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Inhibition of *in vitro* Tumor Cell Invasion by Ginsenoside Rg_3

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The effect of plant glycosides on tumor cell invasion was examined. Among the glycosides tested, ginsenoside Rg_3 was found to be a potent inhibitor of invasion by rat ascites hepatoma cells (MM1), B16FE7 melanoma cells, human small cell lung carcinoma (OC10), and human pancreatic adenocarcinoma (PSN-1) cells, when examined in a cell monolayer invasion model. Structurally analogous ginsenosides, Rb_2, 20(R)-ginsenoside Rg_2 and 20(S)-ginsenoside Rg_3 (a stereoisomer of Rg_3), showed little inhibitory activity. Neither Rh_1, Rh_2, 20(R)-ginsenosides Rh_1, Rb_1, Rc nor Re had any effect. The effective ginsenoside, Rg_3, tended to inhibit experimental pulmonary metastasis by highly metastatic mouse melanoma B16FE7 cells as well. Taking account of our previous finding that 1-oleoyl-lysophosphatidic acid (LPA) induced invasion by MM1 cells in the monolayer invasion model, the effect of Rg_3 on molecular events associated with the invasion induced by LPA was analyzed in order to understand the mechanism of the inhibition. Rg_3, which suppressed the invasion induced by LPA, dose-dependently inhibited the LPA-triggered rise of intracellular Ca^{2+}. Protein tyrosine phosphorylation triggered by LPA was not inhibited by Rg_3.

Key words: Ginsenoside — Tumor cell invasion — Ca^{2+}

There is currently great interest in means for prevention of cancer metastasis, in order to improve the prognosis of cancer patients. Cancer metastasis develops by multiple and sequential steps, including (1) release of tumor cells from the primary site, (2) tumor cell invasion of surrounding tissues and vasculature, (3) arrest of the circulating tumor cells in the microvasculature of target organs and (4) invasion of the parenchyma of the organ and growth in the secondary sites.[1–3] Among these steps, the invasion is the most characteristic process, control of which is likely to provide a new strategy for metastasis prevention.

We have developed an invasion model for estimating tumor cell invasion ability (transcellular migration) *in vitro*.[4,5] In this model, tumor cells are seeded on a primary cultured monolayer of host cells, such as mesothelial or endothelial cells. The tumor cells penetrate the monolayer, grow and form tumor cell colonies underneath the monolayer. The invasive ability of the tumor cells is expressed as the number of tumor cells that penetrate the monolayer. This invasion by tumor cells *in vitro* corresponded well with their invasive capacity *in vivo*, when tested by their implantation into test animals. Thus, this model has allowed us to study the effects of various substances (both potentiators and inhibitors) on tumor cell invasion.[6–10]

Since our invasion model is a cell culture system, 10% fetal calf serum (FCS) is added to the culture medium. It was surprising to find that the omission of FCS from the medium completely abolished the invasion by tumor cells *in vitro*.[11] The addition of FCS restored this ability. 1-Oleoyl-lysophosphatidic acid (LPA) was found to be able to replace FCS in this respect.[12] Exogenous LPA induces smooth muscle contraction, platelet aggregation, cell motility and growth.[13,14] Ridley and Hall[15] reported that LPA characteristically induced focal adhesion and stress fiber formation in quiescent Swiss 3T3 cells, and suggested that this response was mediated by a small GTP-binding protein, rho p21, which received a signal from LPA and transduced it to a system regulating cell adhesion. Our recent observation suggests that the activation of rho p21 and subsequent protein tyrosine phosphorylation are essential for tumor cell invasion of the mesothelial cell monolayer.[16,17] Thus, the use of LPA as an invasion trigger in this invasion model appears beneficial for analyzing the action mechanisms of invasion-potentiators and inhibitors.

Odashima *et al.* previously reported that a crude fraction of plant glycosides extracted from the roots of *Panax ginseng* C. A. Meyer induced phenotypic reverse transformation of cultured hepatoma cells.[18] Later, they found that one of the ginsenosides, Rh_2 (**1**), a protopanaxadiol glucoside, inhibited the growth and stimulated the melanogenesis of B16 melanoma cells, while another structurally analogous ginsenoside, Rh_1 (**4**), enhanced melanogenesis without inhibiting the growth of the cells.[19] These findings indicated that some of the ginsenosides can affect differentiation phenotypes of

cancer cells without impairing cell growth, and led us to study the effects of ginsenosides on tumor cell invasion. This paper deals with the anti-invasive and anti-metastatic potentials of ginsenoside Rg_3 (2), one of the characteristic constituents of red ginseng, and we propose a mechanism by which it may inhibit the invasion.

MATERIALS AND METHODS

Materials N-2-Hydroxyethylpiperazine-N″-2-ethanesulfonic acid (Hepes), bovine serum albumin (BSA) and LPA were purchased from Sigma Chemical Co. (St. Louis, MO); 1-[2-(5′-carboxyoxazol-2′-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)ethane-N,N,N′,N′-tetraacetic acid (fura-2) and its penta-acetoxymethyl ester (fura-2-AM) from Wako Pure Chemical Industries (Osaka); FCS from Cell Culture Laboratories (Cleveland, OH); and Eagle's minimum essential medium from Nissui Pharmaceutical Co. Ltd. (Tokyo).

Cell lines and culture Rat mesothelial cells were isolated from Donryu rat (Japan SLC, Inc., Hamamatsu) mesentery and cultured in minimum essential medium containing 2-fold amino acids and vitamins (MEM) supplemented with 10% FCS as reported previously.[4] Highly invasive clone MM1 cells were obtained from rat ascites hepatoma cells (AH 130 cells), and human lung cancer cells (OC10) were established from a pulmonary tumor of a patient with small cell lung cancer. Mouse melanoma (B16FE7) and human pancreatic adenocarcinoma cells (PSN-1) were generous gifts from Dr. S. Nozawa, Keio University, Medical School and Dr. H. Sakamoto, National Cancer Center Research Institute, respectively. MM1 and OC10 were cultured in MEM supplemented with 10% FCS; B16FE7 and PSN-1 were cultured in RPMI 1640 supplemented with 10% FCS.

Preparation of ginsenosides Ginsenosides were isolated from the unprocessed and processed root of *Panax ginseng* C. A. MEYER, white ginseng and red ginseng, respectively, and identified on the basis of their chemical and physicochemical properties as ginsenosides **4**,[20] **5**, **1–3**, **6**,[21] **7**, **8**, **9**[22] and **10**[23] (Fig. 1). The purified ginsenosides were dissolved in dimethyl sulfoxide (DMSO) immediately before use.

***In vitro* invasion assay** The assay procedure of *in vitro* invasive capacity of tumor cells was essentially the same as described in previous reports.[5–9] Briefly, mesothelial cells isolated from rat mesentery were cultured to confluency to prepare a mesothelial cell monolayer (MCL). The tumor cell suspension (0.5×10^5–1×10^5 cells/ml) was seeded on the MCL and incubated for 20 h at 37°C with or without ginsenosides. The medium usually consisted of MEM supplemented with 10% FCS. In a specific experiment shown in Table V, FCS was omitted (FCS-free) and the invasion was measured in the absence of the serum or FCS was replaced by LPA. The number of penetrated single tumor cells and colonies under the MCL were counted under a phase-contrast microscope. The invasive capacity was expressed as the number of invasive foci/cm^2 (an invasion focus was defined as a penetrated single tumor cell or a tumor cell colony).

Experimental metastasis assay B16FE7 cells in culture were harvested by a brief treatment with 0.25% trypsin and 0.05% EDTA and resuspended in MEM supplemented with 10% FCS. The cell suspension was mixed with or without ginsenosides and injected into the lateral tail vein of the C57BL/6 mice. On the 14th day after the tumor cell injection, the mice were killed and the lungs were examined. The number of metastatic foci on the lung surface was counted under a dissecting microscope.

Determination of cytoplasmic, free Ca^{2+} concentration Intracellular $[Ca^{2+}]$ was monitored by measuring the fluorescence intensity of Ca^{2+}-bound fura 2. To load the cells with fura-2 AM, tumor cells (1×10^7/ml) were suspended in Hepes-glucose buffer (pH 7.4) supplemented with 2% BSA and 6 μM fura-2-AM, and incubated for 30 min at 37°C. The medium was removed and the cells were washed twice and resuspended in the same buffer (Hepes-glucose-BSA) supplemented with 1 mM $CaCl_2$ to be 1.0×10^6 cells/ml. To measure LPA-dependent change of intracellular $[Ca^{2+}]$, ginsenoside (25 μM) or DMSO as a control was added to the cell suspension and the mixture was incubated for 5 min, then

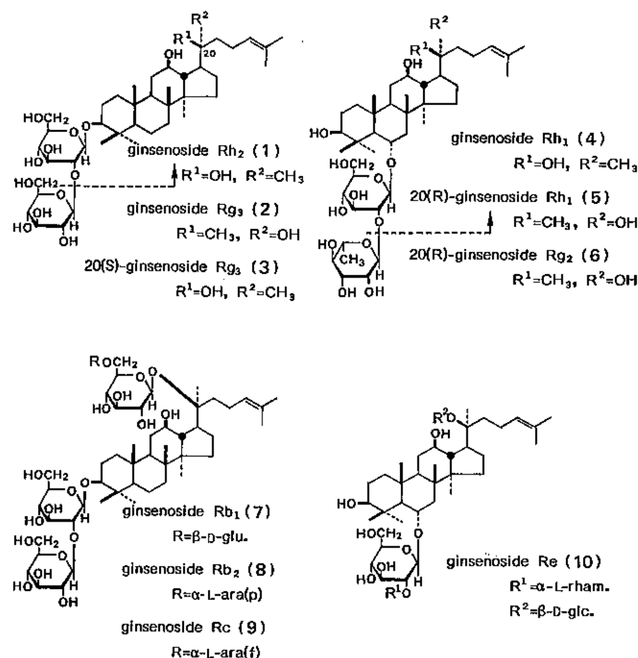

Fig. 1. Structural formulae of ginsenosides.

LPA (125 μM) was introduced and the fluorescence intensity of the cell suspension (1×10^5/ml) was monitored with a spectrofluorometer (excitation wavelength, 335 nm; emission wavelength, 500 nm).

RESULTS

Inhibition of *in vitro* invasion by ginsenosides The effect of ginsenosides on the invasion by MM1 cells *in vitro* is shown in Table I. Almost 99% inhibition of invasion was observed with 32 μM ginsenoside Rg_3 (**2**). Ginsenoside **3**, a C-20 stereoisomer of **2** (Fig. 1), showed much weaker inhibitory activity, suggesting a stereospecific effect of **2**. No appreciable inhibition was observed with **1**, **4** or **5**. Ginsenosides **8** and **6** were effective, but much less potent than **2**. The inhibitory effect of **2** was dose-dependent (Table II). Half-maximum inhibition was estimated to occur at about 6 μM. This ginsenoside, however, did not inhibit the proliferation of the tumor cells, at least during the period of the *in vitro* invasion assay, as shown in the same table.

To examine the cell type specificity of inhibition, we looked at the effect of **2** on a variety of tumor cells. It inhibited the invasion by human lung cancer cell line OC-10, human pancreatic adenocarcinoma cell line PSN-1 and mouse melanoma cell line B16FE7, though the percent inhibition varied with cell line (Table III), suggesting that the inhibition was not specific to MM1 cells.

Inhibition of pulmonary metastasis of B16FE7 cells by ginsenoside Rg_3 (2) Since invasion is one of the important steps in metastasis, ginsenoside Rg_3 (**2**) was expected to prevent cancer metastasis. Therefore, we examined its effect on pulmonary metastasis by highly metastatic mouse melanoma cells (B16FE7). Tumor cells (1.7×10^5 cells) that had been mixed with or without **2** were injected into the tail vein of C57BL6 mice. The number

Table I. Effect of Various Ginsenosides on Tumor Cell Invasion *in vitro*

Ginsenoside	μM	No. of invasion foci/cm^2	Inhibition (%)
None	0	738±47[a]	—
1	32	861±58	0
2	32	9±5	98.8
3	32	577±44	21.8
4	32	752±47	0
5	32	1043±75	0
6	32	480±20	35.0
7	50	790±19	0
8	50	550±21	25.1
9	50	644±10	12.3
10	50	822±10	0

MM1 (1×10^5 cells/ml) cells were seeded on MCL with or without the indicated ginsenosides. The invasion assay medium contained 10% FCS.
a) Mean±SD of at least 3 determinations.

Table II. Concentration-dependent Inhibition of Tumor Cell Invasion *in vitro* by Ginsenoside Rg_3 (**2**)

Ginsenoside Rg_3 (μM)	No. of invasion foci/cm^2	Inhibition (%)	Proliferation[a] ($\times10^5$ cells/ml)
None	738±47[b]	0.0	2.3±0.20[b]
1.6	428±61	42.0	2.3±0.17
8.0	259±24	64.9	2.3±0.14
32.0	9±5	98.8	2.7±0.23
64.0	9±5	98.8	2.0±0.18

MM1 (1×10^5/ml) cells were seeded on MCL in the presence and the absence of ginsenoside Rg_3 (**2**).
a) The tumor cells (1×10^5 cells/ml) were cultured with or without **2** for 24 h and then proliferation was measured as the increase in cell number. The assay medium contained 10% FCS.
b) Mean±SD of at least 3 determinations.

Table III. Effect of Ginsenoside Rg_3 (**2**) on Invasion by Various Tumor Cell Lines

Cell line	No. of invasion foci/cm^2		
	Control[a]	Ginsenoside Rg_3[a]	Inhibition (%)
MM1	752±98[b]	83±3[b]	89.0
B16FE7	1900±190	499±15	73.7
OC-10	1180±65	186±20	84.2
PSN-1	1114±180	456±112	59.1

MM1, OC-10 and PSN-1 (1×10^5 cells/ml), B16FE7 (0.5×10^5 cells/ml) were seeded on MCL.
a) Invasion assay medium contained 10% FCS with or without 25 μM ginsenoside Rg_3 (**2**).
b) Mean±SD of at least 3 determinations.

Table IV. Effect of Ginsenoside Rg_3 (**2**) on Lung-colonizing Potential of B16FE7

Ginsenoside Rg_3 (μM)	No. of mice	No. of metastatic foci/lung
None	6	244.3±71.9
1.3	6	252.4±58.5
4.3	6	128.6±30.8*
13.0	6	100.8±18.1**
29.0	4	104.8±10.0***

B16FE7 cell suspension (0.3 ml, 1.7×10^5 cells) was mixed with or without the indicated concentrations of ginsenoside Rg_3 (**2**) and was injected into the lateral tail vein of C57BL/6 mice. At 14 days after the inoculation, mice were killed and the lungs were examined.
* $P<0.07$. ** $P<0.06$. *** $P<0.04$.

Table V. Effect of Ginsenoside Rg_3 (2) on LPA-induced Invasion by Various Tumor Cell Lines *in vitro*

Cells	No. of invasion foci/cm²			
	FCS-free MEM	LPA[a]	Ginsenoside Rg_3 (2)[a]	Inhibition by 2 (%)
MM1	16±2[b]	763±83[b]	47±7[b]	93.8
B16FE7	361±30	3270±15	360±21	89.0
OC-10	92±4	1600±17	160±2	90.0
PSN-1	410±31	2390±150	263±15	89.0

MM1, OC-10 and PSN-1 (1×10^5 cells/ml), B16FE7 (0.5×10^5 cells/ml) were seeded on MCL.
a) The invasion assay medium contained 20 *μM* LPA in place of 10% FCS with or without 25 *μM* ginsenoside Rg_3 (2).
b) Mean±SD of at least 3 determinations.

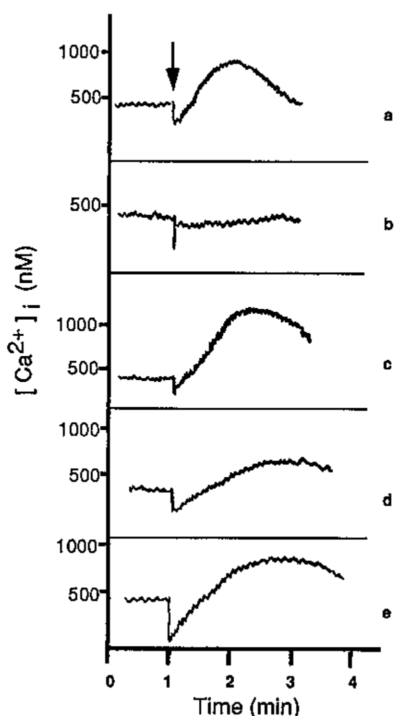

Fig. 2. Effect of ginsenosides on LPA-induced intracellular $[Ca^{2+}]_i$ increase. The fluorescence intensity of fura-2-loaded cells (1×10^5/ml) in Hepes-glucose-BSA buffer supplemented with 1 m*M* $CaCl_2$ was measured in the presence or absence of ginsenosides. The loaded cells were pretreated for 5 min with DMSO (a), or 25 *μM* ginsenoside **2** (b), **10** (c), **6** (d), or **7** (e) prior to the addition of LPA. The arrow indicates the addition of 125 *μM* LPA.

of metastatic foci that developed on the lung surface was counted 14 days after the injection. The metastasis tended to be inhibited by **2**, although the inhibition was statistically insignificant (Table IV). This reduction was not the result of a direct cytotoxic effect of the ginsenoside on the tumor cells, because the viability of the treated tumor cells was more than 95% when estimated by a trypan blue dye exclusion test (data not shown).

Inhibition of LPA-induced invasion by ginsenoside Rg_3 (2) In the cell monolayer invasion model, tumor cell invasion is induced by the addition of either FCS or LPA to the assay medium.[12] We examined the effect of ginsenoside Rg_3 (**2**) in the invasion-assay medium in which 10% FCS was replaced by 25 *μM* LPA. As shown in Table V, approximately 90% inhibition of LPA-induced invasion was seen with 32 *μM* **2**, irrespective of the kind of tumor cells. This indicates that the ginsenoside (**2**) inhibits the invasion, whether the trigger is FCS or LPA.

Effect of ginsenoside Rg_3 (2) on LPA-induced intracellular Ca^{2+} spike and protein tyrosine phosphorylation LPA is known to stimulate cells as an exogenous ligand and to trigger several signaling pathways, including a rise of intracellular Ca^{2+}, and protein tyrosine phosphorylation through ras and rho activation.[14, 24] We therefore examined the effect of ginsenoside Rg_3 (**2**) on $[Ca^{2+}]_i$ and protein tyrosine phosphorylation.

Fig. 2 shows the effect on $[Ca^{2+}]_i$ of MM1 cells. The addition of LPA to the cell suspension resulted in an almost instantaneous rise of $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$, which was 400 n*M* before the stimulation, reached a maximal level (1 m*M*) within 1–1.5 min, followed by a slow decrease to the original level (Fig. 2a). When MM1 cells were pretreated with 25 *μM* **2**, the LPA-induced Ca^{2+} spike was completely abolished (Fig. 2b). The inhibitory effect amounted to 10%, 70%, and 98% at 5 *μM*, 12.5 *μM* and 25 *μM* **2**, respectively. Ginsenosides **1**, **4**, **7** and **10**, which had no effect on invasion (Table I), did not inhibit the Ca^{2+} mobilization by LPA (see data on **10** and **7** in Fig. 2c and e, respectively). Ginsenosides **3**, **6** and **8**, which had a slight invasion-inhibitory effect (Table I), showed 30–40% suppression of $[Ca^{2+}]_i$ (see data on **6** in Fig. 2d). Ginsenosides **5** and **9**, however, inhibited the Ca^{2+} spike by 50%, although they did not suppress the invasion (Table I). LPA induced tyrosine phosphoryla-

tion of 110–130 kDa and 70 kDa proteins of MM1 cells, as reported previously[17]; nevertheless, ginsenoside Rg_3 (2) had no effect on the phosphorylation (data not shown).

DISCUSSION

A decoction of the roots of *Panax ginseng* C. A. Meyer (ginseng root), has been traditionally used as an oriental medicine with analeptic, stomachic and erythropoietic potency. Extensive analyses of the extract of ginseng root have indicated that ginsenosides mediate the biological activities of ginseng root.[25–28] The ginsenosides are plant glycosides with an aglycone (protopanaxadiol or protopanaxatriol) possessing a dammarane skeleton.

In the present study, ginsenoside Rg_3 (**2**) was found to inhibit invasion and metastasis by certain tumor cells without impairing cell growth (Table II). The stereoisomer (**3**) was less effective. Both ginsenosides (**2** and **3**) have a protopanaxadiol moiety as the aglycone, and differ only in the C-20 configuration in the aglycone, which is stereostructurally very similar to cholesterol, an important cell membrane constituent. Ginsenosides **4** and **1**, which were reported to have growth-inhibitory and melanogenesis-inducing activities on B16 melanoma cells[17] had no invasion-inhibiting activity. These results indicate that ginsenosides exert stereo- and structure-specific biological actions and suggest that the mechanisms of their actions on cell growth and invasive locomotion are not necessarily the same. This is in agreement with the results reported by Chen *et al.*, who found that epidermal growth factor (EGF)-induced mitogenesis and cell motility are mediated through different signal transduction pathways.[29]

The molecular mechanism by which ginsenoside Rg_3 (**2**) impairs tumor cell invasion is unclear at present. However, since it inhibits the LPA-induced invasion, it presumably interferes with signaling pathway(s) triggered by the phospholipid. LPA is reported to stimulate the cells via its specific receptor, which is believed to couple with heterotrimeric G-proteins.[24] In fibroblasts such as Rat-l cells, LPA transmits signals to the ras p21-MAPK pathway and the pathway which activates rho p21, possibly via phospholipase C stimulation.[29] The signaling to the latter is considered to evoke the $[Ca^{2+}]_i$ transient, and to stimulate protein tyrosine phosphorylation through rho p21 activation.[30] Our recent observation suggests that activation of rho p21 and subsequent tyrosine phosphorylation of focal adhesion kinase and paxillin (especially regulated by rho p21) are essential for tumor cells to invade MCL.[17] Genistein, an inhibitor of protein tyrosine phosphorylation, was reported to impair tumor cell invasion through Matrigel-coated membranes.[31]

Ginsenoside Rg_3 (**2**), however, did not suppress the signaling of LPA to protein tyrosine phosphorylation. Instead, it completely inhibited the $[Ca^{2+}]_i$ transient induced by LPA at concentrations of the ginsenoside sufficient to inhibit MM1 cell invasion. It is generally accepted that Ca^{2+} mobilization is closely associated with cell motility through stimulation of protein kinase C and actin reorganization. Ca^{2+} mobilization appears to be located upstream of, or to be independent of, rho activation, since the inactivation of rho p21 by a specific inhibitor (ADP-ribosyltransferase C3) does not suppress the Ca^{2+} rise, whereas the inhibitor blocks the phosphorylation.[29] Thus, ginsenoside Rg_3 (**2**) seems to impair the invasion by inhibiting $[Ca^{2+}]_i$ increase without affecting the protein phosphorylation.

Suppression of the $[Ca^{2+}]_i$ rise by guanidine analogues such as the diuretic amiloride has been reported elsewhere to inhibit tumor cell invasion.[32, 33]

Although much work remains to be done to understand the molecular mechanism of tumor cell invasion and its inhibition by ginsenosides, ginsenoside Rg_3 (**2**) could be a useful tool for further research, and for developing a therapeutic strategy for the control of metastasis.

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