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Eosinophil recruitment and mucus hypersecretion are characteristic of asthmatic airway inflammation, but eosinophils have not been shown to induce mucin production. Because an epidermal growth factor receptor (EGFR) cascade induces MUC5AC mucin in airways, and because EGFR is up-regulated in asthmatic airways, we examined the effect of eosinophils on MUC5AC mucin production in NCI-H292 cells (a human airway epithelial cell line that produces mucins). Eosinophils were isolated from the peripheral blood of allergic patients, and their effects on MUC5AC mucin gene and protein synthesis were assessed using in situ hybridization and ELISAs. When IL-3 plus GM-CSF or IL-3 plus IL-5 were added to eosinophils cultured with NCI-H292 cells, MUC5AC mucin production increased; eosinophils or cytokines alone had no effect. Eosinophil supernatant obtained by culturing eosinophils with IL-3 plus GM-CSF or IL-3 plus IL-5 also increased MUC5AC synthesis in NCI-H292 cells, an effect that was prevented by selective EGFR inhibitors (AG1478, BIBX1522). Supernatant of activated eosinophils induced EGFR phosphorylation in NCI-H292 cells. Supernatant of activated eosinophils contained increased concentrations of TGF-α protein (an EGFR ligand) and induced up-regulation of TGF-α expression and release in NCI-H292 cells. A blocking Ab to TGF-α reduced activated eosinophil-induced MUC5AC synthesis in NCI-H292 cells. These results show that activated eosinophils induce mucin synthesis in human airway epithelial cells via EGFR activation, and they implicate TGF-α produced by eosinophils and epithelial cells in the EGFR activation that results in mucin production in human airway epithelium. The Journal of Immunology, 2001, 167: 5948–5954.

Prominent eosinophilic infiltration into airway epithelium is characteristic of airway inflammation in asthma (1) and in nasal polyposis (2). Excessive mucus production from hyperplastic goblet cells is also reported in acute (3) and chronic (4, 5) asthma and in nasal polyps (6). Human tissues in fatal asthma are reported to show extensive eosinophil degranulation, especially in mucus plugs and in surface epithelium (7), and a positive correlation between eosinophil counts in bronchoalveolar lavage fluid and sputum production has been reported in asthmatic patients (8). Because Ag challenge in animal models of allergic asthma resulted in marked eosinophilic airway inflammation and goblet cell metaplasia (9), it was suggested that eosinophils play a role in mucus production in allergic diseases. However, the finding that Th2-induced goblet cell metaplasia occurred in the absence of eosinophil recruitment in IL-5 knockout mice led to the conclusion that eosinophils do not contribute to allergen-induced goblet cell metaplasia in mice (10). Recent studies suggest that differences exist in eosinophil effector functions between mice and humans (11, 12), so the role of eosinophils in mucus production in humans remains unknown.

Mucin production in airways is induced by an epidermal growth factor receptor (EGFR) cascade (13). Activation of EGFR by its ligands (e.g., EGF, TGF-α) resulted in MUC5AC mucin gene and protein synthesis in human airway epithelial (NCI-H292) cells in vitro, and selective inhibitors of EGFR tyrosine kinase phosphorylation prevented ligand-induced mucin synthesis in vitro and prevented allergen-induced goblet cell metaplasia in pathogen-free rats (13).

When human eosinophils are recruited to the site of inflammation, they become activated (14). Eosinophils recruited to tissue express TGF-α in carcinomas (15) and human nasal polyps (16). Because human eosinophils isolated from peripheral blood, when activated with cytokines in vitro, produce and release TGF-α (17–19) in a regulated process, we hypothesized that activated eosinophils stimulate mucin synthesis in human airway epithelial cells via EGFR activation. Therefore, we studied the effect of human eosinophil activation on mucin MUC5AC production in human airway epithelial (NCI-H292) cells, and we studied the role of EGFR activation in eosinophil-induced mucin production in these cells. Some of these results were reported in abstract form (20).

Materials and Methods

Human subjects

Eosinophils were isolated from the peripheral blood of eight allergic subjects with a previous history of blood eosinophilia (eosinophil count range, 8–12%). Subjects ranged in age from 28 to 62 years. Immediate hypersensitivity was confirmed by a positive skin test reaction using a prick-puncture technique, applying extracts of common allergens (ragweed, house dust mite, grass pollens, cat and dog dander, cockroach, alternaria).

1 This work was supported by private funds.
2 Address correspondence and reprint requests to Dr. Jay A. Nadel, Cardiovascular Research Institute, Box 0130, University of California, San Francisco, CA 94143-0130. E-mail address: janadel@itsa.ucsf.edu
3 Abbreviations used in this paper: EGFR, epidermal growth factor receptor; PDGFR, platelet-derived growth factor receptor.
None of the subjects was being treated with oral corticosteroids. Informed, written consent was obtained before participation. The study was approved by the University of California Committee on Human Research.

Isolation of human eosinophils

Eosinophil isolation was performed by MACS (Miltenyi Biotec, Auburn, CA), with minor modifications of the method described previously (21). To prevent bacterial contamination, all solutions were passed through a 0.1-μm pore size filter. Briefly, venous blood was anticoagulated with EDTA. After 45 min of sedimentation on 6% dextran (M, 140,000; Sigma-Aldrich, St. Louis, MO), plasma was recovered, diluted with calcium- and magnesium-free PBS, overlaid onto isotonic Percoll solution (density, 1.077 g/ml; Sigma-Aldrich), and centrifuged at 1000 g for 30 min at 4°C. The supernatant and mononuclear cells at the interface were carefully removed, and erythrocytes in the sediment were removed by hypotonic lysis. Isolated granulocytes were resuspended in 50 μl of buffer (PBS, pH 7.2, supplemented with 0.5% BSA and 2 mM EDTA)/5 × 105 cells. An equal volume of anti-CD16-conjugated magnetic beads (Miltenyi Biotec) was added to the cell pellet. After 30 min of incubation at 6°C, the cells were pipetted to a separation column positioned in the strong magnetica field of the MACS. Cells were eluted with buffer containing BSA. Eosinophil purity counted on a cytopsin smear stained with Diff-Quick Stain Set (Baxter Healthcare, Miami, FL) was consistently >98%. Purified eosinophils were washed twice with PBS and suspended in RPMI 1640 containing 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and HEPES (25 mM).

Activation of eosinophils by incubation with cytokines

Freshly isolated eosinophils (2 × 106 cells/ml) were cultured with NCI-H292 cells in RPMI 1640 with 10% FBS in the absence or the presence of various combinations of IL-3, IL-5, and GM-CSF (25 ng/ml each; BioSource International, Camarillo, CA) for 24 h. These cytokine concentrations are reported to induce TGF-α expression in eosinophils (18, 22). In preliminary studies, we found that when eosinophils were activated with a combination of IL-3 plus either GM-CSF or IL-5, the effect of production was more pronounced than when eosinophils were activated with each cytokine alone (data not shown). Because the number of eosinophils available for each experiment was limited, we used only IL-3 plus GM-CSF or IL-3 plus IL-5 in the experiments.

For the preparation of eosinophil supernatant, freshly purified eosinophils were cultured for 16 h in 24-well plates at 2 × 105 cells/ml in RPMI 1640 containing 5% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and HEPES (25 mM). When the baseline level is low, NCI-H292 cells were serum-starved for 24 h to decrease the baseline level of EGFR phosphorylation and then stimulated with eosinophil supernatant or TGF-α (0.1 ng/ml) (25) for 30 min before stimulation. After stimulation, cells were placed on ice and lysed for 15 min with lysis buffer containing 1% Triton X-100, 1% deoxycholic acid, 50 mM NaCl, 1 mM sodium orthovanadate, and proteinase inhibitors (Complete Mini; Roche, Indianapolis, IN). To remove insoluble materials, the cell lysate was centrifuged at 10,000 rpm for 20 min at 4°C. Aliquots of cell lysates containing equal amounts of protein were incubated with an EGFR Ab (Ab-3, 1 μg/ml; Calbiochem) and with protein A-agarose beads (Santa Cruz Biotechnology), which bind to mouse IgG, overnight at 4°C. Immunoprecipitates were washed four times with cold PBS-0.05% Tween 20. Beads were resuspended in 40 μl of Laemmli sample buffer (Bio-Rad, Hercules, CA), heated to 95°C for 5 min, and centrifuged. The supernatant was electrophoresed on 7.5% polyacrylamide gel. The proteins were then transferred electrophoretically to polyvinylidene difluoride membranes (Bio-Rad), which were incubated with 5% fat-free skimmed milk in PBS containing 0.05% Tween 20 for 1 h and then incubated with an anti-phosphotyrosine mAb (PY99, 2 μg/ml; Santa Cruz Biotechnology). Bound Ab was visualized according to the standard procedure for protein-biotin-peroxidase complex method (Elite ABC kit; Vector Laboratories). Slides were counterstained with hematoxylin. When Abs to MUC5AC or to TGF-α were omitted, no staining was observed (data not shown).

Immunohistochemical staining for MUC5AC protein and TGF-α protein in NCI-H292 cells

NCI-H292 cells were grown on eight-well chamber slides and exposed to the stimuli for 24 h. At the end of the experiment, the cells were fixed with 4% paraformaldehyde for 1 h and then treated with 0.3% H2O2 in methyl alcohol. PBS containing 0.05% Tween 20 and 2% BSA was used as diluent for the Abs. Cells were incubated with a mouse mAb to MUC5AC (clone 45 M1, 1/500; Neo Markers) or an mAb to TGF-α (Ab2, 1/250; Calbiochem) at room temperature for 1 h. After removing excess Ab by washing with PBS, cells were incubated with a biotinylated horse anti-mouse Ab (dilution 1/250; Vector Laboratories, Burlingame, CA) at room temperature for 1 h. Bound Ab was visualized according to the standard procedure for protein-biotin-peroxidase complex method (Elite ABC kit; Vector Laboratories). Slides were counterstained with hematoxylin. When Abs to MUC5AC or to TGF-α were omitted, no staining was observed (data not shown).

Immunoblotting for phosphorylated EGFR

Because EGFR phosphorylation in response to a stimulus is easier to detect when the baseline level is low, NCI-H292 cells were serum-starved for 24 h (to decrease the baseline level of EGFR phosphorylation) and then stimulated with eosinophil supernatant or TGF-α (0.1 ng/ml) (24) for 10 min. In inhibition studies, the epithelial cells were pretreated with BIBX1522 (10 μM) for 30 min before stimulation. After stimulation, cells were placed on ice and lysed for 15 min with lysis buffer containing 1% Triton X-100, 1% deoxycholic acid, 50 mM NaCl, 1 mM sodium orthovanadate, and proteinase inhibitors (Complete Mini; Roche, Indianapolis, IN). To remove insoluble materials, the cell lysate was centrifuged at 10,000 rpm for 20 min at 4°C. Aliquots of cell lysates containing equal amounts of protein were incubated with an EGFR Ab (Ab-3, 1 μg/ml; Calbiochem) and with protein A-agarose beads (Santa Cruz Biotechnology), which bind to mouse IgG, overnight at 4°C. Immunoprecipitates were washed four times with cold PBS-0.05% Tween 20. Beads were resuspended in 40 μl of Laemmli sample buffer (Bio-Rad, Hercules, CA), heated to 95°C for 5 min, and centrifuged. The supernatant was electrophoresed on 7.5% polyacrylamide gel. The proteins were then transferred electrophoretically to polyvinylidene difluoride membranes (Bio-Rad), which were incubated with 5% fat-free skimmed milk in PBS containing 0.05% Tween 20 for 1 h and then incubated with an anti-phosphotyrosine mAb (PY99, 2 μg/ml; Santa Cruz Biotechnology). Bound Ab was visualized according to standard procedures for the avidin-biotin-alkaline phosphatase complex method (ABC kit; Vector Laboratories).

Immunohistochemical staining for MUC5AC protein and TGF-α protein in NCI-H292 cells

NCI-H292 cells were grown on eight-well chamber slides and exposed to the stimuli for 24 h. At the end of the experiment, the cells were fixed with 4% paraformaldehyde for 1 h and then treated with 0.3% H2O2 in methyl alcohol. PBS containing 0.05% Tween 20 and 2% BSA was used as diluent for the Abs. Cells were incubated with a mouse mAb to MUC5AC (clone 45 M1, 1/500; Neo Markers) or an mAb to TGF-α (Ab2, 1/250; Calbiochem) at room temperature for 1 h. After removing excess Ab by washing with PBS, cells were incubated with a biotinylated horse anti-mouse Ab (dilution 1/250; Vector Laboratories, Burlingame, CA) at room temperature for 1 h. Bound Ab was visualized according to the standard procedure for protein-biotin-peroxidase complex method (Elite ABC kit; Vector Laboratories). Slides were counterstained with hematoxylin. When Abs to MUC5AC or to TGF-α were omitted, no staining was observed (data not shown).

Immunoassay of MUC5AC protein

MUC5AC protein in eosinophil supernatant and in NCI-H292 cell supernatant

TGF-α protein in eosinophil supernatant and in NCI-H292 cell supernatant was measured using a quantitative ELISA kit (Oncogene Research Products, Boston, MA), following the manufacturer’s instructions. Each sample was tested in duplicate.

Cell culture

NCI-H292 cells, a human pulmonary mucopidermoid carcinoma cell line capable of expressing mucins (23), were grown in RPMI 1640 containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 mM HEPES at 37°C in a humidified 5% CO2 water-jacketed incubator. When confluent, NCI-H292 cells were incubated with either freshly isolated eosinophils or eosinophil supernatant. As controls, NCI-H292 cells were incubated with culture medium alone (baseline) or with culture medium and IL-3 plus IL-5. After 6 h of culture, eosinophils were centrifuged, and the supernatant was collected; these eosinophils were routinely >95% viable by trypan blue dye exclusion.

In situ hybridization for MUC5AC mRNA

MUC5AC gene expression was assessed by in situ hybridization with 35S-labeled riboprobe. Preparation of the probes and in situ hybridization were performed as described previously (26).

Activation of eosinophils by incubation with cytokines

Freshly isolated eosinophils (2 × 106 cells/ml) were cultured with NCI-H292 cells in RPMI 1640 at 5% FBS in the absence or the presence of the mucin produced by NCI-H292 cells was determined (obtained by calculating the total amount of MUC5AC protein in cell lysate plus the amount in cell culture supernatant at 24 h), using bovine submaxillary gland mucin (type I; Sigma-Aldrich) as a standard. The results were reported as milligrams of MUC5AC protein. Because the amount of mucin produced by NCI-H292 cells varied with cell passage, we expressed the data as a percentage of baseline (NCI-H292 cells cultured alone) on the same experimental day.
Immuno-histochemical staining for TGF-α in eosinophils

Freshly isolated eosinophils were cultured for 16 h in RPMI 1640 containing serum alone or with IL-3 plus IL-5. After the supernatants were harvested, the cells were fixed for 30 min in 4% formaldehyde. Cytospin smears were prepared, and immunohistochemical staining was performed with an mAb to TGF-α (Ab2, 1:250; Calbiochem). As a negative control we used an irrelevant mouse IgG Ab (DAKO, Carpinteria, CA). For the staining technique, see description above of immunohistochemical staining in NCI-H292 cells. Slides were counterstained with hemotoxylin.

Statistical analysis

For analysis of the results of ELISA measurements of MUC5AC protein, one-way ANOVA for repeated measurements was used on raw data (milligrams of mucin) to determine statistically significant differences among groups. When statistical significance was identified in the ANOVA, the Student-Newman-Keuls test was used for multiple comparisons. Data obtained from TGF-α protein measurements in eosinophil supernatant were analyzed using a paired t test. Data obtained from ELISA measurements of TGF-α protein in NCI-H292 cell supernatant were not normally distributed (unequal SDs) and were analyzed using the nonparametric Wilcoxon test. A p < 0.05 for the null hypothesis was accepted as indicating a statistically significant difference. Because NCI-H292 cells produced variable amounts of mucin at baseline, which varied with cell passage, we expressed the data as a percentage of the baseline (NCI-H292 cells alone on the same experimental day) ± SEM.

Results

Activated eosinophils up-regulate MUC5AC gene and protein expression

When NCI-H292 cells were cultured with eosinophils in the presence of IL-3 plus GM-CSF or IL-3 plus IL-5, mucin production increased significantly above baseline (+33.4 ± 9.4% and +37.2 ± 9.6%, respectively; p < 0.05; n = 5). However, when NCI-H292 cells were cultured with eosinophils alone, no significant increase in MUC5AC protein in NCI-H292 cells occurred (+7 ± 3.7%; p > 0.1; n = 5). Similarly, when NCI-H292 cells were cultured with IL-3 plus GM-CSF alone or with IL-3 plus IL-5 alone (no eosinophils), there was no significant change in MUC5AC mucin production (+1.3 ± 1.83% and +2.5 ± 2.7%, respectively; p > 0.1; n = 5).

Treatment of NCI-H292 cells with TGF-α (1 ng/ml), a positive control for mucin production (13), also increased MUC5AC protein production significantly above baseline (+36 ± 8%; p < 0.05; n = 5).

Next, we examined whether substances secreted from eosinophils cause mucin synthesis. Eosinophils were cultured for 16 h, and then the supernatant was separated from the eosinophils and incubated with NCI-H292 cells. The supernatant of eosinophils cultured with IL-3 plus GM-CSF or with IL-3 plus IL-5 increased MUC5AC protein production in NCI-H292 cells significantly, but the supernatant of eosinophils cultured alone had no significant effect (Fig. 1). Similarly, when NCI-H292 cells were cultured with supernatant of eosinophils activated with IL-3 plus IL-5, immunostaining for MUC5AC protein increased, but not when NCI-H292 cells were cultured with supernatant of eosinophils cultured alone (Fig. 2, upper panel). Because MUC5AC mucin synthesis in NCI-H292 cells in response to the supernatant of activated eosinophils was comparable to the response to activated eosinophils themselves, we concluded that the effect of activated eosinophils on mucin synthesis was related to a secreted product. Therefore, subsequent studies were performed with eosinophil supernatant.

In situ hybridization was performed to assess the effect of eosinophil supernatant on MUC5AC mRNA synthesis in NCI-H292 cells. The supernatant of eosinophils cultured with IL-3 plus IL-5 increased MUC5AC mRNA expression in NCI-H292 cells, whereas the supernatant of eosinophils cultured alone had no significant effect (Fig. 2, lower panel). Sense probe for MUC5AC mRNA showed no expression of MUC5AC under all conditions (data not shown).

Selective EGFR tyrosine kinase inhibitors prevent MUC5AC synthesis induced by supernatant of activated eosinophils

Because EGFR tyrosine kinase activation causes mucin production in NCI-H292 cells (13), we examined the effects of selective inhibitors of EGFR tyrosine kinase phosphorylation on activated eosinophil-induced MUC5AC production. Pretreatment of NCI-H292 cells with selective EGFR tyrosine kinase inhibitors (BIBX1522, AG1478) prevented both constitutive and activated eosinophil-induced MUC5AC protein production (Fig. 3), but a selective PDGFR tyrosine kinase inhibitor (AG1295) and a negative control for tyrophostin (AG9) were without effect. Similarly, the selective EGFR inhibitor BIBX1522 prevented supernatant-induced up-regulation of immunostaining for MUC5AC protein (Fig. 2, upper panel) and prevented up-regulation of MUC5AC mRNA expression (Fig. 2, lower panel) in NCI-H292 cells.

EGFR tyrosine kinase phosphorylation by supernatant of activated eosinophils

As reported previously, TGF-α, an EGFR ligand, induces EGFR phosphorylation in NCI-H292 cells (24). The supernatant of eosinophils cultured with IL-3 plus IL-5 also induced EGFR phosphorylation (Fig. 4), an effect that was not observed with supernatant of eosinophils cultured alone. BIBX1522 prevented activated eosinophil-induced EGFR phosphorylation.
Role of the EGFR ligand TGF-α in activated eosinophil-induced MUC5AC protein synthesis

In a series of experiments supernatants of eosinophils cultured with IL-3 plus IL-5 increased MUC5AC mucin production measured by ELISA in NCI-H292 cells (+36.4 ± 9.34% above baseline; n = 5 eosinophil donors); when these supernatants were preincubated with an anti-TGF-α blocking Ab, MUC5AC mucin production decreased significantly (+14.8 ± 6.6% above baseline; p < 0.05 compared with the effect of activated eosinophils). These results implicate TGF-α in mucin production induced by activated eosinophils. Therefore, we examined potential sources of TGF-α. First, we measured TGF-α in eosinophil supernatant by ELISA.

The supernatant of eosinophils cultured alone contained a small amount of TGF-α (Fig. 5) and had sparse staining with an Ab to TGF-α (Fig. 6A, left panel), but supernatant of eosinophils activated with IL-3 plus IL-5 contained increased amounts of TGF-α protein (Fig. 5) and showed intense granular staining for TGF-α protein (Fig. 6A, middle panel). Next, we hypothesized that the TGF-α-mediated effect of activated eosinophils on mucin production could be related in part to an effect of activated eosinophils on TGF-α production by airway epithelial cells. Therefore, we incubated NCI-H292 cells with eosinophil supernatant and performed immunohistochemical staining for TGF-α. NCI-H292 cells expressed TGF-α protein constitutively (Fig. 6B, left panel). Supernatant of eosinophils cultured with IL-3 plus IL-5 induced prominent TGF-α staining in NCI-H292 cells (Fig. 6B, right panel), whereas treatment of NCI-H292 cells with supernatant of eosinophils cultured alone (Fig. 6B, middle panel) or with IL-3 plus IL-5 alone (not shown) was without effect. Next, we measured soluble TGF-α by ELISA in NCI-H292 cell supernatant; TGF-α concentrations in NCI-H292 cell supernatant were 1.86 ± 0.54 pg/ml (range, 0.64–4.42 pg/ml) at baseline and were 13.87 ± 7.69 pg/ml (range, 0.84–50.35 pg/ml) after incubation for 24 h with supernatant of eosinophils cultured with IL-3 plus IL-5 (p < 0.05; n = 6). Cytokine alone or supernatant of eosinophils cultured alone had no significant effect (data not shown).

FIGURE 2. Immunohistochemical staining for MUC5AC protein (upper panel) in NCI-H292 cells and in situ hybridization with an antisense probe for MUC5AC mRNA (lower panel). NCI-H292 cells cultured alone served as a control (baseline), and NCI-H292 cells treated with TGF-α (1 ng/ml) served as a positive control. Eosinophils were cultured alone (EOS alone) or with IL-3 plus IL-5 (EOS IL-3 + IL-5), and the supernatant was harvested and incubated with NCI-H292 cells. Some cultured NCI-H292 cells were pretreated with the selective EGFR tyrosine kinase inhibitor BIBX1522 (BIBX; 10 μM). Staining for MUC5AC is shown as dark areas in lightfield, and hybridization with the MUC5AC mRNA probe is shown as light areas in darkfield. Photomicrographs are representative of three separate experiments. Bar, 50 μm.

FIGURE 3. Effect of pretreatment of NCI-H292 cells with selective EGFR tyrosine kinase inhibitors (BIBX1522 and AG1478, 10 μM each) on MUC5AC protein production by supernatant of eosinophils (EOS) preincubated alone (left columns) or with IL-3 plus IL-5 (right columns). A PDGFR tyrosine kinase inhibitor (AG1295) and a negative control for tyrosphostin (AG9; 10 μM each) served as negative controls. Results are reported as a percentage of baseline (NCI-H292 cells alone) mean ± SEM. *p < 0.05 (compared with baseline); †, p < 0.01 (compared with EOS alone); ††, p < 0.01 (compared with EOS IL-3 plus IL-5). n = 6 EOS donors.

FIGURE 4. Tyrosine phosphorylation of EGFR induced by eosinophil supernatant and TGF-α. NCI-H292 cells cultured alone served as the control (baseline). The cells were treated with TGF-α (0.1 ng/ml) or with supernatant of eosinophils cultured alone (EOS alone) or with IL-3 plus IL-5 (EOS IL-3 + IL-5). Some cultured NCI-H292 cells were pretreated with the selective EGFR tyrosine kinase inhibitor BIBX1522 (10 μM). Cell lysates containing equal amounts of proteins were immunoprecipitated with an mAb to EGFR and analyzed by Western blotting with an antiphosphotyrosine Ab (PY-99). Results are representative of three separate experiments.

FIGURE 5. MUC5AC protein synthesis in activated eosinophil-induced airway epithelial cell. Therefore, we incubated NCI-H292 cells with eosinophil supernatant and performed immunohistochemical staining for TGF-α. NCI-H292 cells expressed TGF-α protein constitutively (Fig. 6B, left panel). Supernatant of eosinophils cultured with IL-3 plus IL-5 induced prominent TGF-α staining in NCI-H292 cells (Fig. 6B, right panel), whereas treatment of NCI-H292 cells with supernatant of eosinophils cultured alone (Fig. 6B, middle panel) or with IL-3 plus IL-5 alone (not shown) was without effect. Next, we measured soluble TGF-α by ELISA in NCI-H292 cell supernatant; TGF-α concentrations in NCI-H292 cell supernatant were 1.86 ± 0.54 pg/ml (range, 0.64–4.42 pg/ml) at baseline and were 13.87 ± 7.69 pg/ml (range, 0.84–50.35 pg/ml) after incubation for 24 h with supernatant of eosinophils cultured with IL-3 plus IL-5 (p < 0.05; n = 6). Cytokine alone or supernatant of eosinophils cultured alone had no significant effect (data not shown).
The supernatant of activated eosinophils caused prominent TGF-α production. Airway epithelial cells such as NCI-H292 cells (27) are reported to phosphorylate to produce mucin (13). Because eosinophils (15) and eosinophil donors. Values from individual isolations are indicated by connecting lines. Means are shown as horizontal lines. *, p < 0.001 (compared with supernatants (SUP) of EOS alone).

Discussion
In the present study, we examined whether eosinophils induce MUC5AC mucin synthesis in airway epithelial cells. Our results showed that activated human eosinophils caused MUC5AC mucin synthesis in NCI-H292 cells. Similarly, the supernatant of activated eosinophils induced MUC5AC gene and protein synthesis in NCI-H292 cells, indicating that this effect of activated eosinophils was related to a secreted product.

Because EGFR activation is reported to cause mucin synthesis (13), we examined whether EGFR activation is required for activated eosinophil-induced mucin synthesis. Activated eosinophil supernatant induced EGFR phosphorylation in NCI-H292 cells, and selective inhibitors of EGFR tyrosine kinase blocked activated eosinophil-induced EGFR phosphorylation and MUC5AC synthesis completely; a selective PDGFR inhibitor (AG1295) and a negative control for tyrphostins (AG9) were without effect. These findings implicate EGFR tyrosine kinase phosphorylation in mucin synthesis induced by activated eosinophils. Furthermore, selective inhibitors of EGFR tyrosine kinase inhibited baseline mucin production in airway epithelial (NCI-H292) cells, demonstrating that baseline mucin production is dependent on EGFR activation. This latter finding suggests that autocrine activation of the EGFR in airway epithelium is responsible for baseline mucin production.

EGFR ligands (e.g., TGF-α) bind to EGFR and activate phosphorylation to produce mucin (13). Because eosinophils (15) and airway epithelial cells such as NCI-H292 cells (27) are reported to produce TGF-α, we examined the role of TGF-α in eosinophil-induced mucin synthesis. First, we found that activated eosinophils release TGF-α. Next, we examined the effect of eosinophil supernatant on TGF-α expression and release by airway epithelial cells. The supernatant of activated eosinophils caused prominent TGF-α protein staining in airway epithelium and increased concentrations of soluble TGF-α in NCI-H292 cell supernatant. Importantly, blocking Ab to TGF-α reduced eosinophil-induced mucin production. Thus, the present study shows for the first time that activated human eosinophils cause mucin production in human airway epithelium by EGFR activation due at least in part to the production of TGF-α. However, the present study did not assess how much of the TGF-α effect was due to its release from eosinophils vs epithelial cells. Blockade of EGFR tyrosine kinase activity prevented eosinophil-induced mucin production completely, but treatment with a TGF-α mAb only reduced eosinophil-induced mucin production, suggesting the existence of other mechanisms of EGFR activation. Other EGFR ligands (e.g., heparin-binding EGF, which is reported to be up-regulated in activated eosinophils (28)) or ligand-independent effects (e.g., oxygen free radicals (24)) could also play roles in the responses.

The 50-aa mature TGF-α is synthesized as a part of the extracellular domain of 160 aa (29) membrane-bound precursor pro-TGF-α (30). Newly synthesized proTGF-α is cleaved by proteolytic enzymes, resulting in the release of soluble TGF-α (31, 32). The soluble, mature form of TGF-α is more potent than the membrane-bound form of TGF-α (33). In this study we found that supernatant of activated eosinophils induces prominent TGF-α staining in airway epithelial cells, suggesting that a product of eosinophils causes TGF-α protein production in airway epithelium. Next, we measured soluble TGF-α in the supernatant of NCI-H292 cells, and we found low, but increased, concentrations of soluble TGF-α after incubation with supernatant of activated eosinophils. Because NCI-H292 cells express EGFR (27), and because after binding to EGFR, TGF-α is internalized and degraded (34), concentrations of soluble TGF-α in NCI-H292 cell supernatant depend on both release of soluble TGF-α (by cleavage of

![FIGURE 5](image-url). Effect of incubation of eosinophils with IL-3 plus IL-5 on release of TGF-α. Isolated human eosinophils (EOS) were cultured for 16 h in RPMI 1640 alone or with IL-3 plus IL-5. After centrifugation, supernatants (SUP) were harvested, and TGF-α protein was measured by ELISA in the supernatants. Results include nine eosinophil isolations from six EOS donors. Values from individual isolations are indicated by connecting lines. Means are shown as horizontal lines. *, p < 0.001 (compared with supernatants (SUP) of EOS alone).

![FIGURE 6](image-url). Immunohistochemical staining for TGF-α in eosinophils and NCI-H292 (epithelial) cells. A. Isolated human eosinophils. Human eosinophils (EOS) were isolated from peripheral blood, cultured for 16 h in RPMI alone (left panel) or with IL-3 plus IL-5 (middle and right panels), and then harvested and fixed. Cytospin smears were obtained and stained with an Ab to TGF-α or to mouse IgG (negative control) and counterstained with hematoxylin as described in Materials and Methods. Arrows, Granular staining for TGF-α. Note the change in shape of eosinophils incubated with IL-3 plus IL-5. Arrowheads, Bilobed nuclei of eosinophils. Photomicrographs are representative of three separate experiments (three EOS donors). Original magnification, ×1000. Bar, 20 μm. B, NCI-H292 cells. NCI-H292 cells were treated for 24 h with supernatant of eosinophils cultured alone or eosinophils cultured with IL-3 plus IL-5. After fixation, NCI-H292 cells alone (baseline, left panel) or NCI-H292 cells treated with supernatant of eosinphils cultured alone (EOS alone, middle panel) or with supernatant of activated eosinophils (EOS IL-3 plus IL-5, right panel), were stained with an Ab to TGF-α. Photomicrographs are representative of three separate experiments; arrows, granular staining for TGF-α. Original magnification, ×1000. Bar, 20 μm.

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proTGF-α and TGF-α binding to the EGFR. Therefore, measured concentrations of TGF-α in NCI-H292 cell supernatant are relative and do not reflect the actual amount of soluble TGF-α released from epithelial cells. Nevertheless, these findings suggest that a product of activated eosinophils cleaves membrane-bound pro-TGF-α in airway epithelial cells, resulting in the release of the active soluble TGF-α. Eosinophils may release elastase (35) and metalloproteases (36) that could play a role in the proteolytic cleavage of pro-TGF-α. However, mechanisms of TGF-α release from airway epithelial cells in response to eosinophil supernatant remain to be investigated.

Previous studies reported a positive correlation between eosinophilia (in blood (37) and in airways (8)) and increased sputum production in asthma, suggesting that eosinophils might be involved in mucus hypersecretion. Similarly, in chronic obstructive pulmonary disease, Saetta et al. (38) reported increased numbers of sputum eosinophils during exacerbations, and Haraguchi et al. (39) reported a positive correlation between airway eosinophils and the presence of goblet cell metaplasia in patients dying of asthma-like attacks. Negative correlations have also been used to argue against the role of eosinophils in mucus hypersecretion. Allergen sensitization in mice results in both infiltration of eosinophils into the airways and goblet cell metaplasia, originally suggesting a possible relationship (9). However, in C57BL/6 IL-5 knockout mice, allergen sensitization no longer caused eosinophil recruitment, but still induced goblet cell metaplasia, leading the authors to conclude that eosinophils are not essential for the induction of mucus production (10). However, mouse eosinophils (including C57BL/6) do not appear to degranulate after Ag stimulation in vivo (11, 40), whereas in asthmatic airway tissue eosinophils show degranulation (7). In vitro, human, but not mouse, eosinophils degranulate upon stimulation (11, 12). These results indicate that findings in mice may not reflect effects in humans.

Healthy subjects have few circulating eosinophils, making studies difficult to perform. Therefore, the present studies were conducted with eosinophils isolated from allergic patients with blood eosinophilia. Because many asthmatic patients are allergic, results obtained with their eosinophils are relevant to allergic airway diseases. Furthermore, in the present study eosinophils isolated from allergic subjects and cultured alone had no significant effect on mucin production by airway epithelial cells, whereas activated eosinophils or supernatant of activated eosinophils induced increased mucin production by airway epithelial cells. Thus, activation of eosinophils appears to be key to their effect on mucin production.

How do the present studies relate to human disease? The present studies were performed with eosinophils isolated from allergic patients with blood eosinophilia. Because many asthmatic patients are allergic, results obtained with their eosinophils are relevant to allergic airway diseases. Furthermore, in the present study eosinophils isolated from allergic subjects and cultured alone had no significant effect on mucin production by airway epithelial cells, whereas activated eosinophils or supernatant of activated eosinophils induced increased mucin production by airway epithelial cells. Thus, activation of eosinophils appears to be key to their effect on mucin production.

References


