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Rhizopus nigricans polysaccharide activated macrophages and suppressed tumor growth in CT26 tumor-bearing mice

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ABSTRACT

In this study, a homogeneous polysaccharide (RPS-1) was extracted from liquid-cultured mycelia of *Rhizopus nigricans*. The weight-average molecular weight of RPS-1 was 1.617×10^7 g/mol and structural characterization indicated that RPS-1 was a non-starch glucan which consisted of a backbone structure of (1→4)-linked α-D-glucopyranosyl residues substituted at the O-6 position with α-D-glucopyranosyl branches. RPS-1 stimulated the production of nitric oxide and tumor necrosis factor-α by triggering phosphorylation of mitogen-activated protein kinases and nuclear translocation of nuclear factor kappa B p65 in RAW 264.7 macrophage cells. Moreover, intragastric administration of RPS-1 improved the immune function of CT26 tumor-bearing mice and significantly inhibited the growth of transplanted tumor. In combination with 5-FU, RPS-1 enhanced antitumor activity of 5-FU and alleviated its toxicity on immune system. These findings suggested that RPS-1 has the potential for the development of functional foods and dietary supplements.

1. Introduction

Fungal polysaccharides have long been appreciated for their biological and pharmacological activities, including immunomodulatory, antitumor, antioxidant, antiviral and anti-infection (Wasser, 2010; Yu, Shen, Song, & Xie, 2018). A wide range of fungal glucans showed immunomodulatory and antitumor activities have been obtained from fruit bodies, mycelia and liquid culture broth. These glucans present diverse structures with (1→3)-, (1→4)- and/or (1→6)-linked linear or branched α-glucans, β-glucans and mixed α/β-glucans, and molecular weight ranged from thousands to millions Da (Synytsya & Novak, 2013). As the most studied fungal glucan, β-glucans are considered as potent immunomodulators and responsible for the beneficial effects in the outcome of malignant tumor (Ina, Kataoka, & Ando, 2013; Namikawa et al., 2015). Upon specific interactions with several cell surface receptors, such as toll-like receptor, complement receptor 3 and dectin-1, β-glucans can trigger a wide spectrum of immune responses through various signaling pathway (Brown & Gordon, 2001; Gantner, Simmons, Canavera, Akira, & Underhill, 2003; Thornton, Vetvicka, Pitman, Goldman, & Ross, 1996).

Despite the well documented β-glucans, little is known about the immunoregulatory activities of fungus derived α-(1→4)-glucans and their underlying mechanisms. Macrophages are one of the major types of phagocytes and play vital roles in innate and adaptive immune system. More important, macrophages orchestrate immune response against cancer by phagocytosing aberrant cells, presenting tumor antigens and secreting heterologous proinflammatory cytokines (Long & Beatty, 2013). Recently research reported that maitake α-(1→4)-glucans served as biological response modifiers with the capability to activate macrophages and dendritic cells and inhibited tumor growth in vivo (Masuda, Nakayama, Tanaka, Naito, & Konishi, 2017; Sun et al., 2016; Wang et al., 2017). YCP, a homogeneous α-(1→6)-branched α-(1→4)-glucan purified from the mycelium of marine fungus, was found to be able to increase phagocytic activity in mice and stimulate NO

Abbreviations: MAPKs, mitogen-activated protein kinases; NF-κB, nuclear factor-κB; NO, nitric oxide; HPSEC-MALLS, high performance size-exclusion chromatography with multiangle laser light scattering; PMB, polymyxin B; 5-FU, 5-fluorouracil

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release in macrophages through p38 signaling axis (Chen et al., 2009; Yang et al., 2005). Macrophage activation may partially underlie immunoregulatory and antitumor activities of α-(1→4)-glucans.

Rhizopus nigricans is a zygomycete filamentous fungus and widely used in food and pharmaceutical industry. In this study, we obtained a novel bioactive glucan from liquid-cultured mycelia of *R. nigricans* and explored the underlying molecular mechanisms of macrophage activation. The antitumor effects of *R. nigricans* polysaccharide were further investigated in CT26 colon cancer-bearing mice.

2. Materials and methods

2.1. Materials

DEAE sepharose Fast Flow and Sephacryl S-500 HR were obtained from General Electric Healthcare Life Sciences (Pittsburgh, USA). Fetal bovine serum, dulbecco's modified eagle medium (DMEM), Lipofectamine® 2000, penicillin and streptomycin were provided by Thermo Fisher Scientific (Waltham, USA). Monosaccharides, lipopolysaccharide (LPS), 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI), polymyxin B (PMB) and 5-fluorouracil (5-FU) were purchased from Sigma-Aldrich (St. Louis, USA). 1-Phenyl-3-methyl-5-pyrazolone (PMP) was obtained from Acros Organics (Geel, Belgium). Antibodies against IκB-α, NF-κB p65 (RelA), JNK1/2, phospho-JNK1/2, and β-actin were purchased from Santa Cruz Biotechnology (Dallas, USA). Antibodies against p38, phospho-p38, p44/42 (ERK1/2), phospho-p44/42 (ERK1/2), and histone H2A were provided by Cell Signaling Technology (Danvers, USA). The cell lysis buffer, nuclear and cytoplasmic protein extraction kit, NF-κB nuclear translocation assay kit and horseradish peroxidase (HRP)-labeled second antibodies were supplied by Beyotime Institute of Biotechnology (Shanghai, China). The pNL3.2.NF-κB-RE and pRL-TK plasmids and dual-luciferase® reporter assay system were obtained from Promega (Beijing, China). Mouse TNF-α and interleukin-2 (IL-2) ELISA kits were purchased from RayBiotech (Norcross, USA).

2.2. Organism and growth conditions

R. nigricans was preserved in Laboratory of Biomass Resources, Shandong University (Jinan, China). The strain was cultured in shaking flask containing potato dextrose broth under controlled conditions (28 °C, 130 rpm) for 10 days.

2.3. Extraction and purification of the R. nigricans polysaccharides

Liquid-cultured mycelia of *R. nigricans* were extracted in boiling water for 2 h and the extract was treated with 3 volumes of ethanol at 4 °C overnight. The precipitate was dissolved in distilled water and deproteinated according to the method of Sevag (Sevag, 1938). The resulted polysaccharide solution was decolorized with D301R resin, followed by dialyzed and lyophilized to obtain the crude polysaccharides. The crude polysaccharides were loaded into DEAE Sepharose Fast Flow column (1.6 cm × 20 cm) and eluted with a linear gradient of sodium chloride solution (0–0.5 M). The unabsorbed fraction was collected and further purified using Sephacryl S-500 HR column (1.6 cm × 60 cm) and the fraction of major peak was collected and lyophilized to powder, named as RPS-1. The total sugar content of RPS-1 was measured by phenol-sulfuric acid method and glucose was used as standard. The endotoxin contamination was tested by the Limulus amoebocyte lysate assay, showing negative value.

2.4. Homogeneity, molecular weight and root mean square radius

The purity, molecular weight (M_w) and root mean square (RMS) radius of RPS-1 were determined by using high performance size-exclusion chromatography coupled with multiangle laser light scattering (HPSEC-MALLS). HPSEC-MALLS measurements were performed on a Waters 2695 instrument (Waters, USA) equipped with OHpak SB-806M (Shodex, Japan) at 25 °C. RPS-1 was dissolved in distilled water and incubated for 3 h. All the solutions were purified by a 0.45 μm filter. The elution was maintained at a flow rate of 0.5 ml/min and monitored by Optilab T-rEX differential refractive index detector (Wyatt, USA) and multiangle laser light scattering instrument equipped with DAWN HELEOS II light scattering (Wyatt, USA) at angles of 35°, 43°, 52°, 60°, 69°, 80°, 90°, 100°, 111°, 121°, 132°, 142°, and 152°. The Astra software was utilized for data acquisition and analysis.

2.5. Monosaccharide composition analysis

Monosaccharide analysis was performed as described previously (Chen et al., 2013).

2.6. Methylation analysis

Methylation of RPS-1 was performed as previous described (Ciucanu & Kerek, 1984). The resulting methylated product was hydrolyzed with 2 M TFA at 120 °C for 2 h followed by reduction with $NaBD_4$ and acetylation with acetic anhydride to yield partially methylated alditol acetates which were further quantified by gas chromatograph-mass spectrometer (GC–MS) using a HP-5 MS fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm, Agillent, USA). The column temperature was set at 120 °C when injected, then increased to 200 °C at 4 °C/min, then to 280 °C at 10 °C/min, and kept at 280 °C for 5 min. Helium was used as the carrier gas.

2.7. Nuclear magnetic resonance analysis

A total of 10 mg of RPS-1 was dissolved in 0.5 ml of D_2O. The ^1H and ^{13}C NMR spectra were recorded on a Bruker AVANCE-300 MHz NMR spectrometer (Bruker, Germany) at 25 °C.

2.8. Cells and animals

Murine macrophage cell line RAW 264.7 and CT26 mouse colon cell line were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cell cultures were incubated in humidified atmosphere of 5% CO_2 at 37 °C. Female BALB/c mice weighing 18–22 g were purchased from Nanjing Qinglong mountain farm (Nanjing, China) and acclimatized for 1 week before used. All the animals were kept according to the National Institute of Health Guide for the Care and Use of Laboratory Animals, and all experimental protocols were in accordance with the institutional guidelines of the Animal Care and Use Committee at Shandong University.

2.9. Measurement of NO and TNF-α

Logarithmically growing RAW 264.7 cells were placed in 96-well plate and treated by a series of concentrations of RPS-1 for 24 h. LPS (100 ng/ml) was used as positive control. After treatment, the levels of NO and TNF-α were assayed by Griess reagent and enzyme-linked immunosorbent assay kit according to the manufacturer's protocols, respectively.

2.10. Immunofluorescence staining

RAW 264.7 cells were plated in 6-well plate and exposed to RPS-1 (400 μg/ml) or LPS (100 ng/ml) for 30 min. After treatment, cells were rinsed with cold phosphate buffer solution (PBS), fixed by paraformaldehyde and incubated with primary antibody against NF-κB p65 overnight at 4 °C overnight. And then cells were incubation with a Cy-3