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Decreased level of osteopontin in children with allergic rhinitis during sublingual immunotherapy

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ABSTRACT

Objective: Sublingual immunotherapy (SLIT) is proven to be very effective in the treatment of allergic rhinitis (AR), but its regulatory mechanism and biomarkers for predicting efficacy are still unknown. Osteopontin (OPN), as a recently described Th2 inflammation related protein, plays key role in the pathogenesis of AR. The aim of this study was to identify the expression and role of OPN during SLIT in children.

Methods: Fifty house dust mite (HDM)-sensitized children with AR were enrolled in this study. AR children received HDM allergen extract or placebo for SLIT. Serum of different time points during treatment was collected and used for enzyme-linked immuno sorbent assay (ELISA) of OPN and related cytokines, respectively. Peripheral blood mononuclear cells from children after SLIT or placebo treatment were collected and stimulated with HDM with or without OPN/anti-OPN after one year's treatment.

Results: Our results showed that expression of OPN protein was decreased after one year's therapy. The decreased OPN expression was positively related to decreased Th2 cytokines and negatively related to enhanced IL-10 and TGF-β expression. *In vitro* experiments confirmed that children received SLIT treatment showed decreased production of Th2 cytokines by PBMCs after HDM stimulation.

Conclusion: During SLIT, decreased OPN expression was related to low Th2 cytokine expression and enhanced IL-10 and TGF-β expression. High serum OPN expression predicts poor treatment efficacy. OPN may be used as a biomarker for SLIT treatment.

1. Introduction

Allergic rhinitis (AR) is one the most common chronic diseases in children with increased incidence [1,2]. Allergen-specific sublingual immunotherapy (SLIT) is the only treatment so far that intervene the natural course of allergic disease and it can prevent the development of asthma in AR children [3]. In the past, immunotherapy was believed to change the immune response from a Th2-predominant response dominated by cytokines IL-4, IL-5, and IL-13, eosinophils, and IgE, toward a Th1 response characterized by the absence of eosinophils and an increased IgG response, with raised IgG4 levels. However, recent studies showed that regulatory T cells (Treg) may be key regulators of immunological processes in peripheral tolerance to allergens during immunotherapy. Skewing of allergen-specific effector T cells to a regulatory phenotype appears as a key event in the development of healthy immune response to allergens and successful outcome in SLIT [4]. However, the underlying mechanism in the process and potential biomarkers is still not fully characterized.

Osteopontin (OPN) is a phosphorylated acidic glycoprotein that can bind with certain CD44 variants and integrin receptors and mediate cell-matrix interactions and cellular signaling [5]. Previously, OPN was described as a Th1 cytokine, and elevated levels of OPN were observed in several Th1-associated diseases, including sarcoidosis, tuberculosis, rheumatoid arthritis, multiple sclerosis, and Crohn's disease [6–10]. Recent studies have showed that OPN is

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also involved in Th2-mediated diseases [11]. Increased OPN level was found in the tissues of nasal polyps in patients with chronic rhinosinusitis plus nasal polyps [12]. All these studies suggested that OPN may play different roles in different disease background due to its complicated structure and several functional motifs.

As the role of OPN in AR and asthma becomes more and more clear, we postulated that OPN may be a potential candidate marker for SLIT treatment. The aim of this study was to evaluate OPN expression in the process of SLIT in children and the direct effect of OPN on peripheral blood mononuclear cells (PBMCs) during SLIT.

2. Materials and methods

2.1. Patients

Fifty children aged 6–16 years with a clinical history of mono-house dust mite (HDM) induced AR for at least 2 years were included. Skin prick test (SPT) or specific immunoglobulin IgE was performed to screen children only allergic to HDM. The cut-off value of specific is 3.5 IU/mL and positive SPT was defined as mean wheal diameter $\geq$2 mm. Those children with chronic diseases (*e.g.* asthma, malnutrition, anatomic malformation of the respiratory system, chronic lung disease, heart disease, gastro-oesophageal reflux disease, cystic fibrosis) were excluded from the study. All these patients were unresponsive to nasal steroids treatment. Patients with a history of chronic drug use (*e.g.* oral or nasal corticosteroids, antiepileptics, immune suppressives, antihistamines, *etc.*) were excluded from the study. The study was performed with the approval of the local ethics committee and with the parent's written informed consent.

2.2. Sublingual immunotherapy

Fifty AR children were divided into SLIT and placebo group randomly with 25 cases in each group. The drugs were labeled with patient code numbers, and the investigator assigned patients in a sequential randomized fashion to a study code number. Individual drug bottles were identity masked such that both patients and researchers were blind to treatment assignment. Study blinding was preserved at the study sites until all subjects completed the study. The HDM allergen extract (CHANLLERNGEN, Dermatophagoides Farinae Drops) for SLIT was manufactured by Wolwopharma Biotechnology Company (Zhejiang, China) and used in the form of drops (no. 1, 1 mg/mL; no. 2, 10 mg/mL; no. 3, 100 mg/mL and no. 4, 333 mg/mL). According to the manufacture's instruction, the patients were asked to take increasing doses (from no. 1 to 3) during the first three weeks up-dosing phase, and then were instructed to have 3 drops of no. 4 solution once daily during the maintenance phase. Children in the placebo group received a diluent containing 50% glycerol and 50% saline buffer and the usage was similar to that of SLIT group with increasing doses given first three weeks and maintenance dose after that. Drops were instructed to be kept under the tongue for 2–3 min before swallowed.

2.3. Clinical evaluation

A 7-day run-in period for all patients with diary symptom and medication monitoring of AR symptoms, frequency and amount of rescue medications (antihistamines and corticosteroids, *etc.*) was finished before study. Following the run-in period, patients were re-evaluated for eligibility in an enrolment visit before treatment with HDM extract SLIT. The symptoms and medications were recorded using a Patient Diary as described earlier [13]. All of the patients recorded their daily symptom score and drug requirement before and throughout the 12-month SLIT study.

The severity of individual nasal symptom score (INSS), including nasal rhinorrhea, sneezing, itching and congestion, was assessed on a scale of 0 to 3 (0 = no symptom, 1 = mild, 2 = moderate, 3 = severe). Total nasal symptom score (TNSS) was defined as the sum of the scores of nasal rhinorrhea, sneezing, itching and congestion. The maximal cumulated clinical score per patient was 12. Although some patients had ocular symptoms associated with rhinitis, only nasal symptoms were evaluated for analysis of efficacy.

Patients were allowed to use the following anti-allergic drugs: oral antihistamine, intranasal corticosteroid, and betamethasone 0.5-mg tablets. Other medications, such as long-lasting antihistamines, cromones, and parenteral corticosteroids, were not permitted during the study. The patients had to record on the same diary card whenever they used medications, and a specific weekly score (AMS) was assigned (1 point: one puff of nasal corticosteroid per nostril, 2 points: one oral antihistamine tablet, 3 points: one betamethasone tablet).

2.4. Blood and nasal lavage samples preparation and analysis

Venous blood samples were collected into Vacuette tubes and centrifuged at 3000 $\times$ *g* for 15 min at 4 °C. Serum samples were stored at −80 °C. These samples were used for Enzyme-linked immunosorbent assay (ELISA) measurement. The whole blood cell counts were measured by LH-785 system (Beckman Coulter, Mervue, Galway, Ireland). Total serum IgE was measured by ECLIA (Electrochemiluminescence) method using an ELX-800 system. Unicap system was used for measuring ECP levels, according to the manufacturer's protocols.

Nasal lavage was performed using saline warmed to 37 °C. The process was performed according to method described elsewhere [14]. The samples were centrifuged to remove cellular debris and aliquots of the supernatants were stored at −20 °C in eppendorff tubes until analysis. Total protein concentrations were determined with Bio-Rad protein assays according to Bradford.

Both the blood and nasal samples were taken at 0, 6, and 12 months for determination.

2.5. ELISA for protein expression

ELISA kits were used for measuring serum OPN, IL-4, IL-13, IL-5, IL-12, IFN-α, IL-10, TGF-β (R&D systems, USA) according to the manufacturer's protocols. The detection limits of the assays were as follows: OPN, 0.312 ng/mL, IL-4, 1.56 pg/mL, IL-13, 93.8 pg/mL, IL-5, 7.8 pg/mL, IL-12, 2.5 pg/mL, IFN-α, 12.5 pg/mL, IL-10, 3.9 pg/mL, TGF-β, 15.4 pg/mL.

2.6. Peripheral blood mononuclear cells (PBMCs) preparation

PBMCs were isolated by Lymphoprep (Fresenius Kabi Norge AS, Oslo, Norway) density-gradient centrifugation from heparinized leucocyte-enriched buffy coats, which were obtained from 14 children (7 received SLIT and 7 received placebo) one year after the treatment. PBMCs were isolated by means of density gradient centrifugation and cultured at 2×10^6/mL in 24-well plates in culture medium: RPMI 1640 supplemented with 5% human AB serum, 5 mmol/L glutamine, and 13 penicillin and streptomycin solution (all from Invitrogen, except serum from Sigma-Aldrich). Stimulation was through addition of allergen (100 ng/mL HDM extract) + PHA (1 μg/mL), with or without anti-OPN (100 ng/mL) or rhOPN (100 ng/mL) for 24 h.

2.7. Statistical analysis

Statistical analysis was performed using Mann–Whitney or Wilcoxon signed rank test. The pre-test Friedman test was done for