Hot Water Extract of *Scutellaria baicalensis* Inhibits Migration, Invasion and Tube Formation in a Human Umbilical Vein Endothelial Cell Model and a Rat Aortic Ring Sprouting Model

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Angiogenesis is essential for the pathophysiological processes of embryogenesis, tissue growth, diabetic retinopathy, psoriasis, wound healing, rheumatoid arthritis, cardiovascular diseases, and tumor growth. Inhibition of angiogenesis represents an attractive therapeutic approach for the treatment of angiogenic diseases such as cancer. However, uncontrolled angiogenesis is also necessary for tumor development and metastasis. Inhibition of vascular endothelial growth factor (VEGF) signaling, a critical factor in the induction of angiogenesis, cause robust and rapid changes in blood vessels of tumors and therefore VEGF constitutes a target for such anti-angiogenic therapy. Recently, since natural compounds pose significantly less risk of deleterious side effects than synthetic compounds, a great many natural resources have been assessed for useful substance for anti-angiogenic treatment. Here we evaluated the anti-angiogenic effects of a hot water extract of *Scutellaria baicalensis* (SBHWE) using *in vitro* assays and *ex vivo* animal experiments. Our results show that SBHWE dose-dependently abrogated vascular endothelial responses by inhibiting VEGF-stimulated migration and invasion as well as tube formation in a human umbilical vein endothelial cell (HUVEC) model, without cytotoxicity, as determined by a cell viability assay. Further study revealed that SBHWE prevented VEGF-induced neovascularization in a rat aortic ring sprouting model. Taken together, our findings reveal an anti-angiogenic activity of *Scutellaria baicalensis* and suggest that SBHWE is a novel candidate inhibitor of VEGF-induced angiogenesis.

Key words: Angiogenesis, Endothelial cell, *Scutellaria baicalensis*

Introduction

Angiogenesis, a neovascularization from pre-existing vessels, is a key step for tumor growth and metastasis and is a complex process that includes degradation of the extracellular matrix, migration, proliferation, sprouting, elongation, and tube formation of endothelial cells, controlled by a delicate balance between angiogenic inducers and angiogenic inhibitors [6, 7, 10, 41]. Therefore, anti-angiogenic agents that can interfere with the essential steps of cancer development are a promising strategy for human cancer treatment [2, 33, 43]. Numerous studies in cell culture and in animal models demonstrate that natural products derived from medicinal plants are able to exert chemopreventive and antitumor activities targeting angiogenesis in the tumor environment [32, 40].

*Scutellariae baicalensis*, a medicinal plant, grows in various regions of Asia, including Siberia, the far east of Russia, Mongolia, China, Japan, and Korea, where it has been used as an ingredient in botanical formulations for thousands of years with positive results for the treatment of inflammatory diseases, allergies, hyperlipemia, diabetes, arteriosclerosis, and bacterial and viral infections [5, 17, 23, 25, 51, 52, 53, 57]. This herb has been included as an important ingredient in various combination therapies such as Ger-Gen-Chyn-Tang [15], Soshiho-tang [28], and Shuanghuanglian [61]. *S. baicalensis* extracts have also been shown to modulate production of...
cytokines related to antiviral activity [3], exert chemopreventive effects against a variety of cancers [4, 22, 36, 56], induce apoptosis of human monocytic leukemia and osteogenic sarcoma cells [14], and inhibit metastasis in hepatocellular carcinoma cells [37]. These beneficial effects are due to its active constituents, which include baicalin, baicalein, wogonoside, and wogonin [4, 11, 53, 55, 56]. Although numerous biological activities of S. baicalensis have been reported, no studies have examined the anti-angiogenic effect of the extract.

Therefore, the purpose of the present study was to investigate the anti-angiogenic activity of SBHWE on VEGF-induced angiogenesis through measurement of migration, invasion, and differentiation of HUVECs in vitro, and an aortic ring sprouting angiogenesis assay ex vivo.

Materials and Methods

Animals

Seven-week-old Sprague-Dawley rats were purchased from Orient Co. (Sungnam, Korea). The rats were housed in a temperature-controlled room with a 12 hr light and 12 hr dark schedule. They were given food and water ad libitum. All experiments were conducted in accordance with the “Guide for the Care and Use of Laboratory Animals” adopted by the United States National Institutes of Health and reviewed and approved by the Ethics Committee, Institutional Animal Care and Use Committee (IACUC, Approval # YWC-131127-1) of Yonsei University (Wonju, Korea).

Plant extract and reagents

The S. baicalensis extract used in the current investigation was provided by the Korea Rural Development Administration. The extract was prepared in distilled deionized water as a stock solution at a concentration of 100 mg/ml, and was stored at -80°C. Before use in the experiments, the stock solution was clarified and diluted with M199 medium at different concentrations.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from USB Corporation (Cleveland, OH, USA). Matrigel basement membrane matrix was obtained from BD Biosciences (Bedford, MA, USA). hVEGF165 recombinant human VEGF165 which has cross reactivity for rat, was purchased from PeproTech (Rocky Hill, NJ, USA). Antibodies to VEGFR2, phospho-VEGFR2 (Tyr 1175), phospho-ERK (Thr 202/Tyr 204), ERK, phospho-p38 MAP kinase (Thr 180/Tyr 182), and p38 MAP kinase were obtained from Cell Signaling Technology (Beverly, MA, USA). Horseradish peroxidase-conjugated secondary antibody was purchased from Thermo Scientific Inc. (Rockford, IL, USA). Cell culture reagents and most other biochemical reagents were purchased from Sigma (St. Louis, MO, USA), unless otherwise specified.

Cell culture

Human umbilical vein endothelial cells (HUVECs) purchased from the American Type Culture Collection (ATCC®, Manassas, VA, USA) were maintained on gelatin-coated plates in M199 medium (GIBCO®, Grand Island, NY, USA) supplemented with 20% fetal bovine serum (FBS) (GIBCO®, Gaithersburg, MD, USA), 3 ng/ml basic fibroblast growth factor (KOMABIOTECH, Seoul, Korea), 100 U/ml penicillin, 100 mg/ml streptomycin (GIBCO®, Grand Island, NY, USA), and 5 U/ml heparin (Sigma) at 37°C in 5% CO2 in air, and used at early passages (passages 6-10) in all experiments. For in vitro experiments, HUVECs were serum- and growth factor-starved for 6 hr, after which the effects of SBHWE were assessed.

Cell viability assay

An MTT assay, a measure of cell viability, was used to assess the cytotoxicity of SBHWE. Briefly, confluent HUVECs in gelatin-coated 24-well plates were treated with different concentrations (1-100 μg/ml) of SBHWE in triplicate. The treated cells were incubated with the extract for 24 hr, followed by the addition of 100 μl of MTT solution (5 mg/ml in PBS) to each well. The plates were further incubated at 37°C for 4 hr and subsequently the medium was carefully removed from the plates, and dimethyl sulfoxide (DMSO) was added to solubilize the formazan produced from MTT by viable cells. The optical density (OD) was read using a plate reader (Molecular Devices, Sunnyvale, CA, USA) at 595 nm. The cytotoxicity of SBHWE was calculated as follows: cell viability (%) = (average absorbance of SBHWE-treated group / average absorbance of control group)×100.

Cellular proliferation assay

HUVECs were seeded at a density of 4×104 cells per well in gelatinized 12-well plates containing M199 medium with 20% FBS and incubated for 24 hr. These cells were serum- and growth factor-starved and treated either with VEGF (20 ng/ml) or VEGF (20 ng/ml) + SBHWE (1-100 μg/ml), and
incubated at 37°C for 24 hr. Thereafter, proliferation of these cells was measured using an MTT assay. OD readings were taken at 595 nm with a spectrophotometer (Molecular Devices) and the absorbance values were normalized to untreated controls to calculate growth percentages.

Migration assay
To evaluate the effects of SBHWE on VEGF-induced HUVEC migration, endothelial cell migration was assessed using gelatin-coated 3-μm pore Transwell inserts (Costar, Corning, NY, USA) placed in 24-well plates. HUVECs (5×10^4 cells/100 μl/insert) were plated in M199 medium (1% FBS) containing various concentrations of SBHWE in the upper chamber of the transwell and M199 medium (1% FBS, 600 μl) containing VEGF (20 ng/ml) was added into the lower chamber as a chemoattractant. After 4 hr, non-migrated cells were removed by cotton swab and migrated cells were stained with H&E and examined under a microscope. The number of migrated cells was quantified by counting the cells under a 40× objective. Each treatment was done in triplicate and eight images were quantified per each transwell membrane.

Cell invasion assays
The invasion assay was performed using a Transwell chamber containing a polycarbonate filter with a pore size of 3 μm as described [20]. HUVECs were suspended in the Matrigel-coated upper chambers in M199 medium (1% FBS) containing S BHWE, at a final concentration of 5×10^4 cells/100 μl. The bottom chambers (600 μl) were filled with M199 (1% FBS) containing VEGF (20 ng/ml). After incubation for 24 hr, the noninvasive cells that remained on the upper surface of the filter were removed by a cotton swab. Cells that traversed through the Matrigel were attached to the underside of the insert filter. The cells were stained with H&E and then eight fields of each membrane were counted under an inverted optical microscope (200×), and the average number of cells in each field was calculated. The data were expressed as percent invasion compared with the control.

Tube formation analysis
This study examined endothelial tube formation using an in vitro angiogenesis assay. Matrigel Growth Factor Reduced (GFR) (BD Biosciences, Bedford, MA) was used as a substrate for the study of angiogenesis. Matrigel GFR solution was thawed overnight at 4°C, and the Matrigel GFR solution (150 μl) was distributed onto each 24-well plate and allowed to solidify for 30 min at 37°C. Cells were trypsinized, resuspended in M199 medium (1% FBS) at 2×10^5 cells/ml with either VEGF (20 ng/ml) or VEGF (20 ng/ml) + SBHWE, and the cell suspension was seeded onto a 24-well plate pre-coated with Matrigel and allowed to form capillary tubes. The capillaries formed were photographed after 20 hr using an inverted microscope, and the average tube areas were quantified using Image-Pro Plus (Media Cybernetics, Bethesda, MD, USA).

Rat aortic ring sprouting assay
The standard rat aortic ring assay was used as a model for our ex vivo angiogenesis study. freshly sacrificed dorsal aortas from seven-week-old male Sprague Dawley rats (n=3) were rinsed with ice-cold HBSS, separated from fibroadipose tissue, and cut into 1 mm-long pieces using a sterile surgical blade. Each aorta ring was placed in a well of a 48-well plate pre-coated with Matrigel matrix (120 μl), and then covered with another layer of Matrigel matrix (50 μl). After polymerization for 30 min, serum-free M199 media was added to each well in the presence or absence of SBHWE with or without 20 ng/ml VEGF. On day 7 the rings were photographed at 40× magnification by phase-contrast microscopy and microvessel outgrowth was quantified. Sprouting was measured using the following scale: 0 = no sprouting; 1 = migrated cells without sprouting; 2 = isolated sprouting; 3 = sprouting in 25-50% of the arterial ring circumference; 4 = sprouting in 50-75% of the circumference; and 5 = sprouting in 75-100% of the circumference. The assay was scored from 0 to 5 in a double-blinded manner, and each data point was assessed six times.

Statistical analysis
All numerical values are represented as the mean ± standard deviations. Statistical significance was determined using Student’s t-test. Each experiment was repeated at least three times to yield comparable results. Values with p<0.05 and p<0.01 were considered to indicate a statistically significant difference.

Results
Effect of SBHWE on viability and proliferation of HUVECs.
To rule out the possibility that inhibition of angiogenesis
was due to cytotoxic effects, we analyzed the viability of HUVECs treated with SBHWE extract by MTT assay. As shown in Fig. 1A, following 24 hr of incubation with various concentrations of SBHWE, endothelial cell viabilities were not significantly changed by treatment with this extract. The results indicate that exposure to SBHWE does not induce cytotoxic effects in the conditions of this study.

To characterize the anti-angiogenic activity of SBHWE, we first determined whether SBHWE inhibited growth factor-induced endothelial cell proliferation. HUVECs were exposed to various concentrations of SBHWE, then cultured with or without VEGF (20 ng/ml) for 24 hr. HUVEC proliferation was significantly increased in response to VEGF treatment, but it was not suppressed as the concentration of SBHWE increased (Fig. 1B). This result indicates that SBHWE does not affect the growth of HUVECs in response to VEGF at these concentrations.

**Effect of SBHWE on motility of HUVECs**

Migration is representative of the ability of endothelial cells to form new blood vessels. We next studied the effects of SBHWE on VEGF-induced cell migration using a Transwell plate. Fig. 2A shows a photomicrograph of endothelial cells migrating across the filter membrane after 4 hr of incubation. In the absence of SBHWE, a large number of HUVECs migrated to the lower side of the filter in the Transwell chamber following stimulation with VEGF (20 ng/ml). This VEGF-induced migration of endothelial cells was dose-dependently inhibited by SBHWE treatment (Fig. 1B). SBHWE alone had no significant effect on basal migration of these endothelial cells. These results revealed that SBHWE significantly inhibited the motility of HUVECs.

**Effect of SBHWE on invasion of HUVECs**

Since invasion of vascular endothelial cells into the extracellular matrix is an important step for neovascular formation, we used Matrigel-coated polycarbonate filters in Transwell chambers to elucidate the anti-invasive effect of SBHWE on HUVECs. HUVECs plated in the upper chamber, which contained M199 media plus various concentrations of SBHWE, were allowed to invade the lower chamber, which contained VEGF acting as a chemoattractant. Our results showed that SBHWE dose-dependently abolished the VEGF-stimulated invasion of HUVECs across the Matrigel-coated filter (Fig. 3). This result indicates that SBHWE markedly inhibited invasion of HUVECs in response to the pro-angiogenic factor, VEGF.

**Effect of SBHWE on tube formation by HUVECs**

To verify its anti-angiogenic activity, we examined the effects of SBHWE on VEGF-induced tube formation by HUVECs on Matrigel, a well-established angiogenesis assay. Our study showed that VEGF stimulation of HUVECs seeded on Matrigel promotes differentiation to form capillary-like tubes within 20 hr; however, in the presence of SBHWE, the width and length of the endothelial tubes in-

![Fig. 1. Effects of SBHWE on the viability and VEGF-induced proliferation of HUVECs. (A) HUVECs were incubated with various concentrations (1, 5, 10, 25, 50, and 100 μg/ml) of SBHWE. After 24 hr, cytotoxicity was quantified by an MTT assay. (B) HUVECs were pretreated for 30 min with various concentrations (1, 5, 10, 25, 50, and 100 μg/ml) of SBHWE before exposure to VEGF (20 ng/ml). After 24 hr, the number of proliferated cells was determined by an MTT assay. VEGF treatment alone served as a positive control. Each bar represents the average ± SE of three independent experiments.](image-url)
Fig. 2. SBHWE inhibits the VEGF-induced migration of HUVECs. HUVECs were treated with VEGF (20 ng/ml) in the presence or absence of SBHWE (1, 5, 10, 25, 50, and 100 μg/ml). Chemotactic migration was assayed after incubation in Transwell plates for 4 hr. (A) Representative migrated cells were photographed. (B) Cells that migrated to the bottom of the filter were counted using optical microscopy. The in vitro angiogenesis assay was performed as described in Materials and Methods. VEGF treatment alone served as a positive control. Data are expressed as mean ± SE (n = 3). *p<0.05 and **p<0.01 versus VEGF alone.

Fig. 3. SBHWE inhibits VEGF-induced invasion of endothelial cells. Effect of SBHWE on HUVEC invasion measured using Transwell culture plates. HUVECs were treated for 24 hr with VEGF (20 ng/ml) and various concentrations (10, 50, and 100 μg/ml) of SBHWE. (A) Representative invaded cells were photographed. (B) Cells that invaded to the bottom of the filter were counted using optical microscopy. VEGF treatment alone served as a positive control. **p<0.01 versus VEGF alone.

Produced by VEGF were reduced dose-dependently. At a concentration of 100 μg/ml, SBHWE completely abrogated endothelial tube formation. To quantify the degree of tube formation, photographs were taken under an inverted microscope (Fig. 4A), and the tube area was calculated using image analysis software (Fig. 4B). This result suggests that SBHWE may have an inhibitory effect on angiogenesis.
Effect of SBHWE on angiogenesis ex vivo

To further characterize its anti-angiogenic activity, we performed the aortic ring sprout formation assay, a widely used ex vivo angiogenesis assay that mimics several stages of angiogenesis, including endothelial cell proliferation, migration, invasion, and tube formation. Treatment with VEGF significantly stimulated vessel sprouting from aortic rings embedded in Matrigel, compared to the results with medium alone. However, compared with the VEGF alone-treated group, SBHWE significantly inhibited the growth of blood vessels in the aortic rings of rats in a concentration-dependent manner (Fig. 5). These results demonstrate that SBHWE caused a dramatic decrease in sprout length and density from the aortic ring. In conclusion, SBHWE exhibited

Fig. 4. SBHWE inhibits VEGF-induced tube formation by endothelial cells. HUVECs were preincubated for 30 min with SBHWE (10, 50, or 100 μg/ml) and plated on Matrigel-coated plates at a density of 2×10^5 cells per well. They were incubated in the presence or absence of 20 ng/ml VEGF, and microphotographs were obtained after 20 hr (40×). (A) Representative endothelial tubes are shown. (B) The area covered by the tube network was measured using Image-Pro Plus software. The experiments were repeated three times, and values represent mean ± SE values of triplicate determinations. **p<0.01 versus VEGF alone.

Fig. 5. SBHWE inhibits VEGF-induced vessel sprouting ex vivo. Aortas in Matrigel were cultured with or without VEGF (20 ng/ml) in the presence or absence of SBHWE, and analyzed on day 7. (A) Representative aortic rings were photographed. (B) SBHWE blocks VEGF-induced vessel sprouting. The assay was scored from 0 (least positive) to 5 (most positive), and the data represent mean ± SE values (n = 6). **p<0.01 versus VEGF alone.
anti-angiogenic activity both in vitro and ex vivo.

Discussion

Natural products are potential sources of alternative medicines for chemoprevention in humans [46, 47]. These products have various chemical properties and can reduce the development of tumors through multiple mechanisms, which include inhibition of angiogenesis and stimulation of apoptosis [49]. *Scutellaria baicalensis* has been widely used as a traditional medicine for the treatment of various ailments, such as inflammation, cancer, infectious diseases, hypercholesterolemia, and hypertension in East Asia since ancient times [8, 21, 44, 60, 62]. Additional studies have demonstrated that baicalin, baicalein, and wogonin, the main bioactive flavonoids derived from *S. baicalensis*, show antitumor and anti-angiogenic effects on various cancer cells [29-31]. Recent studies have reported that an aqueous extract of *S. baicalensis* has a neuroprotective effect on excitotoxic primary neuronal cell death in cortical cells [54], can ameliorate drug addiction-related behavior through functional regulation of dopamine receptors in rat [58], can prevent hematuria and blood-brain barrier disruption in rats because of its anti-inflammatory effects [19, 45], has a hepatoprotective effect in mice fed a high-fat diet [27], and promotes a strong chemotherapeutic effect in lung cancer cells [38]. Although numerous studies on the physiological and pharmacological functions of *S. baicalensis* have been conducted, the detailed functions and mechanisms thereof have not been elucidated thus far.

Angiogenesis plays a critical role in tumor progression and is a tightly regulated process that involves proliferation, migration, invasion, and organization into capillaries by endothelial cells [9, 12]. Currently, one of the most thriving fields in drug development, with a plethora of new drugs on the market, is inhibition of angiogenesis through the specific targeting of VEGF, a potent stimulator of angiogenesis, and of its receptors expressed on the cell surface, which are the major mediators of VEGF-induced proangiogenic signaling in endothelial cells, by therapeutic antibodies and natural products [1, 16, 24, 35, 50]. Thus in the present study, we examined the effects of SBHWE on VEGF-induced angiogenesis both in vitro and ex vivo.

Since endothelial cells play major roles in each step of VEGF-mediated angiogenesis, in order to investigate the anti-angiogenic effects of SBHWE in HUVECs, we first determined the non-toxic concentration of SBHWE to be used for our experiments by examining the cytotoxicity of various concentrations of SBHWE (1-100 μg/ml). No cytotoxic effects of SBHWE were observed at the given concentrations (Fig. 1A). We then examined the effects of a non-toxic concentration of SBHWE on the process of angiogenesis, in the form of endothelial cell proliferation, migration, and invasion in response to VEGF, resulting in the formation of capillary tubes by HUVECs. Our in vitro study demonstrated that SBHWE significantly inhibited the migratory properties of cells toward a chemottractant, VEGF (Fig. 2), and of the ability of VEGF-stimulated cells to pass through a Matrigel-coated membrane (Fig. 3) and differentiate into capillary-like structures formed of VEGF-induced endothelial cells (Fig. 4), although VEGF-induced proliferation was not affected by SBHWE treatment (Fig. 1B). It is to be noted that SBHWE alone had no effects on proliferation of the endothelial cells. These results indicate that the inhibitory angiogenic activity of SBHWE is due to suppression of migration, invasion, and tube formation, but not of proliferation. To determine the underlying molecular mechanism of the anti-angiogenic effects of SBHWE, we examined its effects on phosphorylation of key signal transduction pathways. Binding of VEGF to the receptor tyrosine kinase VEGFR2 leads to activation of VEGFR2’s cytoplasmic domain and initiation of intracellular signaling cascades, including activation of extracellular signal-regulated kinase (ERK), phosphoinositide 3-kinase (PI3K)-AKT, c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (p38 MAPK), which are necessary for essential angiogenic processes [18, 26, 34, 39, 42, 48, 59]. In this study, we found that the phosphorylation of VEGFR2, ERK1/2, and p38 MAPK in response to VEGF were not downregulated by SBHWE treatment (data not shown). Although we did not investigate the effect of this extract on activation of the PI3K-AKT and JNK pathways by VEGF, at the cellular level SBHWE completely suppressed the migration, invasion, and tube formation of VEGF-treated endothelial cells. These results indicate that the inhibitory effect of SBHWE on angiogenesis is not via ERK1/2 and p38 MAPK, and that the anti-angiogenic activity of SBHWE may be due to the blockade of another activation pathway. More experiments are in progress to understand the molecular targets and mechanisms underlying the inhibitory effect of SBHWE on VEGF-induced angiogenesis. To further confirm the anti-angiogenic potential of SBHWE, we conducted an ex vivo rat aortic ring assay. Neoangiogen-
esis, the growth of new capillaries, in response to VEGF synthesized by tumor cells plays a crucial role in tumor growth and metastasis [13]. Similar to the results of the tube formation assay, the VEGF-stimulated outgrowth of vessels from the fragmented aorta ring is suppressed as the dose of SBHWE escalates. This implies that SBHWE significantly inhibited the formation of new vessels.

Based on the data provided in the present study, we conclude that a hot water extract of *S. baicalensis* can significantly inhibit VEGF-induced angiogenesis via suppression of endothelial cell migration, invasion, and tube formation *in vitro*, and sprout formation from aortic rings *ex vivo*. Consequently, *S. baicalensis* may be a promising chemopreventive and therapeutic agent for the treatment of angiogenesis-related diseases such as cancer.

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초록: 혈관내피세포와 환절 대동맥 미세혈관 발아 모델을 이용한 황금 열수추출물의 세포의 이동, 침투 및 관형성 억제 연구

김억천1 · 배기호1 · 김한성2 · 유영만3 · 갤린스키 미철3 · 김택중1*
(1인 생명과학기술학부, 2인 의공학과, 3인 생과학과)

혈관신생의 억제는 염증과 같은 신생혈관형성 질환의 치료를 위해 유용한 접근법이다. 신생혈관형성의 핵심인 자인 혈관내피세포성장인자는 신생혈관형성 질환의 치료를 위한 주요한 표적이다. 본 연구에서는 in vitro 테스트와 ex vivo 동물 실험을 통해 황금 열수추출물의 신생혈관형성 억제효과를 연구했다. 본 연구결과에서 황금 열수추출물이 혈관내피세포성장인자에 의한 활성화된 혈관내피세포에 있어 세포독성 없이 세포의 이동, 침투, 관형성을 높게 억제하였다. 이는 염증과 같은 신생혈관형성 질환의 치료를 위한 유용한 접근법이 될 수 있음을 제안한다.