Lipid-Soluble Ginseng Extract Inhibits Invasion and Metastasis of B16F10 Melanoma Cells

Jieun Yun,^{1,*} Bo Geun Kim,^{1,*} Jong Soon Kang,¹ Song-Kyu Park,² Kiho Lee,²
Dong-Hoon Hyun,³ Hwan Mook Kim,⁴ Man-Jin In,⁵ and Dong Chung Kim⁵

¹Bio-Evaluation Center, Korea Research Institute of Bioscience and Biotechnology, Ochang, Republic of Korea.

²College of Pharmacy, Korea University, Sejong, Republic of Korea.

³Department of Life Science, Ewha Womans University, Seoul, Republic of Korea.

⁴College of Pharmacy, Gachon University, Incheon, Republic of Korea.

⁵Department of Human Nutrition and Food Science, Chungwoon University, Hongseong, Republic of Korea.

ABSTRACT This study was performed to elucidate the effect of a lipid-soluble ginseng extract (LSGE) on cancer invasion and metastasis. The LSGE, even at noncytotoxic concentrations, potently inhibited invasion and migration of B16F10 mouse melanoma cells in a dose-dependent manner. In the presence of 3 $\mu\text{g/mL}$ of LSGE, the invasion and migration of B16F10 cells were significantly inhibited by 98.1% and 71.4%, respectively. Furthermore, the LSGE decreased mRNA and protein levels of matrix metalloproteinase (MMP)-2 in B16F10 cells, leading to a decrease in MMP-2 activity. After B16F10 cells were intravenously injected in the tail vein of C57BL/6 mice, 1000 mg/kg/day of LSGE was orally administered for 13 days, after which lung metastasis of cancer cells was inhibited by 59.3%. These findings indicate that LSGE inhibits cancer cell invasion and migration *in vitro* and lung metastasis of melanoma cells *in vivo* by inhibiting MMP-2 expression.

KEY WORDS: • cancer invasion • lipid-soluble ginseng extract • lung metastasis • matrix metalloprotease-2

INTRODUCTION

GINSENG (*PANAX GINSENG*) HAS been traditionally used as a herbal medicine in Far Eastern countries.¹ An antitumor effect has been considered a major pharmacological function of *Panax ginseng*.^{1,2} In particular, ginsenosides such as ginsenoside Rb2 and Rg3 have been reported to inhibit tumor-induced angiogenesis, tumor invasion, and metastasis.^{2–4} Ginsenoside metabolites inhibited lung metastasis of melanoma cancer cells *in vivo*.⁵ A water extract of ginseng possessed an anticancer activity through the induction of apoptosis and a decrease in telomerase activity in cancer cells.⁶ Aqueous extracts of white and red ginseng consist primarily of ginsenosides and polysaccharides.^{7,8} Water extracts of red ginseng reduced metastasis of human colon cancer cells both *in vitro* and *in vivo*.⁹ Ginsenoside Rh2 inhibited tumor growth in nude mice bearing human

ovarian cancer cells.^{10,11} The main subjects for the anticancer activity of ginseng have been water-soluble extracts of white or red ginseng.

In contrast, since lipid-soluble ginseng extracts (LSGEs) were reported to inhibit the proliferation of various human cancer cells *in vitro* and to extend the survival rate of tumor-bearing mice *in vivo*,^{12,13} our studies have concentrated on the anticancer effects of LSGEs both *in vitro* and *in vivo*.^{7,8,14,15} In particular, the anticancer activity of ginseng was reported to originate mainly from lipid-soluble components.⁷ Since LSGE contained a very small amount of ginsenosides and no polysaccharides, it was assumed that the antitumor effect of LSGE is due to nonpolar components such as polyacetylenes and polyphenolics.^{7,8} Polyacetylenes containing panaxynol, panaxydol, and panaxytriol have been regarded as main components responsible for anticancer activity of LSGE.^{13,15,16} The polyacetylene content of LSGE was considered to be between 2.38% and 6.88%, while panaxynol was identified as a dominant polyacetylene of LSGE.⁸ In our previous reports, LSGE inhibited the growth of initial and advanced human lung tumors in murine xenografts models without any apparent toxicity.^{8,14} LSGE also possessed the anticancer activity through the induction of caspase-mediated apoptosis and G0/G1 cell cycle arrest in NCI-H460 human lung cancer cells.¹⁵

*These authors contributed equally to this work.

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Address correspondence to: Dong Chung Kim, PhD, Department of Human Nutrition and Food Science, Chungwoon University, Hongseong 350-701, Republic of Korea, E-mail: kimdc@chungwoon.ac.kr or Hwan Mook Kim, PhD, College of Pharmacy, Gachon University, Incheon 406-799, Republic of Korea, E-mail: hwanmook@gachon.ac.kr

Tumor progression is a multistep process consisting of uncontrolled proliferation, invasion, and metastasis of cancer cells.¹⁷ Since metastasis is a major contributor to the mortality of most cancers, cancer invasion and metastasis have been considered critical targets for therapeutic treatment of malignant solid tumors.¹⁸ Here, we describe an effect of LSGE on cancer cell invasion and metastasis as well as the expression of matrix metalloproteinase (MMP)-2 in B16F10 melanoma cells.

MATERIALS AND METHODS

Preparation of LSGE

Red ginseng (first grade, 6 years old) was purchased from Korea Ginseng Corporation (Seoul, Korea). LSGE was prepared according to previous reports.^{7,8} LSGE was extracted from red ginseng powder (particle size <400 μm) by using *n*-hexane, which was then eliminated from LSGE using a rotary vacuum evaporator (Eyela, Tokyo, Japan).⁸ For *in vitro* tests, LSGE was dissolved in dimethyl sulfoxide (DMSO) and further diluted with the culture media such that the final concentration of DMSO did not exceed 0.1%.¹⁵ For *in vivo* tests, LSGE was dissolved in ethanol and further diluted in sterilized distilled water such that the final ethanol concentration was 4% before oral administration.^{8,14}

Proliferation assay

B16F10 mouse melanoma cell line (American Type Culture Collection, Manassa, VA) was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 100 $\mu\text{g}/\text{mL}$ of streptomycin sulfate. Cell proliferation assays were performed using an XTT (sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) Cell Proliferation Kit II (Roche, Indianapolis, IN) as previously described.¹⁵ B16F10 cells were plated at 1×10^4 cells/well in 96-well culture plates, incubated overnight, and treated with 0.1% DMSO (vehicle control), 0.3, 1.0, 3.0, or 10.0 $\mu\text{g}/\text{mL}$ of LSGE for 24 h. Then, a XTT labeling reagent and electron-coupling reagent were added to each well according to the manufacturer's instructions. After the mixtures were incubated for 2 h at 37°C, the formazan dye was measured at 490 and 650 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

Invasion and migration assays

The invasive capability of B16F10 cells was performed using a BD matrigel invasion chamber (BD Biosciences, Bedford, MA) as previously described.¹⁸ B16F10 cells (1×10^5) per well, resuspended in serum-free DMEM with 0.1% DMSO (vehicle control), 0.3, 1.0, or 3.0 $\mu\text{g}/\text{mL}$ of LSGE, were transferred into the upper wells of 8-mm-pore-size polycarbonate membranes. The lower wells were filled with a chemo-attractant medium, which was obtained by incubating human fibroblasts (HT-1080). The matrigel chambers were then incubated for 24 h at 37°C with 5% CO₂. After incubation, the cells on the upper surfaces of the

membranes were washed with phosphate-buffered saline (PBS) and then, the membranes were fixed with 4% paraformaldehyde for 10 min. The cells that actively invaded into the bottom surfaces of the membranes were stained with 0.5% crystal violet for 20 min. Invasion was quantified by the number of penetrating cells under a microscope at 100 $\times$ magnification in each membrane.

B16F10 cell migration was evaluated by using a μ -Dish^{35mm, low} culture insert (Thistle Scientific, Glasgow, United Kingdom). Cells (5×10^4) per well were seeded and cultured into both wells of the culture insert in DMEM containing 5% FBS. When the cells reached 100% confluence, the culture insert was removed aseptically with sterile forceps. The cells were treated with 0.1% DMSO (vehicle control), 0.3, 1.0, or 3.0 $\mu\text{g}/\text{mL}$ of LSGE for 14 h at 37°C. Images of cells migration were captured at the indicated times.

Gelatin zymography and western blot analysis

MMP-2 and MMP-9 activities were determined by gelatin zymography as previously described.⁹ B16F10 cells were seeded at 7×10^5 cells/100 mm dish and stabilized in DMEM with 5% FBS at 37°C for 12 h. The culture medium was changed to a serum-free DMEM. After 2 h, the cells were treated with either 0.1% DMSO (vehicle control) or 3 $\mu\text{g}/\text{mL}$ of LSGE for 24 h. The conditioned medium was harvested and then concentrated by a Centricon Plus-20 centrifugal filter device (Millipore, Bedford, MA). The conditioned media with the same amount of protein were mixed with an equal volume of sodium dodecyl sulfate (SDS)-gel loading buffer without boiling and then subjected to 10% SDS-polyacrylamide gels containing 0.1% gelatin under nonreducing conditions. After electrophoresis at 4°C, the gels were soaked in zymogen renaturation buffer (Bio-Rad, Hercules, CA) for 1.5 h to remove residual SDS and rinsed in distilled water. The gels were incubated in a developing buffer (40 mM Tris-HCl, pH 8.0, 10 mM CaCl₂, and 0.02% NaN₃) for 24 h at 37°C, stained with 0.125% Coomassie blue R-250, and visualized by destaining solution (20% methanol, 10% acetic acid). The intensity of bands was quantified by Bio-Rad Image Lab 3.0 (Bio-Rad).

Protein levels of MMP-2 and MMP-9 were determined by western blot analysis as previously described.¹⁹ For western blotting, the conditioned media with the same amount of protein were mixed with an equal volume of SDS-gel loading buffer (125 mM Tris-HCl, pH 6.8, 3% SDS, 40% glycerol, and 0.02% bromophenol blue), boiled for 10 min, and then analyzed on 10% SDS-polyacrylamide gels. The separated proteins were transferred to a polyvinylidene difluoride membrane. After soaking in a blocking solution (10 mM Tris-HCl, pH 7.0, 100 mM NaCl, and 0.1% Tween-20) with 3% skim milk for 1 h, the membrane was incubated with the primary antibodies specific for MMP-2 (Santa Cruz Biotechnology, Santa Cruz, CA), for MMP-9 (Cell Signaling Technology, Danvers, MA), and for actin (Abcam, Cambridge, MA), followed by horseradish peroxidase-conjugated secondary antibody. Beta-actin was used as a loading control.

The antibody binding was detected using enhanced chemoluminescence western reagents (GE Healthcare, Piscataway, NJ). The intensity of bands was quantified by Bio-Rad Image Lab 3.0 (Bio-Rad).

Reverse transcriptase-polymerase chain reaction

Messenger RNA level of MMP-2 and tissue inhibitor of metalloproteinase-2 (TIMP-2) was measured as previously described.²⁰ B16F10 cells were seeded at 7×10^5 cells/100 mm dish and stabilized in DMEM with 5% FBS at 37°C for 12 h. The culture medium was changed to a serum-free DMEM. After 2 h, the cells were treated with either 0.1% DMSO (vehicle control) or 3 $\mu\text{g}/\text{mL}$ of LSGE for 24 h, washed by ice-cold PBS, and harvested. Total RNA was isolated from B16F10 cells using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Then, cDNA was synthesized from 1 μg of total RNA using a PrimeScript™ first-strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan), and amplified using an EmeraldAmp™ PCR Master Mix (Takara Bio) according to the manufacturer's instructions. Beta-actin gene was used as a control. For amplification of cDNA, primers for MMP-2 (upstream primer, 5'-CACCTACACCAAGAACTCC-3'; downstream primer, 5'-AACACAGCCTTCTCCTCCTG-3'), for TIMP-2 (upstream primer, 5'-CTCGCTGGACGTTGGAGGAA-3'; downstream primer, 5'-CACGCGCAAGAACCATCACT-3'), and for actin (upstream primer, 5'-TGGAATCCTGTGGCATCCATGAAAC-3'; downstream primer, 5'-TAAACGCAGCTCAGTAACAGTCCG-3') were used. The polymerase chain reaction (PCR) products were resolved on a 1.5% agarose gel, stained with ethidium bromide, and analyzed using a Bio-profile Bio-IL application (Vilber-Lourmat, Eberhardzell, Germany). The intensity of bands was quantified by Bio-Rad Image Lab 3.0 (Bio-Rad).

Experimental murine lung metastasis

Experimental lung metastasis was performed as previously described.⁹ Seven-week-C57BL/6 female mice (Koatech, Pyungtaek, Korea) were acclimated to laboratory conditions for 1 week. All animal experiments were approved by the Institutional Animal Care and Use Committee, Korea Research Institute of Bioscience and Biotechnology (Ochang, Korea).⁸ The mice were randomly assigned to five groups ($n=6/\text{group}$), all of which were intravenously injected with 0.2 mL of B16F10 cells at a density of 2×10^6 cells/mL in the tail of mice. After 3 h postinoculation, the mice in the first group as a vehicle control were treated with 0.2 mL of 4% ethanol solution once per day by oral gavage. The mice in the second and third groups were orally administered with LSGE at doses of 300 and 1000 mg/kg in 0.2 mL of 4% ethanol solution once per day for 13 days. In the fourth group, used as a positive control, Adriamycin hydrochloride (Sigma-Aldrich, St. Louis, MO) was intraperitoneally administered at a dosage of 2 mg/kg every other day for 13 days. On the 14th day, mice were sacrificed by CO₂ asphyxiation, and their lungs were dissected. The number of metastatic colonies was counted.

Statistical analysis

All data were expressed as mean $\pm$ standard deviation of at least three independent experiments. GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA) was used for statistical analysis. Statistical significance was determined by the two-sample Student's *t*-test assuming unequal variances.

RESULTS AND DISCUSSION

LSGE was prepared using *n*-hexane as an extraction solvent, and about 0.8% (w/w) of red ginseng was recovered as LSGE. To examine the cytotoxicity of LSGE, B16F10 melanoma cells were cultured with between 0 and 10 $\mu\text{g}/\text{mL}$ of LSGE for 24 h. As shown in Figure 1, LSGE significantly inhibited the proliferation of B16F10 cells at concentrations above 3 $\mu\text{g}/\text{mL}$. This result was consistent with our previous reports, showing that LSGE exhibited a potent anticancer effect against a variety of cancer cells both *in vitro* and *in vivo*.^{7,8,14} When B16F10 cells were incubated in the presence of 10 $\mu\text{g}/\text{mL}$ of LSGE, the inhibition rate was 90.6% ($P<.001$). On the other hand, at concentrations ranging between 0 and 3 $\mu\text{g}/\text{mL}$, LSGE showed a mild toxicity against B16F10 cell proliferation. GI₅₀ value of LSGE against B16F10 cells was 5.1 $\mu\text{g}/\text{mL}$. To demonstrate that the anti-metastatic effect of LSGE is independent of its anti-proliferative activity, the concentration of LSGE, which was used in anti-invasive and anti-migratory experiments, should not reach the cytotoxic level.²⁰ Thus, LSGE at concentrations ranging between 0 and 3 $\mu\text{g}/\text{mL}$ was used in all further *in vitro* experiments for elucidating its anti-invasive and anti-migratory effects in B16F10 mouse melanoma cells.

Cancer cell invasion is an essential step in the process of tumor metastasis.^{19,21} To determine whether LSGE affects

FIG. 1. Effects of lipid-soluble ginseng extract (LSGE) on the proliferation of B16F10 melanoma cells. The cells were treated with 0.1% DMSO (vehicle control [V.C]), 0.3, 1.0, 3.0, or 10 $\mu\text{g}/\text{mL}$ of LSGE for 24 h. Data represent the mean $\pm$ SD ($n=3$). The significance was determined using Student's *t*-test: *** $P<.001$. DMSO, dimethyl sulfoxide.

the invasion of cancer cells *in vitro*, the invasive capability of B16F10 cells after treatment with LSGE was evaluated. In the absence of LSGE, B16F10 cells possessed highly invasive activity as demonstrated by their ability to penetrate the membranes. LSGE potently inhibited the invasion of B16F10 cells in a dose-dependent manner (Fig. 2). In the presence of 0.3, 1.0, and 3.0 $\mu\text{g/mL}$ of LSGE, cancer cell invasion was inhibited by 52.4% ($P < .05$), 72.6% ($P < .001$), and 98.1% ($P < .001$), respectively (Fig. 2).

Since cell migration is a critical process of invasion enabling tumor metastasis,¹⁹ we evaluated the effect of LSGE on cancer cell migration. The relative migration was determined by subtracting the final gap width from the initial gap width. Figure 3A showed the initial gap at 0 h. As shown in the vehicle control of Figure 3B, B16F10 cells not treated with LSGE rapidly migrated and completely filled the gap after 14 h of incubation. In contrast, LSGE potently blocked the migration of B16F10 cells in a dose-dependent manner (Fig. 3B). In the presence of 0.3, 1.0, and 3.0 $\mu\text{g/mL}$ of LSGE, the cell migration was inhibited by 21.4% ($P < .05$), 42.9% ($P < .01$), and 71.4% ($P < .001$), respectively (Fig. 3C). Cancer cell invasion and migration were significantly reduced by LSGE, even at noncytotoxic concentrations. These results showed that LSGE possesses an anti-invasive and anti-migratory activity in B16F10 cells. Extracts of several kinds of plants have exhibited an anti-invasiveness effect.^{18,22,23} In particular, a water-soluble red ginseng extract (WSGE) decreased the motility and invasion of SW480 human colon cancer cells.⁹ While WSGE significantly inhibited the invasion of SW480 cancer cells at concentrations above 100 $\mu\text{g/mL}$,⁹ LSGE showed potent anti-invasive activity against B16F10 cancer cells at concentrations below 3 $\mu\text{g/mL}$. The polarity and dielectric constant of extraction solvents influenced the chemical compositions of ginseng extracts.^{7,8} Most saponins and water-soluble carbohydrates

FIG. 2. Effects of LSGE on the invasiveness of B16F10 melanoma cells. The cells were treated with 0.1% DMSO (V.C), 0.3, 1.0, or 3.0 $\mu\text{g/mL}$ of LSGE. Data represent the mean $\pm$ SD ($n=3$). The significance was determined using Student's *t*-test: * $P < .05$, *** $P < .001$.

FIG. 3. Effects of LSGE on the migration of B16F10 melanoma cells. (A) Photograph of the initial gap and cells at 0 h. (B) Photographs of cells treated with LSGE for 14 h. The images are representative of three independent experiments. The cells were treated with 0.1% DMSO (V.C), 0.3, 1.0, or 3.0 $\mu\text{g/mL}$ of LSGE. (C) Quantitative analysis of B16F10 cancer cell migration. Data represent the mean $\pm$ SD ($n=3$). The significance was determined using Student's *t*-test: * $P < .05$, ** $P < .01$, and *** $P < .001$. Color images available online at www.liebertpub.com/jmf

of ginseng are extracted under aqueous condition, but are almost insoluble in hexane.⁷ The contents of carbohydrate and crude saponin in the water extract of ginseng were 81% and 4.84%, respectively.⁷ The hexane extract of ginseng contained only 0.09% crude saponin and no carbohydrates.⁷ Aqueous extracts of ginseng include saponins, amino acids, minerals, and water-soluble carbohydrates; whereas its oil-soluble extracts mainly contain lipids, polyacetylenes, and polyphenolics.^{7,8} Ginsenoside Rg3 and Rh2 have been regarded as the main anticancer active ingredients in WSGE.^{3,15,24} LSGE has been reported to possess a potent anticancer activity that mainly originates from nonpolar polyacetylenes and polyphenolics.^{7,12,13,16} WSGE was reported to possess a relatively low anti-proliferative activity against human hepatoma (HepG2, $\text{GI}_{50}=268.5$ $\mu\text{g/mL}$) and breast (MCF-7, $\text{GI}_{50}=466.6$ $\mu\text{g/mL}$) cancer cells,⁷ whereas LSGE showed potent anti-proliferative activity against various human cancer cell lines, including human hepatoma (HepG2, $\text{GI}_{50}=41.7$ $\mu\text{g/mL}$), breast (MCF-7, $\text{GI}_{50}=54.4$ $\mu\text{g/mL}$), adenocarcinoma lung (NCI-H23, $\text{GI}_{50}=16.7$ $\mu\text{g/mL}$), small cell lung (DMS114, $\text{GI}_{50}=27.0$ $\mu\text{g/mL}$), gastric (MKN74, $\text{GI}_{50}=28.2$ $\mu\text{g/mL}$), prostate (PC-3, $\text{GI}_{50}=28.5$ $\mu\text{g/mL}$), and large cell lung (NCI-H460, $\text{GI}_{50}=90.4$ $\mu\text{g/mL}$) cancer cells.^{7,14} LSGE was more potent than WSGE in

FIG. 4. Effects of LSGE on the activity and expression of MMP-2 in B16F10 melanoma cells. The cells were treated either with LSGE at the concentration of 3 μ g/mL or with the V.C. The band images are representative of three independent experiments. Data of each graph represent the mean $\pm$ SD ($n=3$). The significance was determined using Student's *t*-test: * $P < .05$. (A) The activity of MMP-2 was analyzed by gelatin zymography. The band intensity ratio of MMP-2 activity is shown in the graph. (B) The protein expression of MMP-2 was determined by western immunoblot analysis using the indicated antibodies. Beta-actin was used as a loading control for quantitative western blotting. The band intensity ratio of MMP-2 protein normalized by beta-actin is shown in the graph. (C) The mRNA expression of MMP-2 and TIMP-2 was determined by RT-PCR using the indicated primers. Beta-actin gene was used as a loading control for quantitative RT-PCR. The band intensity ratio of MMP-2 and TIMP-2 mRNAs normalized by Actin is shown in the graphs. MMP, matrix metalloproteinase; RT-PCR, reverse transcriptase-polymerase chain reaction; TIMP-2, tissue inhibitor of metalloproteinase-2.

inhibiting the proliferation of human cancer cells. It has also been reported that both LSGE and WSGE induced apoptosis of cancer cells, but their mechanisms for the induction of tumor cell death were different.^{6,15} LSGE initiated the activation of caspase-8 and -9 followed by activation of caspase-3.¹⁵ WSGE increased the expression of active forms of caspase-3, but seemed not to affect the expression levels of pro-caspase-8 and -9 proteins.⁶

MMPs play a fundamental role in extracellular matrix degradation related to cancer cell invasion, tumor metastasis, and angiogenesis.²⁵ Among them, the MMP-2 and MMP-9 have been reported to be most closely associated with metastatic potential.²⁰ Cancer cell invasion and migration into surrounding tissues are mediated by MMP-2 and MMP-9.²² Therefore, regulation of MMP-2 and MMP-9 are essential for preventing cancer invasion and metastasis.²⁶ In this study, the gelatinolytic activity of both MMP-2 and MMP-9 was measured in B16F10 cancer cells in the absence and presence of LSGE. As shown in Figure 4A, LSGE significantly inhibited MMP-2 activity in B16F10 cells. Treatment with 3.0 μ g/mL of LSGE caused a 39.0% reduction in MMP-2 activity. Similar to MMP-2 activity, LSGE decreased MMP-2 protein level in LSGE-treated cells. The addition of 3.0 μ g/mL of LSGE led to a 38.0% reduction in the protein level of MMP-2 (Fig. 4B). These results indicated that the low activity of MMP-2 in LSGE-treated cells was caused by decreased MMP-2 protein levels. No significant difference in protein level and activity of MMP-9 was observed in LSGE-treated cells (data not shown).

To examine whether the decrease of MMP-2 protein level in LSGE-treated cells was caused by the change of MMP-2 mRNA level, we analyzed the expression of MMP-2 gene using reverse transcriptase-PCR, as well as actin, which served as a loading control. As shown in Figure 4C, LSGE significantly inhibited transcription of MMP-2 DNA. The addition of 3.0 μ g/mL of LSGE caused a 44.0% decrease in the mRNA level of MMP-2. It seems that LSGE suppressed the transcription of MMP-2 DNA, which led to reducing the expression of MMP-2 protein. These results indicated that inhibiting the transcription of MMP-2 DNA caused the low activity and protein level of MMP-2 in LSGE-treated cells. MMP-2 is produced as an inactive pro-MMP-2 requiring activation for catalytic activity.²⁷ TIMP-2 has been reported to play a critical role in regulating MMP-2 activation by forming a ternary complex with pro-MMP-2 and its activator MT1-MMP on the cell surface.²⁷ There is no notable change in mRNA level of TIMP-2 in LSGE-treated cells (Fig. 4C). LSGE did not affect the transcription of TIMP-2 DNA. Since prevention of invasion and metastasis is critical for successful cancer therapy,²⁸ and MMP-2 plays an important role in metastasis of human cancers,^{25,29} MMP-2 has been considered an important therapeutic target for human cancers.^{20,25,26,30} Further work is required to clarify the effect of LSGE on different molecular changes and signaling pathways related to tumor metastasis.

Our results showed that LSGE significantly inhibited cancer cell invasion and migration *in vitro* through inhibition of MMP-2 expression. Since it was reported that the

FIG. 5. Effects of LSGE on the lung metastasis of B16F10 melanoma cells *in vivo*. (A) Photographs of lung colonization in C57BL/6 mice orally administered with LSGE at doses of 300 and 1000 mg/kg/day compared with the V.C and Adriamycin (ADR)-treated group. (B) Quantitative analysis of lung colonization of B16F10 melanoma cells. Data represent the mean $\pm$ SD ($n=6$). Statistical significance of differences between the vehicle control and treatment groups was evaluated using Student's *t*-test: * $P < .05$, *** $P < .001$. Color images available online at www.liebertpub.com/jmf

oral intake of LSGE at doses of 0.3 and 1.0 g/kg/day potently inhibited tumor growth in mice without any apparent toxicity,⁸ LSGE at the same daily dose was orally administered to mice intravenously injected with B16F10 cells in order to confirm that LSGE could inhibit the formation of distant metastasis *in vivo*. As shown in Figure 5A, their lungs were harvested on day 14 and the number of metastatic colonies was counted. In the vehicle control without LSGE treatment, 194.2 ± 64.72 metastatic colonies were generated. In contrast, the corresponding colony numbers in mice orally administered with 0.3 and 1.0 g/kg/day of LSGE were 164.5 ± 67.48 and 79.0 ± 5.00 ($P < .05$), respectively (Fig. 5B). The treatment of 0.3 and 1.0 g/kg/day of LSGE to the mice intravenously injected with B16F10 cells resulted in 15.3% and 59.3% inhibition of lung metastasis, respectively, compared with the vehicle control (Fig. 5B). These data suggest that LSGE significantly inhibits *in vivo* metastasis of B16F10 cancer cells to the lung. In the

Adriamycin-treated group, 28.7 ± 10.56 ($P < .001$) metastatic colonies occurred. Adriamycin as a reference drug, which was intraperitoneally administered at a dosage of 2 mg/kg every other day, strongly inhibited the lung metastasis of B16F10 cancer cells with an 85.2% ($P < .001$) inhibition rate. However, Adriamycin exhibits the typical characteristics of cytotoxic anticancer chemicals.³¹ In previous reports, the oral administration of LSGE at concentrations above 0.3 g/kg/day showed a potent antitumor effect in human lung tumor xenografts *in vivo* without any apparent toxicity.^{8,14} Oral administration has been the most simple treatment of anticancer drugs, irrespective of time and place, and long-term treatment without patients' pain caused by parental administration.^{8,32} In this study, the oral intake of LSGE showed a high efficacy in suppressing tumor metastasis. Therefore, LSGE could be considered a nontoxic antitumor supplement for inhibition of metastasis as well as for growth inhibition of tumors.

It can be concluded that LSGE possesses anti-invasive, anti-migratory, and anti-metastatic activity both *in vitro* and *in vivo*. Even at noncytotoxic concentrations, LSGE significantly inhibited the invasion and migration of B16F10 cells in a dose-dependent manner. The oral administration of LSGE inhibited lung metastasis of cancer cells *in vivo*. In addition, LSGE inhibited transcription of MMP-2 DNA in B16F10 cells, leading to reduction of MMP-2 protein level and activity. In conclusion, LSGE potently inhibits cancer cell invasion and migration *in vitro*, and lung metastasis of melanoma cells *in vivo* through inhibition of MMP-2 expression.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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