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Anti-inflammatory effects of low-molecular weight chitosan oligosaccharides in IgE–antigen complex-stimulated RBL-2H3 cells and asthma model mice

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ABSTRACT

The anti-inflammatory effects of low-molecular weight chitosan oligosaccharides (LM-COS) prepared from high-molecular weight chitosan by enzymatic digestion were investigated against allergic reaction and allergic asthma *in vivo* and *in vitro*. Allergic asthma is an inflammatory disease of the airways associated with enhanced degranulation and cytokine generation. The LM-COS (<1 kDa), consisting of glucosamine $(GlcN)_n$, n = 3–5, were capable of inhibiting both antigen-stimulated degranulation and cytokine generation in rat basophilic leukemia RBL-2H3 cells. The protective effect of LM-COS against ovalbumin (OVA)-induced lung inflammation in asthma model mice was also examined. Oral administration of LM-COS (16 mg/kg body weight/day) resulted in a significant reduction in both mRNA and protein levels of interleukin (IL)-4, IL-5, IL-13, tumor necrosis factor (TNF)-α in the lung tissue and bronchoalveolar lavage fluid (BALF); The protein levels of IL-4, IL-13 and TNF-α in BALF were decreased by 5.8-fold, 3.0-fold and 9.9-fold, respectively, compared to those in the OVA-sensitized/challenged asthma control group. These results suggest that the oral administration of LM-COS is effective in alleviating the allergic inflammation *in vivo* and thus can be a good source material for the development of a potent therapeutic agent against mast cell-mediated allergic inflammatory responses and airway inflammation in allergic inflammatory diseases, including asthma.

1. Introduction

Asthma is a chronic inflammatory disease characterized by variable airflow obstruction, and the associated inflammatory responses are mediated by T-helper type (Th)2 cells, mast cells, B cells, and eosinophils [1,2]. Upon challenge with various allergens, these inflammatory cells infiltrate the airway and produce Th2 cytokines such as interleukin (IL)-4, IL-5, and IL-13 [1,2]. Therefore, targeted therapies have been directed toward preventing Th2 responses. Asthmatic animals are reported to have a high concentration of Th2 cytokines (IL-4 and IL-13) and the proinflammatory cytokine tumor necrosis factor (TNF)-α, in the bronchoalveolar lavage fluid (BALF) and increased levels of Th2 and TNF-α cytokine mRNA and protein in the lung tissues [1,2]. Several drugs for asthma therapy are currently available, but most of these agents induce adverse side effects and are relatively inefficient [3]. Therefore, natural compounds with therapeutic benefits and fewer side effects are necessary for the development of novel anti-asthma drugs and functional foods.

Chitin is the second most abundant naturally occurring polysaccharide composed of β-(1,4)-linked-D-*N*-acetylglucosamine (GlcNAc) after cellulose. Chitosan is a linear heteropolysaccharide composed of α-(1,4)-linked-D-glucosamine (GlcN) and GlcNAc, which can be derived from chitin. There have been numerous reports on the chitinous materials (chitins, chitosan and their derivatives) due to their remarkable biological activities, including antibacterial, antifungal, antitumor and stimulating immunoenhancing properties and also their non-toxic, biocompatible and biodegradable natures [4–9]. For these reasons, the chitinous materials have been a topic of intensive researches for several decades for potential industrial applications such as the production of functional foods, food additive, drugs and cosmetic products [4,10]. However, in spite of their interest as biologically active compounds, the high molecular mass and poor solubility of these polysaccharides have hampered their industrial applications especially as a therapeutic agent. To overcome these problems, preparation of chitosan oligosaccharides in active form is obviously gaining importance in many biomedical applications. Accordingly, there have been many reports on preparation and biological activities of various chitooligosaccharides (COS) (or chitosan oligosaccharides depending on the degree of deacetylation) [4,10]. It has been reported that the biological activities of COS are also significantly dependent on their solubility, molecular weight and degree of deacetylation (DDA, the ratio of glucosamine to *N*-acetylglucosamine, GlcN/GlcNAc) [4,11,12]. The COS are readily soluble in water due to their shorter chain lengths and free amino groups of D-glucosamine units [11] and exhibit higher oral absorption with various biological effects, including anti-bacterial, anti-tumor and anti-

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oxidant effects [13]. A recent study has also shown that the COS with a molecular weight of 1–10 kDa possess *in vitro* inhibitory effects on degranulation and cytokine generation in rat basophilic leukemia cells [10]. However, the effects of low-molecular weight chitosan oligosaccharides (LM-COS) on *in vivo* anti-asthmatic activity against allergic airway inflammation have not been demonstrated.

In this context, we previously reported on the preparation of LM-COS [glucosamine $(GlcN)_n$, n = 3–5, DDA of 100% and molecular weight less than 1 kDa] by enzymatic degradation of high-molecular weight chitosan polysaccharide, whose structure, size and DDA were confirmed mainly by gel-filtration column chromatography and matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-TOF MS) analysis [14]. In the present study, we investigated possible anti-asthmatic activity of LM-COS against allergic airway inflammation *in vivo* and *in vitro* as well. To do this, the effects of LM-COS on degranulation and production of asthma-related cytokines (IL-4, IL-13 and TNF-α) in IgE–antigen complex-stimulated rat basophilic leukemia RBL-2H3 cells and asthma-related cytokines generation in BALF, lung tissue and serum of ovalbumin (OVA)-sensitized/challenged mouse asthma model, were examined.

2. Materials and methods

2.1. Preparation of LM-COS

The high-molecular weight chitosan of approximately 1900 kDa and 98.5% DDA and the high molecular COS (HM-COS) of approximately 70 kDa and 98.5% DDA were kindly provided by Prof. R. D. Park at Cheonnam University, Korea. The LM-COS were prepared as previously described [14]. Briefly, the chitosan was fully dissolved in 1% acetic acid to be a 1% solution (w/v) at room temperature, and the pH of the solution was adjusted to pH 5.0 with $NaHCO_3$. Chitosan polymer was then hydrolyzed by recombinant chitosanase (Kyowa Chemical Ltd., Tokyo, Japan) in a reaction mixture as previously reported [14]. The resulting oligosaccharides mixture was fractionated through Bio Gel-P4 (Bio-RAD, USA) gel-filtration column and finally, one of the two major carbohydrate-positive fractions was obtained. The structure, size and DDA of this fraction were confirmed mainly by gel-filtration column chromatography and matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-TOF MS) analysis, showing that it consists of glucosamine $(GlcN)_n$, n = 3–5, DDA of 100% with molecular weight less than 1 kDa [14]. This low-molecular weight chitosan oligosaccharide fraction was thus named LM-COS and used in the present study for its anti-asthmatic activity.

2.2. Cell culture and MTT assay

RBL-2H3 cells were obtained from the Korean Cell Line Bank (Seoul, Korea) and were grown in MEM containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (PEST) at 37 °C in a humidified incubator (5% CO_2, 95% air). To evaluate the cytotoxic effect of the tested LM-COS and HM-COS against RBL-2H3 cells, treatments for the cytotoxicity test were performed according to the method reported by Huang et al. [15]. Cell viability was determined using an MTT [3-(3,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay.

2.3. β-Hexosaminidase release assay and preparation of RT-PCR samples

The β-hexosaminidase release assay was performed as previously described [15], with slight modifications. RBL-2H3 cells (2×10^5 cells) in 24-well plates were preincubated with 1 ml MEM consisting of 0, 125, 250, 1000 and 1500 μg/ml of LM-COS or HM-COS for 24 h and were stimulated with 0.5 μg/ml anti-DNP IgE for 24 h. The cells were then washed with Siraganian buffer (pH 7.2, 119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, and 25 mM PIPES) and incubated in Siraganian buffer containing 5.6 mM $CaCl_2$ and 0.1% BSA for an additional 10 min. Thereafter, the cells were stimulated for 2 h with DNP-HSA (10 μg/ml), which activates RBL-2H3 cells to produce allergic reactions, and the cells and cell culture supernatants were used for RT-PCR or ELISA. The supernatant (20 μl) was added to the substrate solution (*p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide 2 mM in 0.1 M sodium citrate buffer, pH 1.5) (20 μl), and the mixture was incubated for 1 h at 37 °C. The reaction was terminated by adding 200 μl of stopping buffer (0.1 M $Na_2CO_3/NaHCO_3$, pH 10). The absorbance at 405 nm was measured with a microplate reader (Molecular Devices, USA).

The LM-COS-mediated inhibition of β-hexosaminidase release was expressed as the inhibition percentage and was calculated using the following formula:

$$Inhibition\,(\%) = [1 - T(405\,nm)/C(405\,nm)] \times 100$$

where C is IgE (+) + DNP-HSA (+) + test sample (−), and T is IgE (+) + DNP-HSA (+) + test sample (+).

2.4. Animals, diets and experimental protocol

For *in vivo* experiments, female BALB/c mice (age 6 weeks; KOATECH, Gyeonggi-do, Korea) were used. The mice were housed in an air-conditioned (21–25 °C) and humidity-controlled room with a 12 h on/12 h off light. The mice were maintained under standard laboratory conditions for 1 week prior to the experiments and were then divided into three groups of 10–16 mice. Animal care and handling were performed under protocols approved by the Committee on Animal Experimentation of the Catholic University of Korea. A schematic diagram of the treatment schedule is shown in Fig. 1. A total of 500 μg/ml of OVA was complexed with 500 μg/ml of alum, and the final volume ratio of alum (500 μg/ml) to OVA (500 μg/ml) was 1:3. Each mouse was immunized by intraperitoneal (IP) injection of 14.7 ml/kg body weight of OVA complexed with alum on days 0, 6, and 12 (Fig. 1). The control group received 14.7 ml/kg body weight of phosphate-buffered saline (PBS) with alum by IP injection.

The OVA-challenged mice were exposed to 2% OVA (w/v, in PBS) for 10 min (keeping: 5 min) by inhalation using a compressor nebulizer (0.4 ml/min, NE-C28, Omrom, Tokyo, Japan) and the LM-COS (16.5 mg/kg body weight) were orally administered everyday from day 13 to day 27 consecutively. After feeding, the mice were fasted overnight (16–19 h) and sacrificed on day 29. The mice were euthanized with an IP injection of a Zoletil 50 (Virbac S.A, France) and Rompun (Bayer, Germany) mixture (3:2 mixture). The blood samples were collected into tubes. The lungs were lavaged with ice-cold PBS (0.5 ml), and BALF was obtained with three lavages (a total volume of 1.5 ml). The BALF was centrifuged, and the supernatant was stored at −80 °C until the cytokine assays were performed. The lungs were rapidly removed, frozen on liquid nitrogen and stored at −80 °C for total RNA and protein extraction.

2.5. RNA extraction and RT-PCR analysis of cytokine mRNA production

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad CA, USA) according to the manufacturer's instructions. The RNA was reverse-transcribed using a Power cDNA synthesis kit (Intron, Gyeonggi-do, Korea) with an oligo $(dT)_{15}$ primer according to the manufacturer's recommendations. The polymerase chain reaction (PCR) was performed with a Maxime PCR PreMix kit (Intron, Gyeonggi-do, Korea) in 20 μl of total reaction mixture containing 1 μl of the RT-reaction mixture and 2 μl of each primer (forward and reverse, 10 pmol/μl).

The following *in vitro* primers were used: TNF-α, F, 5′-CGGAATTCGGCTCCCTCTCATCAGTTC and R, 5′-GCTCTAGACCCTT