

Effects of Interleukin-31 on MUC5AC Gene Expression in Nasal Allergic Inflammation

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Key Words

Interleukin-31 · Th2 cytokines · MUC5AC · Allergic rhinitis

Abstract

Background: Interleukin-31 (IL-31) is a newly discovered T helper lymphocyte-derived cytokine that plays an important role in allergic inflammation. However, the effects of IL-31 on mucus production in nasal allergic inflammation are completely unknown. **Objective:** To investigate the effects of IL-31 on mucin gene expression (MUC5AC) in patients with allergic rhinitis and in human airway epithelial cells. **Methods:** Expression levels of IL-31 and IL-31 receptor A (IL-31RA) were evaluated in the inferior turbinate of patients with allergic rhinitis and non-allergic rhinitis with immunohistochemistry. IL-31-induced MUC5AC gene expression was measured with a MUC5AC luciferase reporter assay in human epithelial HM3-MUC5AC cells and quantified by quantitative real-time polymerase chain reaction in human airway epithelial A549 cells. **Results:** IL-31RA was primarily localized in submucosal glands and upregulated in allergic rhinitis. IL-31 was detected in submucosal tissue and increased in allergic inflammation. MUC5AC gene expression was induced by IL-31 stimulation both in HM3-MUC5AC and A549 cells. Additionally, IL-31 cooperated with Th2 cytokines on MUC5AC gene expression

in HM3-MUC5AC cells. **Conclusion:** IL-31 and IL-31RA are up-regulated in patients with allergic rhinitis, and induce MUC5AC gene expression in human airway epithelial cells. These findings suggest that IL-31 plays an important role in mucus overproduction in nasal allergic inflammation.

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Introduction

Allergic inflammatory disease of the airways, including allergic rhinitis and allergic asthma, are characterized by eosinophil infiltration, elevated plasma concentrations of allergen-specific immunoglobulin E and T helper type 2 (Th2) cytokines, and mucus hypersecretion [1]. Allergic symptoms result from the release of inflammatory factors and mediators from immune cells, such as mast cells and basophils, triggered by an allergen and allergen-specific IgE [2]. Th2 cytokines, such as IL-4, IL-5, IL-9, and IL-13, are involved in the allergic response [3, 4].

Interleukin-31 (IL-31) is a novel T cell-derived pro-inflammatory cytokine that possesses a close relationship with atopic and allergic contact dermatitis [5]. Transgenic mice overexpressing IL-31 have a phenotype resembling allergic dermatitis in human subjects and an in-

creased expression of IL-31 in allergic contact dermatitis [6]. Recently, it has been reported that IL-31 was significantly elevated in asthmatic subjects, suggesting that IL-31 may be a useful indicator of asthma [7]. In upper airway diseases, we have recently shown that pollen antigen-induced IL-31 production by peripheral blood mononuclear cells was significantly higher in patients with Japanese cedar/cypress pollinosis as compared to healthy controls and patients treated with specific immunotherapy [8]. In this report, we further demonstrated that the antigen-specific IL-31 production was significantly and positively correlated with the disease severity, including quality of life. However, whether and how IL-31 acts in airway inflammation, especially in humans, remains unknown.

Mucins, which are major components of mucus, are very-high-molecular-weight glycoproteins that can either be membrane associated or secreted into the extracellular space [9]. Secreted mucins are expressed by non-ciliated epithelial cells in the respiratory epithelium and are stored in intracellular secretory granules until stimulated for release by regulated exocytosis [9]. Mucus production is a primary defense mechanism for maintaining airway health. The thickness of the mucus layer depends on the luminal conditions and functional requirements of the secreting surface. In the airway tract, the optimal mucociliary clearance requires a shallow mucus layer that can be transported along with the entrapped inhaled foreign particles. However, the overproduction of mucin is a common pathological feature in chronic rhinosinusitis, allergic rhinitis, asthma, and chronic obstructive pulmonary disease, which leads to mucociliary dysfunction in the upper and lower airways. Five homologous human genes encode the gel-forming mucins. Four of these (MUC2, MUC5AC, MUC5B, and MUC6) are present in tandem as a conserved cluster on human chromosome 11p15 [10]. Among them, MUC5AC is considered to be the predominant mucin in the airway and can be induced by various pathogens including *Pseudomonas aeruginosa* [11] and nontypeable *Haemophilus influenzae* [12]. In regard to Th2 cytokines, including IL-4, IL-9, and IL-13, which are strongly involved in allergic inflammation [13–15], it has been previously shown that these cytokines increase MUC5AC expression in epithelial cells in human cell culture systems [16–18].

In the present study, we investigated the effects of IL-31 on mucus hypersecretion in allergic rhinitis patients. We analyzed the expression levels of IL-31 receptor A (IL-31RA) and IL-31 in the inferior turbinate of either non-allergic rhinitis patients or allergic rhinitis patients. Additionally, IL-31-induced MUC5AC gene expression was

evaluated via a luciferase assay and real-time reverse transcription polymerase chain reaction (PCR) in human cultured epithelial cells.

Methods

Materials

Human IL-4, IL-9, IL-13, and IL-31 were purchased from R&D Systems (Minneapolis, Minn., USA). The Dual-Luciferase™ reporter assay system was obtained from Promega (Madison, Wisc., USA).

Cell Culture

HM3-MUC5AC was generously provided by Carol Basbaum (University of California, San Francisco, Calif., USA). HM3-MUC5AC is a subclone of the LS174T adenocarcinoma cell line, which is stably transfected with an MUC5AC promoter [a 3.7-kb segment of the 5'-flanking region of the human MUC5AC gene pGL3-basic luciferase vector (Promega)]. HM3-MUC5AC was maintained at 37°C and 5% CO₂ atmosphere in Dulbecco's modified Eagle's medium/Ham's F12 medium containing 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). A549 cells, a human pulmonary adenocarcinoma cell line, purchased from the American Type Culture Collection, were cultured in RPMI 1640 medium containing 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml).

RNA-Mediated Interference

IL-31R and control small interfering RNA oligonucleotides were purchased from Santa Cruz Biotechnology, Inc. (Calif., USA). The siRNA was transfected into HM3-MUC5AC cells using siRNA transfection reagent (Santa Cruz Biotechnology, Inc.) according to the manufacturer's instructions.

Luciferase Assay

HM3 cells stably transfected with the MUC5AC-luciferase plasmid were treated with IL-31 and IL-4, IL-9, and IL-13 for 3 h, and harvested for the luciferase assays. Luciferase assays were performed on a Luminescencer-PSN (Bio-Instrument Atto, Bunkyo, Tokyo). The IL-31-dependent fold induction was calculated relative to the luciferase light units in the absence of IL-31 treatment. The normalized luciferase activity was then expressed as the relative luciferase activity (fold induction).

Real-Time Quantitative PCR Analysis

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer's instructions. For the reverse transcription reaction, TaqMan reverse transcription reagents (Applied Biosystems, Foster City, Calif., USA) were used. Briefly, the reverse transcription reaction was performed for 60 min at 37°C, followed by 60 min at 42°C with oligo(dT) and random hexamers. PCR amplification was performed by using TaqMan Universal Master Mix. Pre-developed TaqMan assay reagents (probe and primer mixtures for human MUC5AC and GAPDH) were used to detect the expression of each gene. Reactions were performed in triplicate containing 2× Universal Master Mix, 2 µl of template cDNA, 200 nM of primers, and 100 nM of probe in a final volume of 25 µl, and analyzed in a

96-well optical-reaction plate (Applied Biosystems). To allow for the direct detection of the PCR product, probes included a fluorescent reporter dye, 6-carboxyfluorescein (FAM), on the 5'-end and were labeled with a fluorescent quencher dye, 6-carboxytetramethyl-rhodamine (TAMRA), on the 3'-end. Reactions were amplified and quantified on an ABI 7700 sequence detector and the manufacturer's corresponding software (Applied Biosystems). Relative quantities of mRNAs were obtained by using the comparative Ct Method (for details, see User Bulletin 2 for the ABI PRISM 7500 sequence detection system) and were normalized by using TaqMan Pre-Developed Assay Reagent human GAPDH as an endogenous control (Applied Biosystems).

Tissue Preparation of the Inferior Turbinate from Patients

This study was approved by the Institutional Review Board of Okayama University Hospital, Okayama, Japan. At the time of surgery, 1% lidocaine hydrochloride and 0.02% epinephrine were applied topically in nasal packs. The turbinates were injected with 2–4 ml of 1% lidocaine with 1:100,000 epinephrine. Then, the lateral wall of the inferior turbinate was harvested. The tissue was fixed immediately in 4% paraformaldehyde/PBS solution for several days.

Immunohistochemistry

The expression of IL-31 and IL-31RA in human inferior turbinate was assessed via immunohistochemistry. Paraffin-embedded tissues were cut into 4- μ m thick sections. Sections were deparaffinized, rehydrated, and antigen-retrieved by microwave boiling for 5 min (IL-31) or with 0.1% trypsin at 37°C for 15 min (IL-31RA, Wako Pure Chemicals, Osaka, Japan). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide (H₂O₂) in methanol, and non-specific protein binding was blocked with 10% normal goat serum (Dako Japan, Tokyo, Japan). Tissue sections were then incubated with primary antibodies [IL-31: rabbit polyclonal antibody (2 μ g/ml); LifeSpan Biosciences, Wash., USA; and IL-31RA: mouse monoclonal antibody (clone 3A10, 5 μ g/ml); Abnova Corp., Taipei, Taiwan] or control antibodies (Dako, Japan) overnight at 4°C. A Histofine Simple Stain MAX-PO(R) (Nichirei Biosciences Inc., Tokyo, Japan) or Vectastain elite ABC Mouse IgG kit (Vector Laboratories Inc., Calif., USA) with a diaminobenzidine substrate were used according to the manufacturer's instructions. After washing with deionized distilled H₂O, the sections were counterstained with hematoxylin, dehydrated in ethanol, and coverslips were mounted in xylene with resin.

Statistical Analysis

Data are expressed as means $\pm$ SE. Statistical analysis was performed using ANOVA followed by a post hoc test. *p* values <0.05 were considered statistically significant.

Results

Analysis of IL-31 and IL-31RA in the Inferior Turbinate of Patients with Allergic Rhinitis

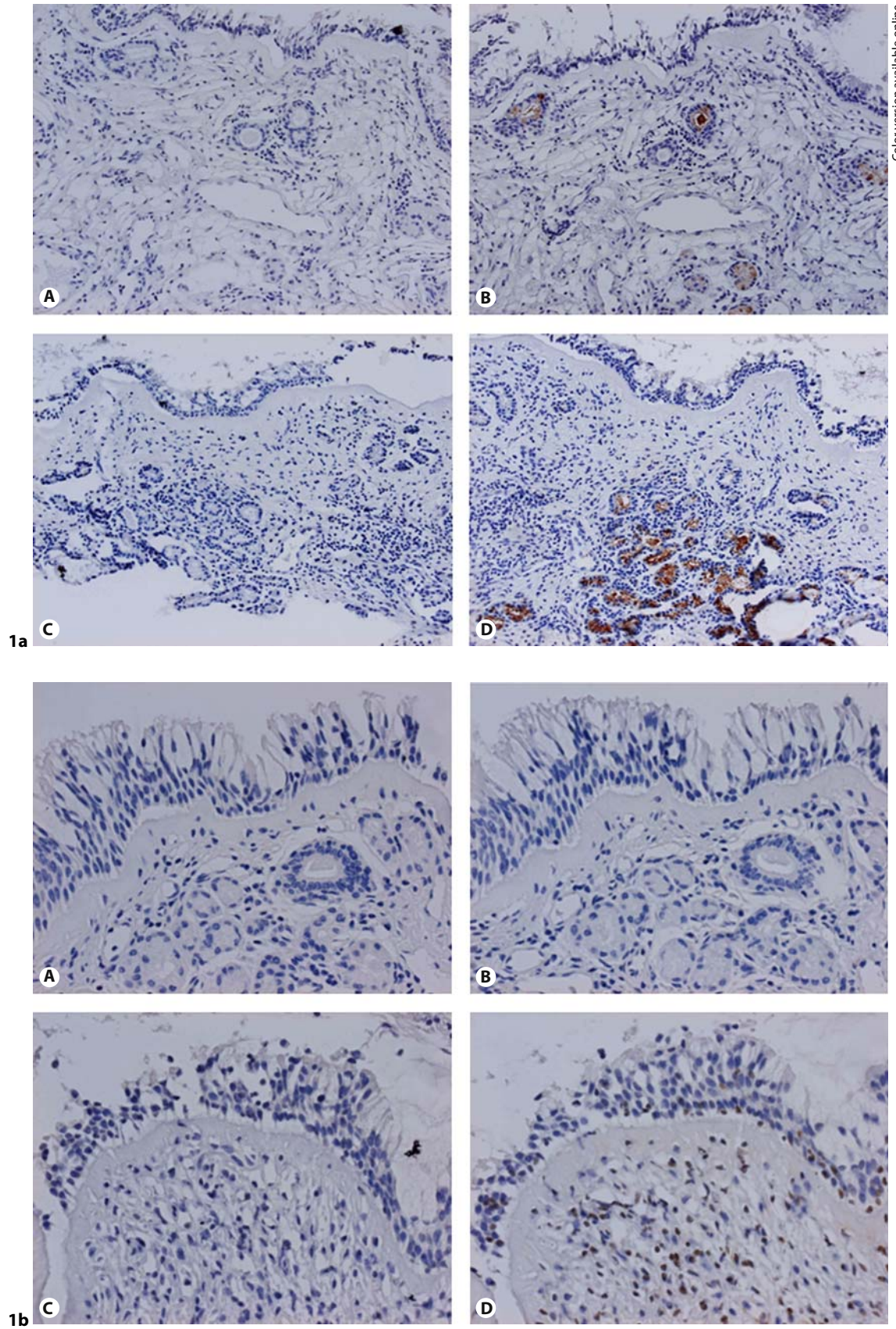
Although it is well known that IL-31 plays an important role in atopic dermatitis, little is known about the

localization of IL-31 and its receptor in human airways. Therefore, we investigated the local expression of IL-31 and IL-31RA in patients with allergic rhinitis or non-allergic rhinitis by performing immunohistochemistry. As shown in figure 1a, IL-31RA was primarily localized in submucosal glands and highly expressed in patients with allergic rhinitis compared to those with non-allergic rhinitis. In figure 1b, IL-31 expression levels were also up-regulated in allergic rhinitis patients compared to non-allergic rhinitis patients. These results suggest that IL-31 is strongly involved in nasal allergic inflammation, especially in secretion.

Functional Effects of IL-31 on MUC5AC Gene Expression

Despite that IL-31 was found to be increased in patients with allergic rhinitis, the functional effects of IL-31 on mucus hypersecretion in human airway epithelial cells remain unknown. Therefore, in order to determine whether IL-31 induces MUC5AC gene expression in human epithelial cells, we first evaluated mucin gene expression after 3 h of IL-31 stimulation (i.e. from 50 to 200 ng/ml) by measuring the luciferase reporter activity of MUC5AC in HM3-MUC5AC cells. As shown in figure 2a, IL-31 induced MUC5AC gene transcription in a dose-dependent manner. Next, we demonstrated that 200 ng/ml of IL-31 together with 50 ng/ml of TGF α induced MUC5AC gene expression in order to show the effect of IL-31 on MUC5AC gene induction (fig. 2b). The result showed both stimulants could induce MUC5AC gene expression significantly. We then assessed whether IL-31 induced MUC5AC gene transcription in human airway epithelial cells by performing real-time quantitative PCR analysis in A549 cells. Consistent with the findings presented in figure 2a, 200 ng/ml of IL-31 also induced MUC5AC gene transcription, as shown in figure 2c. Furthermore, we hypothesized that IL-31R was essential for IL-31-induced MUC5AC gene expression. Therefore, we assessed the

Fig. 1. Immunohistochemical analysis of IL-31 and IL-31RA expression in human nasal mucosa. **a** IL-31RA expression was increased in the inferior turbinate of patients with allergic rhinitis and was primarily found to be localized in submucosal glands. **b** IL-31 expression was increased in the inferior turbinate of patients with allergic rhinitis and was primarily found to be localized in submucosal tissue. **A** and **C** were treated with an isotype control antibody and **B** and **D** were treated with anti-IL-31 RA (**a**) or anti-IL-31 (**b**) antibody. **A, B** Inferior turbinate from non-allergic rhinitis. **C, D** Inferior turbinate from allergic rhinitis. $\times 40$ magnifications of sections.



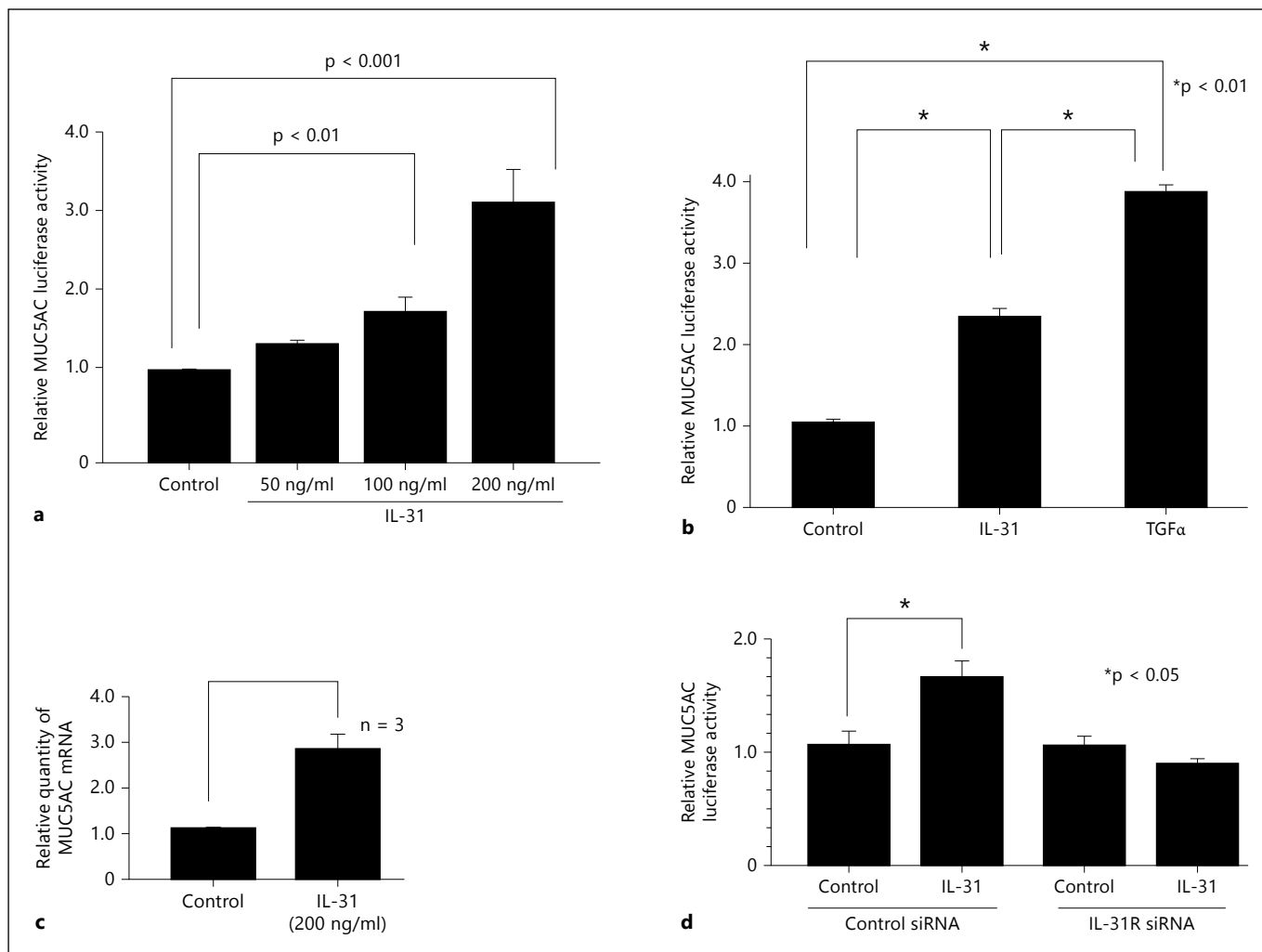


Fig. 2. Functional effect of IL-31 on MUC5AC gene expression. **a** Expression of MUC5AC was evaluated following a 3-hour treatment with various concentrations of IL-31 in HM3-MUC5AC cells. MUC5AC gene expression was upregulated by IL-31 in a dose-dependent manner. Luciferase activity was determined by assaying for chemiluminescence. Values are means $\pm$ SD ($n = 6$). **b** Both IL-31 and TGF α induced MUC5AC gene expression sig-

nificantly. Values are means $\pm$ SD ($n = 6$). **c** IL-31 induced MUC5AC gene expression in human airway epithelial A549 cells by performing quantitative PCR. Values are means $\pm$ SD ($n = 3$). **d** IL-31R siRNA inhibited IL-31-induced MUC5AC gene expression compared with control siRNA by luciferase assay in HM3-MUC5AC cells. Values are means $\pm$ SD ($n = 6$). Data are representative of three independent experiments.

role of IL-31R on IL-31-induced MUC5AC gene expression using an siRNA approach. Figure 2d shows that IL-31R siRNA suppressed IL-31-induced MUC5AC gene expression. This result indicated that IL-31-induced MUC5AC gene expression was mediated through IL-31R.

IL-31 Enhanced Th2 Cytokine-Induced MUC5AC Gene Expression

We have demonstrated that IL-31 was upregulated in allergic rhinitis and MUC5AC gene expression was in-

creased by IL-31 stimulation. Additionally, it has been shown that Th2 cytokines are produced in allergic inflammation. Thus, we hypothesized that IL-31 may junction together with other Th2 cytokines and consequently lead to exacerbated mucus overproduction. We further investigated whether there was a direct interaction between IL-31 (200 ng/ml) and IL-4 (100 ng/ml), IL-9 (100 ng/ml) or IL-13 (100 ng/ml) on MUC5AC gene expression in HM3-MUC5AC cells via a MUC5AC luciferase reporter assay. As expected, Th2 cytokine-induced

MUC5AC gene expression was enhanced by IL-31 treatment, as shown in figure 3. Taken together, our data clearly demonstrate that IL-31 plays an important role in mucus hypersecretion in allergic rhinitis.

Discussion

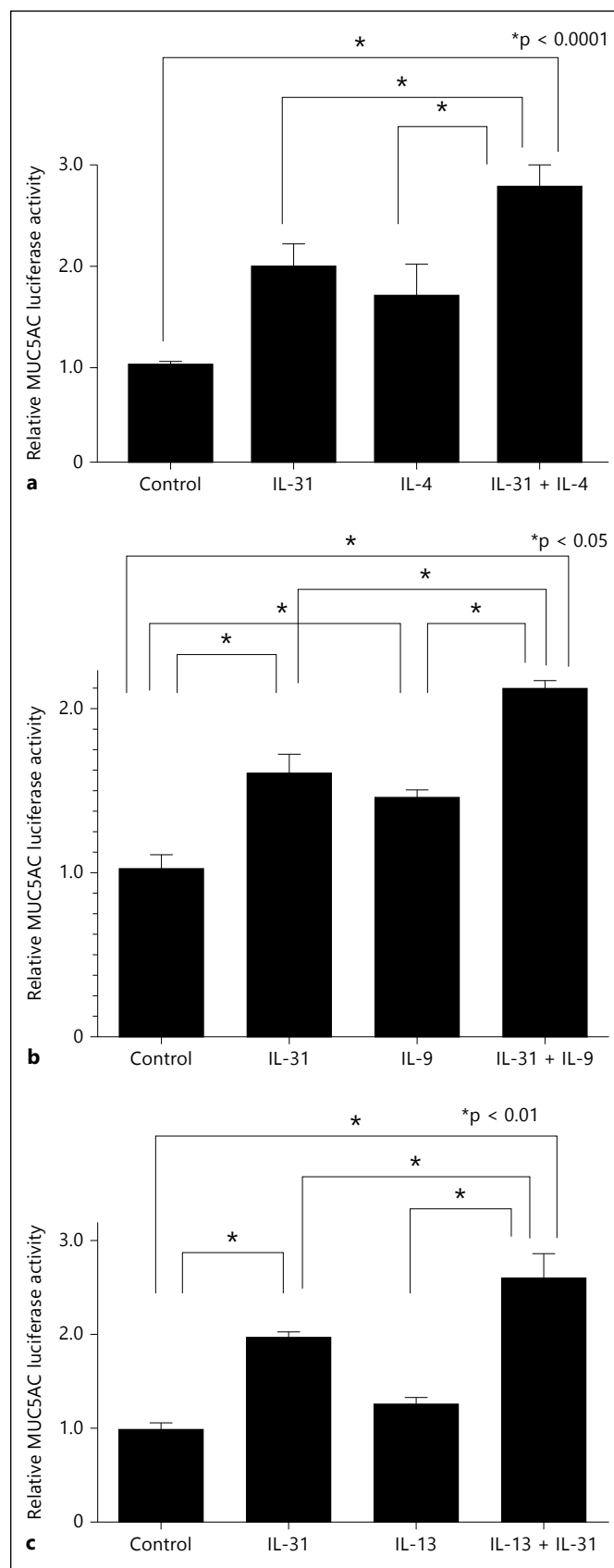
In the present study, we provided direct evidence that both IL-31 and IL-31RA were increased in human nasal mucosa in patients with allergic rhinitis versus those with non-allergic rhinitis. Moreover, we found that IL-31 functionally induced MUC5AC gene expression in HM3-MUC5AC cells and human airway A549 cells, which was enhanced in conjunction with Th2 cytokine.

The findings of this study provide direct evidence that both IL-31 and IL-31RA were upregulated in human nasal allergic inflammation. Although several studies demonstrated that IL-31 was highly expressed in human skin in atopic dermatitis and deeply associated with the pathogenesis of AD [5, 6, 19], there have been no reports that IL-31 expression is increased in patients with allergic rhinitis. This result, although rather unexpected, may suggest a possible role of IL-31 in allergic rhinitis.

Another important finding of the present study is that IL-31 induces MUC5AC gene expression in human airway epithelial cells. MUC5AC is one of the major respiratory mucins and has been shown to be induced by a wide variety of stimuli, including not only pro-inflammatory cytokines [11], epidermal growth factor receptor (EGFR) ligand [20], and bacterial pathogens [11, 12], but also Th2 cytokines, including IL-4, IL-9, and IL-13 [16–18]. Mucus hypersecretion is one of the major symptoms in allergic inflammation. However, the functional role of IL-31 in mucin gene expression, especially in human airway epithelial cells, still remains largely unknown. We clearly demonstrated that IL-31 induced MUC5AC gene expression in human airway epithelial cells. Our findings suggest that IL-31 may play an important role in mucin overproduction in human airway epithelial cells.

IL-4 and IL-13 are significantly expressed and have been shown to play a critical role in nasal allergic inflammation [13, 15]. A recent report had demonstrated that

Fig. 3. IL-31 enhanced Th2 cytokine-induced MUC5AC gene expression. MUC5AC gene expression was enhanced by a combination treatment of IL-31 with IL-4 (a), IL-9 (b), and IL-13 (c) in HM3-MUC5AC cells, as determined by luciferase assay, respectively. Values are means $\pm$ SD (n = 6).



IL-31 expression was significantly increased and correlated with IL-4 and IL-13 in allergic contact dermatitis [21]. Furthermore, it has been shown that IL-31 induces cytokine and chemokine from human bronchial epithelial cells and is enhanced by IL-4 and IL-13 stimulation [22]. Therefore, we examined the effects of IL-31 on IL-4- or IL-13-induced MUC5AC gene expression and found that IL-4- or IL-13-induced MUC5AC gene expression was enhanced by IL-31. These results emphasize the importance of IL-31 in the pathogenesis of nasal allergic inflammation.

In conclusion, the present study demonstrated for the first time that IL-31 and IL-31RA expression are upregu-

lated in nasal mucosa of patients with allergic rhinitis. Additionally, IL-31 induces MUC5AC gene expression in human airway epithelial cells. These findings provided new insights into the novel roles of IL-31 in regulating mucus hypersecretion in nasal allergic inflammation.

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