Inhibitory Effect of Tumor Metastasis in Mice by Saponins, Ginsenoside-Rb2, 20(R)- and 20(S)-Ginsenoside-Rg3, of Red ginseng

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We examined the inhibitory effect of two saponin preparations from Red ginseng, 20(R)- and 20(S)-ginsenoside-Rg3, in comparison with that of ginsenoside-Rb2, on lung metastasis produced by two highly metastatic tumor cells, B16-BL6 melanoma and colon 26-M3.1 carcinoma, in syngeneic mice.

In an in vitro analysis, both saponin preparations showed a significant inhibition of adhesion to fibronectin (FN) and laminin (LM) by B16-BL6 melanoma. Similarly, they significantly inhibited the invasion of B16-BL6 cells into the reconstituted basement membrane (Matrigel)/FN in a dose-dependent manner. In an experimental metastasis model using B16-BL6 melanoma, consecutive intravenous (i.v.) administrations of 100 μg/mouse of 20(R)- or 20(S)-ginsenoside-Rg3 1, 2, 3 and 4 d after tumor inoculation led to a significant decrease in lung metastasis. The inhibitory effect of i.v. administration of both ginseng saponins on the tumor metastasis of B16-BL6 melanoma was also recognized in a low dose of 10 μg/mouse. The oral administration (p.o.) of both saponins (100—1000 μg/mouse) induced a significant decrease in lung metastasis of B16-BL6 melanoma. Moreover, both ginseng saponins were effective in inhibiting lung metastasis produced by colon 26-M3.1 carcinoma. When 20(R)- or 20(S)-ginsenoside-Rg3 was orally administered consecutively after tumor inoculation in a spontaneous metastasis model using B16-BL6 melanoma, both of them significantly inhibited lung metastasis. In the experiment involving neovascularization by tumor cells in vivo, both mice groups given each saponin preparation after tumor inoculation exhibited a significant decrease in the number of blood vessels oriented toward the tumor mass, with no repression of tumor size. These findings suggest that both ginseng saponins, 20(R)- and 20(S)-ginsenoside-Rg3, possess an ability to inhibit the lung metastasis of tumor cells, and the mechanism of their ant metastatic effect is related to inhibition of the adhesion and invasion of tumor cells, and also to anti-angiogenesis activity.

Key words ginseng saponin; 20(R)-, 20(S)-ginsenoside-Rg3; tumor metastasis; tumor invasion

Metastasis is one of the major causes of mortality in cancer. During the metastatic cascade, metastasizing tumor cells interact with various host cells, extracellular matrices and basement membrane components. Such adhesive interaction may enhance the survival or invasiveness of the tumor cells. Therefore, regulation of tumor cell metastasis may help in the development of ant metastatic therapies.

Angiogenesis, the formation of new blood vessels that is a physiological process in embryogenesis and wound healing, has been shown to be an essential participant in the growth and metastasis of most tumor cells. Since the inhibition of tumor neovascularization may be effective for preventing tumor growth and metastasis, several applications using several anti-angiogenesis agents such as protamine, tumor necrosis factor-α (TNF-α) and pentosan polysulfate have been examined.

Extracts of Red ginseng have been shown to possess various biological activities such as immunomodulatory and antitumoral activity. Since the biological activities of Red ginseng are largely attributed to its triterpenoid saponin components (ginsenosides), many types of saponins have been isolated and chemically modified, and their chemical properties and biological effects have been widely studied. Odashima et al. reported that the treatment of Morris hepatoma cells (MH1C1) with ginsenosides extracted from Panax ginseng induced a reverse transformation of MH1C1 cells and retarded the doubling time of the cells. In addition, ginsenoside-Rh2, a glycoside having a dammarane skeleton resembling a steroid skeleton as an aglycone, inhibited the growth of B16 melanoma cells, causing morphological alterations and stimulating melanogenesis in vitro. However, even though many investigators have shown the effects of ginsenosides on the suppression of tumor growth and the inhibition of tumor metastasis, there are few reports on their ability to inhibit the adhesion and invasion of tumor cells. Previously we have reported that ginsenoside-Rb2, a dammarane-type saponin, inhibited the tumor metastasis of B16-BL6, and that the effect was due to the inhibition of tumor-induced angiogenesis.

In the present study, we examined the inhibitory effect of two epimeric saponin preparations, 20(R)- and 20(S)-ginsenoside-Rg3 as well as ginsenoside-Rb2, on lung metastasis produced by B16-BL6 melanoma and colon 26-M3.1 carcinoma cells in syngeneic mice, and looked at the mechanisms of their ant metastatic effects with special reference to the inhibition of adhesion and invasion of tumor cells and the suppression of tumor-induced angiogenesis.

MATERIALS AND METHODS

Ginsenosides and Chemical Reagents Authentic samples of ginsenoside-Rb2 and 20(R,S)-ginsenoside-Rg3 were obtained from 6-year old Red Ginseng in Korea.
Fig. 1. Structure of Ginsenosides

and were kindly provided by Korea Tobacco and Ginseng Corporation and Japan-Korea Red Ginseng Co., Ltd. The respective structures had been confirmed (Fig. 1) and the purity of saponins was above 99.9% as estimated by high performance liquid chromatography. An appropriate amount of each ginsenoside was suspended in 0.1 M phosphate-buffered saline (PBS, Ca²⁺ and Mg²⁺ free, pH 7.4) before use.

The solubility of each ginsenoside was different: soluble to the concentration of 100 μg/ml for ginsenoside-Rb2, less than 25 μg/ml for 20(S)-ginsenoside-Rg3 and almost insoluble for 20(R)-ginsenoside-Rg3. The suspension of each ginsenoside was used for in vivo experiment and in vitro analysis. Purified mouse plasma fibronectin (FN) was purchased from Biomedical Technologies, Stoughton, MA, U.S.A. Purified mouse laminin (LN) and reconstituted basement membrane Matrigel (containing LN, collagen type IV, heparin sulfate proteoglycan and entactin) were purchased from Collaborative Research Inc., MA, U.S.A.

Animals Specific pathogen-free female C57BL/6 and Balb/c mice, 7–8 weeks old, were purchased from Shizuoka Laboratory Animal Center, Hamamatsu, Japan. Mice were maintained in the Laboratory of Animal Experiments, the Institute of Immunological Science, Hokkaido University, under laminar air-flow conditions. Water and pelleted diets (Nihon Nosan Kogyo Co., Ltd., Yokohama, Japan) were supplied ad libitum. All animals were treated according to the Laboratory Animal Control Guidelines in our institute which basically conform to those of the National Institutes of Health-American Association of Laboratory Animal Control.

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 highly metastatic line of colon 26 carcinoma, colon 26-M3.1, which forms nodules of tumor colonies excellently on the surface of the lungs, was isolated by the in vivo selection method of Dr. Fidler. Both tumor cell lines were maintained as monolayer cultures in Eagle’s minimum essential medium (MEM) supplemented with 7.5% fetal bovine serum (FBS), vitamin solution, sodium pyruvate, nonessential amino acids and L-glutamine.

Assay for Experimental Lung Metastasis Experimental lung metastasis was assessed by means of the inoculation of tumor cells into the lateral tail vein of syngeneic mice. Four C57BL/6 mice per group were given an i.v. injection of 5 × 10⁴ melanoma cells. Various doses of each ginsenoside were successively injected i.v. or p.o. 4 times into the tumor-bearing mice from the day after tumor inoculation. The mice were killed 14 d after tumor inoculation. The lungs were fixed in Bouin’s solution and the lung tumor colonies were counted under a dissecting microscope.

Assay for Spontaneous Lung Metastasis C57BL/6 mice were given subcutaneous (s.c.) injections of B16-BL6 melanoma cells (5 × 10⁵) into the right hind footpad. Ginsenosides were administered orally on various days before or after the surgical excision of the primary tumor was carried out on day 21. Mice were killed 14 d after the surgical excision. The lungs were fixed in Bouin’s solution and the lung tumor colonies were counted under a dissecting microscope.

Microassay for Cell Adhesion B16-BL6 melanoma cells were suspended in serum-free MEM to form a single-cell suspension. The tumor cell suspensions (3.5 × 10⁶) with or without various doses (0.1–100 μg/ml) of ginsenosides in a volume of 50 μl/well were added to microwell culture wells that had been pre-coated with 50 μl of fibronectin or LN (5 μg/ml). After a 30-min incubation at 37°C, the wells were washed three times with PBS to remove unattached cells. Then the attached cells were stained with 0.5% crystal violet in 20% methanol for 30 min. After being washed with tap-water, the stained cells were dissolved in 100 μl/well of 30% acetic acid, and the absorbance of each well was measured at 600 nm.

Cell Invasion Assay The invasive activity of tumor cells was assayed in Transwell cell culture chambers (Costar 3422, Cambridge, MA) as described previously. The lower surface of the filters with an 8.0 μm pore size (Nucleopore, Pleasanton, CA) in a Transwell cell culture chamber was precoated with 5 μg/10 μl of fibronectin, and thereafter 5 μg/10 μl of Matrigel was applied to the upper surface of the filters. The filters thus prepared were designated as Matrigel/fibronectin-coated filters. The log-phase cell culture of B16-BL6 melanoma cells was harvested with 0.1 mM EDTA, washed 3 times with serum-free MEM, and resuspended to a final concentration of 2 × 10⁶/ml in MEM containing 0.1% bovine serum albumin (BSA). Then the cells were pretreated with various concentrations, from 0.1 to 100 μg/ml, of ginsenosides at 37°C for 30 min. Each cell suspension was added to the upper compartment of the chamber. After a 4-h incubation period, the filters were fixed with methanol and stained with hematoxylin and eosin. The cells on the upper surface of the filters were removed by wiping with cotton swabs. The cells that had invaded through the Matrigel and the filters into the lower surface were manually counted under a microscope at a magnification of ×400. Each assay was performed in triplicate. The data were expressed as the number of invaded cells/field.

Assay for Tumor-Induced Angiogenesis The assay of tumor angiogenesis in syngeneic mice was carried out...
according to the method described by Kreisler and Ershler\textsuperscript{30} with some modifications.\textsuperscript{31} Three C57BL/6 mice per group were inoculated intradermally (i.d.) with B16-BL6 melanoma cells ($5 \times 10^5$) at two sites on the back. Each ginsenoside was administered i.v. (100 µg/200 µl/mouse) or p.o. (300 µg/400 µl/mouse) 1, 2, 3 and 4 d after tumor inoculation. Two days after the last administration, the mice were killed immediately after i.v. injection of 1% Evan's blue solution, and the skin was separated from the underlying tissues. Angiogenesis was quantitated by counting the number of vessels oriented toward the tumor mass under a dissecting microscope. The tumor size was approximated by averaging the diameters of the short and long axes of the remnant of inoculated cells. All counts were made by a single observer in a blinded manner.

**Statistical Analysis** The statistical significance of differences between groups was calculated by applying the Student's two-tailed $t$ test.

**RESULT**

**Screening of Ginsenosides by in Vitro Invasion Assay** In the previous study, we showed that ginsenoside-Rb2 inhibited tumor metastasis by suppressing the invasion of endothelial cells orienting toward the tumor mass.\textsuperscript{26} To further study the antitumor effect of ginsenosides from Red ginseng, we screened various ginsenosides using an in vitro assay for inhibition of tumor invasion. Figure 2A shows that five ginsenosides, ginsenoside-Rb2, -Rd, -Rg1, 20(R)- and 20(S)-Rg3, exhibited a prominent ability to inhibit the invasion of B16-BL6 melanoma cells. Since ginsenoside-Rb2, 20(R)- and 20(S)-ginsenoside-Rg3 share a common structural unit and all of them inhibited tumor cell invasion similarly in a dose-dependent manner at the dose of 0.1—100 µg/ml (Fig. 2B), we selected and used these three ginsenosides for further analysis.

**Effect of Ginsenosides on Adhesion of B16-BL6 Melanoma Cells** Tumor cell adhesion to the components, such as FN and LN, of extracellular matrices and the basement membrane has been shown to be an important step in tumor metastasis. When we examined the inhibitory effect of ginsenosides on tumor cell adhesion to FN or LN in vitro, 20(R)- and 20(S)-ginsenoside-Rg3 showed an inhibitory effect on the adhesion of tumor cells to both substrates in a dose-dependent manner (Fig. 3). However, ginsenoside-Rb2 showed no inhibitory effect on tumor cell adhesion. In an in vitro analysis, 20(R)- and 20(S)-ginsenoside-Rg3 at the concentration of 0.1—100 µg/ml

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**Fig. 2. Effect of Ginsenosides on the Invasion of B16-BL6 Melanoma Cells into Matrigel/Fibronectin-Coated Filters**

Filters were precoated with 5 µg of FN on their lower surfaces, and then with Matrigel (5 µg) on their surfaces. B16-BL6 melanoma cells ($2 \times 10^5$/well) in 0.1% BSA medium were seeded with or without each ginsenoside [A: 100 µg/ml; B: 0.1—100 µg/ml (□ 0.1 µg/ml; □ 1 µg/ml; □ 10 µg/ml; □ 100 µg/ml)] into the upper compartment of the Transwell cell culture chamber. After 4 h of incubation, the invaded cells on the lower surface were visually counted. a) $p<0.001$; b) $p<0.05$, compared with control group (by Student's two-tailed $t$ test).

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**Fig. 3. Inhibition of Tumor Cell Adhesion to Fibronectin or Laminin by Ginsenosides**

- $\triangle$, Rb2; $\square$, (R)Rg3; $\square$, (S)Rg3. B16-BL6 melanoma cells ($3.5 \times 10^6$) were added to each well which had been pre-coated with 50 µl of 5 µg/ml FN (A) or LN (B) and incubated with or without various concentrations of ginsenosides. After 30 min of incubation, nonadherent cells were washed away and the adherent cells were measured.
did not show any cytotoxic effect against B16-BL6 cells (data not shown).

**Effect of Ginsenosides on Experimental Lung Metastasis of B16-BL6 Cells**

The effect of the ginsenoside preparations on the inhibition of tumor metastasis was examined in an experimental lung metastasis model using B16-BL6 melanoma cells. As shown in Table 1, the i.v. administration of ginsenoside-Rb2, 20(R)- or 20(S)-ginsenoside-Rg3 at the dose of 100 μg/mouse after tumor inoculation significantly inhibited the lung metastasis of B16-BL6 melanoma cells. The significance of their antimitastatic effects in i.v. administration was recognized even at a dose of 10 μg/mouse. Interestingly, the p.o. administration of each ginsenoside was also effective in inhibiting tumor metastasis, although dose-dependency was not observed (Table 2).

**Effect of Ginsenosides on Experimental Lung Metastasis of Colon 26-M3.1 Cells**

Since it was ascertained from Tables 1 and 2 that ginsenosides inhibited lung metastasis of B16-BL6 melanoma cells, we next examined their effect on lung metastasis by another tumor cell, colon 26-M3.1 carcinoma. As shown in Table 3, the i.v., as well as p.o., administration of 20(R)- and 20(S)-ginsenoside-Rg3 significantly inhibited lung metastasis produced by colon 26-M3.1 carcinoma cells. However, ginsenoside-Rb2 exhibited an inhibitory effect only by i.v. administration. These findings indicated that the two saponin preparations, 20(R)- and 20(S)-ginsenoside-Rg3, are able to inhibit the lung metastasis of two different tumor cells.

**Effect of Ginsenosides on Spontaneous Lung Metastasis of B16-BL6 Cells**

The therapeutic effect of multiple administrations of ginsenosides was examined on the spontaneous lung metastasis produced by intrafootpad injection of B16-BL6 melanoma cells. Tumor-bearing mice received multiple administrations p.o. of saponin preparations before or after the primary tumor amputation. All ginsenosides significantly inhibited lung metastasis of B16-BL6 melanoma cells, but did not suppress the growth of the primary tumor (Table 4).

**Inhibitory Effect of Ginsenosides on Tumor-Induced Angiogenesis**

In the previous study, we showed that the treatment with ginsenoside-Rb2 during the early period of tumor inoculation inhibited tumor-induced angiogenesis. To examine whether the antimitastatic activity

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### Table 1. Effect of Various Doses of Ginsenosides on Lung Metastasis Produced by B16-BL6 Melanoma Cells in C57BL/6 Mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Route</th>
<th>Dose (μg/mouse)</th>
<th>No. of lung colonies on day 14</th>
<th>Mean ± S.D. (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (PBS)</td>
<td>i.v.</td>
<td>—</td>
<td>63 ± 8 (57–77)</td>
<td></td>
</tr>
<tr>
<td>Ginsenoside-Rb2</td>
<td>i.v.</td>
<td>100</td>
<td>28 ± 7* (21–38)</td>
<td></td>
</tr>
<tr>
<td>Ginsenoside-20(R)Rg3</td>
<td>100</td>
<td>33 ± 8* (23–42)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ginsenoside-20(S)Rg3</td>
<td>10</td>
<td>34 ± 15* (20–54)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ginsenoside-20(S)Rg3</td>
<td>i.v.</td>
<td>100</td>
<td>46 ± 13* (31–57)</td>
<td></td>
</tr>
<tr>
<td>Ginsenoside-20(S)Rg3</td>
<td>30</td>
<td>40 ± 14* (15–50)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ginsenoside-20(S)Rg3</td>
<td>10</td>
<td>36 ± 12* (24–50)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ginsenoside-20(S)Rg3</td>
<td>30</td>
<td>35 ± 11* (24–51)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ginsenoside-20(S)Rg3</td>
<td>10</td>
<td>28 ± 14* (17–50)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ginsenoside-20(S)Rg3</td>
<td>20</td>
<td>12 ± 2* (21–26)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Five C57BL/6 mice per group were given i.v. administration of various doses of ginsenosides 1, 2, 3 and 4 d after i.v. inoculation of B16-BL6 melanoma cells (5 × 10⁶). Mice were killed 14 d after tumor inoculation for evaluation. a) p < 0.05, b) p < 0.01, c) p < 0.001 as compared with untreated control by Student's two-tailed t test.

### Table 2. Effect of p.o. Administration of Ginsenosides on Lung Metastasis Produced by B16-BL6 Melanoma Cells in C57BL/6 Mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Route</th>
<th>Dose (μg/mouse)</th>
<th>No. of lung colonies on day 14</th>
<th>Mean ± S.D. (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (PBS)</td>
<td>p.o.</td>
<td>—</td>
<td>46 ± 19 (33–74)</td>
<td></td>
</tr>
<tr>
<td>Ginsenoside-Rb2</td>
<td>p.o.</td>
<td>100</td>
<td>39 ± 18 (24–63)</td>
<td></td>
</tr>
<tr>
<td>Ginsenoside-20(R)Rg3</td>
<td>300</td>
<td>21 ± 12* (10–38)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ginsenoside-20(R)Rg3</td>
<td>100</td>
<td>44 ± 9* (34–53)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ginsenoside-20(R)Rg3</td>
<td>1000</td>
<td>23 ± 5* (18–29)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ginsenoside-20(S)Rg3</td>
<td>300</td>
<td>23 ± 2* (21–24)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ginsenoside-20(S)Rg3</td>
<td>1000</td>
<td>43 ± 20 (26–71)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ginsenoside-20(S)Rg3</td>
<td>1000</td>
<td>30 ± 14* (16–48)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ginsenoside-20(S)Rg3</td>
<td>300</td>
<td>32 ± 13* (8–35)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ginsenoside-20(S)Rg3</td>
<td>100</td>
<td>20 ± 3* (18–24)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Five C57BL/6 mice per group were given p.o. administration of various doses of ginsenosides 1, 2, 3 and 4 d after i.v. inoculation of B16-BL6 melanoma cells (5 × 10⁶). Mice were killed 14 d after tumor inoculation for evaluation. a) p < 0.05, b) p < 0.01 as compared with untreated control by Student's two-tailed t test.

### Table 3. Effect of i.v. or p.o. Administration of Ginsenosides on Lung Metastasis Produced by Colon 26-M3.1 Carcinoma Cells in Balb/c Mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Route</th>
<th>Dose (μg/mouse)</th>
<th>No. of lung colonies on day 14</th>
<th>Mean ± S.D. (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (PBS)</td>
<td>—</td>
<td>—</td>
<td>89 ± 20 (68–110)</td>
<td></td>
</tr>
<tr>
<td>Ginsenoside-Rb2</td>
<td>i.v.</td>
<td>100</td>
<td>67 ± 7* (53–68)</td>
<td></td>
</tr>
<tr>
<td>Ginsenoside-20(R)Rg3</td>
<td>300</td>
<td>68 ± 10 (58–79)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ginsenoside-20(S)Rg3</td>
<td>i.v.</td>
<td>100</td>
<td>44 ± 4* (39–47)</td>
<td></td>
</tr>
<tr>
<td>Ginsenoside-20(S)Rg3</td>
<td>300</td>
<td>48 ± 17* (33–65)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ginsenoside-20(S)Rg3</td>
<td>i.v.</td>
<td>100</td>
<td>54 ± 22* (27–76)</td>
<td></td>
</tr>
<tr>
<td>Ginsenoside-20(S)Rg3</td>
<td>300</td>
<td>40 ± 23* (11–65)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Five Balb/c mice per group were given i.v. or p.o. administration of the indicated doses of ginsenosides 1, 2, 3 and 4 d after i.v. inoculation of Colon-26-M3.1 carcinoma cells (5 × 10⁶). Mice were killed 14 d after tumor inoculation for evaluation. a) p < 0.05, b) p < 0.01 as compared with untreated control by Student's two-tailed t test.

### Table 4. Therapeutic Effect of Multiple p.o. Administrations of Ginsenosides on Spontaneous Lung Metastasis Produced by B16-BL6 Melanoma Cells in C57BL/6 Mice

<table>
<thead>
<tr>
<th>Treatment after tumor inoculation</th>
<th>Dose</th>
<th>Size of primary tumor (mm ± S.D.)</th>
<th>No. of lung colonies on day 35</th>
<th>Mean ± S.D. (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (PBS)</td>
<td>—</td>
<td>10.6 ± 1.0</td>
<td>97 ± 12 (78–109)</td>
<td></td>
</tr>
<tr>
<td>Expt. 1</td>
<td></td>
<td>10.4 ± 0.8</td>
<td>44 ± 16* (15–59)</td>
<td></td>
</tr>
<tr>
<td>Expt. 2</td>
<td></td>
<td>10.2 ± 0.9</td>
<td>38 ± 12* (28–58)</td>
<td></td>
</tr>
<tr>
<td>Expt. 3</td>
<td></td>
<td>10.0 ± 1.1</td>
<td>64 ± 23* (27–91)</td>
<td></td>
</tr>
<tr>
<td>Expt. 4</td>
<td></td>
<td>10.0 ± 1.4</td>
<td>23 ± 9* (15–39)</td>
<td></td>
</tr>
<tr>
<td>Expt. 5</td>
<td></td>
<td>10.0 ± 1.3</td>
<td>21 ± 9* (11–35)</td>
<td></td>
</tr>
</tbody>
</table>

Six C57BL/6 mice per group were administered p.o. 300 μg of various ginsenosides 5, 6, 7, 8, 13, 14, 15 and 16 d (Expt. 1) or 22, 23, 24 and 25d (Expt. 2) after intrafootpad injection of B16-BL6 melanoma cells (5 × 10⁶). The primary tumors were surgically amputated 21 d after tumor inoculation. Mice were killed 14 d after the primary tumor amputation and tumor colonies were counted. a) p < 0.05, b) p < 0.01 as compared with untreated control by Student's two-tailed t test.
of 20(R)- and 20(S)-ginsenoside-Rg3 is related to the suppression of neovascularization by tumor cells, we examined their effect on the inhibition of tumor-induced angiogenesis in vivo. As shown in Table 5, the multiple administration of the two saponin preparations significantly inhibited tumor-induced angiogenesis, without the suppression of tumor growth. Moreover, the inhibitory activity of tumor-induced angiogenesis was effective in not only with i.v. administration but also with the p.o. administration of ginsenosides.

DISCUSSION

The present study demonstrated that two saponin preparations, 20(R)- and 20(S)-ginsenoside-Rg3, as well as ginsenoside-Rb2 purified from Red ginseng inhibited lung metastasis produced by B16-BL6 melanoma and colon 26-M3.1 carcinoma cells.

Tumor invasion and adhesion to an extracellular matrix and basement membrane components such as FN and LN are important events in the process of tumor metastasis. 3,4,32,33 In an in vitro study, ginsenoside-Rb2 and two ginsenoside-Rg3 preparations, 20(R)- and 20(S)-ginsenoside-Rg3, exhibited a higher ability to inhibit the invasion of B16-BL6 melanoma cells to Matrigel/FN, showing a dose-dependent manner, than the other ginseng saponin preparations (Fig. 2). Even though ginsenoside-Rb2 and both ginsenoside-Rg3 preparations showed almost the same effect on the inhibition of tumor invasion, only the ginsenoside-Rg3, but not ginsenoside-Rb2, inhibited the adhesion of tumor cells to FN and LN. However, none of these ginsenosides had any effect to inhibit the haptotactic migration of tumor cells in vitro (data not shown). These findings suggested that the antimeatstatic effect of ginsenosides used in this study is commonly related to inhibition of tumor invasion, but considering that ginsenoside-Rb2 could also inhibit the lung metastasis of B16-BL6 melanoma cells (Tables 1, 2 and 3), other functions might be associated with the effect.

Previous studies have revealed that saponin preparations from Red ginseng possessed immunomodulatory activities such as the enhancement of cytokine induction, cell-mediated immunity and natural killer (NK) cell activity. 15-18,34 However, not all studies on the immunomodulatory activities of ginseng have been positive. Yeung et al. 35 reported that ginseng saponins, injected i.v. into mice, suppressed a delayed-type hypersensitivity (DTH) reaction to influenza virus and had no effect on cytotoxic T-lymphocyte (CTL) and NK cell activity. Also, we found that ginsenoside-Rb2 and ginsenoside-Rg3 preparations used in this study could not induce NK cell activity or cytokines such as interleukin-1 (IL-1) and TNF-α from macrophages, and did not not an immunoadjuvant activity to enhance antibody titers to keyhole limpet hemocyanin (KLH) when they were co-immunized with KLH (data not shown).

On the other hand, some ginsenosides have been demonstrated to suppress tumor cell growth in vitro by inducing the reverse transformation of Morris hepatic carcinoma.23,24 B16 melanoma cells 36,37 and human ovarian cells.38 Furthermore, Kubo et al. 39 reported that the methanolic extract from Panax ginseng synergistically enhanced the cytotoxic activity of mitomycin C to Ehrlich carcinoma cells. In the previous study, we reported that the administration of ginsenoside-Rb2 inhibit lung metastasis of B16-BL6 melanoma cells, and that its antimeatstatic effect was due to the suppression of tumor-induced angiogenesis.26 Two ginsenoside-Rg3 preparations and ginsenoside-Rb2 were shown to also significantly inhibit tumor-induced angiogenesis (Table 5).

Of particular significance was the finding that the p.o. administration of ginsenoside-Rb2 and 20(R,S)-ginsenoside-Rg3 inhibited the lung metastasis of tumor cells. Since stimulation at the mucosal tissue has recently been shown to elicit the activation of a common mucosal immune system (CMIS), 40 the activation of immunity at a mucosal tissue, i.e., Peyer’s patch, by the oral administration of ginsenosides may induce and potentiate immune responses in lungs against tumor cells that are destined to metastasize to the tissues. To elucidate such a mechanism, further study should be carried out.

In conclusion, this study demonstrated that the systemic, as well as oral, multiple administration of ginsenoside-Rb2, 20(R)- and 20(S)-ginsenoside-Rg3, isolated from Red ginseng inhibited lung metastasis produced by B16-BL6 melanoma and Colon 26-M3.1 carcinoma cells in mice, and that the antimeatstatic effect was associated with the inhibition of the invasion and adhesion by tumor cells as well as suppression of tumor-induced angiogenesis.

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