# Matrix Metalloproteinase-14 Mediates a Phenotypic Shift in the Airways to Increase Mucin Production

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*Rationale*: **Induced mainly by cigarette smoking, chronic obstructive pulmonary disease (COPD) is a global public health problem characterized by progressive difficulty in breathing and increased mucin production. Previously, we reported that acrolein levels found in COPD sputum could activate matrix metalloproteinase-9 (MMP9).**

*Objectives*: **To determine whether acrolein increases expression and activity of MMP14, a critical membrane-bound endopeptidase that can initial a MMP-activation cascade.**

*Methods*: **MMP14 activity and adduct formation were measured following direct acrolein treatment. MMP14 expression and activity was measured in human airway epithelial cells. MMP14 immunohistochemistry was performed with COPD tissue, and in acrolein- or tobacco-exposed mice.**

*Measurements and Main Results*: **In a cell-free system, acrolein, in concentrations equal to those found in COPD sputum, directly adducted cysteine 319 in the MMP14 hemopexin-like domain and activated MMP14. In cells, acrolein increased MMP14 activity, which was inhibited by a proprotein convertase inhibitor, hexa-D-arginine. In the airway epithelium of COPD subjects, immunoreactive MMP14 protein increased. In mouse lung, acrolein or tobacco smoke increased lung MMP14 activity and protein. In cells, acrolein-induced MMP14 transcripts were inhibited by an epidermal growth factor receptor (EGFR) neutralizing antibody, EGFR kinase inhibitor, metalloproteinase inhibitor, or mitogen-activated protein kinase (MAPK) 3/2 or MAPK8 inhibitors, but not a MAPK14 inhibitor. Decreasing the MMP14 protein and activity *in vitro* by small interfering (si)RNA to MMP14 diminished the acrolein-induced MUC5AC transcripts. In acrolein-exposed mice or transgenic mice with lung-specific transforming growth factor-α (an EGFR ligand) expression, lung MMP14 and MUC5AC levels increased and these effects were inhibited by a EGFR inhibitor, erlotinib.**

*Conclusions*: **Taken together, these findings implicate acrolein-induced MMP14 expression and activity in mucin production in COPD.**

**Keywords:** cigarette smoke; acrolein; erlotinib; mucous cell metaplasia; chronic obstructive pulmonary disease

A leading cause of morbidity (>14 million cases) and mortality (>110,000 deaths/yr) in the United States (1), chronic obstructive pulmonary disease (COPD) is marked by excessive mucin production, chronic cough, shortness of breath, and labored breathing (2–4). The pathogenesis of COPD involves proteinase/antiproteinase imbalance that leads to disruption of the alveolar structure (emphysema) and alteration of the airway architecture (bronchitis) (3, 5), the latter is marked by decreased ciliated and Clara cells and increased mucin-producing cells. The etiology of COPD has been studied extensively and it is clearly linked to cigarette smoking and other environmental exposures (2–4, 6). Cigarette smoke contains numerous irritants but none stronger than acrolein (7–10), a potent inducer of excessive mucin production in laboratory animals (11–13). Excessive mucin production in more advanced COPD is associated with rapid declines in lung function and more frequent exacerbations (including hospitalization and death) (5, 14).

In the lung, the major proteinases include the matrix metalloproteinases (MMPs) and a disintegrin and metallopeptidase domain proteins (including ADAM17 [a disintegrin and metalloproteinase domain-17], also called TACE [tumor necrosis factor-α converting enzyme]) (15–18). Secreted MMPs are typically inactive zymogens (pro-MMPs) and are activated through initial cleavage by the other MMPs or serine endopeptidases (including neutrophil elastase) and subsequent autocatalytic cleavage. However, certain pro-MMPs lack sequences susceptible to proteolytic activation (19) and are activated by the membrane-bound MMP14 (also known as membrane type 1-MMP), an event that triggers an MMP activation cascade (20, 21). Unlike secreted MMPs, MMP14 is activated by proprotein convertases in the trans-Golgi network, allowing cell-specific control of secreted MMP activation. The cell surface localization of MMP14 permits

## AT A GLANCE COMMENTARY

### Scientific Knowledge on the Subject

Acrolein is a component of cigarette smoke and also can be endogenously generated in the airways of persons with chronic obstructive pulmonary disease (COPD).

### What This Study Adds to the Field

Low-level acrolein concentrations (equivalent to those present in COPD sputum) activated and increased matrix metalloproteinase-14 (MMP14) transcripts, protein, and activity. MMP14 immunostaining increased in the airway epithelium of subjects with COPD. Inhibition of MMP14 induction, by epidermal growth factor receptor kinase inhibitors, reduced acrolein-induced mucin levels in mouse lung. Thus, local pharmacological inhibition of MMP14 in the airway epithelium could be useful in the treatment of COPD-related mucin overproduction.

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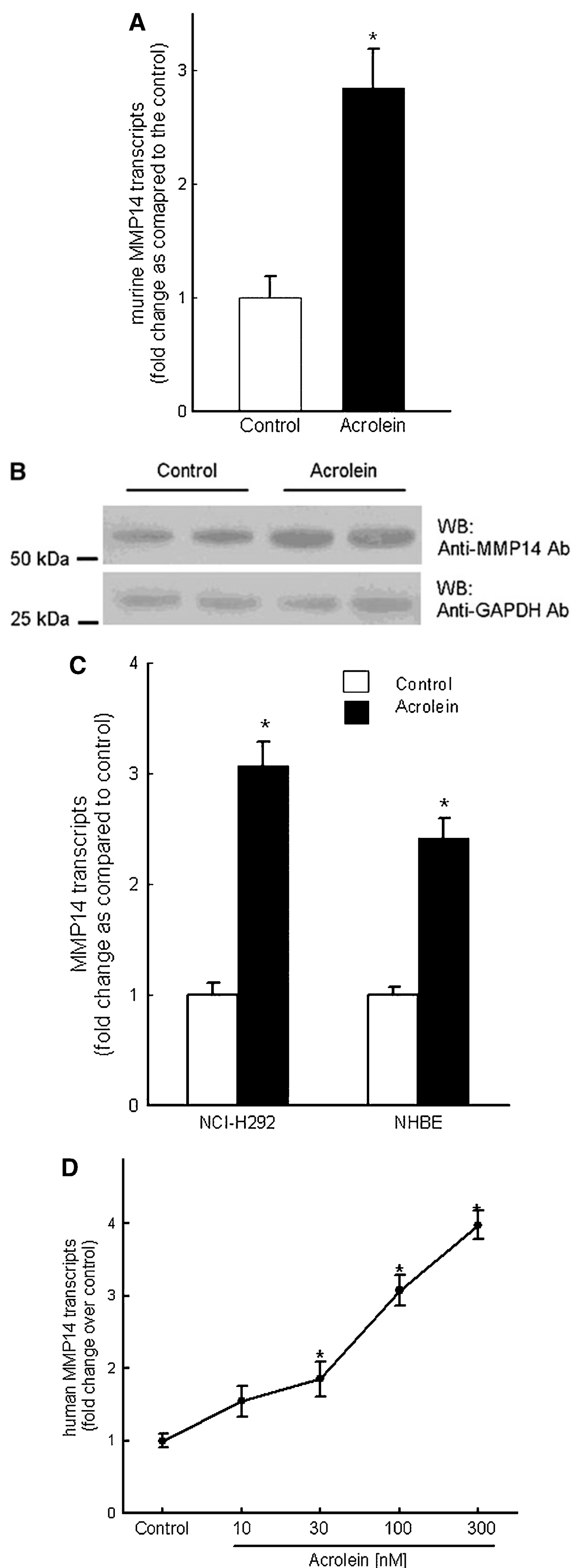


*Figure 1.* Acrolein increases matrix metalloproteinase-14 (MMP14) transcripts and protein levels. (*A*) Lung MMP14 transcript levels increased in FVB/NJ mice exposed to acrolein compared with control mice. FVB/NJ mice were exposed to acrolein (2.0 ppm × 6 h/d × 5 d/wk × 4 wk) or filtered air (control mice) and lung MMP14 levels were measured by quantitative real-time polymerase chain reaction (qRT-PCR). The results are expressed as fold change in the level of transcript after normalizing to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (*B*) Lung MMP14 protein levels increased in FVB/NJ mice exposed to acrolein. MMP14 protein level as determined by Western blot increased in acrolein-exposed FVB/NJ mouse lung after acrolein exposure. Each lane was loaded with 60 μg of protein obtained from an individual mouse that is representative of each group (n = 5 mice per treatment). (*C*) MMP14 transcript levels increased in NCI-H292 cells or normal bronchial epithelial (NHBE) cells treated with 100 nM acrolein (4 h). The level of MMP14 transcript was determined by qRT-PCR. Results are expressed as fold change in the level of MMP14 transcripts after normalizing to ribosomal protein L32 (RPL32). (*D*) MMP14 transcripts increased in NCI-H292 cells in a concentration-dependent manner after acrolein treatment. Confluent serum-starved NCI-H292 cells or NHBE cells were treated (4 h, 37°C) with 10–300 nM acrolein. Values represent means ± SEM (n = 5–9). *Significantly different from control mice, using an all-pairwise multiple-comparison analysis of variance procedure (Holm-Sidak method).

targeting protease activation to pericellular regions. The expression of MMP14 and subsequent activation of MMPs is seen in normal lung fibroblasts exposed to cigarette smoke extract *in vitro* (22).

Previously, MMP9 and ADAM17 have been found to mediate increased mucin production, especially mucin 5AC, oligomeric mucus/gel-forming (MUC5AC) (12, 23, 24), through mobilization of epidermal growth factor (EGF) family ligands that bind to and activate receptor-type protein tyrosine kinases, including epidermal growth factor receptor (EGFR) (18, 23–30). However, small interfering RNA (siRNA) directed against ADAM17 and MMP9 did not completely inhibit the acrolein-induced increase in MUC5AC transcripts. In addition, gene-targeted mice lacking MMP9 had only a partial reversal of phenotype (24), suggesting a role of other MMPs in increased MUC5AC transcript levels. Inasmuch as cell surface mobilization of EGF ligands requires localized protease activation and because MMP14 activation can initiate MMP activation cascades at the cell surface, we sought to examine whether MMP14 transcripts are increased in airway epithelial cells and the role of MMP14 activation in acrolein-induced MUC5AC expression.

## METHODS

### Experimental Design

In a cell-free system, MMP14 activity was measured after submicromolar acrolein exposures at concentrations similar to those found in COPD sputum and acrolein protein adducts were measured by mass spectrometry. In human airway epithelial cells, MMP14 protein activity was measured and the role of proprotein convertase processes evaluated after treatment with an inhibitor, hexa-D-arginine. Immunoreactive MMP14 protein and periodic acid–Schiff staining for mucus glycoprotein was measured in the airway of subjects with COPD and control subjects. Immunoreactive MMP14 protein was measured in the airway of mice exposed to acrolein or cigarette smoke. To determine the role of EGFR and mitogen-activated protein kinase (MAPK) signaling in MMP14 expression, MMP14 transcripts were measured in human airway cells treated with neutralizing antibody (LA1), EGFR kinase inhibitor (AG1478), metalloproteinase inhibitor (GM6001), or MAPK3/2 (PD98059), MAPK8 (SP600125), or MAPK14 (ML3403) inhibitor. In human airway epithelial cells, siRNA to MMP14 was used to determine whether increases in acrolein-induced MUC5AC transcript levels were mediated by MMP14. Last, acrolein-exposed FVB/NJ (non-

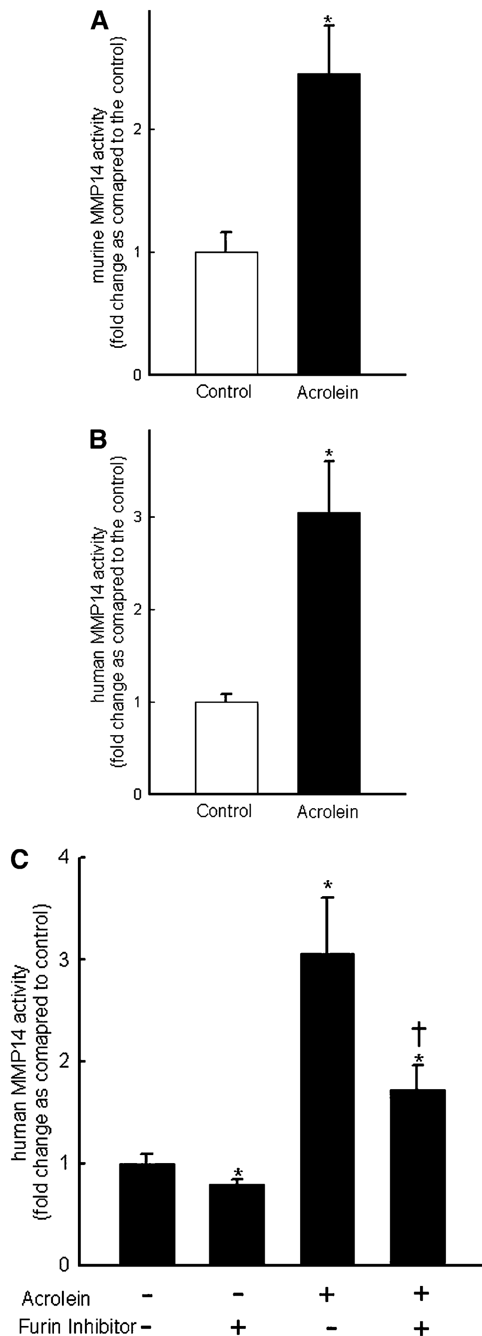


*Figure 2.* Acrolein increases matrix metalloproteinase-14 (MMP14) activity. (*A*) Acrolein increased MMP14 activity in mouse lung. FVB/NJ mice were exposed to acrolein (2.0 ppm × 6 h/d × 5 d/wk × 4 wk) or filtered air (control). MMP14 activity was determined by capturing active MMP14 with anti-MMP14 antibody, which was then used to activate a modified prourokinase detection enzyme to cleave a chromomeric peptide substrate and the resultant color was read spectrophotometrically at 405 nm. A serial dilution of known sample of active MMP14 was run simultaneously to obtain a reference curve. Each sample was analyzed in the linear portion of the curve and the relative amount of active MMP14 was determined by comparing the $OD_{405}$ of each sample against the standard curve. (*B*) Acrolein increased MMP14 activity in NCI-H292 cells. Confluent serum-starved NCI-H292 cells were treated with acrolein (300 nM, 4 h, 37°C) and MMP14 activity was determined. (*C*) A proprotein convertase inhibitor, hexa-D-arginine, inhibited acrolein-induced MMP14 activity by 61 ± 8%. Confluent serum-starved NCI-H292 cells were pretreated with 0.625 μM hexa-D-arginine and incubated with acrolein (300 nM, 4 h, 37°C), and MMP14 activity was determined. Values represent means ± SEM (n = 5–10). *Significantly different from control ($P < 0.05$), using an all-pairwise multiple-comparison analysis of variance (ANOVA) procedure (Holm-Sidak method). †Significantly different from acrolein treatment ($P < 0.05$), using an all-pairwise multiple-comparison ANOVA procedure (Holm-Sidak method).

transgenic) strain mice or doxycycline-regulatable transgenic mice with induced lung-specific transforming growth factor-α (an EGFR ligand) expression were treated with vehicle (control) or with an EGFR kinase inhibitor, erlotinib, and lung MMP14 and MUC5AC transcript levels measured. Values are presented as means ± standard errors and were considered significant when $P < 0.05$ as determined by analysis of variance using an all-pairwise multiple-comparison procedure (Holm-Sidak method) (SigmaStat 3.5; Systat Software, Inc., San Jose, CA). *See* the online supplement for additional details of the methods used.

## RESULTS

### Acrolein Increases Airway Epithelial MMP14 Transcript, Protein, and Activity

Repetitive acrolein exposure increased murine MMP14 transcript levels in FVB/NJ mouse lung (Figure 1A). MMP14 protein in the whole lung homogenates in acrolein-exposed FVB/NJ mice increased as compared with control (unexposed) mice (Figure 1B). MMP14 transcripts increased in acrolein-treated normal bronchial epithelial (NHBE) cells (Figure 1C) and acrolein increased MMP14 transcripts in airway epithelial (NCI-H292) cells in a concentration-dependent manner (Figure 1D). Thus MMP14 transcripts and protein increased after acrolein treatment. In addition, MMP14 activity also increased in acrolein-treated FVB/NJ mouse lung (1.6 ± 0.43 ng/ml) as compared with control mouse lung (0.52 ± 0.043 ng/ml) (Figure 2A). MMP14 activity increased in acrolein-treated NCI-H292 cells after acrolein exposure (8.78 ± 0.24 ng/ml) as compared with the control (2.87 ± 0.24 ng/ml) (Figure 2B). Pretreatment with a proprotein convertase (furin) inhibitor, hexa-D-arginine, diminished the acrolein-induced increase in MMP14 activity (Figure 2C).

### Acrolein Binds to Cysteine-319 of MMP14 Protein

The mass spectral data generated from the full-scan analysis of the digestion of MMP14 were searched against the Uniprot-T database, subset human (Uniprot-T Consortium). This search resulted in the identification of 60 different peptides, including the peptide NPTYGNICDGFDTVAMLR that corresponds to amino acid residues 311 to 328 of the sequence of MMP14. The mass spectral data collected from the analysis of acrolein-exposed MMP14 was searched, using the same database. Twenty-four peptides were identified, and again the peptide NPTYGNICDGFDTVAMLR was identified. When these peptides were compared, the 2+ charge states displayed a mass difference of 28.51067 Da, a mass corresponding to an addition of acrolein to the peptide (Figure 3). The acrolein treatment of MMP14 was repeated and analyzed by single-ion monitoring mass spectrometry. When the mass ranges corresponding to the 2+ and 3+ charge states were scanned, in each case a base peak was observed at 42.95 minutes. This peak eluted at the same time as a peak in the full-scan mass spectrum and displayed mass spectra of the doubly and triply charged acrolein–adducted MMP14 (Figure 4). These data are consistent with an acrolein adduction occurring at the position 319

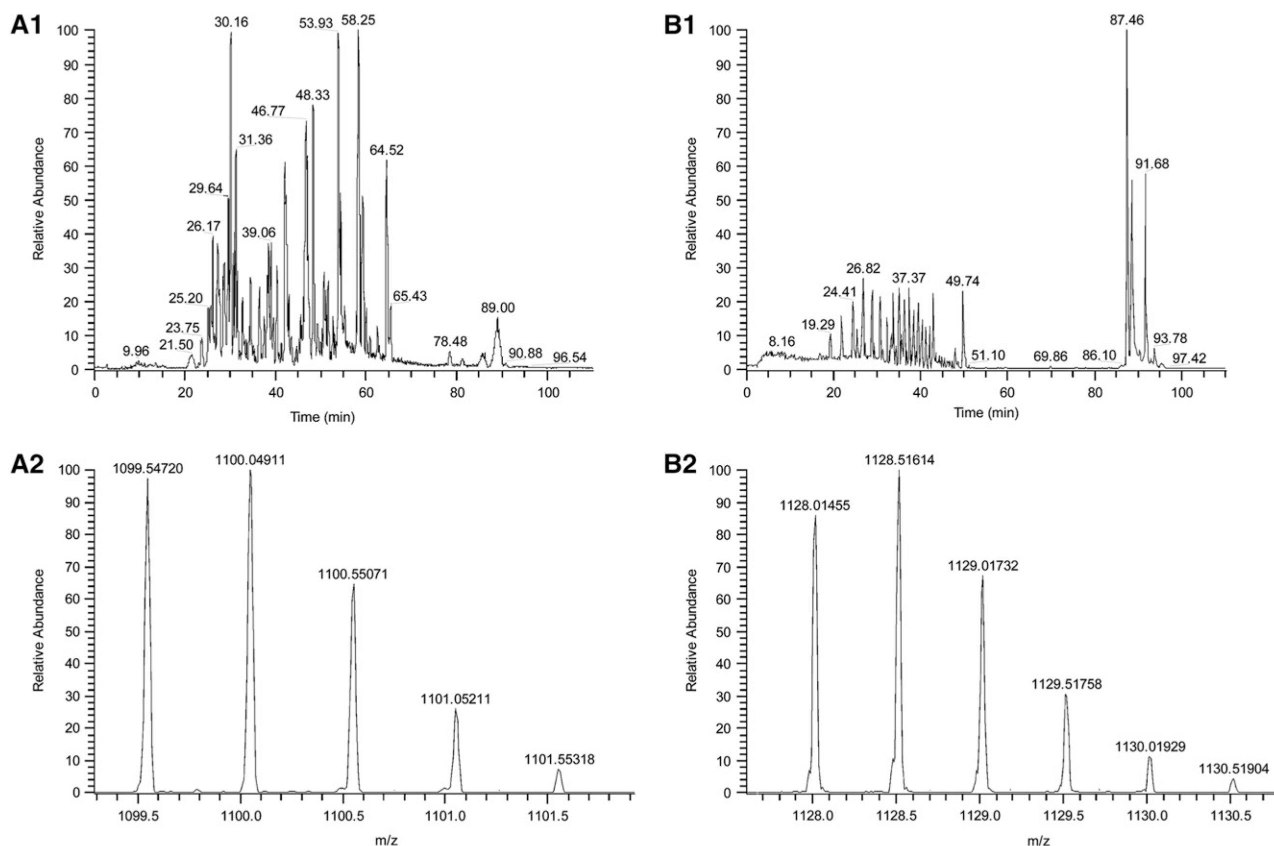


*Figure 3.* Mass spectrum of matrix metalloproteinase-14 (MMP-14) or acrolein-treated MMP-14 tryptic digests. Mass spectrum of MMP14 before and after acrolein treatment demonstrates a change in the 2+ charge state of the NPTYGNICDGFDTVAMLR peptide consistent with acrolein adduct formation. (*A1*) Full scan of MMP-14. (*A2*) Mass spectrum of the peptide NPTYGNICDGFDTVAMLR, 2+ charge state. (*B1*) Full scan of MMP-14 that has been exposed to acrolein. (*B2*) Mass spectrum of the peptide NPTYGNICDGFDTVAMLR with the adduction of acrolein, 2+ charge state. The mass spectral data generated from the full-scan analysis of the digestion of MMP-14 were searched against the Uniprot-T database, subset human (Uniprot-T Consortium).

cysteine. Thus, acrolein not only increases MMP14 production and activity but directly conjugates to a cysteine residue known to form a disulfide bond within the hemopexin domain of MMP14.

### MMP14 Mediates Increased MUC5AC after Acrolein Exposure

Immunostaining for MMP14 protein increased in the airways of subjects with COPD (Figures 5A–5D) and MMP14 staining accompanied increased mucus granule–containing (goblet) cells in the airway epithelium (Figures 5E and 5F). MMP14 immunostaining also increased in the airway epithelium of acrolein-exposed mice (Figures 6A–6D) or tobacco smoke-exposed mice (Figures 6E–6H). To determine the role of MMP14 in acrolein-induced MUC5AC increase, we transfected NCI-H292 cells with siRNA directed against MMP14. MMP14 siRNA decreased MMP14 transcript and protein levels, and activity (Figure 7A). NCI-H292 cells transfected with MMP14 siRNA had reduced levels of constitutive MUC5AC transcripts and demonstrated no increase in MUC5AC transcripts after acrolein treatment (Figure 7B). In contrast, nontransfected cells and cells transfected with scrambled siRNA demonstrated a normal response to acrolein (Figure 7B). Thus, NCI-H292 cells transfected with MMP14 siRNA responded less to acrolein treatment, supporting the hypothesis that acrolein-induced increases in MUC5AC are mediated by MMP14.

### MMP14 Transcript Levels Increase after Acrolein Treatment through EGFR/MAPK3/2/MAPK8 Signaling

Pretreatment with EGFR tyrosine kinase inhibitor (AG1478) (Figure 8A) or LA1 (*see* Figure E1 in the online supplement), a neutralizing antibody against EGFR, diminished the acrolein-induced increase in MMP14 transcripts in NCI-H292 cells. EGFR activation leads to activation of downstream mitogen-activated protein kinase (MAPK). Pretreating the cells with MAPK3/2 inhibitor PD98059 (Figure 8A), with MAPK8 (c-Jun N-terminal kinase or JNK) inhibitor SP600125 (Figure 8B), or with metalloproteinase inhibitor GM6001 (Figure E1) diminished the acrolein-induced increase in MMP14 transcripts. Pretreatment with an MAPK14 (p38) inhibitor had no effect on the acrolein-induced increase in MMP14 transcripts (Figure 8B). Previously, we found that an EGFR antagonist, erlotinib, inhibited acrolein-induced MUC5AC transcripts and immunoreactive mucin protein in FVB/NJ mice (24). Lung MMP14 transcripts increased in acrolein-exposed mice and this effect was inhibited by erlotinib (Figure 9A). Likewise, acrolein-induced increased MUC5AC was inhibited by erlotinib (Figure 9B). In addition, lung MMP14 (Figure 9C) and MUC5AC (Figure 9D) transcripts increased with conditional doxycycline-inducible transforming growth factor-$\alpha$, an EGFR ligand, and erlotinib attenuated this effect in doxycycline-treated mice. Thus, increased MMP14 and MUC5AC transcript levels in acrolein-treated airway epithe-

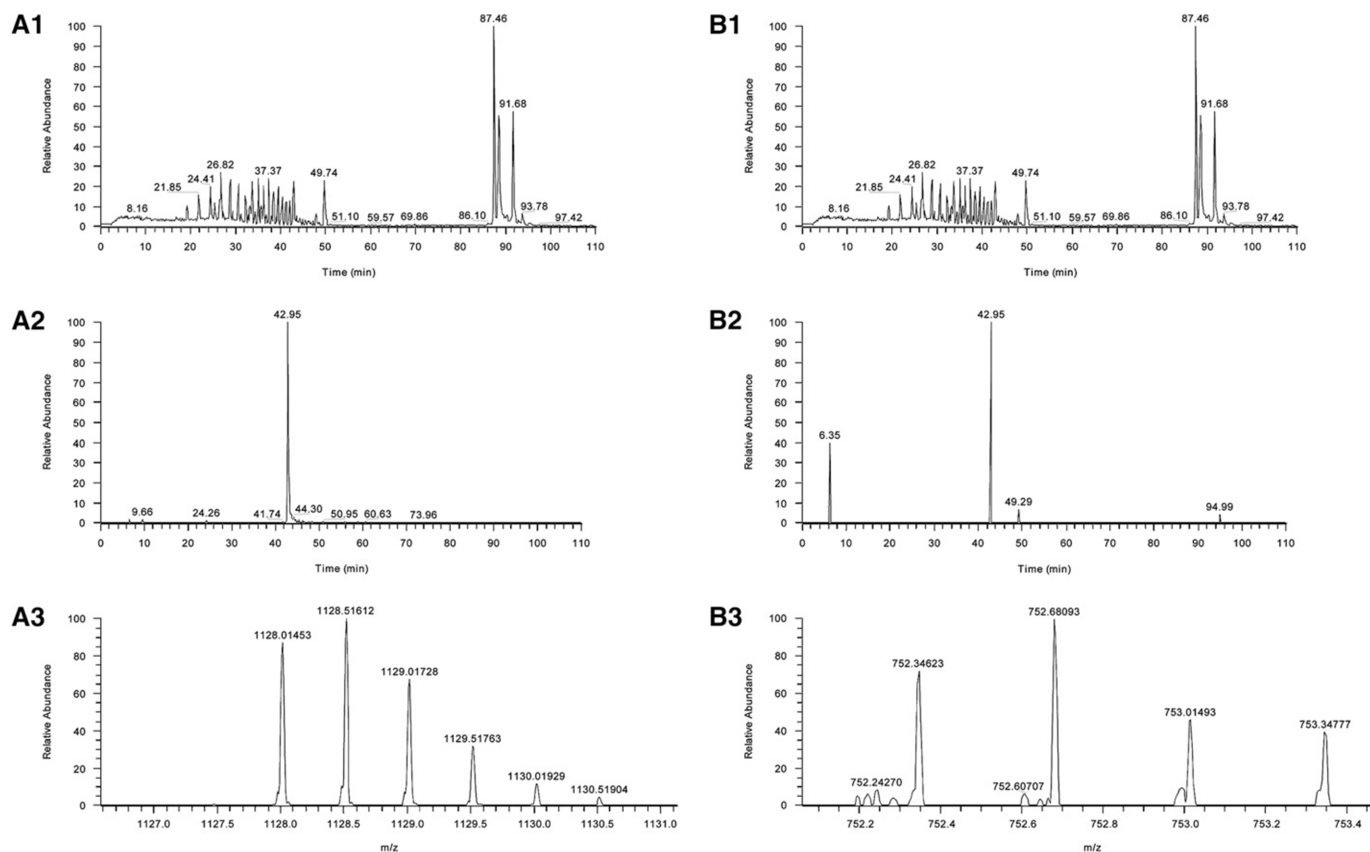


***Figure 4.*** Mass spectrum of matrix metalloproteinase-14 (MMP14) or acrolein-treated MMP-14 tryptic digests. Mass spectrum before and after acrolein treatment demonstrates that adduct formation occurs at cysteine-319 in the hemopexin domain of MMP14. (*A1*) Full scan of acrolein-treated MMP14. (*A2*) Extracted ion scan for masses 1127.99 to 1128.03 Da, showing the presence of a single peak at 42.95 minutes. (*A3*) Mass spectrum of the peak eluting at 42.95 minutes, which is the 2+ charge state of the mass corresponding to acrolein adduction to MMP14. (*B1*) Full scan of acrolein-treated MMP14. (*B2*) Extracted ion scan for masses 752.34 to 752.36 Da, showing the presence of a base peak at 42.95 minutes. (*B3*) Mass spectrum of the peak eluting at 42.95 minutes, which is the 3+ charge state of the mass corresponding to acrolein adduction to MMP14. Analyses were performed by single-ion monitoring mass spectrometry.

lial cells are accompanied by metalloproteinase-mediated, EGFR ligand–dependent MAPK3/2 and MAKP8 signaling.

## DISCUSSION

It is clear that acrolein can initiate mucin overproduction *in vivo*. Animals exposed repeatedly to acrolein develop histological changes including epithelial damage, mucous cell metaplasia, and bronchiolitis, accompanied by excessive macrophage accumulation in the airways (11, 31, 32). Acrolein exposure increased mucus-producing cells in airways and increased MUC5AC transcripts in the lungs of Sprague-Dawley rats (11) and MUC5AC and immunoreactive mucin in the lungs of FVB/NJ mice (12, 24). MMPs have been proposed to play an important role in pathogenesis of COPD with several articles describing the role of MMPs in various lung pathologies (33). In this study, we examine whether acrolein alters expression and activation of MMP14, a critical membrane-bound endopeptidase that can initiate an MMP activation cascade.

The acrolein levels used in this study (submicromolar *in vitro* and 2 ppm *in vivo*) are relevant to common human exposures. Acrolein levels in second-hand tobacco smoke are elevated compared with mainstream smoke, because concentrations are increased in side-stream smoke due to altered tobacco combustion at lower temperatures (34–36). More than 30 million nonsmokers in the United States are exposed to acrolein concentrations in indoor air ranging from 0.8 to 1.5 ppm and levels between 0.1 and 10 ppm have been detected in bars and restaurants (35, 37–39). Acrolein is also generated by biomass fuel combustion and high-temperature cooking with oils (especially in woks) and is the major irritant in grassland and forest fires, and diesel exhaust (34, 35, 40, 41). In addition to exogenous exposure, acrolein is endogenously generated in inflamed tissues from threonine by myeloperoxidase activation (42–45), spermine or spermidine by amine oxidase–mediated catabolism (46–50), or possibly membrane fatty acids by oxidative degradation (35, 51–53).

Because it forms a highly reactive zwitterion ($^{+}CH_2CH{=}CHO^{-}$) through electron rearrangement of the $\alpha,\beta$-unsaturated bond, acrolein readily reacts with various molecules on the airway surface and thus it is nearly completely retained in the respiratory epithelium (10, 54). Acrolein readily attacks nucleophiles, especially thiol-containing proteins (10, 53, 55, 56). Of all the $\alpha,\beta$-unsaturated 2-alkenals, acrolein is among the strongest electrophiles (51), the most irritating (i.e., concentrations as low as 0.06 ppm can cause eye irritation within 5 min) (7, 57, 58), and share in the ability to covalently modify macromolecules, which disrupt critical cellular functions or cause mutations (51, 59–62). Acrolein–protein adducts accumulate in ischemic tissue (52, 63) and in atherosclerotic lesions (45, 64), and we found that acrolein can directly bind to and activate MMP14 in this study.

Previously we reported a role for MMP9 and MMP12 in acrolein-induced MUC5AC expression (12, 23, 24). Acrolein increased MUC5AC transcripts and mucin protein in strain-

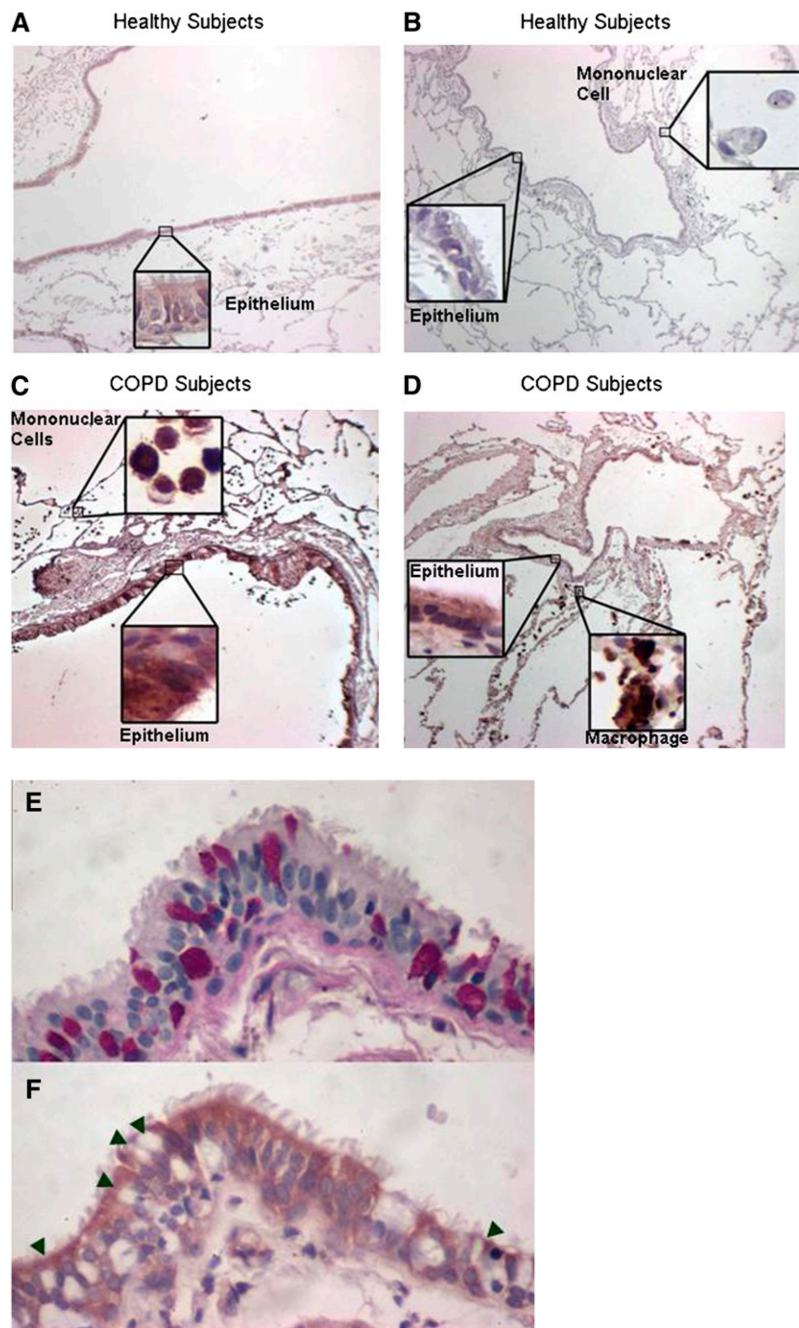


***Figure 5.*** Matrix metalloproteinase-14 (MMP14) immunostaining increases in subjects with chronic obstructive pulmonary disease (COPD). Lung specimens were obtained from human subjects undergoing lung transplant surgery for COPD treatment under institutional review board–approved protocols at the Washington University Medical Center (St. Louis, MO) and immunostaining with anti-MMP14 was performed. Immunostaining with anti-MMP14 antibody (*red stain*) increased in the lungs from (*C* and *D*) subjects with COPD as compared with (*A* and *B*) healthy subjects. The localization was notable for the presence of MMP14 in columnar airway epithelial cells, mononuclear cells in the alveolus, including pigmented macrophages (*insets*). In subjects with COPD, areas of mucous cell metaplasia were present when stained for (*E*) periodic acid–Schiff-positive mucus glycoprotein (*red-purple*) and corresponded with (*F*) translucent-appearing unstained cells (*arrowheads*) when immunostained with anti-MMP14 antibody in serial sections.

matched control *Mmp9*$^{+/+}$ mice more than gene-targeted *Mmp9*$^{-/-}$ mice (23, 24). Similarly, acrolein increased MUC5AC transcripts and macrophage accumulation in lungs of strain-matched control *Mmp12*$^{+/+}$ mice more than in gene-targeted *Mmp12*$^{-/-}$ mice (12). Acrolein increased the transcript levels of MUC5AC in NCI-H292 cells (31) and normal bronchial epithelial (NHBE) cells (23, 24). This increase in MUC5AC transcripts is mediated through an EGFR–MAPK pathway that is initiated by ectodomain shedding of EGFR ligands mediated by metalloproteinases ADAM17 and MMP9 (23). However, siRNA directed against ADAM17 and MMP9 did not completely inhibit the acrolein-induced increase in MUC5AC transcripts. Similarly, the inhibition of acrolein-induced MUC5AC increase was partial in the lungs of gene-targeted *Mmp9*$^{-/-}$ as compared with *Mmp9*$^{+/+}$ mice, suggesting a role for another MMP in acrolein-induced MUC5AC increase. Previously, Ning and colleagues (22) demonstrated that cigarette smoke extract increased MMP14 transcript levels in human lung fibroblasts, so MMP14 was a reasonable candidate for further study in human airway epithelial cells and *in vivo* in mice.

Unlike secreted MMPs, MMP14 is associated with the cell surface through a type 1 transmembrane domain (65). MMP14 is important in lung development as evidenced by a defect in formation of alveolar septae in *Mmp14*$^{-/-}$ mice (66, 67). MMP14 can activate pro-MMP2 (68) and pro-MMP13 (69), which in turn can cleave pro-MMP9 (70). Here we found that acrolein increased MMP14 transcript (Figure 1A), protein (Figure 1B), and activity (Figure 2A) in the lungs of FVB/NJ mice. The inhaled acrolein concentration (2 ppm × 6 h/d) is estimated to

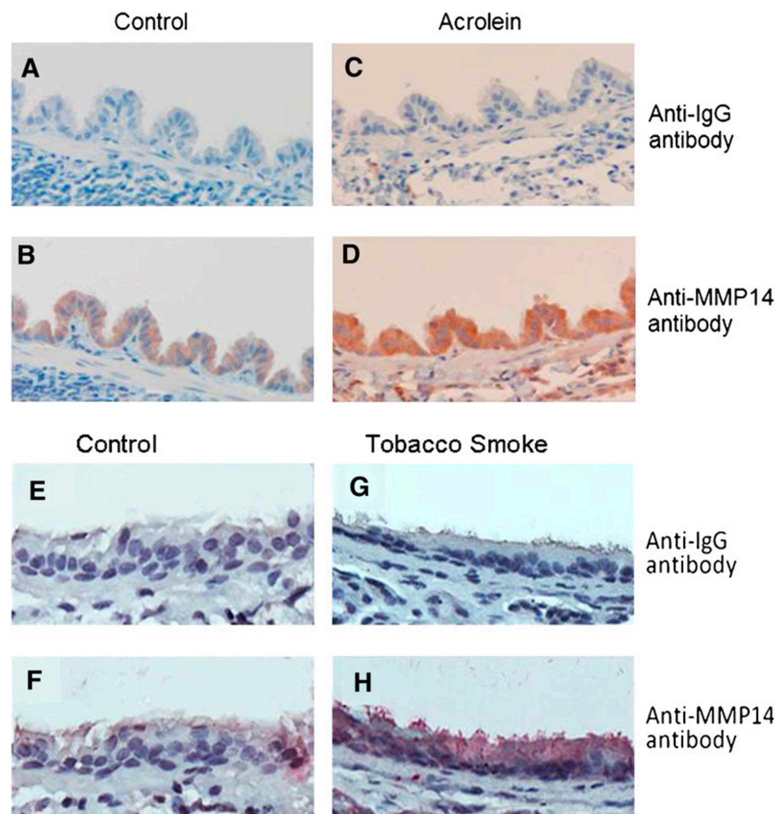


*Figure 6.* Matrix metalloproteinase-14 (MMP14) immunostaining increases in FVB/NJ mouse airway epithelium after acrolein or tobacco smoke exposure. FVB/NJ mice were exposed to (*A*, *B*, *E*, and *F*) filtered air (control), (*C* and *D*) acrolein (2.0 ppm × 6 h/d × 5 d/wk × 4 wk), or (*G* and *H*) tobacco smoke (100 mg/m$^3$ total suspended particulates × 6 h/d × 5 d/wk × 13 wk) and lung sections were incubated with (*A*, *C*, *E*, and *G*) control anti-IgG (diluted 1:100) or (*B*, *D*, *F*, and *H*) anti-MMP14 (diluted 1:100) antibody. In each image, the airway lumen is above the epithelium, which stains red-brown in the presence of MMP14. Endogenous peroxidase activity was quenched and specimens were incubated with horseradish peroxidase–labeled goat anti-mouse secondary antibody (diluted 1:5,000) in antibody dilution buffer, twice rinsed with phosphate-buffered saline (PBS), incubated with chromogen 3,3′-diaminobenzidine tetrachloride (0.05% in PBS), and counterstained with hematoxylin. The sections were visualized with a SPOT 2000 microscope (×40 objective) and the images were captured with a cooled charge-coupled device camera.

deliver an acrolein dose to the lung equivalent to 0.5–1.0 cigarette pack per day. Acrolein increased MMP14 transcripts in NHBE and NCI-H292 cells (Figure 1C). Moreover, the acrolein concentration necessary to increase MMP14 was as low as 30 nM (Figure 1D), which is a concentration well within the concentrations we previously measured in sputum from subjects with COPD (131 ± 24 nM) (24).

MMP14 activity is tightly controlled at the transcriptional and posttranslational levels. MMP14 is produced as a latent propeptide that keeps the enzyme latent through the interaction of a cysteine residue with a zinc ion in the catalytic domain. MMP14 has a unique regulatory mechanism in which the active enzyme undergoes a series of processing steps, either autocatalytic (71, 72) or mediated by other proteases (73, 74), initially to an enzymatically active (~56-kD) species and ultimately to an inactive membrane-tethered (~44-kD) species lacking the entire catalytic domain, thereby regulating the activity and nature of MMP14 proteins at the cell surface and at the pericellular space. MMP14 contains an RXK/RR proprotein convertase enzyme recognition motif between the propeptide and catalytic domain, which can be activated by intracellular subtilisin-type serine proteinases (e.g., furin) before MMP14 reaches the cell surface (73). Pretreatment with a furin inhibitor partially decreased the acrolein-induced increase in MMP14 activity (Figure 2C), suggesting the presence of an additional mechanism for increased MMP14 activity after acrolein treatment.

Inhaled or endogenously generated acrolein reacts directly with protein and nonprotein sulfhydryl groups, mainly at the cell surface, and with primary and secondary amines found in the intracellular proteins (34, 35). In lungs, MMP14 is expressed on surface epithelial cells (75, 76) (Figures 5 and 6). Conjugation of the carbon of acrolein with sulfhydryl groups by a Michael addition reaction is rapid and essentially irreversible (35, 77). Cysteine residues near the transmembrane domain or in the catalytic domain could potentially interfere with the autocatalytic processing and thus increase the amount of active MMP14 present on the cell surface and thus potentially increase MMP14 activity. When MMP14 was treated with acrolein, we identified a cysteine-319 adduct (Figures 3 and 4), which is contained within a hemopexin-like domain and not the conserved "cysteine switch" domain that is cleaved by proprotein convertases. Hemopexin domains are usually involved in substrate recognition of large matrix molecules at sites distant from the catalytic domain (78, 79). However, MMP14 is membrane localized and hemopexin domains appear to be critical for MMP14 dimerization. One process that would require MMP14 self-interaction is the major form of enzyme inactivation by autocatalytic cleavage (72, 74, 79). We propose that our data suggest that MMP14 surface activity is preserved by interference with hemopexin domain–mediated dimerization and autocatalytic inactivation resulting in persistence of active MMP14 on the cell surface of acrolein-treated airway epithelial cells.

MMP14 activity is also regulated at the transcriptional level and can be controlled at the protein level via anti-proteinase inhibitors (80). Acrolein treatment increased the transcript levels of MMP14 in NCI-H292 cells and NHBE cells (Figure 1C). Cytokines (including IL-2, IL-8, and monocyte chemokine protein-1) (81, 82) and growth factors (including EGF [83], fibroblast growth factor-1 [84], vascular endothelial growth factor [85], and insulin-like growth factor-1 [86]) can induce MMP14 expression in various cell lines. Previously, MMP14 has been found to be expressed on rabbit surface airway epithelial cells (75) and alveolar type II cells (76) and in human adenocarcinoma cells (87). We found that MMP14 transcripts increased in the lungs of FVB/NJ mice exposed to

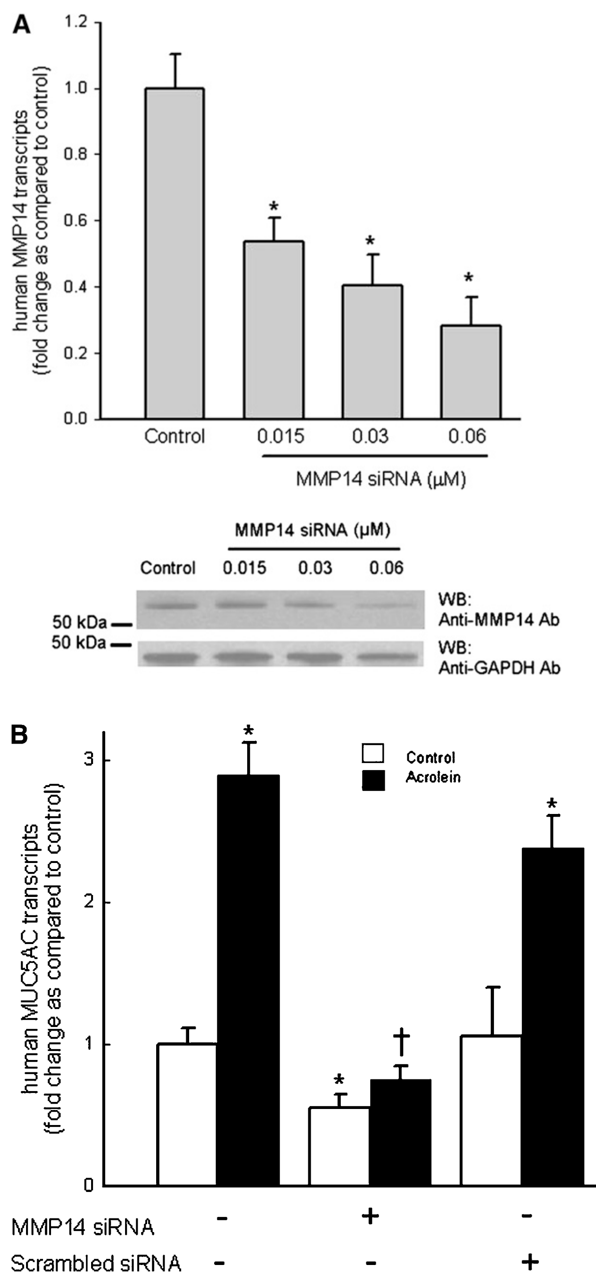


***Figure 7.*** Matrix metalloproteinase-14 (MMP14) mediates acrolein-induced increases in mucin 5AC, oligomeric mucus/gel-forming (MUC5AC) transcripts in human airway epithelial (NCI-H292) cells. (*A*) *Top*: MMP14 transcript levels were diminished in NCI-H292 cells transfected with small interfering RNA (siRNA) directed against MMP14 as compared with cells transfected with scrambled siRNA (negative control) or untransfected cells. *Bottom*: To determine whether siRNA-diminished transcript levels were accompanied by decreased MMP14 protein and activity, protein was isolated and subjected to Western blotting. (*B*) Acrolein-induced MUC5AC transcripts were diminished in NCI-H292 cells transfected with siRNA against MMP14 as compared with cells transfected with scrambled (nonsense) siRNA. NCI-H292 cells (40% confluent) were transfected with siRNA against MMP14 or scrambled siRNA (negative control) and compared with cells not transfected with siRNA. Cells were incubated (37°C, 36 h) and then treated with vehicle or acrolein (300 nM, 4 h, 37°C). RNA was isolated and the level of MUC5AC transcript was determined by quantitative real-time polymerase chain reaction. The results are expressed as fold change in the level of MMP14 or MUC5AC transcripts after normalizing to ribosomal protein L32 (RPL32). Values represent means ± SEM (n = 6–9). *Significantly different from control ($P < 0.05$), using analysis of variance (ANOVA) with all-pairwise multiple-comparison ANOVA procedure (Holm-Sidak method). †Significantly different from acrolein treatment ($P < 0.05$), using an all-pairwise multiple-comparison ANOVA procedure (Holm-Sidak method).

acrolein (Figure 1A). Immunostaining for MMP14 increased in the lungs of FVB/NJ mice exposed to acrolein or tobacco smoke (Figure 6) and in the airways of human subjects with COPD (Figure 5). It is important to note that the subjects with COPD were not current smokers, which suggests that increased MMP14 can be persistent (possibly due to endogenously generated acrolein).

We used siRNA to confirm the role of MMP14 transcripts in acrolein-induced MUC5AC increase (Figure 7). siRNA directed against MMP14 efficiently decreased the transcript and protein levels. NCI-H292 cells transfected with siRNA had lower constitutive levels of MUC5AC transcripts as compared with untransfected cells or cells transfected with scrambled siRNA. Transcript levels of MUC5AC in NCI-H292 cells transfected with MMP14 siRNA after acrolein treatment were not significantly different from control cells. Untransfected cells responded appropriately to acrolein treatment. These results indicate that MMP14 plays a critical role in acrolein-induced MUC5AC increase. As noted previously, MMP14 can activate MMP13 and MMP2, which in turn could activate MMP9. Thus, several MMPs are likely to contribute to MUC5AC increases.

Past investigations of MMP14 regulation have focused on protein processing (as noted previously), and therefore less is known about the signal transduction pathways involved in increased MMP14 expression in the lung. Inhibition of MAPK3/2 (ERK1/2) decreased MMP14 expression in fibrosarcoma cells (88). MAPK3/2 but not MAPK8 (JNK) or MAPK14 (p38) regulates increased MMP14 expression in rat endothelial cells (89) and lung fibroblasts (22). Moreover, constitutively active MAP2K increased MMP14 expression in MDK cells (90) and an MAP2K1/2 (MEK1/2) inhibitor diminished cigarette smoke extract–induced MMP14 expression in lung fibroblasts (22). Here we report that an EGFR kinase inhibitor diminished the acrolein-induced increase in MMP14 transcripts, confirming the role of EGFR in the acrolein-induced increase in NCI-H292 cell (treated with AG1478) (Figure 8A) and mouse lung (treated with erlotinib) (Figure 9) MMP14 transcripts. Treatment with MAP3/2 inhibitor (PD98059) and the MAPK8 (JNK) inhibitor (SP600125), but not the MAPK14 (p38) inhibitor (ML3403), decreased the acrolein-induced increase in MMP14 transcripts, suggesting that MAPK3/2 (ERK1/2) and MAPK8 (JNK), but not MAPK14 (p38), are involved in the response initiated by acrolein in the airway epithelium. Thus, regulation of MMP14 expression in the airway epithelium (which includes MAPK8) differs from that in lung fibroblasts.

The MMP proteinase activity can be regulated by a counterbalance with antiproteinase. For example, the tissue inhibitors of metalloproteinase proteins (TIMPs) represent a family of at least four 20- to 29-kD secreted proteins (TIMPs 1–4) that reversibly inhibit the MMPs in a 1:1 stoichiometric fashion (91). TIMP1 (92), TIMP2, TIMP3, and TIMP4 (93) are expressed in bronchial epithelium. TIMP2 (80) and TIMP3 (94), but not TIMP1 (95), inhibit MMP14 activity. TIMP3 also has the unique ability to bind via its C-terminal domain to heparin sulfate

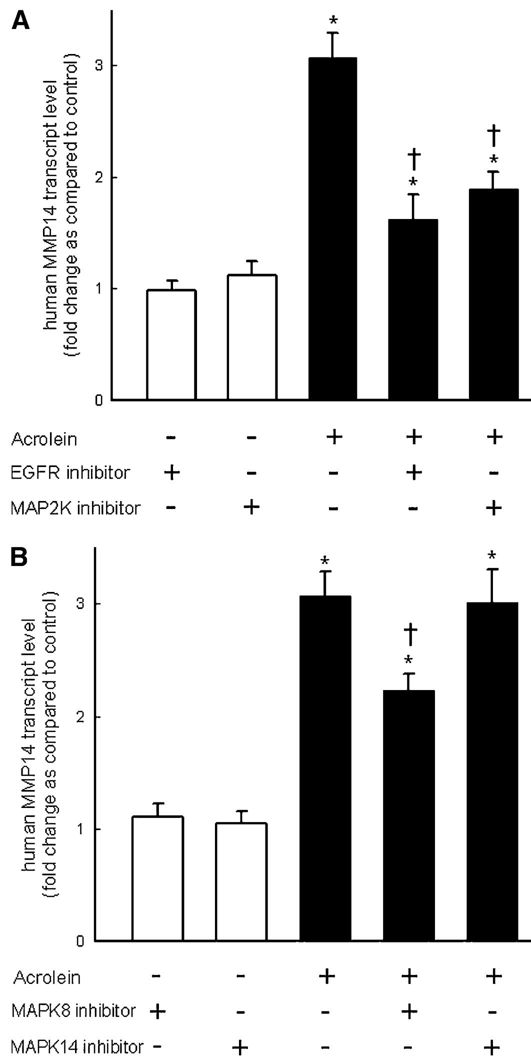


*Figure 8.* Acrolein-induced increases in matrix metalloproteinase-14 (MMP14) transcript levels are mediated by epidermal growth factor receptor (EGFR) and mitogen-activated protein kinase (MAPK) signaling in human airway epithelial (NCI-H292) cells. (*A*) Acrolein-induced increases in transcript levels of MMP14 were diminished by an EGFR kinase inhibitor (0.250 μM AG1478) or an MAPK3/2 inhibitor (5 μM PD98059), by 72 and 57%, respectively. (*B*) MMP14 transcript levels were also decreased in cells treated with an MAPK8 (also called c-Jun N-terminal kinase, JNK) inhibitor (5 μM SP600125) by 41%, but not in cells treated with MAPK14 (also called p38 MAPK) inhibitor (5 μM ML3403) by 2%. Confluent NCI-H292 cells were pretreated (37°C, 1 h) with inhibitor and then incubated with vehicle or acrolein (100 nM, 4 h, 37°C). RNA was isolated and the level of MMP14 transcript was determined by quantitative real-time polymerase chain reaction. The results are expressed as fold change in the level of MMP14 transcripts after normalizing to ribosomal protein L32 (RPL32). Values represent means ± SEM (n = 4–6). *Significantly different from control ($P < 0.05$), using an all-pairwise multiple-comparison ANOVA procedure (Holm-Sidak method). †Significantly different from acrolein treatment ($P < 0.05$), using an all-pairwise multiple-comparison ANOVA procedure (Holm-Sidak method).

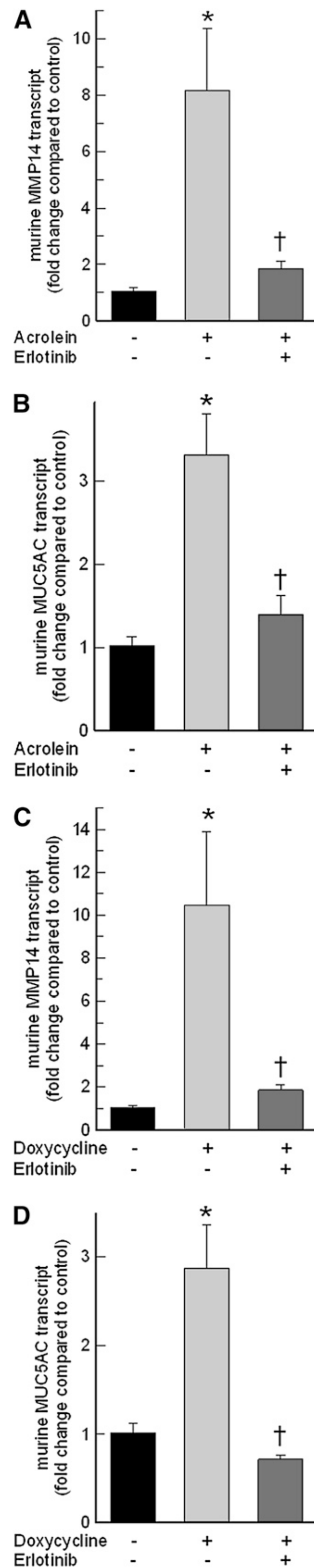


*Figure 9.* Epidermal growth factor receptor (EGFR) inhibition diminishes acrolein- or transforming growth factor (TGF)-α–induced increases in matrix metalloproteinase-14 (MMP14) or mucin 5AC, oligomeric mucus/gel-forming (MUC5AC) transcripts in FVB/NJ mouse lung. (*A*) Acrolein-induced increases in MMP14 transcripts were diminished in mice pretreated with an EGFR inhibitor (erlotinib) by 89% compared with mice treated with sterile vehicle control. FVB/NJ mice were pretreated with erlotinib (100 mg/kg/d by gavage) or vehicle and exposed to acrolein (2.0 ppm × 6 h/d × 5 d/wk × 4 wk). (*B*) Acrolein-induced increases in MUC5AC transcripts were diminished by 86% in mice pretreated with an EGFR inhibitor (erlotinib) compared with mice treated with vehicle control. (*C*) TGF-α induced increases in MMP14 transcripts in conditional transgenic mice after doxycycline induction as compared with the littermate controls maintained without doxycycline for 8 weeks. This effect was inhibited by 91% in mice pretreated with an EGFR inhibitor (erlotinib, 100 mg/kg/d). (*D*) TGF-α induced increases in MUC5AC transcripts in conditional transgenic mice after doxycycline induction as compared with the littermate controls maintained without doxycycline for 8 weeks. This effect was inhibited about 100% in mice pretreated with an EGFR inhibitor (erlotinib). RNA was isolated and the levels of MMP14 or MUC5AC transcripts were determined by quantitative real-time polymerase chain reaction. The results are expressed as fold change in the level of MMP14 or MUC5AC transcripts after normalizing to ribosomal protein L32 (RPL32). Values represent means ± SEM (n = 4–9 mice per group). *Significantly different from control ($P < 0.05$), using an all-pairwise multiple-comparison analysis of variance (ANOVA) procedure (Holm-Sidak method). †Significantly different from acrolein or TGF-α treatment ($P < 0.05$), using an all-pairwise multiple-comparison ANOVA procedure (Holm-Sidak method).

proteoglycans within the extracellular matrix, thereby concentrating it to specific regions within tissues and basement membranes (96). Unlike other TIMPs, TIMP3 is subject to a high degree of transcriptional regulation (97). Previously, we determined that TIMP3 transcript levels decreased in the lungs of FVB/NJ mice after acrolein exposure (23), which also could contribute to an increase in MMP14 and other proteinase activity.

In summary, these findings implicate acrolein-induced MMP14 expression and activity in mucin production in COPD.

Low-level acrolein concentrations (equivalent to those present in COPD sputum) activated and increased MMP14 transcripts, protein, and activity. MMP14 immunostaining increased in the airway epithelium of subjects with COPD. Inhibition of MMP14 induction, by EGFR kinase inhibitors, reduced acrolein-induced mucin levels in mouse lung. Thus, local pharmacological inhibition of MMP14 in the airway epithelium could be useful in the treatment of COPD-related mucin overproduction.

***Conflict of Interest Statement*:** None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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