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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.

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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 chitoooligosaccharides (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 NaHCO₃. 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% CO₂, 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 μ g/ml of LM-COS or HM-COS for 24 h and were stimulated with 0.5 μ g/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 MgCl₂, and 25 mM PIPES) and incubated in

Siraganian buffer containing 5.6 mM CaCl₂ and 0.1% BSA for an additional 10 min. Thereafter, the cells were stimulated for 2 h with DNP-HSA (10 μ g/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 μ l) 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 μ l), and the mixture was incubated for 1 h at 37 °C. The reaction was terminated by adding 200 μ l of stopping buffer (0.1 M Na₂CO₃/NaHCO₃, 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:

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

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 μ g/ml of OVA was complexed with 500 μ g/ml of alum, and the final volume ratio of alum (500 μ g/ml) to OVA (500 μ g/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, Omron, 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 μ l of total reaction mixture containing 1 μ l of the RT-reaction mixture and 2 μ l of each primer (forward and reverse, 10 pmol/ μ l).

The following *in vitro* primers were used: TNF- α , F, 5'-CGGAATTCGGCTCCTCTCATCAGTTC and R, 5'-GCTCTAGACCTT

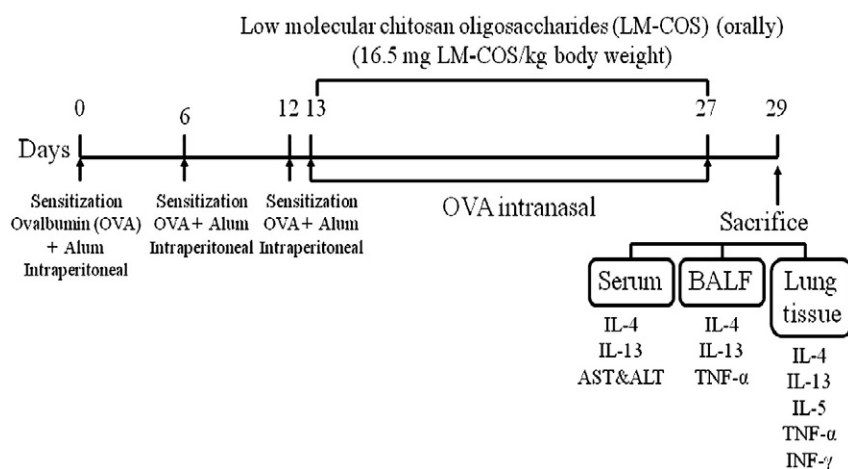


Fig. 1. Protocol for animal treatment. BALF, bronchoalveolar lavage fluid; OVA, ovalbumin.

GAAGAGAACCTGGG; IL-4, F, 5'-ACCTTGCTGTACCCCTGTTC and R, 5'-TTGTGAGCGTGACTCATTC; and IL-13, F, 5'-GCTCTCGCTTGCTTGGTGGTC and R, 5'-CATCCGAGGCCTTTTGGTTAGAG. The GAPDH (F, 5'-ACCACAGTCATGCCATCAC and R, 5'-TCCACCACCTGTTGCTGTA) transcripts were used as internal references. PCR using the TNF- α , IL-4 and IL-13 primers was performed with an initial cycle of 2 min at 94 °C, followed by 35 cycles of 20 s at 94 °C, 30 s at 60 °C, and 40 s at 72 °C, and a final extension for 5 min at 72 °C; PCR using the GAPDH primers was performed using the same settings, with the exception of the annealing temperature (GAPDH, 55 °C).

The sequences of the *in vivo* primers are as follows: IL-4, F, 5'-TCATCCTGCTCTTCTTTC-3' and R, 5'-TGGACTTGGACTCATTC-3'; IL-13, F, 5'-TTGCTTGCCCTTGGTGGTC-3' and R, 5'-GGCTGGAGACCGTAGTGG-3'; TNF- α , F, 5'-GGCAGGTCTACTTTGGAGTCATTGC-3' and R, 5'-ACATTCGAGGCTCCAGTG AATTCGG-3'; IL-5, F, 5'-AAGCAATGAGACGATGAG-3' and R, 5'-CATCACACCAAGGAATC-3'; INF- γ , F, 5'-TGTTTCTGGCTGTACTG-3' and R, 5'-TTGCTGTTGCTGAAGAAG-3'; and β -actin, F, 5'-TGCTGTCCTGTATGCCTCT-3' and R, 5'-AGGCTTTACGGATGTCAACG.

The PCRs with the IL-13 and β -actin primers were performed with an initial cycle of 2 min at 94 °C, followed by 38 cycles (IL-13) or 25 cycles (β -actin) for 20 s at 94 °C, 20 s at 60 °C, and 40 s at 72 °C, and a final extension for 5 min at 72 °C; the PCR using the IL-4 primers was performed similarly, except that the annealing temperature was 54 °C, and the number of cycles was 40; the PCRs using the IL-4 and INF- γ primers were performed similarly, with the exception of the annealing temperature (IL-4 and INF- γ , 50 °C) and number of cycles (IL-4 and INF- γ , 40 cycles). The GAPDH transcripts were used as internal references for the *in vitro* experiments. The β -actin transcripts served as internal controls for the *in vivo* experiments. The PCR products were analyzed by 2% agarose gel electrophoresis.

2.6. Enzyme-linked immunosorbent assay (ELISA)

For the ELISAs, the lung tissues were homogenized at 4 °C in PBS including a protease inhibitor cocktail (Sigma, St. Louis, USA). The lysate was clarified by centrifugation at 13,000 rpm for 20 min at 4 °C. The protein concentration was determined using a Bio-Rad protein kit (Hercules, CA, USA) with bovine serum albumin (BSA, Sigma) used as the standard. The protein levels of IL-4, IL-13 and TNF- α in BALF, lung tissue and serum were also measured. The concentrations of IL-4, IL-13 and TNF- α (eBioscience, San Diego, CA) in BALF, lung tissue and serum were measured using ELISA kits according to the manufacturer's instructions. The production of IL-4, IL-13 and TNF- α in cell culture supernatants or cell lysates was quantified by ELISA kits (IL-4

and TNF- α ; Abcam, Cambridge, UK, IL-13; RayBiotech, Norcross, GA) using the manufacturer's instructions.

2.7. Measurement of serum alanine transaminase (ALT) and aspartate transaminase (AST)

The ALT and AST levels in the serum of OVA-induced asthma mice were measured using commercial kits (Asan Chemical, Seoul, Korea), according to the manufacturer's instructions, after feeding the mice with LM-COS (16.5 mg/kg body weight) for 14 days.

2.8. Statistical analysis

Data from three independent experiments were expressed as mean $\pm$ S.D. One-way analysis of variance (ANOVA) followed by Turkey's test was used to compare the results from different treatments. Data were considered to have statistical significances at $P < 0.05$.

3. Results

3.1. Cytotoxicity and effects of LM-COS and HM-COS on degranulation in IgE-antigen complex-stimulated RBL-2H3 cells

To examine cytotoxicity of LM-COS and HM-COS, RBL-2H3 cells were stimulated with IgE-antigen complexes, cultured with various concentrations of LM-COS or HM-COS (0, 125, 250, 500, 1000, 1500 μ g/ml) and then the cell viability was determined by MTT assay. As shown in Fig. 2C, the viability of RBL-2H3 cells was nearly affected by LM-COS at up to 1500 μ g/ml, showing no significant cytotoxicity of LM-COS. However, the cytotoxicity was increased, compared with control, in cells exposed to 1500 μ g/ml HM-COS ($73.15 \pm 2.13\%$ cell survival). According to this result, the LM-COS concentrations from 125 to 1500 μ g/ml and HM-COS concentration from 125 to 1000 μ g/ml were selected for subsequent experiments. The effect on cytokine protein and β -hexosaminidase release of LM-COS and HM-COS was compared in cell treated with 250, 500 and 1000 μ g/ml LM-COS or HM-COS due to cytotoxicity of 1500 μ g/ml HM-COS.

To assess the effect of LM-COS and HM-COS on degranulation in RBL-2H3 cells, the granule release from mast cells was determined by measuring the extracellular activity of β -hexosaminidase, a marker enzyme for histamine-containing granules [10,16]. As shown in Fig. 2D, the LM-COS dose-dependently attenuated the β -hexosaminidase release in IgE-antigen complex-stimulated mast cells, showing the suppressive effect of LM-COS on degranulation. The β -hexosaminidase levels in 500, 1000 or 1500 μ g/ml LM-COS-treated cells were decreased by 1.19-,

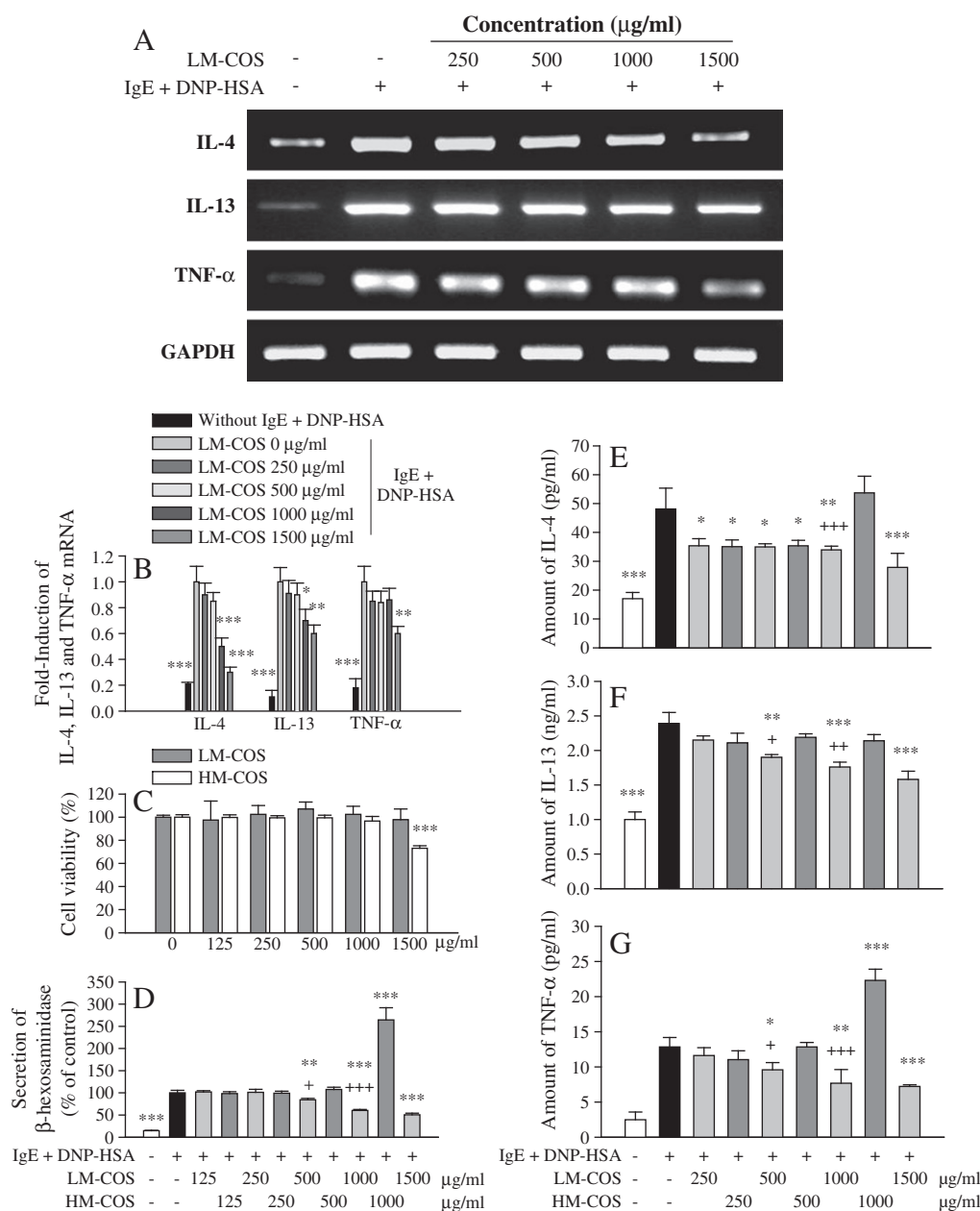


Fig. 2. Effect of low-molecular weight chitosan oligosaccharides (LM-COS) and high-molecular weight chitosan oligosaccharides (HM-COS) on the cytotoxicity, β -hexosaminidase release, cytokine levels in IgE-antigen complex-stimulated rat basophilic leukemia RBL-2H3 cells. The cytokine levels in each sample were normalized to the GAPDH levels. The density of each mRNA was quantified by using SigmaGel software (Jandel Scientific, San Rafael, CA). The cytokines in the cell culture supernatants and cell lysates were analyzed using ELISA. Results are from three experiments and expressed as the mean $\pm$ SD ($n = 3-5$). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to control; + $P < 0.05$, ++ $P < 0.01$ and +++ $P < 0.001$ compared to HM-COS at the same concentration.

1.66- and 1.98-fold, respectively, compared to the LM-COS-untreated control cells.

However, there was no obvious change in the β -hexosaminidase release level in cells treated with 125–1000 $\mu\text{g/ml}$ HM-COS and treatment of cells with 1500 $\mu\text{g/ml}$ HM-COS increased the secretion of β -hexosaminidase above that of the untreated control (Fig. 2D). Lower levels of β -hexosaminidase release were obtained when the cells were treated with 500 and 1000 $\mu\text{g/ml}$ LM-COS than when they were exposed to HM-COS at the same concentration (Fig. 2D).

3.2. Effects of LM-COS on cytokine expression in IgE-antigen complex-stimulated RBL-2H3 cells

The inhibitory effects of LM-COS on the increased level of IL-4, IL-13 and TNF- α mRNA and protein induced by IgE-antigen complex are

shown in Fig. 2A, B and E–G. The IL-4, IL-13 and TNF- α mRNA levels in the control group (IgE-antigen complex-stimulated cells with 0 mg/ml LM-COS) were significantly increased by 4.76-fold, 9.09-fold and 5.55-fold, respectively, compared to the IgE-antigen complex-untreated cells (cells without IgE + DNP-HSA). The LM-COS at 1500 $\mu\text{g/ml}$ significantly inhibited the expressions of IL-4, IL-13 and TNF- α cytokines by 3.33-fold, 1.67-fold and 1.67-fold, respectively, compared to the control group, clearly showing *in vitro* anti-inflammatory effects of LM-COS (Fig. 2A and B).

Their effects on IL-4, IL-13 and TNF- α protein levels were also investigated and 500, 1000 and 1500 $\mu\text{g/ml}$ LM-COS-treated cells were all down-regulated compared to controls (Fig. 2E–G). Protein levels of IL-4, IL-13 and TNF- α of LM-COS group were not significantly different from those of HM-COS group at the concentration of 250 $\mu\text{g/ml}$. However, lower levels of IL-13 and TNF- α protein were obtained when

the cells were treated with 500 and 1000 $\mu\text{g/ml}$ LM-COS than when they were exposed to 500 and 1000 $\mu\text{g/ml}$ HM-COS. In cells treated with 1000 $\mu\text{g/ml}$ LM-COS, protein levels of IL-4, IL-13 and TNF- α were reduced to 1.58-, 1.22- and 2.91-fold, respectively, compared to those exposed to 1000 $\mu\text{g/ml}$ HM-COS (Fig. 2E–G).

3.3. Effects of LM-COS on cytokine production in the BALF and serum of asthma model mice

The *in vivo* anti-asthma effect of LM-COS was examined in asthma mouse model by determining the protein levels of secreted cytokines in the BALF and serum by ELISA. The OVA challenge resulted in 5.8-, 3.0- and 6.0-fold increases in the protein levels of IL-4, IL-13 and TNF- α , respectively, in BALF fluid compared to PBS-challenged control mice (Fig. 3A). After LM-COS consumption (16.5 mg LM-COS/kg body weight/day) for 2 weeks, the protein levels of IL-4, IL-13, IL-5 and TNF- α in the lung tissues of the LM-COS experiment group (OVA + LM-COS) were significantly lower than in the OVA-sensitized/challenged control group (OVA) (Fig. 3A–E). The detection limit of the ELISA is approximately 4 pg/ml (eBioscience, San Diego, CA). However, the sensitivities of the standards and samples, following an overnight incubation at 4 °C, were increased to approximately 2 pg/ml. Therefore, we set the detection limit for the serum cytokines at 4 pg/ml. Sensitization of mice with OVA (asthma-induced control group) resulted in increased production of serum IL-4 and IL-13 proteins. However, LM-COS treatment (16.5 mg LM-COS/kg body weight, OVA + LM-COS mice group) significantly reduced both IL-4 and IL-13 production. In LM-COS treated mice, the IL-4 was detectable in only one out of 10 mice (Fig. 3B) and the IL-13 was not detectable in all the mice (10 mice) tested (Fig. 3C). The results clearly showed that the oral administration of LM-COS is effective in alleviating the allergic inflammation *in vivo*.

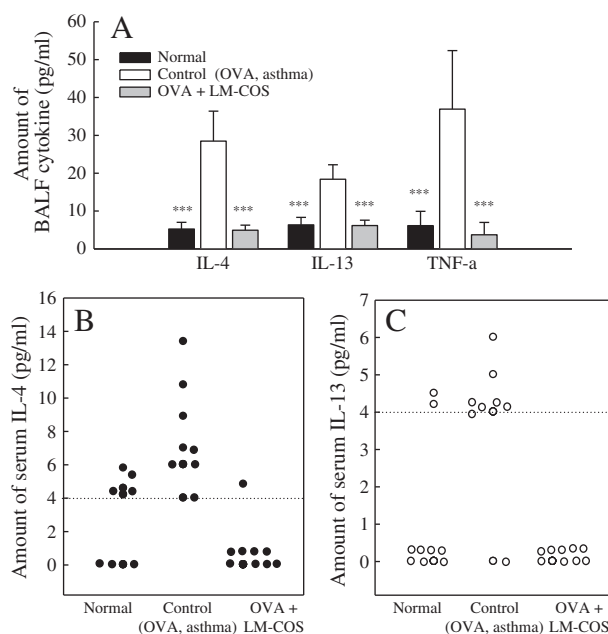


Fig. 3. Effect of low-molecular weight chitosan oligosaccharides (LM-COS) on the cytokine levels in bronchoalveolar lavage fluid (BALF) and serum of asthma model mice. Serum and BALF was obtained on day 29 from PBS-treated mice (normal group), ovalbumin (OVA)-treated mice [control (OVA, asthma) group], and OVA-treated mice administered LM-COS (16.5 mg/kg body weight) (OVA + LM-COS). The cytokines in the BALF and serum were analyzed using ELISA. The mean value was significantly different from the control (OVA, asthma), *** $P < 0.001$.

3.4. Effects of LM-COS on expression and production of IL-4, IL-13 and TNF- α in lung tissue

When animals received LM-COS, the IL-4, IL-5, IL-13, and TNF- α mRNA levels significantly decreased compared to the OVA-sensitized/challenged control group (Fig. 4A–F). The LM-COS significantly attenuated the OVA challenge-mediated increase in IL-4, IL-13, IL-5 and TNF- α mRNA by 1.51-fold, 10.71-fold, 1.85-fold and 1.57-fold, respectively. However, the level of the Th1 cytokine, IFN- γ , was not changed as compared to PBS-challenged control mice (Fig. 4F). Administration of LM-COS led to a significant reduction in the levels of Th2-type cytokines (i.e., IL-4 and IL-3) in the lung tissue (Fig. 4G). The IL-4 and IL-13 protein levels were suppressed by 1.23-fold and 1.38-fold, respectively, after LM-COS consumption (16.5 mg/kg body weight/day), compared to the OVA-sensitized/challenged control group (Fig. 4G). These results clearly demonstrated that LM-COS can abrogate *in vivo* asthma symptoms by inhibiting the expression of Th2-type cytokines during OVA-induced airway inflammation.

3.5. Effects of LM-COS on serum ALT and AST levels in mice with OVA-induced asthma

To confirm the absence of liver toxicity by the LM-COS, the effects of LM-COS on the serum ALT and AST levels in OVA-induced asthma mice were determined. The LM-COS-treated asthma group (OVA + LM-COS) showed no significant differences in serum ALT and AST levels as compared to the OVA-induced control group (OVA, asthma) (Fig. 4H), demonstrating no detectable level of liver toxicity by the LM-COS.

4. Discussion

Although the chitinous materials (chitin, chitosan and their derivatives) have been attracting much attention due to their diverse biological activities, including antibacterial, antifungal, antitumor and immune enhancing properties [4–9,12,17], the high molecular mass and poor solubility of these polysaccharides have hampered their industrial applications especially as a therapeutic agent. Accordingly, recent studies have focused on preparation and biological activities of various chitoooligosaccharides (COS) (or chitosan oligosaccharides depending on the degree of deacetylation) [4,10], since they exhibit higher oral absorption and enhanced solubility in water mainly due to their shorter chain lengths and free amino groups of D-glucosamine units [11]. Various chitoooligosaccharides (COS) (or chitosan oligosaccharides depending on degree of deacetylation) have been reported for their diverse biological activities, including anti-bacterial, anti-tumor and anti-oxidant effects [13]. Recently, *in vitro* anti-inflammatory effects of chitoooligosaccharides (COS) with molecular weight of 1–10 kDa have been reported [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 yet. Therefore, in the present study, we tested possible both *in vitro* and *in vivo* anti-inflammatory and anti-asthmatic effects of low-molecular weight chitosan oligosaccharides (LM-COS) [glucosamine (GlcN)_n, n = 3–5, DDA of 100% and molecular weight of less than 1 kDa], which were prepared in our lab by enzymatic degradation of high-molecular weight chitosan polysaccharide, whose structure, size and DDA were previously confirmed mainly by gel-filtration column chromatography and matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-TOF MS) analysis [14].

Allergy is induced by the interaction between antigen and antigen-specific IgE, and asthma is considered an allergic disease [15,18]. Asthma is a chronic inflammatory disease characterized by an increase in airway responsiveness, such as airway obstruction in response to allergens, chronic eosinophilic airway inflammation, mucin hypersecretion, and non-specific airway hyper-responsiveness

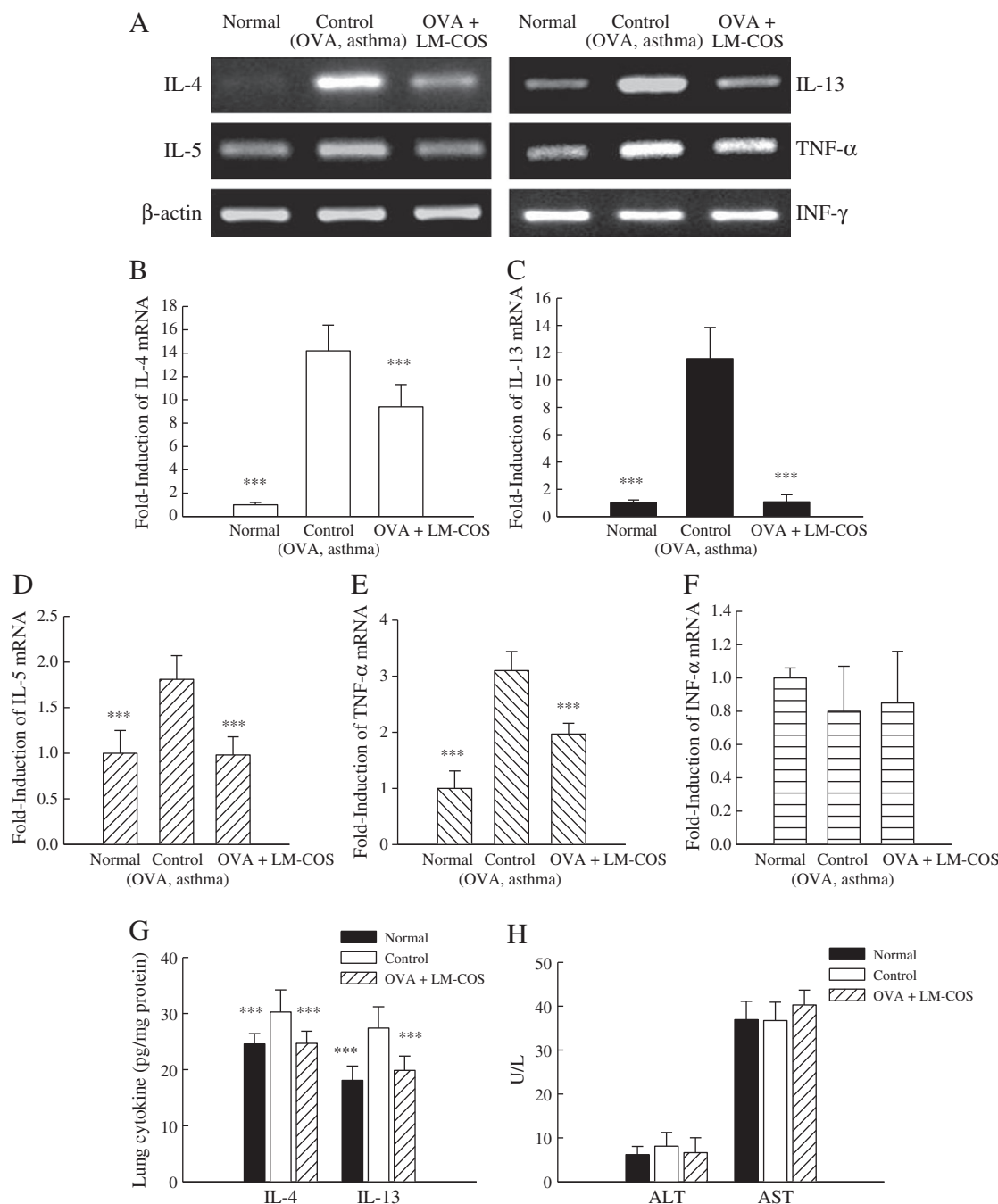


Fig. 4. Effect of low-molecular weight chitosan oligosaccharides (LM-COS) on lung tissue cytokines, ALT and AST levels in asthma model mice. The groups were divided as described in Fig. 3. The cytokine mRNA levels in each sample were normalized to the β-actin levels. The density of each mRNA was quantified by using SigmaGel software (Jandel Scientific, San Rafael, CA). The mean value was significantly different from the control, *** $P < 0.001$.

(AHR). These inflammatory responses are attributed to Th2 cells and other inflammatory factors including mast cells, B cells, eosinophils, cytokines and chemokines [19]. In chronic asthma, it is well characterized that Th2 cells infiltrate the lungs and produce inflammatory cytokines including IL-4, IL-5 and IL-13. Th2 cells play important roles in the initiation and progression of allergic diseases, including asthma, through their ability to produce IL-4, IL-5 and IL-13 [20]. TNF-α is a pro-inflammatory cytokine and has been detected in the BALF of asthmatics. Thus, TNF-α is an important element in determining the severity of asthma.

The LM-COS were found to inhibit β-hexosaminidase release from the RBL-2H3 cells stimulated with IgE-antigen complexes, which have been used as FcεRI-mediated mast cell activator, showing their inhibitory effect on degranulation of histamine-

containing granules in mast cells (Fig. 2C). Mast cell degranulation is important in the initiation of immediate responses following exposure to allergens [10,18]. β-Hexosaminidase, which is associated with a variety of inflammatory diseases, is released along with histamine in mast cells [21]. Thus, histamine and β-hexosaminidase are major targets for effective anti-allergic drugs and are often used as markers of mast cell degranulation [10,21]. On the other hand, aggregation of the high-affinity IgE receptor (FcεRI) on mast cells and basophils leads to the synthesis and secretion of Th2-type cytokines, such as IL-4, IL-5 and IL-13, and proinflammatory cytokines such as TNF-α [22]. Our LM-COS also significantly inhibited the expression of asthma-related cytokines (IL-4, IL-13 and TNF-α) in IgE-antigen complex-stimulated rat basophilic leukemia RBL-2H3 cells (Fig. 2A and D). Vo et al. reported

that among the COS tested (1–3 kDa, 3–5 kDa, and 5–10 kDa), the lower molecular weight COS (1–3 kDa) were the most effective in attenuating the histamine and β -hexosaminidase release from the calcium ionophore A23187-stimulated RBL-2H3 cells and that these COS also inhibited the production and mRNA expression of TNF- α , IL-1b, IL-4, and IL-6 cytokines [10]. We examined, in the present study, much smaller COS [<1 kDa, glucosamine (GlcN)_n, n = 3–5, DDA of 100%], thus named LM-COS, against allergic reactions in IgE-antigen complex-stimulated RBL-2H3 cells and we compared the effects of LM-COS with HM-COS (70 kDa) in this study to clarify the difference of the effects among COS and also emphasize the merit of LM. Since the biological activities of chitinous materials including COS are 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 *in vitro* results observed in this study (Fig. 2) and other research group's results as well [10] suggested that the quite smaller-sized COS (like our LM-COS of less than 1 kDa) can be a good, or even better in terms of molecular sizes, source than larger chitinous materials for the development of pharmaceuticals with anti-asthma effect.

Subsequently, we tested LM-COS for their *in vivo* effect against allergic asthma using ovalbumin (OVA)-sensitized/challenged asthma model mice. The mice fed with LM-COS (16.5 mg/kg body weight) showed significantly reduced levels of asthmatic cytokines (IL-4, IL-13) and TNF- α proteins in the bronchoalveolar lavage fluid (BALF) (Fig. 3A). The LM-COS also significantly attenuated the OVA challenge-mediated increase in Th2-type cytokine protein production (Fig. 4G) and mRNA expression of asthmatic cytokines (IL-4, IL-5, IL-13) and TNF- α in the lung tissues (Fig. 4A–E), whereas the mRNA level of IFN- γ , a Th1 cytokine [23], was not significantly changed (Fig. 4F). On the other hand, ALT and AST levels, known indicators of hepatocellular damage, were not altered by the oral administration of LM-COS, suggesting that the dose of LM-COS used in the present study had no cytotoxic effects (Fig. 4H). Taken collectively, these results suggest that LM-COS can abrogate *in vivo* asthma symptoms, with no detectable liver cytotoxicity and these effects could be mediated by inhibiting the expression of Th2-type cytokines during OVA-induced airway inflammation.

On the other hand, it has been reported that the transcription factor nuclear factor-kappa B (NF- κ B) regulates the expression of the pro-inflammatory cytokines and the anti-asthmatic natural compounds inhibited the activation of NF- κ B in RBL-2H3 cells stimulated by the IgE-antigen complex [18]. The mitogen-activated protein kinases (MAPKs) are appropriate targets for pharmacological treatment of allergic inflammation because the MAPK pathway is involved in expression of inflammatory cytokines [10]. Vo et al. [10] reported that chitoooligosaccharides (COS) inhibited cytokine generation and the phosphorylation of MAPKs pathways, including extracellular signal-regulated kinase (MEK/ERK), p38 kinase, and c-jun-N-terminal kinase (JNK) in RBL-2H3 cells stimulated by calcium ionophore A23187 and 12-myristate 13-acetate (PMA). Therefore, these observations may provide a strong possibility that our LM-COS may also inhibit cytokine generation in asthma model cells and animals through suppression of MAPKs pathways.

In conclusion, the results from the present study demonstrated for the first time that LM-COS [<1 kDa, glucosamine (GlcN)_n, n = 3–5, DDA of 100%], thus small glucosamine oligomers, attenuate the production and expression of Th2 cytokines (IL-4, IL-5 and IL-13) as well as proinflammatory cytokine (TNF- α) in IgE-antigen complex-stimulated rat basophilic leukemia RBL-2H3 cells and in OVA-sensitized/challenged allergic asthma model mice. These results suggest that LM-COS can abrogate the

symptoms of asthma *via* anti-inflammatory activities by down regulating Th2 cytokines and proinflammatory cytokine *in vitro* and *in vivo*. Our findings suggest that LM-COS may be promising candidates for the development of a potent therapeutic agent for allergic asthma, especially due to their smaller sizes.

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