Antitumor effect of a polysaccharide isolated from *Phellinus pullus* as an immunostimulant

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Received August 14, 2015; Accepted January 13, 2016

DOI: 10.3892/br.2016.587

**Abstract.** The antitumor function of fungal polysaccharides is a popular area of interest in the research field due to their high efficiency and low side effects. The main mechanism of fungal polysaccharides is immune enhancement. The polysaccharose (APS-3) was extracted from the fruit body of *Phellinus pullus*. The proliferation inhibition to mouse sarcoma 180 (S180) tumor cells was studied by the MTT method. Mice models of transplanted S180 tumor were established and treated with APS-3 to verify the antitumor activity in vivo. Natural killer (NK) and lymphokine-activated killer (LAK) cytotoxicities of the mice were evaluated by the lactate dehydrogenase method. APS-3 can significantly inhibit the proliferation of the S180 cells. Cells could be completely inhibited by 1.6 mg/ml APS-3 after 24 h treatment. After 18 days of treatment, the antitumor rate of the high-dose group was 85.47%. Histopathology detection showed that for the APS-3-treated mice, the tumor cells dissolved, and exhibited a large range of structureless necrotic areas. NK and LAK cytotoxicities of the APS-3 treated mice increased by 61.85 and 56.16%, respectively, compared with the normal control mice. APS-3 can be used as an antitumor agent by way of immune enhancement.

**Introduction**

In conventional tumor therapies, chemotherapy, radiotherapy and surgical treatment all have limitations and side effects that affect normal cells and endanger to the immunity system (1,2). Patients even succumb of serious side effects, such as myocardial injury. To search for novel effective antitumor agents with less toxic effects, natural products have been focused on previously (3,4). In the 1960s, the antitumor function of fungal polysaccharides was discovered and became a popular area of the research field (5). Since then, it has been widely studied owing to the immunostimulating and low toxicity side effects in the host. Fungal polysaccharides, such as lentian and *Ganoderma lucidum* polysaccharide have previously been used in clinical cancer therapies (6).

Natural killer (NK) cells are innate lymphocytes that are capable of eliminating tumor cells and are therefore used for cancer therapy (7). Lymphokine-activated killer (LAK) cells are activated NK cells that are traditionally prepared from isolated T cells cultured with interleukin-2 (IL-2) and are capable of recognizing cancer cells in a non-major histocompatibility complex-restricted manner (8). LAK cells have been utilized in vivo in animals and in humans to treat cancer and can kill NK-non-sensitive tumor cells (9).

*Phellinus pullus* is a wood rot fungi widely distributed in China that has not been used as a traditional herbal medicine. To the best of our knowledge, there are no pharmacological studies on *Phellinus pullus*. The present study isolated a crude polysaccharide (APS-3) from *Phellinus pullus*. Its antitumor activity was evaluated in vitro and in vivo. In addition, the immunomodulatory effects of APS-3 were also assessed to analyze the underlying mechanisms of its antitumor activity.

**Materials and methods**

**Ethical statement.** The present study was carried out in strict accordance with the recommendations of the Ethical Review Committee of Affiliate Jinan Central Hospital Affiliated to Shandong University (Shandong, China).

**Preparation of polysaccharoses.** The polysaccharose APS-3 was obtained from the fruiting bodies of wildly grown *Phellinus pullus* in the Shandong province of China. The dry fruit body was cleaned, crushed and extracted with boiling NaOH (1.5%) for 30 min three times. The extract was combined and precipitated with 80% ethanol. The precipitates were collected and dissolved with distilled water. The Savage method (10) and activated carbon were used to remove protein and pigments from the extract, and subsequently the polysaccharide APS-3 was obtained. The APS-3 content was measured by the phenol-sulfate acid method and was prepared as a 2-mg/ml stock solution. The stock solution was sterilized.
Tumor tissues were fixed in formalin, and further diluted with cell culture medium to the defined concentrations as indicated.

Cell culture and growth inhibition test. Mouse sarcoma 180 (S180) tumor cells (Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai, China) were cultured and harvested from ascites of the infected mouse. In vitro culture was performed in RPMI-1640 medium (Gibco Life Technologies, Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Zhejiang Tianhang Biological Technology Co., Ltd., Huzhou, China), and 300 mg/l L-glutamine (Gibco Life Technologies, Thermo Fisher Scientific, Inc.), and antibiotics in a humidified 5% CO₂ at 37°C. A total of 1x10⁵ cells/ml of exponentially growing cells were cultured in the medium with 0.1, 0.2, 0.4, 0.8 and 1.6 mg/ml APS-3 for 24 h, and subsequently, cell growth inhibition was tested by the MTT method.

APS-3 treatment in vivo
Animal treatment and experimental design. A total of 25 female Swiss mice (18-22 g) were provided by the Laboratory Animal Center of Shandong University [animal license number: SCXK (Lu) 200900001]. The animals were allowed free access to a standard diet and sterile water, and were maintained in a sterile and ventilated room under controlled environmental conditions (25±1˚C, 50±10% humidity and 12-h light/dark cycle).

The exponentially growing S180 cells were washed with Hank's balanced salt solution and adjusted to a suspension containing 1x10⁵ cells/ml in serum-free RPMI-1640 medium. A total of 20 mice were implanted with 200 μl of the cell suspension by subcutaneous injection to the fore right subaxillary. Seven days after tumor cell inoculation, the tumor-bearing mice were randomly divided into 4 groups (5 mice in each group): Mice received APS-3 intragastrically at the respective doses of 1.5, 3 and 6 g/kg/day (the 3 treated groups, respectively); and the control group - survival time of the MC)

Determination of survival time. After 18 days of treatment, the number of surviving mice in each group was recorded and the percentage of life prolongation was calculated as follows: Inhibition rate (%) = (mean tumor weight in the model control mice - mean tumor weight in the treated mice)/mean tumor weight in the model control mice x 100.

Immune-stimulant function of APS-3
Evaluation of NK cytotoxicity. Spleens of the mice were removed under sterile conditions, disaggregated in D-hanks and filtered through a 200-mesh stainless-steel sieve to obtain a single-cell suspension. Lymphocytes were collected and suspended in RPMI-1640 medium at a concentration of 1x10⁶ cells/ml following treatment with red blood cell lysis buffer. The activity of NK cells was tested using the cytotoxicity assay on YAC-1 cells (Cell Bank of Type Culture Collection of Chinese Academy of Sciences). A total of 100 μl NK and YAC-1 cells were added to a 96-well plate in triplicate to obtain an effector/target (E/T) ratio of 20:1 and co-incubated for 12 h. The amount of released lactate dehydrogenase (LDH) in culture supernatants was determined using the LDH Cytotoxicity assay kit (Biovision, Inc., Milpitas, CA, USA) according to the manufacturer's protocol. The optical density (OD) was read at 490 nm with a microplate reader. The percentage of NK cell cytotoxicity was calculated with the formula: Cytotoxicity (%) = (experimental release - spontaneous release)/(maximum release - spontaneous release) x 100. Spontaneous release was spontaneous LDH release from target cells incubated with medium alone, and maximum release was obtained from target cells lysed with NP-40.

Evaluation of LAK cytotoxicity. Lymphocytes were collected at a concentration of 1x10⁵ cells/ml, as described for the NK cells, and were cultured in the medium with 1,000 U/ml of IL-2. The cells collected by centrifuge 72 h later were LAK cells. The concentration of the LAK cells was adjusted to 2x10⁵ cells/ml. Mouse mast cell tumor P815 cells (Cell Bank of Type Culture Collection of Chinese Academy of Sciences) were used as target cells and the concentration was 1x10⁶ cells/ml. LAK cells and P815 cells were co-incubated for 24 h with an E/T ratio of 20:1. A single effector cell and single target cell were used as controls. Each well was measured with the MTT method. The percentage of LAK cell cytotoxicity was calculated with the formula: Cytotoxicity (%) = [1- (ODtarget cell - ODco-incubated effector cell and target cell)/ODtarget cell] x 100.

Statistical analysis. Each experiment was performed at least in triplicate. All the results are expressed as the mean ± standard deviation. The data were analyzed by the Student's unpaired t-test and P<0.05 was considered to indicate a statistically significant difference.

Results
Growth inhibition of S180 cell by APS-3 in vitro. The proliferation of the S180 cells was evidently inhibited after 24-h treatment by APS-3 (P<0.05) (Fig. 1). The results also indicated that there was a marked dose-dependent inhibition of cell viability.

Antitumor effect in vivo
Determination of survival time. On day 19 of the treatment, the model control mice were ill or had died. However, the majority of the mice in the treated group survived. All doses of APS-3 significantly prolonged survival time of the mice, showing life prolongation ratios of 36, 80 and 85% at APS-3.
doses of 1.5, 3 and 6 g/kg/day, respectively. However, as all the mice were sacrificed on day 19 of the treatment, the life prolongation ratios do not signify the final result.

**Tumor inhibition rate.** Seven days after S180 cell injection, tumors of ~500 mm³ formed in 20 mice. As shown in Fig. 2, APS-3 inhibited tumor growth in a time-dependent manner. The tumor volume decreased after 3 days of APS-3 treatment, but a significant decrease was observed after 9 days of treatment. On day 10 of treatment, the tumor in 1 treated mouse completely disappeared. The tumor volume in the model control mice increased to 1,902 mm³ at day 16, while the tumors in the APS-3-treated mice were only 995.7, 476.4 and 348.0 mm³, respectively. After APS-3 treatment for 18 days, all mice were sacrificed and the tumors were removed. The tumor weight in the model control mice was 1.79 g, while that in the APS-3-treated mice was only 1.09, 0.35 and 0.26 g at the dose of 1.5, 3 and 6 g/kg/day, and the tumor inhibition ratio was 39.11, 80.45 and 85.47%, respectively.

**Histopathology.** Fig. 3 shows that in the paraffin sections of the tumor tissues stained with H&E, regularly arranged tumor cells were observed in the model control mice, and a few necrotic areas and no inflammatory cell infiltrations were observed. However, in the APS-3-treated mice, tumor cells disappeared, and a large range of structureless necrotic areas were observed. Furthermore, inflammatory cell infiltration and granulation tissues were observed in these areas. These findings correlated with the tumor volume and weight in the model control mice and APS-3-treated mice.

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**Figure 1.** Effect of APS-3 on viability and proliferation of the sarcoma 180 cells. Cells (1x10⁵ cells/ml) were incubated with APS-3 at the concentrations of 0.1, 0.2, 0.4, 0.8 and 1.6 mg/ml for 24 h. The data are based on the mean of 3 independent experiments, in each of which determinations were in triplicate.

**Figure 2.** Growth curves in tumor-transplanted mice. Compared with the model control mice, the tumor volume in the treated mice decreased during the first 3 days of treatment, but from day 8 of treatment, the tumor volume decreased significantly.

**Figure 3.** Histopathology of the tumor tissue. Tumor tissues were stained with hematoxylin and eosin and observed under a microscope. In the (A) model control mice, regularly arranged tumor cells were observed, while in the (B) APS-3-treated mice, tumor cells disappeared and inflammatory cell infiltration was observed.

**Figure 4.** Enhancement of NK cytotoxicity in tumor-bearing mice by APS-3. NK cells were isolated from the spleens of mice with sarcoma 180 tumor and NK cytotoxicity was tested using the lactate dehydrogenase cytotoxicity assay kit. NK cytotoxicity of the APS-3-treated mice was higher compared to the model control mice and normal control mice. NK, natural killer.

**Figure 5.** Enhancement of LAK cytotoxicity in tumor-bearing mice by APS-3. LAK cells of the mice with sarcoma 180 tumor were induced and the cytotoxicity of the APS-3 treated mice was higher than that of the model control mice. LAK, lymphokine-activated killer.
Acknowledgements

The authors would like to acknowledge the financial support of the Medical and Health Technology Development Plan of Shandong province (grant no. 2014WS0002) and the China Postdoctoral Science Foundation (grant no. 2014M551913).

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