

Epidermal growth factor receptor signaling mediates regranulation of rat nasal goblet cells

Heung-Man Lee, MD, PhD, Lars Malm, MD, Karim Dabbagh, PhD, Trang Dao-Pick, Iris F. Ueki, Suil Kim, MD, PhD, Jae Jeong Shim, MD, and Jay A. Nadel, MD
San Francisco, Calif

Background: Mucus hypersecretion is a common response to inflammation in the lower airways and is a hallmark of chronic rhinitis.

Objective: The purpose of this study was to elucidate the mechanisms of regranulation (mucus production) of goblet cells in nasal epithelium.

Methods: Because neutrophils induce an epidermal growth factor (EGFR) cascade, we induced degranulation of goblet cells in rat nasal respiratory epithelium by means of intranasal inhalation of N-formyl-methionyl-leucyl-phenylalanine (fMLP), and we examined regranulation of the goblet cells and the role of EGFR inhibitors and neutrophils in the regranulation process.

Results: In the control state Alcian blue/periodic acid–Schiff and mucin MUC5AC staining was present. Degranulation was induced in the nasal septal epithelium 4 hours after intranasal inhalation of fMLP (10^{-7} mol/L); 48 hours later, goblet-cell regranulation was complete. In the control state EGFR protein staining was absent in the epithelium, but after fMLP-induced degranulation, EGFR protein was expressed. After pretreatment with BIBX1522, a selective EGFR tyrosine kinase inhibitor, fMLP-induced degranulation was unaffected, but goblet-cell regranulation was prevented completely.

Conclusion: These data suggest a role for the EGFR cascade in neutrophil-dependent production of goblet-cell mucins. Proving this theory will require the use of selective EGFR inhibitors in clinical studies of nasal hypersecretory states. (J Allergy Clin Immunol 2001;107:1046-50.)

Key words: Secretory cell growth, epidermal growth factor receptor cascade, epidermal growth factor receptor tyrosine kinase activation, nasal hypersecretion, mucin expression

Chronic nasal inflammatory diseases are associated with hypersecretion of mucus, mucin gene expression,¹ goblet-cell metaplasia,² and infiltration of inflammatory cells.³ Goblet-cell development is mediated by epidermal growth factor receptor (EGFR) in the lower airways in vivo and in vitro. Takeyama et al⁴ reported that an EGFR cascade regulates mucin production in airways. Lee et al⁵

Abbreviations used

AB/PAS: Alcian blue/periodic acid–Schiff
EGFR: Epidermal growth factor receptor
fMLP: N-formyl-methionyl-leucyl-phenylalanine
NGSC: Nongranulated secretory cell

reported that instillation of agarose plugs in rat airways causes goblet-cell metaplasia by activation of EGF receptors and that neutrophils play an important role in agarose-induced mucin production. Takeyama et al⁶ showed that activated neutrophils induce mucin expression through EGFR activation in human airway epithelial cells in vitro. Rat nasal epithelium normally contains goblet cells constitutively, but EGFR expression is absent in the epithelium. We hypothesize that the airways of these animals are not inflamed, that they are not actively degranulating, and thus that EGFR expression is downregulated. In a rat model we hypothesized that N-formyl-methionyl-leucyl-phenylalanine (fMLP), by recruiting neutrophils, degranulates goblet cells (by means of an action of elastase⁷) and also causes EGFR expression and activation, resulting in regranulation. To test this hypothesis, we degranulated goblet cells in rat nasal epithelium by using local delivery of fMLP, and we examined the role of EGFR in goblet-cell regranulation by administering a selective EGFR tyrosine kinase inhibitor (BIBX1522).

METHODS

Animals

The experimental animal protocol was approved by the Committee on Animal Research of the University of California, San Francisco. Specific pathogen-free male F344 rats (body weight, 200-230 g; Simonsen Lab, Gilroy, Calif) were used. The animals were housed in pathogen-free BioClean cages with environmentally controlled laminar flow hoods and had free access to sterile food and water.

Nasal tissue preparation

At various times after inhalation, rats were anesthetized with pentobarbital sodium (65 mg/kg administered intraperitoneally). The heart of the animal was exposed, a blunt-ended needle was inserted from the apex of the left ventricle into the ascending aorta, and the systemic circulation was perfused with 4% paraformaldehyde. An incision in the right atrium provided an outlet for the fixative. The eyes, lower jaws, skin, and musculature were removed, and the head was immersed in a large volume of the same fixative for 24 hours. After fixation, the head was decalcified with Surgipath (Decalcifier II; Surgical Medical Industries, Inc, Richmond, Ill) for

From the Cardiovascular Research Institute and the Departments of Medicine and Physiology, University of California, San Francisco, Calif.

Supported by private sources.

Received for publication November 30, 2000; revised February 13, 2001; accepted for publication February 15, 2001.

Reprint requests: Jay A. Nadel, MD, Cardiovascular Research Institute, Box 0130, University of California, San Francisco, CA 94143-0130.

Copyright © 2001 by Mosby, Inc.

0091-6749/2001 \$35.00 + 0 1/83/115140

doi:10.1067/mai.2001.115140

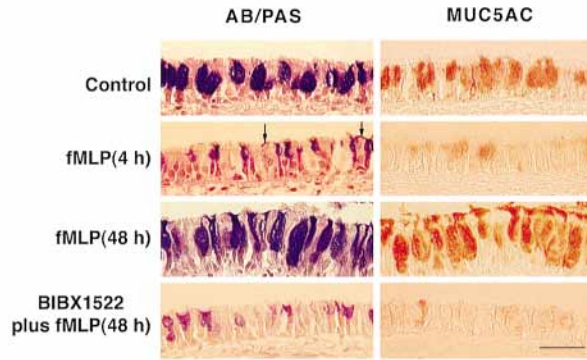


FIG 1. Photomicrographs showing the effect of fMLP alone or with BIBX1522 pretreatment on goblet-cell degranulation and regranulation in nasal epithelium stained with AB/PAS and on MUC5AC protein expression in pathogen-free rats (bar = 50 μ m).

4 to 5 days and rinsed in PBS. The nasal cavity was sectioned transversely at the level of the incisive papilla of the nasal palate. The frontal tissue block was embedded in glycol methacrylate (JB4 Plus; Polysciences, Inc, Warrington, Pa), and the other blocks were embedded in paraffin or in optimal cutting temperature compound (Sakura Finetek, USA, Inc, Torrance, Calif) for frozen sections. Five-micrometer thick sections were cut from the anterior surface of glycol methacrylate-embedded blocks and stained with either Alcian blue (pH 2.5)/periodic acid-Schiff (AB/PAS) to demonstrate acid and neutral glycoconjugates or 3,3'-diaminobenzidine (Sigma Chemical, St Louis, Mo) to visualize leukocytes that had migrated into the epithelium. Five-micrometer thick sections were cut from the anterior surfaces of frozen-embedded blocks and stained with AB/PAS or used for immunostaining for EGFR and MUC5AC.

Counting of neutrophils in nasal epithelium

Neutrophils were counted in high-power fields of the epithelial layer stained with 3,3'-diaminobenzidine at 400 $\times$ magnification. The number of neutrophils within the nasal septal epithelium (from the basement membrane to cell apices) was determined by counting the number of nuclear profiles per unit of basal lamina length.

Quantification of goblet-cell degranulation and regranulation

To assess goblet-cell degranulation and regranulation, we measured the volume density of AB/PAS-stained mucosubstances on the mucosa surface epithelium by using a semiautomatic imaging system according to a previously published method. We examined the stained slides with an Axioplan microscope (Zeiss, Thornwood, NY) connected to a video camera control unit (DXC7550MD; Sony Corp of America, Park Ridge, NJ). Images of the nasal epithelium were recorded in high-power fields with a phase-contrast lens at 400 $\times$ magnification by using an IMAXX Video System (PDI, Redmond, Wash). The intracellular mucin in superficial epithelial secretory cells appears as oval-shaped purple granules of varying size. We measured AB/PAS⁺-stained area and total epithelial area, and we expressed the data as the percentage of AB/PAS area to total area. The analysis was performed on a Macintosh 9500/120 computer (Apple Computer, Inc, Cupertino, Calif) with the public domain National Institutes of Health Image program (developed at the National Institutes of Health and available from the Internet by anonymous FTP or on floppy disk from the National Technical Information Service, Springfield, Va, part no. PB 95-500195GEI).

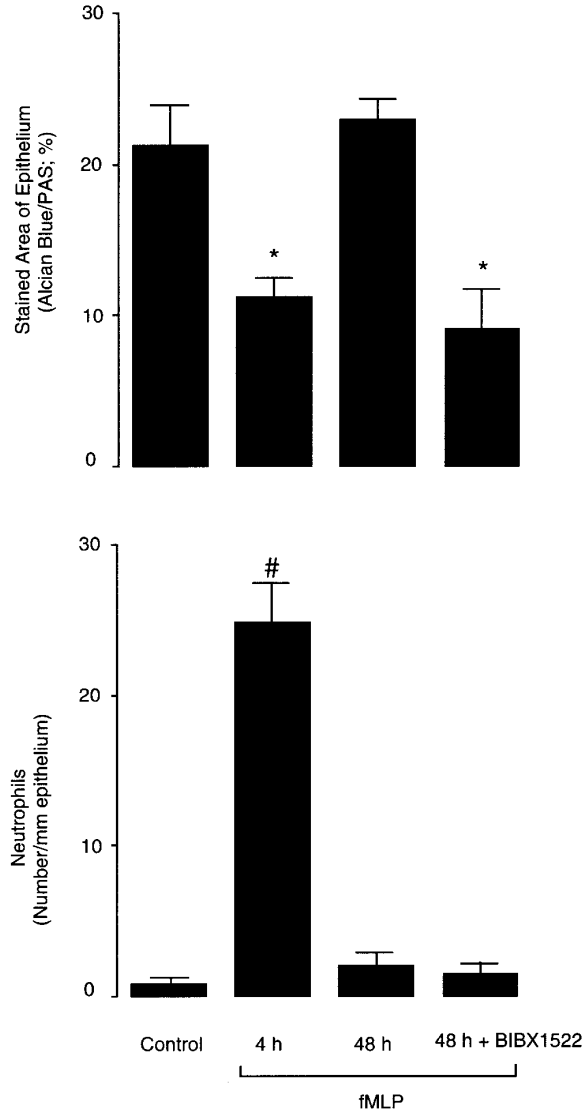


FIG 2. Effect of fMLP alone or with BIBX1522 pretreatment on goblet-cell degranulation and regranulation (expressed as percentage area of nasal epithelium stained with AB/PAS; *upper columns*) and on neutrophil recruitment in the nasal epithelium (expressed as the number of neutrophils per millimeter of epithelium; *lower columns*) in pathogen-free rats. Results are expressed as means $\pm$ SEM. * P < .001 and # P < .01 compared with control animals (n = 5 rats for each condition).

Immunolocalization of EGFR and MUC5AC protein

Paraffin sections from the paraformaldehyde-fixed nasal tissues were treated with 3% H₂O₂/methanol to block endogenous peroxide and were incubated with a mouse mAb to EGFR (Calbiochem, San Diego, Calif) or MUC5AC (NeoMarkers Inc, Fremont, Calif) for 1 hour at a dilution of 1:100. Immunoreactive EGFR or MUC5AC was visualized with the Vectastain Elite ABC kit (Vector Lab, Inc, Burlingame, Calif) by using 3,3'-diaminobenzidine tetrahydrochloride as a chromogen. Controls included the substitution of primary or secondary antibody with PBS.

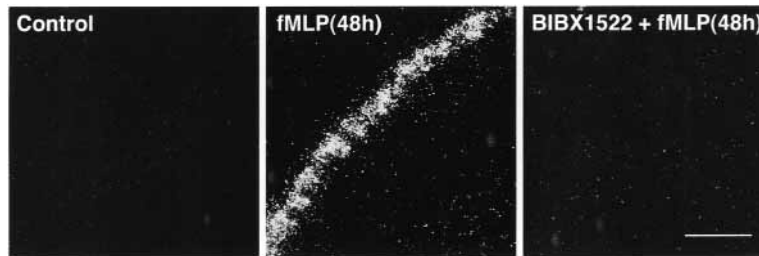


FIG 3. Effect of fMLP alone or with BIBX1522 pretreatment on *MUC5AC* mucin gene expression in rat nose 48 hours after intranasal inhalation of fMLP (10^{-7} mol/L, 5 minutes). The exposures are shown in the *dark field*. Results were similar in each of 3 studies (bar = 75 μ m).

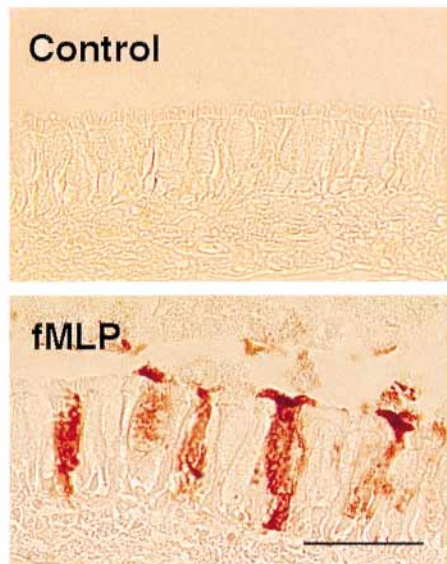


FIG 4. Immunohistochemical staining of EGFR in rat nasal epithelium in control rats 48 hours after intranasal inhalation of fMLP. EGFR⁺ staining was present in goblet cells but not in ciliated cells (fMLP). Results shown are typical of each of 3 studies (bar = 25 μ m).

Protocol of experiments

We studied pathogen-free rats, which normally have many goblet cells in the nasal septal epithelium. The animals were anesthetized with pentobarbital sodium (65 mg/kg administered intraperitoneally) and received fMLP (10^{-7} mol/L, Sigma) in pyrogen-free saline intranasally by means of aerosol spray for 5 minutes to determine the effect of aerosolized fMLP on goblet-cell degranulation and on neutrophil migration into nasal mucosa epithelium. Aerosol exposure was accomplished by placing the rats in a modified chamber connected to an ultrasonic nebulizer (PulmoSonic, DeVilbiss Co, Somerset, Pa) that generated an aerosol mist at rate of 0.3 mL/min. As controls, animals were given saline aerosol alone intranasally.

The rats were killed 48 hours after intranasal delivery of fMLP to study regranulation of nasal goblet cells after inhalation of fMLP aerosol.

To evaluate the effect of EGFR tyrosine kinase activation on goblet-cell regranulation, animals were pretreated with an EGFR tyrosine kinase inhibitor (BIBX1522, 15 mg/kg administered intraperitoneally; generously provided by Boehringer Ingelheim Pharma KG, Ingelheim, Germany) 30 minutes before the inhalation of fMLP. BIBX1522 administration was repeated twice a day until the animals were killed.

Statistical analysis

Results are expressed as means $\pm$ SEM. Data from AB/PAS-stained areas were normally distributed (ie, equal SDs). Therefore differences among groups were tested by using the parametric one-way ANOVA test. Because the data on neutrophil recruitment in the nasal epithelium were not normally distributed (ie, unequal SDs), differences among groups were tested by using the nonparametric Kruskal-Wallis test. When a difference was identified in ANOVA (AB/PAS) or in the Kruskal-Wallis (neutrophil recruitment) test, a Student-Newman-Keuls test was used. A probability of less than .05 for the null hypothesis was accepted as a statistically significant difference.

RESULTS

Effects of fMLP on goblet-cell degranulation and regranulation

In the control state the nasal epithelium contained a significant area of AB/PAS-stained goblet cells (Figs 1 and 2, control). Immunostaining for MUC5AC protein corresponded to the area of staining with AB/PAS (Fig 1, control). There was no evidence of active degranulation (no mucin staining) on the luminal surface (Fig 1, control). Mucin *MUC5AC* gene expression (Fig 3, control) and EGFR expression (Fig 4, control) were absent. We aerosolized fMLP (a neutrophil chemoattractant) because neutrophil recruitment and activation causes goblet-cell degranulation by means of an action of neutrophil elastase.⁷ In rats killed 4 hours after aerosolization of fMLP (10^{-7} mol/L for 5 minutes), the AB/PAS⁺ and MUC5AC⁺ staining was markedly decreased (Fig 1, fMLP, 4 hours; Fig 2, upper columns), and neutrophil recruitment was present in the airway epithelium (Fig 2, lower columns). AB/PAS staining was prominent on the nasal airway luminal surface (Fig 1, arrows; fMLP, 4 hours), confirming the morphologic data (Fig 2, upper columns) that showed a decrease in the AB/PAS-stained area of the epithelium. *MUC5AC* gene expression was still absent (data not shown).

In rats killed 48 hours after fMLP, the area of AB/PAS- and MUC5AC-immunopositive staining had returned to the control state (Fig 1, fMLP, 48 hours; Fig 2, upper columns), and *MUC5AC* gene expression was visible in the area occupied by the goblet cells (Fig 3, fMLP, 48 hours), but neutrophil recruitment was no longer present (Fig 2, lower columns). EGFR expression was now present in pregoblet and goblet cells (Fig 4, fMLP), suggesting that EGFR might be involved in fMLP-induced mucin expression.

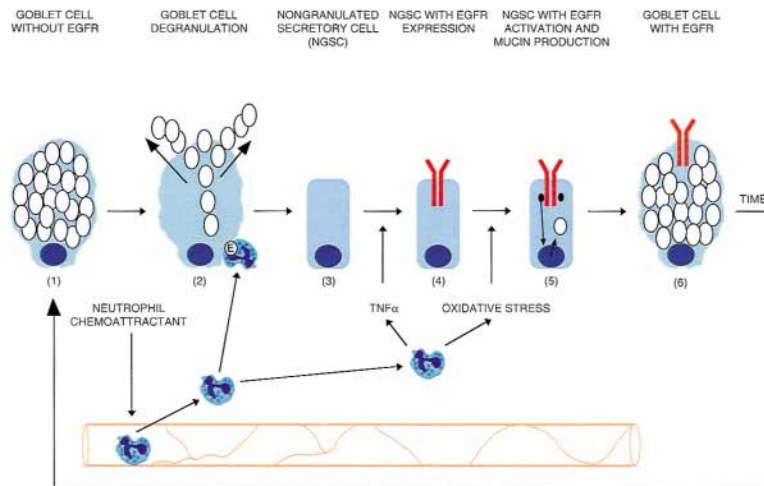


FIG 5. Hypothetical mechanism for goblet-cell degranulation and regranulation in rat nasal epithelium. (1) Nasal epithelium contains resting (nonsecreting) goblet cells without EGFR expression. (2) Chemoattractant causes neutrophil recruitment from the vascular space. Neutrophil elastase (*E*) moves to the surface of activated neutrophils, expressing elastase on their surface, and goblet cells results in degranulation. (3) After degranulation, the goblet cells become nongranulated secretory cells. (4) Neutrophils, on recruitment and activation, release $\text{TNF-}\alpha$, which induces EGFR expression in nongranulated secretory cells. (5) Activated neutrophils induce EGFR tyrosine kinase phosphorylation (indicated by *black dot*) through transphosphorylation by oxidative stress. Phosphorylation of downstream target molecules (including rat sarcoma protein, cytoplasmic serine threonine protein kinase, mitogen-activated protein kinase, and p44/p42 mitogen-activated protein kinase) results in a nuclear signal (*arrow from black dot*) leading to mucin synthesis (*small arrow from nucleus*). (6) Nongranulated secretory cells become goblet cells after mucin synthesis and storage has occurred. Resolution of the inflammatory process results in downregulation of the EGFR. The goblet cells become quiescent (resting cells) and are ready for another cycle of neutrophil-induced goblet-cell degranulation.

Role of EGFR tyrosine kinase phosphorylation in goblet-cell regranulation

In rats ($n = 5$) treated with the selective EGFR tyrosine kinase inhibitor BIBX1522 (15 mg/kg administered intraperitoneally), aerosolization of fMLP still caused goblet-cell degranulation (data not shown), but 48 hours later, the areas of AB/PAS staining and MUC5AC-immunopositive staining remained decreased (Figs 1 and 2, 48 hours and BIBX1522). Pretreatment with BIBX1522 also prevented fMLP-induced MUC5AC gene expression (Fig 3, BIBX1522 and fMLP).

DISCUSSION

Here we show that neutrophil recruitment into rat nasal epithelium (by fMLP) causes goblet-cell degranulation, confirming previous studies in the lower airways of rats that showed that degranulation is due to close contact between neutrophils and goblet cells caused by an action of neutrophil elastase.⁷ Furthermore, the present studies show that this degranulation is followed by regranulation, which is complete within 48 hours. Previous studies have shown that goblet-cell production of mucins is dependent on the expression and activation of EGFR when the epithelium is stimulated by a wide variety of stimuli.^{4-6,9,10} The lower airways of pathogen-free rats contain few or no goblet cells. Here we show that

control rat nasal epithelium contains a large number of goblet cells constitutively, but these goblet cells do not appear to be secreting, and they do not express EGFR. However, we found that fMLP-induced degranulation was followed by upregulation of EGFR and mucin gene expression and that a selective inhibitor of EGFR tyrosine kinase phosphorylation prevented fMLP-induced regranulation. From these studies, we conclude that control nasal goblet cells, which appear to be inactive, do not express EGFR; that neutrophil recruitment induces goblet-cell degranulation and regranulation; and that the process of regranulation appears to be dependent on EGFR activation.

Some goblet cells exist in the epithelium of the upper and lower airways of healthy human subjects, but such epithelium does not express EGFR^{11,12}; here we find that rat nasal epithelium behaves similarly. EGFR activation induces mucin gene and protein production in airways. We speculate that EGFR is downregulated in resting cells and that inflammation may upregulate mucin production rapidly. In pathogen-free rats, airway epithelium devoid of goblet cells is capable of producing well-developed, neutrophil-dependent, EGFR-dependent goblet cells in 48 to 72 hours.⁵

On the basis of the present findings, we suggest that EGFRs are expressed in inflammatory conditions and are downregulated when inflammation wanes. In fact, EGFR expression and activation may be an indication that an

inflammatory state is present. In the present study we show that neutrophil recruitment is capable of upregulating the EGFR cascade and subsequent production of mucins. Upregulation of EGFR occurs in inflammatory diseases, such as nasal polyps, presumably in the presence of active inflammation.^{11,12} Mucin production in the epithelium of other organs (eg, the stomach) also involves EGFRs,¹³ and understanding their regulation of EGFRs may provide novel insights into the pathogenesis of disease. On the basis of previous and present findings, we propose a sequence of events to explain EGFR-dependent goblet-cell production (Fig 5). It is hypothesized that goblet cells may remain inactive in the epithelium until an inflammatory stimulus (eg, viral, bacterial, or immunologic) causes neutrophil recruitment. When neutrophils are recruited to the airways by chemoattractants, they cause goblet-cell degranulation, involving a close-contact adhesive interaction between the neutrophils and goblet cells.⁷ Recruited neutrophils also induce mucin synthesis in airways by activating EGFRs through the production of oxidative stress.⁶ Neutrophils are also implicated in mucin production by cigarette smoke,¹⁰ mechanical wounding,⁵ and T_H2 cytokines.⁹ Neutrophils are recruited into the airways in diseases such as cystic fibrosis, bronchiectasis, and chronic bronchitis,^{14,15} and neutrophil chemoattractants are present in the airways in diseases associated with hypersecretion.¹⁶⁻¹⁸ In these diseases neutrophils in the airway epithelium could be responsible for the large number of goblet cells present in the epithelium and for the resulting degranulation and hypersecretion.

In some cancer cells EGFR activation leads to cell multiplication.¹⁹ Cell multiplication may also be involved in wound healing.²⁰ In normal airway epithelial cells, goblet cells appear to form from precursor cells in the epithelium. The mechanisms that differentiate these processes from one another remain a challenge. The present study implicates the EGFR system in nasal goblet-cell production. Inhibitors of EGFR tyrosine kinase phosphorylation may be useful in diseases associated with nasal hypersecretion. Proof of concept will require testing in patients with these diseases.

We thank Boehringer Ingelheim Pharma KG for providing BIBX1522 and Stanton Glantz, PhD, University of California San Francisco, for advice on the statistical analysis.

REFERENCES

1. Voynow JA, Selby DM, Rose MC. Mucin gene expression (MUC1, MUC2, and MUC5/SAC) in nasal epithelial cells of cystic fibrosis, allergic rhinitis, and normal individuals. *Lung* 1998;176:345-54.
2. Chapelain C, Coste A, Gilain L, Poron F, Verra F, Escudier E. Modified epithelial cell distribution in chronic airways inflammation. *Eur Respir J* 1996;9:2474-8.
3. Rowe-Jones JM, Trendell-Smith N, Shembekar M, Mackay IS. Polypoid rhinosinusitis in patients with host defence deficiencies: cellular infiltration and disease severity. *Rhinology* 1997;35:113-7.
4. Takeyama K, Dabbagh K, Lee HM, Agusti C, Lausier JA, Ueki IF, et al. Epidermal growth factor system regulates mucin production in airways. *Proc Natl Acad Sci U S A* 1999;96:3081-6.
5. Lee HM, Takeyama K, Dabbagh K, Lausier JA, Ueki IF, Nadel JA. Agarose plug instillation causes goblet cell metaplasia by activating EGF receptors in rat airways. *Am J Physiol Lung Cell Mol Physiol* 2000;278:L185-92.
6. Takeyama K, Dabbagh K, Shim JJ, Dao-Pick T, Ueki IF, Nadel JA. Oxidative stress causes mucin synthesis via transactivation of epidermal growth factor receptor: role of neutrophils. *J Immunol* 2000;164:1546-52.
7. Takeyama K, Agusti C, Ueki I, Lausier J, Cardell LO, Nadel JA. Neutrophil-dependent goblet cell degranulation: role of membrane-bound elastase and adhesion molecules. *Lung Cell Mol Physiol* 1998;275:L294-302.
8. Lou YP, Takeyama K, Grattan KM, Lausier JA, Ueki IF, Agusti C, et al. Platelet-activating factor induces goblet cell hyperplasia and mucin gene expression in airways. *Am J Respir Crit Care Med* 1998;157:1927-34.
9. Shim JJ, Dabbagh K, Ueki IF, Dao-Pick T, Burgel P-R, Takeyama K, et al. Interleukin-13 induces mucus production by stimulating epidermal growth factor receptors and by activating neutrophils. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L134-40.
10. Takeyama K, Jung B, Shim JJ, Burgel P-R, Dao-Pick T, Ueki IF, et al. Activation of epidermal growth factor receptors is responsible for mucin synthesis induced by cigarette smoke. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L165-72.
11. Burgel P-R, Escudier E, Coste A, Dao-Pick T, Ueki IF, Takeyama K, et al. Relation of epidermal growth factor receptor expression to goblet cell hyperplasia in nasal polyps. *J Allergy Clin Immunol* 2000;106:705-12.
12. Takeyama K, Fahy JV, Nadel JA. Relationship of epidermal growth factor receptors to airway goblet cell production. *Am J Respir Crit Care Med* 2001;163:511-6.
13. Ichikawa T, Endoh H, Hotta K, Ishihara K. The mucin biosynthesis stimulated by epidermal growth factor occurs in surface mucus cells, but not in gland mucus cells, of rat stomach. *Life Sci* 2000;67:1095-101.
14. Boat TF, Boucher RC. Cystic fibrosis. In: Murray JF, Nadel JA, editors. *Textbook of respiratory medicine*. 2nd ed. Philadelphia: Saunders; 1994. p. 1418-50.
15. Richman-Eisenstat JB, Jorens PG, Hebert CA, Ueki I, Nadel JA. Interleukin-8: an important chemoattractant in sputum of patients with chronic inflammatory airway diseases. *Lung Cell Mol Physiol* 1993;264:L413-8.
16. Keatings VM, Collins PD, Scott DM, Barnes PJ. Differences in interleukin-8 and tumor necrosis factor- α in induced sputum from patients with chronic obstructive pulmonary disease or asthma. *Am J Respir Crit Care Med* 1996;153:530-4.
17. Nocker RE, Schoonbrood DF, van de Graaf EA, Hack CE, Lutter R, Jansen HM, et al. Interleukin-8 in airway inflammation in patients with asthma and chronic obstructive pulmonary disease. *Int Arch Allergy Immunol* 1996;109:183-91.
18. Ozaki T, Hayashi H, Tani K, Ogushi F, Yasuoka S, Ogura T. Neutrophil chemotactic factors in the respiratory tract of patients with chronic airway diseases or idiopathic pulmonary fibrosis. *Am Rev Respir Dis* 1992;145:85-91.
19. Hackel PO, Zwick E, Prenzel N, Ullrich A. Epidermal growth factor receptors: critical mediators of multiple pathways. *Curr Opin Cell Biol* 1999;11:184-9.
20. Puddicombe SM, Polosa R, Richter A, Krishna MT, Howarth PH, Holgate ST, et al. Involvement of the epidermal growth factor receptor in epithelial repair in asthma. *FASEB J* 2000;14:1362-74.