

## Anti-tumor activity of *Gastrodia elata* Blume is closely associated with a GTP-Ras-dependent pathway

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Received March 2, 2007; Accepted May 28, 2007

**Abstract.** *Gastrodia elata* Blume (GEB) is an important medicinal plant in Korea. In order to confirm the anti-tumor activities of GEB extracts, we carried out various *in vitro* anti-tumor assays, including a wound assay and an invasion assay using an ethyl ether extract of GEB. The results showed that the GEB extract exhibits potent anti-tumor activity *in vitro* in a dose-dependent manner. The expression of CD44, cdc42, Timp-2 or RhoA mRNA did not change by GEB treatment, compared to that of the control. GTP-Ras, an active form of a G-coupled protein family, however, is associated with the anti-tumor activity of GEB extracts. We examined various molecular markers related to metastasis by reverse transcriptase-polymerase chain reaction with the extract of GEB-treated B16 cells. There was an increase in GTP-Ras expression by the *Gastrodia elata* Blume extract. Together, these results suggest that the *Gastrodia elata* Blume extract could have potential in alleviating tumorigenesis, by a GTP-Ras-dependent pathway; although the precise molecular mechanisms are still being examined.

### Introduction

*Gastrodia elata* Blume (GEB) is a traditional herb that has been used in East Asian for centuries. It has been used as an anticonvulsant, analgesic and sedative to combat vertigo,

hypertension, general paralysis and tetanus. Vanillyl alcohol and gastrodin, derived from GEB are known to have anticonvulsive actions (1). Recently, it was reported that compounds found in GEB inhibited glutamate-induced apoptosis in neuronal cells (2). In addition, after pentylene-tetrazole-induced seizure activity, the ether fraction of GEB has been shown to attenuate a decrease in  $\gamma$ -aminobutyric acid (GABA) and an increase in glutamate content, as well as having anticonvulsant effects (3).

GEB is also used as sub-material for food or food-related industry. In 2001, the Korea Food and Drug Administration approved that GEB extracts as food ingredients; however, an *in vivo* toxicological study is still needed to determine its safety in food. We have already reported that GEB power-supplemented (0.5-1.0%) dough had a membrane-like structure that was more developed than that of the control, resulting in increased bread volumes (4). These results suggest that bread baked with 0.5-1.0% GEB exhibited an increase in loaf volume due to the more complete development of a gluten matrix.

In our study, we determined that GEB protected cell damage by  $\beta$ -amyloid in neuroblastoma cells (5). The ethyl ether fraction of GEB has potent activity toward  $\beta$ -amyloid-induced cell damage via reducing caspase-3 activity (6,7; data not shown). In the course of a mechanistic study of the methanolic extract of GEB, we found that it has potent anti-tumor activity *in vitro*. The major finding of this report is that GEB exhibits anti-metastatic activities which were confirmed by wound and invasion assay in B16 melanoma cells. We further demonstrated that GEB increased anti-tumor activity via a GTP-Ras-dependent pathway.

### Materials and methods

**Cell culture.** A murine melanoma cell line B16-F1 (B16; Catalog No. CRL-6323) and human umbilical vein endothelial cells (HUVECs) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The B16 cells were cultured at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air in RPMI-1640 medium supplemented with 10% FBS, while the HUVECs were cultured in EGM-2 medium

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**Abbreviations:** HUVECs, human umbilical vein endothelial cells; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT-PCR, reverse transcriptase-polymerase chain reaction; Rho, Ras homologous; GEB, *Gastrodia elata* Blume

**Key words:** *Gastrodia elata* Blume, anti-tumor, GTP-Ras-dependent pathway, B16 melanoma cells

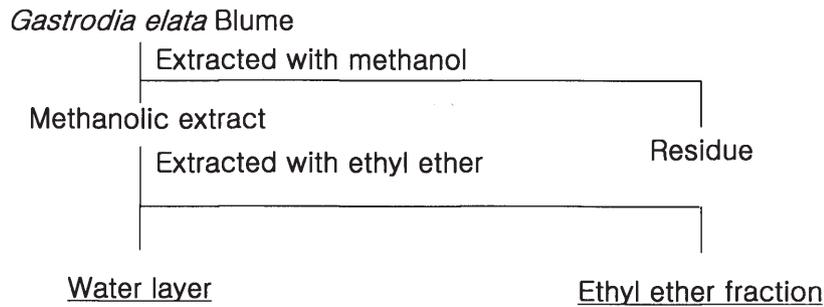


Figure 1. A fractionation procedure for the *Gastrodia elata* Blume extract.

(BD Bioscience, Franklin Lakes, NJ). The B16 cells were maintained in RPMI-1640 medium and subcultured by trypsinization every 3-4 days. The HUVECs were also subcultured every 3-4 days and were used in the experiments.

**Chemicals.** Matrigel (354234, Beckton-Dickinson, San Jose, CA), and an invasion chamber (3401, Costar, Bethesda, MD) were used for an invasion assay. All other materials were commercially available.

**Preparation and fractionation of samples.** Three-year-old rhizoma of *Gastrodia elata* Blume were obtained from a Gastrodia Farm in Youngyang, Kyoungbuk province, Korea. The washed and chopped pieces of GEB were deep-frozen ( $-70^{\circ}\text{C}$ ) until use. Freeze-dried GEB was powdered with a homogenizer. GEB was extracted with methanol, and the methanol extract was resuspended in ethyl ether. The fraction was divided into an ethyl ether fraction and a water fraction. Each fraction was evaporated at  $30^{\circ}\text{C}$  under reduced pressure (Fig. 1). The color of the fraction was light to dark brown. The plant used in this experiment was collected between June and October, 2005 and was identified by senior staff of the Department of Biology, Kyungpook National University, Daegu, Korea. Voucher specimens of the plant have been deposited in Enzyme Biotechnology Lab., KNU under the accession No. 2005-02.

**MTT assay.** A MTT assay was carried out as follows: cells ( $5 \times 10^5$  cells/ml) were split in 96-well plates and incubated for 24 h in  $100\text{-}\mu\text{l}$  of RPMI medium. Various concentrations of extracts were added, and the cells were incubated for an additional 48 h. Then,  $10\text{-}\mu\text{l}$  of MTT solution (5 mg/ml MTT in PBS) was added to each well and incubated at  $37^{\circ}\text{C}$  for 4 h. In order to stop the reaction,  $100\text{-}\mu\text{l}$  of 0.04 M of HCl, in isopropanol, was added with vigorous mixing. The absorbance was determined by a multilabel counter (Victor3; Wallac, Turku, Finland) at 564 nm (8).

**Wound healing assay.** Strips of thin tape (2 mm x 2 cm; 3 M, Seoul, Korea) were attached to the bottom of each well of the 6-well plates (Greiner, Frickenhausen, Germany), and B16 cells were plated at  $1 \times 10^7$  cells/well and allowed to attach at  $37^{\circ}\text{C}$  for 3-5 h in a 5%  $\text{CO}_2$  atmosphere. The tape strips were then removed, creating linear wounds. The plates were photographed and incubated, as above, with media containing various concentrations of GEB. The plates were photographed

at 16, 24 and 40 h and the exact wound width was calculated by a microruler (<http://www.eeob.iastate.edu/faculty/DrewsC/htdocs/microruler-links.htm>) (9).

**Invasion assay.** Transwell plates (pore size  $8\text{-}\mu\text{m}$ ; Costar) were loaded with  $100\text{-}\mu\text{l}$  of Matrigel (BD Biosciences), which was allowed to solidify for 2 h at  $37^{\circ}\text{C}$ , and then the plates were coated with  $10\text{-}\mu\text{l}$  of fibronectin ( $200\text{-}\mu\text{g/ml}$ ; BD Biosciences). The plates were loaded with B16 cells suspended in a 10% FBS ( $1 \times 10^5$  cells/well), the samples were exposed to GEB extracts, and the plates were incubated for 24 h in 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ . The migrated cells were fixed with methanol, stained with hematoxylin, and counted under an inverted microscope (Nikon, Tokyo, Japan) (10).

**Tube formation assay.** HUVECs ( $2 \times 10^4$  cells/well) were dispensed into Matrigel-coated 24-well plates (Beckton-Dickinson) in 0.5 ml of EGM-2 medium containing various concentrations of GEB extracts, and the cells were incubated for 24 h. The cells were then visualized by microscopy and tube formation was scored by counting the number of tubes formed (11).

**RNA isolation and reverse transcriptase polymerase chain reaction.** RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR) were carried out, as described previously, with slight modifications (12-15). Briefly, the total cellular RNA was extracted from B16 cells using the TRIzol reagent (Gibco BRL, Grand Island, NY). First-strand cDNA was synthesized from  $1\text{-}\mu\text{g}$  of total-RNA using oligo(dT) reverse transcriptase (iNtRON Biotechnology, Sungnam, Korea), and a PCR amplified with specific primers for mice CD44 (forward, 5'-TCG ATT AGA ATG TAA CCT GCC-3'; reverse, 5'-TGG TGT GTT CTA TAC TCG CCC-3'), Paxillin (forward, 5'-ACT ACT GCA ACG GAC CCA TC-3'; reverse, 5'-TCG TGG TAG TGG ACC TCA CA-3'), CDC42 (forward, 5'-TTG TTG GTG ATG GTG CTG TT-3'; reverse, 5'-CCC AAC AAG CAA GAA AGG AG-3'), Timp-2 (forward, 5'-GCA TCA CCC AGA AGA AGA GC-3'; reverse, 5'-GGG TCC TCG ATG TCA AGA AA-3'), c-Src (forward, 5'-GCA GCT GTA TGC TGT GGT GT -3'; reverse, 5'-TCA GCA GAA TCC CAA AGG AC-3') and GAPDH (forward 5'-ATG TTC CAG TAT GAC TCC AC-3'; reverse, 5'-GCC AAA GTT GTC ATG GAT GA-3') as an internal control. The resulting PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining.

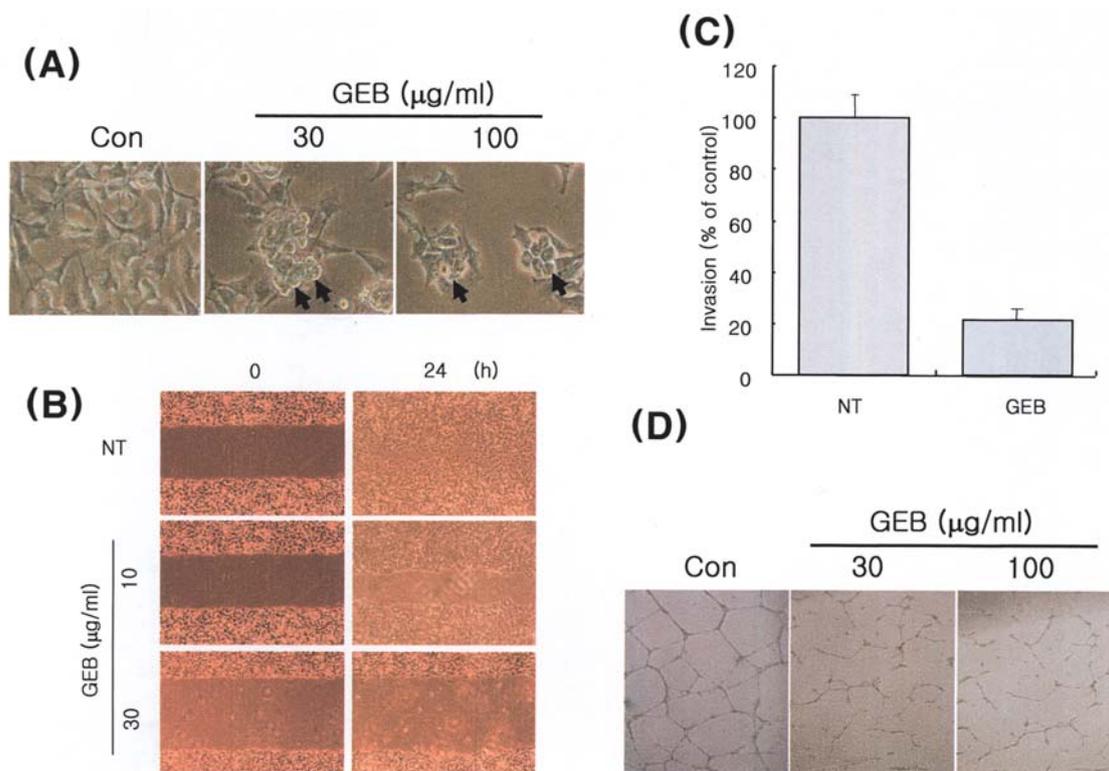


Figure 2. *Gastrodia elata* Blume exhibits potent anti-tumor activity. (A) MTT assay of the GEB extract. Arrows show the GEB-damaged cells (black arrows) when treated with GEB in a dose-dependent manner. (B) Wound healing assay. Confluent monolayers of B16 cells were pretreated with or without 30 or 100  $\mu\text{g/ml}$  of GEB extract. The monolayers were then wounded and stimulated with 75 nM PMA or left untreated NT. The plates were photographed 0, 24 and 48 h post-wounding. (C) Invasion assay. Cell invasion was assayed in Matrigel-coated Transwell chambers. Invasion was compared among B16 cells exposed to GEB extracts. Cell migration was quantified 24 h after the cells were exposed to GEB extracts. Migrated cells were counted from five randomly selected microscopic fields, and the results are given as the average per field  $\pm$ SD of 3 independent experiments. (D) Tube formation assay. HUVECs were plated at  $2 \times 10^4$  cells/well in Matrigel-coated 24-well plates, and then exposed to 0, 30 or 100  $\mu\text{g/ml}$  GEB extract. After 24 h, the culture supernatants were removed and the cells were fixed with 10% formalin. Representative results are given of 3 independent experiments.

**Western blot analysis.** Ras activity was measured using a GTP-Ras Kit (Pierce, Rockford, IL), according to the slight modifications of the manufacturer's instructions. Briefly, B16 cells ( $1 \times 10^6$  cells) were centrifuged at 16,000  $\times$  g, and the supernatant was incubated at 4°C for 1 h with the provided GST-Rhotekin-RBD, before loading (25  $\mu\text{l/lane}$ ) onto a SwellGel immobilized glutathione disk (Pierce). Thereafter, the glutathione disk-bound proteins were solubilized with 2X SDS [125 mM of Tris-HCl, pH 6.8, 2% glycerol, 4% SDS (w/v), 0.05% mercaptoethanol, and 0.05% bromophenol blue], eluted from the disk, resolved by 12% SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell GmbH, Dassel, Germany). The membranes, which were blocked for 1 h with a Tween (0.1%)/TBS, were incubated with the primary antibody (Pierce) and then incubated with HRP-conjugated secondary antibody (Pierce). This was done in order to visualize bands by using the ECL detection system (Amersham Pharmacia, Buckinghamshire, UK) (10,11).

**Statistical analysis.** Data were expressed as the means  $\pm$  standard deviation of the mean values. Statistical significance was determined by Student-Newman-Keuls method for independent means, using the Sigma Plot program. The critical level for significance was set at  $P < 0.05$ .

## Results and Discussion

In this study, we examined whether *Gastrodia elata* Blume (GEB) inhibited cancer cell growth *in vitro*. GEB is a traditional medicinal herb which has been used in East Asian countries for centuries. GEB has been used as an anticonvulsant, analgesic and sedative against vertigo, hypertension, general paralysis and tetanus. Recently, it was reported that fractions of this herb inhibited glutamate-induced apoptosis in neuronal cells (2). Moreover, the herb can protect amyloid  $\beta$ -peptide-induced cell damage in neuroblastoma cells (5,6). GEB has been strongly advocated as a supplement, its extracts can be used as food sub-ingredient; however, an *in vivo* toxicological study is still needed to verify its biosafety. With a dose between 6 g/60 kg/day, the *in vivo* toxicity of GEB did not affect any morphological changes in tissue level (data not shown). It has been determined that GEB extracts exhibited potent anti-oxidant activity (1,18).

On the other hand, cancer progression/metastasis is a complex sequence of events whereby tumor cells invade adjacent tissue (19). The cells penetrate into lymphatics or blood vessels, are transported to near or distant sites and finally, become lodged, extravasate or proliferate in order to form metastatic lesions. The process can cause major complications in chemotherapeutic treatment. Clinical

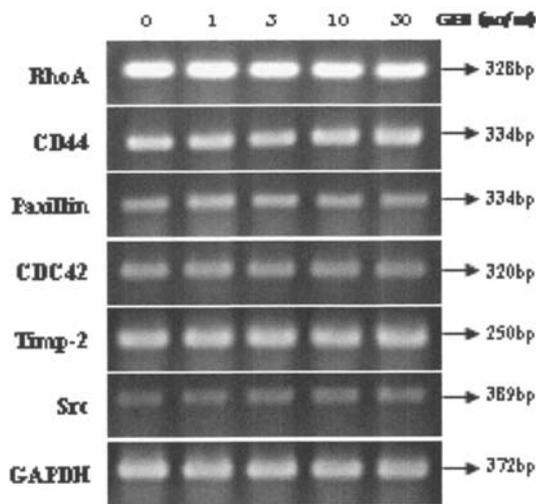


Figure 3. The mRNA expression profile of various molecular targets, by RT-PCR, with or without *Gastrodia elata* Blume extracts. Data of each primer sequence are shown in Materials and Methods.

oncologists have discovered various molecular markers such as CD44, a uterine plasminogen activator receptor (uPAR), ICAM, VCAM, and matrix metalloproteinases (MMPs) during the progression of cancer (20). In order to improve cancer treatment, a greater understanding of the progression of cancer and/or the metastatic process at the molecular and cellular levels is required.

Therefore, in order to examine the inhibitory effects of GEB in B16 cells, we carried out wound healing and invasion assays to test its *in vitro* effects on cell migration, which is an important facet of cell motility, particularly in relation to cancer. A wound healing assay revealed that GEB exhibited clear inhibitory wound width, whereas the untreated control showed dense cell growth between wound regions, suggesting that GEB inhibited cell migration *in vitro* (Fig. 2B). Our invasion assay demonstrated that GEB inhibited invasive activity by >75% versus the control group (Fig. 2C). Collectively, these results indicate that GEB inhibited the wound healing of B16 cells and suppressed invasion *in vitro* in a dose-dependent manner (Fig. 2C and D).

Next, we examined the effects of GEB on angiogenesis, which is a pivotal process of metastasis. HUVECs were treated with GEB, and tube formation was assessed in terms of tube size and numbers (Fig. 2D). Our results revealed that GEB dose-dependently inhibited HUVEC tube formation *in vitro* at 0-100 µg/ml (Fig. 2D). The control showed the highest levels of proliferation, indicating that endothelial cell growth was not restricted by contact inhibition under our experimental conditions (data not shown). Collectively, these findings indicated that GEB inhibited cell migration, cell invasion and tube formation, *in vitro*, in a dose-dependent manner. This suggests that the fraction of GEB might function as an anti-tumor component, *in vivo*, by decreasing metastasis and angiogenesis.

We investigated the effects of GEB on the levels of its potential signaling partners, Rho GTPases. Signal transduction during invasion or angiogenesis is fairly well-documented. Many transcription factors (e.g., EST-1, c-fos and c-jun) and

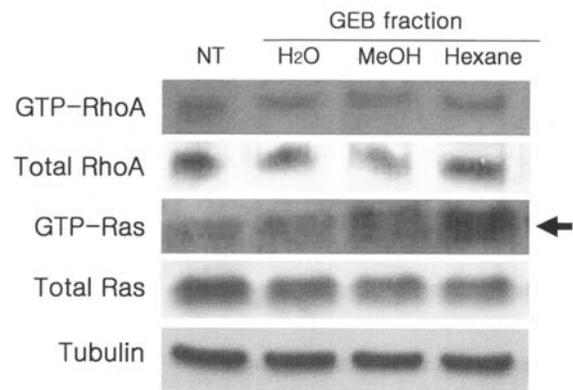


Figure 4. The involvement of the increased GTP-Ras expression by *Gastrodia elata* Blume treatment. Each lane was loaded with 30 µg of GEB-treated cell lysates and the lanes were transblotted to primary antibody in order to detect expressed protein levels.

migration-related proteins are associated with gene expression in endothelial cells during angiogenesis (21,22). VEGF acts as a central regulator, while many morphological changes are overseen by members of the Rho protein family of GTPases. The Rho GTPases such as RhoA, Ras, Rac1, and cdc42, are localized at cell membranes and become activated upon the stimulation of cell-surface receptors (23). The association/dissociation of GTP to Rho protein families triggers specific cellular responses by mediating cell contractility and organizing actin filaments into stress fibers, which thereby influence the motility and migration of endothelial cells, as well as the formation of new blood vessels.

We compared the mRNA levels of the GEB extracts in order to confirm which molecular mechanisms are involved in B16 cell inhibition. In Fig. 3, we showed that the mRNA expression of the GEB treatment operated in a dose-dependent fashion (0-30 µg/ml). RhoA, Paxillin, CD44, cdc42, Timp-2 or c-Src did not show changed expression levels by GEB. Further investigation regarding the precise molecular mechanism is still needed.

We also examined the effects of GEB on GTP-Ras levels in B16 cells. Our Western blot analysis revealed that the treatment of cells with GEB water, or ethyl ether (30 µg/ml) increased GTP-Ras activity to approximately 90, 250%, respectively (Fig. 4, arrow), suggesting that GEB had potential in increasing the active forms of Ras rather than the activity level *per se*.

In conclusion, we demonstrated that GEB extracts inhibited B16 cell growth via a GTP-Ras expression-dependent mechanism, which was confirmed by comparing the Ras-homologous GTPases using Western blotting. If we can control B16 growth suppression by regulating the clinical molecular marker(s), the precise anti-tumor mechanism could be proven, in that the molecular marker(s) could be regulated by the hit(s), including that of GEB extracts.

#### Acknowledgements

This study was supported in part by a grant of Oriental Medicine R&D Project, Ministry of Health & Welfare, Republic of Korea (HMP-B050042).

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