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Suppressive Activity of Histamine H1 Receptor Antagonists, Desloratadine and Levocetirizine, on the Production of Periostin from Nasal Epithelial Cells *In vitro*

Masayo Asano[1], Tomomi Mizuyoshi[1], Shintaro Ishikawa[2], Kazuhito Asano[3*] and Hitome Kobayashi[1]

[1]*Department of Otolaryngology, School of Medicine, Showa University, Tokyo, Japan*

[2]*Department of Physiology, School of Medicine, Showa University, Tokyo, Japan*

[3]*Division of Physiology, School of Nursing and Rehabilitation Sciences, Showa University, Yokohama, Japan*

[*]**Corresponding author:** Kazuhito Asano, Division of Physiology, School of Nursing and Rehabilitation Sciences, Showa University, 1865 Touka-Ichiba, Midori-ku, Yokohama 226-8555, Japan, Tel: +81 45 985 6538; Fax: +81 45 985 7583; E-mail: asanok@med.showa-u.ac.jp

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Abstract

Background: Periostin, a 90-kDa endogenous extracellular matrix protein, is well known to be involved in the development and persistence of allergic rhinitis. Although histamine H1 receptor antagonists are recommended as first choice of agents in the treatment of allergic rhinitis, the influence of the agents on periostin production is not well understood. The present study was undertaken to examine the influence of histamine H1 receptor antagonists on periostin production from nasal epithelial cells after IL-4 stimulation *in vitro.*

Methods: Human nasal epithelial cells (HNEpC) at a concentration of 1×10^5 cells/ml were stimulated with 10.0 ng/ml IL-4 in combination with either desloratadine (DLT), loratadine (LT), levocetirizine (LCT) or cetirizine (CT). After 48 h, culture supernatants were collected and assayed for periostin levels by ELISA. The influence of LCT on transcription factor, STAT6, activation and periostin mRNA expression were also examined by ELISA and real-time RT-PCR, respectively.

Results: Treatment of cells with DLT, LT, LCT and CT suppressed the ability of HNEpC to produce periostin in response to IL-4 stimulation in dose-dependent manner. The minimum concentration that caused significant suppression is 0.01 μM for DLT, 0.05 μM for LT, 0.05 μM for LCT and 0.1 μM for CT. Treatment of HNEpC with LCT at more than 0.05 μM also suppressed STAT6 activation and periostin mRNA expression induced by IL-4 stimulation.

Conclusion: The present results strongly suggest that histamine H1 receptor antagonists favorably modify the clinical conditions of allergic rhinitis through the suppression of periostin production from nasal epithelial cells after IL-4 stimulation.

Keywords: Periostin; Nasal epithelial cells; Histamine H1 receptor antagonists; Suppression; *In vitro*

Introduction

Allergic rhinitis (AR) is well accepted to be an inflammatory disease of the nasal mucosa characterized by IgE-mediated allergic immune responses. After inhalation of aeroallergen such as pollen, house dust and animal dander, they are captured antigen-presenting cells, processed and presented to Th2 type helper T cells [1,2]. Activated Th2 type helper T cells secret interleukin (IL)-4 and IL-13 and stimulate B cells to produce allergen specific IgE. Allergen specific IgE then binds to receptors on the surface of mast cells. On re-exposure to the relevant allergens, they attach IgE on mast cells and cause degranulation and release of wide variety of inflammatory mediators such as histamine, prostaglandins and leukotriene [1,2]. Among these mediators, histamine, the most important mediator of AR, stimulates the sensory nerve ending and induces sneezing [1-4]. Histamine also stimulates the mucus glands causing rhinorrhea and acts on blood vessels to induce plasma exudation leading to nasal congestion [2,5]. From these established concepts, histamine H1 receptor antagonists are recommended as first-line therapy for controlling all rhinitis symptoms in mild and moderate AR.

Periostin is well accepted to be a 90-kDa endogenous extracellular matrix protein secreted from epithelial cells and fibroblast in response to IL-4/IL-13 stimulation [6,7]. In animal experimental models and patients, periostin is reported to play essential roles in the pathobiology of various diseases such as fibrosis, wound healing and myocardial repair as well as tumorigenesis and metastasis [8,9]. In regard to the development of allergic diseases, periostin increases the ability of cells, including fibroblasts and macrophages, to produce chemokines, which are essential factors for migration of inflammatory cells such as eosinophils and macrophages, which are contributed to the development of allergic inflammatory diseases [6,10]. It is also reported that periostin induces goblet cell metaplasia, sub-epithelial fibrosis, basement membrane thickening and collagen deposition in nasal mucosa [7,11], which are called tissue remodeling and the characteristic feature of AR [12,13]. Furthermore, periostin strongly promotes the secretion of matrix metalloproteinases (MMP), MMP-2 and -9 from macrophages and epidermal cells, which are effector molecules for tissue remodeling in AR [13]. From these reports, periostin has been attracted attention as a novel biomarker of AR, but

the influence of histamine H1 receptor antagonists on the production of periostin is not well understood. In the present study, therefore, we examined the influence of histamine H1 receptor antagonists on periostin production from human nasal epithelial cells using an *in vitro* cell culture technique.

Materials and Methods

Reagents

Histamine H1 receptor antagonists used in this study were cetirizine (CT), levocetirizine (LCT), loratadine (LT) and desloratadine (DLT), which were synthetized in Toronto Research Chem., Inc. (North York, ON, Canada). They were dissolved in Airway Epithelial Cell Growth Media (AECG medium; Promo Cell GmbH, Heidelberg, Germany), sterilized by passing through 0.2 µm pore size filters and stored at 4°C until used, respectively. Recombinant human IL-4 (R & D Systems, Inc., Minneapolis, MN, USA) was dissolved in AECG medium, sterilized similar manner and stored at 4°C until used. The reagents used for mRNA isolation and real-time reverse transcription-polymerase chain reaction (RT-PCR) were purchased from Applied Biosystems (Foster City, CA, USA).

Cell culture

Nasal epithelial cells isolated from a healthy human nasal mucosa (HNEpC) in Promo Cell GmbH were suspended in AECG medium at a concentration of 1×10^5 cells/ml and used as a target cell. To examine the influence of IL-4 on periostin production from HNEpC, cell suspensions (1.0 ml) were introduced into 24-well culture plates in triplicate and incubated with various concentrations (5.0 ng/ml to 20.0 ng/ml) of IL-4 in a final volume of 2.0 ml for 24 to 96 h. In cases of examining the influence of histamine H1 receptor antagonists and leflunomide on periostin production from HNEpC after IL-4 stimulation, cells (1.0 ml) were cultured in triplicate with 10.0 ng/ml IL-4 in combination with/without various concentrations of the agents or leflunomide in 24-well plates for 48 h. After culture, supernatants were obtained and stored at -40°C until used. For examining the influence of the agents on signal transducer and activator of transcription (STAT6) activation and mRNA expression in HNEpC, cells (1.0 ml) were cultured in similar manner for 12 and 24 h, respectively. In all experiments, the agents and leflunomide were added to cell cultures 2 h before stimulation.

Assay for factors

Periostin content in culture supernatants was measured by human periostin ELISA test kits (Phoenix Pharmaceuticals, Inc., Burlingame, Calif., USA) according to the manufacturer's instructions. The minimum detectable level of this ELISA kit was 0.027 ng/ml.

Assay for transcription factor activation

STAT6 activity in cultured cells was assessed by examining the levels of phosphorylated STAT6 with ELISA test kits (Abcam plc., Cambridge, MA, USA) according to the manufacturer's recommended procedures.

Assay for mRNA expression

Periostin mRNA expression in cultured cells was examined by real-time RT-PCR. Total RNA was separated from 1×10^5 cells using a 50 µl lysis solution (P/N4383583; Applied Biosystems), according to the manufacturer's protocols. The first-strand cDNA was synthetized from total RNA using a 20x RT enzyme mix (P/N 4383585; Applied Biosystems) and a 2x RT buffer (P/N43833586; Applied Biosystems) with a T100 thermal cycler (Bio-Rad Co., Hercules, CA, USA). Polymerase chain reaction (PCR) was then carried out in duplicate using TaqMan Gene Expression Assays, PCR primers and RT master mix, which were purchased from Applied Biosystems. Predesigned and validated gene-specific TaqMan Gene Expression Assays [14-16] were used for quantitative RT-PCR, according to the manufacturer's protocols. The reaction was conducted as follows: 10 min denaturation at 95°C, 40 cycles of 15s, denaturation at 95°C, and 1 min annealing and extension at 60°C. Samples were analyzed using an ABI Prism 7900HT Fast RT-PCR System (Applied Biosystems) [16,17]. Relative quantification (RQ) studies [18] were prepared from collected data [threshold cycle numbers (Ct)] with ABI Prism 7900HT Sequence-Detection System (SDS) software v. 2.3 (Applied Biosystem). The primers used for periostin mRNA expression (ID: Hs01566734_m1) and 18S ribosomal RNA (ID: Hs99999901_s1), an internal control, were purchased from Applied Biosystem.

Statistical analysis

Statistical significance between control and experimental groups was examined by ANOVA followed by Dunette's multiple comparison tests. Data analysis was performed by using ANOVA for Mac (SPSS Inc., Chicago, IL, USA). The level of significance was considered at a P value of less than 0.05.

Results

Influence of DLT and LCT on periostin production from HNEpC *in vitro*

The first experiments were designed to examine the influence of IL-4 stimulation on periostin production from HNEpC *in vitro*. An IL-4 dose-response profile was firstly examined using different concentrations (2.0 ng/ml, 5.0 ng/ml, 10.0 ng/ml and 15.0 ng/ml) of IL-4 for 48 h treatment; a time course response was then examined using 10.0 ng/ml IL-4 for 24 h, 48 h, and 72 h. Periostin concentrations in culture supernatants, which were measured with ELISA, peaked at more than 10.0 ng/ml of IL-4 (Figure 1a) and for intervals of 48 h (Figure 1b). The third experiments were carried out to examine whether histamine H1 receptor antagonists could inhibit the periostin production by HNEpC in response to IL-4 stimulation. As shown in Figure 2a, addition of DLT into cell cultures at more than 0.01 µM, but not less than 0.005 µM, significantly suppressed periostin production from HNEpC after IL-4 stimulation. LCT also showed the suppressive effects on periostin production from HNEpC, when the cells were treated with the agent at 0.05 µM and more (Figure 2b).

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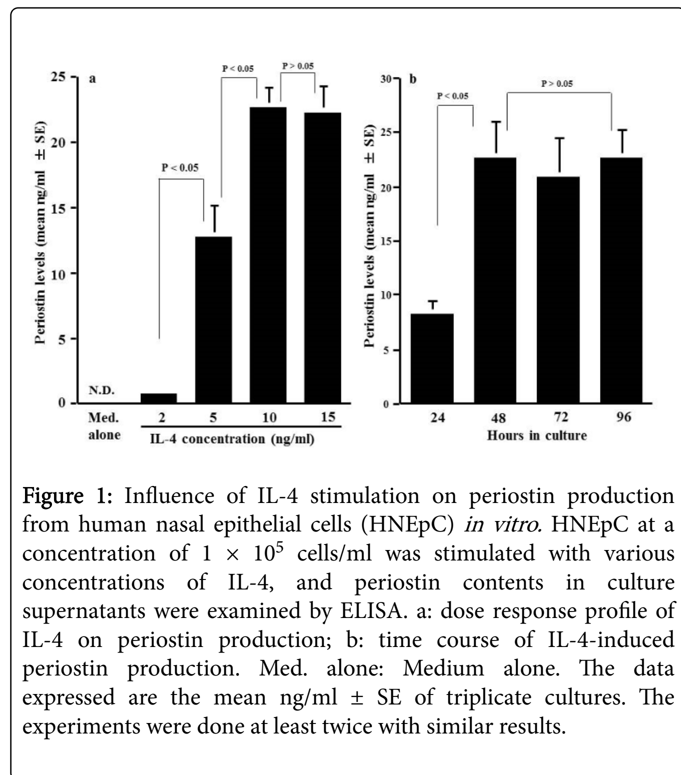

Figure 1: Influence of IL-4 stimulation on periostin production from human nasal epithelial cells (HNEpC) *in vitro.* HNEpC at a concentration of 1 × 10^5 cells/ml was stimulated with various concentrations of IL-4, and periostin contents in culture supernatants were examined by ELISA. a: dose response profile of IL-4 on periostin production; b: time course of IL-4-induced periostin production. Med. alone: Medium alone. The data expressed are the mean ng/ml ± SE of triplicate cultures. The experiments were done at least twice with similar results.

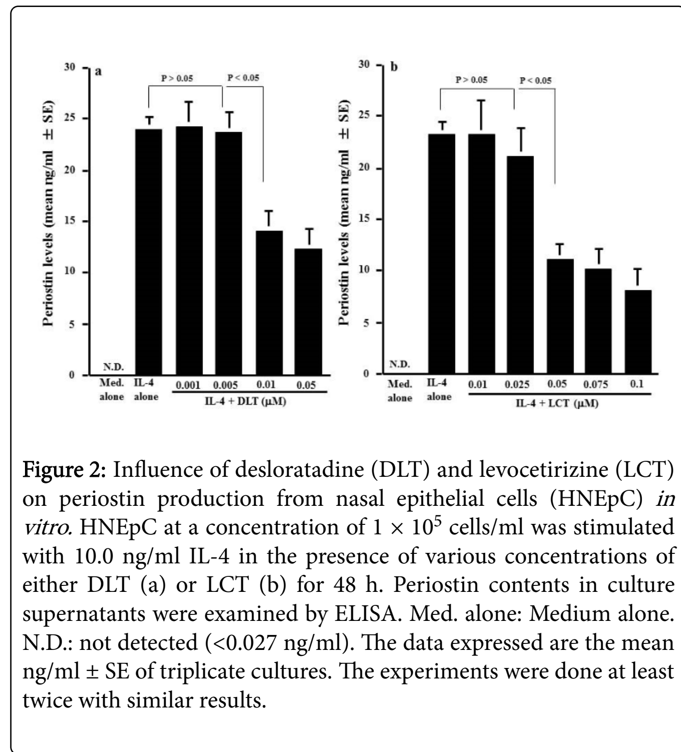

Figure 2: Influence of desloratadine (DLT) and levocetirizine (LCT) on periostin production from nasal epithelial cells (HNEpC) *in vitro.* HNEpC at a concentration of 1 × 10^5 cells/ml was stimulated with 10.0 ng/ml IL-4 in the presence of various concentrations of either DLT (a) or LCT (b) for 48 h. Periostin contents in culture supernatants were examined by ELISA. Med. alone: Medium alone. N.D.: not detected (<0.027 ng/ml). The data expressed are the mean ng/ml ± SE of triplicate cultures. The experiments were done at least twice with similar results.

Next, we examined whether LT and CT, which are mother agents of DLT and LCT, were also suppressed periostin production from HNEpC after IL-4 stimulation. HNEpC were stimulated with 10.0 ng/ml IL-4 in combination with different concentrations of either LT or CT for 48 h. As shown in Figure 3, LT (a) and CT (b) also suppressed periostin production from HNEpC after IL-4 stimulation as in the case of DLT and LCT. However, the minimum concentrations of LT and CT that caused significant suppression of periostin production were much higher than those observed in DLT and LCT.

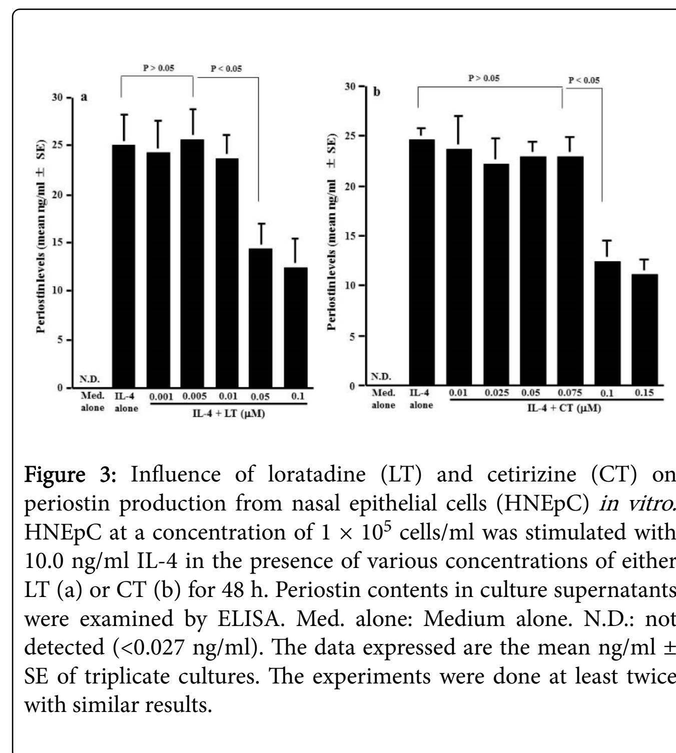

Figure 3: Influence of loratadine (LT) and cetirizine (CT) on periostin production from nasal epithelial cells (HNEpC) *in vitro.* HNEpC at a concentration of 1 × 10^5 cells/ml was stimulated with 10.0 ng/ml IL-4 in the presence of various concentrations of either LT (a) or CT (b) for 48 h. Periostin contents in culture supernatants were examined by ELISA. Med. alone: Medium alone. N.D.: not detected (<0.027 ng/ml). The data expressed are the mean ng/ml ± SE of triplicate cultures. The experiments were done at least twice with similar results.

Influence of LCT on STAT6 activation and periostin mRNA expression in HNEpC *in vitro*

The final experiments were designed to examine the possible suppressive mechanisms of histamine H1 receptor antagonists on periostin production from HNEpC after IL-4 stimulation through the choice of LCT as a target agent. We firstly examined the influence of leflunomide, a STAT6 inhibitor, on periostin production from HNEpC induced by IL-4 stimulation. HNEpC were stimulated with 10.0 ng/ml IL-4 in the presence of different concentrations of leflunomide for 48 h. As shown in Figure 4, treatment of cells with leflunomide at more than 100.0 ng/ml decreased significantly periostin levels in culture supernatants as assessed by ELISA. We next examined the influence of LCT on STAT6 activation in HNEpC after IL-4 stimulation. HNEpC were stimulated with 10.0 ng/ml IL-4 in combination with different concentrations of LCT for 12 h. As shown in Figure 5, LCT at more than 0.05 μM exerted the suppressive effects on IL-4-induced STAT6 activation in HNEpC. We finally examined the influence of LCT on periostin mRNA expression in HNEpC after IL-4 stimulation. HNEpC were cultured with 10.0 ng/ml IL-4 in combination with different concentrations of LCT for 24 h. As shown in Figure 6, addition of LCT into cell cultures at more than 0.05 μM caused significant suppression of periostin mRNA expression in HNEpC as assessed by quantitative RT-PCR.

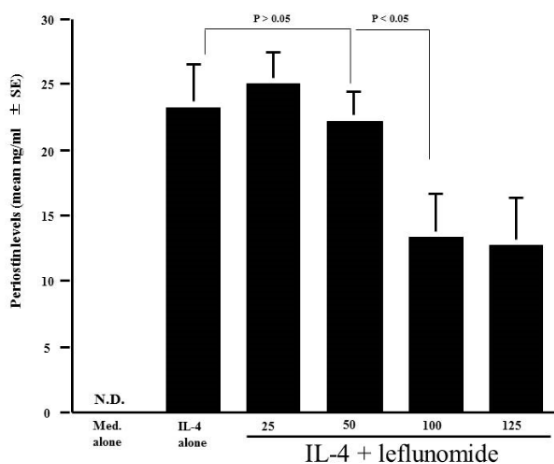

Figure 4: Influence of leflunomide on periostin production from nasal epithelial cells (HNEpC) *in vitro.* HNEpC at a concentration of 1 × 10^5 cells/ml was stimulated with 10.0 ng/ml IL-4 in the presence of various concentrations of leflunomide for 48 h. Periostin contents in culture supernatants were examined by ELISA. Med. alone: Medium alone. N.D.: not detected (<0.027 ng/ml). The data expressed are the mean ng/ml ± SE of triplicate cultures. The experiments were done at least twice with similar results.

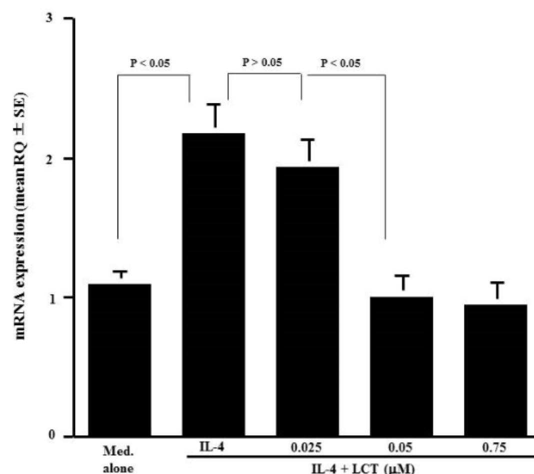

Figure 6: Influence of levocetirizine (LCT) on periostin mRNA expression in nasal epithelial cells (HNEpC) *in vitro.* HNEpC at a concentration of 1 × 10^5 cells/ml was stimulated with 10.0 ng/ml IL-4 in the presence of various concentrations of LCT for 24 h. mRNA expression was examined by real-time RT-PCR. Med. alone: Medium alone. The data expressed are the mean relative quantity (RQ) ± SE of triplicate cultures. The experiments were done at least twice with similar results.

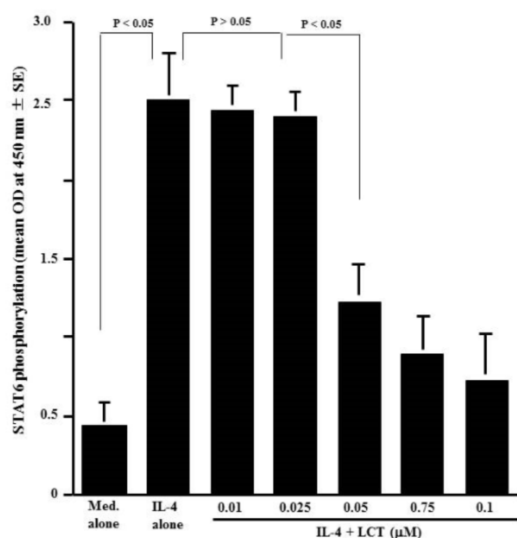

Figure 5: Influence of levocetirizine (LCT) on STAT6 phosphorylation in nasal epithelial cells (HNEpC) *in vitro.* HNEpC at a concentration of 1 × 10^5 cells/ml was stimulated with 10.0 ng/ml IL-4 in the presence of various concentrations of LCT for 12 h. STAT6 activation was examined by ELISA. Med. alone: Medium alone. The data expressed are the mean OD at 450 nm ± SE of triplicate cultures. The experiments were done at least twice with similar results.

Discussion

The present results clearly show that the third-generation histamine H1 receptor antagonists, DLT and LCT, inhibit the ability of nasal epithelial cells to produce periostin induced by IL-4 stimulation as well as their mother drugs, LT and CT *in vitro.* The minimum concentrations that caused significant suppression of periostin production are 0.01 μM for DLT and 0.05 μM for LCT, which are lower levels that observed in their mother drugs and therapeutic blood levels [19, 20].

Histological observation clearly showed the structural abnormalities such as sub-epithelial fibrosis, thickening of the basement membrane, and epithelial injury in nasal mucosa of patients with AR [12,13,21]. A number of studies have also demonstrated goblet cell hyperplasia, enlarged submucosal mucus glands, angiogenesis and lymphangiogenesis in the upper airways, including nasal mucosa of patients with AR [7,13,22]. Furthermore, immunohistochemical analysis revealed the intense deposition of collagen and proteoglycan in the superficial and submucosal layer of allergic nasal mucosa in human and murine AR model [13,23]. These histological changes are called tissue remodeling and may be notorious factors for the development of nasal symptoms, especially nasal obstruction in AR [13]. There is evidence that periostin enhances fibrosis via binding extracellular matrix proteins such as collagen I and fibronectin, and collagen fibrillogenesis through activating lysyl oxidase [24]. Periostin is reported to up-regulate the ability of epithelial cells and fibroblasts to produce collagen by enhancement of regulation of TGF-β signaling pathway [25]. It is also reported that periostin promotes proliferation and migration of both fibroblasts and vascular endothelial cells into inflammatory sites, which are responsible for fibrosis, angiogenesis and

lymphangiogenesis [13]. The influence of periostin on the function of effector cells in allergic inflammatory responses was extensively examined by targeting eosinophils [6,23,26]. Periostin is reported to promote eosinophil migration and increase eosinophil adhesion to the extracellular matrix component fibronectin *in vitro* [6]. Intranasal instillation of allergens extracted from *Aspergillus fumigatus* [6] or ovalbumin [23,26] into periostin-knockout mice cannot induce eosinophil accumulation in airways as compared with wild type mice. Taken together, the results obtained from the present experiments strongly suggest that the suppressive effects of histamine H1 receptor antagonists on periostin production may account, at least in part, for the clinical efficacy of the agents in AR.

IL-4, an inflammatory cytokine mainly secreted from Th2 type T-cell, is well known to be classified into the IL-4/IL-13 family and to have many biological roles such as the stimulation of activated B-cell and T-cell proliferation [27,28]. It is also accepted that IL-4 is essential for class switching of B cells to IgE production and for the maturation of helper T cells to Th2 phenotype, which are responsible for the development and persistence of allergic diseases [27,28]. Stimulation of cells such as B cells and epithelial cells with IL-4 causes the activation of several types of molecules, which are implicated in cellular signal transduction [28,29]. IL-4 first binds to the IL-4 receptor alpha and the complex causes the phosphorylation of Janus kinase (Jak) 1 and 3 which in turn phosphorylate STAT6 [28-30]. STAT6 is then dimerized, migrate to the nucleus and binds to the promoters of the IL-4 responsive genes, which are associated with the production of inflammatory proteins, including cytokines and chemokines [28,30]. Therefore, in the present study, we examined whether histamine H1 receptor antagonists could inhibit periostin production from nasal epithelial cells in response to IL-4 stimulation by the suppression of this signaling pathway using LCT. Addition of leflunomide, a STAT6 inhibitor, at more than 100 μM to cell cultures significantly inhibited periostin production after IL-4 stimulation, indicating that STAT6 activation is essential for periostin production from nasal epithelial cells. We also showed that LCT inhibited STAT6 phosphorylation after IL-4 stimulation in nasal epithelial cells. These results strongly suggest that histamine H1 receptor antagonists, especially LCT inhibit periostin production from nasal epithelial cells after IL-4 stimulation by the suppression of STAT6 phosphorylation, which responsible for periostin mRNA expression. This speculation may be supported by the present observation that addition of LCT into cells cultures significantly inhibited periostin mRNA expression, which was increased by IL-4 stimulation.

Phosphorylation of tyrosine kinases, including Jak 1 and 3 as well as STAT6 requires intracellular free Ca^{2+} ion, which increases in cytosol after stimulations [31,32]. Histamine H1 receptor antagonists are reported to inhibit the signals via Ca^{2+}-dependent mechanisms through inhibition of Ca^{2+} mobilization from both the extracellular space and the endoplasmic reticulum [33,34]. Together with these reports, it is reasonably to speculate that histamine H1 receptor antagonists, especially LCT may suppresses Ca^{2+} mobilization into cytosol after IL-4 stimulation, resulting in suppression of STAT6 activation responsible for periostin mRNA expression. Further experimentations are needed to clarify this point.

In conclusion, the data obtained from the present experiments demonstrate that the suppressive effect of histamine H1 receptor antagonists on periostin production from nasal epithelial cells induced by IL-4 stimulation constitute, at least in part, the therapeutic mode of action of the agents on allergic diseases such as AR and atopic dermatitis.

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