Modulation of mucus production by interleukin-13 receptor α₂ in human airway epithelium

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Short title: IL-13Rα₂ is a mucus modulator in NHBECs.

Abstract

Background   IL-13 induces goblet cell hyperplasia and mucus overproduction in airway epithelial cells. Interleukin-13 receptor α_2 (IL-13Rα_2) has been suggested to act as a 'decoy receptor' in the airway epithelium through inhibiting the IL-13 signal. However, the regulatory mechanisms for mucus production by IL-13Rα_2 remain unclear.

Objectives   The aim of this study was to examine the role of IL-13Rα_2 on the goblet cell hyperplasia and the mucus overproduction by IL-13.

Methods   Bronchi were <u>obtained</u> from the patients who underwent a lung resection due to lung cancer or benign lung tumors. Normal human bronchial epithelial cells (NHBECs) were isolated and cultured using an air-liquid-interface (ALI) method.

Results   The number of periodic acid-Schiff's (PAS)-positive cells, goblet cells and MUC5AC-positive cells increased after adding IL-13 in NHBECs. <u>The concentrations of MUC5AC protein in the supernatant and the mRNA expression of MUC5AC significantly increased</u> after adding IL-13, and returned to control levels at 21 days. <u>The mRNA expression of IL-13Rα_2 significantly increased at 7 days and then continuously increased up-to 21 days. The protein of a soluble form of IL-13Rα_2 in the supernatants significantly increased at 14 and 21 days.</u> Anti-IL-13Rα_1 antibody and recombinant IL-13Rα_2 reduced the number of PAS-positive cells, goblet cells and MUC5AC-positive cells, and MUC5AC mRNA, while anti-IL-13Rα_2 antibody increased the number of these cells and MUC5AC mRNA. <u>The concentration of MUC5AC protein in the supernatant induced by IL-13 was reduced by anti- IL-13Rα_1 antibody and recombinant IL-13Rα_2.</u> IL-13 induced-STAT activation was inhibited by anti-IL-13Rα_1 antibody and recombinant IL-13Rα_2. <u>In contrast, the IL-4-induced mucus production, mucus secretion and STAT activation were not inhibited by recombinant IL-13Rα_2.</u>

Conclusion   The soluble form of IL-13Rα_2 may therefore modulate mucus overproduction by IL-13 through the pathway including IL-13Rα_1 in NHBECs.

Key words: bronchial asthma, goblet cell hyperplasia, IL-13, <u>IL-4,</u> MUC5AC, normal human bronchial epithelial cells

Introduction

Mucus overproduction and goblet cell hyperplasia in the airway are commonly observed in asthma and COPD [1]. Marked goblet cell hyperplasia and an increased mucus production causing airway plugging are known to be pathological feature of deaths from severe asthma attack [2, 3]. Helper T-cell type2 (Th2) cytokines, including interleukin (IL)-13 and IL-4, have been suggested to be implicated in mucus overproduction and goblet cell hyperplasia in asthma [4]. IL-13 induces goblet cell hyperplasia with mucus hypersecretion in the mouse airways and airway hyperreactivity [5]. Airway epithelial hypertrophy, goblet cell hyperplasia and mucus hypersecretion have been shown in IL-13 transgenic mice [6]. IL-13 induces the cell proliferation and goblet cell hyperplasia of human airway epithelial cells in vitro [7, 8]. We previously reported that IL-13 induced MUC5AC expression with the development of goblet cell hyperplasia in human bronchial epithelial cells [9].

IL-13 exerts its biological activities by binding to its receptors on the cell surface. There are two types of IL-13 receptor, a receptor including IL-13 receptor α_1 chain (IL-13Rα_1) and IL-13Rα_2. One IL-13-binding receptor is a heterodimer of the IL-13Rα_1 and IL-4 receptor α chain (IL-4Rα), which also acts as IL-4R, and binds both IL-13 and IL-4 [10]. IL-13 Rα_1 transduces the IL-13 signal via the JAK-STAT pathway and the phosphatidylinositol-3 kinase/insulin receptor substrate-1/2 pathway. IL-13 activates JAK1 and TYK2, followed by the activation of STAT6 and the activated STAT6 induces NFκB activation in the nucleus [11]. IL-13 up-regulates the MUC5AC expression in NHBECs via the JAK-STAT pathway and NFκB activation [12].

Regarding IL-13Rα_2, another IL-13-binding receptor, two forms have been observed, a membranous-bound form and a soluble form [10, 11]. Although the affinity of membranous IL-13Rα_2 and the IL-13Rα_1/IL-4Rα complex with IL-13 is almost the same, the affinity of soluble IL-13Rα_2 with IL-13 is higher than that of IL-13Rα_1/IL-4Rα complex [11]. IL-13Rα_2 has been considered not to transduce the IL-13 signal, while it acts as a 'decoy receptor' and blocks IL-13 signal [10, 11]. However, it remains uncertain which form of IL-13Rα_2 acts as the decoy receptor [11].

We previously demonstrated that IL-13 induces goblet cell hyperplasia in human airway epithelial cells via IL-13Rα_1 [9], but the role of IL-13Rα_2 on the IL-13-induced goblet cell hyperplasia remains to be elucidated.

Therefore, in the present study, we examined the role of IL-13Rα_2 on the

IL-13-induced mucus production and goblet cell hyperplasia in human bronchial epithelial cells.

Materials and Methods

Culture and differentiation of normal human bronchial epithelial cells

Bronchi were obtained from 22 patients (mean age, 67 ± 3.6 years; range, from 35 to 80 years) who underwent a lung resection due to lung cancer or benign lung tumors. The ethics committee of Shinshu University School of Medicine approved the study and the patients provided written informed consent to participate in all aspects of the investigation. The isolation and cultivation of normal human bronchial epithelial cells (NHBECs) have been previously reported [9]. Briefly, passage-2 NHBECs were seeded onto polyester inserts (Costar Transwell clear, Cambridge, MA). Confluence was reached within 7 days and then the cells were cultured with an air-liquid-interface (ALI) method [13].

Culture with IL-13

After the NHBECs reached confluence (Day 0), medium containing IL-13 (recombinant human IL-13, BIOTEC, Hamburg, Germany) or vehicle (PBS) was added to basolateral side of the inserts, and the cells were incubated with 10 ng/ml of IL-13 [9] or vehicle (PBS) with the ALI method. The culture medium was changed every day, and the cells were incubated for 21 days with IL-13 or vehicle (PBS). We previously confirmed that 10 ng/ml IL-13 was the optimal concentration for differentiation into goblet cells under our experimental conditions [9].

Culture with IL-4

We performed the culture with IL-4. The medium containing IL-4 (recombinant human IL-4, Peprotech, London, United Kingdom) was added, and the cells were incubated with 0.1, 1 or 10 ng/ml of IL-4 or vehicle (PBS) with the ALI method. The culture medium was changed every day, and the cells were incubated with IL-4 or vehicle (PBS).

Neutralization with anti-IL-13Rα₁ and anti-IL-13Rα₂ antibodies during IL-13 incubation

To examine the blocking effect of anti-IL-13Rα₁ and anti-IL-13Rα₂ antibodies on goblet cell hyperplasia and MUC5AC expression induced by IL-13, NHBECs were incubated with IL-13 (10 ng/ml) plus anti-IL-13Rα₁ antibody (10 μg/ml, R&D Systems, Inc, Minneapolis, USA), anti-IL-13Rα₂ antibody (2.5 μg/ml, R&D Systems, Inc) or vehicle (PBS) for 7 or 14 days. The culture medium containing

IL-13 plus antibodies was changed everyday. Mouse IgG$_1$ isotype control antibody (10 μg/ml, R&D Systems, Inc) was utilized as a negative control.

Suppression on mucus overproduction with the exogenously added recombinant IL-13Rα$_2$

   To examine the inhibition by a soluble form of IL-13Rα$_2$ on MUC5AC expression induced by IL-13, NHBECs were incubated with IL-13 (10 ng/ml) plus the recombinant human soluble IL-13 Rα$_2$/Fc (0.04, 0.4 or 4 μg/ml) or vehicle (PBS). The culture medium containing IL-13 plus the recombinant human soluble IL-13Rα$_2$/Fc (rsIL-13Rα$_2$) was changed everyday.

Real-Time Quantitative PCR

   To examine the time course of mRNA expression of MUC5AC and IL-13Rα$_2$, RNA was extracted from the cells 7, 14, or 21 days after the culture with IL-13 with ALI methods, and prepared for Real-Time-Quantitative PCR as previously described [9,14]. Briefly, total RNA from the cultured NHBECs on Transwell$^®$ inserts was extracted using ISOGEN (NIPPON GENE, Tokyo, Japan). Matching primers and Hybridization probes (Nihon Gene Research Lab's Inc., Sendai, Japan) were designed for each of the genes as shown in Table 1.

   Real-time reverse transcription (RT)-PCR reaction was carried out according to the manufacturer's instructions (Nihon Gene Research Lab's Inc.). Real-time quantitative PCR curves were analyzed by the Light Cycler 3.5 software (Roche Diagnostics). For the relative quantification of MUC5AC and IL-13Rα$_2$ mRNA expressions, the mRNA expression of GAPDH was used as a control.

Histochemical analysis

   The number of Periodic acid-Schiff's (PAS)-positive cells and goblet cell in NHBECs was histologically examined as previously described [9, 15].   The number of goblet cells with secretory granules was counted as previously reported [7, 9, 15].

Immunohistochemical analysis

   The effects of IL-13 and IL-13Rα antibodies, and a soluble form of IL-13Rα$_2$ on MUC5AC expression was immunohistochemically detected as previously described [9, 11]. The sections of cultured NHBECs on Transwell$^®$ inserts were incubated with either anti MUC5AC antibody (Lab Vision, Fremont, Calif., USA) or

anti-IL-13Rα$_2$ antibody (R&D Systems, Inc). All sections were incubated with anti-IL-13Rα$_2$ antibody as a batch at same time. Either horseradish-peroxidase-conjugated rabbit anti-mouse Immunoglobulins (1:200; DAKO, Glustrup, Denmark) for MUC5AC or peroxidase-conjugated Rabbit Anti-Goat Immunoglobulins (1:200; DAKO) for IL-13Rα$_2$ was added. The antigen-antibody complexes were visualized using diaminobenzidine tetrahydrochloride (Wako).

ELISA for IL-13Rα$_2$ protein in supernatants and cell lysates

Sample preparations of soluble form of IL-13Rα$_2$ in the medium of cultured NHBECs for ELISA were performed according to the method as described with some modification [16]. Passage-2 NHBECs were seeded onto polyester inserts (Transwell), and then cultured in the serum-free medium. After the NHBECs reached confluence (day 0), the cells were incubated with medium containing 10 ng/ml IL-13. The culture supernatants from each well were collected by adding PBS (5 ml) to the apical side of the inserts, and cell lysates were collected by the addition of lysis buffer (1 ml) [RIPA buffer; 50 mM Tris-HCl, 0.15 M sodium chloride, 0.1% sodium dodecylsulfate, 1% sodium deoxycholate, 1 mM phenylmethylsulfonylfluoride (Wako), 1% Triton X-100 (Bio-Rad Laboratories, Calif., USA) and protease inhibitor cocktail (Nakalai Tesque, Kyoto, Japan)] [9]. The concentrations of soluble forms of IL-13Rα$_2$ in 100 µl of culture supernatants and suspension of cell lysates were measured with Human Interleukin-13 soluble Receptor alpha 2 (human IL-13 sRα$_2$) DuoSet ELISA development kit (R&D systems, Inc.) according to the manufacturer's instructions. After the absorbance was measured with a MICROPLATE READER (Model 550, Bio-Rad Laboratories, CA), the data were analyzed with Micrplate Manager III (Bio-Rad). The minimum detection limit of this kit is 5 pg/ml. The concentration of total protein was measured in each sample of the culture supernatants and the suspension of cell lysates using the method described by Lowry et al. [17], and the actual contents of IL-13Rα$_2$ protein in the supernatants and cell lysates in each insert.

ELISA for MUC5AC protein in cell supernatants used to examine the effect of IL-13, IL-4 and IL-13R on the secretion of MUC5AC

The sample preparations of MUC5AC protein in the cell supernatant of cultured NHBECs for ELISA were performed according to the method as described with some modifications [18]. Passage-2 NHBECs were seeded onto polyester inserts

(Transswell), and then cultured in the serum-free medium. After the NHBECs reached confluence (day 0), the cells were incubated with IL-13 (10 ng/ml), IL-13 (10 ng/ml) plus anti-IL-13Rα_1 antibody (10 μg/ml) (R&D Systems, Inc, Minneapolis, USA), IL-13 (10 ng/ml) plus the recombinant human soluble IL-13 Rα_2/Fc (rsIL-13Rα_2, 4 μg/ml, R&D Systems, Inc), IL-4 (1 ng/ml), IL-4 (1 ng/ml) plus the rsIL-13Rα_2 (4 μg/ml) or vehicle (PBS) for 14 days. Anti-human IL-13Rα_1 antibody was selected for its ability to neutralize recombinant human (rh) IL-13Rα_1 bioactivity. This antibody shows less than 5% cross-reactivity with rhIL-13Rα_2, recombinant mouse (rm) IL-13Rα_1, rhIL-5Rα, rhIL-5Rβ, rhIL-4R, and rhIL-9R. Anti-human IL-13Rα_2 antibody has been selected for its ability to block receptor-ligand interaction. This antibody shows approximately a 5% cross-reactivity with rmIL-13Rα_2. Recombinant mouse IL-13Rα_2/Fc chimera has been shown to bind IL-13 with high affinity and is a potent IL-13 antagonist [19, 20].

The culture supernatants from each well were collected by adding PBS (5 ml) to the apical side of the inserts. The concentration of total protein was measured in each samples of the culture supernatants as described by Lowry et al [17]. We corrected the volume of sample to 50 μl by adding a lysis buffer [RIPA buffer; 50 mM Tris-HCl, 0.15 M sodium chloride, 0.1% sodium dodecylsulfate, 1% sodium deoxycholate, 1 mM phenylmethylsulfonylfluoride (Wako), 1% Triton X-100 (Bio-Rad Laboratories, Calif., USA) and protease inhibitor cocktail (Nakalai Tesque, Kyoto, Japan)] according to the protein contents of supernatants. Briefly, 96-well plates (Becton Dickinson Labware, Franklin Lakes, USA) were coated with cell supernatant samples (50 μl) and bicarbonate-carbonate buffer (50 μl). After washing with 0.05% Tween-PBS buffer (PBS containing 0.1% Tween20), MUC5AC monoclonal antibody (Lab Vision, Fremont, Calif., USA) was added and the plate was incubated for 2 hours at room temperature. After washing, horseradish-peroxidase-conjugated rabbit anti-mouse immunoglobulins (DAKO, Glustrup, Denmark) was added and the plate was incubated for 1 hour at room temperature. After washing, 3, 3', 5, 5'-tetramethylbenzine peroxidase solution was added and the plate incubated at room temperature. The reaction was stopped with 1 M H$_3$PO$_4$ and the absorbance was measured at 450 nm with MICROPLATE READER (Model 550, Bio-Rad Laboratories, CA), the data were analyzed using the Micrplate Manager III (Bio-Rad). The data were expressed as the % above the control vehicle (PBS) as previously described [18]

Western blot analysis

A Western blot analysis was performed as previously described [9]. Briefly, for IL-13Rα_2 protein, after the NHBECs were reached confluence (day 0), the cells were incubated with medium containing 10 ng/ml IL-13. The culture supernatants were collected by adding PBS (5 ml) to the apical side of the inserts, and the cell lysates were denatured and then were resolved. In addition, we immunoblotted the recombinant human soluble IL-13Rα_2/Fc (rsIL-13Rα_2, R&D Systems, Inc) (100 ng) as positive loading control sample.

Likewise, for STAT6 and the phosphorylated, active form of STAT6 (p-STAT6) [11], the cells were incubated with medium containing IL-13 (10 ng/ml), IL-13 (10 ng/ml) plus anti-IL-13Rα_1 antibody (10 µg/ml)(R&D Systems, Inc, Minneapolis, USA), IL-13 (10 ng/ml) plus rsIL-13 Rα_2 (4 µg/ml), IL-4 (1 ng/ml), IL-4 (1 ng/ml) plus rsIL-13 Rα_2 (4 µg/ml) or vehicle (PBS) for 14 days. Anti-Phospho-STAT6 (Y641) antibody was raised against a synthetic phosphopeptide containing the phosphorylated tyrosine at position 641 of human STAT6. This antibody detects human STAT6 phosphorylated at Y641 and does not recognize STAT6 unphosphorylated at Y641 (the manufacturer's instructions, R&D Systems, Inc).

The cell lysates were denatured and then were resolved. Thereafter, the samples were transferred to PVDF membranes. For IL-13Rα_2 protein, the membranes were then blocked with 0.1% Tween-PBS buffer (PBS containing 0.1% Tween20) and incubated with mouse anti human IL-13sRα_2 (4.0 µg/ml) (1:1000) (R&D systems, Inc). The membranes were then incubated with peroxidase conjugated Rabbit Anti-Mouse Immunoglobulins (DAKO) (1:500). The spots were developed with 0.02% 3, 3' diaminobenzidine tetrahydrochloride (Wako) containing 4.5 x 10^{-3} % hydrogen peroxide. For STAT6 and the p-STAT6, the membranes were then blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween 20 and incubated with either monoclonal anti-human/mouse STAT6 antibody (R&D Systems, Inc) or affinity-purified rabbit anti-phospho-STAT6 (Y641) antibody (R&D Systems). The membranes were then incubated with HRP-conjugated goat anti-mouse IgG (R&D Systems, Inc) and detected with WesternGlo Chemiluminescent Detection Reagent (R&D Systems, Inc).

Statistical analysis

 All data are expressed as the means±SEM. The data distribution of the variables in the various groups was first assessed with Bartlett's test. When the data for the variables showed a normal distribution, they were compared with a one-way ANOVA, followed by multiple comparisons with the Tukey-Kramer method. When the data for the variables did not show a normal distribution, the variables were compared with the Kruskal-Wallis test, followed by multiple comparisons among groups with the nonparametric Tukey-Kramer method. The changes in the expression of MUC5AC gene, IL-13Rα_2 gene and IL-13Rα_2 protein in the NHBECs cultured with IL-13 was analyzed by a two-way analysis of variance (ANOVA) and the comparison of each point from day 0 was performed by the pair t-test. The comparison among the groups at specific times was analyzed by the pair t-test. Significance was determined to exist at a level of $p<0.05$ (two-tailed test). In the present study, number of data refers to the number of patients from whom bronchial epithelial cells were used.

Results

Time course of IL-13 induced goblet cell hyperplasia in cultured NHBECs

The number of PAS-positive epithelial cells, goblet cells and MUC5AC-positive cells increased in the presence of IL-13 (10 ng/ml) for 7 days (Table 2 and Fig. 1D, 1J) and for 14 days (Table 2 and Fig. 1E, 1K) compared with that in the presence of vehicle of IL-13 (PBS) (Table 2 and Fig. 1A, 1B, 1G, 1H). In contrast, the number of PAS-positive epithelial cells, goblet cells and MUC5AC-positive cells in the presence of IL-13 (10 ng/ml) for 21 days (Table 2 and Fig. 1F, 1L) did not differ from that in the presence of vehicle of IL-13 (PBS) (Table 2 and Fig. 1C, 1I).

Effects of anti-IL-13R antibodies and IL-13Rα₂ on IL-13-induced goblet cell hyperplasia.

Anti-IL-13Rα₁ antibody (10 μg/ml) decreased the number of PAS-positive epithelial cells, goblet cells and MUC5AC-positive cells in the presence of IL-13 for 14 days (Fig. 2B, 2J and Fig. 3A-3C). In contrast, anti-IL-13Rα₂ antibody (2.5 μg/ml) increased the number of PAS-positive epithelial cells, goblet cells and MUC5AC-positive cells in the presence of IL-13 for 14 days (Fig. 2D, 2L and Fig. 3A-3C). IgG₁ isotype-matched control antibody (10 μg/ml) did not change the number of PAS-positive epithelial cells, goblet cells and MUC5AC-positive cells in the presence of IL-13 for 14 days (Fig. 2F, 2N and Fig. 3A-3C). Furthermore, recombinant human soluble IL-13 Rα₂/Fc (rsIL-13Rα₂) decreased the number of PAS-positive epithelial cells, goblet cells and MUC5AC-positive cells in the presence of IL-13 for 14 days (Fig. 2G, 2O) dose-dependently (Fig. 3A-3C), while rsIL-13Rα₂ alone did not affect the number of these cells (Fig. 2H, 2P and Fig. 3A-3C). The number of total cells increased in the presence of IL-13 (10 μg/ml), and anti-IL-13Rα₁ antibody and rsIL-13Rα₂ decreased the number of total cells induced by IL-13 (Fig. 3D). Anti-IL-13Rα₂ antibody did not change the number of total cells induced by IL-13 (Fig. 3D).

Otherwise, the number of PAS-positive cells, goblet cells and MUC5AC-positive cells increased in the presence of IL-4 (1 ng/ml) for 14 days (Fig. 3A-3C and Fig. 4A, 4C) in comparison to that in the presence of vehicle of IL-4 (PBS) (Fig. 1B, 1H and Fig. 3A-3C). RsIL-13Rα₂ (4 μg/ml) did not decreased the number of PAS-positive epithelial cells, goblet cells and MUC5AC-positive cells in the presence of IL-4 for 14 days (Fig. 3A-3C and Fig. 4B, 4D). None of the cells were

positive after incubation with IL-13 or IL-4 without the 1st antibody (Fig. 2Q and 4E).

Effects of anti-IL-13Rα$_1$ antibodies and IL-13Rα$_2$ on IL-13-induced MUC5AC protein secretion.

MUC5AC protein concentrations in supernatant significantly increased in the presence of IL-13 (10 ng/ml) or IL-4 (1 ng/ml) for 14 days compared with that in the presence of control vehicle (PBS) (Fig. 5). Therefore, the MUC5AC protein secretion in NHBECs was induced after adding IL-13 or IL-4. Anti-IL-13Rα$_1$ antibody (10 μg/ml) or rsIL-13Rα$_2$ (4 μg/ml) significantly decreased the MUC5AC protein concentrations in supernatant in the presence of IL-13 for 14 days (Fig. 5). In contrast, rsIL-13Rα$_2$ (4 μg/ml) did not reduce the MUC5AC protein concentration in supernatants in the presence of IL-4 for 14 days (Fig. 5).

MUC5AC and IL-13Rα$_2$ mRNA expression by IL-13

No expression of MUC5AC and only a small amount of expression of IL-13Rα$_2$ mRNA was observed before adding IL-13 (day 0) (Fig. 6A, 6B). The amount of MUC5AC mRNA expression significantly increased from day 0 to day 7 after treatment with IL-13 (10 ng/ml), and returned to baseline levels at day 21 (Fig. 6A). The IL-13Rα$_2$ mRNA expression increased from day 0 to day 7 after adding IL-13 (10 ng/ml). Increased IL-13Rα$_2$ mRNA expression was also observed 21 days after adding IL-13 (Fig. 6B).

Effects of anti-IL-13R antibodies and IL-13Rα$_2$ on MUC5AC mRNA expression

Anti-IL-13Rα$_1$antibody (10 μg/ml) decreased the MUC5AC mRNA expression induced by IL-13 (10 ng/ml) for 7 days (Fig. 7A). In contrast, anti-IL-13Rα$_2$ antibody (2.5 μg/ml) significantly increased the MUC5AC mRNA expression in the presence of IL-13 (10 ng/ml) for 7 days (Fig. 7B). IgG$_1$ isotype-matched control antibody (10 μg/ml) did not change the MUC5AC mRNA expression in the presence of IL-13 or IL-4 for 7 days (Fig. 7A and 7B). On the other hand, rsIL-13 Rα$_2$ dose-dependently decreased the MUC5AC mRNA expression induced by IL-13 (10 ng/ml) for 7 days (Fig. 7C). The amount of MUC5AC mRNA expression significantly increased in the presence of IL-4 (0.1, 1 or 10 ng/ml) for 7 days (Fig. 7D). 1 ng/ml of IL-4 was the optimal concentration for MUC5AC mRNA expression in this study (Fig. 7D). RsIL-13Rα$_2$ (4 μg/ml) did not change the MUC5AC mRNA expression induced by IL-4 (1 ng/ml) (Fig. 7D).

Expression of membranous bound form expression of IL-13Rα₂

Immunohistochemical staining revealed that weak staining of IL-13Rα₂ protein was observed 14 days after adding vehicle of IL-13 (PBS) (Fig. 8A). In contrast, the potency of IL-13Rα₂ protein staining increased in NHBECs 14 days after adding IL-13 (10ng/ml) (Fig. 8B) with strong staining of IL-13Rα₂ protein in some cells. None of the cells were positive after incubation with IL-13 without the 1st antibody (Fig. 8C).

Soluble form of IL-13Rα₂ protein expression by IL-13

In the cell supernatants before adding IL-13 (day 0), a small amount of the soluble form of IL-13Rα₂ protein was detected (Fig. 9A), and the actual contents of IL-13 Rα₂ protein in supernatants were 132.4 ± 45.4 pg (n=4) and the total protein contents were 1.96 ± 0.49 mg (n=4) in each insert. The concentration of the soluble form of IL-13Rα₂ protein in the supernatants significantly increased between day 0 and day 14 after adding IL-13 (10 ng/ml), and continuous increases were observed 21 days after adding IL-13 (Fig. 9A). The concentrations of the soluble form of IL-13Rα₂ protein in supernatants 14 days after adding vehicle of IL-13 (PBS) did not differ from those before adding IL-13 (data not shown).

In the cell lysates before adding IL-13 (day 0), significant amount of IL-13Rα₂ protein was detected (Fig. 9B), and the actual contents of IL-13Rα₂ protein in cell lysates were 173.4 ± 12.5 pg (n=4) and the total protein contents were 2.91 ± 0.85 mg (n=4) in each insert. The amount of IL-13Rα₂ protein in cell lysates 7 days, 14 days and 21 days after adding IL-13 (10 ng/ml) did not differ from that before adding IL-13 (day 0) (Fig. 9B), and that 21 days after adding vehicle of IL-13 (PBS) (data not shown).

Western blot analysis of IL-13Rα₂ protein expression by IL-13

A Western blot analysis shows a small amount of IL-13Rα₂ protein expression in the supernatants of NHBECs before adding IL-13 (day 0) (Fig. 9C). IL-13Rα₂ protein in the supernatants increased from 7 days to 21 days after adding IL-13 (10 ng/ml) (Fig. 9C). IL-13Rα₂ protein in the cell lysates was also observed 14 days after adding IL-13 (Fig. 9C). IL-13Rα₂ protein in the supernatants and cell lysates of NHBECs showed the same bands as that of rsIL-13Rα₂ (100 ng) loaded as a positive control (Fig. 9C).

Western blot analysis of STAT6 and p-STAT6

A Western blot analysis of NHBECs cell lysates showed that STAT expression was observed in the cells 14 days after exposure to vehicle of IL-13 (PBS, Fig. 10A, lane 1), but treatment with IL-13 (10 ng/ml) and IL-4 (1 ng/ml) did not increase the STAT expression (Fig. 10A).

On the other hand, in the cells at 14 days after exposure to vehicle of IL-13 (PBS, Fig. 10B, lane 1), activated STAT (p-STAT) expression was not observed. In contrast, treatment with IL-13 (10 ng/ml) increased the p-STAT expression (Fig. 10B, lane 2). Furthermore, the expression of IL-13-dependent p-STAT decreased by anti-IL-13Rα_1 antibody (10 µg/ml, Fig. 10B, lane 3) and rsIL-13Rα_2 (4 µg/ml, Fig. 10B, lane 4). Treatment with IL-4 (1 ng/ml, Fig. 10B, lane 5) also increased the p-STAT expression, while rsIL-13Rα_2 (4 µg/ml) did not decrease the IL-4-dependent p-STAT expression (Fig. 10B, lane 6).

Discussion

In a present study, we showed that the number of PAS-positive epithelial cells, goblet cells and MUC5AC-positive cells increased in the presence of IL-13 in normal human bronchial epithelial cell (NHBEC) layers cultured with an air-liquid-interface (ALI). In addition, we showed that the MUC5AC protein secretion was induced in the presence of IL-13 in NHBECs. The MUC5AC mRNA expression increased at 7 days after adding IL-13, and then returned to baseline levels at 21 days. Anti-IL-13Rα_1 antibody reduced the number of PAS-positive epithelial cells, goblet cells and MUC5AC-positive cells, and reduced both the protein secretion and mRNA expression of MUC5AC induced by IL-13. These findings suggest that IL-13 induced the development of mucus-producing goblet cell hyperplasia and the MUC5AC protein secretion via IL-13Rα_1, as shown previously [9].

On the other hand, anti-IL-13Rα_2 antibody increased the number of PAS-positive epithelial cells, goblet cells and MUC5AC-positive cells, and MUC5AC mRNA expression in the presence of IL-13. The expression of IL-13Rα_2 mRNA increased from 7 days and continuous increases in IL-13Rα_2 mRNA were observed at 21 days. Immunohistochemical staining revealed that IL-13Rα_2 protein increased in NHBECs 14 days after adding IL-13. The concentrations of the soluble form of IL-13Rα_2 in supernatants significantly increased 7 days after adding IL-13, and continuous increases were observed 21 days. In contrast, the amount of IL-13Rα_2 protein in cell lysates 21 days after adding IL-13 did not differ from that before IL-13 exposure. Furthermore, IL-13Rα_2 reduced the number of PAS-positive cells, goblet cells and MUC5AC-positive cells, and reduced both the protein secretion and mRNA expression of MUC5AC induced by IL-13. These findings suggest that the soluble form of IL-13Rα_2 may modulate goblet cell hyperplasia and mucus secretion by IL-13 in NHBECs.

The goblet cells with secretory granules stained clearly with Periodic Acid-Schiff (PAS) staining method. On the other hand, there were also surface cells, which did not have any goblet cell granules but were still stained with PAS, PAS-positve-but-not-goblet cells. The reason is uncertain. However, well differentiated cells might become goblet cells stained with PAS, while poorly differentiated cells did not become goblet cells, but were stained with PAS. We identified these cells as PAS-positive-but-not-goblet cells, in the present study. Mucins stained with PAS staining method are reported to be composed of various

mucins as well as MUC5AC [21]. Therefore, mucins other than MUC5AC might also be stained with PAS staining.

Differentiated cultures not stimulated with IL-13 show no MUC5AC staining in this study. This might be common features of this culture system. Although the reason is uncertain, the differences in the passage numbers, culture conditions and species of growth factors might be associated with the differences in MUC5AC staining as reported by Mata et al. [22].

An immunohistochemical analysis demonstrated that a membranous-bound form of IL-13Rα_2 protein is induced in airway epithelial cells by various stimuli including IL-4 and IL-13 [23, 24, 25, 26]. Induction of IL-13Rα_2 protein by IL-13 observed in the present study is consistent with the previous reports [11, 25]. However, it remains uncertain which form of IL-13Rα_2 acts as the decoy receptor. Based on an immunohistochemical analysis, we showed the expression of IL-13Rα_2 protein increased in the cytoplasm of cultured NHBECs after exposure to IL-13 for 14 days. Furthermore, the concentrations of the soluble form of IL-13Rα_2 in supernatants increased 7 days after adding IL-13. In contrast, the amount of IL-13Rα_2 protein in the cell lysates, which has been reported to be related to the expression of a membrane-bound form of IL-13Rα_2 protein [11, 27], did not increase after IL-13 exposure. The recombinant IL-13Rα_2 and anti-IL-13Rα_1 antibody reduced the number of PAS-positive epithelial cells, goblet cells and MUC5AC-positive cells, and reduced both the protein secretion and mRNA expression of MUC5AC induced by IL-13. These inhibitory effects of IL-13R on cell functions are consistent with those of previous reports on IL-13 signaling in airway epithelial cells [11, 25] and hepatic fibrosis [24]. A soluble form of IL-13Rα_2 protein might be associated with the down-regulation of goblet cell hyperplasia, MUC5AC expression and MUC5AC protein secretion.

An immunohistochemical analysis has been suggested to demonstrate the expression of a membrane-bound form of IL-13Rα_2 protein [23, 24, 25, 26]. In the present study, an immunohistochemical analysis also demonstrated increases in the IL-13Rα_2 protein expression in the cells after treatment with IL-13, while IL-13 did not increase IL-13 Rα_2 protein expression in cell lysates. In contrast, IL-13 increased concentration of a soluble form of IL-13Rα_2. These findings suggest that IL-13 increased the expression of a soluble form of IL-13Rα_2 protein. On the other hand, the expression of a membrane-bound form of IL-13Rα_2 protein might be increased by IL-13, while the protein turnover might also increase.

We also showed that the number of PAS-positive cells, goblet cells and

MUC5AC-positive cells increased in the presence of IL-4 in NHBECs cultured with ALI. Furthermore, we showed that the MUC5AC protein secretion was induced in the presence of IL-4 in NHBECs. The MUC5AC mRNA expression increased at 7 days after adding IL-4. However, IL-13Rα_2 did not change the number of PAS-positive cells, goblet cells and MUC5AC-positive cells, the MUC5AC protein secretion and the MUC5AC mRNA expression induced by IL-4. These effects of IL-13R on cell function are consistent with those of previous reports on IL-4 signaling in vitro [28].

STAT6 is well known to be a critical transcriptional factor for IL-13 and IL-4 signaling [29, 30]. A previous report [11] suggested that IL-13 induces the expression of IL-13Rα_2 in bronchial epithelial cells, which was STAT6-dependent. A recent report suggested the role of IL-13Rα_2 and TGF-β_1 in the lung fibrosis induced by IL-13 [31]. In the present study, we confirmed that IL-13 activated (phosphorylated) STAT6 which is inhibited by the anti-IL-13Rα_1 antibody and the recombinant IL-13Rα_2 in NHBECs. In contrast, we showed that IL-4 activated (phosphorylated) STAT6 which is not inhibited by the recombinant IL-13Rα_2 in NHBECs.

Soluble IL-13Rα_2 in the serum was reported to act as an inhibitory protein regulating IL-13 responses [32]. A recombinant IL-13Rα_2 protein was capable of binding and neutralizing IL-13 [19] and attenuated airway hyperresponsiveness in mice [33]. Another previous study in vivo reported that recombinant IL-13Ra$_2$ administration inhibited the late phase response in the OVA challenge-induced airway hyperresponsiveness (AHR) as well as mucus hypersecretion and inflammatory change in lung tissue [34]. In the present study, we demonstrated that a rsIL-13Rα_2 attenuated goblet cell hyperplasia, MUC5AC protein secretion and MUC5AC mRNA expression in NHBECs. Therefore, soluble cytokine receptors, including IL-13, may be important regulators of inflammation and may down-regulate cytokine signaling and responses [35], as well as goblet cell hyperplasia.

The findings in the present study suggest that IL-13Rα_2 is expressed in normal human bronchial epithelial cells, and a soluble form of IL-13Rα_2 may play an important role in the modulation of goblet cell hyperplasia and mucus overexpression induced by IL-13. IL-13Rα_2 may therefore be a therapeutic target molecule for airway hypersecretion in bronchial asthma.

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Table 1.

A; Accession numbers of the genes investigated and the primer sequences used for real-time quantitative PCR

Gene	Accession number		Primer
MUC5AC	AF015221	Forward	5'-TGGAACCACGATGACAGCCT-3'
		Reverse	5'-GCTCCTCACAGATGCCAAAG-3'
IL13Rα$_2$	NM_000640	Forward	5'-AGTTCAGGATATGGATTGC-3'
		Reverse	5'-TAACTGGATCTGATAGGC-3'
GAPDH	M33197	Forward	5'-TGAACGGGAAGCTCACTGG-3'
		Reverse	5'-TCCACCACCCTGTTGCTGTA- 3'

B; Hybridization probes used for real-time quantitative PCR

Gene		Hybridization Probe
MUC5AC	Flu	5'-CCAAATACGCCAACAAGACCTGTGGG-3'
	LC	5'-TCTGTGGGGACTTCAACGGGATGC-3'
IL13Rα$_2$	Flu	5'-GCTTGGATCATGCATTACAGTGTGTTGATTACA-3'
	LC	5'-TCTGTGGGGACTTCAACGGGATGC-3'
GAPDH	Flu	5'-TTCAACAGCGACACCCACTCCTCCA-3'
	LC	5'-CTTTGACGCTGGGGCTGGCATTG- 3'

Table 2.

Effect of IL-13 on the cultured NHBECs

Agents	Incubation time (days)	Total cells	PAS-positive cells	Goblet cells	MUC5AC-positive cells
PBS	7	28.3±0.7	1.7±0.6	0.0±0.0	0.0±0.0
	14	29.5±0.9	3.7±0.6	0.2±0.1	0.1±0.1
	21	28.7±1.6	2.2±0.6	0.1±0.2	0.1±0.1
IL-13	7	40.3±0.5*	28.7±0.9***	12.3±0.9***	27.0±1.1***
	14	40.3±0.7*	27.7±0.5***	19.5±1.2***	24.3±0.8***
	21	33.0±1.9	3.5±0.4	0.1±0.1	0.5±0.2

Values are the means±SEM from 6 samples. *p<0.05, ***p<0.001, vs. vehicle of IL-13 (PBS).

Figure Legends

Figure 1.

Periodic acid-Schiff's (PAS) staining (A-F) and MUC5AC immunohistochemical staining (G-L) of normal human bronchial epithelial cells (NHBECs) incubated with IL-13 (10 ng/ml) (D-F, J-L) or vehicle (PBS) (A-C, G-I) for either 7 (A, D, G, J), 14 (B, E, H, K), or 21 days (C, F, I, L) at the air-liquid interface. White arrowheads show PAS-positive goblet cells (D, E). Black arrowheads show MUC5AC-positive cells (J, K). The goblet cells with secretory granules stained clearly with Periodic Acid-Schiff (PAS) staining method. On the other hand, there were also surface cells which did not have any goblet cell granules but were stained with PAS, PAS-positve-but-not-goblet cells. Bar=100 μm.

Figure 2.

Periodic acid-Schiff's (PAS) staining (A-H) and MUC5AC immunohistochemical staining (I-P) of normal human bronchial epithelial cells (NHBECs) incubated at the air-liquid interface with IL-13 (10 ng/ml) (A, B, D, F, G, I, J, L, N, O, Q) or vehicle (PBS) (C, E, H, K, M, P) for 14 days in the presence of either anti-IL-13Rα$_1$ antibody (10 μg/ml) (B, C, J, K), anti-IL-13Rα$_2$ antibody (2.5 μg/ml) (D, E, L, M), anti-IgG$_1$ isotype control antibody (10 μg/ml) (F, N) or recombinant human soluble IL-13Rα$_2$/Fc (rsIL-13Rα$_2$, 4 μg/ml) (G, H, O, P). None of the cells were positive after treatment with IL-13 (10 ng/ml) in the absence of the 1st antibody (Q). White arrowheads show PAS-positive goblet cells (A, D, F). Black arrowheads show MUC5AC-positive cells (I, L, N, O). The goblet cells with secretory granules stained clearly with Periodic Acid-Schiff (PAS) staining method. On the other hand, there were also surface cells which did not have any goblet cell granules but were stained with PAS, PAS-positve-but-not-goblet cells. Bar=100 μm.

Figure 3.

The number of periodic acid-Schiff's (PAS) staining positive cells (A, PAS-positive cells), goblet cells (B), MUC5AC staining positive cells (C, MUC5AC-positive cells) and total cells (D) of normal human bronchial epithelial cells (NHBECs) incubated with either IL-13 (10 ng/ml), IL-4 (1 ng/ml) or vehicle (PBS) for 14 days at the air-liquid interface, in the presence of either anti-IL-13Rα$_1$ antibody (10 μg/ml), anti-IL-13Rα$_2$ antibody (2.5 μg/ml), anti-IgG$_1$ isotype control antibody (10 μg/ml) or recombinant human soluble IL-13Rα$_2$/Fc (rsIL-13Rα$_2$, 0.04, 0.4, or 4

μg/ml). <u>Results are the means ± SEM from 5 different bronchi.</u> Significant differences from PBS alone are indicated by *p<0.05, **p<0.01, and ***p<0.001. Significant differences from IL-13 alone are indicated by +p<0.05, ++p<0.01, and +++p<0.001.

Figure 4.

Periodic acid-Schiff's (PAS) staining (<u>A, B</u>) and MUC5AC immunohistochemical staining (<u>C-E</u>) of normal human bronchial epithelial cells (NHBECs) incubated at the air-liquid interface with IL-4 (1 ng/ml) (<u>A-E</u>) for 14 days in the presence of recombinant human soluble IL-13Rα$_2$/Fc (rsIL-13Rα$_2$, 4 μg/ml) (<u>B, D</u>). None of the cells were positive after treatment with IL-13 (10 ng/ml) in the absence of the 1st antibody (<u>E</u>). <u>White arrowheads show PAS-positive goblet cells (A, B). Black arrowheads show MUC5AC-positive cells (C, D).</u> Bar=100 μm.

Figure 5.

<u>The MUC5AC protein concentrations in supernatants of NHBECs treated with IL-13 (10 ng/ml) or IL-4 (1 ng/ml) in the presence or absence of either anti-IL-13Rα$_1$ antibody (10 μg/ml) or recombinant human soluble IL-13Rα$_2$/Fc (rsIL-13Rα$_2$, 4 μg/ml) for 14 days. Data are expressed as % above control vehicle (PBS). The results are the mean ± SEM from 3 different bronchi. Significant differences from PBS alone are indicated by *p<0.05. Significant differences from IL-13 alone are indicated by $^+$p<0.05.</u>

Figure 6.

A and B: Time course of mRNA expression of MUC5AC (A) and IL-13Rα$_2$ (B) in normal human bronchial epithelial cells (NHBECs) cultured with IL-13 (10 ng/ml). The results are the mean ± SEM from 14 <u>different bronchi.</u> Significant differences from values on day 0 are indicated by * p<0.05, **p<0.02, ***p<0.005.

<u>Figure 7</u>

A and B: The amount of MUC5AC mRNA expression in NHBECs incubated with IL-13 (10 ng/ml) or vehicle of IL-13 (PBS) in the presence of anti-IL-13Rα$_1$ antibody (10 μg/ml) (A), anti-IL-13Rα$_2$ antibody (2.5 μg/ml) (B), <u>anti-IgG$_1$ isotype control antibody (10 μg/ml)</u> or vehicle of antibodies (PBS) for 7 days. The results are the mean ± SEM from 5 <u>different bronchi.</u> Significant differences from the vehicle of IL-13 (PBS) (Control) are indicated by *p<0.01. Significant differences

from IL-13 alone are indicated by +p<0.05.

C: The amount of MUC5AC mRNA expression in NHBECs incubated with IL-13 (10 ng/ml) or vehicle of IL-13 (PBS) in the presence of recombinant human soluble IL-13Rα$_2$/Fc (rsIL-13Rα$_2$, 0.04, 0.4 or 4 µg/ml) or vehicle of IL-13Rα$_2$ (PBS) for 7 days. The results are the mean ± SEM from 5 different bronchi. Significant differences from the vehicle of IL-13 (PBS) (Control) are indicated by *p<0.01. Significant differences from IL-13 alone are indicated by +p<0.05 and ++p<0.01.

D: The amount of MUC5AC mRNA expression in NHBECs incubated with IL-4 (10, 1or 0.1 ng/ml) or vehicle of IL-4 (PBS) in the presence of rsIL-13Rα$_2$ (4 µg/ml) or vehicle of IL-13Rα$_2$ (PBS) for 7 days. The results are the mean ± SEM from 5 different bronchi. Significant differences from the vehicle of IL-4 (PBS) (Control) are indicated by **p<0.01 and *p<0.05.

Figure 8.

Immunohistochemical staining of NHBECs for IL-13Rα$_2$ proteins after exposure to PBS (A, vehicle of IL-13) or IL-13 (10 ng/ml) (B, C) for 14 days in the presence (A, B) or absence (C) of the first antibody. Weak staining of IL-13Rα$_2$ protein (white arrowheads) was observed 14 days after adding the vehicle of IL-13 (PBS) (A). In contrast, the potency of IL-13Rα$_2$ protein staining increased in NHBECs 14 days after adding IL-13 (10ng/ml) (B) with strong staining of IL-13Rα$_2$ protein in some cells (black arrowheads). Arrows shows goblet cells (B,C). Bar = 100 µm.

Figure 9.

A: Time course of a soluble form of IL-13Rα$_2$ proteins concentrations in supernatants of NHBE cells after adding IL-13 (10 ng/ml). The results are the mean ± SEM from 4 different bronchi. Significant differences from values on day 0 are indicated by *p<0.05.

B: Time course of IL-13Rα$_2$ proteins in lysates of NHBECs after adding IL-13 (10 ng/ml). The results are the mean ± SEM from 4 different bronchi.

C: A Western blot analysis of IL-13Rα$_2$ protein in the culture supernatants (lane 1-4) and the cell lysates (lane 6) of NHBECs incubated with IL-13 (10 ng/ml) for 21 days. Lanes shows samples before (day 0, lane 1), and 7 days (day 7, lane 2), 14 days (day 14, lane 3 and lane 6) and 21 days (day 21, lane 4) after adding IL-13. The recombinant human soluble IL-13Rα$_2$/Fc (rsIL-13Rα$_2$, 100 ng) is immunoblotted as positive loading sample (lane 5). The arrow (56kDa) shows IL-13Rα$_2$. Data are representative of three different experiments.

Figure 10.

A Western blot analysis of STAT6 (A) and the phosphorylated, active form of STAT6 (p-STAT6) (B) in the cell lysates of NHBECs incubated with IL-13 (10 ng/ml) or IL-4 (1 ng/ml) for 14 days. Lanes show the data from samples treated with either the vehicle of IL-13 or IL-4 (PBS) (lane 1), IL-13 (10 ng/ml) plus the vehicle of antibody (PBS) (lane 2), IL-13 plus anti-IL-13Rα_1 antibody (10 μg/ml) (lane 3), IL-13 plus recombinant human soluble IL-13Rα_2/Fc (rsIL-13Rα_2, 4 ng/ml) (lane 4), IL-4 (1 ng/ml) plus the vehicle of antibody (PBS) (lane 5), or with IL-4 (1 ng/ml) plus rsIL-13Rα_2 (4 ng/ml) (lane 6). The arrows show STAT6 (a) and p-STAT6 (b) (100～120kDa). Data are representative of three different experiments.