Full Length Research Paper

Inhibition of tumor angiogenesis by *Scutellaria barbata* D. Don via suppressing proliferation, migration and tube formation of endothelial cells and downregulation of the expression of VEGF-A in cancer cells

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Abbreviations: EESB, Ethanol extract of *Scutellaria barbata* D. Don; TCM, traditional Chinese medicine; CAM, chorioallantoic membrane; HUVEC, human umbilical vein endothelial cell; DMSO, dimethyl sulfoxide; MTT, 3-(4, 5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide.

*Scutellaria barbata* D. Don is a medicinal herb that has long been clinically used in China to treat various types of cancer. Extracts of *S. barbata* D. Don have been shown to inhibit the growth of many cancer cells. Previously, we reported that *S. barbata* D. Don promotes cancer cell apoptosis via activation of the mitochondrion-dependent pathway. To further elucidate the precise mechanism of its tumorcidal activity, we investigated the effects of ethanol extract of *S. barbata* D. Don (EESB) on tumor angiogenesis. We found that EESB inhibited angiogenesis in vivo in chick embryo chorioallantoic membrane (CAM). In addition, we observed that EESB dose and time-dependently inhibited the proliferation of human umbilical vein endothelial cells (HUVEC) via blocking the cell cycle G1 to S progression. Moreover, EESB inhibited the migration and tube formation of HUVECs. Furthermore, EESB treatment down-regulated the mRNA and protein expression levels of VEGF-A in both HT-29 human colon carcinoma cells and HUVECs. Our findings suggest that inhibiting tumor angiogenesis is one of the mechanisms by which *S. barbata* D. Don treats cancer.

Key words: Tumor angiogenesis, traditional Chinese medicine, multi-target agents, *Scutellaria barbata* D. Don.

INTRODUCTION

Angiogenesis, a physiological process involving the growth of new blood vessels from the pre-existing vasculature, plays an important role in the development and metastasis of cancers (Folkman, 1971; Folkman, 1995; Folkman, 2006; Cook and Figg, 2010). In the initial stage, tumor cells obtain oxygen and nutrients from nearby blood vessels by simple passive diffusion. However, when tumor grows to reach certain size, oxygen delivery by diffusion is no longer sufficient, which causes tumor cells to induce the sprouting of new blood vessels from pre-existing vasculature, creating a blood supply system within tumor that is essential for continue growth of tumor as well as providing an avenue for hematogenous metastasis (Folkman, 1986; Jain, 1987).

Induction of angiogenesis is mediated by a variety of molecules released by tumor cells (Weidner et al., 1991; Breier and Risau, 1996; Stromblad and Cheresh, 1996). Vascular endothelial growth factor A (VEGF-A) is considered one of the strongest stimulator of angiogenesis (Risau, 1997; Jain, 2002; Ferrara, 2002). VEGF-A is highly expressed in a wide variety of human tumors, which has been associated with tumor...
progression, invasion and metastasis, and poorer survival and prognosis in patients (Maeda et al., 1996; Kaya et al., 2000; Ferrara et al., 2003). When VEGF-A is secreted from tumor cells, it primarily binds to specific receptors located on vascular endothelial cells (EC) (Ferrara, 2002), which in turn triggers a tyrosine kinase signaling cascade that induces EC proliferation, migration, survival, sprouting, and eventually tube formation (Ferrara, 2002; Gille et al., 2001).

Angiogenesis has become an attractive target for anti-cancer chemotherapy due to its essential role for the progression of solid tumors. A variety of anti-angiogenic agents is currently in preclinical development, with some of them now entering the clinic trials. However, the administration of angiogenesis inhibitors usually causes cardiovascular complications, including impaired wound healing, bleeding, hypertension, proteinuria, and thrombosis (Chen and Cleck, 2009; Zangari et al., 2009; Higa and Abrahm, 2009), due to their intrinsic cytotoxicity against non-tumor associated endothelial cells. In addition, multiple signaling pathways are involved in tumor angiogenesis, inhibitors that affect a single pathway may be insufficient and probably lead to resistance (Eikedsal and Kalluri, 2009). These problems highlight the urgent need for the development of multi-target agents with minimal side effects and toxicity. Natural products, including traditional Chinese medicine (TCM), have relatively fewer side effects and have been used clinically to treat various kinds of diseases including cancer for thousands of years (Gordaliza, 2007; Ji et al., 2009; Zhao et al., 2009). TCM formula is a complex combination of many natural products, each of which contains numerous chemical compounds. Therefore, TCM formulas are considered to be multi-component and multi-target agents exerting their therapeutic function in a more holistic way, and discovering naturally occurring agents is a promising approach for anti-cancer treatment.

Scutellaria barbata D. Don is a medicinal herb widely distributed in northeast Asia. As a well known traditional Chinese folk-medicine, it is used for heat-clearing, detoxification, promotion of blood circulation and removal of blood stasis (Chinese Pharmacopoeia Commission, 2010). S. barbata D. Don has long been used as an important component in several TCM formulas for the clinical treatment of various kinds of cancer, since according to the theory of TCM, accumulation of toxic dampness and heat is one of the major causative factors in the pathogenesis of cancers and therefore clearing heat and detoxification is a principle of anti-cancer treatment (Chinese Pharmacopoeia Commission, 2010; Qian, 1987). Extracts of S. barbata D. Don have been shown to inhibit the growth of many cancer cells (Lee et al., 2004; Yin et al., 2004; Cha et al., 2004; Suh et al., 2007) and recently, we reported that S. barbata D. Don can promote cancer cell apoptosis via activation of the mitochondrion-dependent pathway (Wei et al., 2011). However, the precise mechanisms of its tumoricidal activity still remain largely unknown. In this study, we evaluated the effects of ethanol extract of S. barbata D. Don (EESB) on tumor angiogenesis, and investigated the molecular mechanisms mediating its biological effect. We found that EESB inhibited angiogenesis in vivo in chick embryo chorioallantoic membrane (CAM). In addition, we observed that EESB dose and time-dependently inhibited the proliferation of human umbilical vein endothelial cells (HUVEC) via blocking the cell cycle G1 to S progression. Moreover, EESB inhibited the migration and tube formation of HUVECs. Furthermore, EESB treatment down-regulated the mRNA and protein expression levels of VEGF-A in both HT-29 human colon carcinoma cells and HUVECs.

MATERIALS AND METHODS

Materials and reagents

Roswell Park Memorial Institute Medium 1640 (RPMI 1640), Dulbecco's Modified Eagle Medium (DMEM), Fetal bovine serum (FBS), penicillin–streptomycin, Trypsin-EDTA, and TRIzol Reagent were purchased from Invitrogen (Carlsbad, CA, USA). SuperScript II reverse transcriptase was obtained from Promega (Madison, WI, USA). In vitro angiogenesis assay kit was purchased from Millipore (Billericia, MA, USA). Human VEGF-A ELISA kit was obtained from Shanghai Xitang Biological Technology Ltd. (Shanghai, China). All other chemicals, unless otherwise stated, were obtained from Sigma Chemicals (St. Louis, MO, USA).

Preparation of ethanol extract from Scutellaria barbata D. Don (EESB)

The herb was collected in Henan Province, China, in August, 2008. The whole plants of dried S. barbata D. Don were cut into small pieces and EESB was prepared as described before (Wei et al., 2011). Stock solutions of EESB were prepared by dissolving the EESB powder in 50% DMSO to a concentration of 500 mg/ml, and stored at –20°C. The working concentrations of EESB were made by diluting the stock solution in the culture medium. The final concentrations of DMSO in the medium were < 0.5%.

Chick chorioallantoic membrane (CAM) assay

A CAM assay was performed to determine the in vivo antiangiogenic activity of EESB. Briefly, 4 µl of EESB (500 µg/µl) was loaded onto a 0.5 cm diameter Whatman filter paper. The filter was then applied to the CAM of a seven-day embryo. After incubation for 72 h at 37°C, angiogenesis around the filter was photographed with a digital camera. The number of blood vessels was quantified manually in a circular perimeter surrounding the implants, at a distance of 0.25 cm from the edge of the filter. Assays were performed twice, containing totally 10 eggs for each data point.

Cell culture

HUVECs and HT-29 cells were obtained from Xiangya Cell Center, University of Zhongnan (Hunan, China). HUVECs or HT-29 cells were grown in RPMI 1640 or DMEM, respectively. Both RPMI 1640 and DMEM were supplemented with 10% (v/v) FBS, and 100
Units/ml penicillin and 100 µg/ml streptomycin. All cell lines were cultured at 37°C, 5% CO2 under humidified environment.

**Evaluation of cell viability by MTT assay**

Cell viability was assessed by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay. HUVECs were seeded into 96-well plates at a density of 1×10^4 cells/well in 0.1 ml medium. The cells were treated with various concentrations of EESB for 24 h or with 1.5 mg/ml of EESB for different periods of time. At the end of the treatment, 10 µl MTT (5 mg/ml in phosphate buffered saline, PBS) were added to each well, and the samples were incubated for an additional 4 h at 37°C. The purple-blue MTT formazan precipitate was dissolved in 100 µl DMSO. The absorbance was measured at 570 nm using an ELISA reader (BioTek, Model ELX800, USA).

**Cell cycle analysis of HUVECs**

The cell cycle analysis was carried out by flow cytometry using a fluorescence-activated cell sorting (FACS) caliber (Becton Dickinson, CA, USA) and Propidium iodide (PI) staining. After treated with indicated concentrations of EESB for 24 h, HUVECs were harvested and adjusted to a concentration of 1×10^6 cells/ml, and fixed in 70% ethanol at 4°C overnight. The fixed cells were washed twice with cold PBS, and then incubated for 30 min with RNase (8 µg/ml) and PI (10 µg/ml). The fluorescent signal was detected through the FL2 channel and the proportion of DNA in different phases was analyzed using ModfitLT Version 3.0 (Verity Software House, Topsham).

**Migration assay on HUVECs**

Migration of HUVECs was performed by the wound healing method. HUVECs were seeded into 12-well plates at a density of 2×10^5 cells/well in 1 ml medium. After 24 h of incubation, cells were scraped away vertically in each well using a P100 pipette tip. Three randomly selected views along the scraped line were photographed on each well using phase-contrast inverted microscope at a magnification of 100×. Cells were then treated with indicated concentrations of EESB for 24 h, and another set of images were taken by the same method. A reduction in the scraped area indicates a sign of migration.

**Tube formation assay on HUVECs**

The HUVEC tube formation was examined using ECMatrix assay kit (Millipore), following the manufacturer’s instructions. Briefly, confluent HUVECs were harvested and diluted (1×10^6 cells) in 50 µl of medium, containing indicated concentrations of EESB. The harvested cells were then seeded into 1:1 ECMatrix gel (v/v) coated 96-well plates and incubated for 9 h at 37°C. The network-like structures were examined using phase-contrast inverted microscope. The photographs were taken at a magnification of 100×.

**RNA extraction and RT-PCR analysis**

2×10^5 HUVECs or HT-29 cells were seeded into 6-well plates in 2 ml medium and treated with indicated concentrations of EESB for 24 h. Total RNA was isolated with Trizol Reagent. Oligo(dT)-primed RNA (1 µg) was reverse-transcribed with SuperScript II reverse transcriptase (Promega) according to the manufacturer’s instructions. The obtained cDNA was used to determine the mRNA amount of VEGF-A by PCR. GAPDH was used as an internal control.

**Measurement of VEGF-A secretion by ELISA**

The secretion level of VEGF-A was measured using ELISA kit (Xitang) according to the manufacturer’s instructions. The wells were coated with 100 µl capture antibody diluted in coating buffer. The plate was sealed and incubated overnight at 4°C. After three washes, the wells were blocked with 200 µl assay diluents at room temperature for 1 h, followed by another three washes. Then, 100 µl diluted VEGF-A standard and test samples were added and incubated for 2 h at room temperature. After repeated washes, the substrate was added and incubated for 20 min at room temperature, and the absorbance was measured at 450 nm.

**Statistical analysis**

All data are the means of three determinations and data was analyzed using the SPSS package for Windows (Version 11.5). Statistical analysis of the data was performed with Student’s t-test and ANOVA. Differences with P< 0.05 were considered statistically significant.

**RESULTS**

**EESB exhibits anti-angiogenic activity in an in vitro CAM model**

We tested the effect of EESB on in vivo angiogenesis using a CAM model. We observed that EESB treatment significantly reduced the total number of blood vessels in the chicken embryos, compared with untreated control (Figure 1), indicating that EESB is able to suppress angiogenesis in vivo.

**EESB inhibits the proliferation of HUVECs.**

Cell viability was determined using MTT assay. As shown in Figure 2A, treatment with 0.5 to 2.5 mg/ml of EESB for 24 h dose-dependently reduced cell viability by 20 to 80% compared to untreated control cells (P< 0.01). We also evaluated the effect of 1.5 mg/ml of EESB on cell viability with incubation for different periods of time. As shown in Figure 2B, treatment with 1.5 mg/ml of EESB led to a gradual decrease in cell viability with the increase of exposure time. These results suggest that EESB inhibits the proliferation of HUVECs in both dose- and time-dependent manners.

**EESB blocks cell cycle progression of HUVECs**

We investigated the effect of EESB on the G1 to S progression in HUVECs via PI staining followed by FACS.
Figure 1. Effect of EESB on the angiogenesis of chick chorioallantoic membrane (CAM).
(A) A 0.5 cm diameter filter paper loaded with or without 2 mg of EESB was applied to the CAM and incubated at 37°C for 72 h. Angiogenesis around the filter was photographed with a digital camera. The number of blood vessels was quantified manually in a circular perimeter surrounding the implants, at a distance of 0.25 cm from the edge of the filter. Assays were performed twice, containing totally 10 eggs for each data point. Images are representative photographs. (B) Quantification of CAM assay. *P <0.01, versus control cells.

Analysis. As shown in Figure 3, the percentage proportion of S-phase cells following treatment with 0, 0.5, 1.5 and 2.5 mg/ml of EESB was 48.41, 40.07, 37.17, and 31.87%, respectively (P< 0.01), indicating that EESB inhibits HUVEC proliferation by blocking the cell cycle G1 to S progression.
**EESB inhibits HUVEC migration and tube formation**

The effect of EESB on EC migration was determined using the wounding-healing method. As shown in Figure 4, after post-wounding for 24 h, untreated HUVECs migrated into the clear area, whereas EESB treatment dose-dependently inhibited HUVEC migration. We also evaluated the effect of EESB on EC capillary tube formation using ECMatrix system, a solid gel containing mouse basement membrane proteins, in which cultured endothelial cells can rapidly align and form hollow tube-like structures. As shown in Figure 5, when untreated HUVECs were plated on ECMatrix gel, there was a clear formation of elongated tube-like structures. In contrast,
Figure 3. Effect of EESB on the cell cycle progression of HUVECs. Cells were treated with indicated concentrations of EESB for 24 h, stained with PI, and analyzed by FACS. The proportion of DNA in S-phase was calculated using ModfitLT Version 3.0 Software. Data shown are averages with S.D. (error bars) from three independent experiments. *P< 0.01, versus control cells.

Figure 4. Effect of EESB on the migration of HUVECs. After treated with indicated concentrations of EESB for 24 h, the migration pattern of HUVECs was observed using phase-contrast microscopy. The photographs were taken at a magnification of 100 ×. Images are representative of three independent experiments.
EESB treatment significantly resulted in a decrease in capillary tube formation, with a dose-dependent fashion.

**EESB suppresses the expression of VEGF in both HT-29 cells and HUVECs.**

We examined the effect of EESB on the expression of VEGF-A in both HT-29 human colon carcinoma cells and HUVECs. The results of the RT-PCR assay showed that EESB treatment profoundly reduced VEGF-A mRNA expression in both HT-29 cells and HUVECs, with an apparent dose-dependent manner (Figures 6A and B). Data from ELISA assay showed that the protein expression pattern of VEGF-A was similar to its mRNA level (Figures 6C and D).

**DISCUSSION**

Here we reported for the first time that the ethanol extract of *S. barbata* D. Don (EESB) can inhibit angiogenesis
in vivo in CAM and in vitro in HUVECs. Angiogenesis typically consists of several features including proliferation, migration and tube formation of endothelial cells (ECs). Using MTT assay we found that EESB inhibited the proliferation of ECs in both dose- and time-dependent manners. G1/S transition is one of the two main checkpoints used by cell to regulate the cell cycle progress and thus the cell proliferation. Using PI staining followed by FACS analysis we found that EESB treatment dose-dependently blocked G1 to S progression of ECs, suggesting that the anti-proliferative effect of EESB on ECs probably results from cell cycle arrest. Moreover, using wounding-healing method and ECMatrix system we demonstrated that EESB inhibited the migration and capillary tube formation of ECs in a dose-dependent fashion. The process of tumor angiogenesis is highly regulated by growth factors such as VEGF-A which is secreted by tumor cells and ECs and functions via paracrine and autocrine signaling pathways. After secretion VEGF-A primarily binds to its specific receptors located on ECs, triggering the process of angiogenesis. In the present study we found that EESB down-regulated the expression of VEGF-A in both HT-29 human colon carcinoma cells and ECs. In summary, based on present and our previous studies, it is clear that S. barbata D. Don inhibits cancer growth via at least two mechanisms, inducing mitochondrion-dependent apoptosis of cancer cells and inhibiting tumor angiogenesis.

Both apoptosis and angiogenesis are regulated by multi-pathways, such as STAT3, Ras/ERK, PI3K/mTOR and Wnt signalings. In addition, S. barbata D. Don is composed of many chemical compounds including flavonoids, diterpenoids, alkaloids, steroids and polysaccharides. It is unknown how many of these compounds contain anti-cancer activity, and we do not know whether the anti-cancer compounds of S. barbata D. Don target on different sites individually, or act on a single site additively or synergistically. The signaling pathway(s) with which these compounds exert their bioactivity still remains unclear. These intriguing questions must be addressed by future experiments before one can completely elucidate the molecular mechanism by which S. barbata D. Don treats cancer and develop better multi-target drugs for cancer therapy.
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