Inhibition of tumor angiogenesis by TTF1 from extract of herbal medicine

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AIM: To study the inhibition of tumor angiogenesis by 5,2,4′-trihydroxy-6,7,5′-trimethoxyflavone (TTF1) isolated from an extract of herbal medicine Sorbaria sorbifolia.

METHODS: Angiogenic activity was assayed using the chick embryo chorioallantoic membrane (CAM) method. Microvessel density (MVD) was determined by staining tissue sections immunohistochemically for CD34 using the Weidner capillary counting method. The mRNA and protein levels of vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor 2 (VEGFR2, Flk-1/KDR), basic fibroblast growth factor (bFGF), cyclo-oxygenase (COX)-2 and hypoxia-inducible factor (HIF)-1α were detected by quantitative real-time polymerase chain reaction and Western blotting analysis.

RESULTS: The TTF1 inhibition rates for CAM were 30.8%, 38.2% and 47.5% with treatment concentrations of 25, 50 and 100 μg/embryo × 5 d, respectively. The inhibitory rates for tumor size were 43.8%, 49.4% and 59.6% at TTF1 treatment concentrations of 5, 10, and 20 μmol/kg, respectively. The average MVD was 14.2, 11.2 and 8.5 at treatment concentrations of 5 μmol/kg, 10 μmol/kg and 20 μmol/kg TTF1, respectively. The mRNA and protein levels of VEGF, KDR, bFGF, COX-2 and HIF-1α in mice treated with TTF1 were significantly decreased.

CONCLUSION: TTF1 can inhibit tumor angiogenesis, and the mechanism may be associated with the down-regulation of VEGF, KDR, bFGF, HIF-1α and COX-2.

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Key words: Chinese herbal medicine; Sorbaria sorbifolia; TTF1; Inhibition; Tumor angiogenesis

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Able target for tumor treatment[1]. Anti-angiogenesis is an important strategy for tumor therapy[2]. Many studies have demonstrated that tumor angiogenesis can be inhibited by the flavones present in Chinese herbal medicines, including apigenin, silibinin, quercetin, wogonin, genistein and luteolin[3-9]. Previously, we have reported that acetic ether extracts of the medicinal plant Sorbaria sorbifolia (S. sorbifolia) inhibits the growth of HepG-2 cells[10] and mouse S180 sarcoma, down-regulates the levels of tumor necrosis factor (TNF)-α and interleukin (IL)-2, and reduced the cellular activity of natural killer cells[11]. In addition, extracts inhibit the placental glutathione S transferase formation of positive foci in hepatoma precancerous rats and down-regulated the expression of p53 and Bel-2. They increase the activity of superoxide dismutase and glutathione peroxidase and decrease the nitrogen monoxide (NO) synthase activity and malondiadehyde and NO concentrations[12,13]. Six compounds have been identified in the S. sorbifolia acetic ether extracts, including 5,2’,4’-trihydroxy-6,7,5’-trimethoxyflavone (TTF1), 5,7-dihydroxy-8-methoxyflavone, rutin, quercetin, daucosterol, benzoate and p-hydroxybenzoic acid, and TTF1 was the first active flavonoid compound identified[14]. After testing the six compounds, we found that TTF1 inhibited vascular endothelial growth factor (VEGF) expression in HepG-2 cells and VEGF165-induced human umbilical vein endothelial cells proliferation and vascular endothelial growth factor receptor 2 (VEGFR2, Flk-1/KDR) protein expression[15]. This study focused on the effect of TTF1 specifically on the inhibition of tumor angiogenesis.

MATERIALS AND METHODS

Extraction of TTF1

TTF1 was separated using the water extraction and alcohol precipitation method from 10 kg S. sorbifolia (collected from Jilin Province) as previously described[16].

Cell culture

The HepG-2 cell line was purchased from KeyGEN Co., Ltd. (Nanjing, China). Cells were grown in RPMI1640 supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/L streptomycin. Cells were cultured at 37 °C in a humidified incubator containing 5% CO₂. Cells in the logarithmic growth phase were used for tests.

Chick embryo chorioallantoic membrane assay

Angiogenic activity was assayed using a chick embryo chorioallantoic membrane (CAM) as described previously[17]. HepG-2 cell resuspensions (1 × 10⁶) were inoculated into the chick embryo CAM. Using 4-d-old chick embryos in shells, 50 μL of different concentrations of TTF1, apigenin (KeyGEN), and normal saline were added to the chick chorioallantoic membrane once per day for 5 d. Each experimental group included five eggs, and experiments were repeated five times. Chorioallantoic membranes were collected for microscopy and photographic documentation. Five visual fields were randomly chosen for analyzing the angiogenesis inducing rate and inhibitory rate using the SmartScape microscope photography analysis system.

Inducing rate (%) = (vascular branchpoint number after inoculating tumor cells minus the vascular branchpoint number in non-inoculated tumor cells/the vascular branchpoint number in non-inoculated tumor cells) × 100%

Inhibitory rate (%) = (vascular branchpoint number after inoculating tumor cells minus the vascular branchpoint number with drug treatment/the vascular branchpoint number after inoculating tumor cells) × 100%

Nude mouse HepG-2 tumor model

BALB/c nude mice were obtained from the Laboratory Animal Center of the Academy of Military Medical Sciences (Jilin, China). All studies were in compliance with guidelines of the Institutional Animal Care and Use Committee. 0.1 mL HepG-2 cell resuspensions (1× 10⁶) were transplanted into the armpits of test mice subcutaneously as an experimental model. Ten days after HepG-2 cell transplantation, 40 mice bearing tumors were selected and divided into five groups, and orally administered 5, 10 or 20 μmol/kg of TTF1 or 10 μmol/kg of apigenin once a day for 10 d. The control group was treated with normal saline. Mice were sacrificed and the tumors were collected and weighed. The tumor inhibition rate was calculated as follows: inhibition rate (%) = (1- the tumor weight in treatment group/the tumor weight in control group) × 100%. Samples were fixed in a 10% formaldehyde solution to prepare the slides for hematoxylin and eosin staining and microscopy.

Immunohistochemistry

Tissues were fixed in 10% buffered formalin and embedded in paraffin. Immunodetection of blood vessels in mouse tumor sections was performed with an anti-CD34 Ab (Boshide Biotechnology Company, Wuhan, China). Sections were incubated with a biotinylated anti-rat Ab (CD34) and then with peroxidase-conjugated streptavidin (Boshide Biotechnology Company, Wuhan, China). To quantify angiogenesis, microvessel density (MVD) was determined by staining tissue sections immunohistochemically for CD34 using the Weidner capillary counting method[18]. Entire sections were scanned under low magnification, and vascularization was subjectively graded. Three highly vascularized areas per tumor were then evaluated at low magnification (× 200). Any brown-staining CD34 distinct from adjacent microvessels, tumor cells, or other stromal cells was considered a single countable microvessel. The total number of microvessels was determined from five vessels in each area, and the average number was recorded for each tumor. To test TTF1 treatment effect on VEGF and basic fibroblast growth factor (bFGF) expression in tumor, the slides were prepared by following the protocol of S-P Kit. Using the double-blind method, the pictures from at least five representative high-power fields were observed in each slice, and no less than 100 cells in each field were counted for analysis.
Western blotting analysis

Tumors were lysed in lysis buffer (Pierce Roche, United States) and then centrifuged at 12,000 g for 15 min. Protein concentration was determined using the BCA kit (Pierce Rockford, United States) following the manufacturer’s instructions. Seventy µg of protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to a polyvinylidene fluoride membrane ( Pall Corporation, Port Washington, NY, United States). After blocking for 1 h with 5% milk in tris-buffered saline and tween 20, the primary antibody (anti-VEGF; KDR, bFGF; COX-2 or HIF-1α; 1:400) (Boshide Biotechnology Company) was added and incubated at 4 °C overnight. After incubation with secondary antibodies (1:5000), membranes were visualized by chemiluminescence. The intensity of protein bands was quantitatively determined using a ultraviolet crosslinkers (Bio-Rad, United States) and normalized with the intensity of Actin band in each gel.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from tumors using the RNeasy Plus Mini Kit (KeyGEN) following the manufacturer’s instructions. cDNA was generated with the iScript Select cDNA Synthesis Kit (KeyGEN) and analyzed by quantitative real-time polymerase chain reaction (PCR) using SyberGreen qPCR primer assays (KeyGEN) and the iCycler iQ multicolor real time PCR detection system (KeyGEN). Relative expression levels were normalized against β-actin expression run concurrently as a reference control. The primers used were as follows: VEGF (forward, 5'-TACGGTTGTTGCCCTGCTGTG-3'; reverse, 5'-GGCCTCCGGACCAAGTGC-3'; amplicon size of 400 bp), KDR (forward, 5'-AGCCTGTGGCACCCACGATC-3'; reverse, 5'-GGCAATCACCGCTGCCTA-3'; amplicon length of 338 bp); COX-2 (forward, 5'-TTGCCGGACTCCCTTGGGTGTG-3'; reverse, 5'-CTCTCCGGCCACACGAAACCG-3'; amplicon length of 397 bp); HIF1-α (forward, 5'-ACAGCAAGCCAGAGCATGGCAG-3'; reverse, 5'-TGGCTACACGTCTCTGCGCA-3'; amplicon length of 724 bp); β-actin (forward, 5'-GGTCGTGTGTCGCAACCGGCTC-3'; reverse, 5'-CAAACATGATCTGGGTCA TCTTTC-3'; amplicon length of 353 bp).
of 5 μmol/kg, 10 μmol/kg and 20 μmol/kg TTF1, respectively, and it decreased in a dose-dependent manner (Figure 4C). These results indicated that TTF1 inhibited HepG-2 cell-induced angiogenesis in mouse tumors.

**Effect of TTF1 on angiogenesis regulation factors**

To test whether TTF1 affects the expression of the angiogenesis regulation factors including VEGF, KDR, bFGF, COX-2 and HIF-1α, we analyzed the protein levels of these factors in HepG-2 cell-induced tumors in mice. Immunohistochemistry results (as shown in Figures 5 and 6) showed the effect of TTF1 on the expression of VEGF and bFGF. In the control group, expression of VEGF and bFGF was demonstrated by brown staining of the cytoplasm and membrane of cancer cells, with a focal or diffuse distribution (Figures 5E and 6E). In the TTF1 treatment group, the brown-stained VEGF and bFGF cancer cells were significantly reduced, and most of the cells were stained blue (negative), as shown in Figures 5C and 6C. Combining these results showed that treatment with TTF1 resulted in significant down-regulation of VEGF and bFGF expression in tumors (Figure 7). Western blotting indicated that the protein levels of VEGF, KDR, bFGF, COX-2 and HIF-1α were lower in tumors that were treated with TTF1 than in control tumors (Figure 8A and B). We found that the decrease in protein levels occurred in a dose-dependent manner and showed significant differences at the 10 μmol/kg and 20 μmol/kg doses (as shown in Figure 8A and B) when compared to the controls. To explore whether TTF1
inhibits gene transcription to decrease the expression of these angiogenesis regulation factors, quantitative real-time PCR (qRT-PCR) was performed to determine the mRNA levels of VEGF, KDR, bFGF, COX-2 and HIF-1α in mice treated with TTF1. Representative qRT-PCR graphs for these genes is shown in Figure 8C-E. The effect of TTF1 on the mRNA levels of VEGF, KDR, bFGF, COX-2 and HIF-1α was consistent with the effect TTF1 on their protein levels. Our results indicate that TTF1 inhibits tumor angiogenesis by decreasing the RNA and protein levels of angiogenesis regulation factors (VEGF, KDR, bFGF, COX-2 and HIF-1α).

DISCUSSION

*S. sorbifolia* is a Chinese medicinal plant that grows on

Figure 4  Microvessels in tumor tissue angiogenesis in HepG-2-transplanted nude mice. Brown staining indicates CD34 positive cells. A: Control, HepG-2-transplanted nude mouse treated with normal saline; B: TTF1, HepG-2-transplanted nude mouse treated with TTF1; C: Different doses of compound were used as treatments in the HepG-2-transplanted nude mice as follows: sTTF1 (5 μmol/kg); mTTF1 (10 μmol/kg); ITTF1 (20 μmol/kg); apigenin (40 μmol/kg); and Con (control group treated with normal saline). *P < 0.05, **P < 0.01 vs control group.

Figure 5  Down-regulation of expression of vascular endothelial growth factor by TTF1 (*× 200*). Brown staining indicates vascular endothelial growth factor positive. Different doses of compound were used as treatments in the HepG-2-transplanted nude mice as follows: A: sTTF1 (5 μmol/kg); B: TTF1 (10 μmol/kg); C: ITTF1 (20 μmol/kg); D: Apigenin (40 μmol/kg); E: Con (control group treated with normal saline).
Changbai Mountain. Our group began systematic research on its medicinal properties in 2002. An earlier study showed that an acetic ether extract of *S. sorbifolia* has anti-tumor, liver protective and anti-inflammatory effects. Six chemicals were identified in the acetic ether extract, and the novel monomeric compound TTF1 was separated for the first time.

Angiogenesis mainly occurs during embryo development as well as in some pathological conditions, such as damage repair, inflammation, and in particular, tumor growth and metastasis\(^ {16} \). CAM is the ideal *in vivo* model to study angiogenesis and anti-vascular formation. Our research demonstrated that TTF1 inhibited HepG-2-induced CAM angiogenesis. We also found that TTF1 inhibited tumor growth in HepG-2-transplanted nude mice with an inhibition rate similar to that of apigenin, a flavone extracted from another Chinese medicinal plant that is currently in clinical use.

MVD is a marker to assess the level of tumor angiogenesis. An increase in MVD in tumor tissue suggests a fast-growing and potentially more metastatic tumor. After treatment with TTF1 on the transplanted tumors of nude mice, MVD decreased, suggesting that it inhibited tumor angiogenesis. VEGF is the most important inducing factor for angiogenesis, which specifically stimulates the proliferation of vascular endothelial cells and angiogenesis. VEGF primarily functions through dimerization with KDR, and its intracellular tyrosine residues autophosphorylate after VEGF and KDR bind together. bFGF is another important inducing factor for angiogenesis. Tumor cells produce bFGF, and induce the vascular endothelial cells to produce bFGF, at the same time, increasing angiogenesis\(^ {17,18} \). The expression levels of VEGF, VEGFR and bFGF were down-regulated after treatment with TTF1, suggesting that TTF1 may inhibit tumor growth through decreasing angiogenesis-inducing factors in HepG-2-transplanted nude mice.
An insufficient blood supply in fast-growing tumor tissues may cause hypoxia. HIF-1α is the transcription factor that regulates gene transcription during tissue hypoxia. TTF1 may inhibit expression of HIF-1α by suppressing its association with the regulatory sequences of VEGF and bFGF, and therefore resulting in decreased transcription. The expression of VEGF and bFGF may further decrease the expression of KDR through negative feedback. Recent studies have shown that COX-2 is associated with tumor formation, development, and angiogenesis \[19\]. COX-2 was down-regulated after TTF1 treatment in tumor tissues, in accordance with the down-regulation of the other angiogenesis-inducing factors VEGF, bFGF, and therefore resulting in decreased VEGF and bFGF, and HIF-1α expression. TTF1 may inhibit expression of HIF-1α by suppressing its association with the regulatory sequences of VEGF and bFGF, and therefore resulting in decreased transcription. The expression of VEGF and bFGF may further decrease the expression of KDR through negative feedback. Recent studies have shown that COX-2 is associated with tumor formation, development, and angiogenesis \[19\]. COX-2 was down-regulated after TTF1 treatment in tumor tissues, in accordance with the down-regulation of the other angiogenesis-inducing factors VEGF, bFGF, and therefore resulting in decreased VEGF and bFGF, and HIF-1α expression. TTF1 inhibits tumor growth.

S. sorbifolia is a rosaceous plant that grows extensively in Changbai Mountain, in Yunnan, Guizhou, Sichuan, Hubei, Gansu and Ningxia Provinces. It is traditionally used in activating blood, dissolving stasis, reducing swelling, easing pain, and healing fractures and injuries from falls \[20\]. It is a perennial herbaceous plant that has low toxicity and is liver-protective. Our study explored the inhibitory effect of TTF1 on tumor growth and angiogenesis. Further study needs to focus on the different regulatory factors and their interaction using molecular biological techniques after TTF1 inhibition of tumor angiogenesis. The relationship of the chemical structure of TTF1 to its activity should be studied, so that further structural modification may lead to new inhibitors of tumor angiogenesis with better curative effect and easier production. Moreover, further study is also needed to determine whether there are other pathways (such as inducing apoptosis, regulation of nuclear factor-kB or mitogen-activated protein kinase pathways) through which TTF1 inhibits tumor growth.

Figure 8 TTF1 decreases the gene and protein level of angiogenesis regulation factors. Western blotting analysis was used to determine the protein levels of VEGF, KDR, bFGF, COX-2, and HIF-1α. A: Tumor tissues were centrifuged at 12,000 g for 15 min and the supernatant (70 μg/lane) was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Western blotting analysis was performed to detect the protein levels of VEGF, KDR, bFGF, COX-2, and HIF-1α. Different compounds and dosages were used as treatments in the HepG-2-transplanted nude mice as follows: sTTF1 (5 μmol/kg); mTTF1 (10 μmol/kg); ITTF1 (20 μmol/kg); and Con (control group treated with normal saline): B: The intensity of the VEGF, KDR, bFGF, COX-2, and HIF-1α protein bands were determined and normalized with β-actin’s intensity by using the ultraviolet crosslinkers imager and plotted (\(*P < 0.05\) vs control group). C-E: Quantitative real-time PCR was performed to determine the mRNA expression levels of VEGF, KDR, bFGF, COX-2, and HIF-1α (\(*P < 0.05\) vs control group).
COMMENTS

Background

Anti-angiogenesis is an important strategy for tumor therapy. Many studies have demonstrated that tumor angiogenesis can be inhibited by the flavones present in Chinese herbal medicine. Previously, the authors reported that acetic ether extracts of the medicinal plant Sorbaria sorbifolia (S. sorbifolia) inhibits the growth of HepG-2 cells and mouse S180 sarcoma, down-regulates the levels of tumor necrosis factor-α and interleukin-2, and reduces the activity of natural killer cells. 5,7,4′-trihydroxy-6,7,5′-trimethoxyflavone (TTF1) was the first active flavonoid compound identified in S. sorbifolia. This study focused on the effect of TTF1 specifically on the inhibition of tumor angiogenesis.

Research frontiers

Identification of the compounds responsible for the anti-tumor angiogenesis effects of Chinese herbal medicine is a research hotspot. The study used the anti-tumor drug apigenin, which is currently used clinically, as a positive control and comparison for TTF1 treatment. Its mechanism of anti-tumor activity includes inhibition of tumor angiogenesis, induction of tumor cell apoptosis, disturbing cellular signal pathways, and anti-oxidation. The experiments showed that TTF1 had an inhibitory effect on tumor angiogenesis, as did apigenin.

Innovations and breakthroughs

Six compounds were identified in acetic ether extracts of S. sorbifolia, including TTF1, 5,7,4′-trihydroxy-6,7,5′-trimethoxyflavone (TTF1), and p-hydroxybenzoic acid and TTF1 was the first active flavonoid compound identified in S. sorbifolia. After testing the six compounds, the authors found that TTF1 inhibited vascular endothelial growth factor (VEGF) expression in HepG-2 cells and VEGF165-induced human umbilical vein endothelial cell proliferation and vascular endothelial growth factor receptor 2 (VEGFR2, Flk-1/KDR) protein expression. The study explored the inhibitory effect of TTF1 on tumor growth and tumor angiogenesis.

Applications

The study results suggest that the TTF1 extracts of the medicinal plant S. sorbifolia is a potential therapeutic compound that could be used for tumor inhibition.

Terminology

Sorbaria sorbifolia (S. sorbifolia) is a Chinese medicinal plant that grows on Changbai Mountain. An earlier study has shown that an acetic ether extract of S. sorbifolia has anti-tumor, liver protective and anti-inflammatory effects. Chick embryo chorionicallantoic membrane, is the ideal in vivo model for studying angiogenesis and anti-vascular formation. Microvessel density (MVD), is a marker to assess the level of tumor angiogenesis. An increase in MVD in tumor tissue suggests a fast-growing and potentially more metastatic tumor.

Peer review

The present paper examining the effects of extracts of the Chinese herb S. sorbifolia (TTF1) on tumor growth is work that extends and builds upon previously published work by this research group. The paper will gather a lot of interest amongst practicing gastroenterologists and oncologists.

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