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In vivo anti-metastatic action of Ginseng Saponins is based on their intestinal bacterial metabolites after oral administration

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Abstract : We found that the main bacterial metabolite M1 is an active component of orally administered protopanxadiol-type ginsenosides, and that the anti-metastatic effect by oral administration of ginsenosides may be primarily mediated through the inhibition of tumor invasion, migration and growth of tumor cells by their metabolite M1. Pharmacokinetic study after oral administration of ginsenoside Rb1 revealed that M1 was detected in serum for 24 h by HPLC analysis but Rb1 was not detected. M1, with anti-metastatic property, inhibited the proliferation of murine and human tumor cells in a time- and concentration-dependent manner *in vitro*, and also induced apoptotic cell death (the ladder fragmentation of the extracted DNA). The induction of apoptosis by M1 involved the up-regulation of the cyclin-dependent kinase (CDK) inhibitor $p27^{Kip1}$ as well as the down-regulation of a proto-oncogene product c-Myc and cyclin D1 in a time-dependent manner. Thus, M1 might cause the cell-cycle arrest (G1 phase arrest) in tumor cells through the up/down-regulation of these cell-growth related molecules, and consequently induce apoptosis. The nucleosomal distribution of fluorescence-labeled M1 suggests that the modification of these molecules is induced by transcriptional regulation. Tumor-induced angiogenesis (neovascularization) is one of the most important events concerning tumor growth and metastasis. Neovascularization toward and into tumor is a crucial step for the delivery of nutrition and oxygen to tumors, and also functions as the metastatic pathway to distant organs. M1 inhibited the tube-like formation of hepatic sinusoidal endothelial (HSE) cells induced by the conditioned medium of colon 26-L5 cells in a concentration-dependent manner. However, M1 at the concentrations used in this study did not affect the growth of HSE cells *in vitro*.

Key word: ginsenoside, M1, cancer metastasis, invasion, apoptosis, angiogenesis, cyclin-dependent kinase inhibitor, c-Myc, cyclin D.

INTRODUCTION

Ginseng (the root of *Panax ginseng* C. A. MEYER, Araliaceae) has been used for 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 and anti-inflammatory activity.[1-5] Several studies using ginsenosides have also reported antitumor effects, particularly the inhibition of tumor-induced angiogenesis[6], tumor invasion and metastasis[7,8], and the control of phenotypic expression and differentiation of tumor cells.[9,10]

Previously, it was reported that protopanaxadiol-type and protopanaxatriol-type ginsenisides are metabolized by intestinal bacteria after oral administration to their final derivative 20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol [referred to as M1[11] or compound K[12,13]] or 20(S)-protopanaxatriol [referred to as M4[11]] (Fig. 1). This made it unclear whether or not the expression of anti-metastatic effect by oral administration of ginsenosides can be induced by their metabolites.

We have recently reported that protopanxadiol- or protopanxatriol-type ginsenosides and their major metabolites M1 and M4 markedly inhibited lung metastasis of

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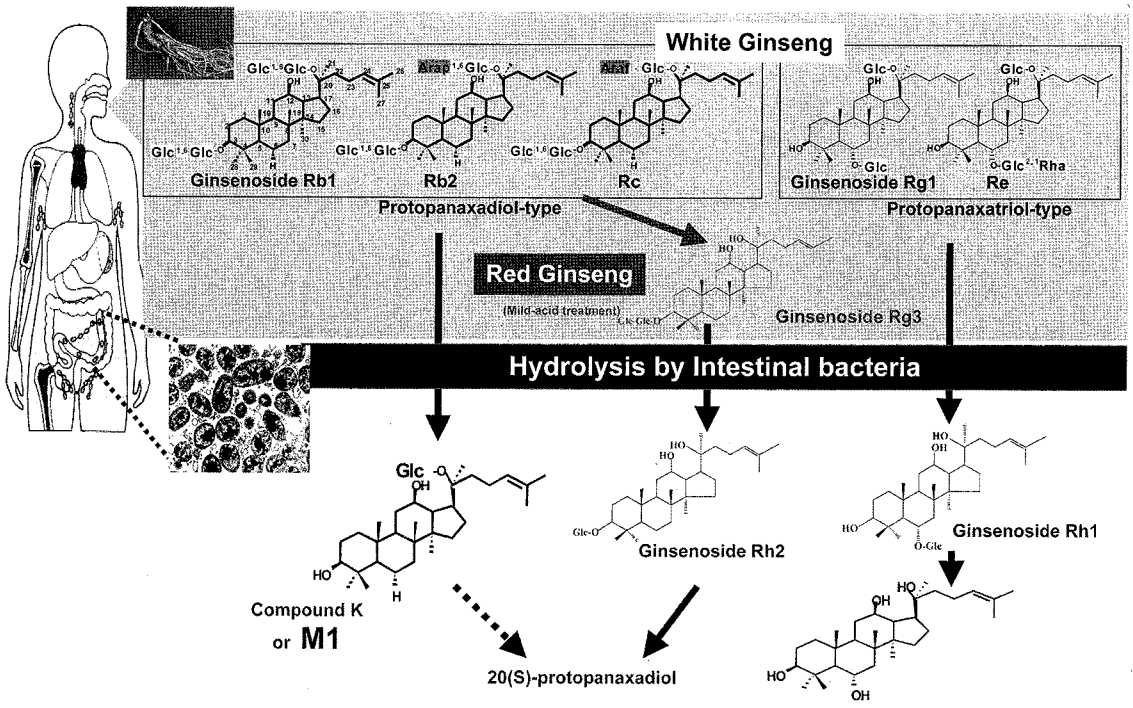

Fig. 1. Isolation and chemical structure of ginsenosides and their intestinal bacterial metabolites.

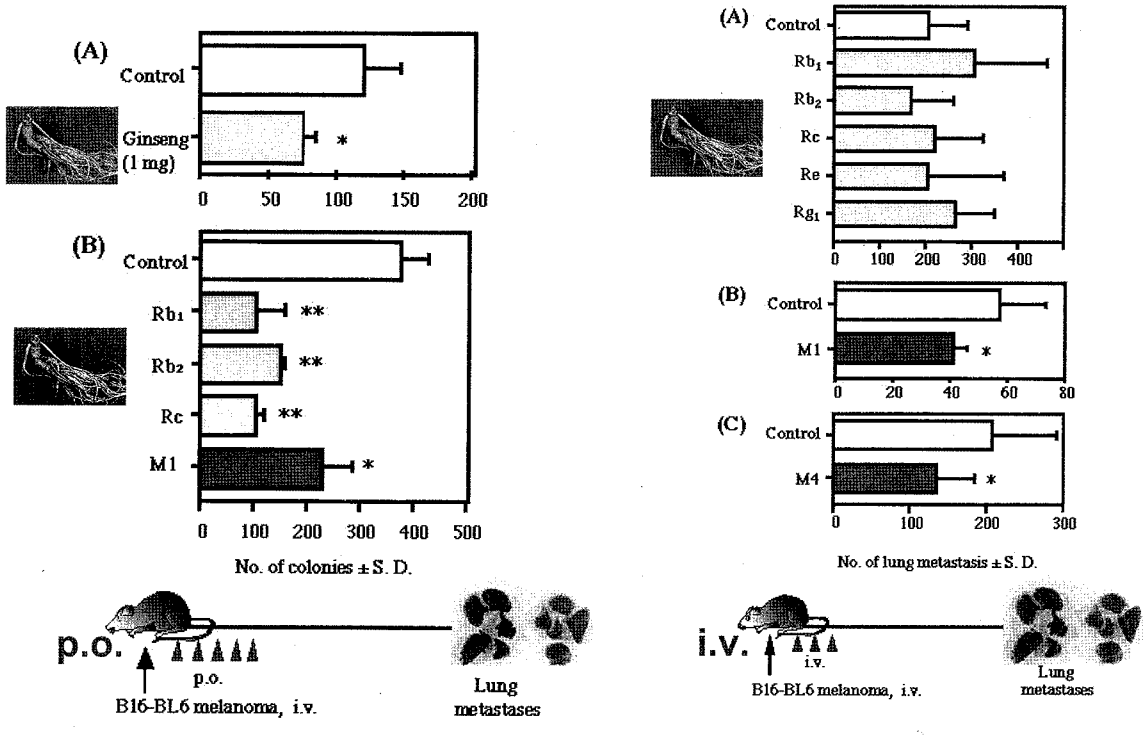

Fig. 2. Effect of oral or i.v. administration of ginsenosides and their metabolites on lung metastasis of B16-BL6 melanoma cells.

B16-BL6 melanoma cells when they were administered 5 times orally.[14,15] (Fig. 2). In contrast, three consecutive i.v. administration of metabolite M1 and M4 after tumor inoculation resulted in a significant inhibition of lung metastasis, whereas ginsenosides Rb1, Rb2, Rc, Re and Rg_1 did not show any inhibitory effect (Fig. 2).

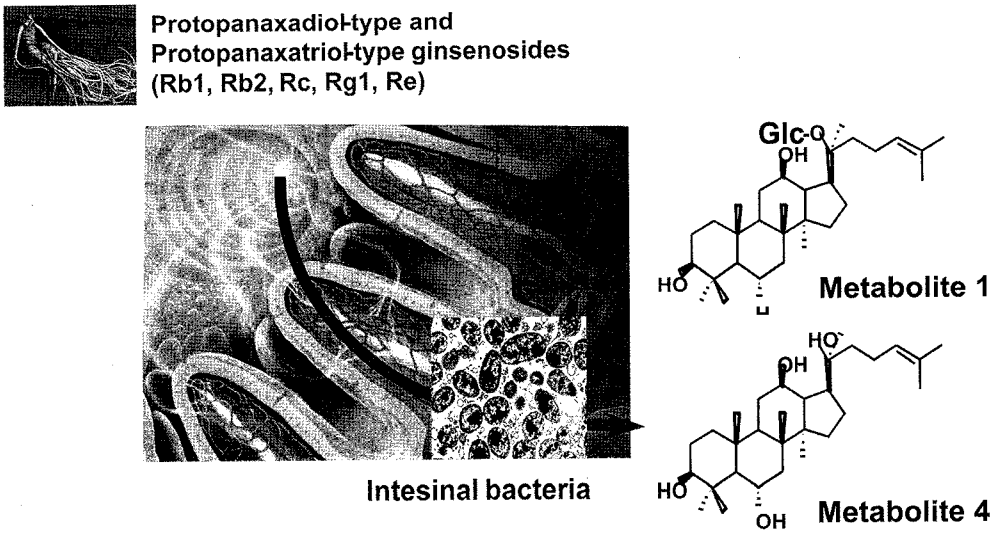

Fig. 3. Anti-metastatic effect by orall administration of ginsenosides is primarily mediated by their intestinal bacterial metabolite M1 and M4.

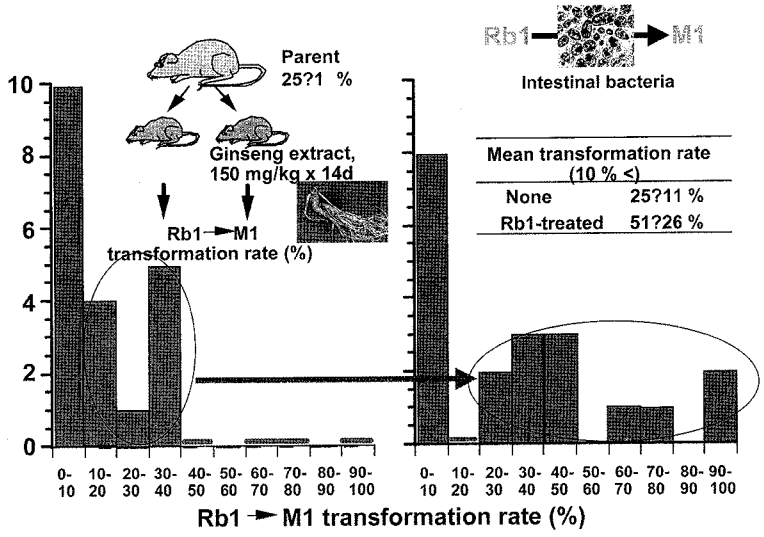

Fig. 4. Effect of oral administration of Rb1 on intestinal bacterial transformation rate of Rb1 to M1.

These findings suggest that the expression of the *in vivo* anti-metastatic effect by orall administration of both types of ginsenosides was primarily based on their metabolite M1 and M4 (Fig. 3). These results may be also supported by the finding that metabilites were detected in the serum from mice orally given ginsenosides, but ginsenosides were not detected by HPLC analysis. This pharmacokinetic study is in good agreement with previous reports on the low absorption rate of Rb1 from the intestines[16,17] and high metabolic rate of Rb1 to M1[17] in rat and human by using HPLC and enzyme-immunoassay methods.[11,12] Moreover, it has also been noted that ginsenosides are hardly decomposed by gastric juice with the exception of slight oxygenation.[13] Therefore, our findings support the notion that ginsenosides may act as natural pro-drug which can be transformed to M1 by intestinal anaerobe(s) after oral administration and consequently induce *in vivo* anti-metastatic effect.

To investigate the incidence of intesinal bacteria possessing ginsenoside Rb1-hydrolyzing potential, hydrolyzing potential of intestinal bacteria, expressed as the transformation rate of Rb1 to metabolite M1, was carried out by using fecal specimens of mice. There were some correlations of the transformation rate of Rb1 to M1 in between mother mice and their litters, particularly, statistically significant between the groups of litters born from

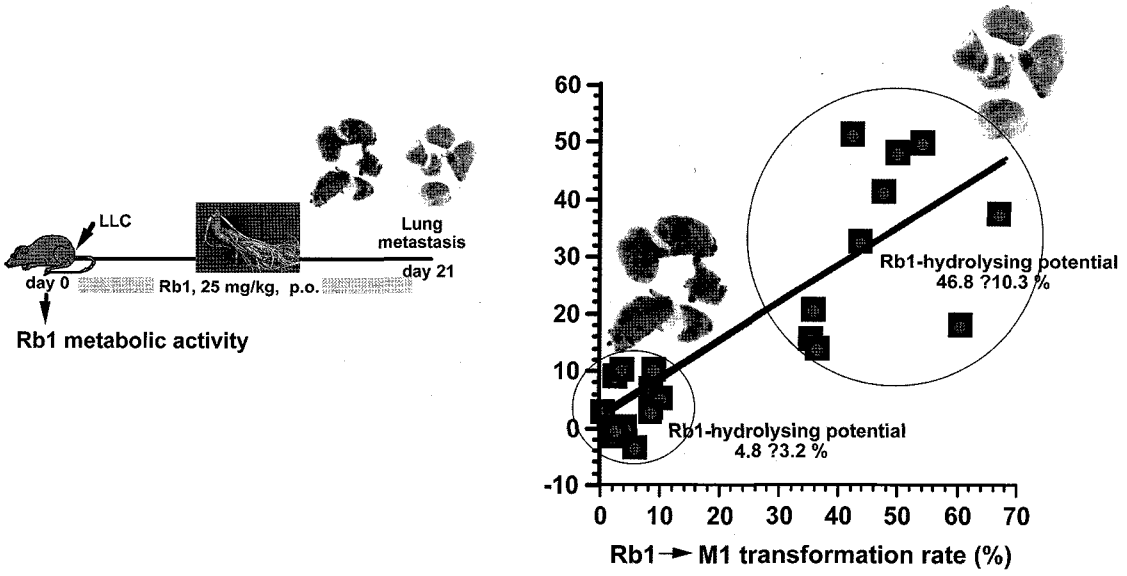

Fig. 5. Positive relationship between ant-metastatic potential and Rb1 metabolic activity.

mothers with different rates of hydrolyzing potential. This suggests that the intestinal microflora of litter are primarily infected from mother. On the other hand, consecutive administration of Ginseng extract to the mice with transformation rate from Rb1 to M1 of 25 ±11%, resulted in a significant increase in the transformation rate, as compared with untreated group (Fig. 4). However, induction of Rb1-hydrolyzng potential by the administration of Ginseng extract was hardly effective for the mice with hydrolyzing potential of less than 10%. For such mice, the inoculation of fecal microflora from mice with high hydrolyzing potential was also ineffective. Therefore, the location of the bacteria capable of hydrolyzing Rb1 on intestinal epithelium cells may be associated with genetic factors of hosts.

To examine the influence of Rb1-hydrilyzing potential on anti-metastatic efficacy of Rb1, Rb1 was orally administered to two sets of mice with low and high hydrolyzing potential after s.c. inoculation of LLC tumor. A significant difference between active and inactive groups and also the tendancy of a positive relationship between hydrolysing potential and inhibition of lung metastasis were observed. These findings indicate that the transformation rate of Rb1 to its active metabolite M1 was dependent on Rb1-hydrolyzing potential of intestinal bacteria, which consequently affected the expression of anti-metastatic efficacy of orally administered Rb1 (Fig. 5). Thus, hydrolyzing potential of intestinal bacteria for crude drug or the formulation may be an important factor influencing the holistic pattern of symptoms and individual pathogenic alterations, so-called **SHO**, by which the diagnosis of disease state and the ways of treatment in Kampo are determined.

On the other hand, these ginsenosides hardly inhibited the invasion, migration, and growth of tumor cells *in vitro*, whereas intestinal bacterial metabolites M1 and M4 showed the inhibitiory effects dose-dependently.[14,15] (Fig. 6). These findings indicate that M1 produced in the serum after the oral administration of ginsenoside Rb1 may induce the *in vivo* anti-metastatic effect partly through the inhibition of tumor invasion, migration and growth of tumor cells. Even if direct addition of ginsenosides into the culture *in vitro*, which is referred to as **FURIKAKE** (in Japanese) assay, was effective at inhibiting tumor cell invasion and proliferation, these results should not be apparently accepted for the explanation of *in vivo* efficacy by oral administration of ginsenosides.

However, the detail of how M1 affects the growth of tumor cells has not been clear. Co-incubation of tumor cells with M1 at concentrations ranging from 5 to 40 μM resulted in a time- and concentration-dependent inhibition of tumor cell proliferation, with accompanying morphological changes (spindle-shape) at the concentration of 20 μM.[18] In addition, M1 at a concentration of 40 μM caused the cytotoxic response in tumor cells at an earlier time period (within 24 h) in the culture. Since the swelling-shape morphology of tumor cells is considered to be an apoptotic character, the cell death by treatment with M1 (40 μM) was due to the induction of apoptosis. The ladder fragmentation of the extracted DNA and swollen-

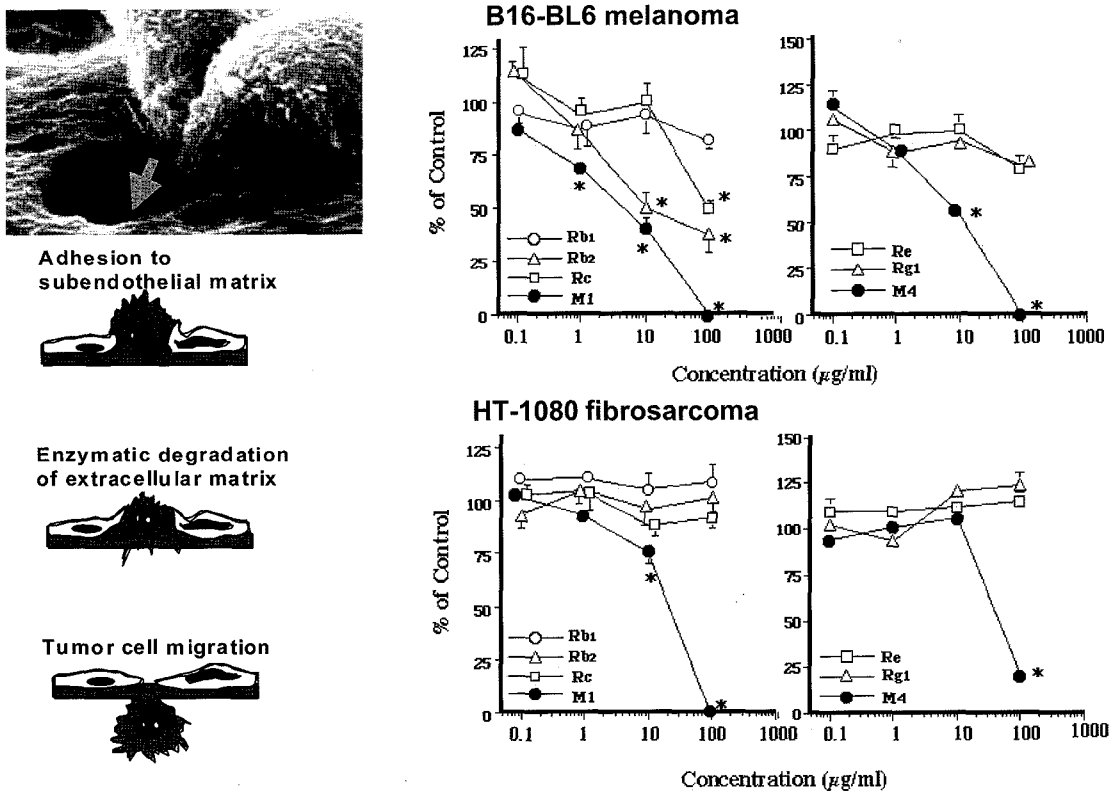

Fig. 6. Effect of ginsenosides or their metabolite (M1, M4) on the invasion of B16-BL6 melanoma and HT-1080 fibrosarcoma cells into reconstituted basement membrane Martigel.

round morphology indicated that the cell death was caused by apoptosis. In contrast, the incubation with ginsenoside-Rb_1 (40 μM) did not affect the morphology of tumor cells or cell proliferation.[14,18]

Although the molecular events that drive the apoptotic signaling pathway are not entirely clear, some apoptosis-related proteins such as cyclin D1, c-Myc or cyclin-dependent kinase (CDK) inhibitors have been reported to be associated with cell division and proliferation.[19,20,21] Therefore, in order to clarify the mechanism of M1-induced apoptosis, we investigated the effect of M1 on the expression of the apoptosis-related proteins, p21, $p27^{Kip1}$, c-Myc and cyclin D1. M1 treatment (40 μM) markedly increased the expression of $p27^{Kip1}$ as compared with the untreated control (Fig. 7). No expression of the other CDK inhibitor, p21 was detected in B16-BL6 cells in this experiment (data not shown). The up-regulation of $p27^{Kip1}$, which is known to inhibit the CDK activity, was observed during the apoptotic process caused by anti-cancer agents including etoposide and camptotecin. On the other hand, a proto-oncogene product c-Myc as well as cyclin D1 have been reported to be overexpressed in the proliferative phase of various types of tumor cells.[19,20,21] The expression of c-Myc and cyclin D1 was down-regulated by M1 treatment in a time-dependent manner. Thus, M1 might cause the cell-cycle arrest in tumor cells through the up/down-regulation of these cell-growth related molecules, and consequently induce apoptosis.

It has been reported that various molecules such as Bcl-2 (an inhibitor of apoptotic cell death), Bax (promotion of apoptosis by antagonizing the function of Bcl-2) and caspases (interleukin-1β converting enzymes to trigger the execution of cell death) are involved in positively or negatively regulating apoptosis signaling.[22,23,24] Recent studies have proposed some signaling pathways for apoptosis mediated by different regulatory molecules.[22,25] Therefore, the possibility that M1 inhibits or promotes these apoptosis-related molecules will be needed to be examined.

We also examined the intra-cellular distribution of M1 after the incubation of tumor cells with dansyl M1. The fluorescent signal of dansyl M1 was detected in the cytosol and nuclei 15-min after incubation, and thereafter was observed predominantly in the nuclei. These findings sug-

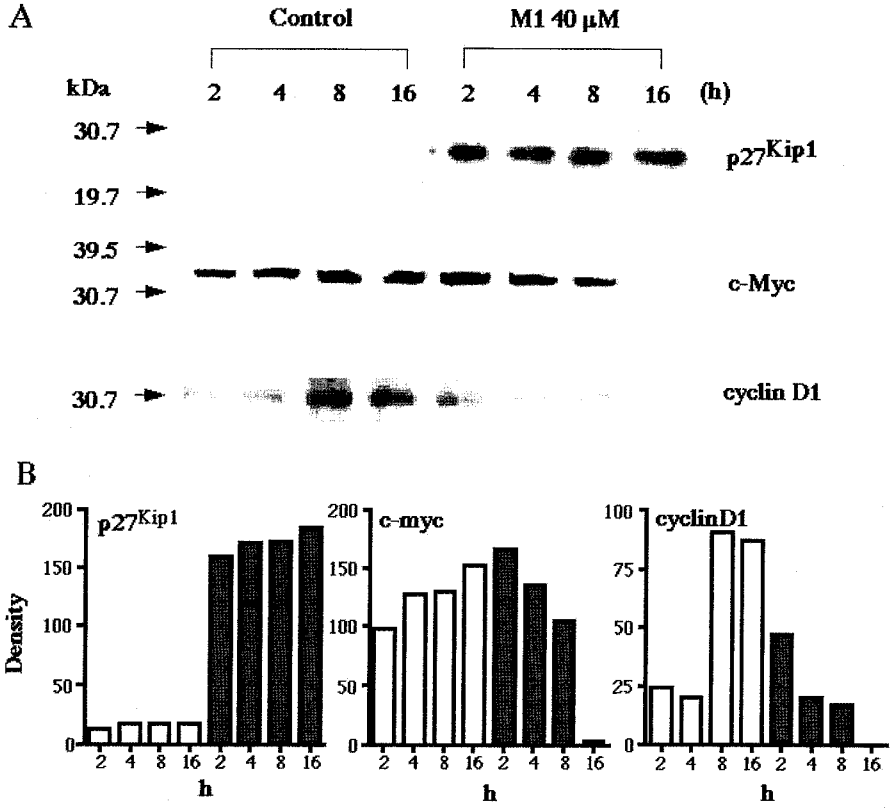

Fig. 7. Western blot analysis of p27Kip1, c-Myc and cyclin D1 in B16-Bl6 cells treated with M1

gest that the apoptotic cell death is induced by intra-cellular M1 through the transcriptional regulation of several cell-growth associated proteins. 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.[26] The regulatory mechanisms of M1 at the transcriptional level will be needed to investigated in detail.

In summary, we demonstrated that a metabolite of ginseng protopanaxadiol saponin (M1), with anti-metastatic property, inhibited the proliferation of tumor cells in a time- and concentration-dependent manner, and in addition induced apoptotic cell death (Fig. 8). The induction of apoptosis by M1 involved the up-regulation of the CDK-inhibtor p27Kip1 as well as the down-regulation of c-Myc and cyclin D1 (Fig. 8). The nucleosomal distribution of M1 suggests that the modification of these molecules is induced by transcriptional regulation.

The above results demonstrated the direct effects of M1 on metastatic cell functions. Tumor-induced angiogenesis (neovascularization) is one of the most important events concerning tumor growth and metastasis (Fig. 9). Neovascularization toward and into tumor is a crucial step for the delivery of nutrition and oxygen to tumors, and also functions as the metastatic pathway to distant organs.[27] The angiogenic response is considered to be composed of a series of sequential steps that endothelial cells degrade the surrounding basement membranes, migrate into the stroma, proliferate and finally differentiate to give rise to new capillary vessels.[28]

The induction of tumor angiogenesis is believed to reflect a balance between positive and negative regulatory factors.[29] Some angiogenesis-related molecules, such as platelet derived growth factor[30], vascular endotherial cell growth factor[31] and fibrobrast growth factor[32] play an important role in positively regulating the formation of tumor angiogenesis. On the contrary, angiostatin[33], thrombospondin[34], palatelet factor 4[35], interferon-g, endostatin[36], interferon-a[37] and nitric oxide[38] inhibit tumor angiogenesis. Therefore, we examined whether or not M1 can affect tumor-induced angiogenesis.

Recently, Tanigawa *et al.*[39] reported that hepatic sinusoidal endothelial (HSE) cells are associated with tumor-induced angiogenesis in the liver. The proliferation and tube formation of HSE cells are stimulated by vascular

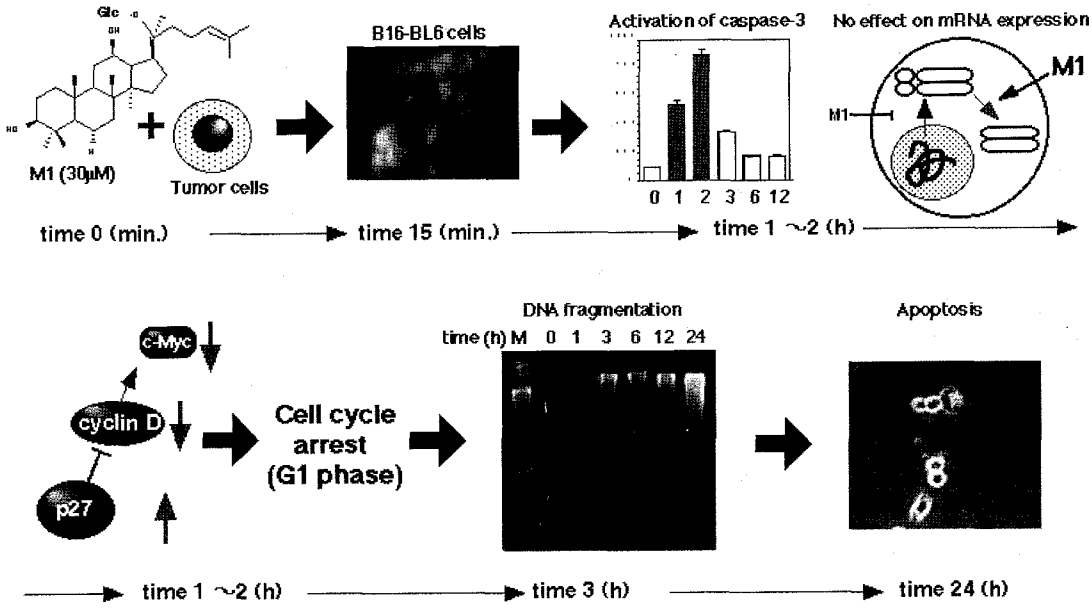

Fig. 8. Proposed mechanism of M1-induced inhibition of tumor growth.

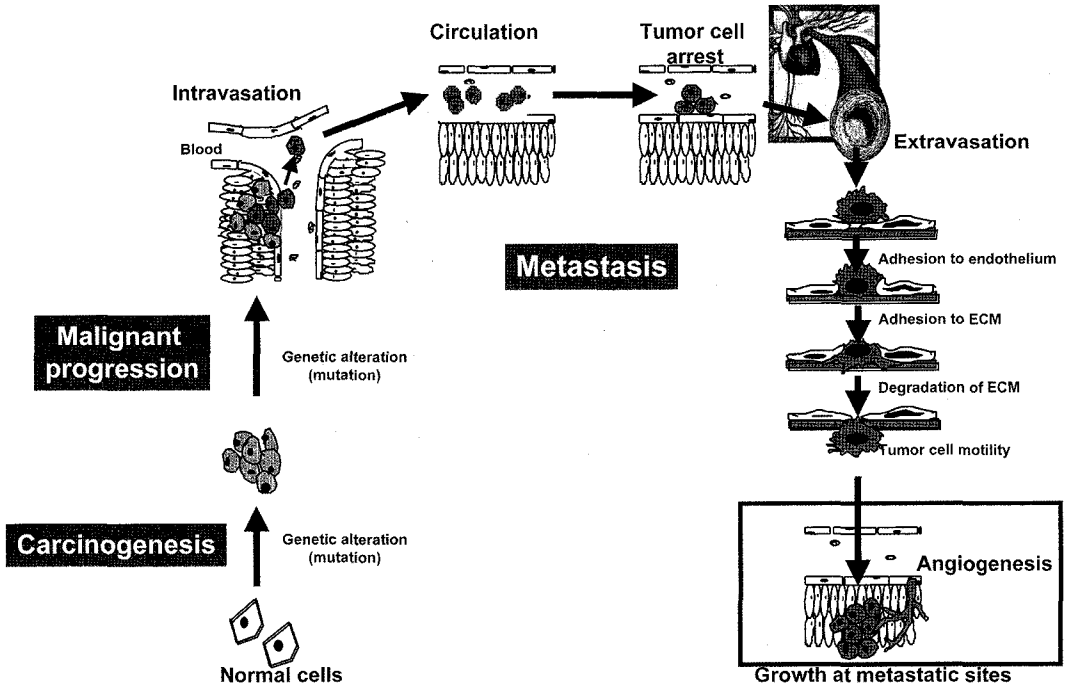

Fig. 9. Tumor-induced angiogenesis in metastatic formation.

endothelial growth factor in conditioned medium of colon 26-L5 cells (CM-L5).[40] Thus, tumor-induced angiogenesis was assessed following tube formation of HSE cells formed on Matrigel-coated plates.

The incubation of HSE cells with 50% CM-L5 caused tube-like structures of HSE cells within 4 h (Fig. 10). Addition of CM-L5 obtained from the cultures of colon 26-L5 cells with M1 (2.5, 5 or 10 μM) resulted in a concentration-dependent inhibition of the tube formation of HSE cells. The incubation of colon 26-L5 cells with M1 at 0-10 μM for 24 h did not affect the growth of HSE cells (data not shown). Moreover, the incubation of HSE cells with M1 at 0-20 μM for 72 h did not affect the growth of HSE cells, nor the formation of tube-like structures (data

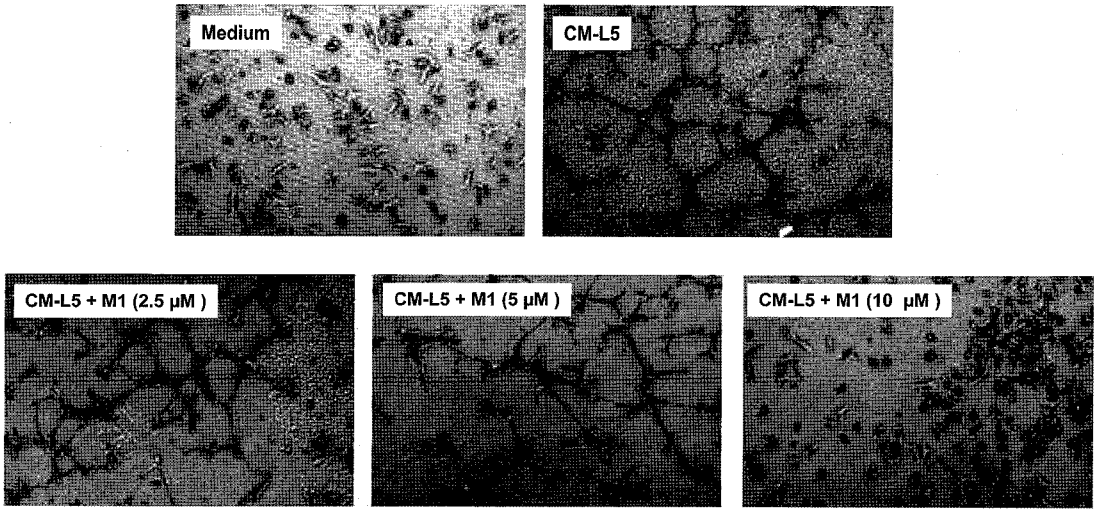

Fig. 10. Effect of M1 on tube formation by hepatic sinusoidal endothelial (HSE) cells induced by conditioned medium of colon 26-L5 cells (CM-L5).

not shown).

The incubation with CM-L5 markedly enhanced the migration of HSE cells as compared with the 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. However, M1 at the concentrations used in this study did not directly affect the inhibition of HSE cell migration (date not shown).

The above results indicated that orally administered Rb_1 is absorbed as its metabolic form by intestinal bacteria, *i.e.* M1. We next analyzed the biodistribution, excretion and metabolism of M1 following its absorption, using mice. First, we macroscopically observed the biodistribution of M1, using dansyl M1 that showed almost the same physiological properties as M1. Dansyl M1 was administered i.v. to a mouse (25 mg/kg). Fifteen min later, the mouse was sacrificed, the abdominal cavity was opened and the gastrointestinal tract was resected. As shown in Fig. 11, the fluorescent signal of dansyl M1 was detected strongly in the liver (panel A) and upper part of the small intestine (panels B and C), slightly in the lung and bladder (panel B) but hardly at all in the kidney (panel B). This observation showed that the fluorescent M1 was taken up into the liver and excreted as bile immediately after its i.v. injection.

To quantify M1 in organs, biological samples resected from mice 40 min after the i.v. injection of M1 (25 mg/kg) were analyzed by HPLC (Fig. 11). Consistent with the macroscopic observation using dansyl M1, much of the M1 was recovered from the liver and contents of the small intestine and a slight amount from the blood, lung and kidney (a total of 50% of the dose was recovered). The similar pharmacokinetic behavior of dansyl M1 and M1 was well consistent with their similar biological properties *in vitro*. The results from fluorescent and HPLC analyses indicated that M1 is distributed in the liver and small intestine within 15 min after i.v. administration with a high degree of selectivity. M1 chemically consists of a hydrophobic dammaranediol moiety similar to cholesterol, and a hydrophilic glucose moiety. Hepatocytes are reported to recognize glucose moiety *via* a receptor[41] except for galactose receptor.[42] Therefore, this specific function of hepatocytes must be partly associated with the selective accumulation of M1 into the liver.

We next analyzed the pharmacokinetics of M1 after i.v. and oral administration (Fig. 12). Immediately following a single i.v. injection of M1 (25 mg/kg), this metabolite disappeared from the blood ($t_{1/2}$, 3 min; $t_{1/2}$, 23 min; *AUC*, 2815 min μg/ml), and instead increased in the liver. The level of M1 peaked at 10 min (C_{max}, 65% recovery) and thereafter gradually disappeared with time ($t_{1/2}$, 25 min; $t_{1/2}$, 75 min; *AUC*, 481.4 hmg/g) (Fig. 12). On the other hand, orally administered M1 was detected in the contents of the stomach, small intestine, caecum and colorectum with the passage of time (Fig. 12), closely related to the movement of M1 in the gastrointestinal tract. Although M1 was undetectable in the blood (data not shown), it was detected in the liver, where it reached the maximal level (8% recovery) 2 h after administration and then gradually decreased (*AUC*, 115.5 hμg/g). Therefore, these results indicated that orally administered M1 was absorbed mainly from the small intestine into the blood followed by

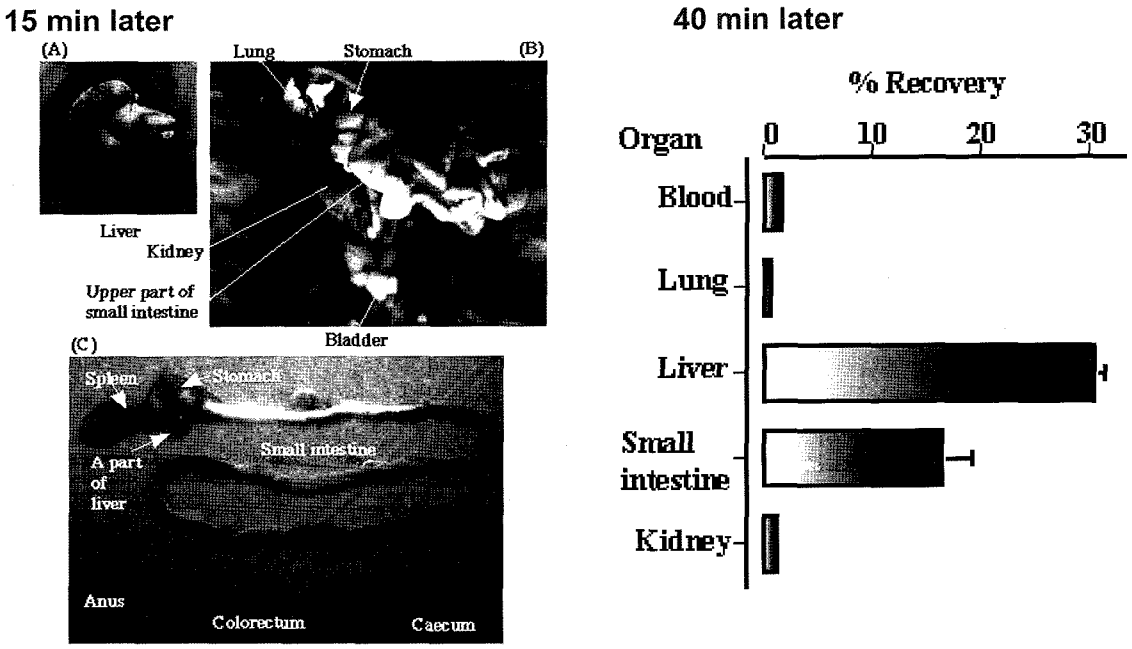

Fig. 11. Biodistrbution of dansyl M1 and quantification of M1 in the organ after i.v. administration.

absorption from its accumulation in the liver.

To examine the metabolism of M1, the liver and contents of the small intestine from M1-treated mice were compared with those of untreated animals using TLC. A band, referred to as EM1 (M1 esterified mainly with stearic and oleic acids), was newly detected 20 min after the i.v. injection of M1. Its color density peaked at 40 min, the turning point of the pharmacokinetic curve of M1 in the liver from α-phase to β-phase (Fig. 13A) and thereafter gradually decreased. At 160 min, the band of EM1 was still detectable, but not M1 (Fig. 13A). EM1 was also detected in the liver together with M1 after the oral administration of the latter (Fig. 13C). EM1 was hardly detected in the small intestine tissue; however, M1 was slightly detected (data not shown). In contrast with the liver, neither the band of EM1 nor any other new band except for M1 was detected in the contents of the small intestine (Fig. 13B), indicating that neither EM1 nor its

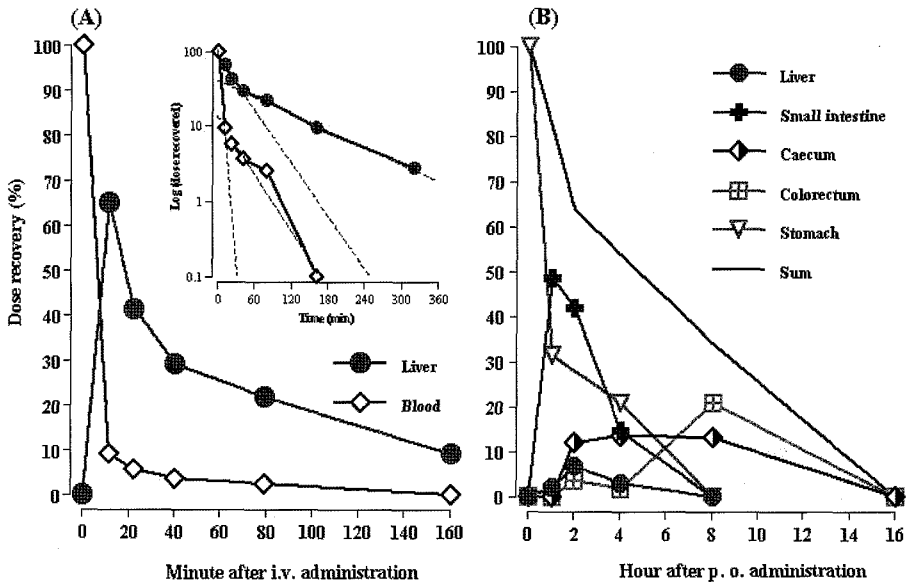

Fig. 12. Distribution of M1 after administration to mice.

metabolites except M1 was excreted as bile.

To examine whether the metabolism of M1 can include variation of aglycone structure or conjugation with acids such as glucuronic and sulfuric acids or with other cellular or tissue components, the liver resected from 40 min after i.v. injection of M1 was divided into three equal parts: one part was untreated control and the others were treated with alkaline or β-glucuronidase, followed by TLC and HPLC analyses. Although both M1 and EM1 were detected in the untreated control, no products except them were newly detected in the samples treated with either alkaline or β-glucuronidase. The amounts of M1 increased in the alkaline-treated sample compared to the untreated control, however. These results showed that M1 was mainly metabolized to esters without structural variation.

We studied the absorption, distribution, excretion, and metabolism of intestinal bacterial metabolites of ginsenosides. Putative metabolic pathways of ginsenosides after oral administration is proposed in Fig. 14. Our study illustrates several novel findings concerning the pharmacodynamics of M1 and M4: (a) M1 was selectively accumulated into the liver and mostly excreted as bile; but (b) free from the excretion, *ca.* 24 mol% of dosed M1 was esterified with fatty acids in the liver; (c) esterified M1 (EM1) was not excreted in the small intestine and so (e) EM1 was accumulated in the liver longer than M1. Therefore, metabolic regulation in the liver may differ between M1 and EM1. In the case of M4, 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 spreading to other organs in the body and excretion as bile.

The abovestudy demonstrated that intestinal bacterial metabolites (M1 and M4) are further biotransformed to fatty acid esters (*i.e.* EM1 and EM4) in the tissues. An issue raised here is whether esterified metabolites, EM1 and EM4, are biologically active or not. We therefore examined the antitumor activities of M1 and EM1, using a solitary tumor model by intrahepatic implantation of B16-F10 cells in C57BL/6 mice.

Tumor cells with or without liposome-encapsulated M1 or EM1were injected into liver capsule. As shown in Fig. 15, the growth of implanted tumor tended to be inhibited in the mice administered with M1 by 23% (not significant compared to the untreated control). In contrast, the treatment of liposome-encapsulated EM1 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 ($p < 0.02$). Thus, the enhanced antitumor

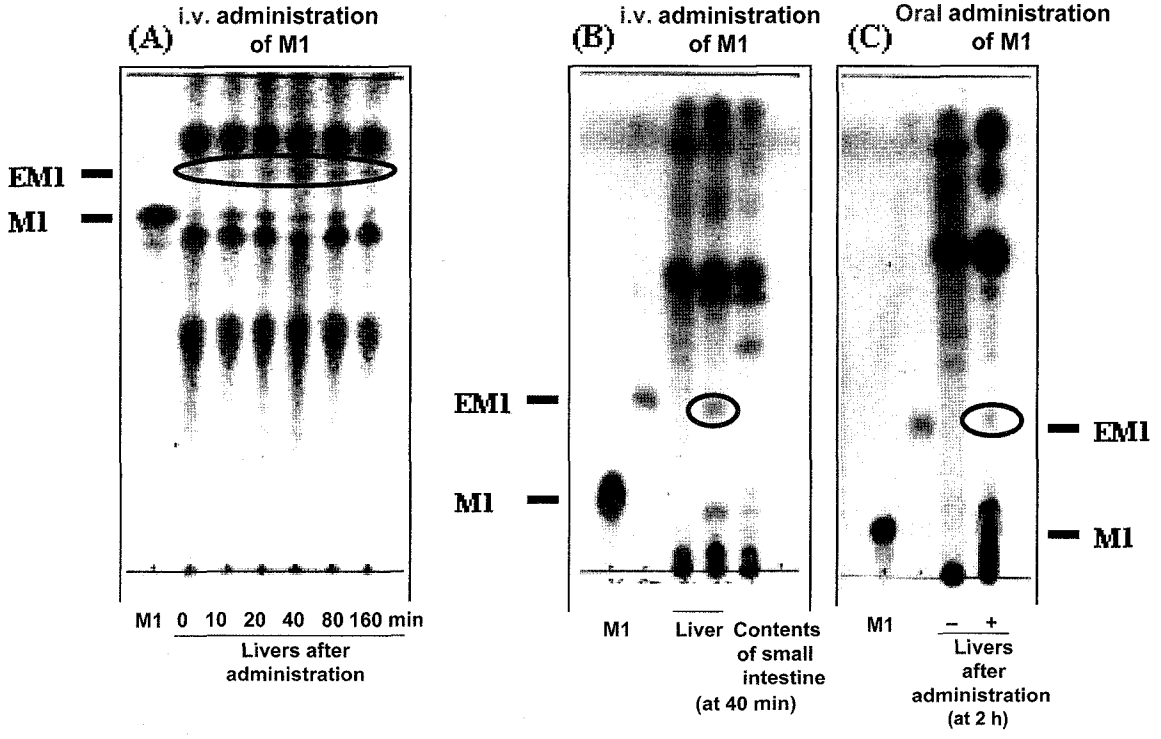

Fig. 13. TLC profiles of M1-metabolites in the liver.

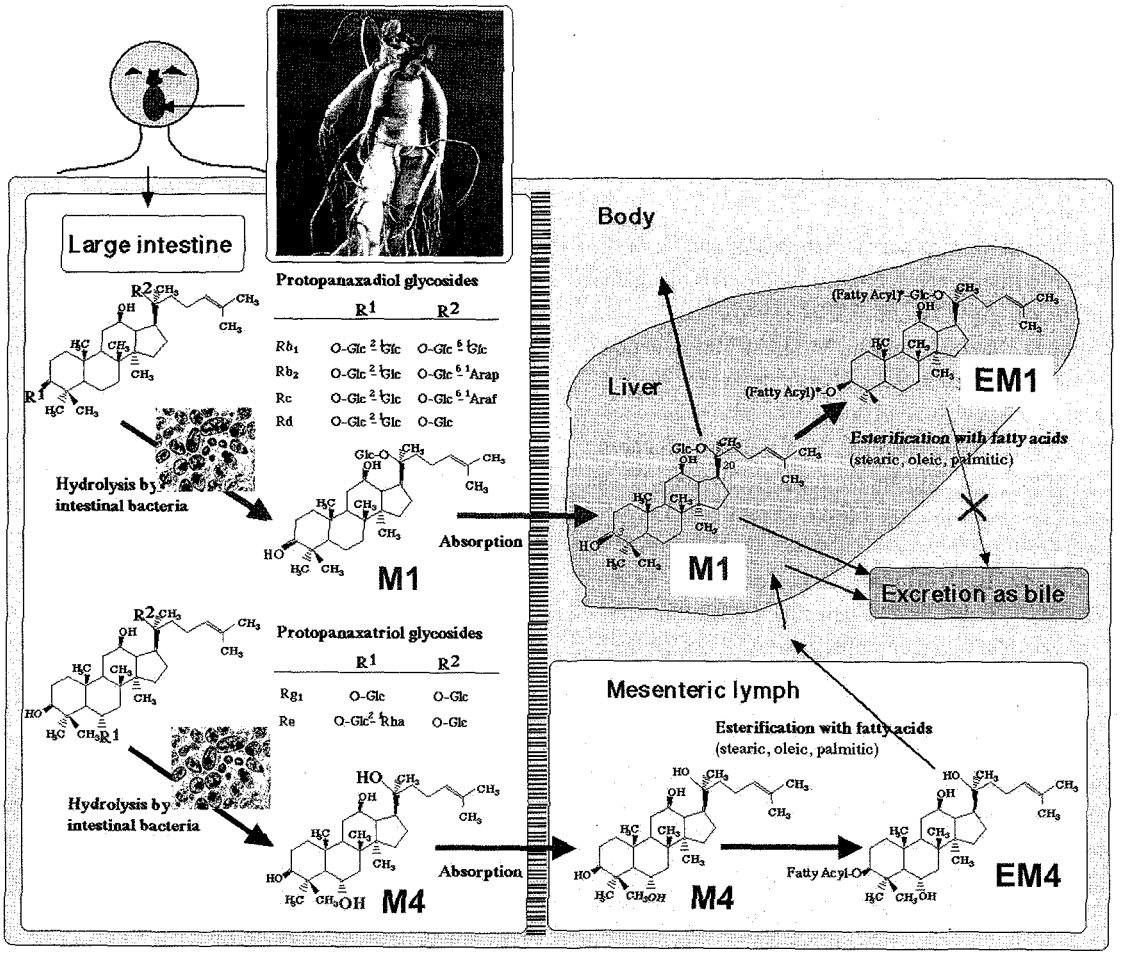

Fig. 14. Putative metabolic pathways of ginsenosides after oral administration.

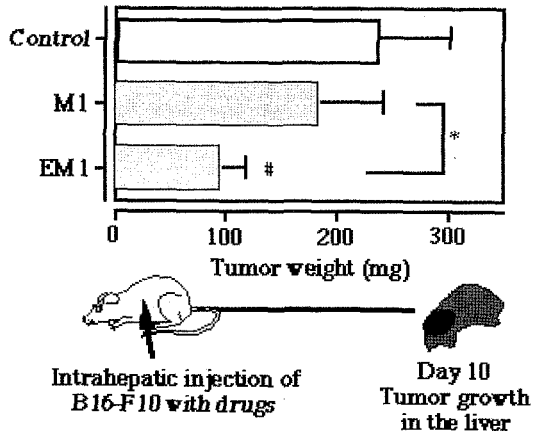

Fig. 15. Effect of liposome-encapsulated M1 and EM1 on the growth of B16-BL6 melanoma cells after intrahepatic co-implantation.

effect of EM1 may be closely associated with its persistent retention in the liver, because EM1 was accumulated in the liver longer than M1. Since EM1 itself was the less cytotoxic against tumor cells than M1 *in vitro*, fatty acid esterification of M1 may lead to detoxification of M1. These results indicate that the suppression of tumor growth by EM1 treatment may involve other mechanisms including the stimulation of the immune system. When B16-F10 cells were incubated for 3 days with hepatic or splenic lymphocytes in the presence or absence of EM1, lymphocyte-mediated tumor cell lysis increased in concentration- and E/T ratio-dependent manners. This demonstrated that EM1-induced inhibition of tumor growth and metastasis may be partly related to the stimulation/activation of lymphocytes to the tumoricidal state.

Thus, some orally administered ginsenosides were metabilized by intestinal bacteria to their metabolite M1, and subsequently resultant M1 was transformed by its esterification reaction to biologically active forms (EM1), which can potentiate M1 through the efficient accumulation.

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