Epidermal growth factor system regulates mucin production in airways

Kiyoshi Takeyama, Karim Dabbagh, Heung-Man Lee, Carlos Agusti, James A. Lausier, Iris F. Ueki, Kathleen M. Grattan, and Jay A. Nadel*

Cardiovascular Research Institute and Departments of Medicine and Physiology, University of California, San Francisco, CA 94143-0130

Communicated by John A. Clements, University of California, San Francisco, CA, December 31, 1998 (received for review October 21, 1998)

ABSTRACT Goblet-cell hyperplasia is a critical pathological feature in hypersecretory diseases of airways. However, the underlying mechanisms are unknown, and no effective therapy exists. Here we show that stimulation of epidermal growth factor receptors (EGF-R) by its ligands, EGF and transforming growth factor α (TGFα), causes MUC5AC expression in airway epithelial cells both in vitro and in vivo. We found that a MUC5AC-inducing epithelial cell line, NCI-H292, expresses EGF-R constitutively; EGF-R gene expression was stimulated further by tumor necrosis factor α (TNFα). EGF-R ligands increased the expression of MUC5AC at both gene and protein levels, and this effect was potentiated by TNFα. Selective EGF-R tyrosine kinase inhibitors blocked MUC5AC expression induced by EGF-R ligands. Pathogen-free rats expressed little EGF-R protein in airway epithelial cells; intratracheal instillation of TNFα induced EGF-R in airway epithelial cells, and subsequent instillation of EGF-R ligands increased the number of goblet cells, Alcian blue–periodic acid–Schiff staining (reflecting mucous glycoconjugates), and MUC5AC gene expression, whereas TNFα, EGF, or TGFα alone was without effect. In sensitized rats, three intratracheal instillations of ovalbumin resulted in EGF-R expression and goblet-cell production in airway epithelium. Pretreatment with EGF-R tyrosine kinase inhibitor, BIBX1522, prevented goblet-cell production both in rats stimulated by TNFα-EGF-R ligands and in an asthma model. These findings suggest potential roles for inhibitors of the EGF-R cascade in hypersecretory diseases of airways.

In Vivo Studies. Cell culture. A human pulmonary mucoid carcinoma cell line, NCI-H292, were grown in RPMI 1640 medium containing 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and Heps (25 mM) at 37°C in a humidified 5% CO₂, water-jacketed incubator. When confluent, cells were incubated with EGF (recombinant human EGF, 25 ng/ml, Genzyme), TGFα (recombinant human TGFA, 25 mg/ml, Genzyme), TNFα (recombinant human TNFα, 20 ng/ml, Genzyme), or EGF plus TNFα or TGFα plus TNFα for 12 h, 24 h, or 48 h. In inhibition studies, cells were pretreated with the selective tyrosine kinase inhibitors, BIBX1522 (10 μg/ml, generously provided by Boehringer Ingelheim), tyrphostin AG1478 (10 μM, Calbiochem) or Compound 56 (10 μM, Calbiochem) 30 min before adding growth factors. The effects of a selective tyrosine kinase inhibitor of platelet-derived growth factor (tyrphostin AG1295, 100 μM, Calbiochem) and a negative control for tyrphostins (tyrphostin A1, 100 μM, Calbiochem) were also examined.

METHODS

In Vitro Studies. Cell culture. A human pulmonary mucoid carcinoma cell line, NCI-H292, were grown in RPMI 1640 medium containing 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and Heps (25 mM) at 37°C in a humidified 5% CO₂, water-jacketed incubator. When confluent, cells were incubated with EGF (recombinant human EGF, 25 ng/ml, Genzyme), TGFα (recombinant human TGFA, 25 mg/ml, Genzyme), TNFα (recombinant human TNFα, 20 ng/ml, Genzyme), or EGF plus TNFα or TGFα plus TNFα for 12 h, 24 h, or 48 h. In inhibition studies, cells were pretreated with the selective tyrosine kinase inhibitors, BIBX1522 (10 μg/ml, generously provided by Boehringer Ingelheim), tyrphostin AG1478 (10 μM, Calbiochem) or Compound 56 (10 μM, Calbiochem) 30 min before adding growth factors. The effects of a selective tyrosine kinase inhibitor of platelet-derived growth factor (tyrphostin AG1295, 100 μM, Calbiochem) and a negative control for tyrphostins (tyrphostin A1, 100 μM, Calbiochem) were also examined.

Abbreviations: EGF, epidermal growth factor; EGF-R, EGF receptor(s); TGFα, transforming growth factor α; TNFα, tumor necrosis factor α; AB, Alcian blue; PAS, periodic acid–Schiff.

*To whom reprint requests should be addressed at: Cardiovascular Research Institute, Box 0130, University of California, San Francisco, CA 94143-0130.

PNAS is available online at www.pnas.org.
glycine, and 20% (vol/vol) methanol (pH 8.3). The proteins were then transferred electrophoretically to nitrocellulose membranes. The membranes were incubated with 5% fat-free skim milk in PBS containing 0.05% Tween 20 for 1 h and incubated with monoclonal mouse anti-EGF-R antibody (1:100, Calbiochem) at 4°C overnight. Bound antibody was visualized according to standard protocols for the avidin–biotin–alkaline phosphatase complex method (ABC kit, Vector Laboratories). As a positive control for EGF-R, cell lysates from A431 cells were used (18).

**Immunocytochemical localization of EGF-R and MUC5AC in NCI-H292 cells.** Cells grown on 8-chamber slides were fixed with 4% paraformaldehyde for 1 h. PBS containing 0.05% Tween 20, 2% normal goat serum, and Levamisol (2 mM) was used as diluent for the antibody. Cells were incubated with mouse mAb to EGF-R (1:250) or to MUC5AC (clone 45 M1, 1:100, New Markers, Fremont, CA) overnight at 4°C, and then washed 3 times with PBS to remove excess primary antibody. Cells were then incubated with biotinylated horse anti-mouse IgG (Vector Laboratories) at 1:200 dilution for 1 h at room temperature. Bound antibody was visualized according to standard protocols for avidin–biotin–alkaline phosphatase complex method.

**Mucin analysis.** Cells were fixed similarly to the immunocytochemical technique and stained with Alcian blue/periodic acid–Schiff (AB–PAS). MUC5AC protein was measured by using ELISA. Cell lysates were prepared with PBS at multiple dilutions, and 50 μl of each sample was incubated with bicarbonate–carbonate buffer (50 mM) at 4°C overnight. Bound antibody was visualized according to standard protocols for avidin–biotin–alkaline phosphatase complex method.

**Goblet-cell production.** Goblet-cell production was determined by counting epithelial cell nuclei over 2 mm of the basal lamina with an oil immersion objective lens (×1,000 magnification). The linear length of the basal lamina under each analyzed region of epithelium was determined by tracing the contour of the digitized image of the basal lamina. The epithelial cells were identified as described (20, 21). In brief, goblet cells are goblet to low columnar in shape, with abundant mucous-stained granules filling most of the cytoplasm. Pre-goblet cells contain smaller mucus-stained areas (<1/3 height in epithelium from basement membrane to luminal surface) or sparse granules stained with AB–PAS. Ciliated cells are recognized by their ciliated borders, lightly stained cytoplasm, and large, round nuclei. Nongranulated secretory cells are columnar in shape and extend from the lumen to the basal lamina. The cytoplasm stains light pink, and a few small, PAS-positive and AB-negative granules are observed in the cytoplasm. Basal cells are small, flattened cells with large nuclei located just above the basal lamina but not reaching the airway lumen.

**Quantification of goblet-cell production.** Goblet-cell production was determined by the volume density of AB–PAS-stained mucus glycoconjugates on the epithelial mucosal surface, by using a semiautomatic imaging system described elsewhere (22). We measured the AB–PAS-positive stained area and the total epithelial area and expressed the data as the percentage of the total area stained by AB–PAS. The analysis was performed with the public domain NIH IMAGE program (developed at the U.S. National Institutes of Health and available by anonymous FTP from zippy.nih.gov or on floppy disk from the National Technical Information Service, Springfield, VA, part number PB95–500195-G01).

**Immunohistochemical localization of EGF-R in rat epithelium.** The localization of EGF-R was examined by using immunohistochemical staining with an antibody to EGF-R (Calbiochem) in frozen sections of rat trachea. Immunostaining was performed similarly to the in vitro studies.

In situ hybridization. The cDNA for rat MUC5AC was generously provided by Carol Basbaum. A 320-bp cDNA fragment of rat MUC5AC was subcloned into the XbaI/HindIII vector. The cDNA was then transcribed in vitro using T7 RNA polymerase to generate the cRNA probe. The protocol for in situ hybridization was performed as described by Basbaum and colleagues (23, 24).
site of the transcription vector, pBluescript-SK(−) (Stratagene). The preparation of RNA probes and in situ hybridization were performed as described (20).

Statistics. All data are expressed as mean ± 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

TNFα Stimulates Production of EGF-R in NCI-H292 Cells. First, we determined whether NCI-H292 cells express EGF-R constitutively. Immunoblot analysis of cell lysates identified the presence of EGF-R protein in confluent cultures of NCI-H292 cells (Fig. 1A). Protein from A431 cells provided a positive control for EGF-R (Fig. 1A); these cells express EGF-R constitutively (18). Immunocytochemical analysis with an anti-EGF-R antibody revealed that most cells were positive, although some cells had more intense staining than the others (Fig. 1B). Northern blotting in NCI-H292 cells showed little expression of EGF-R mRNA constitutively, but TNFα up-regulated EGF-R mRNA at 12 h, accentuated at 24 h (Fig. 1C).

EGF-R Ligands Stimulate MUC5AC Protein and Gene Expression in NCI-H292 Cells. EGF-R are expressed constitutively in NCI-H292 cells; we assessed the ability of EGF-R ligands (EGF and TGFα) to induce mucous glycoconjugate production (assayed by AB–PAS staining; Fig. 2, upper column): Some control cells showed AB–PAS staining; incubation with either EGF or with TGFα increased PAS-positive staining; incubation with TNFα alone did not affect staining (data not shown). However, TNFα potentiated the stimulatory effect of EGF-R ligands on mucous glycoconjugate production (Fig. 2). Immunocytochemical analysis with an anti-MUC5AC antibody showed that the cytoplasm of a few control cells stained positive, similar to AB–PAS staining. Incubation with TNFα plus TGFα increased the staining for MUC5AC protein in a pattern similar to AB–PAS staining (Fig. 2, Lower). To quantify the MUC5AC protein production, an ELISA was performed: EGF alone or TGFα alone caused an ∼2-fold increase in MUC5AC production. Thus, EGF-R ligands induce the production of MUC5AC protein in NCI-H292 cells. TNFα alone caused little increase in MUC5AC, but TNFα potentiated the effect of EGF-R ligands (Fig. 3). To examine MUC5AC gene expression, Northern blotting was performed. MUC5AC gene was expressed at low levels in NCI-H292 cells treated with control medium. Incubation with EGF or TGFα increased MUC5AC gene expression beginning at 12 h and reaching a maximum at 24 h. TNFα alone had no effect on MUC5AC gene expression.

Fig. 1.1 Expression of EGF-R in cultured cells. (A) Immunoblotting of EGF-R in A431 and in NCI-H292 cells. Cells were examined after becoming confluent. Lysates were electrophoresed in 8% acrylamide gels and blotted with anti-EGF-R antibody. Molecular mass of marker protein is reported on the right. (B) Immunocytochemical analysis with anti-EGF-R antibody in cultures of NCI-H292 cells. At confluence, positive staining was seen in most of cells, and certain cells had more intense staining (arrows, right side). In the absence of the primary antibody, no staining was seen (left side). (Bar = 25 μm.) (C) Northern analysis of EGF-R in NCI-H292 cells was performed on total RNA extracted from confluent cultures incubated with TNFα (20 ng/ml) for 12 or 24 h. The RNA (10 μg) was electrophoresed on a formaldehyde-agarose gel, transferred to a nylon membrane, and hybridized with the 32P-labeled EGF-R cDNA probe. After hybridization, the membrane was washed and autoradiographed.

Fig. 2. AB–PAS staining of NCI-H292 cells for identification of mucous glycoconjugates (Upper) and immunocytochemical analysis for MUC5AC protein (Lower). Immunocytochemical analysis with an anti-MUC5AC antibody showed positive staining in only a few cells in the control state in a similar pattern to that of AB–PAS staining. However, incubation with TNFα (20 ng/ml) plus TGFα (25 ng/ml) increased both the PAS-positive staining and MUC5AC protein markedly. (Bar = 50 μm.)

Fig. 3. ELISA for MUC5AC in NCI-H292 cells. MUC5AC protein was measured as described in Methods. TNFα caused little expression of MUC5AC protein (8.7 ± 3.8%, n = 3); TGFα alone caused ∼100% increase in MUC5AC protein, an effect that was potentiated by coinubcation with TNFα (149.5 ± 21.7%, n = 6). The stimulatory effect of TNFα plus TGFα was inhibited by each of the three selective EGF-R tyrosine kinase inhibitors (BIBX1522, 2.1 ± 6.7%; tyrphostin AG1478, 1.0 ± 14.5%; A1, 5.5%; Compound 56, 0.0 ± 5.5%; tyrphostin AG1295) were without effect.
expression but greatly potentiated the stimulatory effect of EGF-R ligands; TGFα showed a greater effect than EGF (Fig. 4).

**EGF-R Tyrosine Kinase Inhibitors Prevent MUC5AC Gene and Protein Expression in NCI-H292 Cells.** To test the hypothesis that activation of EGF-R induces MUC5AC gene and protein expression, cells were incubated with various tyrosine kinase inhibitors. Pretreatment of NCI-H292 cells with selective kinase inhibitors for EGF-R (BIBX1522, AG1478, Compound 56) prevented the increased staining for MUC5AC protein that usually occurred with EGF-R ligands (Fig. 3). A selective tyrosine kinase inhibitor of platelet-derived growth factor (AG1295) and a negative control for tyrphostins (A1) were without effect (Fig. 3). On Northern analysis, BIBX1522 completely inhibited EGF-R tyrosine kinase in the induction of MUC5AC gene and protein expression in NCI-H292 cells.

**TNFα Stimulates EGF-R Production in Rat Airways.** In the control state, tracheal epithelium contained few EGF-R-positive cells (Fig. 5A, Left). However, intratracheal instillation of TNFα induced EGF-R protein in the tracheal epithelium (Fig. 5A, Right).

**Role of EGF-R Ligands in Mucous Glycoconjugate Production and MUC5AC Gene Expression in Rats.** In the control state, tracheal epithelium contained few goblet and pre-goblet cells. Intratracheal instillation of EGF-R ligands (EGF or TGFα) alone had no effect on goblet cell production (Table 1). However, when TNFα was given first, followed 24 h later by TGFα or by EGF (data not shown), the numbers of goblet and pre-goblet cells were increased markedly; the numbers of nongranulated secretory cells and basal cells decreased significantly, whereas there was no change in the total number of cells or in the number of ciliated cells (Table 1). In situ hybridization for MUC5AC gene showed no expression in control animals. When TNFα was instilled intratracheally, followed by EGF or TGFα, expression of MUC5AC was visible in the epithelium (data not shown). Thus, induction of EGF-R alone or stimulation by EGF-R ligands alone was insufficient to induce goblet-cell production. However, after the induction of EGF-R by TNFα, instillation of EGF-R ligands stimulated goblet-cell production markedly.

**DISCUSSION**

We investigated the role of EGF-R and its ligands in mucin production in airway epithelium by using NCI-H292 cells (23) and by using pathogen-free rats whose airways contain few goblet cells. TNFα induced expression of EGF-R in both NCI-H292 cells and rat-airway epithelial cells. After induction of EGF-R by TNFα, subsequent stimulation of EGF-R by its
Effects of mediators and ovalbumin sensitization on tracheal epithelial cells in rats. Cells were analyzed as described in Methods; five rats per group. Control airways and airways stimulated by TGFα (250 ng) alone contained few goblet and pre-goblet cells; TNFa (200 ng) followed by TGFα (250 ng) resulted in increased numbers of goblet (P < 0.001) and pre-goblet (P < 0.0001) cells, accompanied by a decrease of nongranulated secretory cells (P < 0.0001) and basal cells (P < 0.05). Sensitization of rats with ovalbumin (OVA) intraperitoneally (i.p.) had no effect on cell distribution, but when OVA was given i.p. followed by intratracheal (i.t.) instillation, the total number of epithelial cells increased significantly (P < 0.01), and there were increased numbers of goblet (P < 0.001) and pre-goblet (P < 0.001) cells. Nongranulated secretory cells decreased significantly (P < 0.01). In all conditions, the number of ciliated cells was unchanged.

The mechanism by which TNFa acted on MUC5AC gene and protein expression in NCI-H292 cells requires discussion. In our studies, TNFa alone had little effect on MUC5AC production, although one study reported that TNFa induces mucin MUC2 gene expression (24). Our results show that TGFα had a large effect on MUC5AC gene expression, and that the effect of TGFα was markedly potentiated by coincubation with TNFa. The effect of TNFa plus TGFα was greater than additive. A possible explanation for the potentiating effect of TNFa is the up-regulation of EGF-R. In fact, TNFa caused EGF-R gene expression in NCI-H292 cells; similar findings are reported in A431 cells (15), human pancreatic cancer cells (14), and epidermal keratinocytes (16). The fact that the inhibition of EGF-R tyrosine kinase completely blocked MUC5AC gene and protein expression caused by TNFa plus EGF-R ligands implicates the EGFR pathway in the TNFa–TGFα responses. Two other selective EGF-R tyrosine kinase inhibitors (AG1478 and C56) also inhibited MUC5AC protein production, similar to BIBX1522. However, a selective platelet-derived growth factor tyrosine kinase inhibitor (AG1295) and a negative control for tyrphostins (A1) were without effect, implicating EGF-R tyrosine phosphorylation as the signaling pathway inducing MUC5AC expression.

Present results also suggest a possible sequence for the evolution of goblet-cell production based on the expression of EGF-R: Stimulation with TNFa induced intense EGF-R staining of nongranulated secretory cells; their subsequent activation by EGF-R ligands caused progressive staining for mucus glycoconjugates, and the cells became “pre-goblet” and then “goblet” cells. Instillation of TNFa followed by EGF-R ligands induced goblet-cell production without altering the total number of epithelial cells, suggesting that EGF-R activation promoted selective cell differentiation (not proliferation). These findings suggest that goblet cells are derived from nongranulated secretory cells that express EGF-R and are stimulated by EGF-R ligands to produce mucins. In our study, sensitization caused goblet cell production associated with an increase of the total number of cells in airway epithelium, whereas instillation of TNFa and TGFα caused goblet cell production but not epithelial hyperplasia. One reason for the difference may be the duration after the stimulus. A second reason for the difference may be because of the presence of another mediator that causes epithelial cell proliferation in the sensitized animal, such as cysteinyi leukotrienes, which are increased in asthmatic airways (25). We show that EGF-R are not expressed in airway epithelium, whereas instillation of TNFa and TGFα caused goblet cell production but not epithelial hyperplasia.

Fig. 6. Effect of EGF-R tyrosine kinase inhibitor (BIBX1522) on production of goblet cells (expressed as % stained area of airway epithelium occupied by AB–PAS-positive stained cells). (A) Stimulation with TNFα (200 ng, 100 μl). Tracheal instillation of TNFa, followed by the EGF-R ligand TGFα, increased goblet-cell production significantly (n = 5; *, P < 0.0001), an effect that was inhibited by pretreatment with BIBX1522 (3–30 mg/kg, i.p.) dose-dependently (n = 5; P compared with TNFa followed by TGFα; †, P = 0.003; ††, P < 0.0001). (B) Ovalbumin sensitization. Animals given ovalbumin intraperitoneally (i.p.) only showed little AB–PAS-positive staining in bronchial epithelium. Animals first sensitized with ovalbumin (OVA) i.p., followed by three intratracheal (i.t.) instillations of OVA, showed a marked increase in AB–PAS-positive staining (n = 5; *, P < 0.0001). Pretreatment with BIBX1522 (10 mg/kg, i.p.) inhibited OVA-induced production of goblet cells (n = 5; ††, P < 0.0001).
remain. (i) The mechanism of expression of EGFR: TNFα is a likely candidate; it is produced by mast cells (26), neutrophils (27), and macrophages (28) and is elevated in bronchoalveolar lavage fluid obtained in subjects with symptomatic asthma (29); and (ii) The source of EGFR ligands: in asthmatics, epithelial cells are reported to express EGF (13); another possible source is leukocytes, because eosinophils (30), neutrophils, and monocytes (31) produce EGFR ligands. In our study, i.p. sensitization with ovalbumin did not cause goblet-cell hyperplasia, but airway instillation of ovalbumin in sensitized rats, which results in leukocyte recruitment (32), caused goblet-cell production. This result suggests a possible role in leukocytes as a source of EGFR ligands.

Previous studies showed that various stimuli such as ozone (33), sulfur dioxide (35), viruses (34), lipopolysaccharide (33, 34), and inflammatory stimuli to the EGFR system. Presently, there is no effective therapy for hypersecretory airway diseases. Our findings provide a mechanism and a strategy for therapy: by inhibiting EGFR activation, goblet-cell production is prevented. Inhibition of EGFR activation is a likely candidate; it is produced by mast cells (26), neutrophils (27), and macrophages (28) and is elevated in bronchoalveolar lavage fluid obtained in subjects with symptomatic asthma (29); and (ii) The source of EGFR ligands: in asthmatics, epithelial cells are reported to express EGF (13); another possible source is leukocytes, because eosinophils (30), neutrophils, and monocytes (31) produce EGFR ligands. In our study, i.p. sensitization with ovalbumin did not cause goblet-cell hyperplasia, but airway instillation of ovalbumin in sensitized rats, which results in leukocyte recruitment (32), caused goblet-cell production. This result suggests a possible role in leukocytes as a source of EGFR ligands.

Previous studies showed that various stimuli such as ozone (33), sulfur dioxide (34), viruses (34), lipopolysaccharide (33, 34), and inflammatory stimuli to the EGFR system. Presently, there is no effective therapy for hypersecretory airway diseases. Our findings provide a mechanism and a strategy for therapy: by inhibiting EGFR activation, goblet-cell production is prevented. Inhibition of EGFR activation is a likely candidate; it is produced by mast cells (26), neutrophils (27), and macrophages (28) and is elevated in bronchoalveolar lavage fluid obtained in subjects with symptomatic asthma (29); and (ii) The source of EGFR ligands: in asthmatics, epithelial cells are reported to express EGF (13); another possible source is leukocytes, because eosinophils (30), neutrophils, and monocytes (31) produce EGFR ligands. In our study, i.p. sensitization with ovalbumin did not cause goblet-cell hyperplasia, but airway instillation of ovalbumin in sensitized rats, which results in leukocyte recruitment (32), caused goblet-cell production. This result suggests a possible role in leukocytes as a source of EGFR ligands.

Hypersecretion is a major manifestation in many chronic inflammatory diseases of airways. Presently, there is no effective therapy to relieve the symptoms and to halt the progression of these diseases. Our findings provide a mechanism and a strategy for therapy: by inhibiting EGFR activation, goblet-cell production is prevented. Inhibition of EGFR activation is proposed as therapy in hypersecretory airway diseases. Proof of concept in humans will require testing in patients with hypersecretory diseases.

The work was supported in part by National Heart, Lung, and Blood Institute Program Project Grant HL-24136.