Agarose plug instillation causes goblet cell metaplasia by activating EGF receptors in rat airways

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Agarose plug instillation causes goblet cell metaplasia by activating EGF receptors in rat airways. Am. J. Physiol. Lung Cell. Mol. Physiol. 278: L185-L192, 2000.—We hypothesized that foreign bodies in airways cause inflammation leading to goblet cell metaplasia. Instilled agarose plugs lodged in the bronchi of pathogen-free rats caused a time-dependent increase in Alcian blue-periodic acid-Schiff staining that was detected within 24 h and markedly increased at 72 h. Control bronchi contained no pregoblet or goblet cells, but plugged bronchi contained many pregoblet and goblet cells and a decrease in nongranulated secretory cells. In situ hybridization showed no expression of MUC5AC in control airways, but plugged airways showed a marked expression. Control bronchi showed sparse staining for epidermal growth factor receptor (EGFR) protein, but plugged bronchi showed intense EGFR staining in the epithelium. Pretreatment with an EGF tyrosine kinase inhibitor (BIBX1522) prevented Alcian blue-periodic acid-Schiff staining and MUC5AC gene expression in plugged bronchi. Pretreatment with tumor necrosis factor-α, neutralizing antibody or pretreatment with cyclophosphamide abolished plug-induced EGFR protein expression and goblet cell metaplasia. Thus instillation of agarose plugs induces profound goblet cell metaplasia by causing EGFR expression and activation.

epidermal growth factor receptor; tyrosine kinase; MUC5AC

A MARKED INCREASE IN THE NUMBER of goblet cells and hypersecretion of mucus occurs in asthma (1–3), bronchitis (27), bronchiectasis (9), and cystic fibrosis (3); this hypersecretion is postulated to be secondary to inflammation, which causes airway epithelial "remodeling." Wounding of the epithelium also leads to repair processes: a denuding mechanical injury to hamster tracheal epithelium is reported to produce an epithelium largely composed of cells with secretory characteristics (18); mechanical injury with a cotton swab resulted in excess mucus production (12); mechanical injury to the rat tracheal epithelium induced secretory cell markers in epithelium (26); and orotracheal intubation in horses resulted in abundant mucus secretion (11). Similar prolonged tracheal intubation in patients could lead to deleterious effects due to hypersecretion. However, the mechanism of hypersecretion is unknown. In the present study, we hypothesized that agarose plugs instilled into airways would lodge chronically in the bronchi without obstructing them. Furthermore, we suggested that resident plugs would cause inflammation, resulting in goblet cell metaplasia. We show that agarose plugs induce a marked local production of goblet cells as shown by Alcian blue-periodic acid-Schiff (PAS)-positive staining and mucin MUC5AC gene expression, associated with local recruitment of inflammatory cells. Pretreatment with the epidermal growth factor (EGF) receptor (EGFR) tyrosine kinase inhibitor BIBX1522 prevented agarose-induced mucin MUC5AC gene expression and goblet cell metaplasia. The results implicate EGFR activation in plug-induced goblet cell metaplasia.

METHODS

Animals. The experimental animal protocol was approved by the Committee on Animal Research (University of California, San Francisco). Specific pathogen-free, male Fischer 344 rats (230–250 g body wt; Simonsen Laboratories, Gilroy, CA) were used. The rats were housed in pathogen-free BioClean cages with environmentally controlled laminar flow hoods and had free access to sterile food and water.

Drugs. Drugs from the following sources were used: cyclophosphamide from Sigma (St. Louis, MO), methohexital sodium (Brevital, 25 mg/kg ip), and pentobarbital sodium (Nembutal) from Abbott Laboratories (North Chicago, IL). BIBX1522, a selective inhibitor of EGFR tyrosine kinase (generously provided by Boehringer Ingelheim, Ingelheim, Germany), was dissolved in the following solution: 2 ml of polyethylene glycol 400 (Sigma), 1 ml of 0.1 N HCl, and 3 ml of a 2% mannitol solution in water (pH 7.0).

Agarose plugs. To make the agarose plugs, we used a modification of the agar bead preparation (7). Agarose plugs (0.7- to 0.8-mm diameter) were made with 4% agarose type II medium electroendosmosis (Sigma) in sterile phosphate-buffered saline (PBS). To visualize the agarose plugs in tissue, a 3% suspension of Monastral blue B (Sigma) was added after the agarose was melted at 50°C.

Protocol of experiments. We studied pathogen-free rats because they normally have few goblet cells in the airways. The animals were anesthetized with methohexital sodium (Brevital, 25 mg/kg ip). The trachea was exposed aseptically with a midline cervical incision, and the agarose plugs were instilled into a bronchus via a 20-gauge Angiocath (Becton Dickinson, Sandy, UT) connected to polyethylene tubing (internal diameter 0.86 mm, outer diameter 1.27 mm; PE-90,
Clay Adams, Parsippany, NJ) threaded into the incised trachea. The polyethylene tube was bent at a 30° angle to allow selective instillation into the right bronchus. After instillation, the incision was closed with a suture.

To evaluate the role of EGFRs on agarose plug-induced goblet cell metaplasia, animals were treated with BIBX1522 (80 mg/kg ip) 1 h before instillation of the agarose plugs, and intraperitoneal injections were repeated daily (40 mg/kg ip twice a day). The animals were euthanized 24, 48, or 72 h after instillation of the agarose plugs.

To evaluate the role of tumor necrosis factor (TNF-α) in agarose plug-induced goblet cell metaplasia, the animals were treated with a TNF-α neutralizing antibody (Genzyme, Boston, MA). The first treatment (100 µl in 0.2 ml of saline ip) was given 1 h before instillation of the agarose plugs, and intraperitoneal injections were repeated daily. In addition, TNF-α neutralizing antibody was infused (10 µl/h) via an osmotic minipump (Alzet 2ML1, Alza, Palo Alto, CA) implanted subcutaneously.

To study the effect of neutrophils on agarose plug-induced goblet cell metaplasia, the rats were pretreated with cyclophosphamide (an inhibitor of bone marrow leukocytes but not of monocytes) (17). Cyclophosphamide (100 mg/kg ip) was given 5 days before instillation of the agarose plugs, and a second injection of cyclophosphamide (50 mg/kg ip) was given 1 day before instillation of the plugs.

To study the effect of macrophages on agarose plug-induced goblet cell metaplasia, the rats received 1 ml of rabbit anti-rat macrophage serum intraperitoneally (Inter-Cell Technologies, Hopewell, NJ) 1 h before instillation of the agarose plugs and then daily for 3 days before being euthanized.

Tissue preparation. At various times after agarose plug instillation, the rats were anesthetized with pentobarbital sodium (65 mg/kg ip), and the systemic circulation was perfused with 1% paraformaldehyde in diethyl pyrocarbonate-treated PBS at a pressure of 120 mmHg. The right lung was removed, and the right caudal lobe was used for histology. For frozen sections, tissues were removed and placed in 4% paraformaldehyde for 1 h and then replaced in 30% sucrose for cryoprotection overnight. The tissues were embedded in optimum cutting temperature compound (Sakura Finetek USA, Torrance, CA). For methacrylate sections, the tissues were placed in 4% paraformaldehyde for 24 h, then dehydrated with graded concentrations of ethanol, and embedded in methacrylate J B-4 (Polysciences, Warrington, PA). Tissue sections (4 µm thick) were stained with Alcian blue-PAS and counterstained with hematoxylin.

Morphometric analysis of bronchial epithelium. The percentage of Alcian blue-PAS-stained area of mucus glycoconjugates in the epithelium was determined with a semiautomatic image-analysis system according to previously published methods (20). The area of epithelium and Alcian blue-PAS-stained mucus conjugates within the epithelium was manually circumscribed and analyzed with the NIH Image program (developed at the National Institutes of Health and available from the National Technical Information Service, Springfield, VA). Data are expressed as the percentage of the total epithelial area occupied by the Alcian blue-PAS stain. To evaluate mucus secretion semiquantitatively, the percentage of the length of epithelial surface occupied by Alcian blue-PAS-stained mucus conjugates within the epithelium was manually circumscribed and analyzed with the NIH Image program (developed at the National Institutes of Health and available from the National Technical Information Service, Springfield, VA). Data are expressed as the percentage of the total epithelial area occupied by the Alcian blue-PAS stain.

Evaluation of cell types in methacrylate sections and cell analysis. The total number of epithelial cells 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 digitalized image of the basal lamina. The epithelial cells were identified as previously described (20). In brief, basal cells were identified as small flattened cells with a large nucleus located just above the basal lamina but not reaching the airway lumen. The cytoplasm stained darkly, and Alcian blue-PAS-positive granules were not present. Ciliated cells were recognized by their ciliated borders, lightly stained cytoplasm, and large, round nucleus. Nonciliated columnar cells (also called nongranulated “secretory” cells (5)) were columnar in shape and extended from the bronchial lumen to the basal lamina. After intrabronchial instillation of agarose plugs, “developing” goblet cells (pregoblet cells) were formed. These cells showed Alcian blue-PAS-positive staining, the granules were small, and the cells were not packed with granules; they contained smaller mucus-stained areas (greater than one-third height in epithelium from basement membrane to luminal surface) or sparsely and lightly Alcian blue-PAS-stained small granules. Cells of indeterminate type were defined as cells profiles lacking sufficient cytoplasmic characteristics for proper categorization.

Immunohistochemical localization of EGFRs and TNF-α. The presence of EGFRs and TNF-α was determined by immunohistochemical localization with a mouse antibody to the EGFR (Calbiochem, San Diego, CA) and a rabbit antibody to TNF-α (Genzyme, Cambridge, MA). Previously prepared 4-µm frozen sections were postfixed with 4% paraformaldehyde and treated with 0.3% H2O2-methanol. Sections were then incubated with an anti-EGFR (1:250 dilution) or anti-TNF-α antibody (1:500 dilution). Biotinylated horse anti-mouse IgG or anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA) followed by a streptavidin-peroxidase complex (ABC Kit, Vector Laboratories, Burlingame, CA) was used to visualize antigen-antibody complexes stained with 3,3′-diaminobenzidine tetrahydrochloride (Sigma). Negative control slides were incubated with either the primary or secondary antibody omitted and replaced with PBS.

In situ hybridization. 35S-labeled riboprobes were generated from a plasmid containing a 320-bp cDNA fragment of rat MUC5AC kindly provided by Dr. Carol Basbaum (University of California, San Francisco, CA). Sections were hybridized with 35S-labeled RNA probes (2,500–3,000 counts·min−1·µl−1 hybridization buffer) and washed under stringent conditions, including treatment with RNase A as previously described (20). After autoradiography for 7–21 days, the photographic emulsion was developed and the slides were counterstained with hematoxylin.

Counting of neutrophils in airway epithelium. Evaluation of neutrophil influx into the bronchi was performed by staining neutrophils with 3,3′-diaminobenzidine tetrahydrochloride, and the number of neutrophils was counted in the airway lumen and in the epithelium; the results are expressed as the number of stained cells per millimeter of basal lamina length.

Counting of macrophages in airway lumen. Evaluation of macrophage influx into the bronchial lumen was performed by staining methacrylate B-4 sections with Diff-Quik (American Scientific Products, McGaw Park, IL). Macrophages were identified morphologically and counted under a light microscope; the results are expressed as the number of stained cells per millimeter of basal lamina length.

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Bronchoalveolar lavage. To assess differential cell counts in each group of animals, lungs were lavaged five times with 3-ml aliquots of sterile PBS, the lavages were pooled, and the volume was measured. Cells in bronchoalveolar lavage (BAL) fluid were collected by spinning the fluid at 1,000 rpm for 10 min. Ten microliters of the cell suspension were then counted with a hemacytometer to determine the cell number in the BAL fluid. Differential cell counts were performed on cytospun preparations stained with Diff-Quik (American Scientific Products). Differential cell counts were obtained by sampling at least 200 cells on each cytospun slide.

Statistical analysis. Data are expressed as means ± SE. For statistical analysis, two-way or one-way analysis of variance followed by Student’s t-test was used as appropriate. A probability of <0.05 was considered a significant difference.

RESULTS

Effect of agarose plugs on airway epithelial structure goblet cell metaplasia. To determine whether agarose plugs affect the structure of airway epithelium, agarose plugs were instilled into the right bronchi in five pathogen-free rats. In control animals, the bronchial epithelium contained few goblet cells. However, in bronchi where agarose plugs resided, Alcian blue-PAS staining showed a time-dependent increase in goblet cell area, which was detectable as early as 24 h and was greatest 72 h after instillation (Fig. 1). At 24 h, agarose plugs produced significant increases in the number of pre-goblet and goblet cells, and at 48 h, more mature goblet cells were found (Table 1). At 72 h, agarose plugs continued to increase the number of goblet cells (P < 0.01); the number of basal and ciliated cells was not changed (P > 0.05). The total number of epithelial cells per millimeter of basal lamina 72 h after instillation was slightly but not significantly increased (P > 0.05; Table 1); the height of the epithelium (measured from basement membrane to luminal surface of epithelium) was increased from 16.0 ± 1.2 µm in control airways to 38.1 ± 9.1 µm 72 h after instillation of the plugs (P < 0.01; n = 5 rats).

In animals with agarose plugs, airways that did not contain plugs were similar to the airways of control animals (no inflammation and few goblet cells).

In the airway lumen of control animals, there was no Alcian blue-PAS staining. However, in airways with agarose plugs, positive staining was seen in the lumen, indicating that secretion of mucus glycoconjugates had occurred. In airways with agarose plugs, staining increased time dependently. The percentage of the total length of epithelium occupied by Alcian blue-PAS-positive staining in airways adjacent to the plugs increased from 0.1 ± 0.1% in control animals to 4.7 ± 1.4, 13.3 ± 0.7, and to 19.1 ± 0.7% at 24, 48, and 72 h, respectively (n = 5 rats/group). Furthermore, the agarose plugs denuded the epithelium of the plugged bronchi by 13.5 ± 2.3, 6.9 ± 2.4, and 5.1 ± 1.5% of the total area at 24, 48, and 72 h, respectively (n = 5 rats/group).

Effect of agarose plugs on mucin gene expression. In control rats, there was no detectable signal with the antisense probe of MUC5AC in bronchi (n = 4 rats/group). In bronchi where agarose plugs were instilled, there was a signal for MUC5AC that increased time dependently from 24 to 72 h (n = 4 rats). MUC5AC gene expression was found preferentially in cells that stained positively with Alcian blue-PAS (Fig. 3). No signals were detected in other cell types (e.g., smooth muscle, connective tissue). Sections examined with the MUC5AC sense probe showed no expression (Fig. 3).

Table 1. Effect of agarose plugs on distribution of bronchial epithelial cells in pathogen-free rats

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Values are means ± SE in no. of cells/mm basal lamina; n = 5 rats/group. Cells were analyzed as described in METHODS. Characterization was aided by Alcian blue-periodic acid-Schiff staining (which stains mucus glycoconjugates). Control cells contained few pre-goblet and goblet cells. Indeterminate cells lacked sufficient cytoplasmic characteristics for categorization. After instillation of agarose plugs, there was a time-dependent increase in no. of pre-goblet and goblet cells and a decrease in no. of nongranulated secretory cells compared with those in control rats: *P < 0.05; †P < 0.01.

Fig. 1. Effect of intrabronchial instillation of agarose plugs on percentage of Alcian blue-periodic blue-Schiff (PAS)-stained area of epithelium without (open bars) and with (solid bars) epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor BIBX1522 (80 mg·kg⁻¹·day⁻¹ ip) pretreatment in pathogen-free rats (n = 5/group). Rats were euthanized 24, 48, or 72 h after instillation of plugs. Values are means ± SE. Significantly different from control animals: *P < 0.05; **P < 0.01. Significantly different from BIBX1522-treated group euthanized at the same time: †P < 0.05; ††P < 0.01.
Effect of agarose plugs on EGFR expression in airway epithelium. In control animals (n = 5; Fig. 4), immunostaining with an antibody to EGFR showed sparse staining in the epithelium. However, after instillation of the agarose plugs, the epithelium adjacent to the agarose plugs showed EGFR-positive staining in cells that stained positively with Alcian blue-PAS (Fig. 4). The staining pattern for EGFRs paralleled the staining for MUC5AC and Alcian blue-PAS (n = 5 animals).

Effect of agarose plugs on TNF-α protein expression. In control rats, there was little TNF-α expression in the airway epithelium and lumen. Agarose plug instillation significantly increased TNF-α expression in the epithelium and infiltrating inflammatory cells from 1 to 3 days compared with that in control cells (Fig. 5).

Effect of EGFR tyrosine kinase inhibitor on agarose plug-induced goblet cell metaplasia and mucin gene expression. In the present studies, instillation of agarose plugs resulted in the expression of EGFRs in the cells that produce mucus. To test the hypothesis that activation of EGFR induces MUC5AC gene expression and mucus glycoconjugate production after instillation of agarose plugs, an EGFR tyrosine kinase inhibitor (BIBX1522) was injected intraperitoneally into the rats. BIBX1522 markedly inhibited the agarose plug-induced Alcian blue-PAS-stained area of epithelium at 24, 48, and 72 h (n = 5 animals/group; Figs. 1 and 6). It also completely inhibited the expression of the MUC5AC gene at 72 h after plug instillation (Fig. 3).

Fig. 2. Local effect of intrabronchial instillation of agarose plugs on epithelial production of mucus glycoconjugates (stained with Alcian blue-PAS; A) and on infiltration of inflammatory cells (stained with diaminobenzidine [DAB]; B). Low-power fields show cross sections of entire plugged bronchus. Original magnification ×5; bars, 100 µm. Insets: magnified fields showing goblet (A) and inflammatory (B) cells. Original magnification, ×20; bars, 50 µm.

Fig. 3. Effect of EGFR tyrosine kinase inhibitor BIBX1522 (80 mg·kg⁻¹·day⁻¹ ip) on agarose plug-induced mucus glycoconjugate production (expressed as Alcian blue-PAS-positive staining; top) and MUC5AC mRNA expression (middle and bottom) at 72 h in pathogen-free rats. Agarose plugs alone (left) caused Alcian blue-PAS-positive staining in epithelium and MUC5AC mRNA expression (MUC5AC antisense). Pretreatment with EGFR tyrosine kinase inhibitor BIBX1522 prevented Alcian blue-PAS staining and MUC5AC expression in plugged bronchi (right). MUC5AC sense probe showed no expression (bottom). Arrows, location of epithelial basal lamina. Results were confirmed in 4 animals.
Effect of TNF-\(\alpha\) neutralizing antibody on agarose plug-induced goblet cell metaplasia and EGFR protein expression. TNF-\(\alpha\) induces EGFR protein expression in the airway epithelium of pathogen-free rats (25) as well as in other cells (26), and TNF-\(\alpha\) is expressed in the airway epithelium and inflammatory cells in loci of agarose plugs (Fig. 4). Therefore, we examined the effect of the pretreatment of rats with a TNF-\(\alpha\) neutralizing antibody on agarose plug-induced goblet cell metaplasia. In animals pretreated with the TNF-\(\alpha\) neutralizing antibody (\(n=5\)), agarose plugs no longer stimulated EGFR protein expression or the production of Alcian blue-PAS positively stained (goblet) cells (Fig. 5).

Inflammatory cell recruitment by agarose plugs. It was noted that agarose plugs cause epithelial damage and inflammatory cell infiltration. Various inflammatory cells can produce both TNF-\(\alpha\) (4, 10, 15, 21, 30) and EGFR ligands (6). Both EGFR and its ligands are involved in the EGFR cascade that leads to goblet cell metaplasia (28). We evaluated the role of leukocytes and macrophages in agarose plug-induced effects in two ways. First, we examined cells in BAL fluid. In control rats, macrophages were the predominant cells recovered (\(n=5\); Fig. 7). After instillation of agarose plugs, the number of macrophages increased (\(P<0.05\)), and significant numbers of neutrophils appeared in the BAL fluid (\(P<0.01\); Fig. 7). The number of lymphocytes was unchanged.

Infiltrating cells were also evaluated in tissue sections. Airways without agarose plugs contained few neutrophils, but airways containing plugs showed the

![Fig. 4. Effect of intrabronchial instillation of agarose plugs on staining for mucus glycoconjugates (left) and on expression of EGFR protein (right) in pathogen-free rats. In control airways, staining with Alcian blue-PAS and anti-EGFR antibody was negative (control), but adjacent to agarose plugs, Alcian blue-PAS and EGFR staining were positive in epithelium (plug alone). After pretreatment with a tumor necrosis factor (TNF)-\(\alpha\) neutralizing antibody (plug plus anti-TNF-\(\alpha\)) or cyclophosphamide (plug plus cyclophosphamide), agarose plug-induced Alcian blue-PAS and EGFR staining were inhibited. Results were confirmed in 5 animals. Bar, 50 \(\mu\)m.](http://ajplung.physiology.org/)

![Fig. 5. Immunohistochemical staining of TNF-\(\alpha\) in rat bronchial tissue. Brown color is positive staining for TNF-\(\alpha\). In control rats, little expression of TNF-\(\alpha\) was observed in epithelium. Agarose plug instillation (bottom) increased expression of TNF-\(\alpha\) in epithelial and inflammatory cells in airway. Bar, 25 \(\mu\)m.](http://ajplung.physiology.org/)

![Fig. 6. Percentage of Alcian blue-PAS-stained area of epithelium in bronchi of pathogen-free rats treated with various drugs. Agarose plugs were instilled intrabronchially, and rats were euthanized 72 h later. Values are means \(\pm\) SE. In animals pretreated with EGFR tyrosine kinase inhibitor BIBX1522 (80 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) day\(^{-1}\)), TNF-\(\alpha\) neutralizing antibody, or cyclophosphamide, Alcian blue-PAS-stained area of epithelium decreased significantly compared with that in animals with plug alone. Values are means \(\pm\) SE for 5 rats/group. Significantly different from plug alone: *\(P<0.05\); **\(P<0.01\).](http://ajplung.physiology.org/)
Fig. 7. Cellular composition of bronchoalveolar lavage fluid recovered from lungs of pathogen-free rats. Values are means ± SE; n = 5 rats/group. In control animals, cells were predominantly macrophages (solid bars). One day after instillation of agarose plugs, no. of macrophages increased and neutrophils (open bars) appeared; lymphocytes (bars too small to visualize) were unchanged. In rats pretreated with cyclophosphamide, no. of macrophages was similar to that in animals with plug alone, but neutrophil recruitment was suppressed. Significantly different from control group: *P < 0.05; **P < 0.01. #Significantly different from plug plus cyclophosphamide pretreatment, P < 0.01.

presence of neutrophils both in the epithelium and in the lumen (Fig. 2). The number of neutrophils in the airway lumen was 0.2 ± 0.2, 42.4 ± 7.1, 40.7 ± 7.7, and 20.1 ± 7.2/mm basal lamina in control airways and at 24, 48, and 72 h after instillation of plugs, respectively (P < 0.05; n = 5 rats/group). In addition, the number of neutrophils in the airway epithelium was 1.3 ± 0.4, 15.6 ± 2.6, 14.9 ± 1.4, and 14.8 ± 2.6 cells/mm basal lamina in control and at 24, 48, and 72 h after instillation of plugs, respectively (P < 0.01; n = 5 rats/group).

Effect of cyclophosphamide on agarose plug-induced neutrophil recruitment, goblet cell metaplasia, and EGFR protein expression. In cyclophosphamide-treated rats, blood neutrophils were depleted (neutrophil count in venous blood after cyclophosphamide, 1.8 ± 0.5%; n = 5 rats), and plug-induced neutrophil recruitment in BAL fluid was inhibited (Fig. 7). The number of neutrophils in the airway lumen (2.6 ± 0.3/mm basal lamina) and in the epithelium (0.8 ± 0.2/mm) also decreased significantly at 24 h. Cyclophosphamide also inhibited agarose plug-induced expression of EGFR protein (Fig. 4) and goblet cell metaplasia (Fig. 6). These results implicate neutrophils in plug-induced goblet cell metaplasia.

Effect of anti-rat macrophage serum on agarose plug-induced expression of EGFR protein and goblet cell metaplasia. Anti-rat macrophage serum significantly decreased the number of macrophages in the airway lumen around the agarose plugs (plug alone vs. anti-rat macrophage serum-treated rats, 40.1 ± 7.2 vs. 3.1 ± 0.5/mm; P < 0.01). Anti-rat macrophage serum pretreatment did not decrease the expression of EGFR protein (data not shown). In addition, instillation of anti-rat macrophage serum did not significantly decrease Alcian blue-PAS staining in goblet cell areas (11.3 ± 1.2%; P > 0.05).

**DISCUSSION**

We examined the effect of instillation of agarose plugs on goblet cell metaplasia in airways of pathogen-free rats, which have very few goblet cells in the control state (16). Epithelial cells in bronchi of control animals and in bronchi of animals in which plugs were instilled in other airways did not stain for mucous glycoconjugates. Instillation of agarose plugs resulted in a profound, time-dependent increase in the goblet cell area of the bronchial epithelium only adjacent to the instilled plugs, which was detectable within 24 h and was greatest ~72 h after instillation. The total cell number and the number of basal and ciliated cells did not change, but the number of goblet cells increased and the number of nongranulated secretory cells decreased time dependently after agarose plug instillation (Table 1). These results suggest that goblet cell metaplasia was the result of the conversion of nongranulated secretory cells to goblet cells.

Rat airway goblet cells are reported to express the MUC5AC gene (20). In the present study, control bronchi did not express the MUC5AC gene, but airways obstructed by plugs or adjacent to the plugs, which stained positively with Alcian blue-PAS, expressed the MUC5AC gene, suggesting that the MUC5AC gene is involved in agarose plug-induced mucus production. These results indicate that agarose plugs induce the expression of mucin genes and the production of mucous glycoconjugates in selected cells in rat airways.

Next, we examined the mechanism of goblet cell metaplasia induced by agarose plugs. Takeyama et al. (28) described an EGFR cascade that results in goblet cell growth. EGFRs are not normally expressed in the airway epithelium of pathogen-free rats but are induced by TNF-α. In the presence of EGFRs in the epithelium, instillation of EGFR ligands (EGF or transforming growth factor-α) resulted in an increase in mucin gene and protein expression. A selective inhibitor of EGFR tyrosine kinase (BIBX1522) completely inhibited these responses (28), implicating EGFR signaling (14) in goblet cell metaplasia. However, disease-induced goblet cell metaplasia was not investigated. Here, we studied the effect of BIBX1522 on agarose plug-induced goblet cell metaplasia. BIBX1522 inhibited agarose plug-induced production of mucus glycoconjugates and MUC5AC gene expression. These results implicate an EGFR cascade in agarose plug-induced goblet cell metaplasia.

We studied the mechanisms by which the EGFR cascade causes goblet cell metaplasia with agarose plugs. First, we studied the expression of EGFR protein in the bronchial epithelium. Control airways did not stain for EGFR, but airways containing agarose plugs showed selective, time-dependent positive staining for EGFR. Positively stained cells included nongranulated...
secretory, pregoblet, and goblet cells. Thus agarose plugs induced EGFR protein expression.

EGFRs are not constitutively expressed in pathogen-free rats, but it is known that TNF-α is capable of inducing EGFRs in the airway epithelium (28) and in other cells (29). In the present study, we showed that agarose plugs cause the release of TNF-α in the airway epithelium and inflammatory cells, thus inducing EGFRs, so we pretreated rats with a neutralizing antibody to TNF-α. This treatment prevented agarose plug-induced goblet cell metaplasia, implicating TNF-α in agarose plug-induced EGFR expression.

Several cell types are known to produce TNF-α, including epithelial cells (28), mast cells (4), neutrophils (15, 30), eosinophils (10), and macrophages (21). Because neutrophils were increased in BAL fluid and in airway epithelial tissue after instillation of agarose plugs, we studied the effect of leukocyte depletion on agarose-plug-induced goblet cell metaplasia. Cyclophosphamide, a drug that selectively depresses leukocyte production (13), prevented neutrophil recruitment into airway lavage fluid and into the airway epithelium after the instillation of agarose plugs. The pretreatment prevented agarose plug-induced EGFR expression and goblet cell metaplasia. Macrophages were also increased after the introduction of agarose plugs, but cyclophosphamide did not inhibit macrophage recruitment (8). Anti-rat macrophage serum did not inhibit EGFR expression or goblet cell metaplasia. These results implicate neutrophils in agarose plug-induced goblet cell metaplasia. Macrophages could play a role in goblet cell metaplasia under other conditions. The fact that cyclophosphamide also decreased EGFR protein expression after agarose plugs suggests that neutrophils contribute, at least in part, to the EGFR expression in this inflammatory condition.

Neutrophils are also capable of producing the EGFR ligands EGF and transforming growth factor-α (21). In addition, epithelial cells (2) are sources of EGFR ligands, and there was striking denudation of epithelium adjacent to the agarose plugs. Thus the epithelium could be an important potential source of both TNF-α and EGFR ligands.

The mechanical and chemical changes in the airways induced by agarose plugs that cause goblet cell metaplasia are unknown. The agarose plugs resided in conducting airways, but they did not completely occlude them; they caused areas of epithelial denudation, perhaps from mechanical irritation in the area of the plugs. It is reasonable to hypothesize that the effective stimulus is related to movement of the plugs during breathing, with subsequent epithelial abrasion. Mechanical injury to airway epithelium (12, 18, 26) is reported to cause hypersecretion. These prior studies lend credence to the hypothesis that mechanical trauma to the airway epithelium leads to hypersecretion.

Orotracheal intubation is reported to result in abundant mucus secretion in horses (11). Chronic intubation in patients could cause mucus hypersecretion and could be responsible for mucus plugging. Inhibitors of EGFR tyrosine kinase could serve to prevent mucus hypersecretion after tracheal intubation.

Epithelial damage is a common finding in studies of patients even with mild asthma, and the damage is increasingly related to worsening of clinical symptoms (19). In a previous study, Takeyama et al. (28) showed that intratracheal instillation of ovalbumin results in goblet cell metaplasia. Pretreatment with an EGFR tyrosine kinase inhibitor prevented goblet cell production, implicating EGFR activation in the goblet cell metaplasia. We suggest that epithelial damage produced by the allergic response induces EGFR activation, which results in abnormal goblet cell production. Pathogen-free rats normally express few EGFRs, but in sensitized animals, goblet cells stain positively for EGFRs (28). Recently, immunocytochemical staining for EGFRs has been found to be elevated in the airway epithelium of asthmatic subjects compared with that in normal controls; the authors did not localize EGFRs to secretory cells (2). They suggested that EGFR expression could be related to the "airway remodeling" that occurs in asthma. Three recent abstracts also implicate EGFRs as a regulator of bronchial epithelial repair. Polosa et al. (22) reported EGFR immunostaining in the epithelium, strongest between the apical surface of basal cells and the basolateral surface of columnar cells. Puddicombe et al. (23) showed that in scraped monolayers of bronchial epithelial cells, epithelial repair is regulated by EGFRs, and Richter et al. (24) reported that oxidant stress-induced DNA synthesis is modulated by EGFR in bronchial epithelial cells. These studies implicate EGFRs in epithelial damage, repair, and wound closure. Our findings implicate EGFR activation in a different response, specifically involving goblet cell metaplasia. Thus we suggest that mechanical epithelial damage and epithelial injury in asthma may involve a similar (EGFR) cascade, resulting in abnormal growth of epithelial secretory cells. This provides a plausible mechanism for the hypersecretion that occurs in fatal cases of acute asthma (1).

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REFERENCES


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