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Antitumor and antiangiogenic activity of *Schisandra chinensis* polysaccharide in a renal cell carcinoma model

Hai-Ming Qu[a,1], Shi-Jian Liu[b,1], Chun-Ying Zhang[a,*]

[a] Department of Urology, Section 2, The Second Affiliated Hospital of Harbin Medical University, Harbin 150040, China
[b] Department of Nephrology, Section 1, The Second Affiliated Hospital of Harbin Medical University, Harbin 150040, China

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ABSTRACT

The aim of this study was to determine the antitumor and antiangiogenic effects of the *Schisandra chinensis* polysaccharides (SCP) in selected renal cell carcinoma (RCC) cells and evaluate its potential mechanism of action. In vitro, endothelial growth factor (VEGF) secretion by Caki-1 was blockaded in response to SCP treatment for 48 h. In vivo, a significant tumor growth inhibition effect was observed after SCP administration for 4 weeks. Moreover, SCP treatment decreased the level of VEGF, CD31 and CD34 in RCC tumor tissues. Further analysis of the tumor inhibition mechanism indicated that the number of apoptotic tumor cells increased significantly; the expression of Bax and p53 increased; and the expression of Bcl-2 decreased dramatically in transplanted tumor tissues following SCP administration. These results indicated that the potential mechanisms involved by which SCP exerted its antitumor and antiangiogenic activity might be associated with the up-regulation of Bax and p53, downregulation of Bcl-2, as well as the reduction of VEGF, CD31 and CD34 in xenografted tumors. These findings demonstrated that the SCP is a potential antitumor agent for RCC treatment.

1. Introduction

Renal cell carcinoma (RCC) is the most common malignancy of the adult kidney, and annual estimates of newly diagnosed cases have been steadily increasing [1,2]. Although nephrectomy is the most effective treatment at an early stage, therapeutic options for unresectable and/or metastatic RCC are limited due to an inherent tumor resistance to conventional chemotherapy and radiotherapy [3,4]. Because RCC is characterized by a lack of specific clinical signs that allow the diagnosis at an early stage, a high proportion of patients will have metastasis at the time of first diagnosis and confront with an often unpredictable course [5]. In view of these conditions, the discovery of new strategies or molecular targeting therapies for metastatic RCC remains a priority.

In recent years, novel agents for RCC targeting cancer-specific pathways have been developed based on the precise understanding of molecular mechanisms underlying the progression of RCC [6,7]. As a result of the hypervascularity in RCC, several new agents targeting the vascular endothelial growth factor (VEGF) pathway have demonstrated significant activity in patients with advanced RCC, such as sunitinib, sorafenib and pazopanib [8]. Although they are currently being used with some success in patients with advanced RCC, the effect is insufficient, and it is therefore necessary to discover new antiangiogenic targets for the treatment of RCC [9,10].

Schisandra chinensis is a traditional Chinese medicine and has been officially listed in the Chinese Pharmacopoeia as a tonic and sedative agent [11]. Chemical investigations of the extracts of *S. chinensis* have revealed the presence of amino acid, polysaccharide, sesquiterpene, vitamin, organic acid, volatile oil, especially bioactivie components lignan and triter-penenoid [12]. Current researches have revealed that polysaccharides from *S. chinensis* possess a large variety of beneficial effects including immunomodulating [13,14], anticancer [15,16], antioxidant [17], anti-diabetic [18] and anti-aging [19], etc. However, the anti-tumor and antiangiogenic activities of polysaccharides from *S. chinensis* on RCC have not been reported. Therefore, the aim of this study is to determine the antitumor and antiangiogenic action of SCP toward RCC, and gained insights into the mechanisms involved through which SCP exerts its effects.

2. Materials and methods

2.1. Materials

The ethanol-insoluble residue generated during lignans industrial production from *S. chinensis* via 75% ethanol extraction was

* Corresponding author. Tel.: +86 451 86605579; fax: +86 451 86605579.
E-mail address: chunyingzhang@outlook.com (C.-Y. Zhang).
[1] Contributed equally to this paper.

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purchased from Zhongxing Pharmaceutical Co., Ltd., Zhenjiang, Jiangsu Province, China.

2.2. Polysaccharide extraction

After the dried ethanol-insoluble residue of *S. chinensis* was rushed or ground into powder with a blender, the powders (500 g) were extracted three times with distilled water (3 h/time). The whole extract was concentrated under reduced pressure at 50 °C and added by ethanol (final concentration 75% (v/v)) to precipitate the crude polysaccharide at 4 °C overnight. The precipitate was collected by centrifugation (6000 rpm, 20 min) and redissolved in distilled water. Finally, the supernatant was lyophilized to give 53 g of crude *S. chinensis* polysaccharides (SCP).

2.3. Cell lines and cell culture

Human RCC cell line Caki-1 was purchased from American Type Culture Collection. They were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2 and subcultured after trypsinization.

2.4. Detection of VEGF secretion produced by Caki-1 cells

Caki-1 cells were seeded at 2×10^4 cells/well in 96-well culture plates in triplicates. Next day, the supernatants were discarded and the cells were washed twice with PBS. Thereafter cells were cultured in the presence/absence of SCP (100, 200 and 400 μg/ml) under hypoxic or normoxic conditions for 24 h. After treatment, the amounts of VEGF in each supernatant was determined using Quantikine human VEGF Immunoassay kits (R&D Systems, Minneapolis, MN) and were normalized versus total protein content as determined using the Bradford assay.

2.5. In vivo xenograft model

Seven-week-old male athymic BALB/c nude (nu/nu) mice were purchased from Experimental Animal Center of Harbin Medical University. Animals were housed in a specific pathogen-free facility under controlled temperature and humidity. The procedures involving animals and their care were conducted in accordance with institutional guidelines for Laboratory Animal Care of Experimental Animal Center, Harbin Medical University. Early passage Caki-1 cells were harvested and 5×10^6 cells were implanted subcutaneously into both flanks of each mouse. Treatment was initiated after 15 days when the tumors reached 100–150 mm^3 in volume. The animals were randomized into normal control and SCP-treated groups (5 mice per group). Group-I received vehicle only (5 ml/kg) via intra-peritoneal injection (i.p.) served as a normal control. Group-II received SCP at a dose of 400 mg/kg i.p. every third day for 4 weeks, respectively. Tumor volume (TV) was measured every 6 days by measuring two perpendicular dimensions (long and short) using a caliper and calculated as $TV = (a \times b^2)/2$, where a is the larger and b is the smaller dimension of the tumor [20]. Four weeks later, mice were sacrificed and the tumors were collected. A portion was fixed in10% formalin and embedded in paraffin to prepare the block for immunohistochemistry, and the other portions were frozen in liquid nitrogen and maintained at −80 °C.

2.6. Western blotting

Tumor tissue was homogenized in order to extract the cytosolic and nuclear contents with a commercially available kit (BD Bioscence). Protein content was measured by the Bradford assay using bovine serum albumin (BSA) as a standard. Equal amount of protein (30 μg) were resolved by 10–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Bio-Rad) by electroblot analysis. Nitrocellulose blots were blocked with 5% (w/v) nonfat dried-milk and incubated with the indicated primary antibody against p53 (1:10,000, Sigma, Alcobendas, Spain), Bax (1:200, Santa Cruz Biotechnology, Santa Cruz, CA) and Bcl-2 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) in Tris-buffered saline (TBS) overnight at 4 °C, and then the blots were washed and stained with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature. Immunostained proteins were visualized on X-ray film using the enhanced chemiluminescence detection system (Amersham–Pharmacia Biotech, Piscataway, NJ). A β-actin antibody (Merck, Darmstadt, Germany) (1:10,000) was used for loading control.

2.7. RNA extraction and reverse transcriptase–polymerase chain reaction (RT-PCR)

Total RNA was extracted from tumor samples using TRI® reagent (Biotech Labs, Houston, TX, USA) and RT-PCR was performed as described previously [21]. cDNA fragments were amplified by polymerase chain reaction using the following VEGF-specific primers: sense 5′-AGGAGGGCAGAATCATCACG-3′ and anti-sense 5′-CAAGGCCCACAG GGATTTTCT-3′.

2.8. Determination of VEGF

VEGF levels in tumor cytosolic extracts were determined using Quantikine human VEGF Immunoassay kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Data were normalized by the protein concentration in each sample.

2.9. Immunohistochemistry

Serial sections (5-μm-thick) were cut from each paraffin block and deparaffinized in xylene and rehydrated through a graded ethanol concentrations. Then, they were incubated with 3% hydrogen peroxide for 20 min at room temperature to inactivate endogenous peroxidase and heated in 0.1 M citrate buffer (pH 6.0) for 5 min in a microwave to retrieve antigens. After rinsing in TBS, non-specific sites binding to the first antibody were blocked with a blocking solution containing 2.5% bovine serum albumin (Sigma-Aldrich, St Louis, MO) and 2% normal goat serum (Vector Laboratories, Burlingame, CA) in TBS (pH 7.4) for 1 h. Afterwards, the sections were incubated overnight at 4 °C with the primary antibody against CD-31 (1:200 dilution in blocking solution; Santa Cruz Biotechnology, Santa Cruz, CA) and CD-34 (1:100 dilution in blocking solution; Santa Cruz Biotechnology, Santa Cruz, CA). Sections of tumor samples processed identically, but incubated with diluents (blocking solution) in the absence of any primary antibodies, were used as negative control. Then, the sections were washed in TBS and incubated for 20 min with the appropriate biotinylated secondary antibodies (Dako LSAB® System-HRP, Dako, Glostrup, Denmark) at a 1:500 dilution for 1 h. After an extensive wash in TBS, the sections were incubated with avidin–biotin–horseradish peroxidase complex (Dako) for 20 min at room temperature to localize bound antibodies. Staining of the protein was visualized by incubating sections with 3,3′-diaminobenzidine tetrahydrochloride (DAB) and lightly counterstaining them with hematoxylin. The different sections were analyzed per xenograft tumor. For histological assessment, CD31-positive and CD34-positive micro vessels were viewed under Nikon 80i microscope at magnifications of ×600 and