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Toll like receptor 4 (TLR4) mediates the stimulating activities of chitosan oligosaccharide on macrophages

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ABSTRACT

The in vivo and in vitro immunostimulating properties of chitosan oligosaccharide (COS) prepared by enzymatic hydrolysis of chitosan and the mechanisms mediating the effects were investigated. Our data showed that the highly active chitosanase isolated could hydrolyze chitosan to the polymerization degree of 3–8. The resulting COS was an efficient immunostimulator. COS markedly enhanced the proliferation and neutral red phagocytosis by RAW 264.7 macrophages. The production of nitric oxide (NO) and tumor necrosis factor alpha (TNF-α) by macrophages was significantly increased after incubation with COS. Oral administration of COS in mice could increase spleen index and serum immunoglobin G (IgG) contents. COS was labeled with FITC to study the pinocytosis by macrophages. Results showed that FITC–COS was phagocyted by macrophages and anti-murine TLR4 antibody completely blocked FITC–COS pinocytosis. RT-PCR indicated that COS treatment of macrophages significantly increased TLR4 and inducible nitric oxide synthase (iNOS) mRNA levels. When cells were pretreated with anti-murine TLR4 antibody, the effect of COS on TLR4 and iNOS mRNA induction was decreased. COS-induced NO secretion by macrophages was also markedly decreased by anti-murine TLR4 antibody pretreatment. In conclusion, the present study revealed that COS possesses potent immune-stimulating properties by activating TLR4 on macrophages.

1. Introduction

Chitin and its derivatives are known to be non-toxic, non-allergenic, and biocompatible, enabling them to be widely used in medical practice. Lee et al. reviewed the immunological effects of chitin and its derivatives and reported complex activities on innate and adaptive immune responses [1]. Chitosan is a partially or fully de-acetylation product of chitin, which could increase humoral response [2] and activate macrophages and natural killer cells [3,4]. Chitosan could also induce tumor necrosis factor alpha (TNF-α) production in cultured human monocytes [5]. Most of the immune-modulating activities of chitosan are seen when used as an immune adjuvant [6].

Even though chitosan has broad biological activities, the high molecular weight, high viscosity and insolubility in neutral solution greatly restrict its in vivo uses. Thus, increasing attention has been given to converting chitosan to oligomers by chemical or enzymatic hydrolysis [7]. Recently, the immuno-stimulating and other biological properties of chitosan oligosaccharide (COS) received more attention because of water solubility and easy absorption. COS, with polymerization degree of 2–8 glucosamines, is more effective in its immuno-stimulation, anti-tumor [8], and anti-inflammation activities [9]. COS could increase Interleukin (IL)-12 and interferon-γ levels, increase IL-1β gene expression, and induce TNF-α production in macrophages [10,11]. COS showed stimulatory effects on macrophages with combination of interferon-γ as manifested by increased nitric oxide (NO) synthesis and enhanced cytotoxicity towards murine fibrosarcoma Meth A cells [12,13].

The stimulatory effects of COS on macrophages are mediated in a size-dependent and pathway-specific manner. However, the exact mechanisms that mediate the activation of macrophages by COS are poorly defined. There is controversy about the receptors that mediate the activation of macrophages by COS. Han et al. showed that mannose receptor was a major receptor responsible for COS uptake by macrophages using confocal laser microscopy [14]. Feng, Zhao et al. also suggested that oligochitosan internalization is mediated by a macrophage lectin receptor with mannose specificity [15]. Otterlei, Varum et al. indicated that lipopolysaccharides (LPS) and neutral-soluble chitosan share a binding site on monocytes which involves CD14 [5]. Wei, Wang et al. isolated chitosan pentamer (COS5) and hexamer (COS6) and found that the gene expression of cell surface CR3 receptor could be promoted by both COS5 and COS6 [16]. Wu et al. showed that CD14, TLR4 and CR3 on RAW264.7 macrophages were all involved in cell activation by COS, as treatment of RAW264.7 macrophages with anti-CD14, anti-TLR4, and anti-CR3 antibodies all significantly blocked NO production [13].

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In order to further clarify the biological activity of COS and its cellular receptor, we prepared COS by enzymatic hydrolysis. Our data showed that the highly active chitosanase hydrolyzed chitosan to COS with polymerization degree of 3–8. The resulting COS was an efficient immunostimulator. The stimulatory effects of COS on RAW 264.7 macrophages were apparently mediated by TLR4.

2. Materials and methods

2.1. Reagents

DMEM and D-Hank's balanced solution were obtained from HyClone (Logan, Utah, USA). LPS and MTT were obtained from Sigma Chemical Co. Polymyxin B was obtained from Solarbio. Cell culture flasks and cell culture plates were purchased from Costar. FBS was obtained from Biochromag Germany. Anti-murine TLR4 monoclonal antibody was purchased from Cell Signaling. Reverse transcription First Strand cDNA Synthesis Kit and PCR Master Mix (2×) were obtained from Fermentas. TNF-α and NO assay kits were obtained from Nanjing Jiancheng Bioengineering Institute. All other chemicals were of analytical grade and were obtained from Qingdao Alp Science and Techenology Co., Ltd.

2.2. Cell culture

The murine MΦ cell line RAW 264.7 macrophages were obtained from the ShangHai cell line bank. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heated-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2/95% air.

2.3. Animals

ICR mice (20–25 g) obtained from Qingdao Experimental Animal Center were used in this study. They were given free access to rodent chow and water ad libitum and allowed to acclimate by maintaining on a natural light environment for a week prior to experiment. Body weights were recorded throughout all the treatments. All experiments were conducted following the University Experimental Animal Committee Regulations.

2.4. Preparation of COS by chitosanase and analysis of the enzymatic hydrolysis products

Chitosan solution (100 ml, 1.0%, W/V) at pH 5.5 was kept in water bath at 45 °C. Then 1 ml chitosanase (EC 3.2.1.99) (10 mg) was added to the chitosan solution and the mixture was incubated for 30 min at t 45 °C to prepare COS. The composition of the enzymatic hydrolysis products was analyzed by waters high performance liquid chromatography (HPLC) system. The detector is 301 type evaporative light scattering detector. The concentration of the sample solution was 1% and 20 μl of the sample was injected to the column. The mobile phase consisted of acetonitrile/water solution (75:25; V/V) with the flow rate of 1 ml/min. The column temperature is 30 °C.

2.5. Labeling of COS with Fluorescein isothiocyanate (FITC)

The labeling of COS for studying cell uptake profile of COS was adapted from previously reported methods with minor modifications [17]. The mechanism of labeling was based on the reaction between the isothiocyanate group of FITC and the primary amino group on COS. COS was dissolved in 10.0 ml of H_2O(15%, W/V) and the pH of the COS solution was adjusted to pH 9.0. FITC (1 mg/ml) was dissolved in absolute methanol and 25 ml of FITC-methanol solution was added into the COS solution (COS:FITC = 60:1). The reaction was allowed to proceed for 3 h in the dark with shaking at ambient temperature. The mixed solution was precipitated with excess 95% ethanol and was allowed to stay for overnight. After centrifugation (6000 *g*, 40 min), the supernatant was discarded and the precipitant was re-dissolved in methanol (1 mg/ml). The labeling efficiency of FITC was determined. Briefly, fluorescence intensity reading of the FITC labeled-COS (FITC–COS) was measured on a fluorescence spectrophotometer (kexc 490 nm, kemi 520 nm) (Fluoroskan Ascent FL, Thermo Lab systems). The weight of FITC in the FITC–COS could be calculated from the FITC standard curve. The degree of labeling (percent) was calculated as the percent weight of FITC to the weight of FITC–COS.

$$\text{Degree of labeling}\,(\%) = \text{FITC(mg)}/\text{FITC–COS(mg)} \times 100\%$$

2.6. Determination of mitogenic activity of macrophages

RAW264.7 macrophages at 2×10^6 cells/ml were seeded onto 96-well plates and allowed to adhere. The culture medium were refreshed with new complete medium containing dilutions of COS at the final concentrations of 10–100 μg/ml. DMEM culture medium without COS served as the control group. Cells were incubated at 37 °C for 72 h and then the cell proliferation was measured by the MTT method. The relative absorbance at 490 nm was obtained with a plate reader. To confirm free LPS in COS, different concentrations of COS pre-incubated with polymyxin B (1 U/ml) for 1 h before adding to macrophages.

2.7. Determination of neutral red phagocytosis by macrophages

RAW 264.7 macrophages were prepared and treated as mentioned in 2.6. After incubation at 37 °C for 48 h, the culture medium was discarded and 200 μl of 0.7% neutral red (prepared in saline and filtered) was added in each well and cultured for another 1 h. Then the solution was discarded and each well was washed three times with D-Hank's. Lysing solution (200 μl, 0.1 M acetic acid: alcohol = 1:1) was added to each well and the plate was kept overnight at 4 °C. The absorbance at 492 nm was measured and D-Hank's served as blank.

2.8. Measurement of the level of nitric oxide (NO) and TNF-α in macrophages

RAW 264.7 macrophages were prepared and treated as mentioned in Section 2.6. After incubation at 37 °C for 48 h, cell culture medium was collected for measurement of NO and TNF-α. NO production was determined by measuring the accumulation of nitrite (NO_2^-) in the culture medium using Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% H_3PO_4). Briefly, 100 μl of culture medium was added to an equal volume of the Griess reagent in 96-well plate. After incubation at room temperature for 10 min, absorbance at 540 nm was determined with microplate reader. NO concentration was determined by using $NaNO_2$ as a standard. TNF-α secretion was measured by enzyme-linked immunosorbent assay (ELISA) method following the manufacturer's instructions.

2.9. Determination of organ weights and serum IgG, IgM contents

Four groups of ICR mice (n = 20 per group) were used in this study. One group was treated with saline and served as the control group. The other three groups were administrated with different doses of COS (100, 250, 500 mg/kg body weight respectively) intragastrically. At the time point of 10 and 20 days, mice were weighed and the blood was collected from the retro-orbital venous plexus with a capillary. Serum were separated by centrifugation at 4 °C (3000 rpm, 10 min) and stored at −20 °C for further test. Serum immunoglobulin G (IgG) and immunoglobulin M (IgM) contents were measured by ELISA method following the manufacturer's instructions. Mice were then sacrificed and the weights of the spleen and thymus were recorded. The spleen