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Anti-tumor and macrophage activation induced by alkali-extracted polysaccharide from *Pleurotus ostreatus*

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ABSTRACT

Pleurotus ostreatus is popularly consumed as traditional medicine and health food for enhancing immune function in China. Polysaccharides from mushroom have been demonstrated to possess a wide range of health beneficial properties. This study was carried out to elucidate the immunomodulating effects and molecular mechanism involved in the *in vivo* and *in vitro* anti-tumor activities of alkali-extracted polysaccharide (WPOP-N1) from the fruiting bodies of *P. ostreatus*. The results showed that WPOP-N1 significantly inhibited the tumor growth of Sarcoma 180 tumor-bearing mice, and markedly increased the secretion level of TNF-α in serum. In addition, WPOP-N1 enhanced the phagocytic capability of peritoneal macrophages *in vitro*. Furthermore, the secretion of TNF-α and NO and the amount of TNF-α and iNOS transcript were increased significantly when the peritoneal macrophages were exposed to WPOP-N1. Meanwhile, Western blot analysis revealed that the stimulation of peritoneal macrophages by WPOP-N1 induced the phosphorylation of p65 and a marked decrease of IκB expression. These results suggest that WPOP-N1 could activate macrophages through NF-κB signaling pathway, and the anti-tumor effects of WPOP-N1 can be achieved by its immunostimulating property.

1. Introduction

The enhancement or potentiation of host defense has been recognized as a possible means of inhibiting tumor growth without any harm to the host [1]. Macrophages are known to be pivotal immunocytes of the host defense against invading pathogen or tumor growth [2]. Activated macrophages release a wide range of mediators including reactive oxygen species (ROS), nitric oxide (NO), hydrolytic enzymes, bioactive lipids, and proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1, which have been identified as the major effector molecules involved in the destruction of tumor cells by macrophages [3]. Various stimuli can bind to pattern recognition receptors (PRRs) on the surface of macrophages, such as Toll-like receptors (TLRs) or Dectin-1, then trigger several different signaling pathways including tyrosine kinases, PKC, PI3K/Akt, MAPKs, and NF-κB, and finally lead to the gene expression of a large spectrum of mediators with anti-tumor activity [4].

In Asian countries like China, Korea and Japan, mushrooms have been collected, cultivated and used as food since ancient times. Traditionally, mushroom has been defined as a umbrella-shaped fruiting body of macrofungi, and some edible mushrooms have been well studied in their nutrition as well as non-toxic medicinal properties by Asian people for over two thousand years [5]. Nowadays, mushrooms are distinguished as important natural resources of immunomodulating and anticancer agents [6]. The majority of immunomodulator compounds from mushrooms belong to polysaccharides, and these polysaccharides, particularly β-D-glucan derivates, glycopeptide/protein complexes, and proteoglycans, can activate immune effecter cells, such as lymphocytes, macrophages, T cells, dendritic cells (DCs) and natural killer (NK) cells involved in the innate and adaptive immunity, to produce some biologic response modifiers (BRM) [7,8].

Pleurotus ostreatus, famous for its delicious taste, high quantities of proteins, carbohydrates, minerals and vitamins, and low fat,

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is a commercially important edible mushroom known as oyster mushroom [9]. The medicinally beneficial effects of *P. ostreatus*, such as antioxidant, immunomodulatory, anti-tumor, antiviral, anti-inflammatory, antimicrobial and cholesterol-lowering activities, are well known worldwide [10–14]. However, there is relatively little information pertaining to alkali-extracted water-soluble polysaccharides, especially about the mechanism of immunomodulation and macrophage activation. Therefore, the purpose of this investigation was to elucidate the mechanism of anti-tumor and macrophage activation activities of alkali-extracted water-soluble polysaccharide isolated from the fruiting bodies of *P. ostreatus*.

2. Materials and methods

2.1. Materials and chemicals

Sepharose CL-6B gel filtration media and chemiluminescent detection kit of ECL plus western blotting reagents were purchased from Amersham Pharmacia Biotech. Cyclophosphamide (CTX), 5-fluorurazil (5-FU), thioglycollate medium, Griess reagent, DEAE-cellulose, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO, USA). Affi-Prep Polymyxin Matrix was purchased from BIO-RAD. RPMI 1640 medium and fetal bovine serum (FBS) were the products of Gibco (Grand Island, USA). All other chemical reagents were of analytical grade.

Anti-NF-κB p65 antibody was obtained from Abcam. Phospho-NF-κB p65 (Ser536) and IκB-α antibodies were purchased from Cell Signaling Technology. Antibody against β-actin (AC-40) was obtained from Sigma-Aldrich.

2.2. Isolation and purification of alkali-extracted polysaccharides

The air-dried fruit bodies of *P. ostreatus* were ground, and then extracted with 80% ethanol for 24 h. After filtered, the residues were air-dried and extracted with distilled water at 80 °C for three times. Then the water-unextractable residue was dried and extracted with 0.5 M NaOH solution containing 0.3% (w/w) KBH_4. The alkali extract was filtered, centrifuged and neutralized with hydrochloric acid (0.1 M), and then the supernatant was concentrated by evaporation under reduced pressure and precipitated with 3 volumes of ethanol. Polysaccharide precipitate was collected by centrifugation and deproteinated by a combination of proteinase and Sevag method [15], and the supernatant was lyophilized to obtain crude alkaline *P. ostreatus* polysaccharides (cWPOP).

The sample (cWPOP) was dissolved in distilled water, then loaded onto DEAE-cellulose column (3 × 30 cm), and eluted successively with distilled water and 0–1 M NaCl. The fractions were collected, and monitored with the phenol–sulfuric acid method [16]. The main fraction was collected, dialyzed, and lyophilized, and further fractioned on Sepharose CL-6B column (2.6 × 100 cm), eluted with 0.15 M NaCl to yield one main fraction, and coded as WPOP-N1. The yield of WPOP-N1 was 3.4% of dried material, and the carbohydrate content was 97.6% determined by phenol–sulfuric acid method. In addition, WPOP-N1 had a negative response to the Bradford assay [17] and no absorption at 260 or 280 nm in UV spectrum was detected, indicating the absence of protein and nucleic acid. Possible contaminants of endotoxin in WPOP-N1 were removed using Affi-Prep Polymyxin Matrix (BIO-RAD).

2.3. Animals

Male BALB/c mice (18–22 g) were purchased from Animal Experimental Center of Jilin University. The mice were housed in plastic cages and kept under standardized conditions at temperature between 22 and 24 °C and 20% humidity with a 12 h light/dark cycle, and had free access to tap water and food throughout the study. They were allowed to acclimatize for 1 week before the experiments started. Animal experiments were conducted under principles of good laboratory animal care and approved by ethical committee for Laboratory Animals Care and Use of Jilin University.

2.4. In vivo antitumor activity and TNF-α secretion in serum

Sixty mice were randomly divided into six groups. Sarcoma 180 cells obtained from the peritoneal cavity of the tumor-inoculated mice were washed twice and re-suspended in PBS. Under sterile condition, 0.2 ml of Sarcoma 180 cell suspension (1×10^6 cells/ml) was inoculated into the right hind limbs at day 0, while the mice in the normal group were not inoculated with tumor cells. After 24 h of the start of tumor inoculation, WPOP-N1 (100, 200, and 400 mg/kg, 0.2 ml), cyclophosphamide (CTX, 30 mg/kg, as positive control), and sterile saline (as negative control) were orally administered to mice in different groups daily. On the 21st day after the tumor inoculation, all mice were weighed and sacrificed, and the solid tumors were carefully extirpated and weighed. The anti-tumor activity was expressed as an inhibitory rate (%) calculated as $[(A–B)/A] \times 100$, where A and B are the average tumor weights of the model control and experimental groups, respectively. Tumor necrosis factor-alpha (TNF-α) in sera collected from the tumor-bearing mice was measured using murine enzyme-linked immunosorbent assay (ELISA) kit.

2.5. In vitro anti-tumor activity

In vitro anti-tumor activity against Sarcoma 180 cells was determined by colorimetric MTT assay. Briefly, tumor cells were seeded in 96-well flat-bottomed plates and allowed to adhere for 24 h at 37 °C with 5% CO_2 atmosphere. Sterilized sample solutions were added into 96-well plate to give a final concentration of 100, 200 and 400 μg/ml, while those in the negative control (NC) group were treated with the complete RPMI-1640 medium only, and those in the positive control group were treated with 5-fluorurazil (5-FU, 25 μg/ml). After the cultivation for 72 h, 20 μl MTT solution (5 mg/ml) was added. After incubated at 37 °C for 4 h, the supernatant was aspirated and 100 μl dimethyl sulphoxide was added to each well. The absorbance was measured at 570 nm by a microplate reader (Bio-Tek EXL800, American).

2.6. Preparation of peritoneal macrophages

Male BALB/c mice were injected intraperitoneally with sterile thioglycollate medium for three consecutive days, and then the resident peritoneal macrophages were harvested by peritoneal lavage and centrifugation. Then peritoneal macrophages were cultured in 96-well plate for 2 h. Non-adherent cells were removed by washing with PBS, the adhered macrophages were cultured for another 24 h with fresh complete RPMI-1640 medium.

2.7. Measurement of phagocytosis capacity of peritoneal macrophage

Peritoneal macrophages prepared as above were incubated with WPOP-N1 (100, 200 and 400 μg/ml) for 24 h, and then 100 μl of aseptic neutral red solution were added (0.075%) into each well and incubated for another 1 h. After washed with PBS, the cells were lysed in 150 μl cell lysis buffer (anhydrous ethanol:acetic acid = 1:1, v/v). The absorbance was measured at 550 nm using Bio-Tek EXL800 microplate reader. The absorbance represented the phagocytic ability of the macrophages.