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A novel polysaccharide, isolated from *Angelica sinensis* (Oliv.) Diels induces the apoptosis of cervical cancer HeLa cells through an intrinsic apoptotic pathway

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ABSTRACT

A novel polysaccharide isolated from *Angelica sinensis*, named APS-1d showed cytotoxic activity towards several cancer cell lines *in vitro*. However, the precise antitumor mechanisms of this compound are unknown. In this study, we investigated the pro-apoptotic effects of APS-1d in human cervical cancer HeLa cells both *in vitro* and *in vivo*, and further elucidated the mechanisms of this action. Inhibition of HeLa cell proliferation was determined by MTT assay and the therapeutic efficacy of APS-1d was evaluated by human cancer xenografts in nude mice. Cell apoptosis was examined with flow cytometry and TUNEL assay. The mechanism of action of APS-1d was investigated by Western blot analysis. APS-1d decreased HeLa cell proliferation in a concentration- and time-dependent manner *in vitro*. In addition, APS-1d significantly inhibited tumor growth in athymic nude mice. Characteristic manifestations of apoptosis including apoptotic morphological features and the sub- G_0/G_1 peaks were observed when the cells were treated with APS-1d. Further analysis showed that APS-1d-induced apoptosis was associated with the regulation of Bcl-2 family protein expression, a decrease in the mitochondrial membrane potential, and an increase in the cytosolic cytochrome *c* levels. Sequentially, APS-1d increased the activities of caspase-9, -3, and poly (ADP-ribose) polymerase in a concentration-dependent manner, however, no obvious activation of Bid and caspase-8 was observed. Pretreatment with Z-LEHD-FMK, a specific inhibitor of caspase-9, significantly attenuated APS-1d-induced cell apoptosis, and activation of caspase-3. Taken together, our studies indicate that APS-1d is capable of inhibiting HeLa cell proliferation and inducing apoptosis in these cells which primarily involves the activation of the intrinsic mitochondrial pathway.

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Introduction

Although the efficacy of chemotherapy for the majority of cancer types has improved over the last three decades, high toxic effects of chemotherapeutic drugs causing a severe reduction in quality of life are still formidable problems in clinical medicine (Rein and Kurbacher, 2001). Therefore, it is important to develop novel potent, but low toxic anti-cancer reagents, including natural products.

Since the discovery that Letinan, a polysaccharide from *Lentinus edodes* (Berk.) Sing inhibited mouse sarcoma 180 and displayed very low toxicity compared with chemical antitumor drugs (Chihara et al., 1969), a number of polysaccharides with antitumor activity have been reported, such as *Panax ginseng, Coriolus versicolor* and *Agaricus blazei* (Kobayashi et al., 2005; Nakazato et al., 1994; Shin et al., 2002). The root of *Angelica sinensis* (Oliv.) (Chinese Danggui), a well-known Chinese herbal medicine, has been used historically in gynecology for thousands of years (Sarker and Nahar, 2004). In the last few years, polysaccharides as one of the main compounds in *Angelica sinensis* have also attracted much attention (Cao et al., 2006a). Previous work has shown that polysaccharides inhibited tumor growth mainly through stimulation of humoral and cell-mediated immunity, so they were regarded as biological response modifiers (Zaidman et al., 2005). We and other investigators also revealed that the crude polysaccharide from *Angelica sinensis* possessed antitumor effects in mice transplanted with sarcoma 180, leukemia L1210 and Ehrlich ascitic cancer *via* activation of the host immune response (Shang et al., 2003).

Our group recently isolated several kinds of polysaccharides from *Angelica sinensis*, and studied their effects on cancer cells. Interestingly, a novel polysaccharide, named APS-1d having a backbone composed of (1,4)-α-D-glucopyranosyl (Glc*p*) residues,

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and branches composed of (1,6)-α-D-Glc*p* residues with a terminal β-L-arabofuranose (Ara*f*) residue exhibited significant anti-tumor effects *in vitro*, especially in human cervical cancer HeLa cells (Cao et al., 2006b). However, the precise molecular and cellular mechanisms remain unclear. An increasing number of reports have confirmed that polysaccharides or their complexes could have cytotoxic effects on various tumor cell lines *in vitro*, but are less toxic to normal cells. Moreover, it is reported that some kinds of polysaccharides with a backbone mainly composed of Glc*p* residues, such as *Cladonia furcata* polysaccharide and Maitake mushroom polysaccharide, could induce apoptosis in cancer cells (Fullerton et al., 2000; Lin et al., 2001). Therefore, we examined whether APS-1d has a similar apoptotic effect on HeLa cells.

Apoptosis is an energy-dependent type of programmed cell death. In general terms, apoptotic pathways can be sub-divided into two categories, the extrinsic pathway and the intrinsic pathway (Wajant, 2002). The extrinsic pathway is initiated by ligands engagement of cell surface receptors (Fas, TNF receptor, and TRAIL receptor) with their respective ligands (FasL, TNF, and TRAIL) to activate membrane-proximal caspases (caspase-8 and -10) (Mehmet, 2000). The intrinsic pathway requires disruption of the mitochondrial membrane and the release of mitochondrial proteins, such as cytochrome c (Cyt c). Once Cyt c is in the cytosol, Cyt c together with Apaf-1 activates caspase-9, and the latter then activates caspase-3 (Desagher and Martinou, 2000).

Therefore, we conducted this study to elucidate the cellular mechanism of APS-1d on HeLa cells both *in vivo* and *in vitro*. Using terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) and flow cytometric analysis, APS-1d was found to induce apoptosis and also tended to induce S phase arrest in HeLa cells. In our research, APS-1d-induced apoptosis was accompanied by the alteration of expression of the Bcl-2 family members, disruption of mitochondrial potential, release of Cyt c from mitochondria and activation of capase-9 and downstream caspase-3. These results might be helpful in understanding the antitumor mechanism of polysaccharides, and to develop a novel antitumor agent.

Materials and methods

Cell lines and reagents

Human cancer cell line HeLa (cervical cancer), obtained from Xi'an Cell Engineering Center (Xi'an, China), was cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin in a humidified 5% CO_2 atmosphere at 37 °C.

APS-1d was prepared in our laboratory as described previously (Cao et al., 2006b). Briefly, the powdered roots of *Angelica sinensis* (Oliv.) Diels (5.5 kg) were defatted with alcohol and then decocted 3 times with 4 volumes of water. The aqueous extract was concentrated and treated with 3 volumes of ethanol for precipitation. The gel-like precipitate was suspended in water and dialyzed against distilled water (exclusion limit 3.5 kDa). The nondialyzable portion was frozen at -20 °C, then thawed and centrifuged to remove insoluble materials. After the freeze-thaw process was repeated 6 times, the supernatant was lyophilized and the brown product (APS-0) was obtained. APS-0 was dissolved in distilled water, and loaded onto a DEAE-sephadex A-25 column. The column was eluted with distilled water followed by 0.3 M and 0.5 M NaCl, respectively. The water-eluted fraction (APS-1) was further fractionated on a column of Sephadex G-100, eluted with 0.1 M NaCl and separated into four fractions. The forth fraction, which molecular weight was lower than the other fractions, was pooled and applied onto the column of Sephadex G-100 once more. The relevant fraction was concentrated, dialysed and lyophilized to get white powder (APS-1d, 2.1 g).

The molecular weight of APS-1d was determined to be 5.1 kDa by high-performance gel-permeation chromatography. The percentage of total sugar was determined to be 91.5% by phenol-sulfuric acid method. The optical rotation was [α] 20D+45.9 (*c* 0.2, H_2O). The component sugars of APS-1d, determined by GC, were glucose and arabinose with a molar ratio of 13.8:1. Using methylation analysis, partial acid hydrolysis, FT-IR, 1D and 2D NMR (H/H-COSY, HSQC and HMBC) experiments, the structure of APS-1d was elucidated to be a backbone composed of 1,4-α-D-glucopyranosyl residues, with branches attached to O-6 of some residues. The branches were composed of 1,6-α-D-glucopyranosyl residues, and terminated with β-L-arabinofuranose residues. Moreover, the percentage of nitrogen content in APS-1d was determined to be 0.12% using a Vario EL III Element analyzer (Elementar, Germany). ^{1}H-NMR spectrum showed no signs of protein or lipopolysaccharide.

Cell proliferation assay

Cell proliferation was analyzed by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay, as previously described (Mosmann, 1983). Briefly, exponentially growing cells in 96-well plates were treated with different concentrations (0.3-300 μg/ml) of APS-1d in complete medium or the medium alone. MTT (5 mg/ml, Sigma-Aldrich, MO, USA) 20 μl was added 24, 48 or 72 h later. After the plates were incubated at 37 °C for 4 h, the supernatant was aspirated, and 150 μl dimethyl sulfoxide (DMSO) was added to each well.

Absorbance was measured at 570 nm by a 96-well microplate reader (Bio-Rad, Tokyo, Japan). The percentage of surviving cells was calculated as follows: the ratio of cell survival (%)=(mean absorbency in test wells)/(mean absorbency in control wells) × 100.

Cell cycle analysis and apoptosis assay

HeLa cells were seeded in six-well plates (5×10^5 cells/well) and allowed to grow for one day before being exposed to APS-1d (0, 3, 30 or 300 μg/ml). Cells were collected after 72 h. For cell cycle analysis, the cells were collected by trypsinization, fixed in 70% ethanol, washed in phosphate buffered saline (PBS), resuspended in 1 ml of PBS containing 1 mg/ml RNase and 50 μg/ml propidium iodide (PI), and then incubated for 30 min in the dark at room temperature. DNA content of the cells was measured by an Elite-ESP flow cytometer (Beckman Coulter, Miami, FL, USA), and the population of each phase was calculated using the Elite Multicycle software (Phoenix Flow Systems, San Diego, CA, USA). For the apoptosis assay, the cells were washed twice with PBS, and then stained with annexin V-FITC and PI, which were included in the Apoptosis Detection Kit (Biovision Research Products, Mountain View, CA, USA), for 30 min in the dark before being analyzed by flow cytometry. Experiments were conducted three times, and the results are reported as the mean of the three experiments.

Assay of antitumor activity in vivo

Female athymic BALB/c nude mice (6 weeks of age) were purchased from Shanghai National Center for Laboratory Animals (Shanghai, China) and maintained in pathogen-free conditions.

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