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Comparative studies on the immunoregulatory effects of three polysaccharides using high content imaging system

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ABSTRACT

In this study, polysaccharides were isolated from *Astragalus membranaceus*, *Ganoderma lucidum* and *Radix ophiopogonis* and named APSII, GLPII and OGPII for comparison of their immunoactivities. MTT assay indicated that these polysaccharides increased the metabolic activity of Raw264.7 macrophages and induced cell differentiation to dendritic like cells. High content screening and mathematical modeling were used to quantify the cell irregularity, a hallmark of cell differentiation by polysaccharides. The results showed that GLPII increased cell irregularity, but APSII and OGPII had slightly less effects. Imaging analysis also revealed that polysaccharides inhibited cell proliferation while inducing the cell differentiation. In addition, APSII and GLPII but not OGPII induced NO production and enhanced cell phagocytic ability. Interestingly, inducible nitric oxide synthase inhibitor blocked polysaccharide-enhanced phagocytosis, indicating NO production is crucial for macrophages to acquire phagocytic ability, which was further confirmed by correlation studies. APSII and GLPII significantly promoted the maturation of macrophages by the increase in the expression of MHCII, CD40, CD80 and CD86, while OGPII had less effects. In summary, we have suggested a practical and economical method to quantify macrophage differentiation (irregularity) induced by polysaccharides for quality assurance and have found the role of NO production on macrophage phagocytic ability.

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1. Introduction

Polysaccharides isolated from traditional Chinese medicine (medicinal plants, mushroom, algae and lichens) have been widely studied in the biomedical arena due to their multiple biological functions and relatively low toxicity, such as anti-tumor, antioxidation, immunoregulation abilities [1,2]. Polysaccharides can invoke the immune system, as they were found to increase thymus and spleen indices (weight of thymus or spleen/body weight) [3], promote lymphocyte proliferation or maturation [4–6] and stimulate cytokine secretion [7,8]. Some of polysaccharides from traditional Chinese medicine, such as *astragalus* polysaccharide, lentinan and krestin have already been used in clinic applications [2,9].

Ganoderma lucidum was believed as an effective tonic for longevity in Asia during the past thousands of years. It was found to be a suitable adjuvant for anti-tumor targeting by down-regulation of T regulatory cells in the immune system [10]. *G. lucidum* polysaccharide (GLP) had diverse chemical features with different

Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Ara, arabinose; APS, *Astragalus membranaceus*; DC, dendritic cells; DMEM, dulbecco's modified eagle medium; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GLP, *Ganoderma lucidum*; GC, gas chromatograph; Gal, galactose; Glu, glucose; HPGPC, high performance gel permeation chromatography; HPLC, high performance liquid chromatography; HCS, high content screening; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; Man, mannose; PBS, phosphate-buffered saline; Rha, rhamnose; OGP, *Radix ophiopogonis*; SMT, s-methylisothiourea sulfate; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TEM, transmission electron microscopy; Xyl, xylose.

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molecular weight, degree of branching, and commonly contains β-glucans, hetero-β-glucans, heteroglycans or α-manno-β-glucans [11]. GLP possesses powerful immune-modulating activities by promoting the maturation of dendritic cells (DCs) and enhancing the cytotoxicity of natural killer cells and activating T helper1 immune responses [12,13]. *Astragalus membranaceus* is another traditional Chinese herb that has been used in the treatment of wounds, chronic fatigue, diabetes, membranous nephropathy and immune-related diseases [14]. Its active ingredient *Astragalus* polysaccharides (APS) can be served as adjuvant of hepatitis B vaccine that could promote the expression of DC surface antigen MHCI/MHCII, CD40, CD80/86, and increase TNF-α, IL-1β and NFATc4 mRNA expression and TNF-α and IL-1β protein expression [15,16]. The structure of APS has been reported by us previously that APS is composed of terminal arabinose, one 1,5-linked arabinose, one 1,3-linked rhamnose, one 1,3,4-linked rhamnose, five 1,4-linked methyl galacturonates and six 1,4-linked methyl glucuronates [17]. *Radix ophiopogonis* has been used to nourish *yin* and moisturize lung that was well documented in Shen Nong's Materia Medica two thousand years ago [18]. It is widely applied in the treatment of disorders such as thrombosis, myocardial ischemia, arrhythmias, respiratory disease and hyperglycemia [19]. *Ophiopogon* polysaccharide (OGP) is the major active compound of *Ophiopogon japonicas*, and was reported to increase the phagocytic function of macrophages and improve the humoral and cellular immunity [20]. The backbone of OGP was delineated to compose 1,6-α-D-glucopyranose and 1,3,6-α-D-glucofuranose, while the branched chains contained 1,3-α-D-glucopyranose and 1-α-D-glucopyranose [20]. Other than the above three commonly used polysaccharides powered with immunoregulatory functions, many polysaccharides extracted from other traditional Chinese herbs also have similar activities. However, there are very few research that have been focused on the comparison of polysaccharides derived from different herbs for their immunoregulatory function. Since there are hundreds types of polysaccharides extracted from natural products in the market, it is urgent to evaluate their immuoregulatory potency and efficacy by setting up a standardized *in vitro* method for better quality assurance of polysaccharides.

Macrophages are one of the major types of antigen presenting cells in innate immune system. As a type of extraordinarily versatile cell, it plays a central role in host defense against pathogen infection through phagocytosis, antigen presenting, cytokine secretion, and activation of adaptive immune response of T and B cells [21,22]. Raw264.7 cells are derived from BALB/c mice cells infected with Abelson leukemia virus that possessed the characteristic of macrophages, and they are generally considered as a suitable cell model for studying the functions of macrophages *in vitro* [23–25]. Raw264.7 cells were commonly used for active compound prediction on immune system. For example, polysaccharide from *Cordyceps militaris* could enhance immunoregulatory activities by the release of toxic molecules like nitric oxide (NO) and reactive oxygen species in Raw264.7 cells [26]. Polysaccharide extracted from the roots of *Actinidia eriantha* significantly enhanced phagocytic activity in Raw264.7 cells, induced the production of NO, TNF-α, IL-10, IL-1β and IL-6, and promoted the expression of accessory and co-stimulatory molecules such as MHC molecules and CD40,CD80 or other surface markers [26,27]. Therefore, Raw264.7 cell line was used as an *in vitro* model in this work to evaluate the immune activities of polysaccharides.

Most previous studies regarding polysaccharides mainly focus on only one type of polysaccharide to investigate its immune response, while in this study we selected APS, GLP and OGP to compare their immunomodulatory effect on Raw264.7 cells. There are over 300 types of polysaccharides that have been extracted from natural products, a few of which being approved for clinical application in China [28,29]. Although most of the polysaccharides were demonstrated to regulate the immune system, there are lack of standard and feasible methods to evaluate their immunomodulatory activity. Through imaging analysis of *in vitro* cell differentiation into DC like cells, we proposed to use high content imaging system and analysis to quantify the efficacy and potency of polysaccharide induced cell differentiation, aiming to standardize the evaluation system of immunoregulatory effects of polysaccharides extracted from Chinese herbs. In addition, we compared the dextran uptake to cell metabolic activity, phenotypic maturation surface markers and NO production. We found the positive correlation between NO level and cellular uptake of dextran, indicating the necessity of NO production on the phagocytic ability of macrophages induced by polysaccharides.

2. Materials and methods

2.1. Chemicals and materials

Lipopolysaccharide (LPS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), fluorescein isothiocyanate-conjugated dextran (FITC-Dextran 40,000) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco. All antibodies used to study the surface markers were purchased from BD Biosciences Pharmingen (San Diego, CA, USA). NO assay kit was purchased from Beyotime Biotechnology (Jiangsu, China). All antibodies used to investigate the phenotypic maturation surface marker were purchased BD Biosciences (San Diego, CA, USA).

2.2. Sample preparation

The crude polysaccharides powder including *Astragalus*, *G. lucidum* and *Ophiopogon* were purchased from Xi'an yuansun Biological Technology. Crude polysaccharides powder was dissolved in ultrapure water and centrifuged. The supernatant was de-proteinized with trichloroacetic acid (TCA) and centrifuged. The de-proteinized supernatant was precipitated by alcohol and washed three times until the supernatant was clear, then dissolved and lyophilized. The dried crude polysaccharides were subjected to chromatography on DEAE-32 anion-exchange chromatography column. Crude polysaccharides were dissolved, centrifuged, and the supernatant was loaded onto the column. Ultrapure water followed by a series of NaCl solution in the order of 0.1, 0.2, 0.5, 1 and 2 M were used for elution of polysaccharides. Each fraction of the eluent were collected and analyzed by the phenol–sulfuric acid method. Two major fractions of each polysaccharide were collected and designated as APSI, GLPI, OGPI (eluted by ultrapure water) and APSII, GLPII, OGPII (eluted by 0.1 M NaCl). Other fractions of the polysaccharide content were at a minimal or non-detectable amount and were not collected. The fraction I and II was dialyzed and lyophilized. APSII, GLPII and OPGII had biological activity by the increase of cell metabolic activities detected by MTT assay, therefore fraction II of each type of polysaccharide were evaluated for their immunoactivities. APSII, GLPII and OPGII were further purified by Sephacryl S-300 high resolution (GE Healthcare Bioscience) column chromatography. Polysaccharides was dissolved ultrapure water, centrifuged and aspirated the supernatant applied onto the column and eluted with 0.2 M NaCl, collected and detected using the phenol–sulfuric acid method [30,31]. Endotoxin level was tested by endotoxin-specific kit (Chinese Horseshoe CrabReagent Manufactory, Co.). Polysaccharide samples were mixed with tachypleus amebocyte lysate reagent and incubated for 1 h at 37 °C followed by examination for gelation. The endotoxin level was estimated to be <0.015 EU/mg for all polysaccharides.

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