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• BASIC RESEARCH •

Inhibitory effect of heparin-derived oligosaccharides on secretion of interleukin-4 and interleukin-5 from human peripheral blood T lymphocytes

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Abstract

AIM: To investigate the inhibitory effect of heparin-derived oligosaccharides (Oligs) on secretion of interleukin-4 (IL-4) and interleukin-5 (IL-5) from human peripheral blood T lymphocytes (PBTLs).

METHODS: Oligs were prepared by three different heparin depolymerization methods and separated by gel filtration chromatography. PBTLs from ten adult patients with allergic eosinophilic gastroenteritis were treated with phytahematoagglutinin (PHA) and Oligs. The supernatants from the cell culture of PBTLs were harvested and subjected to the determination of IL-4 and IL-5 contents by ELISA method.

RESULTS: At the concentration of 5 μg/mL, Oligs with different M_r had different effects on the secretion of IL-4 and IL-5. The tetrasaccharide with M_r of 1 142, produced by depolymerizing heparin with hydrogen peroxide, had the strongest inhibitory effect on the secretion of IL-4. It decreased the IL-4 content from 375.6±39.2 ng/L (PHA group) to 12.5±5.7 ng/L (P<0.01). The hexasaccharide with M_r of 1 806, produced by depolymerizing heparin with β-elimination method, had the strongest inhibitory effect on the secretion of IL-5. It decreased the IL-5 content from 289.2±33.4 ng/L (PHA group) to 22.0±5.2 ng/L (P<0.01).

CONCLUSION: The inhibitory activity of Oligs on the secretion of IL-4 and IL-5 from human PBTLs closely depends on their molecular structure, and there may be an essential structure to act as an inhibitor. The most effective inhibitors of IL-4 and IL-5 secretion are tetrasaccharides and hexasaccharides, respectively.

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INTRODUCTION

Heparin is a highly sulfated, polyanionic glycosaminoglycan. It is composed of the trisulfated disaccharide unit [- *L*-iduronic acid-2-sulfate (IdoA2S) (1→4)-α-*D*- glucosamine-*N*,6 -disulfate (GlcNS6S)-], interrupted by irregular sequences containing undersulfated (or oversulfated) uronic acids and amino sugar residues, with an average relative molecular mass (M_r) of 12 000-15 000[1]. In addition to its anticoagulatory effect, heparin has anti-inflammatory property, which has been used for treatment of ulcerative colitis and other inflammatory bowel diseases (IBD)[2-15]. Although many studies have reported heparin's anti-inflammatory activities, there is little information available in literature about the specific oligosaccharide structures in heparin that inhibits the secretion of IL-4 and IL-5. IL-4 and IL-5 abnormalities have been reported to be consistent with the elevated IgE and eosinophilia in allergic eosinophilic gastroenteritis, suggesting that strategies targeting T lymphocytes may be efficacious in treatment of this kind of diseases[16-19]. Here, we examined whether there was a relationship between the molecular weight of heparin-derived Oligs and their inhibitory effect on IL-4 and IL-5 secretion from PBTLs in allergic eosinophilic gastroenteritis.

MATERIALS AND METHODS

Drugs and reagents

Heparin sodium was donated by Dongying Tiandong Biochemical Industrial Co. Ltd, and 300 mL/L hydrogen peroxide (H_2O_2) was purchased from Laiyang Fine Chemical Regent Co, Ltd. Benzethonium chloride and (4-chloro)-benzyl chloride were purchased from Sigma Co., USA. Sodium nitrite was purchased from Jinan Chemical Reagents Co., Ltd. Superdex-30 was purchased from Amersham Biosciences. Human IL-4 and IL-5 ELISA kits (96 tests) were purchased from Bender Medsystems, USA. Phytahematoagglutinin (PHA) was purchased from Shanghai Yihua Medical Science and Technology Co., Ltd. RPMI 1640 culture medium was purchased from Gibco, Paisley, UK.

Preparation of Oligs

Heparin was degraded by oxidative depolymerization with hydrogen peroxide[20], deaminative cleavages with nitrous acid[21] and β-eliminative cleavages with alkaline[22], respectively. All Oligs were obtained from the products separated by gel filtration chromatography with Superdex-30 column (26 mm×1200 mm), and the oligosaccharides were eluted with 0.25 mol/L ammonium bicarbonate and dried with lyophilization.

Determination of the relative molecular weight of Oligs

The M_r of Oligs was determined by high performance size exclusion chromatography (HPSEC) on two Ultrahydrogel 250 (7.8 mm×300 mm) columns in series. The calibration was based on low molecular mass heparin for calibration CRS (Batch No 1A, European Pharmacopoeia) standards with number average molecular mass (M_n) of 3 700. A refractometer (RI) detector connected in series to an ultraviolet (UV) spectrophotometer

set at 234 nm was used for detection, and 28.4 g/L solution of sodium sulphate (pH 5.0) was used as the mobile phase at a flow rate of 0.5 mL/min[23].

T cell separation and culture

Twenty-five milliliters of edetic acid anticoagulant peripheral blood was taken from ten adult patients with allergic eosinophilic gastroenteritis, and then mixed with 25 mL of PBS (pH 7.4, containing 50 mL/L fetal bovine serum). The diluted peripheral blood was then laid on 50 mL of lymphocyte separating solution carefully and centrifuged for 20 min at 2 000 r/min at room temperature. The peripheral blood lymphocytes (PBL) were collected and washed two times with PBS and suspended in RPMI 1640 solution (containing 200 mL/L fetal bovine serum). One millilitre of PBL suspending solution was added to the column of Nylon cotton (5 mm×16 mm). Then 0.2 mL of RPMI 1640 (containing 200 mL/L fetal bovine serum) was added on the Nylon cotton surface to close the column. The column was incubated for 30 min at 37 ℃ in an incubator. After that, PBTLs were eluted with RPMI 1640 (containing 200 mL/L fetal bovine serum) solution and then centrifuged for 10 min at 2 000 r/min. PBTLs were diluted to the cell density of 1.0×10^6 cells/mL, and 0.2 mL of the diluted cell suspending solution was added to a well of 96-well plate as a blank control. Another well was added into 0.2 mL of diluted cell suspending solution containing 20 μg of PHA as PHA control group. Other wells were added into 0.2 mL of diluted cell suspending solution containing 20 μg of PHA and 1 μL of 1.0 mg/mL heparin and Oligs. The cells were incubated for 48 h at 37 ℃ in a humidified atmosphere containing 50 mL/L CO_2 in air.

Detection of cytokines

The cells were harvested and centrifuged for 10 min at 1 000 r/min at room temperature. IL-4 and IL-5 in the cell culture solution were assayed by ELISA according to the manufacturer's instructions.

Statistical analysis

Data were expressed as mean±SD. Student's *t* test and one-way analysis of variance were used for statistical analysis. *P* values less than 0.05 were considered statistically significant.

RESULTS

Figure 1 shows the Oligs prepared from heparin degradation and separated by gel filtration chromatography. The peaks numbered were Oligs collected for inhibiting IL-4 and IL-5 experiments, and their M_r is listed in Table 1.

Table 1 Oligs obtained from chromatography eluted solution and their M_r

Oligs produced by H_2O_2 oxidation		Oligs produced by β-elimination		Oligs produced by degradation with HNO_2	
Samples	M_r	Samples	M_r	Samples	M_r
Olig H1	6 032	Olig B1	6 621	Olig N1	6 680
Olig H2	3 206	Olig B2	3 375	Olig N2	3 150
Olig H3	2 381	Olig B3	2 447	Olig N3	2 334
Olig H4	1 786	Olig B4	1 804	Olig N4	1 747
Olig H5	1 142	Olig B5	1 344	Olig N5	1 047
Olig H6	632	Olig B6	702	Olig N6	609

The effect of Oligs on secretion of IL-4 showed that all the Oligs had inhibitory activities and Oligs prepared from different methods or with different M_r had different effects. First, the Olig prepared from H_2O_2 depolymerizing method, was Olig H5 with M_r of 1 142 (tetrasaccharides), which had the strongest inhibitory effect and decreased the IL-4 content from 375.6±39.2 ng/L (PHA group) to 12.5±5.7 ng/L (P<0.01). Second, among the Oligs from β-eliminative cleavage heparin, Olig B5 with M_r of 1 344 (tetrasaccharides) had the strongest inhibitory activity and decreased the IL-4 content to 54.4±6.3 ng/L (P<0.01). Third, the Olig from nitrous acid deaminative cleavage heparin, was Olig N5 with M_r of 1 107 (tetrasaccharides), which had the strongest inhibitory activity and decreased the IL-4 content to 47.4±5.8 ng/L (P<0.01). Although all these Oligs were tetrasaccharides, they had different M_r, because the amount of sulfate groups in their structure was different. The IL-4 content in the heparin group was 152.4±17.9 ng/L, and the difference in inhibitory activities between Oligs and heparin group was significant (P<0.01, Figures 2A, 3A).

The inhibitory effect of Oligs on secretion of IL-5 and IL-4 was similar. But there were some differences. Firstly, the Oligs prepared from H_2O_2 oxidation method, were Olig H4 with M_r of 1 786 and Olig H5 with M_r of 1 142, which had strongest inhibitory activities. They decreased the IL-5 content from 289.2±33.4 ng/L (PHA group) to 31.7±5.6 ng/L and 35.5±4.4 ng/L, respectively (P<0.01). Secondly, among the Oligs from β-eliminative cleavage heparin, Olig B4 with M_r of 1 804 (hexasaccharides) had the strongest inhibitory activity and decreased the IL-5 content to 22.0±5.2 ng/L (P<0.01). Thirdly, among the Oligs from nitrous acid deaminative cleavage heparin, Olig N4 with M_r of 1 747 (hexasaccharides) had the strongest inhibitory activity and decreased the IL-5 content to 69.2±6.3 ng/L (P<0.01). The inhibitory activities of these Oligs were stronger than those of unfractioned heparin (P<0.01, Figure 2B). The strongest inhibitor was Olig B4 (Figure 3B).

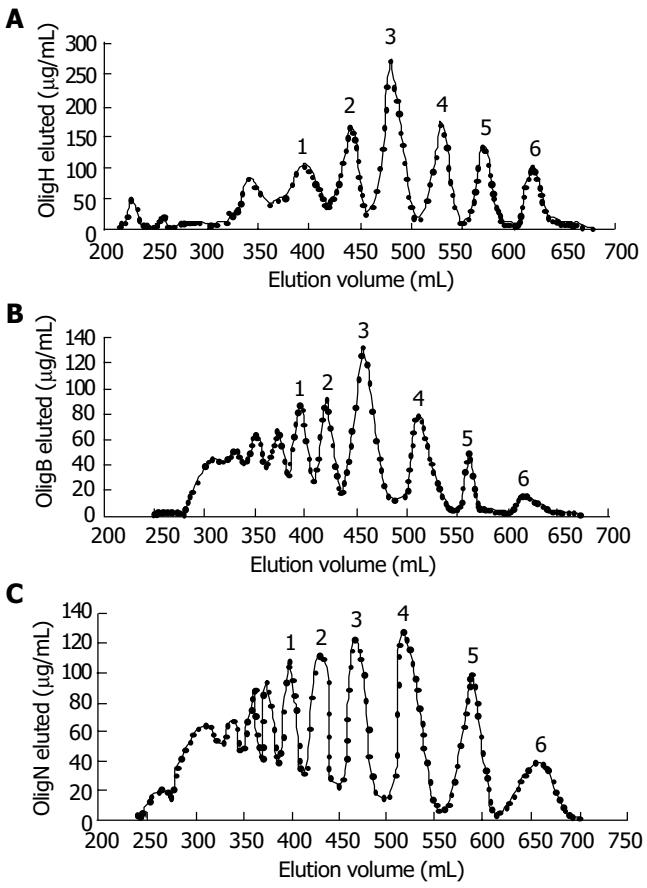

Figure 1 Size separation of heparin oligosaccharides. A: heparin degraded with hydrogen peroxide; B: heparin degraded with β-eliminative cleavage; C: heparin degraded with nitrous acids. The reaction products were separated on a Superdex 30 size exclusion column (26 mm×1 200 mm) at a flow rate of 0.5 mL/min in 0.25 mol/L ammonium bicarbonate. Elution profiles were monitored by carbazole assay.

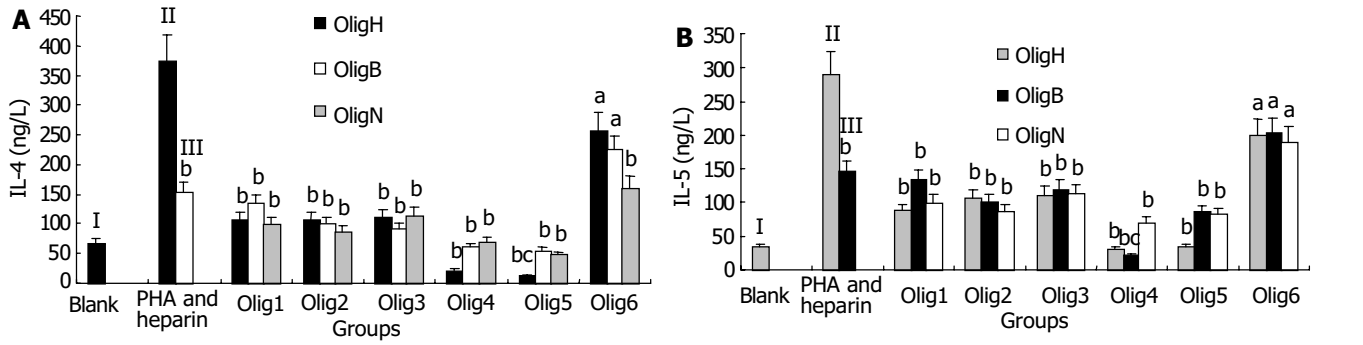

Figure 2 Content of IL-4 (A) and IL-5 (B) in PBTL culture solution of different groups. I, II and III represent blank control group, PHA control group and heparin group respectively. Olig1 to Olig 6 represent corresponding oligasaccharide samples described in Table 1 (from 1 to 6), respectively. [a]$P<0.05$, [b]$P<0.01$ *vs* PHA group; [c]$P<0.05$ *vs* Olig H4 group. (n = 10 for each group).

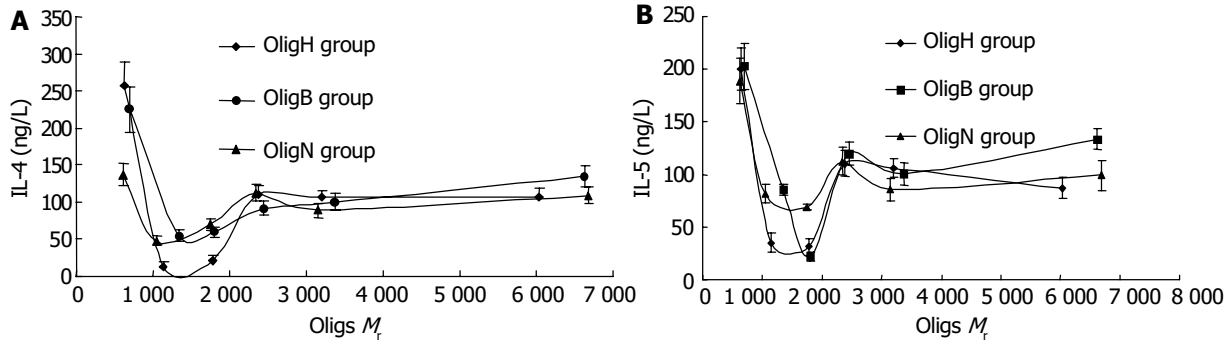

Figure 3 Relationship between M_r of Oligs and their activity on inhibiting IL-4 (A) and IL-5 (B) secretion. [a]$P<0.05$ *vs* Olig H4 group.

DISCUSSION

Allergic eosinophilic gastroenteritis is characterized by elevated total immunoglobulin E (IgE). IgE plays a significant role in allergic IBD by mediating the cross-linking of high-affinity Fc receptors (FcεRI) on mast cells, thus resulting in the release of a vast array of pro-inflammatory mediators including histamine, leukotrienes, and cytokines[24] (Figure 4). In clinical studies, increased secretion of IL-4 and IL-5 by peripheral blood T cells has been reported in patients with eosinophilic gastroenteritis[17]. Furthermore, T cells derived from the duodenum of patients with eosinophilic gastrointestinal disorder could preferentially secrete helper T cell 2 (Th2) cytokines that pre-dominantly include IL-4 and IL-5 when stimulated with milk proteins[17]. IL-4 might play a critical role in mediating IgE-dependent allergic reactions, and regulation of IL-4 production or action might be useful for the prevention or therapy of immediate hypersensitivity disorders[25-27]. IL-5, on the other hand, is most specific to the eosinophil lineage and responsible for the selective expansion of eosinophils and their release from bone marrow. Eosinophil granules contain a crystalloid core composed of major basic proteins (MBP) and a matrix composed of eosinophil cationic protein (ECP)[28] (Figure 4). These cationic proteins share certain pro-inflammatory properties but differ in other aspects. MBP also triggers degranulation of mast cells and basophils. Triggering of eosinophils through engagement of receptors for cytokines could lead to the generation of a wide range of inflammatory cytokines, including IL-1, IL-3, IL-4, IL-5, IL-13, granulocyte-macrophage colony stimulating factor (GM-CSF), transforming growth factors (TGF), TNF-α, RANTES, macrophage inflammatory protein 1α, vascular endothelial cell growth factor, and eotaxin 1, indicating that they have the potential to modulate multiple aspects of the immune response[29]. These findings imply that regulation of IL-5 production or action may also be useful for the prevention or therapy of allergy symptoms and inflammation.

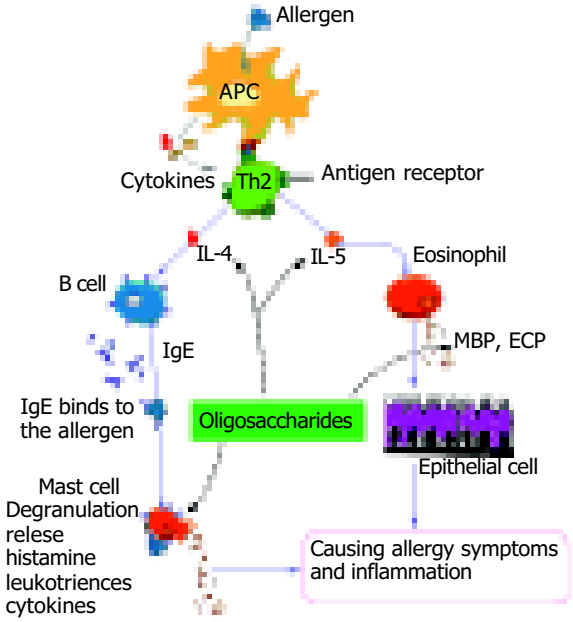

Figure 4 Interactions among inflammatory cells in pathogenesis of allergic eosinophilic gastroenteritis. Broken arrows denote the inhibitory effect of heparin-derived oligosaccharides on the various targets shown. MBP: major basic protein; and ECP: eosinophil cationic protein.

Heparin is a kind of polyanionic polysaccharides. In addition to its anticoagulant activity, heparin has a wide range of biological activities, including inhibition of complement activation[30], regulation of cell proliferation[31], inhibition of angiogenesis and tumor growth[32,33], and antiviral activity[34,35]. Over the last decade, heparin and low molecular mass heparin have been used to treat IBD in clinical practice[36-50]. The mechanisms by

which heparin is able to treat IBD include its ability to inhibit the recruitment of neutrophils, reduce production of pro-inflammatory cytokines[51] and restore the high-affinity receptor binding to antiulcerogenic growth factor[12,13]. The ability of heparin to inhibit neutrophil activation, adhesion, and chemotaxis was also found in a mouse model of IBD[14], suggesting that balanced interactions between mast cells and neutrophils might be important for the development of IBD. Furthermore, unfractioned heparin has potent immunomodulatory effects[52-57]. Administration of unfractioned heparin may therefore be rational in patients with ulcerative colitis or Crohn's disease resistant to conventional forms of treatment[39,40]. The anti-inflammatory effects of heparin can be most probably attributed to its physical binding to a variety of heparin-binding proteins such as TNF-α, IL-4, IL-5, RANTES, secretory leukocyte protease inhibitor, neutrophil-derived elastase and cathepsin G, eosinophil-derived major basic protein, and L- and P-selectins[58-61]. Alternatively, heparin has been shown to specifically inhibit the inositol 1,4, 5-triphosphate signal transduction pathway, which is important for a vast array of inflammatory cellular responses[62,63].

If heparin is to be used as an anti-inflammatory drug, the risk of inducing bleeding must be abrogated. It has been reported that the anti-inflammatory effects of heparin were independent of its anticoagulant activity[64-67], especially when it was used for treatment of allergic inflammation. Partial chemical modifications of heparin, such as depolymerization or partial desulfation, are the aim to develop heparin-derived anti-inflammatory drugs. We degraded heparin and separated the fragments by gel filtration chromatography. The oligosaccharides obtained were used as anti-inflammatory reagents and the results were promising. The hypothetic mechanisms for anti-inflammatory effects, such as inhibiting secretion of IL-4 and IL-5, were studied. Our results showed that Oligs with different M_r had different activities on inhibiting the secretion of IL-4 and IL-5. The Oligs, which had the strongest inhibitory activities on IL-4 secretion, were tetrasaccharides, but they had different M_r corresponding to the production methods. That was because different methods caused different desulfation during the process of degradation. The same phenomenon occured in inhibiting the secretion of IL-5, but the strongest inhibitors were hexasaccharides other than tetrasaccharides. The mechanism needs further studies.

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