



Role of leptin in allergic rhinitis during sublingual immunotherapy

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Abstract

Objective Increasing evidence suggests that leptin is upregulated during allergic reactions in the airway and related to the severity of disease in allergic rhinitis (AR). In this study, we aimed to investigate the expression of leptin during sublingual immunotherapy (SLIT) in AR patients.

Methods Forty AR patients without obesity were recruited in this study. Twenty patients received house dust mite (HDM) allergen extract for SLIT and twenty patients received placebo randomly. Protein expression of leptin in serum and nasal lavage was tested by enzyme-linked immuno sorbent assay (ELISA) 1 and 2 years after SLIT treatment, respectively. Peripheral blood mononuclear cells (PBMCs) and human nasal epithelial cell were prepared and stimulated by recombinant leptin after 24 months' SLIT treatment and the induction of Th2 cytokines (IL-4/IL-5/IL-13) were detected by ELISA.

Results SLIT treatment decreased the expression of leptin protein in serum and nasal lavage significantly compared with placebo group 1 and 2 years after SLIT treatment. Nasal leptin level was correlated to decreased Th2 response (IL-4/IL-5/IL-13) and enhanced Treg (IL-10/TGF- β) response after 2 years' SLIT. We also found that SLIT decreased the ability of leptin in promoting Th2 cytokines expression by PBMCs and human nasal epithelial cell after 2 years' SLIT treatment.

Conclusion Changes of leptin expression in serum and nasal lavage may be correlated with Th2/Treg regulation during SLIT. Our results suggested that leptin served as an important biomarker during SLIT.

Keywords Allergic rhinitis · Sublingual immunotherapy · Leptin

Introduction

Allergic rhinitis (AR) is chronic allergic inflammation of the upper airway and affected 30–40% of total population worldwide [1, 2]. AR patients often present with annoying symptoms

such as blocked nose, itchy nose, sneezing, and runny nose [3]. Most AR patients received good results from traditional medication such as nasal glucocorticoid, antihistamine, etc. However, medication therapy belongs to symptomatic treatment that cannot modify the immunological process of AR. Specific immunotherapy, including sublingual or subcutaneous immunotherapy (SLIT/SCIT), is believed to regulate the allergic response of AR by changing it from a Th2-biased reaction toward a Th1 reaction accompanied by increased production of IgG4, IL-10, TGF- β and decreased IgE levels [4, 5].

Leptin, a 16 kDa non-glycosylated polypeptide, is one of hormones which regulate food intake and energy expenditure. Serum leptin level is closely correlated with body fat percentage and body fat mass [6]. Besides, leptin is found to play important roles in neuroendocrine function, angiogenesis, hematopoiesis and various immune responses. Previous study suggested that leptin is involved in the apoptosis, proliferation, and activation of T cells [7]. Expression of leptin in AR was enhanced according to past reports and leptin level reflects the severity of disease in AR [8, 9]. However, the expression of leptin during SLIT was not established.

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In the current study, we aimed to explore the changes of leptin expression during SLIT as well as its correlation with Th cytokines and provide evidence in the pathogenesis of SLIT.

Materials and methods

Patients

Forty AR patients (18–60 years old, without obesity) only allergic to house dust mite (HDM) for at least 2 years were recruited consecutively in this study. Our study was performed with the approval of the Guangzhou Overseas Chinese Hospital's ethics committee and the parent's written informed consent. All AR patients were diagnosed according to typical symptoms, physical examination, skin prick test, and/or specific IgE measurement for common allergens as described in Allergic Rhinitis and its Impact on Asthma (ARIA) guideline (2010) [10]. The allergic status was determined by skin prick test (SPT) or serum immunoglobulin IgE specific to common inhalant allergens (dust mites, pets, molds, cockroach, etc). The specific IgE value > 3.5 IU/mL or wheal diameter ≥ 2 mm in SPT test was defined as positive. Patients with other nasal disease (chronic rhinosinusitis, nasal tumor, etc.) and systematic chronic diseases (e.g., asthma, chronic lung disease, heart disease, gastroesophageal reflux disease) were excluded from our study.

Administration of the immunotherapy

Forty AR patients were divided as SLIT and control group randomly with 20 cases in each group. During the initial phase, the HDM allergen extract (CHANLLERNGEN, Wolvopharma Biotechnology Company, Zhejiang, China) were given with increasing doses in 5 weeks (No. 1, 1 mg/mL; No. 2, 10 mg/mL; No. 3, 100 mg/mL; No. 4, 333 mg/mL). During the maintenance phase, the patients received two drops of solution (No. 5, 1000 mg/mL) from the sixth week to the end of treatment. In the control group, a diluent (50 mg/mL glycerin saline solution) was supplied. Drops were guided to be kept under the tongue for 2–3 min before swallowed.

Evaluation of disease severity

All of the patients recorded their daily symptom score and averaged in an 8-week observation period at different time points during the 24-month SLIT study as described earlier [11]. Briefly, the nasal symptoms (runny nose, sneezing, blocked nose, itching nose) were scored by the patients on a 0 to 3 scale as follows: 0 = no symptoms, 1 = slight symptoms, 2 = moderate symptoms, and 3 = severe symptoms. The total nasal symptom score (TNSS) was summed.

Blood and nasal lavage samples preparation

Venous blood samples (10 mL) were collected between 11am and 2 pm by vein puncture method at appropriate condition. The samples were centrifuged at 3000g for 15 min at 4 °C, and stored at -80 °C for further analysis. Serum IgE and eosinophil cationic protein (ECP) were measured by ECLIA (Electrochemiluminescence) method and Unicap system, respectively.

Nasal lavage was prepared according to method described elsewhere [12]. In brief, every patient was subjected to nasal lavage using 5 ml/nostril of physiologic saline solution (0.9% NaCl). The samples were centrifuged and the supernatants were stored at -20 °C for analysis. Both the blood and nasal samples were taken at 0, 12, and 24 months for test.

Enzyme-linked immunosorbent assay (ELISA) for protein expression

ELISA kits (R&D systems, USA) were performed for measuring serum and nasal cytokine expression according to the protocol provided by manufacturer. The detection limits of the assays were as follows: leptin, 1.65 pg/mL, IL-5, 7.8 pg/mL, IL-12, 2.5 pg/mL, IL-10, 3.9 pg/mL, TGF- β , 15.4 pg/mL.

Peripheral blood mononuclear cells (PBMCs) preparation

After the blood was obtained from patients 2 years after SLIT, PBMCs were prepared by Lymphoprep density-gradient centrifugation from heparinized leukocyte-enriched buffy coats according to the manufacture's instruction. The sorted cells were cultured at 37 °C in 5% CO₂ for 3 days in 1 mL RPMI 1640 medium (2×10^6 cells/mL) in the presence or absence of house dust mite extract (20 μ g/ml HDM, RayBiotech, USA), recombinant leptin or other stimulators or inhibitors (R&D, USA).

Cell culture and treatment

Human nasal epithelial cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO, USA) and penicillin/streptomycin (Sigma, St. Louis, MO, USA) at 37 °C in a humidified chamber with 5% CO₂. The cells were treated by house dust mite extract (20 μ g/ml HDM, RayBiotech, USA), recombinant leptin or other stimulators or inhibitors (R&D, USA).

Statistical Analysis

Values are presented as mean \pm SD except additional note. Comparisons were performed by nonparametric Mann–Whitney *U* test. The Spearman rank correlation test was done to analyze the correlation between different biomarkers. $p < 0.05$ was defined as significant difference. All analyses were performed using statistical software SPSS (version 18).

Results

Demographic characteristics of study population and clinical efficacy

The baseline characteristics are described in Table 1. This study was conducted with 40 patients, 20 (19–46 years old) of whom enrolled in SLIT group and 20 (21–47 years old)

Table 1 Demographic characteristic of AR patients

	SLIT group	Placebo group
Number	20	20
Sex (male:female)	11:9	12:8
Age (months)	34.6 \pm 15.1	36.8 \pm 16.8
Baseline symptoms (TNSS)	9.5 \pm 2.4	9.1 \pm 3.5
TNSS after 12 months' SLIT	3.4 \pm 1.5*	8.6 \pm 2.9
TNSS after 24 months' SLIT	1.7 \pm 0.8*#	7.7 \pm 2.9
BMI	22.6 \pm 1.9	21.7 \pm 1.5
Smoking history	12 (60%)	13 (65%)
Sensitive to D1	14 (70%)	15 (75%)
Sensitive to D2	16 (80%)	17 (85%)
Nasal steroid	13 (65%)	14 (70%)
Nasal antihistamine	12 (60%)	13 (65%)
Oral antihistamine	16 (80%)	14 (70%)

D 1 dermatophagoides pteronyssinus, D 2 dermatophagoides farinae

*Compared with placebo group, $p < 0.05$

#Compared with score after 12 months' SLIT, $p < 0.05$

of whom enrolled in control group. At the end of study, 16 (80%) patients in SLIT group and 14 (70%) patients in control group completed the 2-year study period. Our results showed that SLIT treatment decreased symptom scores significantly compared with placebo group, confirming

the efficacy of SLIT (Table 1).

Decreased of serum and nasal leptin protein levels during SLIT treatment

As shown in Table 2, both serum and nasal leptin levels decreased significantly after 1 year's SLIT treatment and this trend maintained for at least 2 years. Consistently, we also found that decreased protein expression of nasal ECP and IgE after 1 year and 2 years' SLIT treatment (Table 2). However, serum ECP and IgE were not affected by SLIT. After 2 years' SLIT treatment, nasal leptin expression was found to be positively correlated with nasal IgE and ECP expression, respectively (Table 4). In the control group, the expression of leptin, IgE and ECP did not change significantly during the SLIT treatment conducted by placebo (Table 2).

Nasal Th1/Th2 cytokines expression during SLIT and their correlations with nasal leptin expression

To explore the relation between leptin and Th response, we detected the expression of Th1/Th2 cytokines. After 2 years' SLIT treatment, the changes of serum Th cytokines were not significant (Data not shown). However, nasal Th1-related cytokines (IL-12 and IFN- α) and Treg-related cytokines (IL-10 and TGF- β) expression were enhanced significantly, suggested that SLIT treatment induced Th imbalance (Table 3). On the other side, Th2 polarization (IL-4 and IL-5) was inhibited significantly after 1 and 2 years' SLIT (Table 3). In the control group, serum and nasal Th1/Th2/Treg cytokines were not affected by SLIT treatment (Table 3). Consistently, nasal leptin expression was positively correlated with nasal Th2 cytokines (IL-4 and IL-5) and negatively correlated with nasal Th1 cytokines (IL-12 and IFN- α) and Treg

Table 2 Decreased expression of leptin during SLIT or placebo treatment

Cytokines	SLIT group			Control group		
	Baseline	1 year's SLIT	2 years' SLIT	Baseline	1 year's Placebo	2 years' Placebo
Nasal leptin (ng/mL)	9.8 \pm 2.1	5.5 \pm 1.9*	2.2 \pm 0.9*	8.5 \pm 3.1	9.1 \pm 2.7	9.5 \pm 2.6
Serum leptin (ng/mL)	11.5 \pm 3.7	7.5 \pm 2.1*	3.5 \pm 1.9*	12.4 \pm 4.1	11.9 \pm 3.8	12.8 \pm 4.7
Nasal ECP (ng/mL)	41.5 \pm 11.3	23.6 \pm 4.8*	7.9 \pm 3.2*	43.6 \pm 12.7	38.5 \pm 13.1	37.4 \pm 13.6
Serum ECP (ng/mL)	57.6 \pm 31.8	48.2 \pm 19.5	31.5 \pm 16.7	65.1 \pm 21.5	60.3 \pm 23.4	57.1 \pm 26.9
Nasal IgE (IU/mL)	76.6 \pm 21.8	41.9 \pm 25.6*	18.6 \pm 7.9*	59.2 \pm 17.3	66.2 \pm 18.3	63.3 \pm 16.4
Serum IgE (IU/mL)	135.8 \pm 21.9	124.6 \pm 18.6	127.5 \pm 28.4	119.2 \pm 27.8	124.3 \pm 20.6	109.7 \pm 16.5

*Compared with baseline, $p < 0.05$

cytokines (IL-10 and TGF- β) expression after 2 years' SLIT treatment (Table 4).

Decreased leptin-stimulated Th2 response of PBMCs induced by HDM and human nasal epithelial cells after SLIT

After 2 years' SLIT treatment, both HDM and HDM/leptin induced Th2 cytokines (IL-4, IL-5, IL-13) production by PBMCs and human nasal epithelial cells were significantly inhibited (Figs. 1, 2). However, Th1 and Treg cytokines level were not changed (data not shown) after stimulation.

Discussion

Inhibited Th2 response in SLIT may be attributed to complicated immunological process. Our study provided evidence that decreased leptin expression during SLIT treatment was involved in Th response regulation, suggesting that leptin may be used as a biomarker for SLIT treatment.

Successful SLIT is often accompanied by inhibition of Th2 response and eosinophil recruitment in response to allergen [13, 14]. In this study, our results showed that SLIT reduced symptom scores effectively in Chinese adults, which is similar with previous studies [15, 16]. However, the detailed mechanism in this process is not well defined.

Leptin, which is predominantly made by adipose cells and was first described to regulate energy balance by inhibiting hunger, also plays other biologic functions. Recent studies have suggested that the elevated expression of leptin in AR patients is related to the severity of disease [8, 9, 17]. Therefore, we supposed that leptin may be involved in the progression of SLIT. To reduce the effect of obesity on leptin expression, we excluded obese AR patients from our study. Our results suggested that both serum and nasal leptin expression reduced significantly after 1 year's SLIT and this trend maintained at least for 2 years regardless of sex. Interestingly, Ciprandi's study [18] showed that serum leptin was significantly increased only in males after the

Table 4 Relationship between nasal leptin and Th cytokines

	Nasal leptin	
	<i>r</i>	<i>p</i>
IL-4	0.63	0.01
IL-5	0.68	0.03
IFN- γ	-0.62	0.01
IL-12	-0.55	0.01
IL-10	-0.69	0.002
TGF- β	-0.62	0.03
ECP	0.56	0.01
IgE	0.61	0.003

second SLIT course. This discrepancy maybe attributed to difference of ethic or allergen or the effect of obesity since Ciprandi's study did not exclude obese patients.

To explore the imbalance of Th response during SLIT and its correlation with leptin, we further measured the variations of Th1/Th2/Treg cytokines during SLIT. Our results suggested that nasal Th2 response was inhibited and Th1 and Treg responses increased after 2 year's SLIT, proving the transition from Th2 response to Th1 and Treg responses after SLIT. Correlation analysis showed that nasal leptin was correlated with Th cytokines. Therefore, we stimulated PBMCs and nasal epithelial cells with HDM and rh-leptin to confirm the direct effect of leptin. Our results showed that leptin promote PBMC to differentiate to Th2 cells, manifested as significant up-regulation of Th2 cytokines in PBMCs. We also observed that leptin promote up-regulation of Th2 cytokines by nasal epithelial cells. These results confirmed the direct regulatory effect of leptin on Th balance in PBMCs and the indirect role of leptin in the regulation of Th2 cytokines from nasal epithelial cells in AR. After 2 years' SLIT, the ability of leptin in promoting Th2 cytokines expression by PBMCs and nasal epithelial cells were reduced the significantly, suggesting that the regulation of leptin by SLIT is one the pathways of Th imbalance in immunotherapy. Interestingly, previous reports showed that the overall effect of leptin on T memory cells is to increase Th-1 responses and

Table 3 Variations of Th cytokines levels during SLIT or placebo treatment

Cytokines	SLIT group			Control group		
	Baseline	1 year's SLIT	2 years' SLIT	Baseline	1 year's SLIT	2 years' SLIT
Nasal IL-4 (pg/mL)	4.5 \pm 1.8	2.6 \pm 0.8*	1.2 \pm 0.7*	3.8 \pm 1.6	4.2 \pm 2.2	4.7 \pm 2.1
Nasal IL-5 (pg/mL)	68.2 \pm 16.8	43.1 \pm 17.2*	25.6 \pm 12.3*	61.3 \pm 23.4	73.4 \pm 21.6	65.4 \pm 18.9
Nasal IL-12 (ng/mL)	25.8 \pm 11.2	39.6 \pm 13.8	61.2 \pm 21.5*	28.7 \pm 12.3	33.1 \pm 14.5	24.7 \pm 13.7
Nasal IFN- α (ng/mL)	27.1 \pm 9.8	48.5 \pm 12.3	79.8 \pm 26.5*	31.2 \pm 11.2	25.8 \pm 10.3	33.7 \pm 12.8
Nasal IL-10 (IU/mL)	79.2 \pm 25.6	63.1 \pm 21.2	22.0 \pm 17.3*	79.2 \pm 25.6	79.2 \pm 25.6	79.2 \pm 25.6
Nasal TGF- β (IU/mL)	118.5 \pm 25.9	98.2 \pm 27.5	28.3 \pm 15.1*	101.3 \pm 21.7	93.6 \pm 31.1	106.2 \pm 22.3

*Compared with baseline, $p < 0.05$

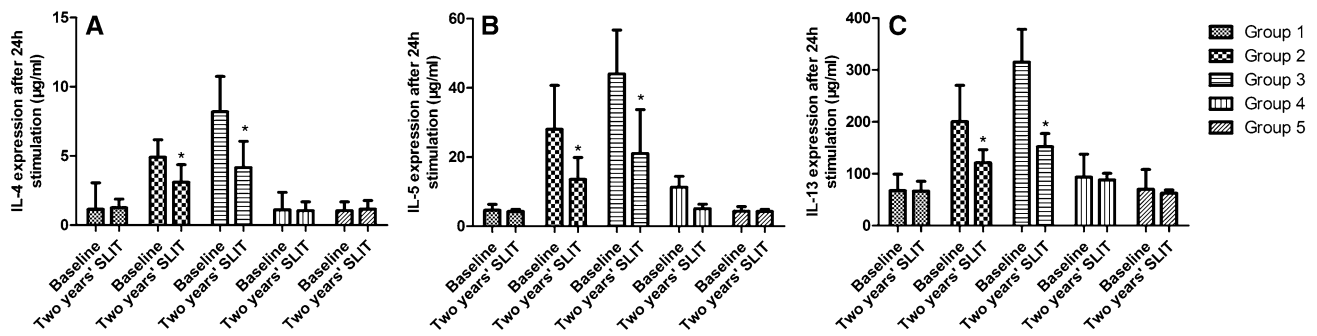


Fig. 1 After stimulated with rh-leptin, significant up-regulation of Th2 cytokines (IL-4, IL-5, IL-13) in PBMCs from AR donor. Two years' SLIT decreased the ability of leptin in promoting Th2 cytokines by PBMCs (a–c). * $p < 0.05$, comparison between

two groups. (Group1: PBS; Group 2: 100 ng/mL HDM; Group 3: 100 ng/mL HDM + 100 ng/mL rh-leptin; Group 4: 100 ng/mL HDM + 100 ng/mL anti-leptin; Group 5: IgG)

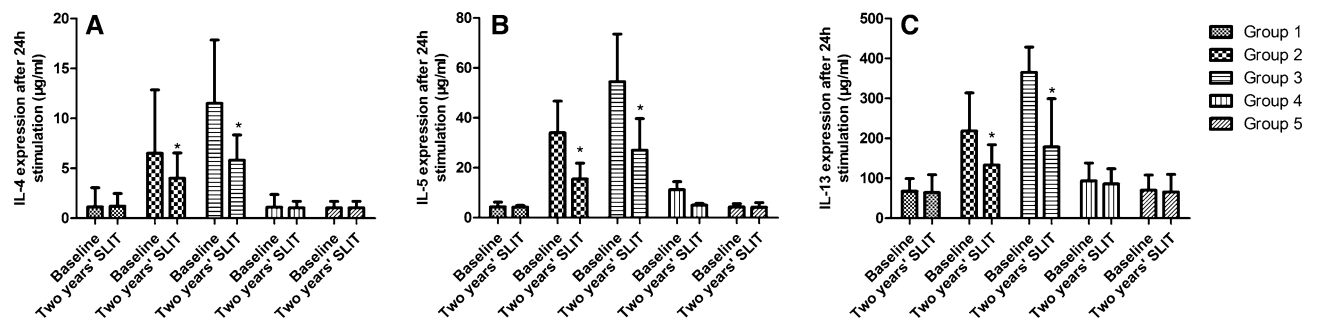


Fig. 2 After stimulated with rh-leptin, significant up-regulation of Th2 cytokines (IL-4, IL-5, IL-13) by nasal epithelial cells from AR patients. Two years' SLIT decreased the ability of leptin in promoting Th2 cytokines by nasal epithelial cells (a–c). * $p < 0.05$, compari-

son between two groups. (Group1: PBS; Group 2: 100 ng/mL HDM; Group 3: 100 ng/mL HDM + 100 ng/mL rh-leptin; Group 4: 100 ng/mL HDM + 100 ng/mL anti-leptin; Group 5: IgG)

decrease Th-2 and regulatory T cells responses [19]. This discrepancy maybe attributed to different signal pathway of leptin in disease backgrounds.

In clinic, AR is often accompanied by respiratory disease (asthma, sinusitis, etc), obesity or other systemic diseases. All those diseases may affect leptin expression; therefore, further studies were needed to explore the role and regulation of leptin in various diseases.

In sum, our finding showed that during SLIT, leptin expression was decreased and this low leptin expression maintained until at least 2 years after treatment. Decreased leptin expression was related to low Th2 cytokine expression and enhanced Th1 and Treg cytokines expression.

Compliance with ethical standards

Conflict of interest All authors declares that they have no conflicts of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with ethical standards of the insti-

tutional and/or national research committee and with 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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