in the absence or presence of a various concentrations of a caspase-3 inhibitor (Z-DEVD-FMK) and incubated for 30 minute prior to the addition of 30 μM of M1. The cultures were incubated for further 24 h. Crystal violet staining was performed at the end of the culture. Each column represents the mean±S.D. of 3 dishes. *, $p<0.05$; **, $p<0.01$ as compared with untreated control by Student's two-tailed *t*-test.

for 30, 60 or 90 min did not affect the mRNA expression in LLC cells (data not shown). Thus, M1-induced apoptosis in LLC cells is due to the increase of caspase-3 activity, but not to elevation of the expression.

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.* In addition, protopanaxadiol-type ginsenosides were not found in serum after they were administered orally, and only M1 was detected in serum and showed inhibitory effects on the proliferation, migration and invasion of tumor cells *in vitro*.[14] These findings clearly indicated that M1, a major metabolite produced from orally administered ginsenosides, is primarily responsible for the induction of the *in vivo* anti-metastatic effect. In the present study, we demonstrated that administration of M1 significantly inhibited the growth of a solitary tumor after intrapulmonary implantation of LLC cells, and suppressed the metastasis to mediastinal lymph nodes (Fig. 2). However, the details of how the active metabolite M1 affects the growth of tumor cells are not yet clear.

Apoptosis, a form of physiological cell death, has been defined by characteristic morphological changes of the nucleus and cytoplasm, including chromatin condensation, cytoplasmic blebbing, and formation of membrane-enclosed apoptotic bodies containing fragments of the nucleus and the cytoplasm.[22] Treatment of LLC cells with M1 inhibited the proliferation of the cells and induced apoptosis (DNA fragmentation) and a swollen-shape in the cells (Fig. 3). This finding is highly consistent with our previous reports using B16-BL6 melanoma cells.[16] However, the detailed mechanism underlying the apoptosis induced by M1 is not yet fully understood.

It has been reported that various molecules such as growth factors, intracellular mediators of signal transduction, and nuclear proteins regulating gene expression and the cell cycle are involved in positively or negatively regulating apoptotic signaling.[23-25] Recent studies have suggested that a sequential proteolytic activation of caspase-like proteases plays a major role in the execution of apoptosis. The caspase-like proteases are subdivided into three groups[26]: caspase-1-, caspase-2- and caspase-3-like proteases. In particular, activation of caspase-3 is also regulated by a number of different mechanisms involved in the process of apoptosis. As shown in Fig. 4, the caspase-3 activity in LLC cells was markedly promoted by the treatment with 30 μM M1. The level of the activity peaked at 2 h, and thereafter decreased with time. Pretreatment of LLC cells with 40 μM Z-DEVD-FMK (a specific inhibitor of caspase-3) for 30 min almost completely abrogated the M1-induced inhibition of tumor cell proliferation (Fig. 5). However, M1 did not affect the expression of the mRNA for caspase-3 in LLC cells (data not shown). These results indicate that the anti-proliferative activity of M1 against LLC cells is due to the induction of apoptosis via the activation of caspase-3.

Although the molecular events that drive the apoptotic signaling pathway are not entirely clear, cell cycle-related proteins such as cyclin D1, c-Myc or cyclin-dependent kinase (CDK) inhibitors ($p21^{WAF1/CIP1}$ and $p27^{KIP1}$) have been reported to be associated with cell division and proliferation.[27-30] For example, $p27^{KIP1}$ caused cell cycle arrest at the G_1-S phase transition by associating with the cyclin D-CDK4 complex followed by potent inhibition of the phosphorylation of retinoblastoma (Rb) protein.[31] Over-expression of $p27^{KIP1}$ resulted in the induction of G1-S arrest and apoptosis in cancer cells.[32] Also, $p21^{WAF1/CIP1(-/-)}$ mutant cells or cells in which the expression of $p21^{WAF1/CIP1}$ was downregulated were shown to be more susceptible to apoptosis in response to PGA_2 or ionizing irradiation than wild-type cells.[33,34] Thus, it appears that CDK inhibitors such as $p21^{WAF1/CIP1}$and $p27^{KIP1}$ are somehow functionally linked apoptotic processes, although there have been some contradictory reports. Our previous study using Western blot analysis showed that the up-regulation of $p27^{KIP1}$ and down-regulation of c-Myc and cyclin D1 by M1 treatment are responsible for the induction of apoptosis in B16-BL6 melanoma cells.[16] Therefore, M1-induced apoptosis in LLC cells may be caused by the up/down-regulation of cell-cycle-related proteins as well as activation of apoptosis-related enzymes. Several *in vitro* studies have reported that the ginsenosides Rh_2

and Rs_3, which are known to characteristically exist in red ginseng, inhibited the growth of human breast and liver cancers by the induction of apoptosis via up-regulation of $p21^{WAF1/CIP1}$ and down-regulation of cyclin D3 *in vitro*.[35-39] Our previous study indicated that M1 was effective at inhibiting the growth and invasion of tumor cells *in vitro* although ginsenosides Rb_1, Rb_2 and Rc did not affect the tumor cell growth *in vitro*.[14] Considering the importance of intestinal bacterial metabolites (including M1) for the expression of the *in vivo* effects of orally administered ginseng saponins, conversely, further studies will be needed to determine whether or not the *in vitro* efficacies of ginsenosides Rh_2 and Rs_3 are reflected by *in vivo* efficacy after their oral administration.

When B16-BL6 melanoma cells were incubated with dansyl M1,[40] the fluorescent signal of dansyl M1 was detected in the cytosol and nuclei within 15 min after addition of the M1, and thereafter was observed predominantly in the nuclei.[16] Since M1 has a steroid-like chemical structure, it may interact with some intracellular receptors, including a steroid receptor, which are known to be involved in the rapid regulation of nuclear proto-oncogene transcription.[41] Several investigators have reported that ginsenosides Rg_1, Rh_1 and Rh_2 act as functional ligands of the glucocorticoid receptor and thereby induce the differentiation of F9 teratocarcinoma cells, growth inhibition of hepatoma cells and expression of mRNA for tyrosine aminotransferase in hepatocytes *in vitro*.[42-44] Therefore, the regulatory mechanisms of M1 at the transcriptional level still need to be investigated in greater detail.

The present study demonstrated that M1 was effective at inhibiting the growth of LLC at the implantation site after orthotopic implantation, and tended to suppress lymph node metastasis. In addition, the anti-proliferative activity of M1 against LLC cells is primarily due to the induction of apoptosis with accompanying characteristic morphological changes, via promotion of caspase-3 activity.

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和文抄録

本研究では，薬用人参のプロトパナキサジオール・サポニン成分の腸内細菌代謝物であるM1を経口投与することにより，ルイス肺癌細胞（LLC細胞）の同所性移植による肺移植局所での腫瘍の増殖及び縦隔リンパ節への転移を有意に抑制した。*In vitro* におけるLLC細胞の増殖に及ぼすM1の抑制効果を検討した結果，M1は濃度依存的にLLC細胞の増殖を抑制し，30 μMの濃度では特徴的な形態変化を伴った抑制がみられた。LLC細胞をM1で処理した後2時間目をピークとしたカスパーゼ-3活性の著しい増加がみられ，引き続いて3-24時間にわたり経時的に，DNAの断片化を指標とするアポトーシスの誘導が観察された。LLC細胞に対するM1の増殖抑制は，カスパーゼ-3の特異的な阻害剤であるZ-DEVD-FMKの前処置により完全に解除されたことから，M1によるアポトーシスの誘導過程にカスパーゼ-3の活性が作用している。以上，M1のLLC細胞に対する増殖抑制機序の一つとして，カスパーゼ-3活性の亢進を介してアポトーシスを誘導し，その結果として *in vivo* の抗腫瘍効果を引き起こすものと考えられる。

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