

Grape Seed Extract Inhibits Angiogenesis via Suppression of the Vascular Endothelial Growth Factor Receptor Signaling Pathway

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Abstract

Blockade of angiogenesis is an important approach for cancer treatment and prevention. Vascular endothelial growth factor (VEGF) is one of the most critical factors that induce angiogenesis and has thus become an attractive target for antiangiogenesis treatment. However, most current anti-VEGF agents often cause some side effects when given chronically. Identification of naturally occurring VEGF inhibitors derived from diet would be one alternative approach with an advantage of known safety. Grape seed extract (GSE), a widely used dietary supplement, is known to have antitumor activity. In this study, we have explored the activity of GSE on VEGF receptor and angiogenesis. We found that GSE could directly inhibit the kinase activity of purified VEGF receptor 2, a novel activity of GSE that has not been characterized. GSE could also inhibit the VEGF receptor/mitogen-activated protein kinase-mediated signaling pathway in endothelial cells. As a result, GSE could inhibit VEGF-induced endothelial cell proliferation and migration as well as sprout formation from aorta ring. *In vivo* assay further showed that GSE could inhibit tumor growth and tumor angiogenesis of MDA-MB-231 breast cancer cells in mice. Consistent with the *in vitro* data, GSE treatment of tumor-bearing mice led to concomitant reduction of blood vessel density and phosphorylation of mitogen-activated protein kinase. Depletion of polyphenol with polyvinylpyrrolidone abolished the antiangiogenic activity of GSE, suggesting a water-soluble fraction of polyphenol in GSE is responsible for the antiangiogenic activity. Taken together, this study indicates that GSE is a well-tolerated and inexpensive natural VEGF inhibitor and could potentially be useful in cancer prevention or treatment.

Angiogenesis, the formation of new blood vessels, plays a critical role in tumor progression. There are multiple steps involved in tumor angiogenesis. Each step provides an opportunity for therapeutic intervention. Although the cellular and molecular mechanisms that govern angiogenesis are only beginning to be understood, it is clear that a balance of proangiogenic and antiangiogenic factors controls the formation of new blood vessels (1). Among these factors, vascular endothelial growth factor (VEGF) is one of the most critical and specific angiogenesis factors (2). The biological function of VEGF on endothelial cells is mainly mediated through binding to receptor tyrosine kinase, VEGF receptor 1 (flt1/VEGFR1) and VEGF receptor 2 (KDR/flk1/VEGFR2), both of which are crucial for

normal vascular development (2). Binding of VEGF to VEGFR induces conformational changes in the receptor, followed by dimerization and autophosphorylation of the tyrosine residues of the receptor (3). Inhibiting VEGF activity by neutralizing antibodies or introduction of dominant negative VEGFRs into endothelial cells often results in inhibition of tumor growth (2). In fact, a humanized monoclonal antibody against VEGF, Avastin, is the first angiogenesis inhibitor that was approved by U.S. Food and Drug Administration for cancer treatment (4).

Whereas many of the inhibitors that efficiently suppress angiogenesis are currently being tested at various stages of clinical development, diet-based approaches to limit angiogenesis are being actively explored (5). This latter approach has a major merit due to the proven safety for human use. Several safe chemopreventive phytochemicals, such as curcumin, resveratrol, and catechins, are known to have antiangiogenic activity as one of the mechanisms to suppress tumor growth (6, 7). Epidemiologic studies indicate that diet and nutrition influence the development of cancer (8, 9). The highest rate of breast cancer is observed in populations with western lifestyles that include relatively high-fat, meat-based, low-fiber diets, whereas the lowest rates are typically observed in Asian populations with mainly plant-based diets. The high content of phytochemicals in these plant-based diets has been proposed as the underlying factor responsible for the low breast

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cancer incidence in Asian women but the mechanisms are relatively unexplored (10).

One of the plants that have high content of phytochemicals is grape. Grape and red wine are consumed worldwide and have been reported to be associated with reduced risk of cancer. Grapes are rich in polyphenols, of which approximately 60% to 70% is found in grape seeds. Commercial preparations of grape seed extract (GSE) contain 75% to 95% procyanidins. GSE is marketed as a dietary supplement in the United States, owing to their powerful protective properties against free radicals and oxidative stress.

GSE has been linked to cancer prevention and therapy. Increased consumption of grapes was reported to be associated with reduced cancer risk (11). Studies in carcinogen-induced and genetically engineered cancer models (12–14) have revealed a chemopreventive role of proanthocyanidins in GSE. GSE was also shown to inhibit the growth of a number of cancer cells *in vitro* (15) and tumor growth in mice (14, 16–21).

Despite the known anticancer activity, the mechanisms of the effect of GSE are not fully understood. Understanding such mechanisms is important for exploring the full potential of GSE in chemoprevention and treatment of cancer. Several studies have shown that GSE could negatively regulate a number of cellular functions or signaling molecules in tumor cells, including aromatase activity (16, 20), cell cycle progression (15), EGF-induced mitogenic signaling (22), and nuclear factor- κ B signaling (23), or could induce caspase activity (24). Recently, GSE was also reported to inhibit endothelial cell proliferation and tube formation on Matrigel (25) and reduce vessel density in human prostate tumor (26). These observations suggest that GSE is likely a natural inhibitor of VEGFR, and antiangiogenesis may be another mechanism of its antitumor activity. In this study, we have done further *in vitro* and *in vivo* experiments to characterize the antiangiogenic and antitumor activities of GSE and explored its possible molecular mechanism. We present evidence that GSE inhibits endothelial cell function, at least in part, via inhibition of the VEGFR/mitogen-activated protein kinase (MAPK) signaling pathway.

Materials and Methods

GSE

GSE standardized preparation, constituting of at least 85% (w/w) procyanidins, was provided by San Joaquin Valley Concentrates (Fresno, California) and dissolved in water for all the experiments. The GSE preparation contains approximately 19, 36, 8, and 22 ng/ μ g of procyanidins B1, B2, B3, and B4.

Cell proliferation

Endothelial cell proliferation in the presence and absence of growth factors was evaluated in both human umbilical vascular endothelial cells (HUVEC; Clonetics, Lonza) and bovine capillary endothelial cells (a generous gift from Catherine Butterfield and Judah Folkman).

Endothelial cells were plated onto a gelatinized 24-well culture plate in 0.5-mL DMEM (Life Technologies, Inc.; for bovine capillary endothelial cells) containing 10% bovine calf serum or serum-free endothelial basal medium-2 (Lonza; for HUVECs) containing 10% FCS and incubated for 24 h. The medium was replaced with 0.25 mL DMEM + 5% bovine calf serum + 1% L-Glutamine/Penicillin/Streptomycin (for bovine capillary endothelial cells) or 0.25 mL endothelial basal medium-2 + 2% FCS + 1% L-Glutamine/Penicillin/Streptomycin (for HUVECs) containing various concentrations of GSE. After 30 min of incubation, additional 0.25 mL of medium was added to achieve a final volume of

0.5 mL of medium with or without basic fibroblast growth factor (1 ng/mL), VEGF (100 ng/mL; PeproTech, Inc.), or endothelial cell growth factor supplements (SingleQuo kit, Lonza). After 48 h, cells were dispersed in trypsin and counted with a Coulter counter.

Cell migration

Endothelial cell migration was assessed by a modified Boyden chamber assay (27). HUVECs (1.5×10^5) were plated in endothelial basal medium-2 containing 0.05% FCS in the upper chamber of the transwell (8- μ m pore, Costar), which was precoated with 200 μ g/mL Matrigel (Becton Dickinson). Cells were then treated with various concentrations of GSE for 30 min at 37°C. Endothelial basal medium-2 containing 0.05% FCS and VEGF (50 ng/mL) was then added to the lower chamber. After 8 h, nonmigrated cells were removed with a cotton swab and migrated cells were stained and examined under a microscope. The number of migrated cells was quantified by counting the cells using 40 \times objectives. Migration was normalized to percent migration, with migration in the presence of VEGF representing the scale of 100%.

Immunoblot

HUVECs were cultured in endothelial basal medium-2 containing 2% FCS for 24 h and then incubated with various concentrations of GSE for 30 min before VEGF (100 ng/mL) stimulation for 5 min. Total cells extracts were prepared in Laemmli sample buffer. Proteins were separated by electrophoresis on SDS gels, transferred onto polyvinylidene difluoride membranes, and incubated with the following primary antibodies (Cell Signaling): anti-VEGFR2, anti-phospho-VEGFR2, anti-MAPKp42/44, and anti-phospho-MAPKp42/44. Binding of the primary antibody was detected with a horseradish peroxidase-conjugated secondary antibody and chemiluminescent substrate (Pierce). Densitometric analysis was done using the AlphaEase FC imaging system (Alpha Innotech Corp.).

In vitro kinase assay

An ELISA assay kit (Sigma) was used to determine the ability of GSE to inhibit VEGFR2 tyrosine kinase activity. Briefly, GSE were incubated with VEGFR2 (Upstate) in HEPES buffer solution containing Mn^{2+} and Mg^{2+} and ATP in 96-well plates coated with a poly-Glu-Tyr substrate. Phosphorylated tyrosine was then detected by sequential incubation with a mouse IgG anti-phosphotyrosine antibody and a horseradish peroxidase-linked sheep anti-mouse immunoglobulin antibody. Color is developed with horseradish peroxidase chromogenic substrate and quantified by an ELISA reader at wavelength 492 nm. The results were expressed as percent kinase activity. IC_{50} values were defined as the drug concentration that resulted in 50% inhibition of enzyme activity.

Chick aortic ring assay

The chick aortic ring assay was modified from a rat aortic ring assay as described previously (28, 29). Briefly, aortic arch was dissected from day 12 to 14 chick embryos and cut into rings and embedded into Matrigel in a four-well plate (Nunc). After incubation for 10 min at 37°C, the aortic rings were fed with MCDB-131 serum-free medium (Life Technologies, Invitrogen) containing various concentrations of GSE with or without VEGF (100 ng/mL) or endothelial cell growth supplement (ECGS) (Sigma; 50 μ g/mL). Sprouts formed within 24 to 72 h. Images were photographed with 4 \times objectives of Olympus inverted IX81 at $\times 40$ magnification. The extent of sprout formation from chick aortic ring was quantified using Image-Pro software as described (30).

Tumor formation

Six- to 8-week-old severe combined immunodeficient mice were used for tumor xenografts. Mice were housed in specific pathogen-free conditions according to the guidelines of the association for Assessment and Accreditation of Laboratory Animal Care. All studies were carried out under approved institutional experimental animal care and use protocols.

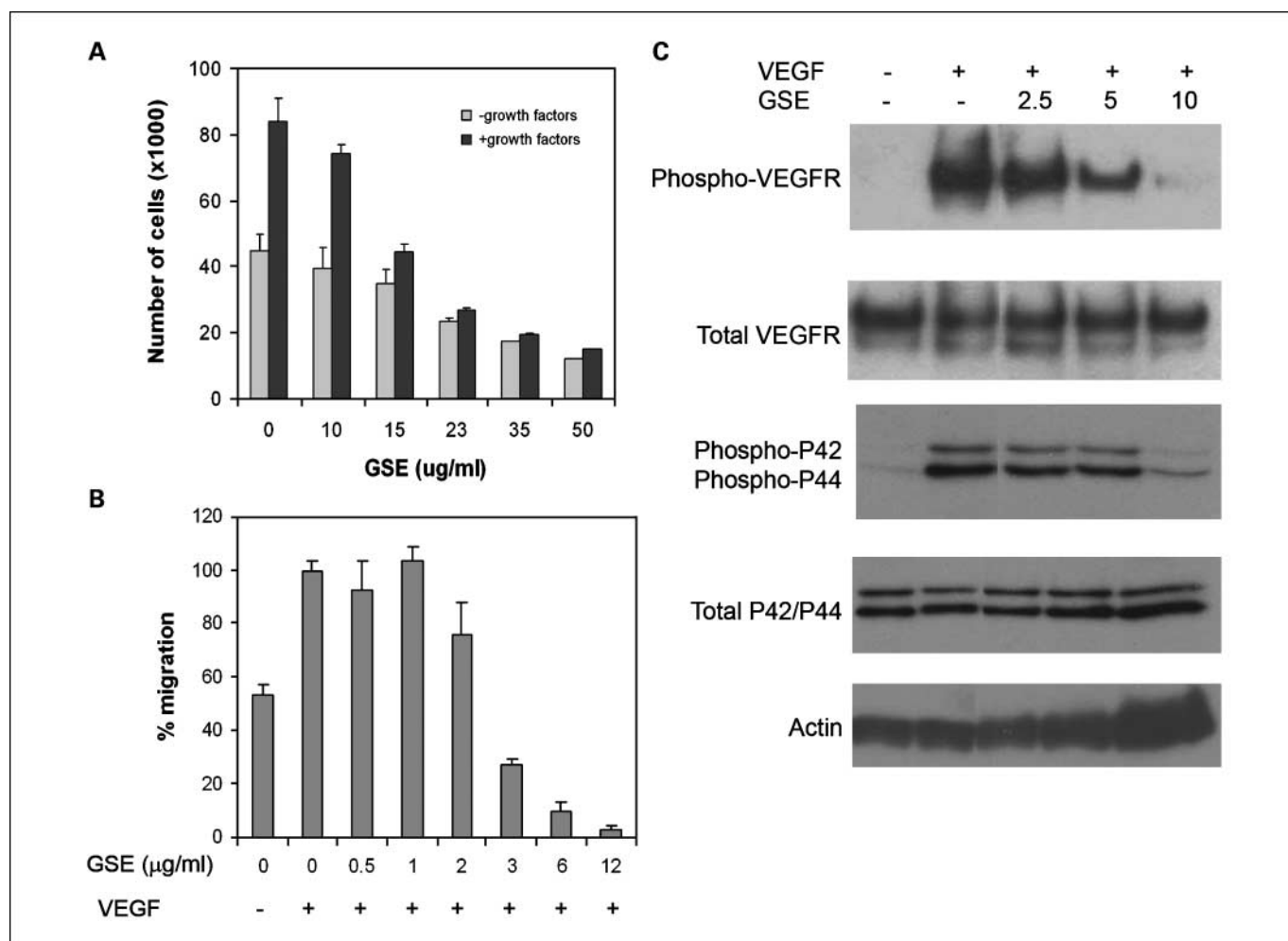


Fig. 1. GSE inhibits VEGFR2 signaling in endothelial cells. **A**, GSE inhibits endothelial cell proliferation. HUVECs were plated onto gelatinized 24-well culture plates and treated with various concentrations of GSE for 30 min. The cells were then stimulated with ECGS. After 48 h, cells were dispersed in trypsin and counted with a Coulter counter. **B**, GSE inhibits endothelial cell migration. Endothelial cell migration was assessed by a modified Boyden chamber assay. HUVECs were plated in the upper chamber of the transwell and treated with various concentrations of GSE for 30 min. VEGF (50 ng/mL) was then added to the lower chamber. After 8 h, nonmigrated cells were removed with a cotton swab, and migrated cells were stained and examined under a microscope. *Columns*, mean percentage of control without GSE treatment in the presence of VEGF; *bars*, SD. **C**, GSE inhibits VEGFR2 signaling. Quiescent HUVECs were incubated in serum-free medium in the presence or absence of GSE (2.5, 5, 10 μ g/ml) for 30 min, followed by stimulation with 100 ng/mL VEGF for 5 min. Phosphorylation of VEGFR-2 or MAPK was assessed by Western blot with anti-phospho-VEGFR or anti-phospho-MAPK antibody. Total VEGFR or MAPK was analyzed with anti-VEGFR antibody or anti-MAPK antibody. The results showed that GSE inhibited phosphorylation of VEGFR2 and MAPK in HUVECs.

Mice were individually gavage fed with 100 μ L of water control ($n = 5$) or GSE (50 mg/kg; $n = 6$) for 3 wk. Mice were then given s.c. injection of MDA-MB-231 (5×10^6 per mouse) and continued to be gavaged daily with GSE or water control. Tumor volume was assessed twice weekly by caliber. Volumes were determined using the formula (width)² \times length \times 0.52. Body weight was monitored weekly as an indicator of overall health of the animals.

Immunohistochemistry

Representative tumor tissues (four of each group) were harvested and fixed in 10% neutral buffered formalin at 4°C for 12 h. All tissues were paraffin embedded. Sections (5 μ m) were first stained with H&E to evaluate tissue viability and quality. Sections were then microwaved in citrate buffer and were costained with antibody against MECA (BD Bioscience) to visualize the vessels and with anti-phospho-MAPK (Cell Signaling) to visualize the phosphorylation status of MAPK. For CD31 staining, sections were permeabilized with 36 μ g/mL proteinase K (Roche Diagnostics Corp.) and

stained with antibody against CD31 (platelet/endothelial cell adhesion molecule; BD Bioscience). Tyramide signal amplification direct and indirect kits (NEN Life Science Products, Inc.) were used to amplify staining signals. Sections were photographed at $\times 200$ magnification using an Olympus AX70 microscope. Vessel density (average of five fields) was determined with Image-Pro software.

Polyvinylpyrrolidone depletion

GSE was incubated with polyvinylpyrrolidone at room temperature for 30 min and then centrifuged at 13,000 rpm for 10 min to remove polyvinylpyrrolidone. The absorbance of supernatant at wavelength of 280 was measured to ensure that polyphenol is removed.

Statistical analysis

Data were expressed as mean \pm SD. Student's *t* test is used to determine statistical significance between control and test groups. $P < 0.05$ is considered to be statistically significant.

Results

Effect of GSE on VEGFR2 signaling

To characterize the activity of GSE on VEGFR and angiogenesis, we started by comparing the effect of GSE on proliferation of endothelial cells cultured in the presence and absence of growth factors, as growth stimulation (such as VEGF) plays a critical role in triggering normal angiogenesis and tumor angiogenesis. HUVECs and bovine capillary endothelial cells were incubated with various concentrations of GSE for 30 minutes before the addition of VEGF (100 ng/mL), basic fibroblast growth factor (1 ng/mL), or growth medium containing various growth factors. The treated cells were then counted after 48 hours. As shown in Fig. 1A, a greater inhibitory activity of GSE was observed in cells treated with growth factors containing VEGF than in cells cultured in the absence of growth stimulation. A similar result was also obtained with bovine capillary endothelial cells (data not shown). This result suggests that GSE may regulate angiogenesis by interfering with the growth factor-induced signaling pathway.

The effect of GSE on VEGF-driven endothelial cell migration was also evaluated by using a modified Boyden chamber assay (27). As shown in Fig. 1B, at a concentration of 3 $\mu\text{g/mL}$, GSE abolished VEGF-induced migration without inhibiting the attachment of endothelial cells to the membrane.

We next evaluated the effect of GSE on VEGF signaling, one of the most potent and specific angiogenesis factors. Strong evidence shows that blocking the activity of VEGFR2 (KDR) can limit the ability of most tumors to stimulate the formation of blood vessels (2). Thus, we tested the effect of GSE on VEGF-induced tyrosine phosphorylation of VEGFR-2. Serum-starved HUVECs were treated with GSE or control buffer for 30 minutes, followed by stimulation with VEGF for 5 minutes. The phosphorylation state of VEGFR-2 was assessed by Western blot with anti-phospho-VEGFR2 antibody. As shown in Fig. 1C, GSE inhibited VEGF-induced tyrosine phosphorylation of VEGFR2 in a dose-dependent manner. The total amount of VEGFR protein in each sample of cells remained comparable, suggesting that inhibition of phosphorylation of VEGFR2 by GSE was not due to reduced VEGFR2 expression. These results suggest that GSE are potent inhibitors of VEGF-induced tyrosine phosphorylation of VEGFR-2.

To determine whether the inhibition of tyrosine phosphorylation of VEGFR2 by GSE is caused by inhibition of the kinase activity of VEGFR, we carried out an ELISA-based *in vitro* tyrosine kinase assay. As a positive control, an inhibitor of VEGFR, SU5416, showed inhibition of kinase activity with an IC_{50} of 1 $\mu\text{mol/L}$ (data not shown), in agreement with a previous report (31). GSE also showed a strong inhibition of VEGFR kinase activity, with an IC_{50} of ~ 10 ng/mL (Fig. 2A).

To define the biochemical mechanism for the inhibition of VEGFR2 by GSE, we examined the effect of increasing concentrations of ATP on the inhibitory activity of GSE (Fig. 2B and C). The Lineweaver-Burk plot showed that increasing concentrations of GSE had a strong effect on the apparent $1/V_{\text{max}}$ (y -axis intersection), indicating that GSE behaves mainly as a noncompetitive inhibitor for ATP binding. The Dixon plot revealed a K_i of 4 ng/mL for the inhibition of GSE.

To further dissect the downstream signaling pathway of VEGFR that might be affected by GSE, we examined the effect of GSE on VEGF-induced activation of MAPK in HUVECs.

MAPK (32) is one of the key components involved in the signaling pathways that support endothelial cell proliferation. As shown in Fig. 1C, VEGF caused a significant up-regulation of phosphorylation of both MAPKp44 and MAPKp42 isoforms

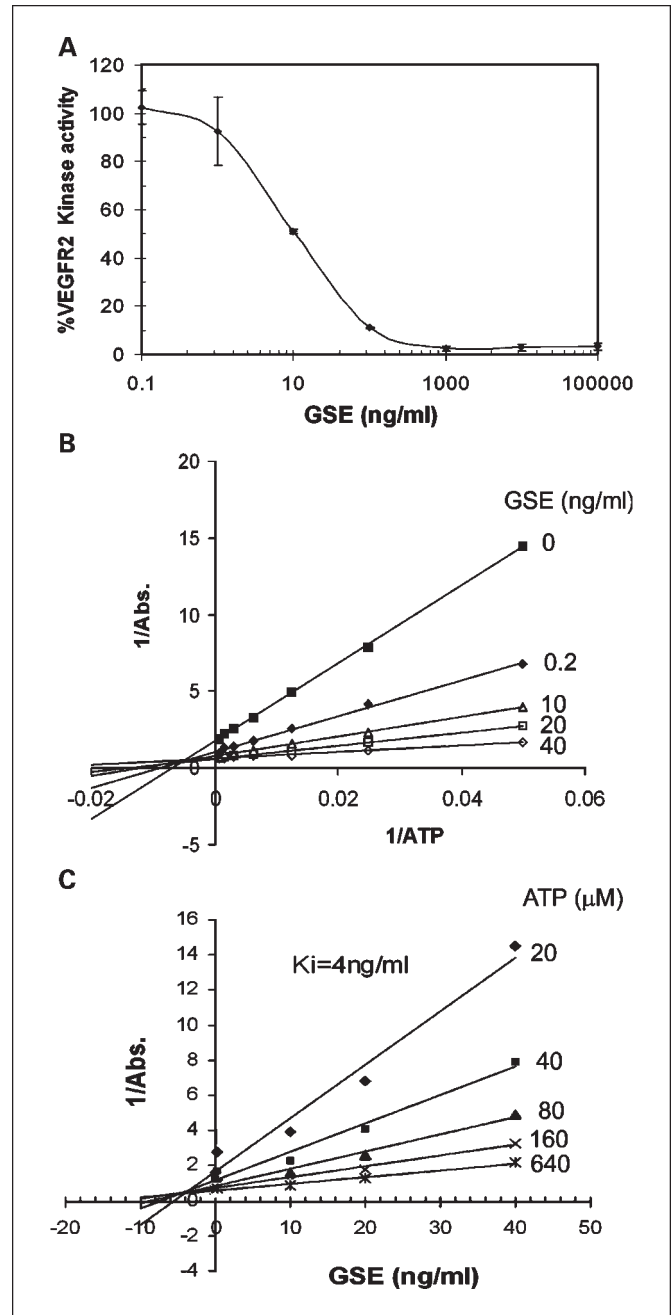


Fig. 2. GSE inhibits VEGFR2 kinase activity. A, GSE inhibited VEGFR2 kinase activity. VEGFR2 and various concentrations of GSE were incubated in kinase reaction buffer containing ATP in 96-well plates coated with a poly-Glu-Tyr substrate. Phosphorylation of the substrate was monitored with a purified phosphotyrosine specific monoclonal antibody conjugated to horseradish peroxidase followed by chromogenic reaction with horseradish peroxidase substrate. Data are represented as percentage of control that was not treated with GSE. Lineweaver-Burk plot (B) and Dixon plot (C) of the inhibition of VEGFR with GSE. ATP at increasing concentrations was incubated with VEGFR2 and various concentrations of GSE and monitored for the effect on kinase activity.

in HUVECs. Treating HUVECs with GSE inhibited VEGF-induced phosphorylation in both of the MAPK proteins, whereas the total amount of MAPK was unaffected.

Inhibition of proliferation and VEGFR activation in endothelial cells seemed to require higher doses of GSE than inhibition of VEGFR kinase activity *in vitro* (Fig. 1 versus Fig. 2). This may be because the active components in GSE are not stable in culture or not able to penetrate well into the cells to exert effect. Nevertheless, these results collectively suggest that GSE might block angiogenesis and tumor growth via a novel activity: direct inhibition of a key receptor tyrosine kinase of the endothelial cells and its downstream signaling cascade.

Effect of GSE on sprout formation of aortic ring

Compared with *in vitro* proliferation and migration assays, organ culture methods, such as rat aorta ring assay (28), are thought to more closely mimic multiple stages of *in vivo* angiogenesis, including endothelial cell proliferation, migration, and tube formation. To further characterize the antiangiogenic activity of GSE, we next performed an aorta ring assay.

To study the effect of GSE on microvessel sprout formation, we used a modified aortic ring sprout formation assay, chick aortic arch assay (29). In this assay, chick aortic rings were embedded in the Matrigel and fed with medium containing different concentrations of GSE. The rings were then stimulated with VEGF or ECGS. Sprout formation was examined under a microscope. Microvessels were initially noticed in VEGF- or ECGS-containing samples. Treatment with GSE resulted in a dose-dependent decrease in capillary sprouting: The growing sprouts were shorter and fewer cells migrated into the matrix (Fig. 3A and B), indicating that GSE could inhibit VEGF- and ECGS-induced microvessel sprouting.

To test whether the inhibition by GSE was reversible, we performed chicken aorta ring assay with the following modification: Aortic ring was cultured first with both ECGS and GSE for the indicated incubation time (2, 24, and 48 hours), then GSE was removed from the culture and aorta ring was continually cultured with ECGS. As shown in Fig. 3C, inhibition of vessel sprouts was released when GSE was removed, suggesting that inhibition of vessel sprouts by GSE is reversible and not likely due to general cytotoxicity.

In vivo antitumor angiogenesis activity of GSE

To study the potential *in vivo* antitumor angiogenesis activity of GSE, we tested the effect of GSE in a tumor model in mice. The experimental mice were prefed with GSE (+GSE) or water only without GSE (-GSE) by oral administration. The mice were then implanted with MD-MBA-231 breast cancer cells. Treatment of mice with GSE markedly inhibited MDA-MB-231 tumor growth compared with the control group treated with water (Fig. 4A and B). GSE treatment seemed to have no obvious toxicity and showed no detectable effect on body weight and behavior of mice bearing MDA-MB-231 (data not shown).

To further evaluate the status of tumor angiogenesis, we examined vessel formation in tumors treated with GSE. Tumor tissues were costained with MECA-32 antibody against pan-endothelial cell antigen and anti-phosphorylated MAPK. These two antibodies detect the vessels and the phosphorylation status of MAPK (33), respectively. Compared with

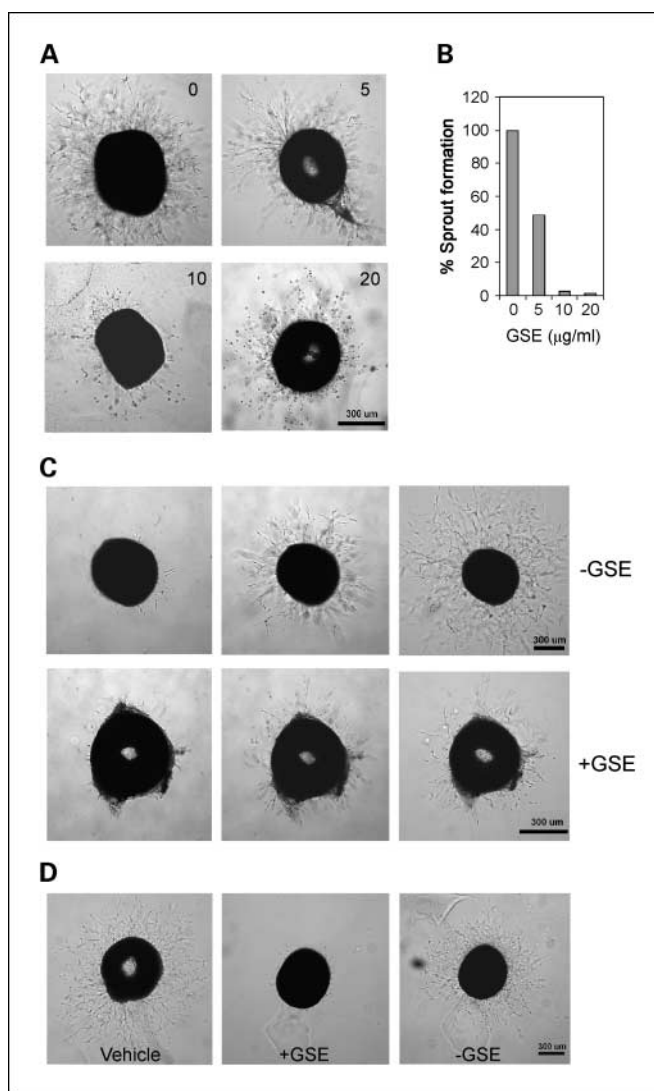


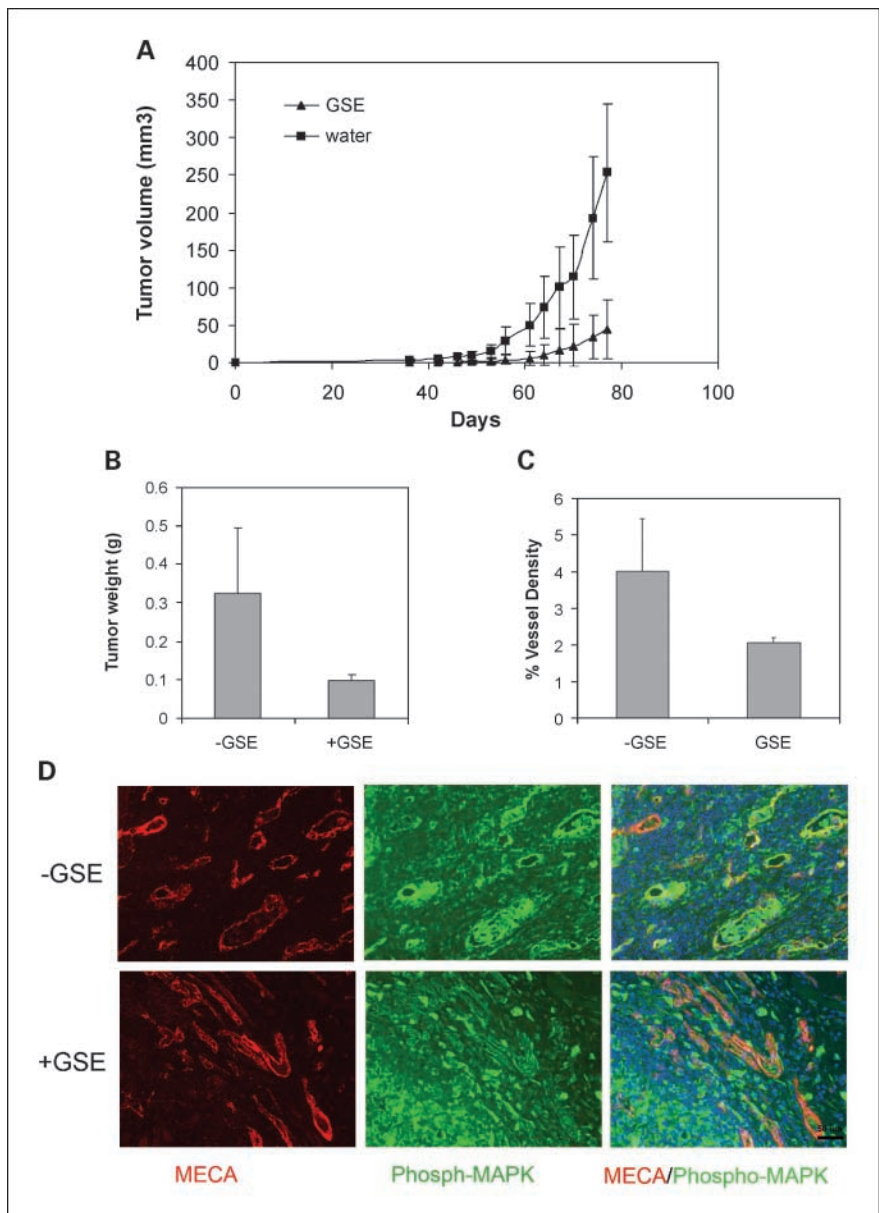
Fig. 3. GSE inhibit sprout formation from chicken aorta. *A*, chick aortic rings were placed in Matrigel gels and fed with MCDB131 serum-free medium containing various concentrations of GSE (5, 10, 20 µg/ml) in the presence or absence of ECGS (50 µg/mL). The effect of GSE on endothelial cell sprout formation from various aorta samples was examined on day 3. *B*, quantification of sprout formation. Results are represented as percentage of control that was not treated with GSE. *C*, chicken aortic rings were incubated with or without GSE (10 µg/mL) in the presence of ECGS. The sprout formation was examined on days 1 to 3. *D*, inhibition of GSE on vessel sprout formation is reversed once GSE is removed from the medium. Aorta rings were first incubated with (II) or without GSE (10 µg/mL; I) for 48 h. The culture was then replaced with fresh medium without GSE and further incubated in the presence of ECGS for 72 h (III).

the control group treated with water, GSE treatment led to a significant decrease in tumor vessel counts (Fig. 4C) and a decrease in the level of MAPK phosphorylation (Fig. 4D). These results further indicate that GSE is an angiogenesis inhibitor likely via interference with the MAPK signaling pathway.

Water-soluble polyphenol of GSE in antiangiogenic activity

The composition of GSE has been studied extensively and is known to be rich in polyphenols. The polyphenols in GSE

Fig. 4. GSE inhibits *in vivo* angiogenesis. **A**, GSE inhibits MDA-MB-231 tumor growth and tumor angiogenesis *in vivo*. MDA-MB-231 cells were s.c. injected into the back of severe combined immunodeficient mice. GSE (50 mg/kg/d; +GSE) or water control (-GSE) were orally given to each mouse before and after the tumor implantation. Tumor volumes were compared between water control and the GSE treated as a function of time. **B**, tumors were excised and weighed after the treatment. **C**, quantification of vessel density in the tumors based on CD31 staining. **D**, blood vessels in the tumors were costained with MECA-32 antibody against pan-endothelial cell antigen (red) and anti-phosphorylated MAPK (green) to assess tumor angiogenesis.



are mainly procyanidins. To test whether polyphenol is responsible for the antiangiogenic activity of GSE, we used polyvinylpyrrolidone to remove polyphenol from GSE. Polyvinylpyrrolidone forms hydrogen bonds with phenolic compounds, yielding a polyvinylpyrrolidone-phenolic precipitate, which can be removed by centrifugation. Polyvinylpyrrolidone-treated GSE was tested in VEGFR kinase assay, endothelial cell proliferation assay, and aorta ring assay. As shown in Fig. 5, polyvinylpyrrolidone-treated GSE had little effect on VEGFR2 kinase activity (Fig. 5A) and cell proliferation (data not shown) and did not inhibit VEGF-induced phosphorylation of VEGFR in endothelial cells (Fig. 5B). Removal of polyphenol also eliminated the inhibitory activity of GSE on aorta ring sprout formation (Fig. 5C). These results suggest that a water-soluble polyphenol in GSE, probably one of the procyanidins, is likely responsible for the antiangiogenic activity of GSE.

Discussion

GSE is widely consumed as a dietary supplement and possesses anticancer activity against various cancers (16–19, 21). Previous reports suggested antiangiogenesis as one possible mechanism for its antitumor activity (18, 25). In this study, we have further characterized the antiangiogenic and antitumor activities of GSE and started to dissect the possible molecular mechanisms underlying these activities. Our data indicate that GSE can inhibit angiogenesis both *in vitro* and *in vivo* via blocking VEGFR-MAPK activation.

Our results showed that GSE could inhibit various aspects of angiogenesis, including growth factor-induced endothelial cell proliferation, migration, tube formation on Matrigel (data not shown), sprout formation from aorta, and angiogenesis in tumor. The inhibitory activity of GSE on endothelial cell functions is not likely due to cell toxicity, although GSE can induce cell apoptosis at higher concentration (data not shown; ref.

25). This was reflected in our observation that GSE has a rather specific activity for actively proliferating endothelial cells (growth factor-stimulated proliferation; Fig. 1) and was further supported by the results that inhibition of vessel sprouting was reversible on removal of GSE (Fig. 3).

As a first step to study the possible molecular mechanism of the antiangiogenic activity of GSE, we have looked into the effect of GSE on the receptor for VEGF, one of the most critical angiogenesis factors. VEGFR2 is a potent target for cancer treatment. Many antibodies and chemical agents that inhibit VEGFR signaling have been developed and tested in the different stages of clinical trials. However, much less is known for the kinase inhibitory activity in natural products. Our results revealed a novel activity of GSE: inhibition of VEGFR2 kinase activity and suppression of its MAPK downstream signaling.

In addition to the study of the *in vitro* antiangiogenic activity of GSE using HUVECs in various assays, we have also examined the effect of GSE on tumor growth and tumor angiogenesis in mice using a xenograph model. Our results showed that GSE treatment inhibited tumor growth in mice, concomitant with a reduction in tumor vessel counts. This effect of GSE could be the result of the blockage of VEGFR-MAPK signaling, as evidenced by the observation that phosphorylation of MAPK was suppressed in the GSE-treated

mice, consistent with our *in vitro* study. Further studies are needed to evaluate the potential of the antiangiogenic activity of GSE in tumorigenesis and tumor progression using a carcinogen-induced or genetically engineered tumor model because such models would more closely mimic the multistage tumor progression in human patients.

A water-soluble fraction of polyphenol from GSE seems to be responsible for its antiangiogenic activity. The major form of polyphenol in commercial preparations of GSE is procyanidins, including procyanidins B1 to B5, procyanidin C1, procyanidin B5-3'-gallate, which compose 75% to 95% of GSE (16, 17, 34, 35). Future studies will be needed to further define the individual polyphenol component of GSE, alone or in combination, for antiangiogenic and anti-tumor activities

Angiogenesis inhibitors have emerged as a new class of drugs that can be used to treat and prevent cancer. The efficacy of antiangiogenic agents may be improved if they can be used chronically. However, most of the current antiangiogenesis agents, approved for clinical use or being tested in trial, result in certain side effects such as hypertension, bleeding, fever, diarrhea, etc. Our study indicates that GSE, a dietary supplement, is a well-tolerated and inexpensive natural angiogenesis inhibitor that suppresses VEGFR kinase activity and its downstream signaling in endothelial cells and could

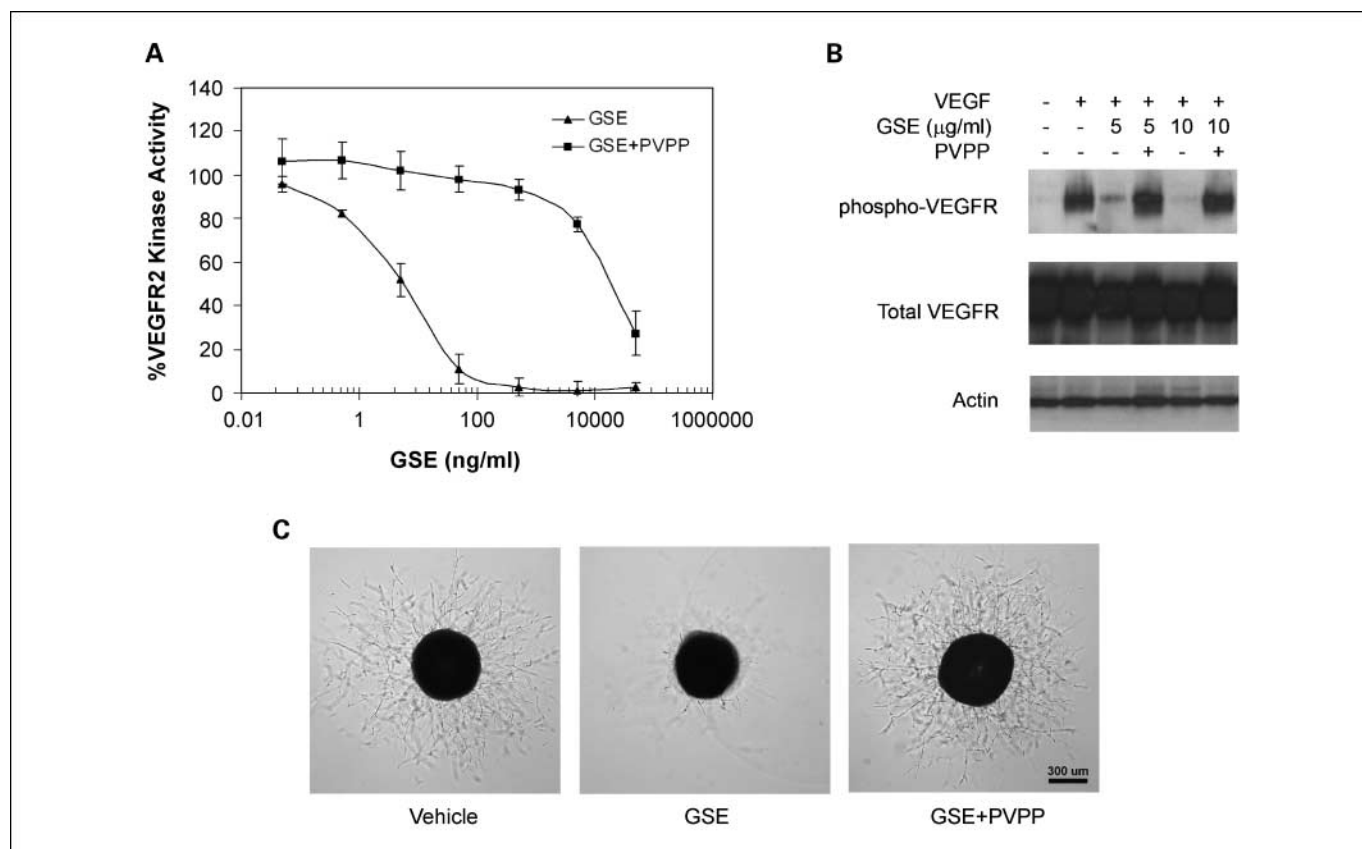


Fig. 5. Polyvinylpyrrolidone depletes antiangiogenic activity in GSE. GSE was incubated with polyvinylpyrrolidone (PVPP) for 30 min and followed by centrifugation to remove polyvinylpyrrolidone. The supernatant was then tested for the effects on the kinase activity of purified VEGFR2 (A), phosphorylation of VEGFR2 in HUVECs (B), and sprout formation from chick aorta (C).

potentially be used chronically to enhance its anticancer activity in cancer prevention or treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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