Herstellung	HE1		tj	Lektorat:	pst	Datum:	13.12.05
File name (6 Digits)		RES529					
File extension (T1, S1)			Template_ZU				
Graphics		figures:	9	tables:	1		

Short title/Running head@0p	IL-13 Induces CLCA1 and MUC5AC in Bronchial Epithelial Cells		
Section title (Original Article, Review Article, Case Report)@2l	Basic Science Investigations		
Citation line	Journal title: Respiration	DOI: 10.1159/0000	XXXXX
Article title {ql}	Relationship between Calcium-Activated Chloride Channel 1 and MUC5AC in Goblet Cell Hyperplasia Induced by Interleukin-13 in Human Bronchial Epithelial Cells		
Subtitle			

Author(s){y}@4w@6a	Initials	First name	Surname/Collaboration
	M.	Masanori	Yasuo[a]
	K.	Keisaku	Fujimoto[a]
	T.	Tsuyoshi	Tanabe[a]
	H.	Hironobu	Yaegashi[a]
	K.	Kenji	Tsushima[a]
	K.	Keiichirou	Takasuna[b]
	T.	Takeshi	Koike[c]
	M.	Mutsuo	Yamaya[d]
	T.	Toshio	Nikaido[e]

Author(s) and City	

Institute(s)	
	[a]First Department of Internal Medicine, [b]Second Department of Surgery and [c]Department of Dentistry and Oral Surgery, Shinshu University School of Medicine, Matsumoto, [d]Department of Geriatric and Respiratory Medicine, Tohoku University School of Medicine, Sendai, and [e]Department of Regenerative Medicine, Toyama Medical and Pharmaceutical University, Faculty of Medicine, Toyama, Japan

DivInfo	

Queries to the author	Page 7: Competitive Ct: please explain Ct. Page 10: OmU3 or OUm3 **Please check words marked in green.**

Received/accepted{y}@c	
	Received: June 20, 2005
	Accepted after revision: November 21, 2005
	Published online: ■■■

Name for correspondence{y}	Keisaku Fujimoto
Full address	First Department of Internal Medicine
	Shinshu University School of Medicine
	3-1-1 Asahi, Matsumoto (Japan)
(Tel., Fax, E-Mail)	Tel. +81 263 37 2631, Fax +81 263 36 3722, E-Mail keisaku@@hsp.md.shinshu-u.ac.jp

ISSN number{y}	0025--7931

	res

Key Words
Bronchial epithelial cells
Goblet cell hyperplasia
hCLCA1
Interleukin-13
MUC5AC

Abstract{zy}

Background: Interleukin (IL)-13 has recently been reported as the major T-helper 2 cytokine involved in mucus overproduction and oversecretion in allergic airways. However, the relationship between human calcium-activated chloride channel-1 (hCLCA1) and MUC5AC induced by IL-13 in vitro has not been fully investigated. **Objectives:** The present study examines whether IL-13 induces the expression of hCLCA1 in normal human bronchial epithelial (NHBE) cells. We also investigated the relationship between hCLCA1 and MUC5AC expression and the development of goblet cell hyperplasia (GCH). **Methods:** NHBE cells were isolated from human bronchi, and cultured with an air-liquid interface. hCLCA1 and MUC5AC gene and protein expression, as well as GCH were examined in the cells after exposure to IL-13. **Results:** Incubation with IL-13 for 14 and 21 days increased the total number of epithelial cells, the number of periodic acid-Schiff (PAS)-stained epithelial cells, the number of goblet cells, as well as expression of mRNA and protein of hCLCA1 and MUC5AC. The number of goblet cells with secretory granules also increased after 21 days of incubation with IL-13. Niflumic acid, a chloride channel inhibitor, reduced mRNA expression of hCLCA1 and MUC5AC, and reduced the number of PAS-positive cells after incubation with IL-13. NHBE cells exposed or not to IL-13 expressed IL-13 receptor α_1 (IL-13Rα_1), and an antibody to IL-13 Rα_1 also reduced the number of PAS-positive cells after exposure to IL-13. **Conclusions:** IL-13 might induce the expression of MUC5AC and hCLCA1 gene and protein in well-differentiated NHBE cells. These cells might also differentiate into goblet cells and become hyperplastic.

Introduction

Goblet cell metaplasia and hyperplasia in airways are associated with persistent airway inflammation. These conditions probably represent an innate host defense to enhance mucus clearance and remove harmful stimuli from the lungs [1, 2]. On the other hand, goblet cell hyperplasia (GCH) is a pathophysiological feature of respiratory diseases characterized by mucus overproduction, such as chronic obstructive pulmonary disease (COPD), asthma, cystic fibrosis and bronchiectasis [3]. Mucus hypersecretion induces obstruction of the peripheral airways in patients with asthma [3] and in patients with diffuse panbronchiolitis [4], and is a major cause of death among patients with severe asthma [3].

Interleukin (IL)-13 is the pleiotropic 12-kDa protein product of a gene on chromosome 5 at q31 that is produced in large quantities from activated CD4+ Th2 cells by various antigens [5, 6]. This cytokine plays an important role in airway inflammation in allergic and non-allergic asthma, as well as in COPD [5, 7, 8], and it shares a receptor component and signaling pathway with IL-4 [5, 7]. The administration of IL-13 to the mouse trachea induces eosinophil influx into the airways, GCH with mucus hypersecretion and airway hyperreactivity [7]. IL-13 transgenic mice also develop airway epithelial hypertrophy, mucus hypersecretion and GCH [9]. Furthermore, a study in vitro has shown that IL-13 induces tracheal epithelial cells to differentiate into mature goblet cells with the production of MUC5AC protein in guinea pigs [10]. Atherton et al. [11] reported that IL-13 induces GCH and MUC5AC protein production in normal human bronchial epithelial (NHBE) cells. Their results suggest that IL-13 directly acts on airway epithelial cells to induce GCH.

Chloride channels of airway epithelial cells play an important role in the regulation of mucus production as well as in airway fluid and electrolyte transport [12, 13]. Among a novel family of proteins that mediates Ca^{2+}-activated conductance (Ca^{2+}-activated Cl^- channel family; CLCA family), human CLCA1 (hCLCA1) protein is expressed in the digestive tract including the small intestine, colon and appendix [14]. This protein plays an important role in fluid and electrolyte transport as well as in mucus secretion [14]. Nakanishi et al. [15] have demonstrated that murine *gob-5*, corresponding to hCLCA1, is expressed at high levels and strictly localizes in airway epithelium, especially in mucus-producing cells in mice sensitized with ovalbumin. Furthermore, adenoviral gene transfer

with an antisense *gob-5* construct suppresses the development of airway hyperreactivity and mucus overproduction in ovalbumin-sensitized mice. In contrast, the overexpression of *gob-5* in airway epithelia transfected with a sense *gob-5* construct via an adenoviral vector results in mucus overproduction and airway hyperreactivity. The expression of hCLCA1 is increased in the epithelial cells of patients with asthma [16] and COPD [17] and in sputum from patients with COPD [17]. A gene transfection study by Kuperman et al. [18] showed that mucus production and murine *Muc5ac* and *Gob-5* expression are induced by IL-13 in *Stat6+/–* mice. In this manner, both MUC5AC and hCLCA1 induced by IL-13 would participate in GCH of the airway epithelium. However, the relationships between these two genes and protein expression induced by IL-13 in human airway epithelium remain obscure.

The present study examines whether IL-13 directly induces the expression of hCLCA1 and MUC5AC in cultured NHBE cells and investigates the relationship between GCH and the expression of hCLCA1 and MUC5AC.

Materials and Methods

NHBE Cell Culture

Bronchi were obtained from 20 patients (mean age, 64 ± 3.1 years; range, 35--77 years) who underwent lung resection due to lung cancer or benign lung tumors. The Ethics Committee of the Shinshu University School of Medicine approved the study, and the patients provided written informed consent to participate in all aspects of the investigation. We isolated and cultured NHBE cells as described by Yamaya et al. [19] and Gray et al. [20]. Briefly, bronchi were rinsed in ice-cold sterile phosphate-buffered saline (PBS) to remove mucus and debris, and then incubated at 37°C for 4–6 h in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, Mo., USA) and Ham's F-12 (F-12; Sigma) containing protease (Dispase, GIBCO, Grand Island, N.Y., 0.1 mg/ml) and 1% antibiotic-antimycotic (GIBCO). After the incubation, the medium was aspirated, and DMEM/F-12 containing 1% antibiotic-antimycotic (DMEM/F12 basic medium) was applied to inhibit the enzyme activity. The bronchi were opened longitudinally in ice-cold DMEM/F12 basic medium, rubbed with blunt-tipped tweezers, and then clusters of NHBE cells were dispersed by repeated aspiration using a 10-ml pipette. Suspended NHBE cells were sedimented at 400 *g* for 5 min, and then resuspended in DMEM/F-12 basic medium. After counting using a hemocytometer and estimating viability by trypan blue staining, the NHBE cells were seeded at a density of 5

× 10^3/cm^2. Primary culture and subculture proceeded in LHC-9 (Biofluids, Rockville, Md., USA). Cells were used for experimentation after two subcultures (passage-2 cells), when the number of viable cells reached approximately 1 × 10^7.

NHBE Cell Differentiation

Passage-2 NHBE cells were seeded at a density of 2.0 × 10^5/cm^2 onto polyester inserts (6.5-mm diameter, 0.4-μm pore size and 10-μm thickness; Costar Transwell Clear; Costar, Cambridge, Mass., USA) coated with type IV collagen (Cellgen, Koken, Tokyo, Japan), and then cultured in serum-free DMEM/F12 medium containing ITS-A (1.0%; GIBCO), triiodothyronine (10 ng/ml; ICN, Irvine, Calif., USA), epidermal growth factor (EGF; recombinant human EGF, 0.5 ng/ml; GIBCO), all-trans retinoic acid (10^{-7} *M*; Wako, Osaka, Japan), hydrocortisone (0.5 μg/ml; Wako), bovine serum albumin (2.0 μg/ml; Itoham Foods, Hyogo, Japan), endothelial cell growth supplement (8.34 μg/ml; UBI, Lake Placid, N.Y., USA) and antibiotic-antimycotic (1.0%; GIBCO). Culture medium was added to both the apical and basolateral side of the inserts, and the cells were cultured by immersed feeding [19]. The culture medium was changed every 2 days. Confluence was reached within 5 days and then the cells were cultured using an air-liquid interface [19]. Briefly, the apical medium was removed and cells were nourished only from the basolateral side. The culture medium was changed every 2 days, and the cells were maintained in an incubator under a humidified 5% CO_2 atmosphere at 37°C.

Culture with IL-13

When the cells reached confluence (day 0) as described above, air-liquid interface culture was started. Medium containing IL-13 (0.1, 1, 10 and 100 ng/ml recombinant human IL-13, Biotec, Hamburg, Germany) or vehicle (PBS) was added to the basolateral side of the inserts. The culture medium was changed every 2 days, and cells were incubated for 14 or 21 days (from day 0 to day 14 or day 21) with IL-13 or vehicle (PBS). IL-13 at the concentrations applied here is effective but not toxic [11, 21--23].

Real-Time Quantitative PCR

Sample preparation for real-time-quantitative PCR of cultured NHBE cells was examined as described [24], with some modification. To examine the time course of mRNA expression of hCLCA1 and MUC5AC, RNA was extracted from the cells after 1, 3, 5, 7, 14 or 21 days in the presence of IL-13. The apical side of the cell sheets on Transwell® culture inserts were gently washed three times with PBS. Total RNA from cultured NHBE cells was extracted using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Because the culture insert was too small to contain

1,000 µl of Isogen per sample, firstly 150 µl of Isogen were added to the culture insert and repeatedly pipetted. Thereafter, 150 µl of samples were mixed with 850 µl of Isogen in centrifuge tubes.

Real-time quantitative PCR proceeded as reported [15, 16], with some modification. Briefly, cDNA samples corresponding to 20 ng of total RNA were measured by real-time quantitative PCR using a Perkin-Elmer Applied Biosystems Prism model 7900 sequence detection instrument according to the manufacturer's instructions. Matching primers and TaqMan probes were designed for each of the genes: hCLCA1, ACCTTGACCCTGACTGTCACGT (forward primer), GTCCTTGTTCGTTTTGGAAGTCAC (reverse primer) and CGTCCAATGCTACCCTGCCTCCAATTAC (TaqMan probe; AF039400) [16], and MUC5AC, TACTCCACAGACTGCACCAACTG (forward primer), CGTGTATTGCTTCCCGTCAA (reverse primer) and TGTGCTTGGAGGTGCCCACTTCTCAA (TaqMan probe; AF015521) [16]. The reporter and quencher dyes for all probes were 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA), respectively. Gene expression levels of hCLCA1 and MUC5AC were compared using the competitive (→comparative) Ct (Ct; threshold cycle) method against the GAPDH gene [25].

Histochemical Analysis

GCH in cultured NHBE cells was histologically examined as described [10]. The cells were fixed in 20% formalin neutral buffer, embedded in paraffin and cut into 3-µm slices. Mucin production was confirmed by periodic acid-Schiff (PAS) staining. We compared the differentiation of NHBE cells incubated with vehicle or IL-13 into goblet cells as follows. Goblet cell density was analyzed according to the method described by Atherton et al. [11], with some modification. The numbers of total epithelial cells, PAS-positive cells and goblet cells were measured by counting epithelial nuclei over 250 µm in five random sites per specimen from 6 of 20 serial patients using a light microscope (BX-51; Olympus, Tokyo, Japan) and by photography using a digital camera system (DP-70; Olympus).

Immunohistochemical Analysis

We analyzed hCLCA1, MUC5AC and IL-13Rα_1 subunit expression in cultured NHBE cells immunohistochemically. Human CLCA1 antibody immunostaining proceeded as described [16]. Takeda Chemical Industries provided a rabbit anti-hCLCA1 antibody [15, 16]. Briefly, the N-terminal region of hCLCA1, corresponding to residues 22--124, was produced in *Escherichia coli* AD494 (DE3) as a hybrid protein fused to thioredoxin

using the pET Trx Fusion System 32 (Novagen, Madison, Wisc., USA). The hCLCA1 fusion protein was purified using the His Bind Purification Kit (Novagen). The antibody directed against hCLCA1 was produced in rabbits injected with 0.7 mg of purified fusion protein mixed with Freund's adjuvant (Sigma). The hCLCA1 antibody was produced by binding to hCLCA1, and then purified by protein A and human CLCA4 column chromatography. Purified hCLCA1 antibody specifically recognized hCLCA1 that was detected by Western blotting and immunohistochemistry at about 90 kDa in human small intestine protein and in Chinese hamster ovary cells. The antibody did not react to *Gob-5*, rat *Clca1*, or human CLCA4 expressed in Chinese hamster ovary cells.

For immunohistochemical analysis of hCLCA1, MUC5AC and IL-13Rα_1 subunit expression, cultured NHBE cells on Transwell inserts were fixed in 20% formalin neutral buffer for 8–12 h, embedded in paraffin and sectioned. The sections were incubated with either anti-hCLCA1 antibody diluted 1:200, anti MUC5AC antibody (Lab Vision, Fremont, Calif., USA) diluted 1:600 or anti IL-13Rα_1 antibody (clone 116730; Techne, Minneapolis, Minn., USA) diluted 1: 600 in PBS containing 1% bovine serum albumin overnight at 4°C, and then washed three times with PBS to remove excess primary antibody. Either horseradish-peroxidase-conjugated swine anti-rabbit IgG (1:100) for hCLCA1, horseradish-peroxidase-conjugated rabbit anti-mouse IgG (1:200; Dako, Glostrup, Denmark) for MUC5AC or horseradish-peroxidase-conjugated anti-mouse IgG (1:200/Dako) for IL-13Rα_1 was added, and the sections were incubated for 30 min at room temperature. After 3 PBS washes to remove excess secondary antibody, antigen-antibody complexes were visualized using diaminobenzidine tetrahydrochloride (Wako).

Immunofluorescent Confocal Microscopy Examination

Co-expressed hCLCA1 and MUC5AC in cultured NHBE cells were identified using dual-immunofluorescence techniques [26] and immunofluorescent confocal microscopy. Because mucin granules tended to swell and coalesce in paraffin-embedded fixed specimens [27], specimens were quick-frozen to circumstantially observe cells producing mucus. Cultured NHBE cells incubated with 10 ng/ml IL-13 for 14 days were embedded in Tissue-Tek OCT Compound (Sakura Finetechnical, Tokyo, Japan), rapidly frozen in liquid nitrogen and stored at –80°C.

The frozen samples were cut into 8-μm slices using a cryostat at –20°C, mounted on slides and warmed to room temperature before fixation in acetone at –4°C. The secondary antibodies were Cy3 donkey anti-rabbit IgG (1: 500) for hCLCA1 and FITC goat anti-mouse IgG (1: 200) for MUC5AC (Jackson ImmunoResearch Laboratories, West Grove, Pa., USA). After incubation with the primary and secondary antibodies for 1

h in the dark, the slides were rinsed with PBS three times, and then nuclei were stained with DAPI (SIGMA) for 10 min in the dark. The slides were again rinsed with PBS three times and immersed in Fluorescent Mounting Medium® (Dako, Carpinteria, Calif., USA). Cover slips were applied and the slides were stored at 4°C in the dark. All slides were examined by confocal laser scanning microscopy (TCS SP2 AOBS; Leica, Wetzlar, Germany) within 24 h of staining. Immunostaining was visualized with a blue diode, argon ion, and helium neon laser using 405, 488 and 543 laser lines, respectively.

Transmission Electron Microscopy

Electron-microscopic procedures have been described in detail elsewhere [28]. In brief, cells on porous filters were fixed with glutaraldehyde/osmium tetroxide, dehydrated in a graded series of ethanol, substituted with propylene oxide and embedded in epoxy resin (Oken, Tokyo, Japan). Ultrathin sections were cut on an OUm3 (→OmU3) ultramicrotome (Reichert-Jung, Vienna, Austria) at 0.1 µm thickness and mounted on copper grids (Veco, Eerbeek, The Netherlands). The sections were observed with a JEM-1200EX transmission electron microscope (Jeol, Tokyo, Japan) at an accelerating voltage of 80 kV.

Western Blotting

Western blot analysis proceeded as described [29]. Briefly, NHBE cells were cultured in medium containing 10 ng/ml of IL-13 on polycarbonate for 4, 7 or 14 days. Cultured NHBE cells were resuspended in lysis buffer [RIPA buffer; 50 m*M* Tris-HCl, 0.15 *M* sodium chloride, 0.1% sodium dodecylsulfate, 1% sodium deoxycholate, 1 m*M* phenylmethylsulfonylfluoride (Wako), 1% Triton X-100 (Bio-Rad Laboratories, Calif., USA) and protease inhibitor cocktail (Nakalai Tesque, Kyoto, Japan)], fractured for 10 s using an ultrasonic cell disrupter (Taitec, Saitama, Japan) and then incubated for 5 min. Lysates were centrifuged at 20,000 *g* for 30 min and the supernatant was stored at –80°C. Harvested cell samples were lysed in RIPA buffer at 4°C for 15 min. For MUC5AC, cell lysates containing 50 µg protein, determined using the method of Lowry et al. [30], were denatured in reducing sample buffer and resolved by electrophoresis on 8% polyacrylamide gels for 150 min at 20 mA with α_2-macroglobulin (720 kDa; Sigma) as the molecular weight standard. Thereafter, samples were transferred to PVDF membranes in transfer buffer composed of 10 m*M* N-cyclohexyl-3-aminopropanesulfonic acid (Dojindo, Kumamoto, Japan) and 10% methanol for 90 min at 100 mA. The membranes were then blocked with 1% Tween-PBS and incubated with mouse monoclonal MUC5AC antibody (1:100 Zymed, San Francisco, Calif., USA) for 24 h. For hCLCA1, cell lysates containing

50 µg of protein were denatured in reducing sample buffer and resolved by electrophoresis on 8% polyacrylamide gels for 50 min at 75 mA. Thereafter, samples were transferred to PVDF membranes in transfer buffer composed of 250 m*M* Tris-HCl, 192 m*M* glycine and 20% (v/v) methanol for 120 min at 100 mA. The membranes were then blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h and incubated with anti-rabbit IgG (1 µg/ml) for hCLCA1 (1:2,300) for 24 h. The membranes were then incubated for 1 h with horseradish-peroxidase-conjugated goat anti-mouse IgM and IgG (1:500) to detect the monoclonal MUC5AC antibody, and with goat anti-rabbit IgG (1:1,200) to detect the monoclonal hCLCA1, respectively. Spots were developed with 0.02% 3,3′ diaminobenzidine tetrahydrochloride (Wako) containing 4.5 × 0.001% hydrogen peroxide. β-Actin confirmed protein extraction.

Statistical Analysis

All data are expressed as means ± SEM. A two-way analysis of variance compared MUC5AC and hCLCA1 gene expression in the NHBE cells incubated with IL-13 and vehicle over time. The paired t test compared groups at specific times. One-way analysis of variance and Student's t test compared variables between vehicle- and IL-13-treated groups. Significance was determined at $p < 0.05$ (two-tailed test).

Results

Relationships between IL-13 Concentration and GCH in Cultured NHBE Cells

When cultured using the air-liquid interface, NHBE cells developed a well-differentiated epithelial structure including ciliated, basal and secretory cells as reported previously [11, 19, 20]. The total number of epithelial cells, PAS-positive epithelial cells and goblet cells did not increase in the presence of 0.1 or 1 ng/ml IL-13 or vehicle (PBS) (table 1, fig. 1b). In contrast, the total number of epithelial cells increased in the presence of 10 ng/ml of IL-13 for 14 days (table 1). Likewise, incubation with IL-13 (10 ng/ml) for 14 days significantly increased the number of PAS-positive epithelial cells and goblet cells (table 1, fig. 1c). PAS-positive cells and goblet cells were regularly arranged 14 days after adding IL-13 (10 ng/ml, fig. 1c). Furthermore, the total number of epithelial cells, and the numbers of PAS-positive and goblet cells significantly increased (data not shown), and PAS-positive cells were also regularly arranged 21 days after adding 10 ng/ml IL-13 (fig. 1d). The total number of epithelial cells, and the numbers of PAS-positive and goblet

cells did not differ 14 and 21 days after exposure to 10 ng/ml IL-13 (data not shown). Thus 10 ng/ml of IL-13 induced the development of mucus-producing GCH.

The numbers of PAS-positive cells and goblet cells in the presence of 100 ng/ml of IL-13 also significantly increased (table 1). Furthermore, the total cell number, and the numbers of PAS-positive epithelial cells and goblet cells did not significantly differ after incubation with 100 and 10 ng/ml IL-13 ($p > 0.05$). In contrast, PAS-positive cells were not always regularly arranged at the apical side of the cell layer 14 days after exposure to 100 ng/ml of IL-13 (fig. 1e), and the arrangement was more haphazard with 100 ng/ml than with 10 ng/ml of IL-13 (fig. 1e). These findings indicated that 10 ng/ml of IL-13 were the optimal concentration for differentiation into goblet cells under our experimental conditions.

GCH Induced by IL-13 in Cultured NHBE Cells

Transmission electron microscopy revealed that GCH with secretory granules was induced by incubation with IL-13 (10 ng/ml) for 21 days (fig. 2a). The number of goblet cells with mucin granules was increased in cell sheets incubated with IL-13 (10 ng/ml; fig. 2a) compared with those exposed to vehicle (PBS; fig. 2b). On the other hand, neither vehicle (PBS; fig. 2b) nor lower concentrations of IL-13 (0.1 and 1 ng/ml; data not shown) induced GCH.

Gene Expression of hCLCA1 and MUC5AC Induced by IL-13 (10 ng/ml)

No hCLCA1 mRNA was expressed after 3 days of incubation with IL-13 (10 ng/ml), but it became detectable on day 5, increased thereafter and peaked on day 14 (fig. 3a). The hCLCA1 mRNA expression was also increased after 21 days of exposure to IL-13 (fig. 3a) whereas MUC5AC mRNA was expressed on day 3. The MUC5AC mRNA expression increased from day 3 and peaked on day 14 (fig. 3b). The expression of MUC5AC mRNA was also increased after 21 days of exposure to IL-13 (fig. 3b).

Protein Expression of hCLCA1 and MUC5AC

Immunohistochemical staining revealed that both hCLCA1 and MUC5AC proteins increased in NHBE cells cultured with IL-13 for 14 days at an air-liquid interface (fig. 4). Cells incubated with vehicle (PBS) were not stained with the antibody to hCLCA1 proteins (fig. 4c). In contrast, cells were stained with antibody to MUC5AC (fig. 4e). On the other hand, IL-13 increased the number of goblet cells stained with antibody for MUC5AC (fig. 4f). In contrast, some goblet cells were stained with antibody for hCLCA1 (fig. 4d, arrowheads), whereas others were negative or weakly stained with hCLCA1 (fig.

4d). None of the cells were positive without the first antibody and incubated with IL-13 (fig. 4b) or vehicle (PBS; fig. 4a).

Localization of hCLCA1 and MUC5AC

To obtain more information about the relationship between hCLCA1 and MUC5AC protein expression in goblet cells, we examined the localization of hCLCA1 and MUC5AC proteins using laser confocal microscopy. Both hCLCA1 and MUC5AC proteins were expressed only at goblet cells (fig. 5a, b, d, e). MUC5AC proteins were detected in all goblet cells, whereas hCLCA1 proteins were expressed in some of them. MUC5AC-negative (and hCLCA1-positive) goblet cells were not observed in cells incubated with IL-13. MUC5AC proteins were localized at mucus granules, and hCLCA1 proteins were localized at the circumference of the MUC5AC proteins and surface of goblet cells (fig. 5d, e).

Western Blot Analysis of hCLCA1 and MUC5AC Protein

The expression of hCLCA1 protein was not significant after 4 days of IL-13 (10 ng/ml) exposure (fig. 6a). In contrast, significant amounts of hCLCA1 protein were expressed in the cells after 7 days of exposure, and the expression of hCLCA1 protein increased from day 7 to day 14 of IL-13 (10 ng/ml) exposure (fig. 6a). On the other hand, MUC5AC protein was expressed after 4 days of incubation with IL-13 (10 ng/ml; fig. 6b). Furthermore, MUC5AC protein expression increased with time (from day 4 to day 14) of exposure to IL-13 (10 ng/ml; fig. 6b).

Effects of Chloride Channel Blockers on the Gene Expression of hCLCA1, MUC5AC and GCH

To estimate the activity of the inhibitor of chloride channel transport on the inhibition of IL-13-induced mRNA expression of hCLCA1, MUC5AC and GCH, cells were incubated with niflumic acid (100 μ*M*), a potent inhibitor of chloride secretion [31] (Calbiochem, Darmstadt, Germany). Our preliminary study showed that incubating the cells with niflumic acid (100 μ*M*) for more than 48 h induced damage such as cell detachment. Therefore, cells were incubated with niflumic acid (100 μ*M*) for 48 h from day 12 to day 14. Another study also used the same time of exposure and concentration of this reagent [31]. Niflumic acid (100 μ*M* for 48 h) obviously reduced mRNA expression of MUC5AC and hCLCA1 induced by IL-13 (10 ng/ml). The expression of hCLCA1 mRNA in the cells incubated with niflumic acid (100 μ*M* for 48 h) plus 10 ng/ml IL-13 (1.2 ± 0.1%/GAPDH, n = 3, $p < 0.01$) was significantly lower than that in cells incubated with 10 ng/ml IL-13 alone (15.8 ± 4.7%/GAPDH, n = 3). Likewise, the expression of MUC5AC

mRNA in cells incubated with niflumic acid (100 μ*M* for 48 h) plus 10 ng/ml IL-13 (19.7 ± 2.3%/GAPDH, n = 3, $p < 0.01$) was significantly lower than that in cells treated with 10 ng/ml IL-13 alone (167.5 ± 23.7%/GAPDH, n = 3).

Furthermore, incubation with 10 ng/ml IL-13 significantly increased the number of goblet cells (fig. 7a). In contrast, niflumic acid (100 μ*M* for 48 h) significantly reduced the number of goblet cells induced by IL-13 (fig. 7b).

Effects of Antibody to the IL-13Rα$_1$ Subunit on IL-13-Reduced GCH

Because a heterodimer of the IL-4Rα and IL-13Rα$_1$ subunits is associated with the activity of IL-13 in airway epithelial cells [5], we performed neutralization studies against IL-13Rα$_1$ and IL-4Rα. IL-13 (10 ng/ml) and either anti IL-13Rα$_1$ (10 μg/ml) or anti IL-4Rα (100 ng/ml) [32] antibodies (R & D Systems, Minneapolis, Minn., USA) were incubated with the cells for 14 days. We initially found that 10 μg/ml of anti-IL-13Rα$_1$ reduced the number of goblet cells after exposure to IL-13 (10 ng/ml), but 1 μg/ml of anti IL-13Rα$_1$ did not reduce this number after exposure to IL-13 (10 ng/ml; data not shown). The anti-IL-13Rα$_1$ (fig. 8b) and anti-IL-4Rα (fig. 8c) antibodies reduced the number of goblet cells induced by IL-13 (fig. 8a).

Effects of IL-13 on IL-13 Receptor Expression

The cells were immunohistochemically stained for IL-13Rα$_1$ using monoclonal antibody for IL-13Rα$_1$ [(1:600) clone 116730, Techne]. The preliminary study showed that the antibody diluted at 1:600 resulted in optimal staining for IL-13Rα$_1$ (data not shown). Immunostaining for IL-13Rα$_1$ was positive in the cytoplasm of all epithelial cells induced by IL-13 (10 ng/ml; fig. 9a) and of those treated with vehicle (PBS; fig. 9b). The magnitude of IL-13Rα$_1$ expression in the epithelial cells induced by IL-13 (10 ng/ml) was similar to that in cells incubated with vehicle (PBS; fig. 9a, b). In the absence of the first antibody (anti-IL-13Rα$_1$ antibody), no positive staining for IL-13Rα$_1$ was evident after incubation with IL-13 (10 ng/ml; fig. 9c).

Discussion

The present study showed that human recombinant IL-13 directly induced the gene and protein expression of MUC5AC and hCLCA1 in well-differentiated NHBE cells in vitro, and that these cells also differentiated into goblet cells and became hyperplastic. These conclusions were based on the findings that exposure to IL-13 for 14 days increased the total number of epithelial cells, the numbers of PAS-stained epithelial cells

and goblet cells, and mRNA and protein expression of hCLCA1 and MUC5AC. Numbers of PAS-positive cells and of goblet cells were also increased 21 days after incubation with IL-13. Transmission electron microscopy revealed increased numbers of goblet cells with secretory granules 21 days after exposure to IL-13. Niflumic acid, a chloride channel inhibitor, reduced mRNA expression of hCLCA1 and MUC5AC, and the number of goblet cells after exposure to IL-13. NHBE cells expressed IL-13Rα_1, and an antibody to IL-13 Rα_1 also reduced the number of goblet cells induced by IL-13.

Peak gene induction of hCLCA1 and MUC5AC was evident 14 days after exposure to IL-13. Therefore, gene expression of hCLCA1 and MUC5AC might be induced by IL-13. GCH was evident after 14 and 21 days of exposure to IL-13. Furthermore, the chloride channel inhibitor niflumic acid reduced mRNA expression of hCLCA1 and MUC5AC, and the number of goblet cells after exposure to IL-13. These findings suggest that the gene expression of hCLCA1 and MUC5AC is associated with the protein expression of hCLCA1, MUC5AC and GCH, as described [18].

On the other hand, immunohistochemical analysis showed that hCLCA1 protein was distributed in some goblet cells, while MUC5AC protein was expressed in all goblet cells. Laser confocal microscopy showed that both hCLCA1 and MUC5AC proteins were expressed only at sites of goblet cells. MUC5AC proteins were localized at mucus granules, and hCLCA1 proteins were localized at the circumference of the MUC5AC proteins and goblet cell surface. ~~On the other hand, MUC5AC and hCLCA1proteins were detected in all and in some goblet cells, respectively.~~ Although hCLCA1 is overexpressed in the airway epithelium of asthma patients [16], Robichaud et al. [33] reported that hCLCA1 is not primarily essential for mucus overproduction. Therefore, mechanisms unrelated to hCLCA1 might also be associated with GCH induced by IL-13.

The MUC genes encode the protein backbones of large, viscoelastic, highly glycosylated macromolecules called mucins that comprise the major macromolecular components of airway mucus [2]. Sixteen genes encoding human mucins have been identified, including the dominant MUC5AC gene that is expressed in airway goblet cells [2]. In bronchial biopsy specimens from asthmatics, MUC5AC expression is ~60% increased compared to non-asthmatic individuals [34].

On the other hand, the novel CLCA family of proteins mediates Ca^{2+}-activated Cl conductance [14--16]. The hCLCA family might play an important role in fluid and electrolyte transport, as well as in mucus secretion in the digestive tract and in the mucus overproduction associated with GCH in the airways of patients with asthma and COPD [14--16]. The development of airway hyperresponsiveness is closely associated with

hCLCA1 in the mouse model of asthma [15]. Zhou et al. [31] have demonstrated that expression of the transfected hCLCA1 gene in NCI-H292 cells induces mucin production and that niflumic acid, a blocker of the Cl efflux, inhibits MUC5AC production in these cells. They also demonstrated that niflumic acid reduces airway inflammation, goblet cell metaplasia and mucus overproduction with the upregulation of *mCLCA3* (that corresponds to hCLCA1) in mice intranasally instilled with *Aspergillus fumigatus* extract antigen [31]. However, whether the Th2 cytokines, including IL-13, can directly induce hCLCA1 expression in cultured NHBE cells was unknown, although IL-13 and IL-4 directly induce MUC5AC gene expression and mucus production in cultured airway epithelial cells [11, 35, 36]. Here, we demonstrated that IL-13 induced the gene and protein expression of hCLCA1 as well as the expression of MUC5AC together with the development of GCH in cultured NHBE cells. Leverkoehne and Gruber [37] found occasional mCLCA3 protein expression in the mucous layer lining the intestinal lumen in both the small and large intestines. The expression of hCLCA1 protein in some goblet cells in the present study is consistent with their findings.

The IL-13 receptor was thought to induce mucin production via IL-13Rα_1 [5] or EGF receptor (EGFR) [38]. The expression of IL-13Rα_1 in cultured NHBE cells was consistent with the findings in normal and asthmatic bronchial epithelial cells [39], and in cells in the presence or absence of IL-13 [40]. Furthermore, GCH was reduced by the neutralization of IL-13Rα_1. Neutralization of IL-4Rα, which is also associated with the activity of IL-13 in airway epithelial cells [5], reduced GCH after the cells were exposed to IL-13. IL-13Rα_1 and IL-4Rα might be associated with IL-13-induced mucus overproduction in cultured NHBE cells.

On the other hand, EGF as well as stimulation of the EGFR and EGFR-tyrosine kinase by tumor necrosis factor-α or transforming growth factor-α [21] may play key roles in epithelial differentiation [20], MUC5AC expression and mucin production [21] in rat and human airways. Shim et al. [38] reported that IL-13 induces mucin production by stimulating EGFR and by activating neutrophils. Further studies are needed to confirm the role of EGFR in the IL-13-induced GCH.

Western blotting revealed hCLCA1 protein expression in the cells 7 days after exposure to IL-13, and this increased until day14, although hCLCA1 protein was not evident 4 days after exposure. In contrast, MUC5AC protein was expressed 4 days after exposure to IL-13. MUC5AC protein expression increased from day 4 until day 14. On the other hand, mRNA expression of hCLCA1 did not increase after 3 days of exposure to IL-13. Increases in hCLCA1 mRNA expression were significant from day 5 after

exposure to IL-13. In contrast, increases in MUC5AC mRNA expression were significant 3 days after exposure to IL-13. IL-13 increased hCLCA1 mRNA from day 5 to day 14, and MUC5AC mRNA from day 3 to day 14. Thus, the time course changes in protein expression of hCLCA1 and MUC5AC were similar to those of hCLCA1 and MUC5AC mRNA.

In summary, IL-13 induced gene and protein expression of hCLCA1 and MUC5AC, and induced GCH in well-differentiated NHBE cells cultured at an air-liquid interface. The protein expression of hCLCA1 and MUC5AC peaked 14 days after exposure to IL-13, and GCH was observed at 14 and 21 days after exposure to IL-13. Niflumic acid, a chloride channel inhibitor, reduced the mRNA expression of hCLCA1 and MUC5AC, and inhibited GCH after exposure to IL-13. Cultured NHBE cells expressed IL-13Rα_1, and an antibody to IL-13 Rα_1 reduced the number of goblet cells after exposure to IL-13. These findings suggest that IL-13 induces hCLCA1 through the pathway of MUC5AC expression, and that hCLCA1 might play an important role in mucus overproduction and GCH.

Acknowledgments

The authors thank Kumiko Nirasawa, Fumie Tezuka, Kayo Suzuki and Kiyokazu Kametani for excellent technical assistance and for providing information about transmission electron microscopy. We also thank Atsushi Nakanishi (Discovery Research Laboratories II, Pharmaceutical Discovery Research Division, Takeda Chemical Industries, Ibaraki, Japan) for providing the hCLCA1 antibody. This study was supported by a grant to the Respiratory Failure Research Group from the Ministry of Health, Labor and Welfare of Japan.

References

1 Widdicombe JH, Widdicombe JG: Regulation of human airway surface liquid. Respir Physiol 1995;99:3--12.

2 Rogers DF: The airway goblet cell. Int J Biochem Cell Biol 2003;35:1--6.

3 Aikawa T, Shimura S, Sasaki H, Ebina M, Takishima T: Marked goblet cell hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack. Chest 1992;101:916--921.

4 Kaneko Y, Yanagihara K, Miyazaki Y, Hirakata Y, Mukae H, Tomono K, Okada Y, Kadota J, Kohno S: Overproduction of MUC5AC core protein in patients with diffuse panbronchiolitis. Respiration 2003;70:475--478.

5 de Vries JE: The role of IL-13 and its receptor in allergy and inflammatory responses. J Allergy Clin Immunol 1998;102:165--169.

6 Minty A, Asselin S, Bensussan A, Shire D, Vita N, Vyakarnam A, Wijdenes J, Ferrara P, Caput D: The related cytokines interleukin-13 and interleukin-4 are distinguished by differential production and differential effects on T lymphocytes. Eur Cytokine Netw 1997;8:203--213.

7 Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, Donaldson DD: Interleukin-13: central mediator of allergic asthma. Science 1998;282:2258--2261.

8 Zheng T, Zhu Z, Wang Z, Homer RJ, Ma Bing, Riese RJ Jr, Chapman HA, Shapiro SD, Elias JA: Inducible targeting of IL-13 to the adult lung causes matrix metalloproteinase- and cathepsin-dependent emphysema. J Clin Invest 2000;106:1081--1093.

9 Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang J, Zhang Y, Elias JA: Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. J Clin Invest 1999;103:779--788.

10 Kondo M, Tamaoki J, Takeyama K, Nakata J, Nagai A: Interleukin-13 induces goblet cell differentiation in primary cell culture from guinea pig tracheal epithelium. Am J Respir Cell Mol Biol 2002;27:536--541.

11 Atherton HC, Jones G, Danahay H: IL-13-induced changes in the goblet cell density of human bronchial epithelial cell cultures: MAP kinase and phosphatidylinositol 3-kinase regulation. Am J Physiol Lung Cell Mol Physiol 2003;285:L730–L739.

12 Knowles MR, Clarke LL, Boucher RC: Activation by extracellular nucleotides of chloride secretion in the airway epithelia of patients with cystic fibrosis. N Engl J Med 1991;325:533--538.

13 Groneberg DA, Eynott PR, Oates T, Lim S, Wu R, Carlstedt I, Nicholson AG, Chung KF: The relationship of chronic mucin secretion to airway disease in normal and CFTR-deficient mice. Am J Respir Cell Mol Biol 1998;19:853--866.

14 Gruber AD, Elble RC, Ji HL, Schreur KD, Fuller CM, Paudi BU: Genomic cloning, molecular characterization, and functional analysis of human CLCA1, the first human member of the family of Ca^{2+}-activated Cl^{-} channel proteins. Genomics. 1998;54:200--214.

15 Nakanishi A, Morita S, Iwashita H, Sagiya Y, Ashida Y, Hideo S, Fujisawa Y, Nishimura O, Fujino M: Role of gob-5 in mucus overproduction and airway hyperresponsiveness in asthma. Proc Natl Acad Sci USA 2001;98:5175--5180.

16 Hoshino M, Morita S, Iwashita H, Sagiya Y, Nagi T, Nakanishi A, Ashida Y, Nishimura O, Fujisawa Y, Fujino M: Increased expression of the human Ca^{2+}-activated Cl^{-} channel 1 (CaCC1) gene in the asthmatic airway. Am J Respir Crit Care Med 2002;165:1132--1136.

17 Fujimoto K, Morita S, Iwashita H, Kimura H, Gono H, Nakanishi A, Kubo K: Gene expression of calcium-dependent chloride channel (*CLCA*) family in the sputum cells from COPD (abstract). Am J Respir Crit Care Med 2002;165:A599.

18 Kuperman DA, Huang X, Koth LL, Chang GH, Dolganov GM, Zhu Z, Elias JA, Sheppard D, Erle DJ: Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. Nat Med 2002;8:885--889.

19 Yamaya M, Finkbeiner WE, Widdicombe JH: Differentiated structure and function of cultures from human tracheal epithelium. Am J Physiol 1992;262:L713--L724.

20 Gray T, Guzman K, Davis CW, Abdullah LH, Nettesheim P: Mucociliary differentiation of serially passaged normal human tracheobronchial epithelial cells. Am J Respir Cell Mol Biol 1996;14:104--112.

21 Takeyama K, Dabbagh K, Lee HM, Agusti C, Lausier JA, Ueki IF, Grattan KM, Nadel JA: Epidermal growth factor system regulates mucin production in airways. Proc Natl Acad Sci USA 1999;96:3081--3086.

22 Rose MC, Piazza FM, Chen YA, Alimam MZ, Bautista MV, Letwin N, Rajput B: Model systems for investigating mucin gene expression in airway diseases. J Aerosol Med 2000;13:245--261.

23 Atherton H, Mesher J, Poll CT, Danahay H: Preliminary pharmacological characterisation of an interleukin-13-enhanced calcium-activated chloride conductance in the human airway epithelium. Naunyn Schmiedebergs Arch Pharmacol 2003;367:214--217.

24 Yoon JH, Gray T, Gutzman K, Koo JS, Nettesheim P: Regulation of the secretory phenotype of human airway epithelium by retinoic acid, triiodothyronine, and extracellular matrix. Am J Respir Cell Mol Biol 1997;16:724--731.

25 Chuanzhong Y, Ming G, Fanglin Z, Haijiao C, Zhen L, Shiping C, YongKang Z: Real-time quantitative reverse transcription-PCR assay for renal cell carcinoma-associated antigen G250. Clin Chim Acta 2002;318:33--40.

26 Hong KU, Reynolds SD, Watkins S, Fuchs E, Stripp BR: Basal cells are a multipotent progenitor capable of renewing the bronchial epithelium. Am J Pathol 2004;164:577--588.

27 Sandoz D, Nicolas G, Laine MC: Two mucous cell types revisited after quick-freezing and cryosubstitution. Biol Cell 1985;54:79--88.

28 ~~Kiyokazu K~~(→Kametani K): Detection of aluminium by energy dispersive X-ray microanalysis at high accelerating voltages with semi-thin sections of biological sample. J Electron Microsc (Tokyo) 2002;51:265--274.

29 Ishibashi Y, Inouye Y, Okano T, Taniguchi A: Regulation of sialyl-Lewis x epitope expression by TNF-α and EGF in an airway carcinoma cell line. Glycoconj J 2005;22:53--62.

30 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265--275.

31 Zhou Y, Shapiro M, Dong Q, Louahead J, Weiss C, Wan SH, Chen Q, Dragwa C, Savio, Huang M, Fuller C, Tomer Y, Nicolaides NC, McLane M, Levitt RC: A calcium-activated chloride channel blocker inhibits goblet cell metaplasia and mucus overproduction. Novartis Found Symp 2002;248:150--165.

32 Davey EJ, Greicius G, Thyberg J, Severinson E: STAT6 is required for the regulation of IL-4-induced cytoskeletal events in B cells. Int Immunol 2000;12:995--1003.

33 Robichaud A, Tuck SA, Kargman S, Tam J, Wong E, Abramovitz M, Mortimer J, Burston HE, Masson P, Hirota J, Slipetz D, Kennedy B, O'Neill G, Xanthoudakis S: Gob-5 is not essential for mucus overproduction in preclinical murine models of allergic asthma. Am J Respir Cell Mol Biol 2005;33:303--314.

34 Fahy JV: Goblet cell and mucin gene abnormalities in asthma. Chest 2002;122(suppl):320S–326S.

35 Kibe A, Inoue H, Fukuyama S, Machida K, Matsumoto K, Koto H, Ikegami T, Aizawa H, Hara N: Differential regulation by glucocorticoid of interleukin-13-induced eosinophilia, hyperresponsiveness, and goblet cell hyperplasia in mouse airways. Am J Respir Crit Care Med 2003;167:50--56.

36 Dabbagh K, Takeyama K, Lee HM, Ueki IF, Lausier JA, Nadel JA: IL-4 induces mucin gene expression and goblet cell metaplasia in vitro and in vivo. J Immunol 1999;162:6233--6237.

37 Leverkoehne I, Gruber AD: The murine mCLCA3 (alias gob-5) protein is located in the mucin granule membranes of intestinal, respiratory, and uterine goblet cells. J Histochem Cytochem 2002;50:829--838.

38 Shim JJ, Dabbagh K, Ueki IF, Dao-Pick T, Burgel PR, Takeyama K, Tam DC, Nadel JA: IL-13 induces mucin production by stimulating epidermal growth factor receptors

and by activating neutrophils. Am J Physiol Lung Cell Mol Physiol 2001;280:L134--L140.

39 Heinzmann A, Mao XQ, Akaiwa M, Kreomer RT, Gao PS, Ohshima K, Umeshita R, Abe Y, Braun S, Yamashita T, Roberts MH, Sugimoto R, Arima K, Arinobu Y, Yu B, Kruse S, Enomoto T, Dake Y, Kawai M, Shimazu S, Sasaki S, Adra CN, Kitaichi M, Inoue H, Yamauchi K, Tomichi N, Kurimoto F, Hamasaki N, Hopkin JM, Izuhara K, Shirakawa T, Deichmann KA: Genetic variants of IL-13 signalling and human asthma and atopy. Hum Mol Genet 2000;9:549--559.

40 Yasunaga S, Yuyama N, Arima K, Tanaka H, Toda S, Maeda M, Matsui K, Goda C, Yang Q, Sugita Y, Nagai H, Izuhara K: The negative-feedback regulation of the IL-13 signal by the IL-13 receptor α_2 chain in bronchial epithelial cells. Cytokine 2003;24:293--303.

Appendix after References (Editorial Comments){zy}

Legends

Fig. 1. PAS staining of NHBE cells cultured at air-liquid interface and incubated with 1 (**b**), 10 (**c, d**) and 100 ng/ml (**e**) of IL-13 or vehicle (PBS, **a**) for 14 days (**a–c**, **e**) or with 10 ng/ml of IL-13 for 21 days (**d**). Bar = 100 μm.

Fig. 2. Transmission electron microscopy of NHBE cells cultured at an air-liquid interface and incubated with 10 ng/ml IL-13 (**a**) or vehicle (PBS; **b**) for 21 days. Cultures contained many mucin granules, and GCH is evident (**a**). * = Artifact; p = polyester microporous membrane; ▼ = cilia. Bar = 2 μm. Original magnification, ×2,500.

Fig. 3. Time course of mRNA expression of hCLCA1 (**a**) and MUC5AC (**b**) in NHBE cells cultured with 10 ng/ml IL-13 or vehicle (PBS). Results are means ± SEM from 6 samples. Significant differences from corresponding control values (vehicle alone) are indicated: [a] $p < 0.05$, [b] $p < 0.005$, vs. day 0 (→1).

Fig. 4. Immunohistochemical staining for hCLCA1 (**c, d**) and MUC5AC (**e, f**) proteins in the presence of 10 ng/ml IL-13 (**b**, **d**, **f**) or vehicle (PBS, **a**, **c**, **e**) for 14 days. Cells were stained with anti-hCLCA1 (1: 200; **c**, **d**), anti-MUC5AC (1: 600; **e**, **f**) or without first antibody (**a**, **b**). **d** Arrowheads show hCLCA1-positive goblet cells; some goblet cells were negative for hCLCA1 immunostaining. Bar = 100 μm.

Fig. 5. **a–c** Laser confocal microscopy to detect hCLCA1 (Cy3, red) and MUC5AC (FITC, green) proteins and nucleus (DAPI, blue) in goblet cells in sheets cultured with 10 ng/ml IL-13 for 14 days at air-liquid interface. **d**, **e** Merged images (**a--c)** showing

different focus in the same field. Arrowheads show MUC5AC-positive and hCLCA1-negative goblet cells. Bar = 20 μm.

Fig. 6. Western blot analysis of hCLCA1 (**a**, 90 kDa, arrow) and MUC5AC (**b**) protein in NHBE cells incubated with 10 ng/ml of IL-13 for 14 days. Lanes show samples 4, 7 and 14 days after exposure to IL-13, respectively. β-Actin confirmed protein extraction (β-actin) and α_2-macroglobulin (720 kDa, arrowhead) served as molecular weight standard for MUC5AC protein detection (**b**).

Fig. 7. PAS staining in NHBE cells incubated with IL-13 (10 ng/ml) for 14 days with niflumic acid (100 μ*M*; **b**) or vehicle (PBS; **a**) for 48 h, from day 12 to day 14. Numbers of PAS-positive cells are remarkably reduced in cells treated with niflumic acid (**b**). Bar = 100 μm.

Fig. 8. PAS staining in NHBE cells incubated with 10 ng/ml IL-13 (**a–c**) for 14 days with anti IL-13Rα1 (**b**), anti-IL-4Rα (**c**) antibody or without antibody (**a**). Bar = 100 μm.

Fig. 9. Immunohistochemical staining for IL-13Rα1 (1: 600; clone 116730) in NHBE cells treated with 10 ng/ml IL-13 (**a**, **c**) or vehicle (PBS; **b**) for 14 days. Cytoplasm of cells in sheets incubated with IL-13 (**a**) and PBS (**b**) was positively stained. Cell sheets stained without first antibody (anti-IL-13Rα1 antibody) were negative (**c**). Bar = 100 μm.

Table{y}

Footnote(s)

Table 1. ■■■

	Total cells, n	PAS-positive cells, n	Goblet cells, n
Control vehicle (PBS)	43.2±3.6	15.7±1.8	10.3±0.1
IL-13			
0.1 ng/ml	45.4±4.8	15.2±0.9	10.3±0.1
1.0 ng/ml	43.5±2.2	15.5±0.7	10.4±2.2
10 ng/ml	55.1±2.4*	13.2±1.9**	13.4±1.3***
100 ng/ml	58.8±2.6**	15.2±2.5**	12.2±1.9**

Values are means ± SEM from 6 samples. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, vs. vehicle of IL-13 (PBS).