Scutellaria barbata D. Don induces G1/S arrest via modulation of p53 and Akt pathways in human colon carcinoma cells

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Abstract. Cancer cells are characterized by an uncontrolled increase in cell proliferation. G1 to S transition is one of the two main checkpoints used by cells to control the cell cycle progress and cell proliferation. G1/S progression is highly regulated by multiple intracellular signaling transduction cascades including Akt and p53 pathways, which therefore becomes a promising target for the development of novel anticancer therapy. *Scutellaria barbata* D. Don (SB) is a major component in many Chinese medicine formulas that have long been used in China to clinically treat various cancers including colorectal cancer (CRC). Recently, we reported that the ethanol extract of SB (EESB) is able to induce cancer cell apoptosis via activation of the mitochondrion-dependent pathway and inhibit tumor angiogenesis through suppression of Hedgehog signaling. To further elucidate the precise mechanisms of its antitumor activity, in the present study we evaluated the effect of EESB on the proliferation of human colon carcinoma HT-29 cells and investigated the underlying molecular mechanism. We found that EESB could inhibit the proliferation of HT-29 cells through blocking the G1/S cell cycle progression. In addition, EESB treatment profoundly promoted antiproliferative p21 expression, but inhibited the expression of pro-proliferative PCNA, cyclin D1 and CDK4 in HT-29 cells. Moreover, the phosphorylation/activation of Akt was significantly suppressed by EESB treatment, whereas that of p53 was enhanced. These results suggest that EESB could effectively induce G1/S arrest in human colon carcinoma cells via modulation of multiple cell cycle-related signaling pathways.

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Abbreviations: EESB, ethanol extract of *Scutellaria barbata* D. Don; CRC, colorectal cancer; DMSO, dimethyl sulfoxide; MTT, 3-(4, 5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide

Key words: *Scutellaria barbata* D. Don, colorectal cancer, proliferation, cell cycle, p53, Akt

Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors and the leading cause of death around the world (1). Despite many advances in the field of cancer therapeutics, chemotherapy remains the main therapeutic approach for patients with advanced CRC. However, drug resistance and toxicity against normal cells limit the effectiveness of currently-used chemotherapies for CRC (2-4). Thus it is necessary to develop novel anticancer agents. Compared to modern chemotherapeutics natural products contain relatively fewer side effects and have been shown to possess beneficial therapeutic effects for cancer (5-7). Therefore, identifying naturally occurring agents is a promising approach for anticancer treatment.

Cancer cells are characterized by an unregulated increase in cell proliferation (8). Besides its significance for tumor biology, the uncontrolled proliferation is an important prognostic indicator for various cancers. Eukaryotic cell proliferation is regulated by the cell cycle, and G1 to S transition is one of the two main checkpoints used by cells to control the cell cycle progress (9). G1/S progression is highly regulated by cyclin D1 and cyclin-dependent kinase 4 (CDK4) (10,11). An unchecked or hyperactivated cyclin D1/CDK4 complex often leads to uncontrolled cell division and malignancy (12,13). As a proliferation inhibitor, p21 protein plays a role in G1 arrest by binding to and inhibiting the activity of Cyclin-CDK complexes (14). In addition, p21 also binds to proliferating cell nuclear antigen (PCNA), a processivity factor for DNA polymerase, inhibiting PCNA-dependent DNA replication (15). The decrease of p21 expression is associated with the promotion of tumor formation and a poor prognosis in many types of cancer (16).

The process of cell cycle is mediated by multiple intracellular signaling transduction cascades including Akt and p53 pathways. PI3K-dependent Akt pathway is essential for cell proliferation and survival and has been shown to be activated in several cancer types (17-22). After activation by extracellular stimuli, PI3K is able to phosphorylate PI(4)P and PI(4,5)

P2 to generate PI(3,4)P2 and PI(3,4,5)P3, respectively. These lipids serve as plasma membrane docking sites for proteins containing pleckstrin-homology (PH) domains, such as Akt and its upstream activator PDK1. The colocalization of PDK1 and Akt in plasma membrane results in the phosphorylation of Akt leading to its activation (23). Akt promotes cell survival by inhibiting apoptosis and/or by promoting cell cycle progression (24,25). Akt upregulates the expression of cyclin D1 through phosphorylating GSK3β. Phosphorylation of GSK3β decreases its kinase activity on cyclin D1, which subsequently prevents the nuclear export and the cytoplasmic proteasomal degradation of cyclin D1 (26,27). In addition, activation of Akt pathway negatively regulates p21 expression (28). The tumor suppressor p53 is a transcription factor that responds to certain stresses to preserve genomic integrity by arresting cell cycle progression (29,30). p53 normally is a short-lived protein that is maintained at low levels in cytoplasm, but in response to DNA-damaging agents and nucleotide depletion, the p53 protein is phosphorylated and accumulates in the nucleus, in which it induces the expression of various critical genes such as p21. Therefore, inhibition of excessive cell proliferation via modulation of Akt and p53 pathways and the expression of the downstream cell cycle-related genes (31) has become a major focus for cancer chemotherapies.

As a well-known traditional Chinese folk medicine, *Scutellaria barbata* D. Don (SB) has long been used as an important component in many Chinese medicine formulas to treat various types of cancer (32-35). Previous studies proposed that extracts of SB (ESB) possess antitumor activity to suppress the growth of many types of cancer including CRC both *in vitro* and *in vivo* (36-42). In addition, we recently reported that ESB is able to induce cancer cell apoptosis via activation of the mitochondrion-dependent pathway and inhibit tumor angiogenesis through suppression of Hedgehog signaling (43-45). To further elucidate the precise mechanism of the potential tumoricidal activity of SB, in the present study we investigated its effect on the proliferation of human colon carcinoma HT-29 cells and investigated the underlying molecular mechanism.

Materials and methods

Materials and reagents. 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). Cyclin D1, CDK4, p21, PCNA antibodies, horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Cell Signaling (Beverly, MA, USA). Bio-Plex phosphoprotein assay kits were purchased from Bio-Rad (Hercules, CA, USA). BCA Protein Assay kit was purchased from Tiangen Biotech Co., Ltd. (Beijing, China). All the other chemicals, unless otherwise stated, were obtained from Sigma Chemicals (St. Louis, MO, USA).

Preparation of ethanol extract of Scutellaria barbata D. Don (EESB). EESB was prepared as previously described (43). 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%.

Cell culture. Human colon carcinoma HT-29 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in DMEM containing 10% (v/v) FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in a 37˚C humidified incubator with 5% CO_2.

Cell viability evaluation. Viability of HT-29 cells was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Cells were seeded into 96-well plates at a density of $1x10^4$ cells/well in 100 μl medium. The cells were treated with various concentrations of EESB for different periods of time. At the end of the treatment, 10 μl MTT (5 mg/ml in phosphate buffered saline, PBS) was 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, model ELX800 (BioTek, USA).

Colony formation assay. HT-29 cells ($2x10^5$) were seeded into 6-well plates in 2 ml medium and treated with various concentrations of EESB for 24 h. The cells were then diluted in fresh medium in the absence of EESB and reseeded into 6-well plates at a density of $1.5x10^3$ cells/well. After incubation for 7 days in a 37˚C humidified incubator with 5% CO_2, the colonies were counted under a microscope. Cell survival was calculated by normalizing the survival of the control cells as 100%.

Cell cycle analysis by flow cytometry. 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, HT-29 cells were harvested and adjusted to a density of $1x10^6$ 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 Inc., Topsham, ME, USA).

RT-PCR analysis. HT-29 cells were seeded into 6-well plates at a density of $2x10^5$ cells/well and treated with various 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 cyclin D1, CDK4, PCNA and p21 by PCR. GAPDH was used as an internal control. The sequences of the primers of cyclin D1, CDK4, PCNA, p21 and GAPDH were: cyclin D1 forward 5'-TGG ATG CTG GAG GTC TGC GAG GAA-3' and reverse 5'-GGC TTC GAT CTG CTC CTG GCA GGC-3' (Tm=55˚C, 573 bp); CDK4 forward 5'-CAT GTA GAC CAG GAC CTA

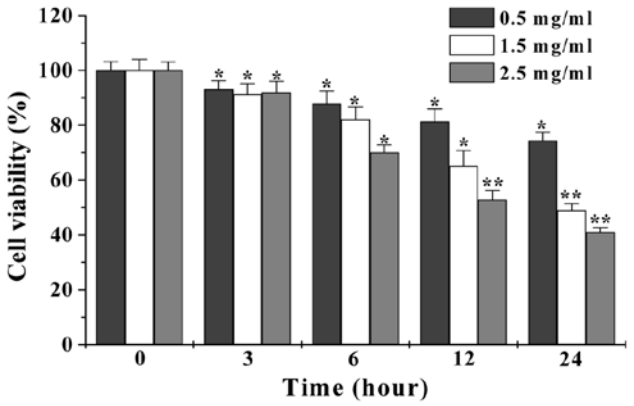

Figure 1. Effect of EESB on HT-29 cell viability. Cells were incubated with different concentrations of EESB for indicated time periods. Cell viability was determined by MTT assay. The data were normalized to the viability of control cells (100%, treated with 0.5% DMSO vehicle). Data shown are averages with SD (error bars) from three independent experiments. *P<0.05, **P<0.01, vs. control cells.

AGC-3' and reverse 5'-AAC TGG CGC ATC AGA TCC TAG-3' (Tm=58˚C, 206 bp); PCNA forward 5'-GCT GAC ATG GGA CAC TTA-3' and reverse 5'-CTC AGG TAC AAA CTT GGT G-3' (Tm=56˚C, 610 bp); p21 forward 5'-GCG ACT GTG ATG CGC TAA TGG-3', reverse 5'-TAG AAA TCT GTC ATG CTG GTC TGC-3' (Tm=55˚C, 358 bp); GAPDH forward 5'-CG ACC ACT TTG TCA AGC TCA-3' and reverse 5'-AG GGG TCT ACA TGG CAA CTG-3' (Tm=58˚C, 240 bp). Samples were analyzed by gel electrophoresis (1.5% agarose).

Western blot analysis. HT-29 cells ($5x10^5$) were seeded into culture flask and treated with various concentrations of EESB for 24 h. The treated cells were lysed with cell lysis buffer and centrifuged at 15,000 x g for 15 min followed by determination of protein concentration in supernatants. Equal protein per lysate was resolved on Tris-glycine gel, transferred onto PVDF membrane, and blocked for 2 h with 5% nonfat dry milk. Membranes were incubated with desired primary antibody cyclin D1, CDK4, p21, PCNA and β-actin (at a dilution of 1:1000) overnight at 4˚C and then with appropriate HRP-conjugated secondary antibody followed by enhanced chemiluminescence detection.

Bio-Plex phosphoprotein assay. HT-29 cells ($2.5x10^5$) were seeded into 25 cm^2 flasks in 5 ml medium and treated with 1.5 mg/ml of EESB for 24 h. Treated cells were lysed using a commercially available lysis kit and centrifuged at 14,000 x g for 15 min. The protein extracts were quantified by BCA protein assay. The presence of p-AKT, p-p53 was detected using a bead-based multiplex assay for phosphoproteins according to the manufacturer's protocol (Bio-Rad). Data were collected and analyzed using the Bio-Plex 200 suspension array system (Bio-Rad).

Statistical analysis. Data were analyzed using the statistical software SPSS13.0. Statistical analysis of the data was performed with Student's t-test and One-way analysis of variance (ANOVA). P-values <0.05 was considered as significant.

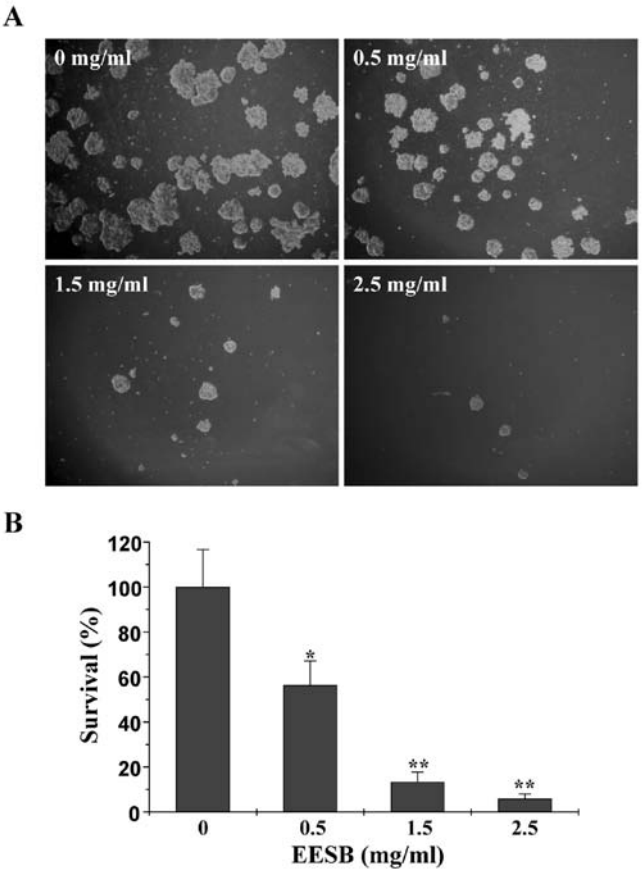

Figure 2. Effect of EESB on HT-29 cell survival. Cells were treated with various concentrations of EESB for 24 h and then incubated in fresh medium without EESB for another 7 days. (A) The photographs were taken at a magnification of x40 using phase-contrast microscopy. Images are representative of three independent experiments. (B) The data were normalized to the survival of control cells. Data are averages with SD (error bars) from three independent experiments. *P<0.05, **P<0.01, vs. control cells.

Results

EESB suppressed HT-29 cell proliferation. We first performed MTT assay to examine the effect of EESB on HT-29 cell viability. As shown in Fig. 1, treatment with 0.5-2.5 mg/ml of EESB for 3-24 h, respectively reduced cell viability by 6.92-25.59, 8.65-51 or 8.07-59.1%, compared to untreated control cells (P<0.01 or 0.05). We further verified these results using a colony formation assay. As shown in Fig. 2A and B, treatment with 0.5, 1.5 and 2.5 mg/ml of EESB for 24 h reduced the cell survival rate by 43.70, 86.67 and 94.07% (P<0.01 or 0.05, vs. control). Thus, EESB inhibits CRC cell proliferation in a dose- and time-dependent manner.

EESB inhibited G1/S cell cycle progression in HT-29 cells. The effect of EESB on cell cycle was evaluated by FACS analysis with PI staining. As shown in Fig. 3, the percentage of HT-29 cells in S-phase following treatment with 0, 0.5, 1.5 or 2.5 mg/ml of EESB was 38.97, 33.22, 29.06 or 24.85%, respectively (P<0.05), suggesting that EESB-caused inhibition of HT-29 cell proliferation is mediated by the blockade of cell cycle G1-S progression.

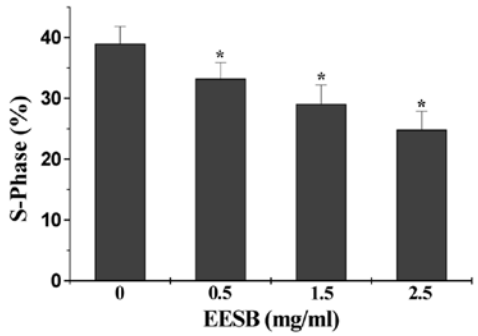

Figure 3. Effect of EESB on the cell cycle progression in HT-29 cells. Cells were treated with various concentrations of EESB for 24 h, stained with PI, and analyzed by FACS. The proportion of DNA in S-phase was calculated using ModfitLT version 3.0 software. Data shown are averages with SD (error bars) from three independent experiments. *P<0.05, vs. control cells.

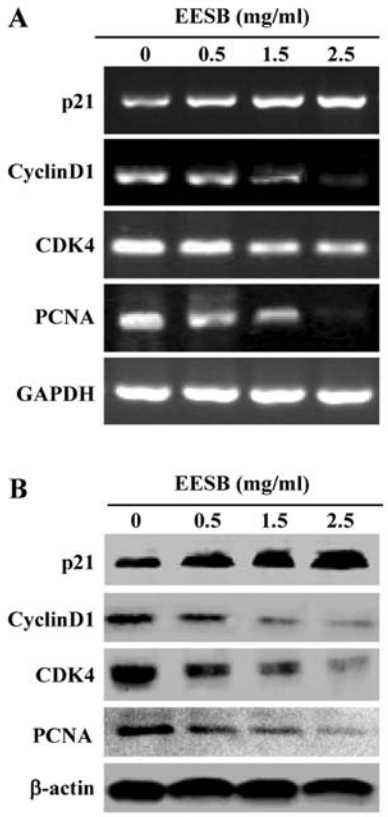

Figure 4. Effect of EESB on the expression of p21, cyclin D1, CDK4 and PCNA in HT-29 cells. Cells were treated with various concentrations of EESB for 24 h. (A) The mRNA levels of p21, cyclin D1, CDK4 and PCNA were determined by RT-PCR. (B) The protein levels of p21, cyclin D1, CDK4 and PCNA were determined by western blotting. GAPDH and β-actin were used as the internal controls for the RT-PCR or western blot assays, respectively. Data are representative of three independent experiments.

EESB altered the expression of cell cycle-regulatory factor in HT-29 cells. We next examined the effect of EESB on the expression of cell cycle-regulatory factors. Data from RT-PCR and western blot analysis showed that EESB treatment profoundly enhanced antiproliferative p21 expression, but suppressed the expression of pro-proliferative PCNA, cyclin D1 and CDK4 in HT-29 cells, at both transcriptional and translational levels (Fig. 4).

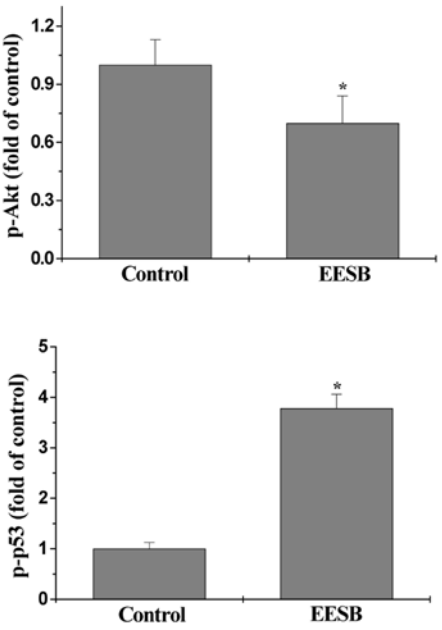

Figure 5. Effect of EESB on the phosphorylation of AKT and p53 in HT-29 cells. After treatment with 1.5 mg/ml of EESB for 24 h, the phosphorylation levels of AKT and p53 in HT-29 cells were determined by Bio-Plex Phosphoprotein assay. The data were normalized to the phosphorylation level within controls and represented as fold of control. Data are averages with SD (error bars) from three independent cell-based experiments. *P<0.05, vs. controls.

EESB modulated Akt and p53 pathways in HT-29 cells. The activation (phosphorylation) of Akt and p53 was determined by Bio-Plex Phospho-protein assay. As shown in Fig. 5A and B, after EESB treatment the phosphorylation level of Akt in HT-29 cells was significantly decreased, whereas that of p53 was significantly increased, as compared to controls (P<0.05). These data suggest that EESB modulates the activation of multiple cell cycle-related signaling pathways.

Discussion

Natural products have been used in China for thousands of years as alternative remedies for a variety of diseases including cancer. Among Chinese traditional medicinal plants, *Scutellaria barbata* D. Don (SB) has been traditionally used for the treatment of inflammation, such as hepatitis, osteomyelitis, gynecological diseases, due to its antibacterial activity. Recently, SB has gained increasingly attention to its usage as an antitumor herb (32-42). Similar to other medicinal herbs, SB is considered to be a multi-target agent that exerts therapeutic function in a holistic way. Previously, we reported that SB promotes the apoptosis of human colorectal carcinoma cells *in vitro* and inhibits tumor angiogenesis *in vivo* via suppression of the Hedgehog pathway (43-45). To further elucidate the mechanism of the tumoricidal activity of SB, herein we investigated its effect on the proliferation of human colon carcinoma HT-29 cells.

In the present study, we found that ethanol extract of SB (EESB) inhibited proliferation of HT-29 cells in a dose- and time-dependent manner. Eukaryotic cell proliferation is regulated by the cell cycle, which consists of four periods: S phase (DNA synthesis phase), M phase (mitosis), G1 and G2 phase.

At different phases, passage through the cell cycle is governed by sequential activation and subsequent inactivation of a series of cyclin-dependent kinases (CDKs), whose activity depends on interactions with timely expressed cyclins and cyclin-dependent kinase inhibitors (CDKIs). By using FACS analysis with PI staining we found that the inhibitory effect of EESB on HT-29 cell proliferation was associated with the blockage of G1 to S progression. As one of the main checkpoints of cell cycle, G1/S transition is responsible for initiation and completion of DNA replication (9), which is strongly regulated by the combined activity of the cyclin D1/CDK4 complex (10,11). The proliferation inhibitor p21 plays an inhibitory role in G1/S progression by inhibiting the activity of cyclin-CDK complexes as well as the PCNA-dependent DNA replication (14,15). Consistent with the effect on G1/S arrest, EESB upregulated p21 expression and downregulated the expression of PCNA, cyclin D1 and CDK4 in HT-29 cells. The process of cell cycle is mediated by multiple intracellular signaling transduction cascades including Akt and p53 pathways. Activation of Akt pathway promotes cell proliferation by positively regulating cyclin D1 expression and downregulating the expression of p21 (26-28). In response to DNA-damaging agents, the tumor suppressor p53 protein is phosphorylated and induces the expression of various critical genes including p21. By using Bio-plex cytokine assay, herein we found that EESB treatment significantly suppressed that activation of Akt but increased the phosphorylation level of p53 in HT-29 cells.

In conclusion, we demonstrated that EESB inhibited the proliferation of HT-29 cells via G1/S cell cycle arrest, which was mediated by the modulation of p53 and Akt pathways. Together with our previous studies, it is suggested that *Scutellaria barbata* D. Don inhibits cancer progression via multiple mechanisms, including induction of cancer cell apoptosis, inhibition of cell proliferation and tumor angiogenesis.

Acknowledgements

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