The expression of *in vivo* anti-metastatic effect of Ginseng protopanaxatriol saponins is mediated by their intestinal bacterial metabolites after oral administration

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Abstract

The present study demonstrated *in vivo* and *in vitro* anti-metastatic activities of a major intestinal bacterial metabolite M4 formed from protopanaxatriol saponins of Ginseng (the root of *Panax ginseng* C.A.MEYER) in comparison with ginsenoside-Re and Rg_1. Ginsenosides and M4 at the dose of 500 μg/mouse showed marked inhibition of lung metastasis of B16-BL6 melanoma cells when they were administered 5 times orally. In contrast, three consecutive i.v. administrations of M4 after tumor inoculation resulted in a significant inhibition of lung metastasis, whereas Re and Rg_1 did not show any inhibitory effect. On the other hand, these ginsenosides hardly inhibited the invasion, migration and the growth of murine B16-BL6 melanoma and human HT1080 fibrosarcoma cells *in vitro,* whereas the intestinal bacterial metabolite M4 showed inhibitory effects dose-dependently. These findings clearly indicated that the induction of *in vivo* anti-metastatic effect by oral administration of ginsenosides may be primarily mediated by their metabolic component M4.

Key words *Panax ginseng,* ginsenosides, intestinal bacterial metabolites, metastasis, tumor invasion.

Introduction

Ginseng (the root of *Panax ginseng* C.A. MEYER, Araliaceae) has been used in the traditional medicine of China, Korea, Japan and other Asian countries for the treatment of various diseases including psychiatric and neurologic diseases and diabetes mellitus. Ginseng saponins (ginsenosides) have been regarded as the principal components responsible for the pharmacological activities of Ginseng so far. Ginsenosides are glycosides containing an aglycone (protopanaxadiol or protopanaxatriol) with a dammarane skeleton and have been shown to possess various biological activities such as the enhancement of cholesterol biosynthesis, stimulation of serum protein synthesis, immunomodulatory effects, anti-inflammatory activity and anti-tumor activity.[1-5] There are many reports on antitumor effects of various ginsenosides, 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]

We have previously shown that ginsenosides Rb_2 and Rg_3 markedly inhibited lung metastasis of B16-BL6 and colon26 cells and tumor-induced angiogenesis when administered orally to mice.[6,7] It has also been noted that ginsenosides, such as Rb_1, Rb_2, Rc and Re, showed no or little inhibiton of *in vitro* tumor cell invasion, which is considered to be an important step in the tumor metastatic process.[11,12] Previously, several investigators have reported that ginsenosides are metabolized by intestinal bacteria after oral administration and that main metabolic components of protopanaxadiol- and protopanaxatriol-type saponins are 20-*O*-β-D-glucopyranosyl-20(*S*)-

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protopanaxadiol (referred to as M1 [13] or compound K [14,15]) or 20(S)-protopanaxatriol (referred to as M4[13]), respectively. This made it unclear whether or not the expression of anti-metastatic effect by oral administration of ginsenosides can be induced by their metabolites. Our recent study indicated that *in vivo* anti-metastatic effect by oral administration of protopanaxadiol-type saponins is mediated by their metabolic component M1.[16] In the present study, we therefore investigated *in vivo* and *in vitro* anti-metastatic activities of protopanaxatriol saponins (ginsenoside-Rg_1 and Re) and their metabolite M4 to determine our previous findings.

Materials and Methods

Chemical reagents : Ginsenoside-Re and Rg_1 and their metabolic component M4 were supplied by Il Hwa Co. Ltd. (Kyonggi-do, Korea). Ginsenosides were isolated from the extract according to the reported procedures [17] and their major intestinal bacterial metabolite M4 was the same as described previously.[13] The isolation and structures of these compounds are shown in Fig. 1

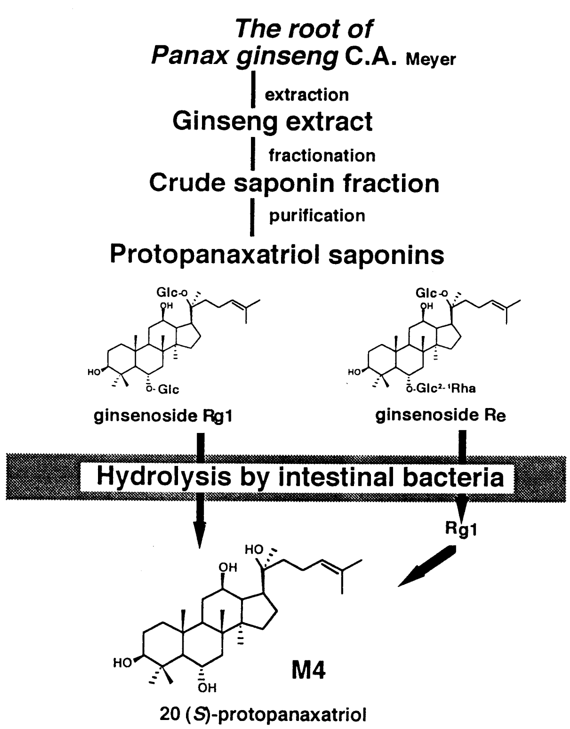

Fig. 1 Isolation and chemical structure of protopanaxatriol saponins and their metabolite.
Glc, β-D-glucopyranosyl ; Rha, α-L-rhamnopyranosyl

Animals : Specific pathogen-free 8-weeks-old female C57BL/6 mice were purchased from Shizuoka Laboratory Animal Center, Hamamatsu, Japan. Mice were maintained in the Laboratory of Animal Experiments, Research Institute for Wakan-yaku, Toyama Medical and Pharmaceutical University, under laminar air-flow conditions.

Cells : A highly metastatic subline of murine B16 melanoma, B16-BL6, was kindly provided by Dr. Fidler, M.D., Anderson Cancer Center, Houston, TX, U.S.A. The B16-BL6 melanoma cells and human fibrosarcoma HT-1080 cells were maintained as monolayer cultures in Eagle's minimum essential medium (MEM : GIBCO BRL, Life Technologies, Inc., NY) supplemented with 5 % fetal bovine serum (FBS: GIBCO BRL, Life Technologies, Inc., NY : Lot No. 37K2043), vitamin solution, sodium pyruvate, non-essential amino acids and L-glutamine (M.A. Bioproducts, Walkersville, MD). Mouse lung fibroblastic (MLF) cells were obtained by treating the minced lungs of C57BL/6 mice with 0.25 % trypsin for 1 h at 37°C and maintained as monolayer cultures in MEM supplemented with 10 % FBS.

Assay for experimental lung metastasis of tumor cells : Log-phase cell cultures of B16-BL6 melanoma cells were harvested with 1 mM EDTA in Ca^{2+}-and Mg^{2+}-free phosphate buffered saline (PBS), washed with serum-free MEM, and resuspended to give appropriate concentrations in PBS. C57BL/6 mice were given by i.v.injection of B16-BL6 ($3\times10^4/200\ \mu l$) cells in serum-free MEM. Ginsenosides and their metabolite were orally or intravenously administered 5 or 3 times after or betore tumor inoculation. The mice were euthanized by cervical dislocation 14 days after tumor inoculation. The lungs were fixed in Bouin's solution, and the lung tumor colonies were manually counted under a dissecting microscope. Metastasis was expressed as the mean number of tumor colonies in lungs±S.D.

Tumor invasion assay : The invasive activity of tumor cells was assessed in a Transwell cell culture chamber (Costar 3422, Cambridge, MA, USA) according to the method described previously.[18] Briefly, polyvinylpyrrolidone-free polycarbonate filters with pore size 8.0 μm (Nucleopore, Pleasanton, CA, USA) were precoated with 1 μg (HT-1080) or 2 μg (B16-BL6) of fibronectin (Iwaki Glass, Tokyo, Japan) in a volume of 50 μl on their lower surface and dried at room temperature. A reconstituted basement membrane Matrigel (containing Iaminin, type IV collagen, heparan sulfate proteoglycan and entactin : Collaborate Research Inc., MA, USA) was applied at 5 μg (B16-BL6) or 10 μg (HT-1080) to the upper surface of the filters and dried at room temperature. These prepared filters were designated as Matrigel/fibronectin-coated filters. The coated filters were dried, washed extensively with PBS and then dried just before use. Log-phase cell cultures of B16-BL6 melanoma cells and HT-1080 cells were harvested with 1 mM EDTA and resuspended to a final concentration of 2×10^6/ml in MEM containing 0.1% bovine serum albumin (BSA). Cell suspension (100 μl) with or without ginsenosides or their metabolite was added to the upper compartment and 0.1 % BSA-MEM (600 μl) was addae to the lower compartment. The culture was incubated for 5 h at 37°C in a 5 % CO_2 atmosphere. The tumor cells on the filters were fixed with methanol and then stained with hematoxylin and eosin. The cells on the upper surface of the filters were removed by wiping them with cotton swabs. The cells that had invaded through the Matrigel barrier and the filters to the lower surface were manually counted under a microscope in five predetermined fields at a magnification of 400. Each assay was performed in triplicate.

Haptotactic migration assay : Tumor cell migration along a gradient of substratum-bound fibronectin (haptotactic migration) was assessed in Transwell cell culture chambers according to the methods previously reported.[19] The lower surface of the filters was precoated with 1 μg (HT-1080) or 2 μg (B16-BL6) of fibronectin, as described above. The following procedures were the same as those of the invasion assay.

Cell growth assay : The growth of tumor cells was assessed by a WST-1 Cell Counting Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Briefly, B16-BL6 or HT-1080 or MLF cells (1×10^3/well) in MEM containing 5 or 10 % FBS were seeded into 96-well culture plates. After a 4-h incubation, various concentrations of ginsenosides and their metabolite were added to the well, and the plates were incubated at 37°C for an additional 48 h. WST-1 solution was added to each well and incucated at 37°C for 4 h before the termination. The absorbance of the culture was measured at 450 nm in an immuno-reader (Immuno Mini NJ-2300, Nippon InterMed K.K. Tokyo).

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

Results and Discussion

We first examined the effect of ginsenosides Re,

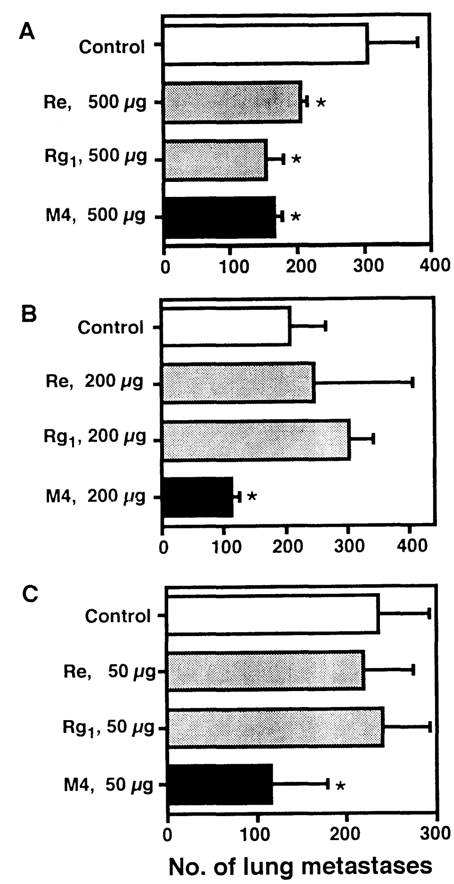

Fig. 2 Effect of ginseng saponins and their metabolite on experimental lung metastasis by i.v. injection of B16-BL6 melanoma cells.
Five to three C57BL/6 mice per group were administered orally (A) or intravenously (B) with ginsenoside-Re, Rg_1 and their metabolic component M4 consecutively for 5 (A) or 3 (B) days after the i.v. injection of B16-BL6 melanoma cells (3×10^4). Mice were administered orally with ginsenoside-Re, Rg_1 and their metabolic component M4 consecutively for 5 days before the tumor inoculation (C). Mice were killed 14 days after tumor inoculation, and the lung tumor colonies were manually measured., *, $p<0.05$ as compared with untreated control by Student's *t*-test.

Rg_1 and their major metabolic derivative M4 on lung metastasis produced by i.v. injection of B16-BL6 melanoma cells. Fig. 2A demonstrates that ginsenosides and M4 at the dose of 500 μg/mouse showed marked inhibition of lung metastasis of B16-BL6 melanoma cells when they were administerd 5 times orally. In contrast, three consecutive i.v. administrations of M4 after tumor inoculation resulted in a significant inhibition of lung metastasis, whereas Re and Rg_1 did not show any inhibitory effect (Fig. 2B). These findings suggest that the main bacterial metabolite M4 is an active component of orally administered ginsenosides, and that the anti-metastatic effect by oral administration of ginsenosides may be primarily mediated by M4. This result may be also supported by the findings that M4 was detected in the serum from mice orally given ginsenoside-Rg_1, but Rg_1 was not detected.[13)]

We next investigated whether or not ginsenosides and their metabolite M4 were able to influence the growth of tumor cells or normal mouse fibroblasts *in vitro*. These cells were cultured for 2 days with ginsenosides or M4 at a concentration ranging from 0.1 to 100 μg/ml. As shown in Fig. 3, ginsenoside-Re and Rg_1 at any concentration did not affect the growth of mouse B16-BL6 melanoma, human HT-1080 fibrosarcoma, or MLF cells. However, M4 at the concentrations of 25 and 100 μg/ml markedly inhibited the growth of both tumor cells. The growth of normal MLF cells was significantly inhibited by 100 μg/ml of M4 but not inhibited by 25 μg/ml.

Tumor invasion into extracellular matrices and basement membranes is a crucial step in the complex multistage process which leads to the metastatic formation. Therefore, we investigated the effects of ginsenosides and M4 on the invasion and migration of B16-BL6 melanoma cells or HT-1080 fibrosarcoma cells. Direct addition of ginsenoside-Re or Rg_1 at the concentrations ranging from 0.1 to 100 μg/ml exhibited no or slight inhibition of the invasion and migration of B16-BL6 melanoma cells but did not affect the invasion or migration of HT-1080 cells (Figs. 4 and 5). In contrast, M4 inhibited the invasion and migration of both tumor cells in a dose-dependent manner. These results strongly suggest taht *in vivo* anti-metastatic effect by oral administration of ginsenosides is due to their resulting metabolite M4, and indicate that such inhibitory mechanism is partly associated with the inhibition of tumor invasion, migration and growth of tumor cells. However, as shown in Fig. 2C, Re and Rg_1 did not inhibit lung metastasis when they were orally administered 5 times before tumor inoculation, whereas M4 showed a significant inhibition of lung metastasis. Therefore, further study will be needed to examine in detail whether the resulting M4 can directly affect the metastatic cell functions *in vivo* or whether M4-induced host responses are indirectly related to the expression of the anti-metastatic effect.

In conclusion, we demonstrated that the oral

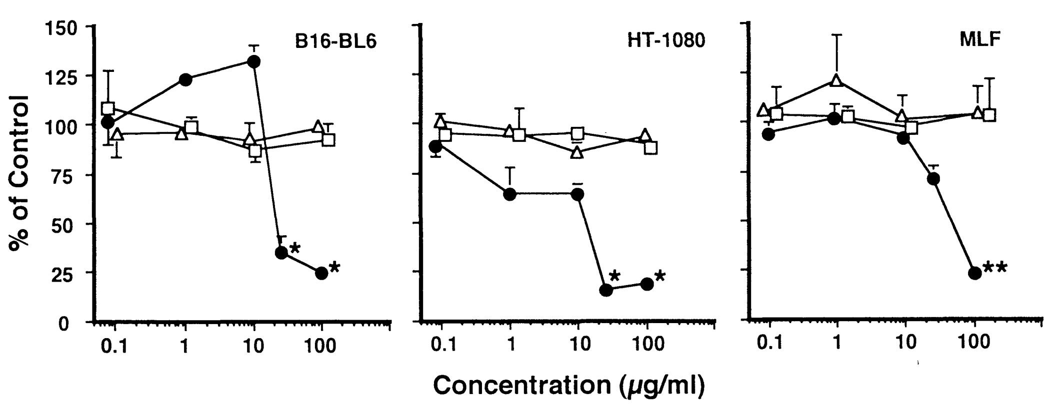

Fig. 3 Effect of ginsenosides and M4 on the growth of B16-BL6, HT-1080 and MLF cells *in vitro*. B16-BL6, HT-1080 or MLF cells (1×10^3) were incubated with various concentrations of ginsenosides Re (□), Rg_1 (△) or M4 (●) for 2 days at 37°C. WST-1 (10 μl) was added to each well and incubated at 37°C for 4 h before the termination. The absorbance of the cultures was measured at 450 nm. *, $p<0.001$; **, $p<0.0001$ as compared with untreated control by Student's t-test.

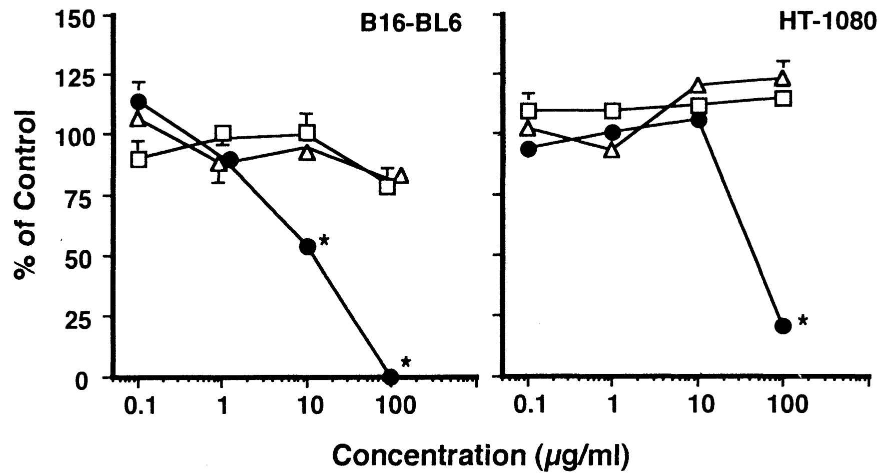

Fig. 4 Effect of ginsenosides and M4 on the invasion of B16-BL6 melanoma and HT-1080 fibrosarcoma cells into Matrigel/fibronectin-coated filters.
Filters were pre-coated with 1 or 2 μg of fibronectin on their lower surfaces and then with 5 or 10 μg of Matrigel on their upper surfaces. B16-BL6 or HT-1080 cells (2×10^5 cells) in 0.1 % BSA medium were seeded with or without the indicated concentrations of ginsenosides Re (□), Rg_1 (△) or M4 (●) into the upper compartment of a Transwell cell culture chamber. After a 5-h incubation, the cells that invaded the lower surfaces were visually counted. *, $p<0.0001$ as compared with untreated control by Student's *t*-test.

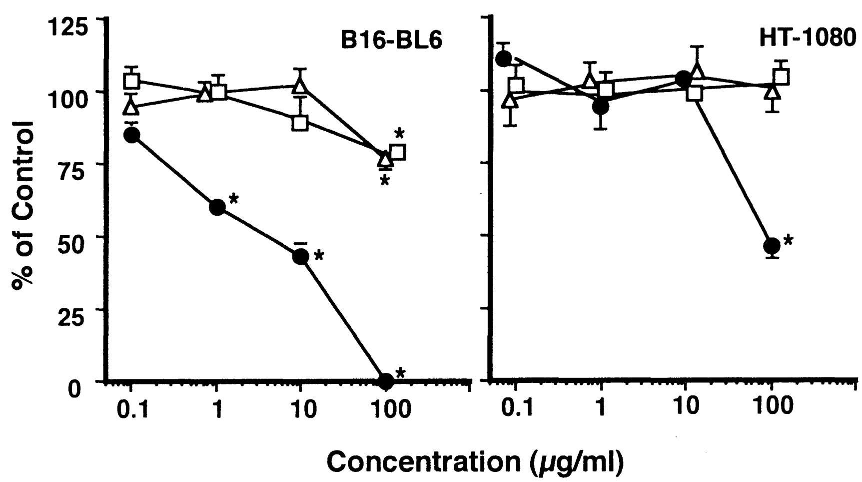

Fig. 5 Effect of ginsenosides and M4 on the haptotactic migration of B16-BL6 and HT-1080 cells to fibronectin-coated filters.
Filters of the chambers were pre-coated with 1 or 2 μg of fibronectin on their lower surfaces. B16-BL6 or HT-1080 cells (2×10^5 cells) in 0.1 % BSA medium were seeded with or without the indicated concentrations of ginsenosides Re (□), Rg_1 (△) or M4 (●) into the upper compartment of the Transwell cell-culture chamber. After a 5-h incubation, the migrated cells on the lower surfaces were visually counted. *, $p<0.0001$ as compared with untreated control by Student's *t*-test.

administration of a major metabolic component M4 as well as ginsenoside-Re or Rg_1 was effective in inhibiting lung metastasis of B16-BL6 melanoma and that the direct addition of M4 into the culture markedly inhibited the invasion, migration, and the growth of tumor cells, as compared with ginsenosides. In addition, i.v. administration of M4 resulted in a significant inhibition of lung metastasis, whereas ginsenosides did not show any activities. These findings clearly indicate that the induction of *in vivo* effects by ginsenosides is primarily based on their metaboilite M4.

Acknowledgments

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和文抄録

本研究では，薬用人参（*Panax ginseng* C.A. MEYER）の protopanaxatriol saponin 成分の主要な腸内細菌代謝物である M4 を用いて，*in vivo* および *in vitro* の癌細胞の転移・浸潤に及ぼす効果を ginsenoside-Re および Rg_1 と比較検討した。その結果，Re, Rg_1 及び M4 は B16-BL6 メラノーマ細胞を静脈内に移入後 5 日間の経口投与により，いずれも癌細胞の肺への転移を有意に抑制した。癌細胞を移植した後 3 日間の静脈内投与では M4 のみが肺転移を有意に抑制し，Re および Rg_1 では抑制効果が認められなかった。一方，*invitro* のマウス B16-BL6 メラノーマ細胞あるいはヒト HT-1080 線維肉腫細胞の増殖，再構成基底膜への浸潤および移動実験において，Re および Rg_1 はいずれの活性に対しても抑制効果を示さず，M4 のみが濃度に依存して顕著に抑制した。以上の成績から，ginsenoside-Re および Rg_1 の経口投与による癌転移の抑制効果の発現は，それらの腸内細菌代謝物である M4 に主として基づいていることが明かとなった。

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