Anti-angiogenic effect of mountain ginseng *in vitro* and *in vivo*: Comparison with farm-cultivated ginseng

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Abstract. Mountain ginseng (*Panax ginseng*) has been used for cancer patient therapy in Northeast Asia. Although it is well known that cancer cells are able to induce angiogenesis, the effect of mountain ginseng on angiogenesis is still unknown. In the present study, we investigated whether ethanolic extract of mountain ginseng (MGE) could inhibit angiogenesis in *in vitro* and *in vivo* models. In comparison with farm-cultivated ginseng extract (FGE), MGE more strongly inhibited cell migration and formation of capillary-like network within non-cytotoxic ranges in SVEC4-10 cells. In addition, MGE dose-dependently suppressed Transwell cell migration of the cells. Moreover, MGE reduced the phosphorylation and expression of VEGF-R2 as well as the phosphorylation of FAK, Src, Akt and ERK, the intermediate proteins in the VEGF-R2 signaling cascade, in the cells. As expected, MGE dramatically decreased hemoglobin content in Matrigel plugs in mice. In conclusion, MGE possesses stronger anti-angiogenic properties than FGE in vascular endothelial cells. Such effect of MGE is correlated with inhibition of activation of the VEGF-R2 signaling pathway. Therefore, the novel features of MGE may be helpful for understanding its anticancer mechanism for the treatment of cancer patients.

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Abbreviations: FAK, focal adhesion kinase; FGE, ethanolic extract of farm-cultivated ginseng; MGE, ethanolic extract of mountain ginseng; Src, steroid receptor coactivator

Key words: angiogenesis, ginsenosides, mountain ginseng, SVEC4-10 cells, VEGF-R2

Introduction

Ginseng (*Panax ginseng*), affiliated with the Araliaceae family, is a perennial plant that is widely distributed in Northeast Asia and North America (1). In particular, among various ginseng species, *Panax ginseng* is known globally as Korean ginseng. Korean ginseng has been generally used globally as a component of traditional medicine and functional foods because it possesses many beneficial health effects, such as antioxidant, anticancer, anti-diabetes, anti-inflammation and neuroprotection (2-4). The beneficial health effects of Korean ginseng are closely associated with its bioactive components including ginsenosides, phenolic acids, flavonoids and polysaccharides (2,4). Korean ginseng, which grows in mountains without any artificial manipulation after sowing, is called mountain ginseng in Northeast Asia, and ethanolic extracts of mountain ginseng (MGE) have been used as a herbal drug in pharmacopuncture treatment for cancer patients in Korea (5). Recently, we found that MGE, which is rich in ginsenosides, possessed anticancer activity against human breast cancer in a xenograft model (6).

Angiogenesis is closely associated with various physiological and pathological states, such as fetal development, wound-healing, inflammation and vascular diseases (7). In addition, cancer cells easily form tumor mass because of their rapid growth compared with the growth of normal cells, and then the inside of the tumor mass becomes hypoxic (8). As a result, cancer cells themselves promote the formation of novel blood vessels under hypoxic conditions by producing and secreting angiogenetic factors, such as VEGF (8). Consequently, cancer cells grow quickly and metastasize to other organs through the neo blood vessels (8). Therefore, the inhibition of neo blood vessel formation may be an important key factor for anticancer therapy. Although MGE has the anticancer properties, the effect of MGE on angiogenesis remains to be unclarified.

In the present study, we investigated whether MGE and an ethanolic extract of farm-cultivated ginseng (FGE) could inhibit angiogenesis in both *in vitro* and *in vivo* models. We found that both MGE and FGE suppressed angiogenesis of vascular endothelial cells stimulated by angiogenetic factors. In addition, the anti-angiogenic action of MGE was stronger than that of FGE in both *in vitro* and *in vivo* models.

Furthermore, the anti-angiogenic effect of MGE was associated with inhibition of the activation of the VEGF-R2 signaling pathway in vascular endothelial cells. These findings may provide novel information for the clinical application of MGE as a cancer dietary supplement, and they may be helpful for understanding the action of MGE in a tumor microenvironment.

Materials and methods

Reagents. DMEM (cat. no. 30-2002) was obtained from the American Type Culture Collection. Antibiotics, FBS and 1X PBS were procured from GE Healthcare Life Sciences. Specific antibodies against focal adhesion kinase (FAK, cat. no. 3285), p-FAK (cat. no. 3281), steroid receptor coactivator (Src, cat. no. 2109), p-Src (cat. no. 6943), Akt (cat. no. 9272), p-Akt (cat. no. 9271), ERK (cat. no. 4695), p-ERK (cat. no. 9101), VEGF-R2 (cat. no. 9698) and p-VEGF-R2 (cat. no. 3817) were purchased from Cell Signaling Technology, Inc. A specific antibody against β-actin (sc-1616) was obtained from Santa Cruz Biotechnology, Inc. VEGF was procured from R&D Systems. Matrigel was purchased from Becton Dickinson (BD Biosciences). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), hemoglobin assay kit, and all other chemicals were obtained from Sigma-Aldrich (Merck KGaA). All other chemicals were of analytical grade.

Preparation of mountain ginseng and farm-cultivated ginseng extracts. The MGE and FGE were prepared following a previous method (6). Briefly, voucher specimens of mountain ginseng and farm-cultivated ginseng were deposited in the National Institute for Korean Medicine Development (NIKOM, Gyeongsan, Korea) after identification by Dr H. Lee, a herbalist. Dried fragments of the herbs were boiled in 30% ethanol solution for about 3 h, and deposited at 4˚C after cooling. Next day, the separated supernatant was filtered through a 0.45 μm filter, and the filtrate was concentrated and then lyophilized. The dried pellets were stored at -20˚C until use. The lyophilized powders of MGE and FGE were dissolved in 4% ethanol solution for *in vitro* and *in vivo* studies.

High-performance liquid chromatography analysis. Ginsenoside contents in MGE and FGE were analyzed according to a previous method (6).

Animals. Male C57BL/6 mice (6 weeks and 19-21 g) were obtained from Koatech Co., and housed in cages (5 mice per cage) under specific pathogen-free conditions (21-24˚C and 40-60% relative humidity) with a 12 h light/dark cycle. They were given free access to standard rodent food (Envigo) and water. All animal experiments were approved by the Committee of Animal Care and Experiment of NIKOM with a reference number (NIKOM-2020-5). Animal studies were performed according to the guidelines of the Animal Care and Use Committee at NIKOM.

In vivo Matrigel plug assay. Matrigel plug assay was performed following a modification of a previous protocol (9). Matrigel including VEGF (400 ng/ml) was mixed with MGE (0-200 μg/ml) or FGE (200 μg/ml). After adaptation, 200 μl of the Matrigel mixtures was subcutaneously injected into the flank of mice. After 2 weeks, mice were euthanized by CO_2 (in 30%/min). Matrigel plugs were isolated from sacrificed mice, and then washed with cold 1X PBS twice. The washed Matrigel plugs were used for hemoglobin content assay and histological analysis.

Hemoglobin content assay. Hemoglobin content in Matrigel plugs was detected by a hemoglobin assay kit in accordance with the manufacturer's instructions.

Histological analysis. Histological analysis was carried out according to a modified method, previously reported (10). To observe aspect of red blood cells in Matrigel plugs, briefly, deparafinized Matrigel plug on slices was stained with hematoxylin-eosin. The stained tissue slices were embedded with the Mounting solution. Histological features in Matrigel plugs were observed under a light microscope with 100x magnification.

Cell culture. SVEC4-10 cells, a murine endothelial cell line (11), were purchased from the American Type Culture Collection. The cells were cultured in DMEM medium containing 10% (v/v) FBS and antibiotics at 37˚C in a humidified atmosphere of 5% CO_2. All *in vitro* tests contained a vehicle control group (0.016% ethanol).

Cell viability assay. Cell viability was determined following a modification of a method previously reported (12). Briefly, SVEC4-10 cells were seeded on a 96-well plate ($1x10^4$ cells/well). Next day, the cells were incubated with MGE or FGE (0-200 μg/ml) for 24 h, and then further incubated with 100 μl culture media containing 300 μg/ml MTT reagent for 2 h. Next 100 μl of dimethyl sulfoxide was added to the plate after removal of the supernatant, and then the plate was incubated for 15 min. Cell viability was determined at 570 nm using a microplate reader (Tecan Sunrise).

Cell migration. Wound healing assay and Transwell migration assay were performed in accordance with a modified method, previously reported (13). SVEC4-10 cells were seeded on a 6-well plate ($1x10^5$ cells/well), and then the cells were incubated until 95% confluence in 10% FBS medium. Next, the cells were wounded with a p20 pipette tip under serum free condition, and then incubated with MGE (0-200 μg/ml) or FGE (200 μg/ml) containing FBS overnight. Wound closure was observed under a light microscope with 100x magnification. To confirm cell migration using the Transwell migration assay, the cells were seeded on a 24-well Transwell insert ($1x10^3$ cells/insert) in serum-free DMEM, and then incubated with MGE containing FBS (lower chamber) overnight. The insert insides were swabbed and then stained with 0.2% crystal violet in a 20% methanol solution. The stained cells were observed under a light microscope with 100x magnification. Wound closure and migrated cells were measured using ImageJ software (version 1.51j8 for Windows; National Institutes of Health).

Tube formation assay. Matrigel was added to a 96-well plate (50 μl/well), which was then incubated for 30 min at 37˚C.

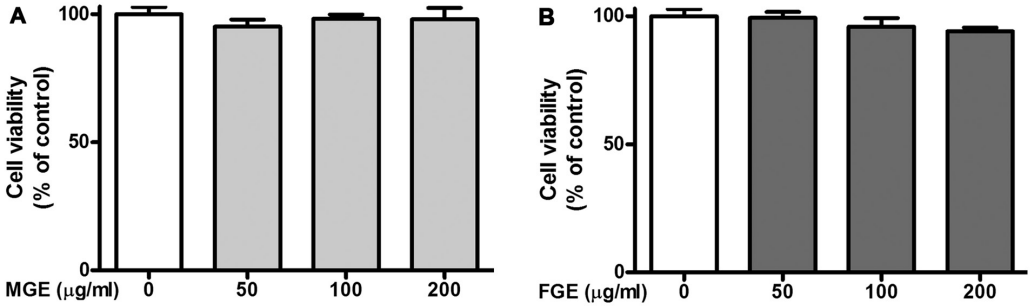

Figure 1. Effects of MGE and FGE on SVEC4-10 cell viability. The viability of SVEC4-10 cells was assessed following (A) MGE and (B) FGE treatment. Data are presented as the mean ± SD (n=8). MGE, mountain ginseng extract; FGE, farm-cultivated ginseng extract.

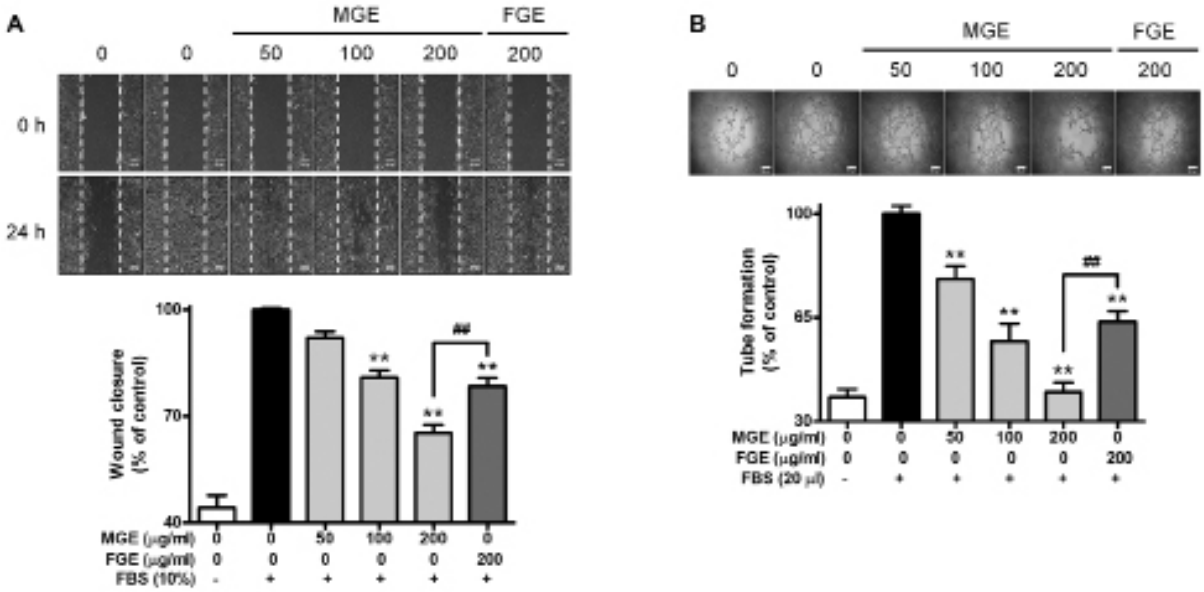

Figure 2. Inhibitory effects of MGE and FGE on the migration and tube formation of SVEC4-10 cells. (A) Cell migration and (B) tube formation were assessed in SVEC4-10 cells following MGE and FGE treatment. Data are expressed as the mean ± SD (n=4). **P<0.01 vs. the FBS-treated group. $^{\#\#}$P<0.01 as indicated. MGE, mountain ginseng extract; FGE, farm-cultivated ginseng extract.

SVEC4-10 cells, pretreated with MGE or FGE for 30 min, were seeded on the Matrigel-coated 96-well plate. After 3 h, the cell morphology was observed under a light microscope with 100x magnification. The tube lengths and areas were measured using ImageJ software (National Institutes of Health).

Immunoblotting analysis. Immunoblotting analysis was evaluated following a method previously reported (14). Briefly, the blotted proteins on PVDF membrane were visualized using a chemiluminescent reaction (Immnobilon Western; Millipore Corporation) with an Imaging system (ImageQuant LAS 4000, GE Healthcare Life Sciences). The level of target proteins was compared to that of a loading control (non-phosphorylated proteins or β-actin), and the results were expressed as a ratio of density of each protein identified by a protein standard size marker (BIOFACT Co., Ltd.). The density of each inverted band was measured using ImageJ software (National Institutes of Health).

Statistical analyses. The experimental results were listed as means ± SD for *in vitro* studies or SEM for *in vivo* studies One-way analysis of variance (ANOVA) was used for multiple comparisons (GraphPad Prism version 5.03 for Windows; GraphPad Software, Inc.). We applied the Dunnett's test or Tukey's test for one-way ANOVA for significant variations between treated groups. Differences at the *P<0.05 and **P<0.01 levels were considered statistically significant.

Results

Inhibitory effects of MGE and FGE on cell migration and capillary-like network in SVEC4-10 cells: Comparison between MGE and FGE. To compare the effects of MGE and FGE on cell migration and tube formation in vascular endothelial cells, we investigated the effects of MGE and FGE on cell migration of SVEC4-10 cells. As presented in Fig. 1, MGE and FGE did not show cytotoxicity within the concentration ranges (0-200 μg/ml). Subsequently, when SVEC4-10 cells were stimulated by FBS, the cells completely covered a wound area (Fig. 2A). In contrast, MGE dose-dependently inhibited the cell migration of FBS-activated SVEC4-10 cells, and the inhibitory effect of MGE at 200 μg/ml was more potent than that of FGE at the same concentration (Fig. 2A). Based on the cell migration results, we examined whether MGE and FGE

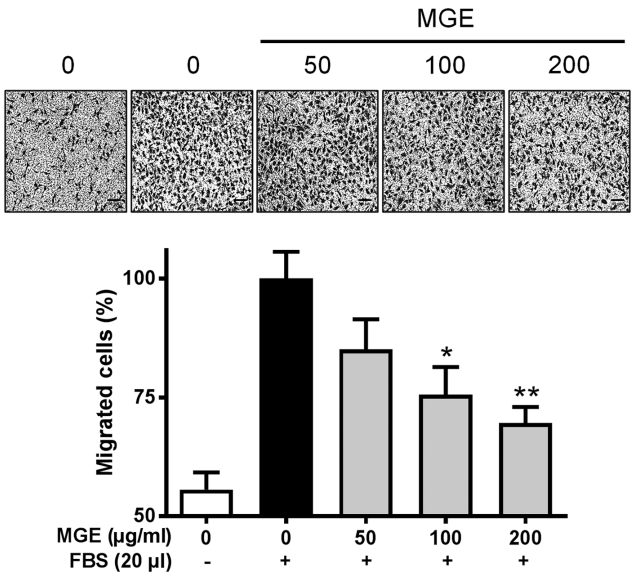

Figure 3. Inhibitory effect of MGE on transwell cell migration. A transwell assay was performed to assessed the migration of SVEC4-10 cells following MGE treatment. Data are expressed as the mean ± SD (n=3). *P<0.05 and **P<0.01 vs. the FBS-treated group. MGE, mountain ginseng extract.

could suppress the formation of capillary-like network of FBS-activated SVEC4-10 cells. The inclusion of MGE significantly reduced the formation of capillary-like network in the cells, and the inhibitory effect of MGE at maximum dose was stronger than that of FGE at the same concentration (Fig. 2B). The results suggest that MGE and FGE have anti-angiogenic properties within non-cytotoxic ranges in vascular endothelial cells. In addition, the anti-angiogenic effect of MGE is stronger than that of FGE.

Inhibitory effect of MGE on Transwell cell migration of SVEC4-10 cells. As we found that MGE was able to inhibit the cell migration and capillary-like network formation of vascular endothelial cells on a 2-D structure, we tried to confirm whether MGE was capable of suppressing the cell migration of FBS-stimulated SVEC4-10 cells on a 3-D structure. Consistent with the cell migration and capillary-like network formation on the 2-D structure, MGE concentration-dependently attenuated Transwell cell migration of the cells (Fig. 3). This finding suggests that MGE may be able to attenuate the formation of neo blood vessels in a whole body system.

Inhibitory effects of MGE on the phosphorylation of proteins related to the VEGF-R2 signaling cascade. After we found that MGE was able to reduce the cell migration and tube formation of vascular endothelial cells, we were interested in the effect of MGE on the VEGF-R2 signaling pathway because angiogenic factor-exposed vascular endothelial cells are capable of formatting neo blood vessels through activation of the VEGF-R2 signaling pathway (15). MGE not only reduced both phosphorylation and expression of VEGF-R2 (Fig. 4A) but also attenuated the phosphorylation of FAK, Src, Akt and ERK, intermediate proteins in the VEGF-R2 signaling pathway (16), in SVEC4-10 cells activated by FBS (Fig. 4B). Also, MGE at 50 μg/ml almost blocked the phosphorylation of FAK, one of the intermediate proteins of the

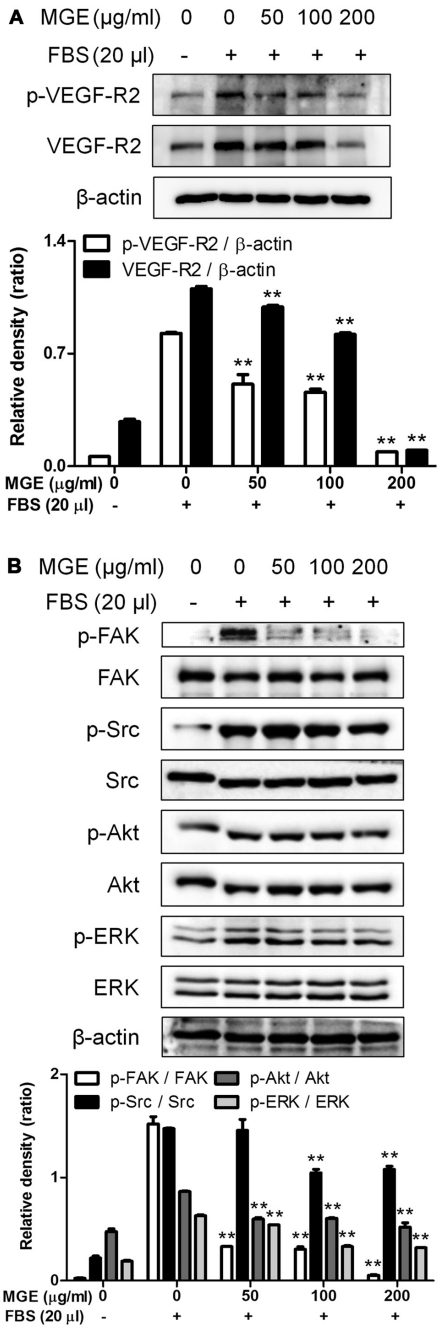

Figure 4. Inhibitory effect of MGE on the phosphorylation of proteins related to tube formation. Immunoblotting was performed to assess the phosphorylation and expression of (A) VEGF-R2 as well as the phosphorylation of (B) FAK, Src, Akt and ERK in SVEC4-10 cells following MGE treatment. Data were obtained from three independent experiments. **P<0.01 vs. the FBS-treated group. MGE, mountain ginseng extract; FAK, focal adhesion kinase; Src, steroid receptor coactivator; p, phosphorylated.

VEGF-R2 signaling pathway (Fig. 4B). These results suggest that MGE has anti-angiogenic properties through inhibition of activation of the VEGF-R2 signaling cascade in vascular endothelial cells.

Inhibitory effect of MGE on neo blood vessels in mice. Finally, because we found that MGE possesses anti-angiogenic properties through inhibiting the phosphorylation of proteins related to the VEGF-R2 signaling cascade in vascular endothelial cells, we tried to confirm the anti-angiogenic action of MGE

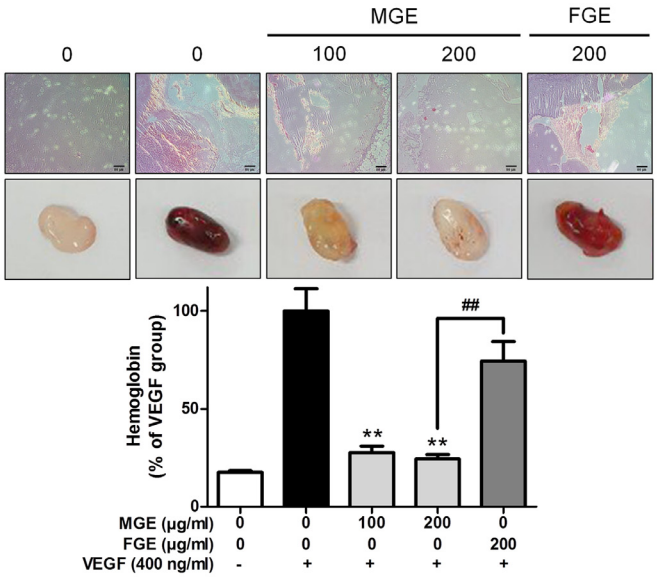

Figure 5. Inhibitory effect of MGE on hemoglobin levels in murine Matrigel plugs. Hemoglobin content was assessed from the Matrigel plugs extracted from sacrificed mice following VEGF, MGE (0-200 μg/ml) and FGE (200 μg/ml) treatment. Data are expressed as the mean ± SEM (n=4). **P<0.01 vs. the VEGF-treated group. $^{\#\#}$P<0.01 as indicated. MGE, mountain ginseng extract; FGE, farm-cultivated ginseng extract.

on a whole body system. In histological observation of a Matrigel plug assay *in vivo*, MGE dose-dependently attenuated accumulation of erythrocytes induced by VEGF in Matrigels. Also, the inhibitory effect of MGE at 200 μg/ml was stronger than that of FGE (Fig. 5). Consistent with the histological results, MGE dramatically reduced the level of hemoglobin, a biomarker of blood vessels, in Matrigel including VEGF (400 ng/ml) in mice (Fig. 5). Surprisingly, MGE at 200 μg/ml almost suppressed the level of hemoglobin in Matrigels to a level similar to that in Matrigels of the control group. In addition, the inhibitory effect of MGE was more potent than that of FGE at the same concentration (Fig. 5). These findings suggest that MGE is able to inhibit neo blood vessels in a whole body system. Overall, MGE may be a possible herbal drug candidate or adjuvant for therapy for cancer patients.

Discussion

Korean ginseng has been commonly used worldwide as an ingredient of functional foods and in traditional medicine because Korean ginseng possesses various beneficial health effects (2-4). Such beneficial health properties are closely correlated with various bioactive compounds in Korean ginseng (2,4). Especially, mountain ginseng is richer in bioactive compounds than farm-cultivated ginseng (17). Currently, MGE has been used as an element of pharmacopuncture for treatment of cancer patients in Korea (5). In addition, we found in a recent study that MGE exerted anticancer activity against human breast cancer (6). Growth and metastasis of cancer cells are closely related to angiogenesis (8). Nevertheless, the effect of MGE on angiogenesis has not been known.

In this study, we examined whether MGE was capable of suppressing angiogenesis in vascular endothelial cells in both *in vitro* and *in vivo* models, and we found that MGE had more potent anti-angiogenic action than FGE. In addition, MGE inhibited the phosphorylation and expression of VEGF-R2 as well as the phosphorylation of FAK, Src, Akt and ERK, which are intermediate proteins in the VEGF-R2 signaling cascade (16), in vascular endothelial cells. Consonant with *in vitro* data, MGE showed stronger anti-angiogenic action than FGE in the formation of neo blood vessels in Matrigels including VEGF in mice. Such an anti-angiogenic action of MGE may be closely associated with its richness in ginsenosides because some ginsenosides possess anti-angiogenic activities (18).

One possible mechanism for the anti-angiogenic action of MGE may be associated with the inhibition of the VEGF-R2 signaling cascade in vascular endothelial cells. It is well known that VEGF is a strong angiogenic activator among various angiogenic activators, and VEGF exists in five isoforms (16). In addition, VEGF is produced from various cells, such as cancer cells, fibroblasts, and inflammatory cells (16). Most importantly, VEGF is able to initiate angiogenesis through promoting the activation of the VEGF-R2 signaling pathway in vascular endothelial cells (16) because VEGF-R2 is expressed in vascular endothelial cells alone (16). When VEGF-R2, which belongs to the receptor tyrosine kinase superfamily, is activated by VEGF, VEGF-R2 can phosphorylate Akt, ERK, FAK, and Src (16). Then, the phosphorylated intermediate proteins promote cell migration, vascular permeability, proliferation, and survival of vascular endothelial cells (16). As a result, neo blood vessels are formed. In particular, cancer cells easily make VEGF under hypoxic conditions (8). Therefore, inhibiting the activation of the VEGF-R2 signaling cascade is an important point for the treatment of cancer patients. In support of this, MGE inhibited cell migration and tube formation of vascular endothelial cells. In addition, MGE reduced the phosphorylation of FAK, Src, Akt and ERK during suppressing both phosphorylation and expression of VEGF-R2 in the cells. Moreover, MGE dramatically suppressed the formation of neo blood vessels in Matrigel plug in mice. This indicates that MGE exerts anti-angiogenic properties through inhibiting the activation of the VEGF-R2 signaling pathway in vascular endothelial cells.

Another possible anti-angiogenic mechanism of MGE may be correlated with the rich ginsenosides in MGE. It is well known that some ginsenosides, such as ginsenoside Rb1, Rb2, Rg3, Rh1 and Rh2, have anti-angiogenic activities (18). Recently, we found that MGE possessed a stronger anticancer action than FGE in human breast cancer, and MGE was richer in total ginsenosides than FGE (6). Remarkably, among the anti-angiogenetic ginsenosides, MGE included higher concentrations of ginsenoside Rb1 and Rb2 than FGE (6). Overall, this study may support the possibility of using MGE as a herbal drug for the treatment of cancer patients in clinics. Nevertheless, this study has some limits in representing a preclinical study for clinical application.

In summary, this study demonstrates that MGE possesses a stronger anti-angiogenic action than FGE in vascular endothelial cells in both *in vitro* and *in vivo* models. The anti-angiogenic mechanism of MGE is closely associated with the activation of the VEGF-R2 signaling cascade. Thus, the intracellular targets of MGE include VEGF-R2, FAK, Src, Akt and ERK in vascular endothelial cells. In addition, such an

anti-angiogenic effect may be correlated with MGE being rich in ginsenosides. These findings may provide novel information for understanding how MGE attenuates the growth of human cancers by regulating the tumor microenvironment. Furthermore, this study may provide support for using MGE as a possible herbal drug for the treatment of cancer patients in clinics. However, other preclinical studies are necessary to confirm the anti-angiogenic action of MGE and to provide complete support for any clinical application because this study has some limits in representing a preclinical study for clinical application. Furthermore, other studies, such as toxicology, metabolism, pharmacokinetics, and phytochemical studies, are necessary to ensure patient safety with MGE and to identify the active phytochemicals of the anti-angiogenic action in MGE.

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Availability of data and materials

All data generated and analyzed during this study are included in this published article.

Authors' contributions

JSK participated in the design of this study, performed most experiments and acquired all the data. JMY participated in the design of this study and interpretation of all the experimental data, improved the design of this study, analyzed statistics of all the experimental data, drafted this manuscript and revised the manuscript. JEP prepared MGE and FGE. JK and SGK participated in *in vivo* studies. YMS and JHS participated in the study design and interpretation of the data. HJK designed this study, interpreted all the experimental data and supervised all experiments. JSK and HJK confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Committee of Animal Care and Experiment of NIKOM with a reference number (NIKOM-2020-5).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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