Phytomedicine 33 (2017) 21–27

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.com/locate/phymed

Angelica polysaccharides inhibit the growth and promote the apoptosis of U251 glioma cells *in vitro* and *in vivo*

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ARTICLE INFO

Keywords:
Angelica polysaccharides
Glioma
Cell proliferation
Cell cycle
Apoptosis

Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Aps, angelica polysaccharides
AS, angelica sinensis (oliv.) diels
Cis, cisplatin
EMT, esenchymal transition
PBS, phosphate-buffered saline
TGF, transforming growth factor
TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling

ABSTRACT

Background: *Angelica sinensis* (Oliv) Diels (Apiaceae) is a traditional medicine that has been used for more than 2000 years in China. It exhibits various therapeutic effects including neuroprotective, anti-oxidant, anti-inflammatory, and immunomodulatory activities. *Angelica* polysaccharides (APs), bioactive constituents of *Angelica* have been shown to be responsible for these effects; however, the utility of APs for the treatment of glioma and their mechanism of action remain to be elucidated.

Purpose: In this study, we investigated the inhibitory effects of APs on a glioma cell line and their molecular mechanism of action.

Study design: U251 cells were utilized to confirm the effects of APs on glioma.

Methods: The human glioblastoma cell line U251 was utilized for both *in vitro* and *in vivo* models, in which we tested the effects of APs. Flow cytometry, gene expression analysis, western blotting, and MTT assays were used to elucidate the effects of APs on cell proliferation, cell cycle, and apoptosis.

Results: The results demonstrated that APs significantly inhibited the growth and proliferation of U251 cells and induced their apoptosis. Furthermore, APs effectively reduced the expression of several cell cycle regulators: cyclins D1, B, and E. The apoptosis suppressor protein Bcl-2 was also downregulated, and the expression of pro-apoptotic proteins Bax and cleaved-caspase-3 increased. Additionally, APs inhibited the transforming growth factor (TGF)-β signaling pathway and stimulated the expression of E-cadherin, thus prohibiting cell growth.

Conclusion: In conclusion, the results indicate that APs attenuate the tumorigenicity of glioma cells and promote their apoptosis by suppressing the TGF-β signaling pathway. The present study therefore provides evidence of the inhibitory effects of APs against glioma progression, and proposes their potential application as alternative therapeutic agents for glioma.

Introduction

Gliomas, which arise from glial cells, are the most common malignant tumors of the central nervous system. They account for more than half of all intracranial tumors, and the 5-year survival rate is less than 5% (Stupp et al., 2005). Malignant glioma is characterized by rapid growth, frequent postoperative relapse, and high mortality (Altieri et al., 2014). Currently, the treatments for glioma include surgery, chemotherapy, and radiotherapy; further, treatments based on gene therapy and immunotherapy are being actively researched (Arrillaga-Romany et al., 2014; Cuddapah et al., 2014). However, the overall therapeutic efficacy remains unsatisfactory, with an average survival time of less than 1 year (Cloughesy et al., 2014; Liu et al., 2009). In addition, the molecular mechanism(s) of glioma development and progression are still poorly understood. However, with recent technological advancements, several traditional Chinese medicines that suppress the development of glioma have been identified (Evirgen et al., 2011; Huang et al., 2013; Jeong et al., 2012; Tsai et al., 2002). Therefore, a combination of traditional knowledge and modern scientific approaches has provided a new direction for research into the diagnosis and treatment of glioma.

Angelica sinensis (Oliv.) Diels (Apiaceae) is a popular traditional medicine that has been used for more than 2000 years in China (Zhao et al., 2003). It exhibits a variety of therapeutic effects including neuroprotective (Kuang et al., 2006), antioxidant (Wu et al., 2004), anti-inflammatory (Han et al., 1998; Yang et al., 2007), and immunomodulatory activities (Ozaki, 1992). Recent studies have shown that *Angelica* polysaccharides (APs), one of the most important group of bioactive components of AS, exert anti-oxidative (Zeng et al., 2015), neuroprotective (Zhou et al., 2015), and immunomodulatory effects (Yang et al., 2006). Furthermore, there is evidence suggesting that a number of pharmacological effects of AS are closely associated with its

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http://dx.doi.org/10.1016/j.phymed.2017.06.007
Received 22 March 2017; Received in revised form 23 May 2017; Accepted 11 June 2017

polysaccharide fractions (Jin et al., 2012). Nevertheless, whether APs could improve the symptoms of glioma, and the mechanism(s) underlying their effects remain to be elucidated.

The current study was performed to investigate the effects of APs on the behavior of glioma cells and the underlying molecular mechanisms, by studying U251 glioma cell proliferation and apoptosis*in vitro* and *in vivo*.

Materials and methods

Drugs

Angelica polysaccharide (AP) was purchased from Shanghai yuanye Bio-Technology Co., Ltd (Shanghai, China). And then were analyzed using UV/VIS spectra (UV-2501PV, Shimadzu, Japan), the percentage of total sugar was determined to be 91.14% by phenol sulfuric acid method. Cisplatin (Cis, produced by Hospira Australia Pty Ltd) was purchased from The First Hospital of Jilin University.

Cell culture

The U251 human glioblastoma cell line was obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM (Thermo Fisher Scientific Inc., Beijing, China) supplemented with 10% fetal calf serum and cultured in a humidified atmosphere containing 5% CO_2 at 37 °C. Only cells in the exponential phase of growth were used in the following experiments.

Colony formation assay

Cells were seeded at a density of 200 cells *per* dish (35 mm dishes), and incubated for 14 days in a humidified atmosphere containing 5% CO_2 at 37 °C. The resulting colonies were rinsed with phosphate-buffered saline (PBS), fixed with paraformaldehyde for 20 min, then stained with Giemsa (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) for 8 min. The number of colonies containing at least 50 cells was counted under a microscope. The colony formation efficiency was calculated as: (number of colonies/number of cells inoculated) × 100.

MTT assay

The 3-(4,5-dimethylthiazol-2-yl) − 2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich, St. Louis, MO, USA) was used to determine cell proliferation and viability. The cells were seeded at a density of 2×10^3 cells/well and were allowed to adhere prior to exposure to test substances for 48 h. Then, MTT was added to each well at a final concentration of 0.2 mg/ml and the cells were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C for 4 h. The supernatant was then removed and formazan crystals were dissolved in 200 μl of dimethyl sulfoxide (Sigma-Aldrich) for 15 min. Optical density was determined at 490 nm using a microplate reader (ELX-800; BioTek, Winooski, VT, USA).

Flow cytometry analysis of the cell cycle

Cells were collected at a density of 90% after culture, fixed in 70% cold ethanol, and kept at 4 °C for 3 h. They were then washed in PBS and resuspended in staining buffer containing 25 μl of propidium iodide and 10 μl of RNase A. The cell suspension was incubated for 45 min in the dark at 37 °C, before analysis of cell cycle markers by fluorescence-activated cell sorting (FACS) Calibur flow cytometry (BD, Franklin Lakes, NJ, USA).

Flow cytometry analysis of apoptosis

Apoptotic cells were identified using the flow cytometry system and an apoptosis detection kit (Beyotime Technology, Nanjing, China), according to the manufacturer's instructions. Cells were harvested, centrifuged, and washed, prior to resuspension in 400 μl of binding buffer. Annexin V-fluorescein isothiocyanate (5 μl) was then added to the cell suspension, and the mixture was incubated at 6 °C for 15 min in the dark. Subsequently, propidium iodide (10 μl) was added to the cell suspension before further incubation at 2–8 °C for 5 min in the dark, followed by flow cytometry analysis within 1 h.

Western blot

Cells were lysed with NP-40 lysis buffer (Beyotime, Haimen, China) and the total protein was extracted using radioimmunoprecipitation assay buffer (Beyotime). Protein concentrations were determined using bicinchoninic acid assay (Beyotime). The total protein (40 μg) content of each sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electro-transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). Membranes were blocked with 5% non-fat milk at room temperature for 1 h, then incubated with primary antibodies (against cleaved-caspase-3, Bax, Bcl-2, cyclin D1, cyclin E, cyclin B, E-cadherin, Smad2, Smad3, p-Smad2, and p-Smad3; 1:500; Bioss, Beijing, China) at 4 °C overnight. Membranes were then incubated with horseradish peroxidase-labeled secondary antibody (Beyotime) at 25 °C for 45 min. Proteins were visualized using enhanced chemiluminescence reagents (Qihai Biotec, Shanghai, China). After exposure, the film was scanned and the images and gray density were analyzed using the Gel-Pro-Analyzer software (Media Cybernetics). Protein expression levels were determined using β-actin as an internal control.

In vivo experiments

Eighteen BABL/c nude mice (20 g, 4–6 weeks of age) were purchased from the animal center of Jilin University. The mice were maintained under pathogen-free conditions at 22 °C and 40–50% humidity, with a 12 h light/dark cycle. The mice had *ad libitum* access food and water. The animal care and treatment protocols were approved by the Experimental Animal Ethics Committee of Jilin University. The mice were assigned randomly to four groups: (1) control, (2) AP (50 mg/kg), (3) Cis (0.05 mg/kg), and (4) AP + Cis. U251 cells (1×10^6) were suspended in 0.2 ml of normal saline and inoculated subcutaneously into the right breast pad of the nude mice. Tumor growth was measured with calipers every 3 days. Tumor volumes in mice were determined using the formula: tumor volume = $(a \times b^2)/2$, where a and b are the larger and smaller of the two dimensions, respectively.

Detection of apoptosis using terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)

Apoptotic cells were detected using the *in situ* Cell Death Detection Kit (Roche, Mannheim, Germany), according to manufacturer's instructions. Paraffin sections of the tumor were inactivated with H_2O_2 and incubated with 50 μl of TUNEL reaction solution at 37 °C for 60 min. After washing with PBS, the sections were incubated with 50 μl of Converter-POD working solution at 37 °C for 30 min. After the sample was developed with 3′-diaminobenzidine, the nuclei were stained with hematoxylin. The prepared samples were observed under a microscope and photographed at 400 × magnification.

Statistical analysis

Data are presented as mean ± standard deviation. Comparisons