

ORIGINAL PAPER

Role of epidermal growth factor receptor in maintaining airway goblet cell hyperplasia in rats sensitized to allergen

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Clinical and Experimental Allergy

Summary

Background Stimulation of epidermal growth factor receptor (EGFR) induces airway goblet cell hyperplasia, but the role of this molecule in the maintenance of this pathologic change remains uncertain.

Objective To determine the mechanisms by which goblet cell hyperplasia is maintained in airway epithelium, we investigated EGFR-induced signalling pathways that lead to both mucin production and antiapoptosis *in vitro*. We also tested whether the inhibition of EGFR tyrosine kinase speeds reversal of established goblet cell hyperplasia to normal epithelial phenotype *in vivo*.

Methods MUC5AC production was measured by immunoassay, and antiapoptotic responses were determined by Bcl-2 expression and terminal deoxynucleotidyl transferase-mediated dUTP-biotin Nick End Labelling staining using NCI-H292 cells. The effect of an inhibitor of EGFR tyrosine kinase (AG1478) on goblet cell hyperplasia was also determined in rats sensitized with ovalbumin (OVA).

Results MUC5AC was constitutively expressed and few apoptotic cells were observed in NCI-H292 cells under non-stimulated condition. TGF- α increased MUC5AC and Bcl-2 expression, an effect that was prevented by inhibitors of EGFR tyrosine kinase (AG1478), MEK (PD98059), and NF- κ B (CAPE). After the addition of TGF- α , AG1478 and an inhibitor of phosphatidylinositol 3 kinase/Akt (LY294002), but not PD98059, induced a marked apoptotic response, which was prevented by the caspase inhibitor Z-VAD fmk. Goblet cell hyperplasia and EGFR expression in airway epithelium were noted in the OVA-sensitized rats. Intratracheal instillation of AG1478 induced apoptosis of goblet cells, reverting the airway epithelium to normal epithelial phenotype.

Conclusion These findings indicate that EGFR plays an important role in the maintenance of goblet cell hyperplasia. We speculate that inhibitors of the EGFR cascade might be an effective therapy of airway remodelling.

Keywords apoptosis, asthma, epithelium, mucin, remodelling, TGF- α , tyrosine kinase

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Introduction

Mucus hypersecretion is one of the characteristic features of various chronic inflammatory diseases of the airways, including chronic bronchitis, cystic fibrosis, bronchiectasis, and asthma [1–4]. The hypersecretory phenotype in asthma is constituted by hyperplasia and hypertrophy of mucus-producing cells, such as mucous gland hypertrophy and goblet cell hyperplasia. In fatal asthma, goblet cell hyperplasia usually develops in small airways where mucus plugs are contiguous with intracellular mucous

glycoconjugates in goblet cells [5–7]. Thus, goblet cell hyperplasia is believed to contribute to the clinical manifestations, morbidity, and mortality of severe asthma. In addition, recent studies of biopsy specimens have revealed the presence of goblet cell hyperplasia and abnormal mucin gene expression in even mild to moderate asthma [8]. Thus, goblet cell hyperplasia is an important target for the treatment of airway hypersecretion in asthma, regardless of the disease severity.

Although there are many drugs that are highly effective in controlling asthma symptoms, such as glucocorticosteroids,

long-acting β_2 agonists, and leukotriene receptor antagonist, the effects of these drugs on airway remodelling are controversial [9–11]. No current method of treatment reverses the established structural changes in airway goblet cells. Considerable efforts have been made to identify the mechanisms that control persistent goblet cell hyperplasia, and both mucin gene expression and antiapoptotic responses have been proposed recently as candidate mechanisms responsible for the maintenance of goblet cell hyperplasia. Several mucin genes are up-regulated in goblet cells in studies on biopsy and autopsy specimens from asthma patients [12]. *MUC5AC* is the most frequently expressed mucin gene in asthma, and because it is specifically expressed in goblet cells [13], up-regulation of *MUC5AC* expression may be critical for the maintenance of goblet cell hyperplasia. In addition, Tesfaijzi *et al.* [14] have reported that the antiapoptotic protein Bcl-2 is up-regulated in hyperplastic goblet cells, and that the proportion of Bcl-2-positive cells decreases before resolution of the hyperplasia. These findings indicate that both mucin gene expression and antiapoptosis may be involved in the maintenance of goblet cell hyperplasia.

Interestingly, mucin gene expression and apoptosis may be related to epidermal growth factor receptor (EGFR), which is known to be expressed in the airways of cystic fibrosis, pulmonary fibrosis, chronic sinusitis, COPD, seasonal allergic rhinitis, and asthma [15–20]. Furthermore, a significant positive correlation between EGFR expression and hyperplastic goblet cells has recently been shown in asthma patients [21]. EGFR activation in airways is involved in mucin synthesis, i.e. TNF- α induction of EGFR and subsequent activation of EGFR by its ligands result in goblet cell production [22]. In addition, possible involvement of EGFR in the antiapoptotic response has been reported in intestinal epithelial cells, suggesting a role of EGFR in the maintenance of mucosal continuity [23]. The expression and subsequent activation of EGFR may thus be involved in the process of airway remodelling. For these reasons, we hypothesized that goblet cell hyperplasia is maintained by both continuous mucin gene expression and antiapoptotic responses that are activated through EGFR signalling pathways. Therefore, the purpose of the present study was to determine whether established goblet cell hyperplasia is reversed by treatment with an EGFR tyrosine kinase inhibitor. We designed *in vitro* studies using NCI-H292 cells and *in vivo* studies using Brown-Norway rats to identify the underlying signalling pathways in each response. As a stimulus to EGFR, we used TGF- α because this ligand has been shown to cause mucin production both in NCI-H292 cells and in rat airways [22]. In inhibition studies of EGFR signalling pathways, we examined two major downstreams, MEK–MAPK and PI-3K–Akt, by the use of their selective inhibitors.

Methods

Cell culture

NCI-H292 cells, a human pulmonary mucoepidermoid carcinoma cell line, were grown in RPMI 1640 medium containing 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μ g/mL) and HEPES (25 mM) at 37 °C in a humidified 5% CO₂ water-jacketed incubator. When confluence had been achieved, cells were incubated for 24 h with TGF- α (human rTGF- α , 10 ng/mL, Calbiochem, San Diego, CA, USA), and control cells were incubated with medium alone for the same time period. For the mucin production inhibition studies, AG1478 (a selective EGFR tyrosine kinase inhibitor, 10 μ M), PD98059 (a selective MEK inhibitor, 30 μ M), LY294002 [a phosphatidylinositol 3 kinase (PI3K) inhibitor, 10 μ M], CAPE (an NF- κ B inhibitor, 30 μ M), U73122 (a phospholipase C γ inhibitor, 30 μ M) and tyrphostin A1 (a negative control for AG1478, 100 μ M) were added to the cultures 30 min before adding TGF- α . For the apoptosis experiments, inhibitors were added to the cultures 24 h after adding TGF- α , because TGF- α required 24 h to induce mucin-confluent conditions in NCI-H292 cells. All inhibitors were purchased from Calbiochem. We selected the optimal concentrations of the inhibitors that have been shown to induce enough responses and not to affect the cell viability [22, 24, 25].

Immunoassay of MUC5AC

At the end of each experiment, cells grown on eight-chamber slides were fixed with 4% paraformaldehyde for 1 h, and then stained with Alcian blue (pH 2.5)/periodic acid-Schiff (PAS) to visualize mucous glycoconjugates [26].

MUC5AC protein was measured as described previously [22]. In brief, cells were lysed with lysis buffer (20 mM sodium phosphate, pH 7.8, 150 mM NaCl, 5 mM EDTA, 50 mM HEPES, 1% Triton-X100, 50 mM NaF, 1 mM sodium orthovanadate, 5 mM PMSF, and 10 μ g/mL each of leupeptin and aprotinin). The cell lysates were prepared with phosphate-buffered saline (PBS) at multiple dilutions that contained the same amount of protein in each condition, and 50 μ L of each sample was incubated at 40 °C with bicarbonate-carbonate buffer (50 μ L) in a 96-well plate (Maxisorp Nunc, Fisher Scientific, Santa Clara, CA, USA) until dry. The plates were washed three times with PBS and then blocked for 1 h at room temperature with 2% bovine serum albumin (BSA), fraction V (Sigma, St Louis, MO, USA). The plates were again washed three times with PBS and then incubated with 50 μ L of *MUC5AC* mAb (clone 45 M1, 1 : 100, Lab Vision Corp., Fremont, CA, USA) diluted with PBS containing 0.05% Tween 20. After 1 h, the wells were washed three times with PBS, and 100 μ L horseradish peroxidase goat anti-mouse IgG conjugate (1 : 10 000) was dispensed into each well. After 1 h, the plates were washed three times with

PBS. The colour reaction was developed with 3,3',5,5'-tetramethylbenzidine peroxidase solution (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) and stopped with 2N H₂SO₄. Absorbance was read at 450 nm.

Analysis of apoptosis

Both NCI-H292 cells and paraffin-embedded tissue sections of rat airways were evaluated for cell death using the Takara *in situ* apoptosis detection kit (Takara Biomedicals, Tokyo, Japan) according to the manufacturer's instructions. The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin Nick End Labelling (TUNEL) assay was performed. Cell death was visualized by labelling of DNA strand breaks by TdT, which catalyses polymerization of labelled nucleotides to free 3'-OH DNA ends in a template-independent manner (TUNEL reaction). Briefly, the cells grown on eight-chamber slides were fixed with 4% paraformaldehyde for 30 min and washed with PBS. The cells and the sections were treated with proteinase K (20 µg/mL) for 15 min at room temperature and then incubated with recombinant terminal deoxynucleotidyl transferase for 1 h at 37 °C. Fluorescein isothiocyanate (FITC)-labelled nucleotides were incorporated at the sites of DNA strand breaks via TdT, reacted with horseradish peroxidase-conjugated anti-FITC antibody, and visualized by a diaminobenzidine substrate reaction for 15 min. The slides were counterstained with haematoxylin. Under a light microscope, apoptotic cells were counted at ×400 magnification in 10 randomly selected areas under each set of conditions. Measurements in tissue sections were normalized to the length of the basement membrane, and the mean values were calculated for each animal.

Animals and study design

The experiments were approved by the Committee on Animal Research, Tokyo Women's Medical University. Specific pathogen-free male Brown-Norway rats weighing 180–220 g (Sankyo Labo Service, Inc., Tokyo, Japan) were sensitized by an intraperitoneal injection with 1 mg ovalbumin (OVA, Sigma) and 200 µg Al(OH)₃ in 1 mL of sterile saline on days 0 and 7. The rats were then exposed to aerosolized 1% (w/v) OVA or sterile saline for 30 min on days 14–16. To evaluate the effect of an EGFR tyrosine kinase inhibitor on reversal of established goblet cell hyperplasia to normal epithelial phenotype, AG1478 (3 mg/kg; intratracheal instillation) or vehicle was administered every 48 h from days 17 to 24. The outcome measurements were made on days 14 (before OVA challenge), 17, and 24 (Fig. 1).

Immunohistochemical analysis of MUC5AC and epidermal growth factor receptor

At a preselected time during anaesthesia, the systemic circulation was perfused with 1% paraformaldehyde

in PBS at a pressure of 120 mmHg. Both the lungs and the trachea were removed and placed in 4% paraformaldehyde for 1 h. They were then embedded in paraffin, cut into cross sections (4 µm thick), and placed on glass slides.

Immunohistochemistry was performed on paraffin-embedded sections. Sections were re-fixed with 4% paraformaldehyde for 5 min, autoclaved in a 0.01 M sodium citrate buffer (pH 6.0) for 5 min at 120 °C to retrieve epitopes, and then immersed in an AutoBlocker solution (Research Genetics, Huntsville, AL, USA) for 2 min at 40 °C to inhibit endogenous peroxidase activity. After blocking the non-specific binding sites with 3% BSA and 2% normal goat serum, the sections were incubated overnight at 4 °C with mouse monoclonal antibody against MUC5AC (clone 45 M1, 1:100, Lab Vision Corp.) and mouse monoclonal antibody against EGFR (1:40, Calbiochem), and then washed three times with PBS to remove excess primary antibody. The sections were then incubated for 15 min at 40 °C with anti-rabbit IgG conjugated with horseradish peroxidase-labelled polymer (EnVision+™ peroxidase, DAKO, Kyoto, Japan). The immunoreaction was amplified using a tyramide-based signal amplification method (Tyramide Signal Amplification™ Biotin System, NEN® Life Science Products Inc., Boston, MA, USA) according to the manufacturer's instructions. Bound antibodies were then visualized using 3,3'-diaminobenzidine as a substrate and counterstained with a haematoxylin solution. All immunohistochemical staining included control sections in which an unrelated antibody of the same isotype was substituted for the primary antibody.

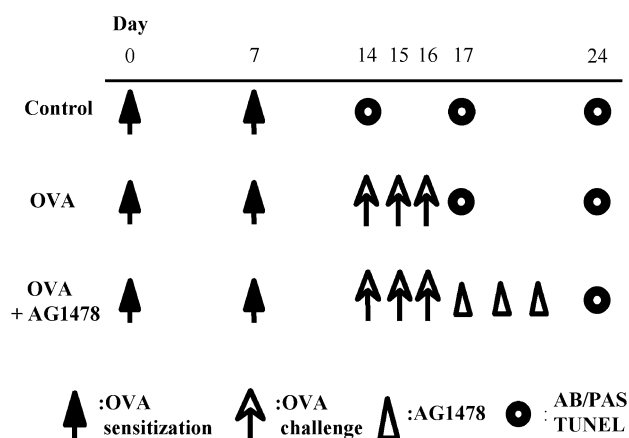


Fig. 1. Schematic diagram of the experimental protocol. Rats were sensitized by intraperitoneal injection of OVA and Al(OH)₃ on days 0 and 7. On days 14, 15, and 16, the rats were challenged for 30 min with aerosolized 1% (w/v) OVA or sterile saline (control group). AG1478 was administered intratracheally every 48 h starting on day 17. The outcome measurements were made on days 14 (before OVA challenge), 17, and 24. OVA, ovalbumin.

Analysis of stained area of the airway epithelium

Goblet cell hyperplasia and expression of both MUC5AC and EGFR were analysed by the volume density of the positive stained area on the epithelial mucosal surface with a semi-automatic imaging system as described elsewhere [22]. The intrapulmonary airways of the left lung lobe from each animal were microdissected, and 4- μ m-thick sections were prepared for analysis of the airway epithelium. The airway sections at generations three to six, respectively, were analysed for each of our data sets. Ten randomly selected areas in each animal were captured at $\times 400$ magnification, and the area that stained positive with Alcian blue (pH 2.5)/PAS, MUC5AC Ab, and EGFR Ab and the total epithelial area were measured with the NIH Image program (National Technical Information Service, Springfield, VA, USA, part number PB95-500195GEI). The data are expressed as percentages of the total area that stained positive by each staining method, and the mean was calculated for each animal. To avoid observer bias, all slides were coded before analysis and read blind.

Statistical analysis

Values for histochemistry of rat tissues were analysed using one-way ANOVA for a factorial experimental design. If significance was achieved by one-way analysis, post-ANOVA comparison of means was performed using Scheffe's *F* test. $P < 0.05$ was considered to be statistically significant. All statistical analyses were performed with Stat-View 4.01 software (Abacus Concepts, Berkeley, CA, USA).

Results

Effects of epidermal growth factor receptor signalling inhibitors on mucin production

The cytoplasm of NCI-H292 cells was stained positive with Alcian blue/PAS under control conditions. Addition of TGF- α increased the Alcian blue/PAS staining (mucous glycoconjugates) within 24 h, and pre-treatment with AG1478, PD98059, or CAPE prevented the increase in the staining, whereas LY294002 and U73122 had no effect on the TGF- α -induced mucus production (Fig. 2a). To analyse mucin production quantitatively, MUC5AC protein production was measured by immunoassay. TGF- α caused a significant increase in MUC5AC protein production within 24 h that was similar to the increase in Alcian blue/PAS staining. The TGF- α -induced MUC5AC production was inhibited by pre-treatment with AG1478, PD98059, or CAPE, but not by LY294002 and U73122 (Fig. 2b). These results suggest that TGF- α induced ligand-dependent activation of EGFR that subsequently activated an MEK/NF- κ B pathway and resulted in MUC5AC production.

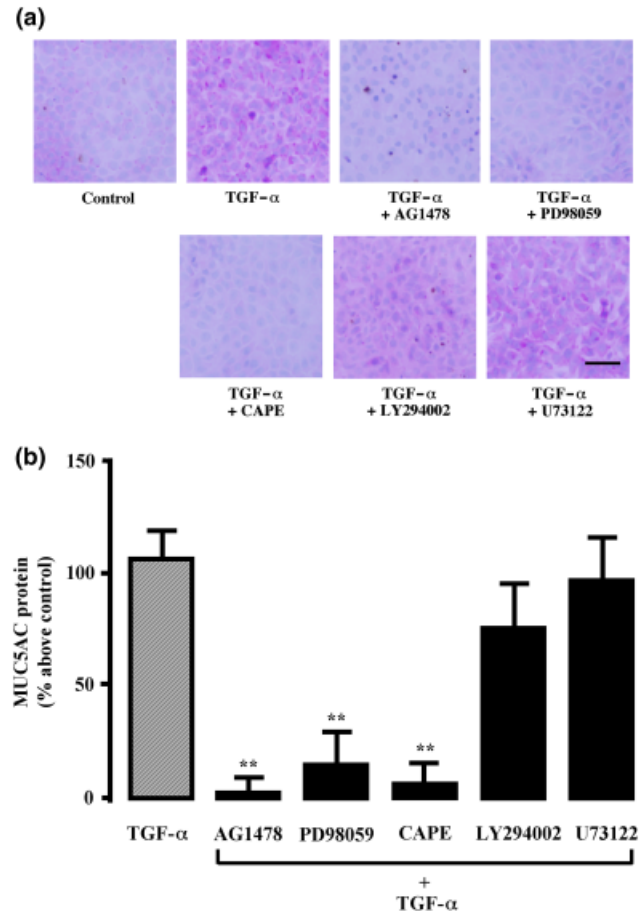


Fig. 2. Alcian blue (2.5)/PAS staining (a) and ELISA for MUC5AC (b) in NCI-H292 cells. The cytoplasm of NCI-H292 cells was stained with Alcian blue/PAS under control conditions. TGF- α increased the area of Alcian blue/PAS-positive staining, an effect that was inhibited by a selective EGFR tyrosine kinase inhibitor (AG1478), a selective MEK inhibitor (PD98059) or a NF- κ B inhibitor (CAPE), whereas a phosphatidylinositol 3 kinase inhibitor (LY294002) and a phospholipase C γ inhibitor (U73122) showed no effect. Similarly, TGF- α increased MUC5AC production within 24 h, an effect that was inhibited by AG1478, PD98059 and CAPE, but not by LY294002 and U73122. Data are means $\pm$ SE. ** $P < 0.01$, significantly different from the response to TGF- α alone. Scale bar = 50 μ m. EGFR, epidermal growth factor receptor; PAS, periodic acid-Schiff.

Role of epidermal growth factor receptor in antiapoptosis in NCI-H292 cells

To determine whether the EGFR system is involved in apoptotic mechanisms in NCI-H292 cells and to identify the signalling pathway that induces antiapoptotic responses, the cells were stimulated with TGF- α for 24 h and then exposed to several inhibitors for 24 h. There were few apoptotic cells in either the control cells or cells treated with TGF- α for 24 h. Both AG1478 and LY294002 induced prominent apoptotic responses in mucus-producing cells (Fig. 3a), and the numbers of apoptotic cells increased significantly, whereas PD98059 had no effect on the apoptotic responses. Furthermore, the apoptotic responses induced by both AG1478 and LY294002 were

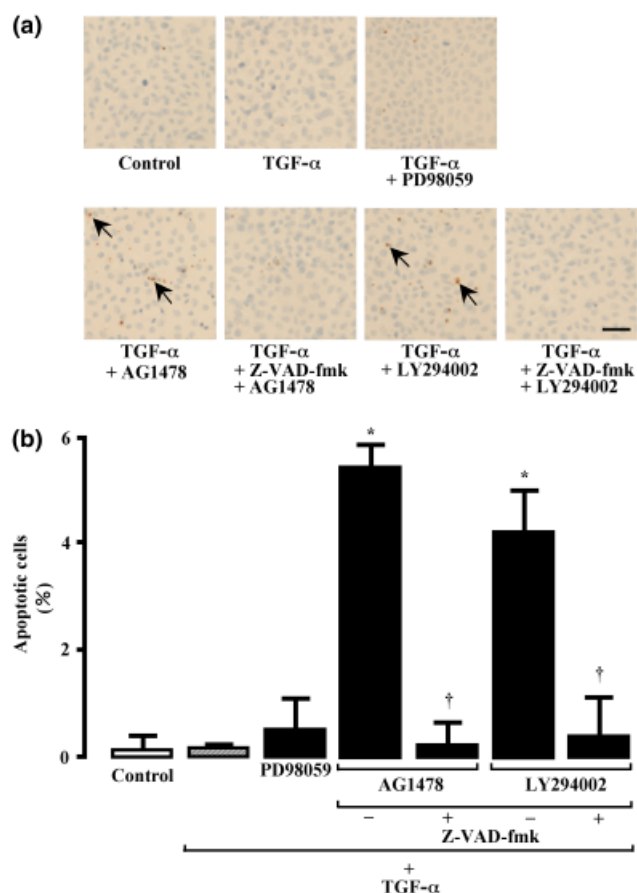


Fig. 3. TUNEL staining (a) and the percentage of apoptotic cells (b) in NCI-H292 cells. Brown nuclei indicate TUNEL staining of apoptotic cells. There were few apoptotic cells under the control (medium alone) and TGF- α -treated conditions. AG1478 and LY294002 both induced an increase in the number of apoptotic cells (arrows), whereas PD98059 had no effect. In addition, Both AG1478- and LY294002-induced apoptosis were prevented by pretreatment with the caspase inhibitor Z-VAD-fmk. Data are means $\pm$ SE. * P <0.05, significantly different from the control value. † P <0.05, significantly different from the response without Z-VAD-fmk. Scale bar = 50 μ m. TUNEL, TdT-mediated dUTP-biotin Nick end labelling.

abolished by pretreatment of cells with a caspase inhibitor, Z-VAD fmk (Fig. 3b). These results indicate that the antiapoptotic responses were induced by activation of an EGFR/Akt/PI3K pathway.

Bcl-2 protein immunoreactivity measured by immunocytochemistry was sparse under control conditions. Addition of TGF- α induced an increase in Bcl-2 expression within 24 h, but the increase was prevented by pretreatment with AG1478 or PD98059 (Fig. 4).

Sensitization-induced goblet cell production and epidermal growth factor receptor expression

Marked goblet cell hyperplasia was observed in the epithelium throughout the airways of the OVA-sensitized

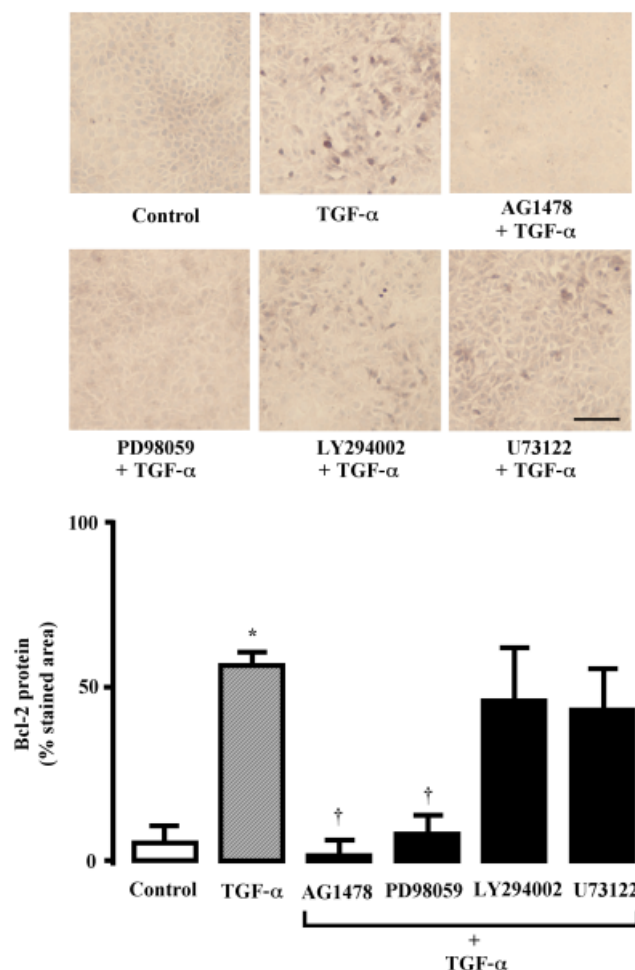


Fig. 4. Immunocytochemical staining of Bcl-2 in NCI-H292 cells. The cytoplasm of NCI-H292 cells showed positive immunoreactivity under control conditions. TGF- α increased the area of immuno-positive cells with Bcl-2 Ab, an effect that was inhibited by AG1478 and PD98059 but not by LY294002 or U73122. Data are means $\pm$ SE. * P <0.05, significantly different from the control value. † P <0.05, significantly different from the response to TGF- α alone. Scale bar = 50 μ m.

and -challenged rats 24 h after the final OVA challenge (on day 17), and the goblet cell hyperplasia progressed further on day 24. Quantitative assessment of the area of Alcian blue/PAS-positive staining of the airway epithelium on day 24 revealed a sevenfold increase in the positive-stained area in the OVA-sensitized and -challenged rats relative to the OVA-sensitized and saline-treated controls. The bronchial epithelium of the OVA-sensitized and saline-treated controls exhibited little Alcian blue/PAS-positive staining (Fig. 5). The immunohistochemical analysis performed on day 24 revealed a marked increase in both MUC5AC and EGFR immunoreactivity in the OVA-sensitized and -challenged rats compared with the OVA-sensitized and saline-treated controls (Fig. 6). MUC5AC immunoreactivity was observed only in goblet cells and the staining pattern was similar to the Alcian blue/PAS staining pattern. EGFR immunoreactivity was mainly

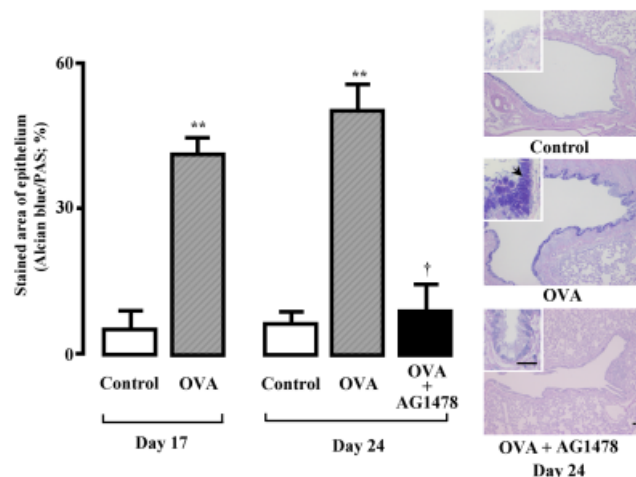


Fig. 5. Alcian blue/PAS staining of OVA-sensitized and saline-treated control, OVA-sensitized and -challenged rats, and OVA-sensitized and -challenged rats treated with AG1478. On day 17 the percentage of airway epithelium stained with Alcian blue/PAS was significantly higher in OVA-sensitized and challenged rats than in OVA-sensitized and saline-treated controls. The Alcian blue/PAS-stained area was further increased in OVA-sensitized and -challenged rats on day 24, whereas intratracheal instillation of AG1478 inhibited the increase in the area of Alcian blue/PAS-positive staining. Data are means $\pm$ SE. ** $P < 0.01$, significantly different from the control values. † $P < 0.05$, significantly different from the values for OVA. An arrow indicates goblet cells. Scale bar = 50 μ m. OVA, ovalbumin; PAS, periodic acid-Schiff.

observed at the apical surface of the airway epithelium, where goblet cells were dominant (Fig. 6, arrows). Some EGFR immunoreactivity was also located in the basal cells, whereas staining was rare in the control group. Sections unexposed to primary antibody or in which an unrelated antibody of the same isotype had been substituted for the primary antibody were negative, and EGFR immunoreactivity was diminished by preadsorption of the antibody with excess EGFR protein (data not shown).

Effect of AG1478 on established goblet cell hyperplasia

Intratracheal instillation of AG1478 three times a week begun after the final OVA challenge (day 17, after establishment of goblet cell hyperplasia) induced a significant reduction in the area of Alcian blue/PAS-positive staining in the airway epithelium within a week (Fig. 5 on day 24), which speeded up the reversal of the goblet cell hyperplasia to the normal epithelial phenotype. A negative compound of AG1478, AG1, had no effect on the reversal of goblet cell hyperplasia (data not shown). The immunoreactivity of MUC5AC showed a pattern similar to Alcian blue/PAS staining. To determine whether apoptotic responses were involved in the reduction of goblet cell hyperplasia, the level of apoptosis in the airway epithelium was assessed by a TUNEL assay. There were few apoptotic cells in the airway epithelium of either the OVA-

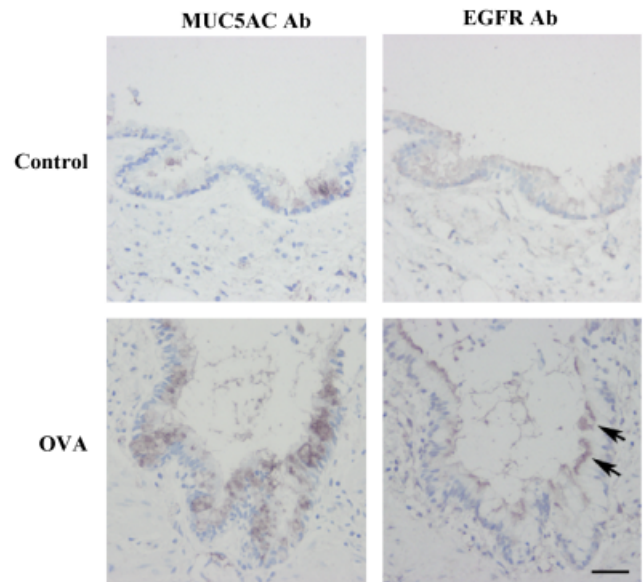


Fig. 6. Immunohistochemical staining of MUC5AC and EGFR in OVA-sensitized and saline-treated controls (upper panels) and in OVA-sensitized and -challenged rats (lower panels). MUC5AC and EGFR immunoreactivity was up-regulated in the airway epithelium of the OVA-sensitized and -challenged rats. Marked MUC5AC immunoreactivity was observed in the goblet cells. EGFR immunoreactivity was mainly observed at the apical surface of the airway epithelium (arrows). Scale bar = 50 μ m. EGFR, epidermal growth factor receptor; OVA, ovalbumin.

sensitized and saline-treated controls or the OVA-sensitized and -challenged rats. By contrast, the apoptotic cells were observed among the airway epithelial cells of the AG1478-treated rats (arrows, Fig. 7a) and the number of apoptotic cells was significantly higher compared with OVA-sensitized and -challenged rats (Fig. 7b).

Discussion

We set out to determine whether the inhibition of EGFR activation might have therapeutic implications for airway remodelling. Goblet cell hyperplasia is a prominent structural change established in the airway epithelium of asthma patients. Excessive mucus secreted by hyperplastic goblet cells causes mucus plugging, which may be related to the morbidity in asthma. Despite the importance of goblet cell hyperplasia, because the exact molecular mechanisms underlying the development and maintenance of goblet cell hyperplasia remain uncertain, there is still no therapeutic approach to reverse the hypersecretory phenotype. In the present study, we showed that MUC5AC production and antiapoptotic mechanisms are involved in the maintenance of the hypersecretory phenotype and that both phenomena are induced via activation of EGFR. Interestingly, the down-stream signalling of EGFR activation is different for each response. The MUC5AC production is promoted by an MEK/MAPK/NF- κ B pathway, whereas the antiapoptotic activity is induced by

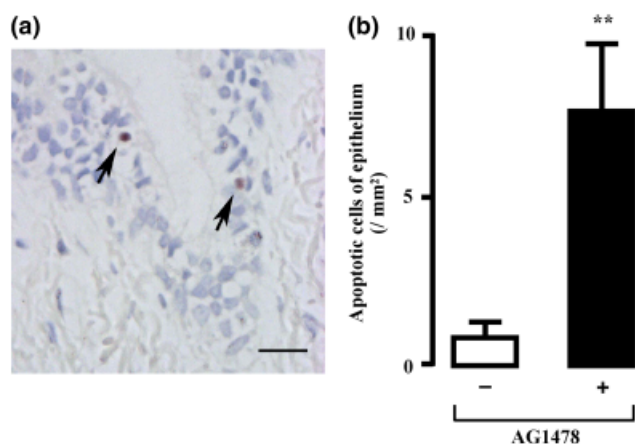


Fig. 7. Effect of AG1478 on induction of apoptosis of the airway epithelial cells in OVA-sensitized and challenged rats. Intratracheal instillation of AG1478 after the final OVA challenge induced an increase in the number of apoptotic cells among the airway epithelial cells (b). Data are means $\pm$ SE. ** $P < 0.01$, significantly different from OVA-sensitized and -challenged rats without AG1478. Brown nuclei (arrows) indicate positive TUNEL staining of apoptotic cells (a). Scale bar = 50 μ m. TUNEL, TdT-mediated dUTP-biotin Nick end labelling; OVA, ovalbumin.

activation of PI3K and subsequent phosphorylation of Akt. Most importantly, the present study showed for the first time that an EGFR tyrosine kinase inhibitor reverts the established goblet cell hyperplasia to the normal epithelial phenotype in rats *in vivo*.

It has been reported that goblet cell hyperplasia in the rat airways induced by sensitization and subsequent challenge with OVA can be prevented by glucocorticoids or selective EGFR tyrosine kinase inhibitors [10, 22]. However, glucocorticoids are unable to restore established goblet cell hyperplasia in humans [10] or in IL-13-treated mice [27]. The present study demonstrated that selective EGFR tyrosine kinase inhibitors are effective not only in preventing the development of goblet cell hyperplasia but also in reversing goblet cell hyperplasia. Because airway remodelling is generally present before treatment in patients with asthma, our *in vivo* findings suggest that the inhibitors play a role in reversing the established structural changes in such patients.

We have shown that two distinct mechanisms, mucin production and antiapoptosis, are involved in the establishment of goblet cell hyperplasia. The relationship between EGFR activation and mucin gene expression in the airways has been reported elsewhere. The ligand-dependent and -independent activation of EGFR up-regulates both MUC5AC mRNA expression and protein synthesis, and these effects are blocked by a selective EGFR tyrosine kinase inhibitor [22, 28]. Our *in vitro* results showed that inhibition of EGFR tyrosine kinase, MEK, or NF- κ B inhibited the TGF- α -induced MUC5AC production, whereas a PI3K inhibitor had no effect. These results corroborate previous studies demonstrating that the

down-stream signalling that causes MUC5AC production occurs through concomitant activation of EGFR/Ras/Raf/ERK [29]. In the present *in vivo* study, expression of both MUC5AC and EGFR was up-regulated in the airway epithelium of OVA-sensitized and -challenged rats, consistent with previous studies on bronchial biopsy sections from asthma patients [30].

An apoptotic response is involved in the reversal of the established goblet cell hyperplasia. It has been shown that phosphorylation of EGFR by its ligands activates the enzyme PI3K, which results in the formation of 3-phosphatidylinositols, which in turn activate serine/threonine kinase Akt. Akt inhibits Fas-induced caspase activity and apoptosis, thereby promoting cell survival [31]. In the present study, the inhibitor of EGFR tyrosine kinase (AG1478) and the inhibitor of PI3K (LY294002) both induced a prominent apoptotic response in TGF- α -treated NCI-H292 cells, whereas inhibition of TGF- α -stimulated MEK activity had no effect. These results indicate the EGFR/PI3K/Akt signalling pathway to the antiapoptotic response induced by TGF- α in NCI-H292 cells. Furthermore, the caspase inhibitor Z-VAD fmK abolished both AG1478- and LY294002-induced apoptosis, suggesting an involvement of caspase activity in the antiapoptotic responses induced by EGFR activation. We found that intratracheal instillation of AG1478 induced both a significant reduction in the area of Alcian blue/PAS-positive staining and an increase in the number of apoptotic cells in the airway epithelium in OVA-sensitized and -challenged rats. Taken together, it is possible that antiapoptotic mechanisms of EGFR contribute to the maintenance of the hypersecretory phenotype of airway epithelial cells in OVA-sensitized rats.

We found that TGF- α up-regulated Bcl-2 expression in NCI-H292 cells. Recent studies have shown that Bcl-2 expression in goblet cells is up-regulated under hypersecretory airway conditions, such as exposure to ozone, endotoxin, cigarette smoke, or OVA sensitization [32], suggesting a role of Bcl-2 in the maintenance of goblet cell hyperplasia. Because these stimuli that up-regulate Bcl-2 expression are also known to transactivate EGFR in airway epithelial cells, an EGFR signalling pathway may be involved in Bcl-2 expression in hypersecretory airway diseases. There is evidence that TGF- α plays an antiapoptotic role by regulating the expression of Bcl-2 family proteins via the NF- κ B-dependent pathway in gastric mucosal cells [33]. Although Bcl-2 is known to have antiapoptotic effects in epithelial cells, our study showed that the inhibition of Bcl-2 production by PD98059 did not induce apoptotic responses in NCI-H292 cells. In the apoptosis experiments, the cells were stimulated with TGF- α for 24 h. Thus, Bcl-2 expression has been up-regulated in the cells when PD98059 was added. These results revealed that the addition of PD98059 might inhibit further expression of Bcl-2, but did not affect the

antiapoptotic response induced by Bcl-2. Previous studies have shown that the ERK pathway does not have a direct antiapoptotic function in breast adenocarcinoma cells (T47D, MCF7) or HEK293 embryonic kidney epithelial cells [34]. However, the ERK pathway prevents TNF- α - and UV-induced apoptosis [35, 36]. Thus, different cell types may use the Akt or ERK signalling pathway for cell survival in response to different pro-apoptotic stimuli. It has been shown recently that Akt phosphorylates both Bad and caspase 9 [37, 38], thereby inhibiting their pro-apoptotic activity. The phosphorylation of these proteins appears to be a mechanism of the antiapoptotic function for Akt. It should be noted that there were few apoptotic NCI-H292 cells even under control conditions in our experiment. Because EGFR and its ligand TGF- α are constitutively expressed in NCI-H292 cells, Akt phosphorylation may be induced endogenously and result in continuous antiapoptotic responses, even under control conditions.

Activation of EGFR is essential to induce inflammatory cell chemoattractants, such as matrix metalloproteinase 9 and IL-8 [39, 40], in asthma. Products of inflammatory cells, TGF- α , elastase, and oxidants, have been shown to up-regulate mucin gene expression [28, 41, 42]. Thus, inhibition of EGFR may influence the maintenance of goblet cell hyperplasia indirectly via inhibition of the recruitment of inflammatory cells. In the future, it should be tested whether the inhibition of EGFR also affects other clinical manifestations of asthma, such as airway hyperresponsiveness, inflammatory cell recruitment, and smooth muscle hypertrophy.

Restitution is the key initial step in the repair of airway desquamation and can restore mucosal continuity in chronic inflammatory airway diseases. However, once the process of restitution is directed abnormally, the pathological structural changes termed 'airway remodelling' may occur. Goblet cell hyperplasia is a prominent feature of airway remodelling and is essential for airway hypersecretion in many chronic inflammatory diseases. Our results confirm that the expression and subsequent activation of EGFR initiate critical signalling events in mucin production and antiapoptosis that maintain goblet cell hyperplasia and contribute to resistance of airway remodelling to therapy. Importantly, inhibition of EGFR activation has been proposed as a therapy to reverse the structural changes in the airways, but demonstration that it does will require tests in patients with hypersecretory phenotypes, especially in asthma.

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