IL-4 Induces Mucin Gene Expression and Goblet Cell Metaplasia In Vitro and In Vivo

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Goblet cell metaplasia and mucous hypersecretion are important features in the pathogenesis of asthma. The cytokine IL-4 has been shown to play a role in animal models of asthma, where it induces Th2 lymphocyte differentiation and B lymphocyte IgE class switch. IL-4 has also been implicated in the differentiation of goblet cells via effects on lymphocytes and eosinophils. In this study we hypothesized that IL-4 induces airway epithelial cell mucin gene expression and mucous glycoconjugate production by direct action on these cells. In vitro, cultured airway epithelial cells (NCI-H292) expressed IL-4R constitutively, and IL-4 (10 ng/ml) induced MUC2 gene expression and mucous glycoconjugate production. In vivo, mouse airway epithelial cells expressed IL-4R constitutively, and IL-4 (250 ng) increased MUC5 gene expression and Alcian blue/periodic acid-Schiff-positive staining at 24 h; IL-4 did not increase inflammatory cell numbers in airway tissue or in bronchoalveolar lavage. TNF-α and IL-1β levels in bronchoalveolar lavage were not increased in response to IL-4 instillation. These results indicate that airway epithelial cells express IL-4R constitutively and that IL-4 directly induces the differentiation of epithelium into mucous glycoconjugate-containing goblet cells. The Journal of Immunology, 1999, 162: 6233–6237.

Mucous hypersecretion has been implicated in the pathogenesis of acute asthma (1). Mucous glycoconjugate-containing goblet cells are an important source of these secretions and have been suggested to contribute to mucous plugging in asthma (2). The airways of healthy subjects contain few goblet cells (3), but their numbers are increased in the airways of patients who die of asthma (2). The mechanisms that control the differentiation of epithelial cells into goblet cells are presently poorly understood.

IL-4 is a pleiotropic cytokine that is believed to play an important role in animal models of asthma by inducing Th2 lymphocyte differentiation (4) and IgE class switch by B lymphocytes (5). IL-4 has also been shown to play a role in allergen-induced goblet cell metaplasia, because pretreatment with a neutralizing IL-4R Ab prevented the production of mucous glycoconjugates (6); in STAT-6-deficient mice (which have impaired IL-4R signaling), allergen-induced goblet cell metaplasia was also inhibited (7). Finally, IL-4 transgenic mice, which specifically express IL-4 in the airways, develop goblet cell metaplasia (8). The mechanism that mediates these effects of IL-4 on airway epithelial cell differentiation remains unknown.

Recent studies have demonstrated the presence of IL-4R in human bronchial epithelial cells in vivo and in vitro (9, 10). We hypothesized that IL-4 induces goblet cell metaplasia, at least in part, via direct actions on epithelial cells. In the present study we found that a human airway epithelial (NCI-H292) cell line expresses IL-4R and that IL-4 causes mucin gene expression and the production of mucous glycoconjugates in vitro. Furthermore, we showed that pathogen-free mice express IL-4R in airway epithelial cells in vivo and that IL-4 instillation causes goblet cell metaplasia within 24 h without evidence of inflammatory cell recruitment into the airways.

Materials and Methods

Cells

A human pulmonary mucoepidermoid carcinoma cell line, NCI-H292, was purchased from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium supplemented with 10% FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified, 5% CO2-supplemented air-containing incubator at 37°C. Upon reaching visual confluence, the cells were treated with control medium (RPMI 1640 and 10% FCS) or with medium supplemented with human rIL-4 (10 ng/ml; Genzyme, Cambridge, MA) for 12, 24, 48, or 72 h.

Determination of IL-4R in NCI-H292 cells

The presence of IL-4R α-chain (part of the IL-4R) was examined by immunocytochemistry using a monoclonal mouse anti-human IL-4R α-chain Ab (Genzyme). Briefly, cells were grown in eight-well chamber slides until reaching confluence and were fixed with 4% paraformaldehyde for 24 h. Cells were then pretreated with 0.3% H2O2/methanol to quench endogenous peroxidase and were incubated with anti-human IL-4R α-chain Ab (1/50 dilution). Biotinylated horse anti-mouse IgG (1/200; Vector, Burlingame, CA) followed by streptavidin-peroxidase complex (ABC kit, Vector Laboratories) were used to visualize Ag-Ab complexes in the cells. Diluent lacking primary Ab, primary Ab blocked with soluble human IL-4R (R&D Systems, Minneapolis, MN), and nonimmune mouse IgG were used as controls.

Determination of mucous glycoconjugate production in NCI-H292 cells

Mucous glycoconjugate production in NCI-H292 cells was assessed using slot blotting and periodic acid-Schiff (PAS)1 staining as previously described (11–13). Briefly, NCI-H292 were cultured in six-well (10-cm2) culture dishes and incubated with 1.5 ml of control medium or IL-4-supplemented medium for 24, 48, or 72 h. At the end of the incubation period,
the culture supernatants were harvested and centrifuged to remove cell debris. The cell layer was scraped in 0.5 ml of RIPA lysis buffer (PBS containing 1% Triton X, 1% sodium deoxycholate, and 10 mg/ml PMSF) and centrifuged. Culture supernatants (250 μl) and cell lysates (50 μl) were blotted onto nitrocellulose membranes (0.2 μm pore size; MSI, Westboro, MA) by vacuum using a dot-blot apparatus (Bio-Rad, Richmond, CA), and mucous glycoconjugates were visualized by PAS reaction. Reflective densitometry was performed to quantify PAS staining using a computerized quantitative image analysis system (Bio-Rad). Values obtained for cell lysates represent the amount of mucous glycoconjugates present in cells, and the values for supernatants represent secreted mucous glycoconjugates. When both values are added together, the resulting value represents the total amount of mucous glycoconjugates produced by the cells.

To investigate whether TNF-α mediates the effects of IL-4 on mucous glycoconjugate production, some cultures treated with IL-4 were also treated with an anti-TNF-α-neutralizing Ab (20 ng/ml; Genzyme) for the entire incubation period.

**Determination of MUC2 and MUC5AC gene expression in NCI-H292 cells**

MUC2 and MUC5AC gene expression in NCI-H292 cells were assessed by in situ hybridization using 35S-labeled riboprobes generated from plasmids containing human MUC2 and MUC5AC cDNA (provided by Dr. Carol Basbaum, University of California, San Francisco, CA) following methods previously described (14). For these experiments, NCI-H292 cells were grown in eight-well chamber slides and were treated with control or IL-4-supplemented medium for 12, 24, and 48 h. Cells were then fixed for 24 h before performing in situ hybridization. In addition, parallel cultures were stained with Alcian blue/PAS (AB/PAS; Sigma, St. Louis, MO) to visualize mucous glycoconjugates in the cell layers.

**Mice**

Pathogen-free, male, BALB/c mice, weighing 20–25 g, were purchased from Simonsen Laboratories (Gilroy, CA). The mice were housed in microisolator cages kept in pathogen-free, environmentally controlled, laminar flow hoods with free access to sterile Chow and water. All procedures were approved by the committee on animal research, University of California, San Francisco, CA.

**IL-4 treatment**

BALB/c mice were anesthetized with inhaled methoxyflurane (Mallinckrodt Veterinary, Mundelein, IL) and were treated with 50 μl of sterile PBS or recombinant mouse IL-4 (250 ng/mouse; Boehringer Mannheim, Indianapolis, IN) by intranasal instillation. Animals received a single dose of PBS or IL-4 and were euthanized 24 h later with a lethal dose of pentobarbital (nembutal sodium, 200 mg/kg; Abbott Laboratories, North Chicago, IL) by intrapericardial injection. The lungs were removed, rinsed in PBS, and fixed by overnight immersion in 4% paraformaldehyde solution (Sakura Finetek U.S.A., Torrance, CA). Some samples were fixed with 5% paraformaldehyde, treated with 0.3% H2O2/methanol, and incubated with various dilutions of the anti-IL-4R Ab (1/250 to 1/50). Biotinylated anti-agarose IgG, followed by streptavidin-peroxidase complex (ABC kit, Vector Laboratories, Burlingame, CA) was used to visualize Ag-Ab complexes in sections, which were then dehydrated as described in the previous section.

**Results**

First, we examined IL-4Rα expression in NCI-H292 cells, which are known to produce mucous glycoconjugates (15). Most cells expressed IL-4Rα chain, although some cells located in areas of dense confluence (left panel, arrows) expressed higher levels. When diluted lacked primary Ab, when primary Ab was blocked with soluble human IL-4R, or when nonimmune mouse IgG was used, no signal was detected (Fig. 1).

**Effect of IL-4 on mucus glycoprotein production and MUC2 gene expression in NCI-H292 cells**

The effect of IL-4 treatment on MUC2 and MUC5AC gene expression in mouse airways was determined by in situ hybridization in sections close to sections used for AB/PAS staining. 35S-labeled riboprobes were generated from a plasmids containing rat MUC2 and MUC5AC cDNA (provided by Dr. Carol Basbaum) and following methods previously described (14).
IL-4 induced the secretion of mucous glycoconjugates into the supernatant by NCI-H292 cells by at least 2-fold at all time points investigated ($p < 0.01$; Fig. 2).

To investigate whether TNF-α was involved in mediating the stimulatory effects of IL-4 on mucous glycoconjugate production in these cells, we used an anti-TNF-α neutralizing Ab in IL-4-treated cell cultures. This Ab had no effect on IL-4-induced mucous glycoconjugate production, indicating that TNF-α was not involved in mediating IL-4’s stimulatory effects (data not shown).

**FIGURE 2.** Effect of IL-4 on mucous glycoconjugate production in NCI-H292 cells. Densitometric analysis of PAS-positive material produced by NCI-H292 cells in response to control and IL-4-supplemented medium (10 ng/ml) at 24, 48, and 72 h. Cell culture supernatant (secreted mucous glycoconjugates, solid bars) and cell lysates (intracellular mucous glycoconjugates, open bars) were blotted onto nitrocellulose membranes and stained with PAS. IL-4 increased the amount of both intracellular and secreted mucous glycoconjugates, which was greatest at 72 h. The figure is representative of three separate experiments ($n = 4$; *, $p < 0.05$ compared with control). Bars are the mean ± SEM. C, control.

IL-4 significantly stimulated MUC2 gene expression in a time-dependent manner, with stimulatory effects only detectable from 12 h onward (Fig. 3, bottom). We performed semiquantitative analysis of the effect of IL-4 on MUC2 gene expression and found that IL-4 caused an approximately 3-fold increase in the amount of positive signal (dark spots, arrows) at 24 h. The intensity of this signal was also more intense in IL-4-treated cells. The location of the signals obtained correlated with areas that displayed increased AB/PAS staining in the cell layers (Fig. 3, top). We also investigated the effect of IL-4 on MUC5AC gene expression in these cells and found no differences compared with controls (data not shown).

**FIGURE 3.** Effect of IL-4 on MUC2 gene expression in NCI-H292 cells. AB/PAS staining (top panels) and in situ hybridization for the MUC2 gene (bottom panels) in NCI-H292 cells treated with control medium (left panels) or IL-4-supplemented medium (10 ng/ml; right panels) for 24 h. NCI-H292 cells displayed basal PAS staining and MUC2 gene expression. IL-4 treatment increased the number and intensity of cells staining positively for PAS (arrows) and increased MUC2 gene expression. The figure shows representative results from three different experiments. Bar = 25 μm.

**FIGURE 4.** IL-4R protein expression in mouse airway epithelium. When the tissue was stained with an anti-IL-4Rα Ab, most airway epithelial cells stained positively (upper panel); sections incubated with isotype control Ab remained unstained (lower panel). Bar = 25 μm.

**FIGURE 5.** Effects of IL-4 on AB/PAS staining (top panels) and MUC5AC gene expression (bottom panels) in mice at 24 h. In control mice (left panels) AB/PAS staining was low, and MUC5AC staining was not visible. Airway instillation of IL-4 (250 ng) increased AB/PAS staining and MUC5AC gene expression; expression colocalized with AB/PAS staining. Bar = 25 μm.
To determine the role of pulmonary inflammation in IL-4-induced goblet cell growth in vivo, we performed 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma) staining of the lung sections. IL-4 did not affect the number of DAB-positive staining cells at 24 h (n = 5; p > 0.1 compared with control), a time when AB/PAS-positive staining of the epithelium occurred. Staining with eosin (for eosinophils) was also negative.

In BAL there were no significant differences in total or differential cell numbers between PBS- and IL-4-treated mice at 24 h after a single IL-4 instillation (n = 5; p > 0.1 compared with control). These results provide no evidence that IL-4 treatment causes pulmonary inflammation at 24 h.

Effect of IL-4 on levels of TNF-α and IL-1β in BAL

ELISA analysis showed that TNF-α and IL-1β levels were below the limits of detection (15 pg/ml) in control mice and that IL-4 did not cause significant increases in either cytokine (n = 5; p > 0.1), supporting the idea that IL-4 does not cause pulmonary inflammation at 24 h.

Discussion

In this study we addressed the hypothesis that the cytokine IL-4 can induce the differentiation of airway epithelial cells into mucous glycoconjugate-containing goblet cells via direct action on these cells. We showed that IL-4 is capable of inducing mucin gene expression and mucous glycoconjugate production in vitro and in vivo. This is, to our knowledge, the first demonstration of a direct effect of IL-4 on the differentiation of airway epithelial cells into mucous glycoconjugate-producing goblet cells.

Because IL-4Rs are known to be present on human bronchial epithelial cells (9, 10), we investigated the presence of IL-4R on cultured NCI-H292 cells. We showed that NCI-H292 cells express the IL-4R α-chain, which is a subunit of the functional IL-4R (16). These cells can therefore respond to IL-4 through their receptors and constitute a valid system to study the direct effect of IL-4 on epithelial cell differentiation.

In our investigation of the role of IL-4 in epithelial cell differentiation, we performed two sets of studies, one in vitro and one in vivo. First, we examined the effects of IL-4 on mucin gene expression and mucous glycoconjugate production by NCI-H292 cells in vitro. IL-4 induced mucin MUC2 gene expression from 12 h onward; IL-4 also increased AB/PAS staining in these cells, showing that the production of mucous glycoconjugates was increased. Furthermore, TNF-α, which has previously been shown to induce mucin gene expression and mucous glycoconjugate production (15), was not involved in mediating the stimulatory effects of IL-4. Thus, IL-4 directly induces the differentiation of epithelial cells into mucous-producing goblet cells in an in vitro system involving a single cell type.

Next, to address the in vivo relevance of the findings obtained from cultured epithelial cells, we investigated IL-4R expression and the short term (24-h) effects of IL-4 on goblet cell metaplasia in the airways of pathogen-free mice. We reasoned that short term studies might not allow time for secondary, chronic inflammatory effects to occur. We found that most (nongranulated secretory, ciliated, and goblet) airway epithelial cells express IL-4R α-chain, thus confirming the presence of IL-4R in airway epithelium in vivo. Instillation of IL-4 resulted in the rapid expression of mucin MUC5AC gene and the production of mucous glycoconjugates. Previous studies on transgenic mice expressing IL-4 in airways showed that these animals develop goblet cell metaplasia and increased MUC5AC gene expression (8, 17). Expression of IL-4 in airway epithelium was reported to be associated with airway inflammation, but the authors could not determine whether the goblet cell metaplasia was due to a direct effect of IL-4 or whether it was a secondary effect of infiltrating cells. Similarly, in a model of asthma where IL-4R signaling was blocked by treatment with an anti-IL-4R antibody, airway goblet cell metaplasia was reported to be blocked. Therefore, direct IL-4-dependent stimulation of goblet cells can induce airway goblet cell metaplasia in vivo.
anti-IL-4R Ab or by gene disruption of the STAT-6 signaling pathway, both airway inflammation and goblet cell metaplasia were prevented (6, 7). Again, this design did not allow the investigators to determine whether the effect of IL-4 on goblet cell metaplasia was direct, indirect, or both. In another asthma model in mice, it was proposed (but not proven) that IL-4 has no direct role in mucous production, but was acting on epithelial cells via lymphocyte homing into the airways and that inflammation was responsible for goblet cell metaplasia (18). These studies provide important insights into the role of T cell subsets in airway inflammation, but they do not demonstrate mechanisms underlying the development of goblet cells.

In this respect these previous studies differ from our present findings. The animals overexpressed IL-4 from birth, allowing a long period for IL-4 to cause inflammatory infiltration, so it was impossible to separate direct from indirect effects of IL-4. In contrast, in our studies, mucin synthesis was present by 24 h after delivery of IL-4, at a time when no inflammatory infiltrate was seen in airway tissue or in BAL. In addition, two inflammatory cytokines have been incriminated in mucin gene expression in vitro, TNF-α and IL-1β (15). Therefore, we examined BAL fluid and found that IL-4 did not increase the concentrations of these cytokines at a time (24 h after IL-4) when goblet cell metaplasia was present.

The present studies show unequivocally that IL-4 can induce goblet cell metaplasia via a direct effect on airway epithelial cells. They do not rule out other important effects on goblet cell growth (mechanisms unknown) that may occur secondary to chronic IL-4 effects.

Mucous plugging has long been recognized as a major factor contributing to the mortality associated with acute severe asthma (3, 19), and goblet cell metaplasia contributes to this hyperssecretion (19). Because airways in normal healthy individuals contain few goblet cells (3), abnormal proliferation of goblet cells is necessary for the symptoms of hyperssecretion to occur. Previous and present studies implicate IL-4 in goblet cell metaplasia. Therapy designed to ablate the effects of IL-4 may provide an important therapeutic strategy.

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References