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ORIGINAL ARTICLE

Elimination of IL-13 Reverses Established Goblet Cell Metaplasia into Ciliated Epithelia in Airway Epithelial Cell Culture

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ABSTRACT

Background: Interleukin (IL)-13 induces goblet cell metaplasia and plays an important role in mucus hypersecretion in asthma. We previously reported that IL-13 induced goblet cell differentiation along with less ciliated cell differentiation in guinea pig tracheal epithelial cells *in vitro*. In this study, we asked whether elimination of IL-13 could reverse the established goblet cell metaplasia into ciliated epithelia.

Methods: Primary epithelial cells from guinea pig tracheas were cultured at an air-liquid interface with the medium containing human recombinant IL-13 for 14 days, and continuously cultured with IL-13-eliminated medium, or cultured under the condition of neutralization of IL-13 with anti IL-13 antibody.

Results: 2 days after elimination of IL-13, the periodic acid-Schiff-positive area as well as MUC5AC protein level rapidly decreased. After 4 days, the number of goblet cells dramatically decreased, while that of ciliated cells inversely increased. The total number of epithelial cells did not change, and 5-bromo-2'-deoxyuridine uptake decreased after IL-13 elimination. Transitional cells with cilia and secretory granules increased after IL-13 elimination. Similarly, the neutralization of IL-13 with anti-IL-13 antibody for 5 days reversed the goblet cell metaplasia into ciliated epithelia, and transitional cells also increased.

Conclusions: Elimination of IL-13 reverses goblet cell metaplasia into ciliated epithelia *in vitro*, and transition of goblet cells to other phenotypes, especially ciliated cells, may be involved in this phenomenon. IL-13 inhibition may be a therapeutic strategy of established goblet cell metaplasia in asthma.

KEY WORDS

anti-IL-13 antibody, ciliated cells, goblet cells, IL-13, transition

INTRODUCTION

Goblet cell metaplasia in airways has been established as one of pathologic characteristics of asthma.[1] Mucus overproduction due to goblet cell metaplasia causes airway narrowing, loss in lung function and occasionally asthmatic death.[2] However, the specific treatments for mucus overproduction are not currently available.

There is increasing evidence that Th2 cytokines, especially IL-13, induce goblet cell metaplasia and play an important role in mucin production. For example, IL-13-transgenic mice and rats treated with intratracheal instillation of IL-13 demonstrate marked goblet cell metaplasia.[3,4] We previously reported that IL-13 induced marked goblet cell differentiation along with inhibition of ciliated cell differentiation, when primary airway epithelial cells from guinea pigs were cultured at air-liquid interface under the condition of the long-term exposure to IL-13.[5] Atherton *et al.* similarly reported that IL-13 increased goblet cell density of cultured human bronchial epithelial cells.[6] It is an important strategy for the treatment of hypersecretion in asthma to elucidate the mechanism of resolu-

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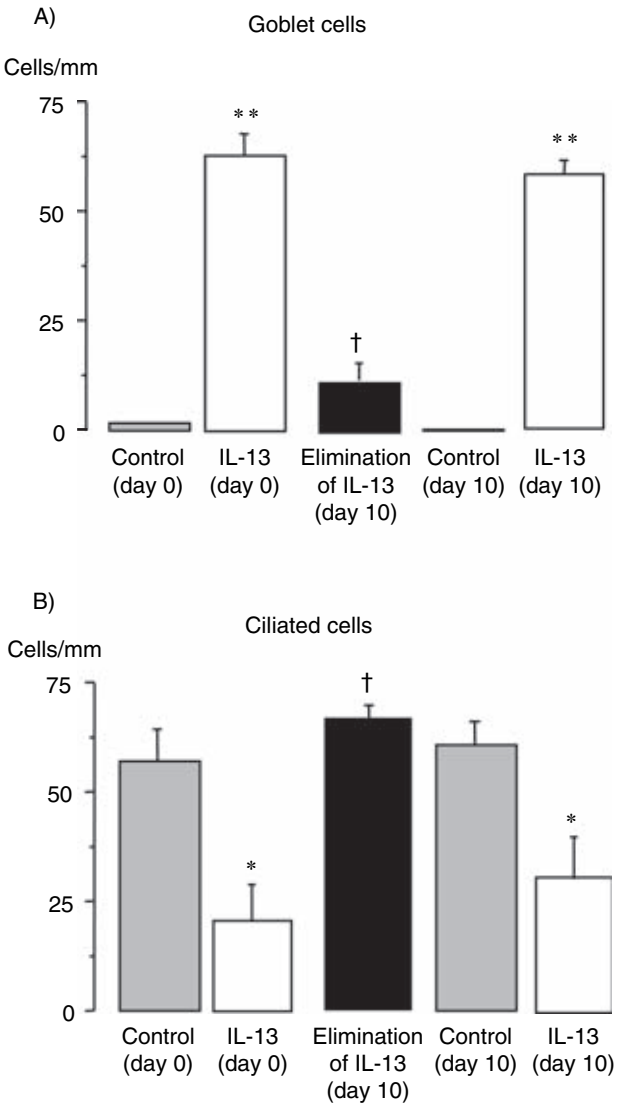

Fig. 1 The effects of elimination of IL-13 on goblet cell number (**A**) and ciliated cell number (**B**). Control (day 0, day 10): the cells were cultured in the absence of IL-13 for 14 days (day 0), and cultured in the absence of IL-13 for more 10 days (day 10). IL-13 (day 0, day 10): the cells were cultured in the presence of IL-13 for 14 days (day 0), and cultured in the presence of IL-13 for more 10 days (day 10). Elimination of IL-13 (day 10): the cells were cultured in the presence of IL-13 for 14 days, and then cultured in the absence of IL-13 for 10 more days. $n=4$. $^{**}P < 0.01$, $^{*}P < 0.05$, IL-13 *vs.* other groups. † $P < 0.05$ *vs.* IL-13 on day 10.

tion of goblet cell metaplasia. Antigen cessation induces resolution of goblet cell metaplasia along with the decrease in IL-13 in the asthmatic animal model.[7] Anti IL-13 antibody and soluble IL-13 receptor alpha 2 (sIL-13 Rα2)-IgG fusion protein reverse established goblet cell metaplasia in animal asthma models.[8-10] However, the mechanism of resolution of goblet cell metaplasia remains unclear. In this study with cultured guinea pig tracheal epithelial cells, we have demonstrated that elimination of IL-13 reverses established goblet cell metaplasia into ciliated epithelia.

METHODS

Epithelial cells from guinea pig trachea were isolated by digestion with 0.05% protease (type XIV, Sigma, St. Louis, MO, USA) at 4℃ overnight. Cells were pelleted (200 g, 10 min) and suspended in a 50 : 50 mixture of Dulbecco's modified Eagle medium (DMEM) and Ham's F12 medium (GIBCO, Grand Island, NY, USA) containing 5% fetal calf serum. Viability was −90% as assessed by trypan blue exclusion. Cells were plated at 2.5×10^5 cells per cm^2 onto polycarbonate inserts of 12 mm diameter, 0.4-μm pore size, and 10-μm thickness (Costar Transwell, Cambridge, MA, USA), which had been coated with human placental collagen (type XVI, Sigma). From the first day after plating, cells were cultured with a serum-free, hormonally defined medium containing insulin (10 μg/ml, Sigma), transferrin (5 μg/ml, Sigma), triiodothyronine (20 ng/ml, Sigma), epithelial cell growth factor (EGF) (25 ng/ml, Sigma), all-trans retinoic acid (5×10^{-8} M, Sigma) and endothelial cell growth supplement (7.5 μg/ml, Sigma). After achieving confluence, the apical medium was removed, and cells were fed from only the basolateral side with 1 ml of medium containing human recombinant IL-13 (10 ng/ml, Biosource International, Camarillo, CA, USA). EGF was eliminated from the medium after confluence to avoid multilayer formation. Cells were cultured at an air-liquid interface for 14 days with a medium containing IL-13 and then continuously cultured maximally for more than 10 days with an IL-13-eliminated medium. In a separate experiment, mouse monoclonal anti-human IL-13 antibody (clone 32116.11, 1 μg/ml, 10 μg/ml, Sigma) or mouse IgG_1 isotype (10 μg/ml, Sigma) was added for 5 days to the epithelial cells that had been exposed to IL-13 (10 ng/ml) for 14 days. The date when elimination of IL-13 or neutralization of IL-13 with anti-IL-13 antibody had started was defined as day 0. Cells were maintained in an incubator under 21% O_2 and 5% CO_2 at 37℃, and culture media were changed once every 2 days.

For light and electron microscopic studies, the cells on porous filters were fixed with glutaraldehyde/osmium tetroxide, and embedded in epon. Thick sections were stained with toluidine blue for light microscopy. Thin sections (with a silver interference color) were stained with lead citrate and uranyl acetate for transmission electron microscopy (TEM).

For cell analysis, the total cell numbers were determined by counting epithelial cell nuclei over 1 mm of the filter in cross sections of cultures with an oil immersion objective lens (×1000 magnification) by light

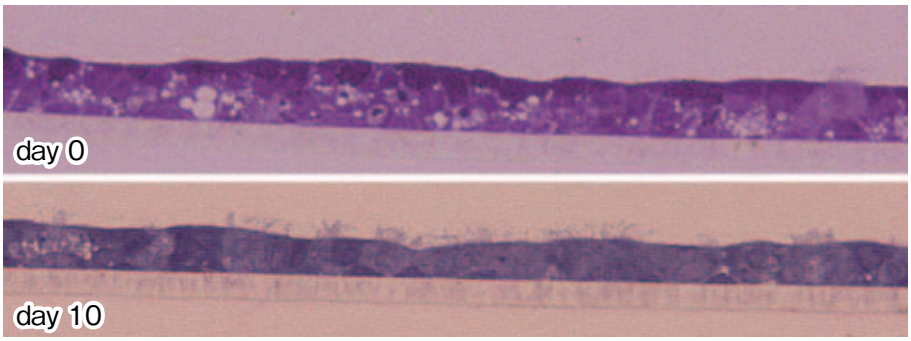

Fig. 2 Light microscopic photographs of the cultured tracheal epithelial cells. Upper panel: The cells treated with IL-13 for 14 days (defined as day 0). Lower panel: IL-13-eliminated cells on day 10. Goblet cells were replaced into ciliated cells on day 10 after elimination of IL-13. Toluidine blue stain. Objective magnification×40.

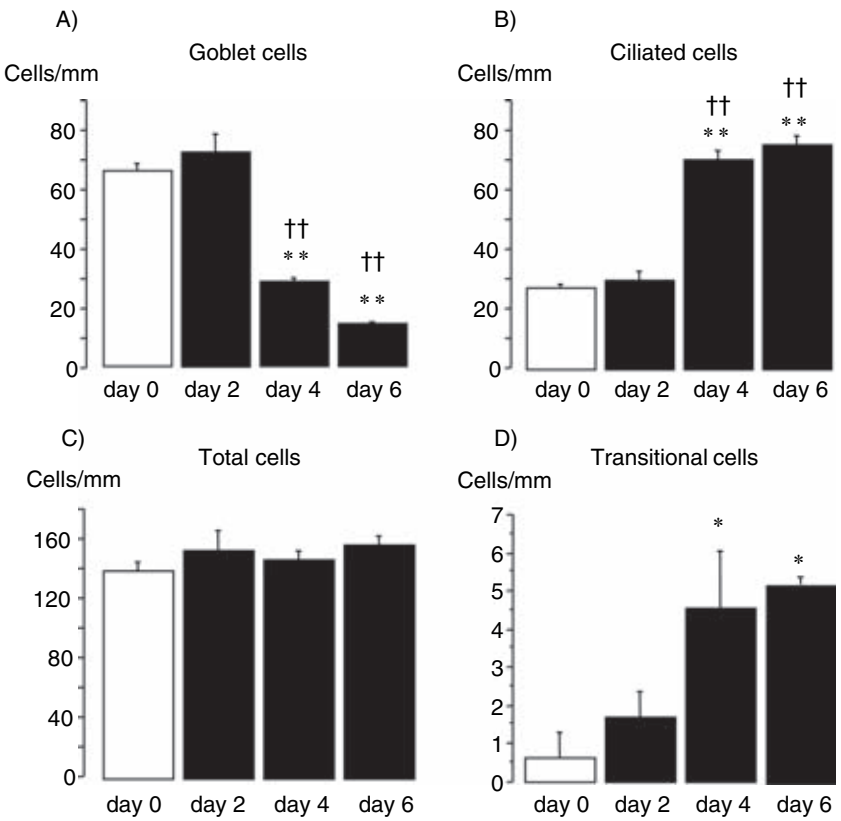

Fig. 3 Time courses of goblet cell number (**A**), ciliated cell number (**B**), total cell number (**C**) and transitional cell number (**D**) after elimination of IL-13. Goblet cell number rapidly decreased on day 4, while ciliated cell number inversely increased. Total cell number was not significantly changed during observation. Transitional cell number increased after day 4. Results were obtained from three different experiments. * $P < 0.05$, ** $P < 0.01$ *vs.* the number of each cell type on day 0. †† $P < 0.01$ *vs.* the number of each cell type on day 2.

microscopy. The epithelial cells were classified as follows. Ciliated cells contained ciliated borders. Goblet cells contained secretory granules. Basal cells were small, flattened cells located just above the filter but not reaching the apical portion. Transitional cells were defined as the cells that contained both ciliated

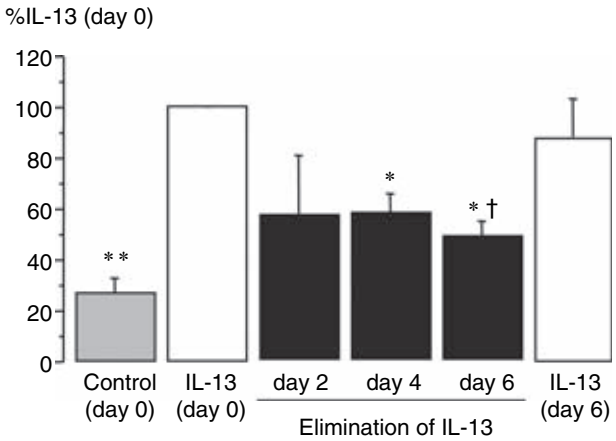

Fig. 4 Time course of BrdU uptake in cultured tracheal epithelial cells after elimination of IL-13. Data are shown as percent uptake of the IL-13-treated cells on day 0. Control (day 0): the cells have been cultured in the absence of IL-13 for 14 days. IL-13 (day 0, day 6): the cells have been cultured in the presence of IL-13 for 14 days (day 0), and cultured in the presence of IL-13 for more 6 days (day 6). Elimination of IL-13 (day 2, day 4, day 6): the cells have been cultured in the presence of IL-13 for 14 days, and then cultured in the absence of IL-13 for 2, 4, 6 days. $n = 3-5$. ** $P < 0.01$ and * $P < 0.05$ *vs.* IL-13-treated cells on day 0. † $P < 0.05$ *vs.* IL-13-treated cells on day 6.

borders and secretory granules. The rest of the cells were defined as others.

For assessment of mucin-specific staining by light microscopy, cross sections of the cell sheet were stained with periodic acid-Schiff (PAS). By using a semiautomatic imaging system (Win Roof, Mitani Co. Fukui, Japan), PAS-positive area and the total epithelial area were measured and the data were expressed as the percentage of the total area stained by PAS.

MUC5AC protein was measured using ELISA as described by Takeyama *et al.*[11] Cell lysates were prepared with phosphate buffered saline (PBS) at multiple dilutions, and 50 µl of each sample was incubated with bicarbonate-carbonate buffer (50 µl) at 40℃ in 96-well plate (Nunc), until dry. Plates were washed three times with PBS and then incubated with 50 µl of mouse monoclonal MUC5AC antibody (clone 45 M1, 1 : 100, New Markers, Fremont, CA, USA), which was diluted with PBS containing 0.05% Tween 20 (Sigma). After 1 hour, the wells were washed three times with PBS, and 100 µl of horseradish peroxidase-goat anti-mouse IgG conjugate (1 : 10,000, Sigma) was added. After 1 hour, plates were washed three times with PBS, color was developed with 3,3', 5,5'-tetramethylbenzidine (TMB) peroxidase solution (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) and stopped with 1 M H_2SO_4. Absorbance was read at 450 nm.

Cell proliferation was assessed by a commercial ELISA kit (Roche Diagnostics GmbH, Mannheim, Germany), based on the measurement of 5-bromo-2'-deoxyuridine (BrdU) incorporation during DNA synthesis in proliferating cells. Epithelial cells were incubated with the diluted BrdU added to the basolateral side prior to 24 hours. Then, the cells were washed well with DMEM/Ham F12 medium and dried with a hair-dryer for about 5 minutes, and stored at 0℃ for maximal 6 days. The cells were fixed and incubated with anti-BrdU-antibody conjugated with peroxidase for 90 minutes. After the antibody was removed, color was developed with TMB peroxidase solution and stopped with 1 M H_2SO_4. Absorbance was read at 450 nm.

STATISTICS

All data are expressed as means ± SEM. One-way ANOVA was used to determine statistically significant differences between groups. Scheffe's *F* test was used to correct for multiple comparisons when statistical significances were identified in the ANOVA. A probability of <0.05 for the null hypothesis was accepted as indicating a statistically significant difference.

RESULTS

The cells treated with IL-13 (10 ng/ml) for 14 days (defined as day 0) consisted of a large number of mature goblet cells with a small number of ciliated cells (Figs. 1, 2). Non-treated, control cells on day 0 mainly consisted of ciliated cells, whereas goblet cells were rarely found (Fig. 1). On day 10 after elimination of IL-13, most of the established goblet cells were replaced by ciliated cells (Figs. 1, 2). In contrast, the number of each cell type was not changed on day 10 in IL-13-treated cells and the non-treated, control cells (Fig. 1). The time course of each cell number after elimination of IL-13 is shown in Figure 3. Goblet cell number rapidly decreased on day 4, while ciliated cell number inversely increased (Figs. 3A, 3B). The total number of the cells did not significantly change after elimination of IL-13 (Fig. 3C). In a cell proliferation assay, BrdU uptake of IL-13-treated cells on day 0 was higher than that of non-treated, control cells on day 0 (Fig. 4). After IL-13 elimination, BrdU uptake decreased with time, and BrdU uptake of IL-13 eliminated cells on day 4 and on day 6 were significantly lower than that of IL-13-treated cells on day 0. BrdU uptake of IL-13-treated cells on day 6 was the same level as that on day 0, suggesting that BrdU uptake level was not changed in IL-13-treated cells for 6 days. Thus, BrdU uptake of IL-13-eliminated cells on day 6 was significantly lower than that of IL-13-treated cells on day 6 (Fig. 4).

Prior to the decrease of goblet cell number, the significant decrease in the PAS-positive area and MUC5AC protein level of cell lysates already oc-

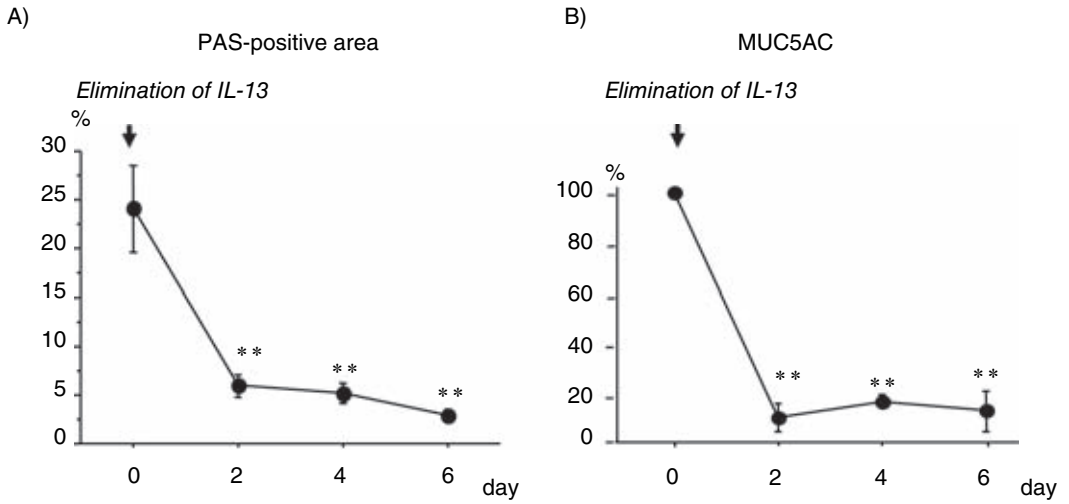

Fig. 5 The time course of PAS-positive areas (**A**) and MUC5AC levels (**B**) of the cultured tracheal epithelial cells after elimination of IL-13. PAS-positive areas and MUC5AC levels significantly decreased after day 2. $n = 4$. ** $P < 0.01$ *vs.* the values on day 0.

curred on day 2 (Figs. 5A, 5B). In electron micrographs, the secretory granules on day 2 were smaller in size and number, and more electrodense than those on day 0 (Figs. 6A, 6B). Degranulation was rarely found. MUC5AC protein levels in the lavage fluid from the apical side on day 1–2 were not increased compared with those on day 0 (data not shown). Interestingly, transitional cells with ciliary borders and electro-dense secretory granules are frequently observed after day 4, and the number was significantly increased after day 4 (Figs. 3D, 6C).

To confirm the reversal from goblet cell metaplasia to ciliated epithelia, we further studied the effect of the neutralization of IL-13 with anti IL-13 antibody on the established goblet cell metaplasia. Treatment with anti IL-13 antibody (1 μg/ml, 10 μg/ml), but not with control IgG_1 isotype (10 μg/ml) for 5 days decreased the goblet cell number and increased the ciliated cell number (Figs. 7A, 7B). Total cell number did not change (Fig. 7C). Transitional cells with ciliary borders and electro-dense secretory granules increased under the condition of anti-IL-13 antibody treatment (Figs. 6D, 7D).

DISCUSSION

In this study, we demonstrated that elimination of IL-13 reversed established goblet cell metaplasia into ciliated epithelia *in vitro*. We also demonstrated that total cell number did not significantly change for 6 days after elimination of IL-13 without the increase in cell proliferation, and the transitional cells with both ciliary borders and secretory granules were increased. These results suggest that newly appeared ciliated cells are derived from transition of other types of cells, especially goblet cells rather than cell proliferation *de novo*. Furthermore, neutralization of IL-13 with anti-IL-13 antibody for 5 days decreased the number of goblet cells and increased that of ciliated cells, and transitional cells were frequently observed. This may provide therapeutic evidence that inhibition of IL-13 such as anti-IL-13 antibody directly affects epithelial cells and resolves established goblet cell metaplasia. Although the airway epithelial cells are classically known to show plasticity,[12] the transition of goblet cells to ciliated cells remains uncertain. Interestingly, You *et al.* have recently reported that transition of ciliated cells to goblet cells is induced by IL-13 at air-liquid interface culture of murine tracheal epithelial cells.[13] Thus, our findings and their results suggest that IL-13 is a key cytokine to transform cell phenotype of airway epithelium. Yoshisue *et al.* have reported that IL-13 inhibits ciliated cell-associated genes and inhibits ciliogenesis, whereas IL-13 stimulates MUC genes and induces goblet cell metaplasia.[14] Therefore, further studies are needed to elucidate that elimination of IL-13 restores expression of ciliated cell-associated genes and downregulates expression of MUC genes.

It is widely accepted that IL-13 is a central mediator in asthma, and strongly associated with airway remodeling.[3,15] IL-13 directly transforms human lung fibroblasts into myofibroblasts,[16] and changes physiological responses to β-adrenergic agonists in human airway smooth muscles.[17] Our data as well as others show that IL-13 interferes with ciliogenesis.[5,6,14,18] Therefore, IL-13 is an important cytokine for inducing transformation in resident cells in the airway, which also results in airway remodeling.

Our *in vitro* study may support several experiments done *in vivo*. For example, the resolution of

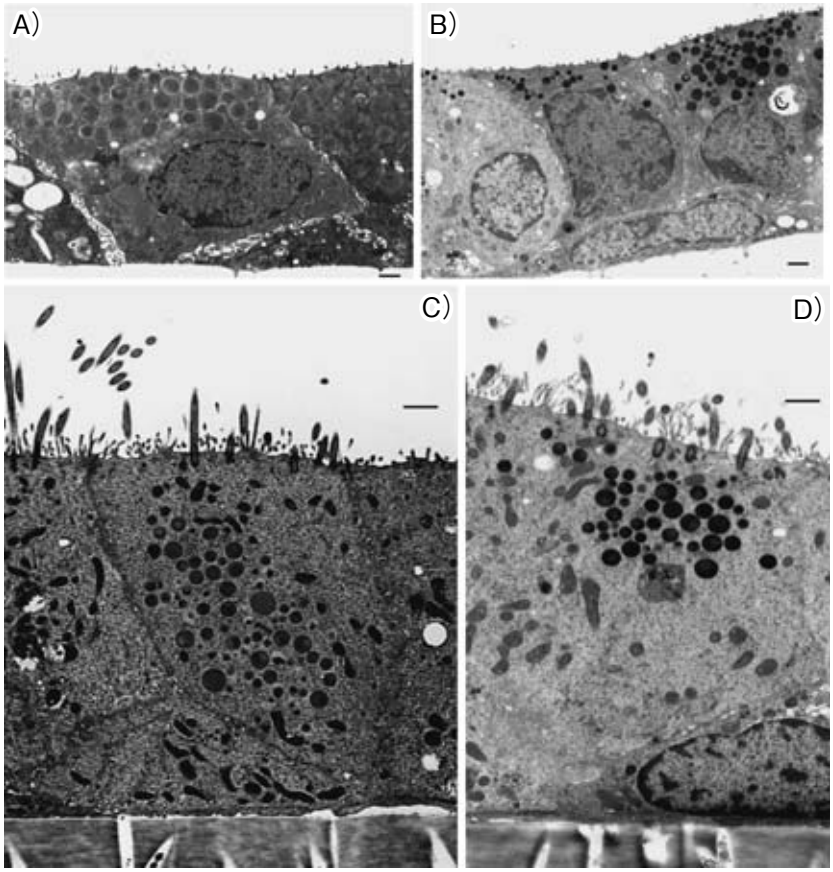

Fig. 6 Transmission electron microscopic photographs of the cultured tracheal epithelial cells. **A:** The cells on day 0. **B:** The cells on day 2. Secretory granules of the cells on day 2 are smaller and more electrodense than those on day 0. **C:** The transitional cell on day 4 after IL-13 elimination that possesses both cilia and secretory granules. **D:** The transitional cell under the condition of neutralization of IL-13 with anti-IL-13 antibody (10 μg/ml) for 5 days. Bar = 1 μm.

goblet cell metaplasia after antigen cessation is observed along with the decrease in IL-13 in allergic asthmatic models.[7] Anti-IL-13 antibody or sIL-13Rα2 instillation into the trachea recovers from established goblet cell metaplasia.[8-10] Therefore, IL-13 elimination or inhibition may be a useful strategy for resolution of established goblet cell metaplasia in asthma.

As shown in Figure 5, decreases in MUC5AC protein levels and PAS-stained areas preceded a decrease in the number of goblet cells. In fact, small, sparse and electron-dense secretory granules were concordant with these biochemical changes. Thus, these data suggest that continuous stimulation with IL-13 is needed to maintain MUC5AC synthesis and secretory granules. An excessive mucin secretion could explain the decrease in MUC5AC protein and PAS-positive areas after IL-13 elimination. However, this possibility is unlikely because morphological findings did not show degranulation, and MUC5AC levels in the supernatant during early phase (day 1–2) did not increase.

Mucus overproduction due to goblet cell metaplasia causes airway narrowing, loss in lung function and occasionally asthmatic death.[2] Specific treatments for mucus overproduction are not currently available. Our study demonstrated the possibility of transition of goblet cells to other cell types, especially ciliated cells *in vitro*. If transition to ciliated cells is shown in animal models, this may provide a new concept for the treatment of established goblet cell metaplasia.

In conclusion, elimination of IL-13 reversed established goblet cell metaplasia into ciliated epithelia in airway epithelial cell culture. Transition may be involved in this phenomenon. IL-13 inhibition may be a therapeutic strategy of established goblet cell metaplasia in asthma.

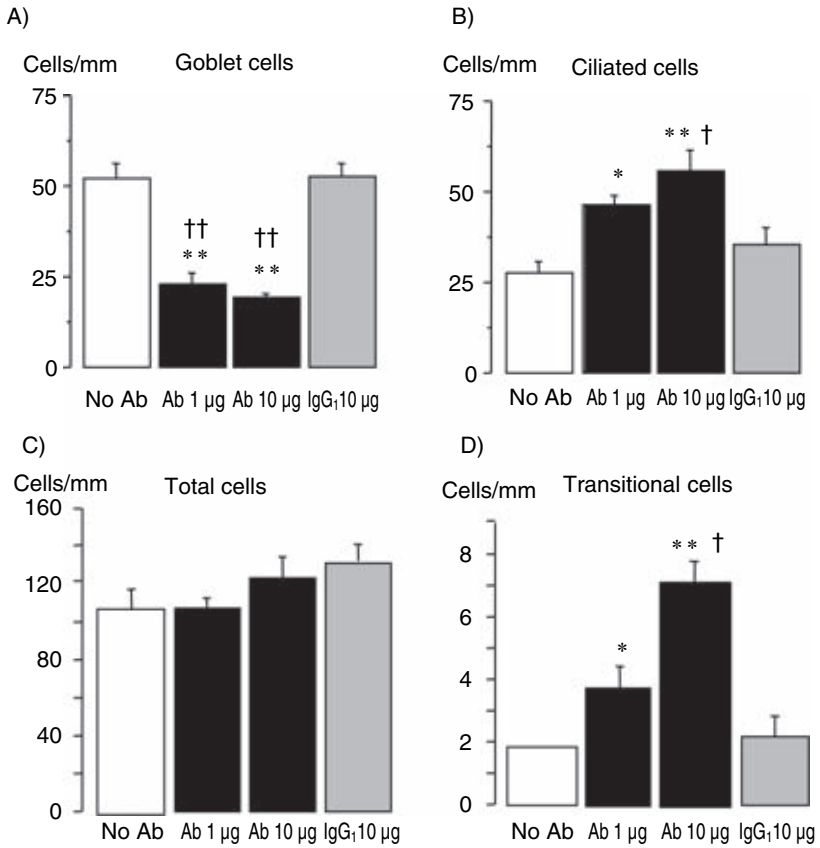

Fig. 7 The effects of anti-IL-13 antibody on goblet cell number (**A**), ciliated cell number (**B**), total cell number (**C**) and transitional cell number (**D**). Anti-IL-13 antibody (Ab 1 μg/ml, 10 μg/ml) or IgG_1 isotype (10 μg/ml) was added for 5 days with IL-13 (10 ng/ml) to the epithelial cells that had been cultured with medium containing IL-13 for 14 days. All groups are the cells on day 5. $n = 4$. ** $P < 0.01$, * $P < 0.05$ *vs.* non-Ab-treated cells. †† $P < 0.01$, † $P < 0.05$ *vs.* IgG_1 isotype-treated cells.

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