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Metabolic activation of ginsenoside against cancer: Intestinal bacterial deglycosylation and hepatic fatty-acid esterification

Hideo HASEGAWA*

Itto Institute of Life Science Research, Happy World Inc., 3-13-8 Shiraitodai, Fuchu, Tokyo, 183-0011, Japan.

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Abstract

Despite the commonly accepted concept that ginsenoside is responsible for the pharmacological effects of ginseng (*Panax ginseng* C. A. MEYER), there were few systematic studies of the metabolic activation of ginsenoside in the body after oral administration of ginseng. Although, ginsenoside was known to be metabolized by intestinal bacteria, it was obscure whether or not the metabolites participate ginseng actions. For this reason, we analyzed the interrelationship between ginsenoside, formation of intestinal bacterial metabolites, pharmacokinetics of metabolites and preventive actions against cancers. Orally administered ginsenoside passed through the stomach and small intestine without decomposition by either gastric juice or liver enzymes into the large intestine, where ginsenoside was deglycosylated by colonic bacteria. 20(*S*)-Protopanaxadiol monoglucoside (M1) was a major metabolite of protopanaxadiol-type ginsenoside. Following absorption of M1 in the blood, it transferred into the liver with a high degree of selectivity. M1 directly exerted antitumor effects such as induction of apoptosis and cell cycle arrest and inhibition of tumor-induced neovascularization; however, much higher doses of M1 were toxic to the host. Thus, most of the absorbed M1 was excreted rapidly as bile, and some residual M1 (approximately 25% of the dose) was esterified with fatty acids. The esterified form (EM1) was sustained in the liver longer than M1. Thus, the *in vivo* antitumor activity paralleled with the pharmacokinetic behavior. EM1 did not directly affect tumor growth *in vitro*, whereas it stimulated lymphocytes to become cytotoxic to tumor cells. The esterification of M1 with fatty acids potentiated the antitumor activity of M1 through delay of the clearance and through immunostimulation. These findings have given a clue to the mechanism underlying expression of ginseng actions in the body.

Key words *Panax ginseng*, ginsenoside, metabolic activation, intestinal bacteria, deglycosylation, fatty acid, esterification.

Abbreviations R*x*, ginsenoside R*x*; M1, 20(*S*)-protopanaxadiol 20-*O*-β-D-glucopyranoside; EM1, fatty acid ester of M1; M4, 20(*S*)-protopanaxatriol; M12, 20(*S*)-protopanaxadiol.

1. Introduction

Ginseng (the roots of *Panax ginseng* C. A. MEYER, Alariaceae) has been used as one of the most valuable traditional medicines in the Orient for over 2000 years. Ginseng was believed to be an elixir, in other word, cure-all and tonic; however, it was disregarded as entirely psychic especially by the scientists and medical doctors in the western world. For the past few decades, scientific investigations on ginseng have remarkably been advanced, and have partly supported its medical efficacy witnessed through clinical experiences and claims in Oriental medicine. At present, ginseng is one of the most deeply and widely investigated herbal medicines among those currently used both in traditional and modern medicine.

The main ingredients of ginseng are ginsenosides, glycosides containing an aglycone (protopanaxadiol or protopanaxatriol) with a dammarane skeleton. So far, 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

*To whom correspondence should be addressed. e-mail : hasegawahideo@hotmail.com

cell cultures *in vitro* and from intraperitoneal (i.p.) or intravenous (i.v.) injection to experimental animals, though ginseng is generally used as a drug taken orally. Besides, there were few systematic studies of the metabolic activation of ginsenoside in the body after oral administration of ginseng. Although, ginsenoside was known to be metabolized by intestinal bacteria, it was obscure whether the metabolites participate ginseng actions. Recently, epidemiological studies have identified an association between ginseng intake and a decreased incidence and growth of cancers.[1-4] Thus, we conducted *in vivo*, *in vitro* and molecular-level investigations to gain a better understanding of the mechanism underlying anticancer actions of ginseng in the body. Studies using metabolites *in vitro* and *in vivo*, in comparison to parent ginsenosides, have clearly indicated that the anticancer activities of ginseng are based on its saponin metabolites formed by intestinal bacterial deglycosylation and hepatic fatty-acid esterification (Fig. 1). The major object of this article is to review the recent advance of studies on metabolic activation of ginsenoside.

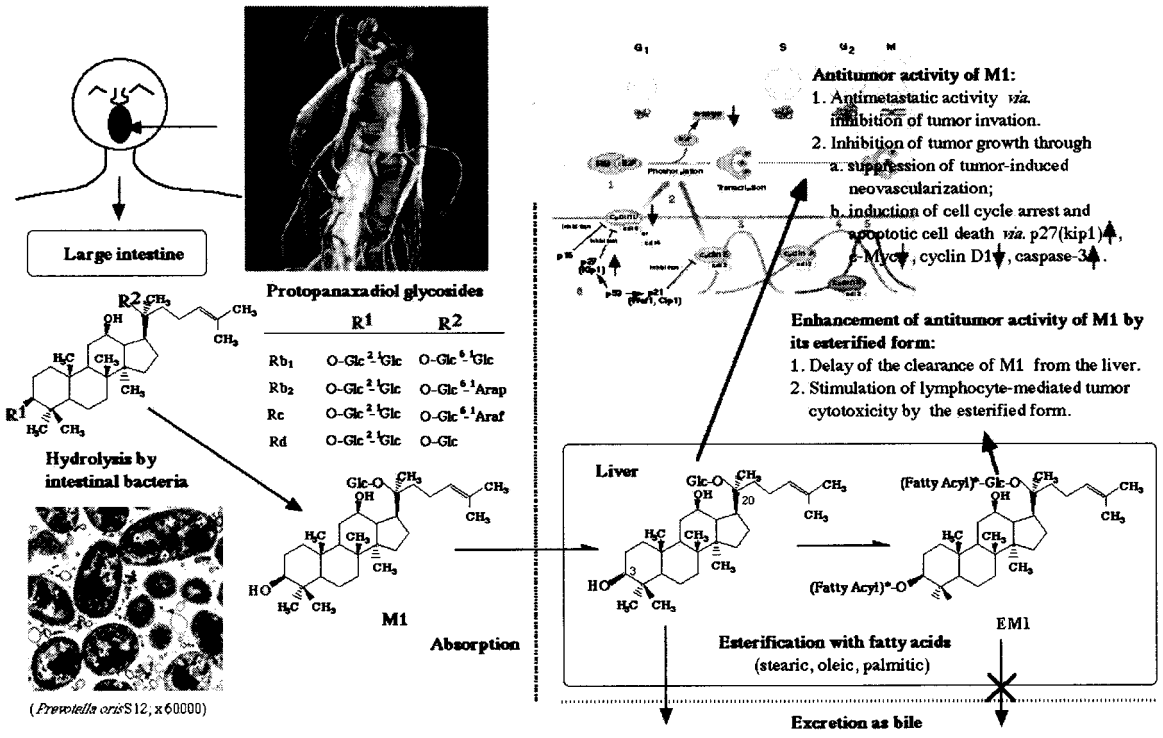

Fig. 1 Metabolic activation of protopanaxadiol-type ginsenosides against cancer through intestinal bacterial deglycosylation followed by hepatic fatty-acid esterification. Ginsenosides are deglycosylated by colonic bacteria and subsequently absorbed into the blood. M1, the major metabolite of protopanaxadiol-type ginsenosides, selectively accumulates in the liver and is mostly excreted as bile; however some residual M1 is esterified with fatty acids at C-3 of the aglycone moiety or C'-6 of the glucose moiety. The esterified form, *i.e.* EM1, is sustained in the liver longer than M1, that potentiates the antitumor activities of M1. Protopanaxatriol-type ginsenosides are activated in almost the same manners as diol-type ginsenosides.

2. Colonic bacterial deglycosylation of ginsenoside

Crude drugs in herbal prescriptions contain many β-glycosides. Orally administered β-glycosides meet gastric juice, digestive and bacterial enzymes in the gastrointestine. Metabolic activation of β-glycosides by intestinal bacteria have been well studied using cycasin[5] (methylazoxymethanol β-glucoside) from the seeds of Cycadaceae or amygdalin[6] (mandelonitrile β-gentiobioside) from the seeds of Rosaceae. Such glycosides become toxic only when deglycosylated: If amygdalin is given i.v. or i.p. to conventional animals or administered orally to the animals treated with antibiotics (without intestinal bacteria), its toxicity is not observed.[6] Besides, the carcinogenic activity of cycasin is expressed in not germ-free but conventional animals.[7] Based on these findings, Kobashi and Namba *et al.* have surveyed the

metabolism of herbal β-glycosides,[8-11] including geniposide from Gardeniae fructus, sennoside from Rhei rhizoma or Sennae folium, glycyrrhizin from Glycyrrhizae radix, paeoniflorin from Paeoniae radix, saikosaponin from Bupleuri radix, barbaloin and aloesin from Aloe, homoorientin from Swertiae herba, mangifelin from Swertiae herba or Anemarrhenae rhizoma and bergenin from Cimicifugae rhizoma or Malloti cortex. Metabolic studies on β-glycosides have revealed that intestinal bacterial enzymes are primarily involved in the metabolism of β-glycosides. Thus, Kobashi has hypothesized that plant β-glycosides may act as natural pro-drugs,[11] which can be transformed to active ingredients by intestinal bacteria after oral administration and consequently induce *in vivo* efficacy.

Some researchers have investigated the metabolism of ginsenoside by using acids, enzymes, intestinal bacteria and animals. Han *et al.* examined the decomposition products of Rb_1, Re and Rg_1 using 0.1 N HCl as gastric acid.[12] Takino *et al.* surveyed the pharmacodynamics of Rb_1, Rb_2 and Rg_1 in rats.[13] The decomposed products taken from the stomach of rats given a gastric bolus of Rb_2 differed from those produced by 0.1 N HCl, and Rb_2 was found to be hardly decomposed by gastric juice with the exception of slight oxygenation.[14] Fig. 2 demonstrates that the *in vitro* metabolism of Rb_1.[15] When Rb_1 was incubated with the contents of mouse gastrointestinal tract, it was metabolized to M1 only by the contents of the large intestine. This phenomenon was also observed in the mice administered with Rb_1 orally.[15] With the passage of time after administration, Rb_1 was detected in the stomach and small intestine followed by M1 in the caecum and colorectum (Fig. 3). These results indicate that colonic bacteria metabolize Rb_1 to M1.

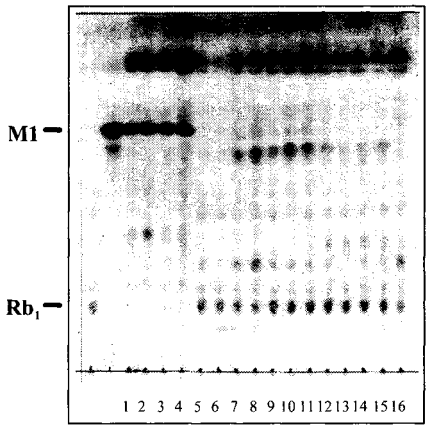

Fig. 2 *In vitro* metabolism of Rb_1 in the contents of gastrointestinal tract. The gastrointestine of C57BL/6 mouse was cut by about 2 cm from the anus to the stomach and the contents were anaerobically cultured in 2 ml of 0.1%Rb_1-GAM broth at 37 ℃ for 30 h. Culture media were extracted with water-saturated *n*-BuOH (1 ml) and centrifuged (1000 x g, 10 min). An aliquot (2 μl) of the *n*-butanol layer was analyzed by TLC [plate, silica gel 70 F_{254} ; developing solvent, $CHCl_3$-MeOH-H_2O (65 : 35 : 10, v/v, lower phase); detection reagent, 8% vanillin in MeOH-72% H_2SO_4 (1 : 5, v/v), with heating at 140 ℃ for 3 min]. **A**: Lanes 1-3, rectum and colon; Lane 4, caecum; Lanes 5-15, small intestine; Lane 16, stomach.

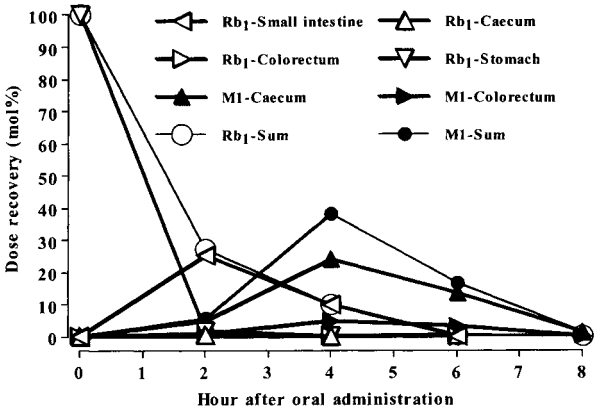

Fig. 3 Gastrointestinal levels of Rb_1 and M1 after oral administration of Rb_1. C57BL/6 mice were orally administered with 43 mg/kg Rb_1, corresponding to 25 mg/kg M1, and sacrificed at the indicated time after administration. The amounts of Rb_1 and M1 in the gastrointestinal tract were determined by HPLC. Each point represents the mean values of dose recovery (mol%) from 3 animals.

Kanaoka *et al.* investigated the metabolism of Rb_1 and Rg_1 by human intestinal bacteria.[16] We also examined the metabolism of ginsenosides in detail.[17] *In vitro* studies have reveled that the nutritious conditions of culture media affect bacterial growth and enzyme production, thus resulting in metabolic variations. The oligosaccharides connected to the C-3 or C-20 hydroxy group of the aglycone were cleaved stepwise from the terminal sugar by bacterial hydrolysis, forming M1 (Fig. 1) or 20(*S*)-protopanaxatriol (M4) *via* intermediates with structural characteristics of each ginsenoside. M1 was gradually hydrolyzed to 20(*S*)-protopanaxadiol (M12).

The bacteria capable of hydrolyzing Rb_1 to M1 was isolated and identified as *Prevotella oris*. This microorganism is a major intestinal bacterial species found together with *P. buccae* in $10^{9.9}$/g wet feces and 73% of fecal specimens.[18] Rb_1-hydrolyzing potential was seen in 79% of human fecal specimens.[19] Therefore, intestinal *P. oris* is believed to participate the metabolism of ginsenosides. However, human fecal flora hydrolyzed

ginsenosides more potently than isolated *P. oris* strains. Recently, *Eubacterium* sp. A-44[20] and *Fusobacterium* K-60[21] have been also shown to exert Rb_1-hydrolyzing potential. In addition, streptococci, bifidobacteria and fusobacteria can weakly hydrolyze Rb_1 and Rb_2.[22] Therefore, many kinds of intestinal bacteria seem to be involved in ginsenoside utilization co-operatively.

The absorption rates of ginsenoside from the intestines are very low. TLC analysis failed to detect intact ginsenoside in the serum obtained from rats administered with Rb_1 orally, and yielded a narrow success in recovering an extremely low proportion (0.05%) of the dose in the urine within 48 h.[23] Moreover, Rb_1 was also undetectable in the plasma from humans given ginseng powder. Takino *et al.* have determined the absorption rate of Rg_1, Rb_1 and Rb_2 to be 1.9%, 0.1% and 3.7%, respectively.[13, 23-25] In spite of poor absorption of ginsenoside, radioisotope assay revealed that serum radioactivity in rats orally given Rb_2 together with its radioactive derivative was 3 times higher than the serum Rb_2-level determined by HPLC.[25] Using GC-MS, Cui *et al.* analyzed the urinary compounds of ginsenosides in humans given ginseng preparations: About 1.2% of the dose was recovered in 5 days and the amounts of sapogenins (M4 and M12) were only 5% of urinary compounds.[26] These results suggested the metabolic ingredients of ginsenosides.[25, 26] Although Takino *et al.* did not show evidence for the absorption of bacterial metabolites from the intestines to the blood, we have detected the metabolites at 0.4-5.1 μg/ml in the peripheral blood from rats administered with ginsenosides (1 g/kg/day).[17] Akao *et al.* has also found that M1, they call compound K, appears in the plasma of gnotobiotic rats after oral administration of Rb_1.[20]

3. Relation of deglycosylation to antimetastatic efficacy of ginsenoside

Intestinal microflora is very changeable in dependence on host conditions, including diet, health and even stress. Indeed, bacterial Rb_1-hydrolyzing potential differed among humans[19] and experimental mice.[27] Orally administered ginsenosides are poorly absorbed from the intestines and metabolized by colonic bacteria, and the bacterial metabolites are absorbed from the intestines. Therefore, it is easily hydrolyzed that the individual differences in bacterial Rb_1-hydrolyzing potential may affect ginseng efficacy. Thus, we evaluated the antimetastatic efficacy of Rb_1, using 2 sets of mice with low and high hydrolyzing potentials of intestinal bacteria. Fig. 4 shows the relation of bacterial Rb_1-hydrolyzing potentials to antimetastatic effects by oral administration of Rb_1. Administration of Rb_1 to the mice with low hydrolyzing potential was ineffective in reducing the number of tumor metastatic colonies in the lung compared to the untreated animals, whereas, lung metastasis was significantly inhibited in the mice with high hydrolyzing potential. This result indicates that the antimetastatic efficacy of Rb_1 was positively correlated with Rb_1-hydrolyzing potentials.

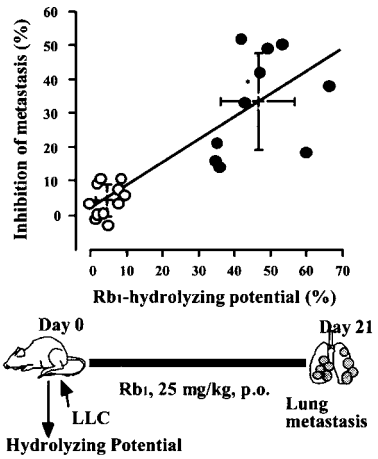

Fig. 4 Relation of Rb_1-hydrolyzing potentials to antimetastatic efficacy of Rb_1. C57BL/6 mice were selected to make 2 groups with low (4.8 $\pm$ 3.2 mol%, n = 11, ○) or high (46.8 $\pm$ 10.3 mol%, n = 10, ●) hydrolyzing potential, and given the drinking water dosed with Rb_1 (90 ppm) for 2 weeks after subcutaneous inoculation of LLC cells (5×10^5) into the abdominal wall. Dosages (25 mg/kg/d) were calculated from the measurements of water intake and animal body weight. Untreated control mice (n = 11) for antimetastatic activity were given drinking water without Rb_1. After 21 days of inoculation, mice were killed and the lungs were examined to check for metastasis. Inhibition of metastasis was expressed as the percent of untreated control. Each point (○ or ●) shows inhibition rate of metastasis (%) and means (+) $\pm$ S.D. of each group. * $p < 0.01$ *vs.* low potential group or untreated control by Student's *t*-test.

Some correlation of Rb_1-hydrolyzing potentials was observed between mother mice and littermates,[15] suggesting bacterial infection from mother to littermate. Microflora analysis has revealed that the frequency (not enzymatic activity) of bacteria regulates Rb_1-hydrolyzing potentials and that consecutive ginseng intake stimulates colonic ginsenoside-hydrolyzing bacteria.

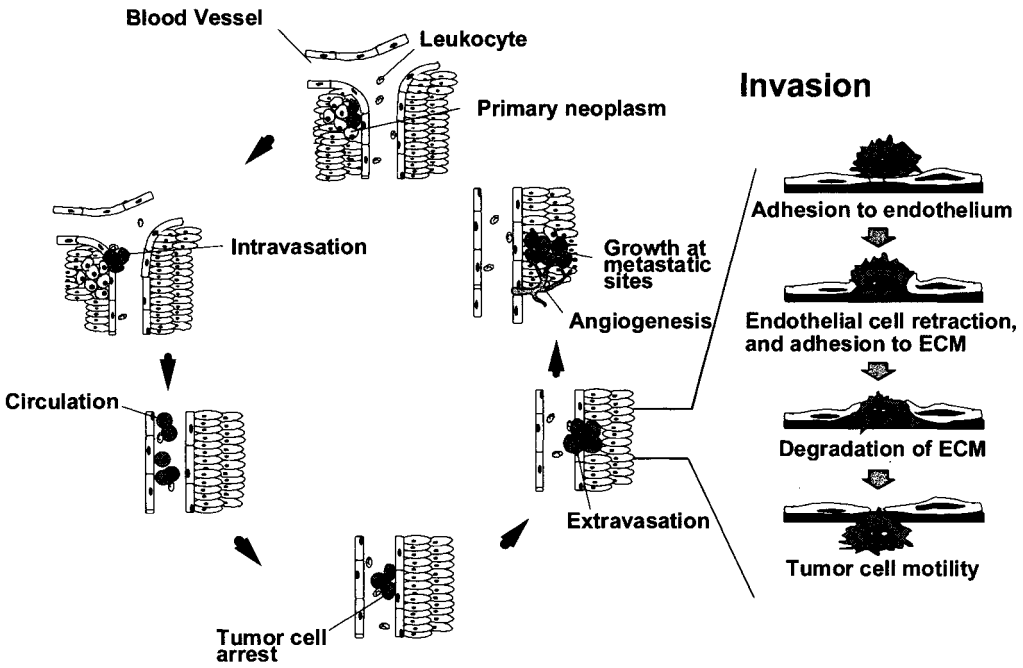

Fig. 5 Schematic illustration of the major steps of tumor metastasis

4. Antimetastatic activity of ginsenoside based on its bacterial metabolite

Metastasis, one of the major causes of mortality in cancer, is a complex cascade of events involving tumor dissemination from the primary site of growth to distant organs. The pathogenesis of metastases following tumor development and progression can be subdivided into a variety of sequential steps (Fig. 5): 1) Release from the primary tumor and invasion of the surrounding tissues. 2) Entry into the vascular or lymphatic circulation. 3) Transit in the circulation. 4) Arrest in the capillary bed of a distant organ. 5) Extravasation from the circulation. 6) Growth at apparently selected sites that are distant from the original tumor site.[28-31] Few cells in a primary tumor can complete all the steps necessary to achieve metastasis. Specific tumor interactions with host cells (platelet, lymphocytes or endothelial cells) and/or extracellular matrix (ECM) and basement membrane components are therefore fundamental events in preferential organ colonization whereby metastases occur in specific organs and not randomly.

To answer the question whether the bacterial metabolites are associated with the expression of antimetastatic efficacy after oral administration of ginsenoside, the activities of bacterial metabolites were examined in comparison with parent ginsenosides. The results obtained from *in vivo* and *in vitro* comparative examinations are summarized in Fig. 6 (typically represented by the data for Rb_1 and M1).

Both of ginsenoside and metabolite exerted a marked inhibition of lung metastasis when administered orally to mice; however, only i.v. administration of metabolite resulted in a significant inhibition of tumor metastasis (Fig. 6).[32] Tumor invasion into extracellular matrices and basement membranes is a crucial step in the complex multistage process that leads to the metastatic formation (Fig. 5). Ginsenosides exerted no or slight

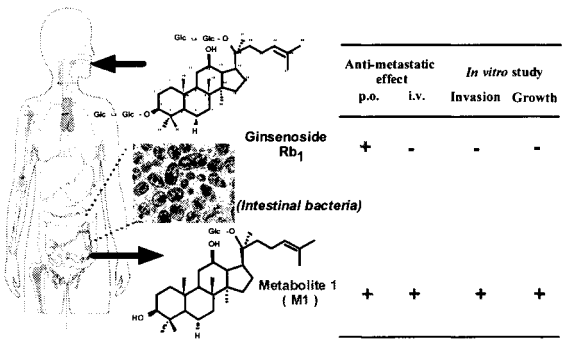

Fig. 6 Antimetastatic effects of Rb_1 and its metabolite, M1.

inhibition of tumor proliferation and invasion in murine B16-BL6 melanoma and human HT-1080 sarcoma cell lines, whereas their metabolites even at non-cytotoxic concentrations inhibited the invasion of both tumor cells more effectively than ginsenosides (Fig. 6).[32, 33] Tumor invasion consists of such distinct events as adhesion to adjacent basement membranes, enzymatic degradation, and migration (Fig. 5). Bacterial metabolite at non-cytotoxic concentrations inhibited the adhesion of B16-BL6 cells and reduced the type IV collagenase secretion from HT1080 cells.[34] The inhibitory activities against the migration of B16-BL6 or HT-1080 cells paralleled with the anti-invasive activities.[32, 33] These findings suggest that the action of bacterial metabolites primarily mediates the antimetastatic effects by orally administered ginsenosides. This concept agrees with the pharmacokinetic findings: not ginsenosides but their bacterial metabolites are absorbed from the intestines after oral administration of ginsenosides (Section 2).

Dansyl M1, a biologically active fluorescent probe of M1,[35] entered the cytosol and quickly reaches the nuclei of B16-BL6 cells after about 15 min of incubation (Fig. 7).[36] M1 inhibited the proliferation of LLC cells, with characteristic morphological changes at 40 μM.[37] Treatment of LLC cells with 30 μM of 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 (Fig. 7). M1-induced growth inhibition of LLC cells was completely abrogated by the pretreatment with Z-DEVD-FMK, a specific inhibitor of caspase-3, suggesting that M1 functions *via* the activation of caspase-3 in the process of apoptosis in LLC cells.[37] In addition, Western blot analysis has demonstrated that M1 rapidly up-regulates the expression of $p27^{Kip1}$, but down-regulates the expression of c-Myc and cyclin D1 in a time-dependent manner (Fig. 7).[36] Thus, the anti-proliferative activity of M1 against tumor cells is principally due to the induction of apoptosis *via* promotion of caspase-3 activity and regulation of apoptosis-related proteins, and this induction may lead to the antitumor activity *in vivo*.

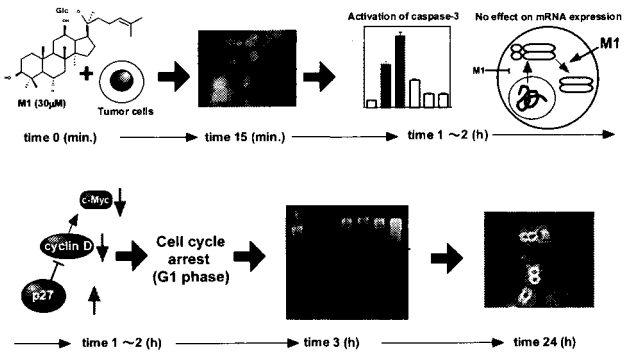

Fig. 7 Mechanism of M1-induced growth inhibition of tumor cells.

Tumor-induced neovascularization (angiogenesis) is also one of the most important events concerning tumor growth and metastasis (Fig. 5). Angiogenesis toward and into tumors is a crucial step for the delivery of nutrition and oxygen to tumors, and also functions as the metastatic pathway to distant organs.[38] 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.[39] Recently, Tanigawa *et al.* have reported that hepatic sinusoidal endothelial (HSE) cells are associated with tumor-induced angiogenesis in the liver.[40] The proliferation and tube formation of HSE cells are stimulated by vascular endothelial growth factor in conditioned medium of colon 26-L5 cells (CM-L5).[41] Addition of CM-L5 obtained from the cultures of colon 26-L5 cells with non-cytotoxic concentrations of M1 resulted in a concentration-dependent inhibition of the tube formation of HSE cells.[42]

5. Link between pharmacokinetics and antitumor activity of M1

Pharmacokinetic analysis of M1 has led to a better understanding of the mechanism underlying *in vivo* antitumor activity of M1. Oral administration of M1 (10 mg/kg) after intrahepatical implantation of colon 26-L5 tumors resulted in a significant inhibition of tumor growth and metastasis compared with control.[42] The hepatic M1-level peaked 2 h after administration (7.1 μg/g liver at the dose of 10 mg/kg; 34 μg/g liver at 25 mg/kg) and then gradually decreased (Fig. 8). Hepatic retention of M1 at more than 5 μM (3 μg/g) for 6 h (Fig. 8) was sufficient to inhibit tube formation of HSE cells.[42] Significantly effective levels of M1 at more than 8 μM (5 μg/g) and 16 μM (10 μg/g) required to prevent the invasion and proliferation of colon 26-L5 cells (Fig. 9)

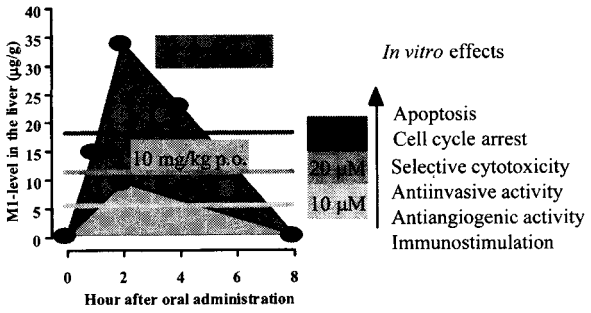

Fig. 8 Hepatic M1-levels and possible *in vitro* effects. C57BL/6 mice were given oral administration of M1 at a dose of 10 or 25 mg/kg. The hepatic M1-levels of M1 at the indicated time after administration were determined by HPLC. Each point represents the mean value of M1-liver level from 2 to 3 animals.

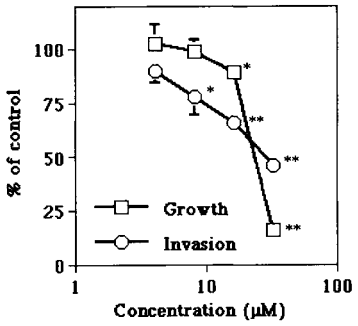

Fig. 9 Cytotoxic and anti-invasive activities of M1 against colon 26-L5 cells. For the cytotoxicity assay, cells ($1x10^4$/well) were incubated with M1 (□) at 37°C for 4 h. Cell viability was assayed by the crystal violet staining method. For the anti-invasion assay, cells ($1x10^4$/well) were seeded with M1 (○) into the upper compartment of a Transwell cell culture chamber. After 4 h of incubation, the cells that invaded the lower surfaces were visually counted. Data are expressed as the mean ± S.D. of 3 dishes. *, $p < 0.05$; **, $p < 0.01$ *vs.* control by Student's two-tailed *t*-test.

were sustained for more than 4 h in the liver (Fig. 8). Moreover, the hepatic accumulation of M1 at more than 20 μM (12 μg/g) might exert relatively selective cytotoxicity against tumor cells compared with normal cells[32, 33] *via* induction of cell cycle arrest and apoptosis (Fig. 8). On the other hand, oral administration of M1 before tumor inoculation also exerted a dose-dependent inhibition of hepatic metastasis produced by intraportal injection of colon 26-L5 cells (Fig. 10). This suggests that the antimetastatic effect is indirectly related to M1-induced host-mediated responses. Therefore, the antimetastatic effects of M1 may be partly associated with its direct efficacy to tumor cell functions (growth and invasion) and with host responses indirectly mediated by M1 (regulation of tumor-induced angiogenesis

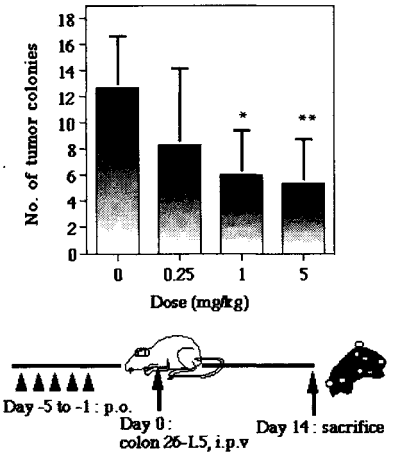

Fig. 10 Antimetastatic effects by oral administration of M1 before tumor inoculation. Female BALB/c mice were inoculated intraportally with colon 26-L5 cells (2×10^4 cells/mouse) after the oral administration of M1 for 5 days (0.25, 1 or 5 mg/kg/day). After 14 days of tumor inoculation, the animals were sacrificed and the number of tumor colonies in the liver was counted manually. The results represent the mean ± S.D. of 6 mice per group. *, $p < 0.05$; **, $p < 0.02$ *vs.* control by Student's two-tailed *t*-test.

and immunomodulation) (Fig. 8).

6. Esterification of M1 with fatty acid in the liver

Our results from fluorescence and HPLC analyses have first illustrated several novel findings concerning the pharmacokinetics of M1 (Fig. 1): (a) M1 selectively transfers into the liver from the blood within 15 min after i.v. administration and is mostly excreted as bile; but (b) free from the excretion, some (*ca.* 24 mol%) of dosed M1 is esterified with fatty acids in the liver; (c) esterified M1 (EM1) is not excreted in the small intestine and so (e) EM1 is accumulated in the liver longer than M1.[43]

The selective accumulation of M1 in the liver was not observed with respect to M4. M1 chemically consists of dammaranediol and glucose (Fig. 1). Hepatocytes can recognize glucose moiety *via* a receptor except for galactose receptor.[44, 45] Therefore, this specific function of hepatocytes must be partly associated with the selective accumulation of M1 into the liver.

Metabolic regulation of M1 in the liver differed from those glycosides which are conjugated with glucronic acid (*ex.* glycyrrhetic acid and baicalein, the sapogenins of glycyrrhizin and baicalin).[10] The transfer of M1 from the blood to the bile *via* the liver means a simple influx and efflux reaction of hepatocytes to M1.

The esterification of M1 shows the presence of another metabolic reaction. Mass spectrometry analysis has defined EM1 as a mixture of various fatty acid M1 monoesters, including stearate, oleate or palmitate. The fragment pattern of EM1 suggests that fatty acid is linked to M1 at C-3 of the aglycone moiety or C'-6 of the glucose moiety. To our knowledge, there have been few reports on animal fatty acid triterpene esters, except for two reports by Tabas *et al.* who isolated the triterpene esters containing 80% stearate and 20% palmitate from the liver of rabbits and humans.[46] They 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.[47] The detection of fatty acid esters of M1 (EM1) in the liver is the first evidence for their hypothesis.

7. Enhancement of antitumor activity of M1 by fatty acid esterification

The esterification of M1 with fatty acids potentiated the *in vivo* antitumor activity of M1 in parallel with the pharmacokinetic behavior (Fig. 11): The growth of implanted hepatoma tended to be inhibited in mice administered with M1 by 23% (not significant compared to the control mice) (Fig. 11A). In contrast, the treatment of 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 ($p < 0.02$). Concerning the pharmacokinetics, 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. 11B). On the other hand, EM1 was also rapidly accumulated in the liver (T_{max}, 40 min; C_{max}, 43% recovery), but the EM1 level decreased gradually with time, and over 25% of the dose (120 μg/g wet tissue) was sustained for 24 h after administration. Since only a small amount of M1 resulting from deacylation of EM1 in the liver was detected, the deacylation rate appears to be very low. 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. Breistøl *et al.* have reported that a fatty acid ester derivative of cytarabine is more effective for the treatment of hematological malignancies than cytarabine.[48] Thus, the enhanced antitumor effect of EM1 may be closely associated with its persistent retention in the liver.

An issue raised here is which active principle, M1 or EM1, is required for expression of the antitumor effects of ginsenoside. The dammaranediol in M1 is structurally similar to cholesterol. Although M1 shows relatively selective cytotoxicity against tumor cells compared with normal cells *in vitro*,[33] excessive intracellular M1 may become toxic to normal cells. Because EM1 was less cytotoxic than M1 *in vitro*,[43] the esterification of M1 may represent a detoxification reaction, just as cholesterol esterification is shown to prevent the cytotoxicity of excess intracellular cholesterol.[49] It is also possible that the effect of EM1 may not depend on

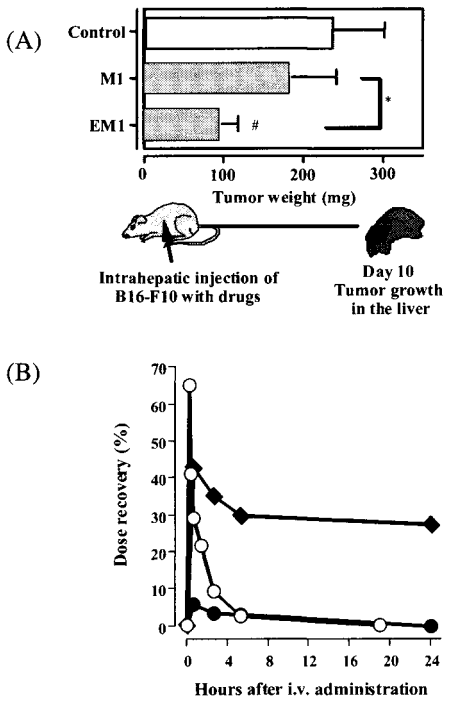

Fig. 11 *In vivo* antitumor activity (**A**) and hepatic levels (**B**) of M1 and EM1. (**A**) The antitumor activities of M1 and EM1 were evaluated, using a solitary hepatoma model by intrahepatic implantation of B16-F10 cells in C57BL/6 mice. Mice were given a single injection of B16-F10 cells with or without M1 or EM1 (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.* control; *$p < 0.02$ *vs.* M1-treated group by Student's two-tailed *t* test. (**B**) Animals were given a single i.v. injection of M1 or EM1 at a dose of 30 mg/kg. At the indicated time after administration, the dose recovery of M1 and EM1 in the liver was determined by HPLC. Each point represents the mean value of % dose recovery from 2 to 3 animals (◆, EM1; ○, M1; ●, M1 deacylated from EM1).

itself but the resultant M1 from deacylation of EM1 by esterase. Neither M1 nor cholesterol oleate stimulated splenic lymphocytes to become cytotoxic to tumor cells, whereas EM1 promoted the tumor cell lysis mediated by lymphocytes, particularly non-adherent splenocytes, in a concentration-dependent manner.[50] These results clearly indicate that EM1 can induce host-mediated responses more strongly that M1.

There are mainly 2 pathways in the tumor cell lysis mediated by immune cells: NK cells destruct tumor cells by injecting perforin into target cells, and macrophages kill tumors through phagocytosis or activation of T cells. Anti-asialo GM1 serum and 2-chloroadenosine can selectively eliminate NK cells and macrophages, respectively.[51] In the lung metastasis produced by i.v. inoculation of B16-BL6 cells, the treatment with anti-asialo GM1 serum or 2-chloroadenosine before tumor inoculation increased the number of tumor metastatic colonies in the lung by 6 fold or 1.5 fold, respectively, compared with the control group (Fig. 12). In this metastasis model, NK cells seem to play more important roles

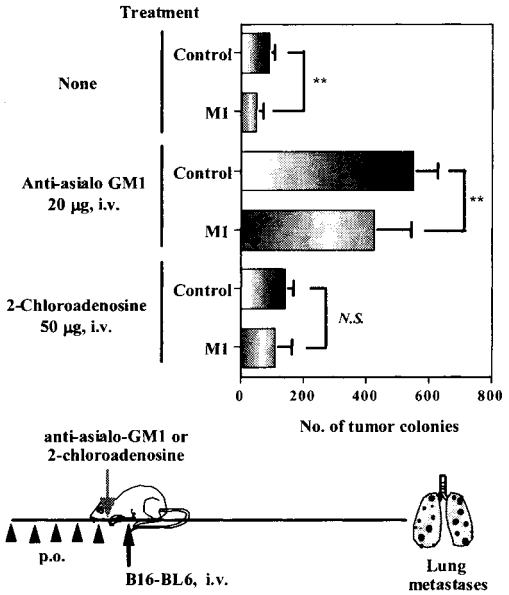

Fig. 12 Antimetastatic effects by oral administration of M1 before tumor inoculation. Female C57BL/6 mice were administered orally with or without M1 for 5 days (5 mg/kg/day) before an i.v. injection of B16-BL6 cells (3 x 10^4 cells/mouse). Anti-asialo GM1 serum or 2-chloroadenosine was administered i.v. at 24 h before tumor inoculation (*i.e.* immediately after the last administration of M1). After 14 days of tumor inoculation, the animals were sacrificed and the number of tumor colonies in the lung was counted manually. The results represent the mean ± S.D. of 6 mice per group. **, $p < 0.01$ *vs.* control by Student's two-tailed *t*-test. *N.S.*, not statistically significant.

in preventing metastatic spread of tumor cells than macrophages. The oral administration of M1 prior to tumor inoculation significantly abrogated the enhanced metastasis in the mice pretreated with anti-asialo GM1 serum (*i.e.* NK cell-eliminated mice), but not in the 2-chloroadenosine-pretreated mice (*i.e.* macrophage-eliminated mice) (Fig. 12). This indicates that the antimetastatic mechanism of M1 is principally associated with the activation of macrophages and T cells. M1 is mostly excreted as bile after administration. Instead, its fatty acid esters (EM1) biotransformed in the liver is accumulated in the body (Section 6) and they promote the tumor cell lysis mediated by non-adherent splenocytes.[50] Therefore, the antimetastatic effect may be based on EM1-mediated activation of T cells. Some glycolipids, such as α-galactosylceramide and a lipid A analogue, have been reported to stimulate host immune responses *via* surface molecules, including CD1, for T cell recognition of glycolipids.[52] EM1 also belong to the category of glycolipid. Therefore, if the esterification of M1 is a detoxification reaction, the resultant esters (*i.e.* EM1) can potentiate M1 through efficient accumulation.

8. Conclusion

In the latter of the 20th century, pharmaceutical studies on traditional medicines started in parallel with structural elucidation of ingredients, in order to give scientific grounds to their medical efficacy. These medicines are administered orally, in general. Therefore, their ingredients must meet gastric juice, digestive and bacterial enzymes in the intestines. Nonetheless, lacking consideration of metabolic behavior, the whole extracts or ingredients have been not a little examined by directly adding to cell cultures *in vitro* or by injecting i.p. or i.v. to experimental animals. Surely, these methods may be useful for intact ingredients but not for their metabolites. Our studies using metabolites *in vitro* and *in vivo*, in comparison to parent ginsenosides, have revealed that the anticancer activities of ginseng after oral administration are based on its saponin metabolites formed by intestinal bacterial deglycosylation and hepatic fatty-acid esterification (Fig. 1): Bacterial metabolites directly exert antitumor effects such as induction of apoptosis and cell cycle arrest and inhibition of tumor-induced angiogenesis, and the fatty acid esters of metabolite

potentiate the antitumor effects through delay of the clearance and through immunostimulation. These findings clearly indicate that the pharmacological studies accompanied by metabolic elucidation of ingredients need to gain a better understanding of the real active principles in the body.

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和文抄録

人参（*Panax ginseng* C. A. MEYER; 和名，オタネニンジン）は滋養強壮，造血，精神安定，腱胃整腸，微小循環の改善，免疫増強など多彩な薬効をもつことから古来より多くの疾患の治療に用いられてきた。人参に含まれる成分の解析が進むとともに，それぞれの成分の薬理活性に関する研究が行われ，代謝系，循環器系，消化器系，中枢神経系，及び免疫系に対する調節作用だけでなく，抗腫瘍及び抗炎症など，様々な方面で薬効を発揮することが実験的に証明されつつある。これらの解析は，主に人参から抽出されたエキス及びその主成分である人参サポニンを中心に行われた。現在，人参の薬効はその主成分である人参サポニンの作用に依拠すると信じられている。しかし，人参サポニンが経口摂取後，生体内でどのようにして活性を発現するようになるのかといった作用機序に関する系統的な研究はこれまでほとんどなされてこなかった。また，人参サポニンが腸内細菌によって代謝されることは知られていたが，その代謝物が薬効発現に関わっているのかどうか明確でなかった。そこで，人参サポニン，腸内細菌による代謝，代謝物の薬物動態，及び抗腫瘍効果の関係を検討した。その結果，人参サポニンは腸内細菌によって代謝され，吸収された後，組織内で脂肪酸抱合されることを見出した。また，人参サポニン自身には抗腫瘍効果はほとんどなく，代謝物に変換されることによって効果を発現すること，さらに，脂肪酸抱合によってその効果が増強されることも明らかにした。これらの結果は，人参サポニンの作用機序を理解する上で基礎となる発見である。

*〒183-0011 府中市白糸台 3-13-8
一都生命科学研究所 長谷川秀夫

References

1) Yun, T. K. and Choi, S. Y. : Preventive effect of ginseng intake against various human cancers: a case-control study on 1987 pairs. *Cancer Epidemiol. Biomarkers Prev.* **4**, 401-8, 1995.
2) Ahn, Y. O. : Diet and stomach cancer in Korea. *Int. J. Cancer Suppl.* **10**, 7-9, 1997.
3) Yun, T. K. : Experimental and epidemiological evidence of the cancer preventive effects *of Panax ginseng* C. A. Meyer. *Nutrition Rev.* **54** (II), S71-S81, 1996.
4) Yun, T. K. and Choi, S. Y. : Non-organ specific cancer prevention of ginseng; Preventive study in Korea. *Int. J. Epidemiol.* **27**, 359-364, 1998.
5) Spatz, M., McDaniel, E. G. and Laqueur, G. L. : Cycasin excretion in conventional and germfree rats. *Proc. Soc. Exp. Biol. Med.* **121**, 417-422, 1966.
6) Drasar, B. S. and Hill, M. J. : Human intestinal flora, pp. 61-63, Academic Press, London, New York, 1974.
7) Morgan, R. W. and Hoffmann, G. R. : Cycasin and its mutagenic metabolites. *Mutation Res.* **114**, 19-58, 1983.
8) Akao, T., Hattori, M., Namba, T. and Kobashi, K. : Metabolic activation of drug components by intestinal bacterial enzymes. Metabolism of drugs by intestinal bacteria. *J. Trad. Med.* **9**, 1-13, 1992 (Engl. Abstract).
9) Kobashi, K., Akao, T., Hattori, M. and Namba, T. : Metabolism of drugs by intestinal bacteria. *Bifidobacteria Microflora* **11**, 9-23, 1992.
10) Kobashi, K. and Akao, T. : Relation of intestinal bacteria to pharmacological effects of glycosides. *Bioscience Microflora* **16**, 1-7, 1997.
11) Kobashi, K. 1998. Glycosides are natural prodrugs : Evidence using germfree and gnotobiotic rats associated with a human intestinal bacterium. *J. Trad. Med.* **15**, 1-13 (Engl. Abstract).
12) Han, B. H., Park, M. H., Han, Y. N., Woo, L. K., Sankawa, U., Yahara, S. and Tanaka, O. : Degradation of ginseng saponins under mild acidic conditions. *Planta Med.* **44**, 146-149, 1982.
13) Takino Y. : Studies on the pharmacodynamics of ginsenoside-Rg_1, -Rb_1 and -Rb_2 in rats. *Yakugaku Zasshi* **114**, 550-564, 1994 (Engl. abstract).
14) Karikura, M., Miyase, T., Tanizawa, H., Taniyama, T. and Takino, Y. : Studies on absorption, distribution, excretion and metabolism of ginseng saponins. VI. The decomposition products of ginsenoside Rb_2 in the stomach of rats. *Chem. Pharm. Bull.*, **39**, 400-404, 1991.
15) Hasegawa, H. and Benno, Y. : Anticarcinogenesis in mice by ginseng-hydrolyzing colonic bacteria. *Microb. Ecol. Health Dis.* **12**, 85-91, 2000.
16) Kanaoka, M., Akao, T. and Kobashi K. : Metabolism of ginseng saponins, ginsenosides, by human intestinal bacteria. *J. Trad. Med.* **11**, 241-245, 1994 (Engl. Abstract).
17) Hasegawa, H., Sung, J. H., Matsumiya, S. and Uchiyama, M. : Main ginseng saponin metabolites formed by intestinal bacteria.

Planta Med. **62**, 453-457, 1996.

18) Benno, Y., Endo, K., Mizutani, T., Namba, Y., Komori, T. and Mitsuoka, T. : Comparison of fecal microflora of elderly persons in rural and urban areas of Japan. *Appl. Environ. Microbiol.* **55**, 1100-1105, 1989.

19) Hasegawa, H., Sung, J. H. and Benno, Y. : Role of human intestinal *Prevotella oris* in hydrolyzing ginseng saponins. *Planta Med.* **63**, 436-440, 1997.

20) Akao, T., Kida, H., Kanaoka, M., Hattori, M. and Kobashi, K.: Intestinal bacterial hydrolysis is required for appearance of compound K in rat plasma after oral administration of ginsenoside Rb_1 from *Panax ginseng*. *J. Pharm. Pharmacol.* **50**, 1155-1160, 1998.

21) Park, S. Y., Bae, E. A., Sung, J. H., Lee, S. K. and Kim, D. H.: Purification and characterization of ginsenoside Rb_1-metabolizing beta-glucosidase from Fusobacterium K-60, a human intestinal anaerobic bacterium. *Biosci. Biotechnol. Biochem.* **65**, 1163-1169, 2001.

22) Bae, E. A., Park, S. Y. and Kim, D. H. : Constitutive beta-glucosidases hydrolyzing ginsenoside Rb_1 and Rb_2 from human intestinal bacteria. *Biol. Pharm. Bull.* **23**, 1481-1485, 2000.

23) Odani, T., Tanizawa, H. and Takino, Y. : Studies on the absorption, distribution, excretion and metabolism of ginseng saponins. III. The absorption, distribution and excretion of ginsenoside Rb_1 in the rat. *Chem. Pharm. Bull.* **31**, 1059-1066, 1983.

24) Odani, T., Tanizawa, H. and Takino, Y. : Studies on the absorption, distribution, excretion and metabolism of ginseng saponins. II. The absorption, distribution and excretion of ginsenoside Rg_1 in the rat. *Chem. Pharm. Bull.* **31**, 292-298, 1983.

25) Tanizawa, H., Karikuma, M., Miyase, T. and Takino, Y. : Studies on the metabolism and/or decomposition and distribution of ginsenoside Rb_2 in rats. *Proc. 6th Int. Ginseng Symp.*, Seoul, pp. 187-194, 1993.

26) Cui, J. F., Bjorkhem, I. and Eneroth, P. : Gas chromatographic-mass spectrometric determination of 20(*S*)-protopanaxadiol and 20(*S*)-protopanaxatriol for study on human urinary excretion of ginsenosides after ingestion of ginseng preparations. *J. Chromatogr. B Biomed. Sci. Appl.* **689**, 349-355, 1997.

27) Hasegawa, H. and Uchiyama, M. : Antimetastatic efficacy of orally administered ginsenoside Rb_1 in dependence on intestinal bacterial hydrolyzing potential and significance of treatment with an active bacterial metabolite. *Planta Med.* **64**, 696-700, 1998.

28) Hart, I. R. : "Seed and soil" revisited: mechanisms of site specific metastasis. *Cancer Metastasis Rev.* **1**, 5-16, 1982.

29) Liotta, L. A., Rao, C. V. and Barsky, S. H. : Tumor invasion and the extracellular matrix. *Lab. Invest.* **49**, 636-649, 1983.

30) Fidler, I. J. : The Ernst W. Bernet memorial award lecture: the evolution of biological heterogeneity in metastatic neoplasms. In: "Cancer invasion and metastases: Biologic and therapeutic aspects" (Eds. by Nicolson, G. L. and Milas, L.), Raven Press, New York, pp. 5-26, 1984.

31) Nicolson, G. L. : Tumor cell instability, diversification, and progression to the metastatic phenotype: from oncogene to oncofetal expression. *Cancer Res.* **47**, 1473-1487, 1987.

32) Wakabayashi, C., Hasegawa, H., Murata, J. and Saiki I. : The expression of *in vivo* anti-metastatic effect of ginseng protopanaxatriol saponins is mediated by their intestinal bacterial metabolites after oral administration. *J. Trad. Med.* **14**, 180-185, 1997.

33) Wakabayashi, C., Hasegawa, H., Murata, J. and Saiki, I. : *In vivo* antimetastatic action of ginseng protopanaxadiol saponins is based on their intestinal bacterial metabolites after oral administration. *Oncol. Res.* **9**, 411-417, 1997.

34) Hasegawa, H., Sung, J. H. and Huh, J. D. : Ginseng intestinal bacterial metabolite IH901 as a new anti-metastatic agent. *Arch. Pharm. Res.* **20**, 539-544, 1997.

35) Hasegawa, H., Suzuki, R., Wakabayashi, C., Murata, J., Tezuka, Y., Saiki, I. and Kadota S. : Synthesis of a biologically active fluorescent derivative of GM1, a main ginseng saponin metabolite formed by intestinal bacteria. *Biol. Pharm. Bull.* **22**, 513-516, 1998.

36) Wakabayashi, C., Murakami, K., Hasegawa, H., Murata, J. and Saiki, I. : An intestinal bacterial metabolite of ginseng protopanaxadiol saponins has the ability to induce apoptosis in tumor cells. *Biochem. Biophys. Res. Commun.* **246**, 725-730, 1998.

37) Suda, K., Murakami, K., Murata, J., Hasegawa, H. and Saiki I. : Induction of apoptosis in Lewis lung carcinoma cells by an intestinal bacterial metabolite resulted from orally administered ginseng protopanaxadiol saponins. *J. Trad. Med.* **17**, 236-244, 2000.

38) Folkman, J. and Cotran, R. : Relation of vascular proliferation to tumor growth. *Int. Rev. Exp. Pathol.* **16**, 207-248.

39) Folkman, J. : How is blood vessel growth regulated in normal and neoplastic tissue? G. H. A. Clowes memorial award lecture. *Cancer Res.* **46**, 467-473, 1986.

40) Tanigawa, N., Lu, C., Mitsui, T. and Miura, S. : Quantitation of sinusoid-like vessels in hepatocellular carcinoma: its clinical and prognostic signification. *Hepatology.* **26**, 1216-1223, 1997.

41) Murakami, K., Sakukawa R., Sano, M., Hashimoto, A., Shibata, J., Yamada, Y. and Saiki, I. : Inhibition of angiogenesis and intrahepatic growth of colon cancer by TAC-101. *Clin. Cancer Res.* **5**, 2304-2310, 1999.

42) Suda, K., Murakami, K., Murata, J., Hasegawa, H. and Saiki I. : An intestinal bacterial metabolite (M1) of ginseng protopanaxadiol saponins inhibits tumor-induced neovascularization. *J. Trad. Med.* **17**, 144-150, 2000.

43) Hasegawa, H., Lee, K. S., Nagaoka, T., Tezuka, Y., Uchiyama, M., Kadota, S. and Saiki, I. : Pharmacokinetics of ginsenoside deglycosylated by intestinal bacteria and its transformation to biologically active fatty acid esters. *Biol. Pharm. Bull.* **23**, 298-304, 2000.

44) Kawaguchi, K., Kuhlenschmidt, M., Roseman, S. and Lee, Y. C.: Differential uptake of D-galactosyl- and D-glucosyl-neoglycoproteins by isolated rat hepatocytes. *J. Biol. Chem.* **256**, 2230-2234, 1981.

45) Shimizu, K., Maitani, Y., Takahashi, N., Takayama, K. and Nagai, T. : Association of liposomes containing a soybean-derived sterylglucoside mixture with rat primary cultured hepatocytes. *Biol. Pharm. Bull.* **21**, 818-822, 1998.

46) Tabas, I., Chen, L. L., Clader, J. W., McPhail,A. T., Burnett, D. A., Bartner, P., Das, P. R., Pramanik, B. N., Puar, M. S., Feinmark, S. J., Zipkin, R. E., Boykow, G., Vita, G. and Tall, A. R. : Rabbit and human liver contain a novel pentacyclic triterpene ester with acyl-CoA: cholesterol acyltransferase inhibitory activity. *J. Biol. Chem.*, **265**, 8042-8051, 1990.

47) Tabas, I., Beatini, N., Chen, L. L., Su, W. C., Puar, M. S., Dugar, S. and Clader, J. W. : Identification and characterization of an acyl-CoA: triterpene acyltransferase activity in rabbit and human tissues. *J. Lipid Res.*, **32**, 1689-1698, 1991.

48) Breistϕl, K., Balzarini, J., Sandvold, M. L., Myhren, F., Martinsen,

M., Clercq, E. D., and Fodstad, Φ. : Antitumor activity of P-4055 (elaidic acid-cytarabine) compared to cytarabine in metastatic and s.c. human tumor xenograft models. *Cancer Res.* **59**, 2944-2949, 1999.

49) Spector, A. S., Mathur, S. N. and Kaduce T. L. : Role of acyl-coenzyme A : cholesterol *O*-acyltransferase in cholesterol metabolism. *Prog. Lipid Res.* **18**, 31-53, 1975.

50) Hasegawa, H. and Saiki I. : Oleoyl triterpene glycoside biosynthesized from ginseng suppresses growth and metastasis of murine melanoma B16-F10 tumor *via* immunostimulation. *J. Trad. Med.* **17**, 186-193, 2000.

51) Schultz, R. M., Tang, J. C., DeLong, D. C., Ades, E. W., Altom, M. G. : Inability of anti-asialo-GM1 and 2-chloroadenosine to abrogate maleic anhydride-divinyl ether-induced resistance against experimental murine lung carcinoma metastases. *Cancer Res.* **46**, 5624-8, 1986.

52) Porcelli, S. A. and Modlin, R. L. : The CD1 system: Antigen-presenting molecules for T cell recognition of lipids and glycolipids. *Annu. Rev. Immunol.* **17**, 297-329, 1999.