Acrolein-Activated Matrix Metalloproteinase 9 Contributes to Persistent Mucin Production

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Chronic obstructive pulmonary disease (COPD), a global public health problem, is characterized by progressive difficulty in breathing, with increased mucin production, especially in the small airways. Acrolein, a constituent of cigarette smoke and an endogenous mediator of oxidative stress, increases airway mucin 5, subtypes A and C (MUC5AC) production; however, the mechanism remains unclear. In this study, increased mMUC5AC transcripts and protein were associated with increased lung matrix metalloproteinase 9 (mMMP9) transcripts, protein, and activity in acrolein-exposed mice. Increased mMUC5AC transcripts and mucin protein were diminished in gene-targeted *Mmp9* mice [*Mmp9*^{-/-}] or in mice treated with an epidermal growth factor receptor (EGFR) inhibitor, erlotinib. Acrolein also decreased mTissue inhibitor of metalloproteinase protein 3 (an MMP9 inhibitor) transcript levels. In a cell-free system, acrolein increased pro-hMMP9 cleavage and activity in concentrations (100–300 nM) found in sputum from subjects with COPD. Acrolein increased hMMP9 transcripts in human airway cells, which was inhibited by an MMP inhibitor, EGFR-neutralizing antibody, or a mitogen-activated protein kinase (MAPK) 3/2 inhibitor. Together these findings indicate that acrolein can initiate cleavage of pro-hMMP9 and EGFR/MAPK signaling that leads to additional MMP9 formation. Augmentation of hMMP9 activity, in turn, could contribute to persistent excessive mucin production.

Keywords: mucus; COPD; matrix metalloproteinase; cigarette smoke; oxidative stress

Chronic obstructive pulmonary disease (COPD), a growing global public health problem, is predicted to be the third leading cause of death by 2020 (1). COPD develops after exposure to noxious agents, with tobacco smoking accounting for over 90% of new cases. Death and disability from COPD are marked by remodeling of the airway, particularly those of small diameter, and alveolar architecture, resulting in accelerated decline in lung function (2). Excessive mucin production in more advanced COPD is associated with rapid declines in lung function and more frequent exacerbations (including hospitalization and death) (2, 3).

The pathogenesis of COPD involves an imbalance between antiproteases and proteinases, especially matrix metalloprotei-

CLINICAL RELEVANCE

Acrolein is a cigarette smoke component and endogenous oxidative stress product. At concentrations in chronic obstructive pulmonary disease sputum, acrolein activates matrix metalloproteinase 9 (MMP9) directly and activates epidermal growth factor receptor signaling, leading to MMP9 and MUC5AC accumulation, features of persistent mucus production.

nases (MMPs) (4). Lung biopsies/sputum demonstrate increased MMP1 (5), 2 (6), 8 (7), 9 (8), and 14 (9). Of these, the predominant form in the airway epithelium is MMP9, with increased activity noted in sputum (8). Macrophages derived from patients with COPD display increased MMP9 mRNA (7) and secrete more MMP9 when stimulated with cigarette smoke (9).

A viscoelastic gel composed of water (95%) and mucins, mucus lines the respiratory epithelium and protects against infectious and environmental agents (10, 11). Large heterogeneous glycoproteins, mucins are encoded by at least 15 genes, but previous analyses indicate that mucin 5, subtypes A and C (hMUC5AC), and mucin 5B (hMUC5B) constitute the majority of the glycoprotein in the airway secretions (11). Although hMUC5B is primarily constitutively expressed, hMUC5AC is highly inducible in specialized epithelial (goblet) cells that are limited in number and found mainly in the large airways of healthy individuals. With smoking, goblet cell number increases markedly, especially in small diameter airways (10–12).

Cigarette smoke consists of many irritants, but one of the most prevalent and potent is acrolein. Second-hand smoke contains high levels because aldehydes are enriched in side-stream smoke and smoking just one cigarette/m³ room-space can lead to concentrations of greater than or equal to 2.0 ppm (13). Acrolein is in wood smoke, diesel exhaust, cooking oil fume, and smog (11). Once inhaled, acrolein can penetrate the nasal airways and deposit throughout the lower respiratory tract. Because it forms a highly reactive zwitterion (⁺CH₂CH = CHO⁻) through electron rearrangement of the α-β unsaturated bond, inhaled acrolein readily reacts with surface macromolecules and is retained in the epithelium (14). In laboratory animals, acrolein decreases lung function (15) and increases goblet cell number and MUC5AC expression (12). In recent years, acrolein also has emerged as an endogenous mediator of oxidative stress (16). Generated by lipid peroxidation and myeloperoxidase-catalyzed threonine peroxidation (17), increased acrolein-adducted proteins have been detected in atherosclerosis (18), end-stage kidney disease (19) and Alzheimer's disease (20).

Surprisingly, the role of MMPs in mucin overproduction is poorly understood. Previously, Borchers and coworkers (21) reported that excessive macrophage accumulation was observed

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in *Mmp12*^(+/+) strain-matched control mice as compared to *Mmp12*^(-/-) gene-targeted mice, and macrophage accumulation correlated lung mMUC5AC transcript levels. In addition, lipopolysaccharide (LPS) causes mucus production by activating epidermal growth factor receptor (EGFR), and an MMP inhibitor decreased this effect in rats (22, 23).

Using human airway epithelial cells, we and others previously demonstrated that acrolein increased MUC5AC transcripts through an EGFR–mitogen-activated protein kinase (MAPK) pathway (22, 24, 25). This response is mediated by ectoshedding of EGFR ligands initiated by MMPs (including hMMP9). This led us to propose that acrolein initiates a prolonged increase in hMUC5AC expression, via a process that involves MMP activation, EGFR ligand release, and EGFR/MAPK signaling. Once activated, EGFR/MAPK signaling can produce increased hMMP9 and decreased tissue inhibitor of metalloproteinase protein (hTIMP) 3 transcripts. In the present study, we measured lung mMUC5AC transcripts and mMMP9 protein/activity of acrolein-exposed gene-targeted *Mmp9*^(-/-) and control *Mmp9*^(+/+) mice. To determine whether acrolein could activate hMMP9, pro-MMP9 was treated in concentrations found in sputum isolated from subjects with COPD. Finally, we assessed the role of EGFR/MAPK signaling in the accumulation of hMMP9 transcripts.

MATERIALS AND METHODS

Mouse Strains and Acrolein Exposures

Gene-targeted *Mmp9*^(-/-) mice (FVB.Cg-*Mmp9*^{tm1Tvu/J} No. 004104; Jackson Laboratory, Bar Harbor, ME) or strain-matched control (*Mmp9*^(+/+)) mice (FVB/NJ No. 001800) (male, 6–8 wk) were exposed to filtered air (control) or acrolein (Cat. No. 36520; Alfa Aesar, Ward Hill, MA) (2.0 ppm × 6 h/d × 5 d/wk × 4 wk) as previously described (12). In additional groups exposed to acrolein, *Mmp9*^(+/+) or *Mmp9*^(-/-) mice were treated with an EGFR tyrosine kinase inhibitor, erlotinib (100 mg/kg by gavage; OSI Pharmaceuticals, Melville, NY) after each acrolein exposure.

Tissue Preparation

After exposure, mice were injected with pentobarbital sodium (50 mg/kg, intraperitoneally; Abbott Labs, Chicago, IL) and the abdominal aorta severed. The right inferior lung lobe and the left lobe were clamped, excised, frozen in liquid nitrogen and stored (–70°C) for mRNA, Western blot, zymography, and MMP9 activity assays. For immunohistochemistry, the trachea was cannulated, lungs instilled (30 cm H₂O, 10% formalin No. SF100; Fisher Scientific, Fair Lawn, NJ), trachea ligated, and the lung was immersed in fixative (24 h, 4°C).

Quantitation of Transcript Levels

RNA was reverse transcribed into complementary cDNA as described (23). The PCR primers and probes (sequences in Table E1 in the online supplement) were used for quantitative real-time PCR (qRT-PCR) with a 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) to analyze samples in quadruplicate, and the results were expressed as fold change compared with control.

mMMP9 Immunohistochemistry

Paraffin sections (5 µm) were placed in citrate buffer solution (10 mM Citric acid, 0.05% Tween 20, pH 6.0, 30 min, 95°C), PBS rinsed, and incubated (30 min, 24°C) in 2% goat serum, 1% bovine serum albumin (BSA), 0.1% Triton X-100, and 0.5% Tween 20 solution. Sections were incubated (30 min, 24°C) with 1:100 MMP9 antibody (No. IM37L; Calbiochem, San Diego, CA) in 1% BSA solution. Peroxidase activity was quenched (15 min, 24°C) with 3% H₂O₂ in methanol. Sections were incubated (30 min, 24°C) with 1:5,000 horseradish peroxidase (HRP)-labeled goat anti-mouse secondary antibody (No. K5355; Dako Cytomation, Fort Collins, CO) in antibody dilution solution, PBS rinsed twice, and incubated (10 min, 25°C) with 0.05% 3,3'-diamino benzidine tetrahydrochloride (No. K5355; Dako Cytomation) in PBS and hematoxylin stained. The sections were visualized (Spot 2000 microscope) and images

captured by CCD-camera with Metamorph (Meta Imaging; Molecular Devices, Downington, PA).

mMMP9 and mGAPDH Western Blot

Frozen lung tissue was homogenized (Tekmar, Cincinnati, OH) and lysed with tissue protein extraction reagent (TPER) (No. 78510; Pierce, Rockford, IL) containing 1× protease inhibitor cocktail (No. 78410; Pierce). Homogenates were centrifuged (10,000 × g, 5 min, 4°C) and the supernatant protein concentration measured (bicinchoninic acid method). Lung protein (50 µg) was mixed with SDS sample buffer (No. 516732; Sigma, St. Louis, MO) and boiled (5 min). Protein was resolved by SDS polyacrylamide gel electrophoresis using 4 to 12% Tris-Glycine gels (No. EC6028; Invitrogen, Carlsbad, CA) and transferred electrophoretically to polyvinylidene dichloride membrane (No. LC2005; Invitrogen), incubated 1 hour with 5% fat-free milk in tris-buffered saline with 0.05% Tween-20 (TBS-T) (No. T9039; Sigma), and incubated with 1:1,000 anti-MMP9 antibody (overnight, 4°C, Cat. No. AB16306; Abcam, Cambridge, MA). The membrane was washed twice with TBS-T and incubated with 1:4,000 goat anti-rabbit IgG HRP-linked secondary antibody (1 h, 24°C, No. 7074; Cell Signaling Technology, Waltham, MA). The membrane was washed twice with TBS-T and bound antibody was visualized using enhanced chemiluminescent (Cat. No. RPN2108; Amersham Biosciences, Piscataway, NJ). The membrane was stripped in a % SDS 16 mM Tris-HCl solution (pH 6.7, 1 h, 60°C), and incubated with loading control 1:1,000 anti-GAPDH antibody (overnight, 4°C, Cat. No. AB9485; Abcam). The membrane was washed twice with TBS-T and incubated with 1:4,000 goat anti-rabbit IgG HRP-linked secondary antibody (1 h, 25°C), and bound antibody was visualized as above.

Mucin Protein Enzyme-Linked Immunosorbent Assay

Mucin protein in mouse lung homogenates was determined using a modification of the MUC5AC enzyme-linked immunosorbent assay method developed by Takeyama and colleagues (25). Briefly, 50 µl lung homogenates containing 100 µg protein, 0.1 to –100 µg/ml bovine mucus standard (Cat. No. M3895; Sigma), or known positive samples (mouse gastric and intestine preparations) or known negative samples (mouse brain preparations) were incubated (2 h, 37°C) with bicarbonate-carbonate buffer (50 µl) in a 96-well plate (Cat. No. 12-565-123; Fisher Scientific). Plates were washed three times with TBS-T and blocked with 2% BSA, fraction V (1 h, 24°C, Cat. No. A3156; Sigma). Plates were again washed three times with TBS-T and then incubated (overnight, 4°C) with MUC5AC monoclonal antibody (1:100, Cat. No. MS-145-P; NeoMarkers, Fremont, CA) or mouse IgG-κ subtype (1:100, Cat. No. AB18447; Abcam) or 0.2% BSA in TBS-T. The wells were washed three times with TBS-T and incubated (1 h, 37°C) with goat-anti-rabbit IgG-HRP linked antibody (1:2,000, Cat. No. SC2004; Santa Cruz Biotechnologies, Santa Cruz, CA) or TBS-T alone. The wells were washed three times with TBS-T and incubated with (1 h, 25°C) with *o*-phenylenediamine (Cat. No. P5412; Sigma) and stopped with 1 N H₂SO₄. Absorbance was read at 492 nm. The hMUC5AC antibody detected mMUC5AC protein in the mouse lung homogenates with sensitivity and cross-specificity. The relative amount of mucin protein was determined by comparing each sample (in duplicate) to the linear portion of the standard curve and expressed as fold change in the mucin level (because bovine mucus was used to generate the standard curve) in the acrolein-exposed animals as compared with the controls.

Gelatin Zymography

Lung protein (50 µg) in 2× Tris-Glycine buffer was electrophoresed on a 10% Tris-Glycine gel containing 0.1% gelatin (No. EC6175; Invitrogen). Gels were washed twice in zymogram renaturing solution (30 min, 24°C, No. LC2670; Invitrogen) and then preincubated (30 min) and incubated in zymogram developing solution (12 h, 37°C, No. LC2671; Invitrogen). Stained in 0.5% Coomassie blue R-250 (No. 20279; Pierce) in 40% CH₃OH:10% CH₃COOH (1 h, 24°C), and destained (rinsed twice) in 40% CH₃OH:10% CH₃COOH (1 h, 24°C) to visualize digested bands in the gelatin matrix.

Mass Spectrometric Analysis of Acrolein Concentrations

Acrolein in the induced sputum and expired breath condensate were analyzed as described previously (26, 27) and data from non- or

ex-smokers re-evaluated for this study. Briefly, expired breath condensate was collected (20 min; α -amylase free) and induced sputum was obtained after hypertonic saline aerosol inhalation from healthy non-smoking control subjects or ex-smoking subjects with COPD (meeting the Global Initiative for Chronic Obstructive Lung Disease criteria). Subjects with COPD had a history of cough and sputum production for over 2 consecutive years and for most days in a consecutive 3-month period, as well as fixed airflow obstruction, which was defined as postbronchodilator forced expiratory volume in one second (FEV₁)/forced vital capacity less than 70% and a postbronchodilator reversibility of FEV₁ less than 12%, measured at baseline and after inhalation of a β_2 -agonist (salbutamol 400 μ g, via metered-dose inhaler). All patients with COPD had a history of cigarette smoking (≥ 50 pack-yr), but at the time of testing all were ex-smokers. Data from current smokers was removed from the data set, so that endogenous acrolein levels could be estimated.

Sputum was mixed 1:1 with 1% dithiothreitol (No. D0632; Sigma) and incubated in 3% BSA solution (15 min, 37°C). PBS (1:1) was added and the sample filtered (70- μ m cell strainer, No. 352350; Becton Dickinson, Franklin Lakes, NJ) and centrifuged (300 $\times$ g, 5 min). Acrolein concentrations of the supernatant were determined by liquid chromatography/atmospheric chemical ionization tandem mass spectrometry (LC/APCI-MS/MS) (API 365; Perkin Elmer, Thornhill, ON, Canada) in negative-ion mode after 2,4-dinitrophenylhydrazine derivatization and separation with a Supelcosil C18 DB column (75 $\times$ 4.6mm inside diameter, 3 μ m; Supelco, Bellefonte, PA) using an CH₃COOH:CH₃OH-gradient (26). This study conformed to the declaration of Helsinki and was approved by the Internal Review Board of the Maugeri Foundation. Informed consent was obtained from all patients.

Cell Culture, Inhibitor Pretreatment, and *In Vitro* Acrolein Treatment

NCI-H292 cells (No. CRL-1848; ATCC, Manassas, VA) were grown in RPMI 1640 medium (No. 30-2001) with 10% fetal calf serum (No. 30-2020) (ATCC), 100 U/ml penicillin, and 100 μ g/ml streptomycin (37°C, pH 7.4; Sigma). For acrolein treatment, 5,000 cells/cm² NCI-H292 cells were seeded into 6-well plates (Cat. No. 3506; Corning, Corning, NY). Once confluent, the cells were incubated (24 h, 37°C, pH 7.4) in serum-free RPMI 1640. To determine whether acrolein treatment increases mMMP9 mRNA, NCI-H292 cells were treated (4 h, 37°C) with increasing concentrations of acrolein or dilution buffer (PBS). To determine the role of MMP/EGFR/MAPK signaling in acrolein-induced increased MMP9 mRNA, cells were treated with: (1) MMP inhibitor (10 μ M GM6001 [No. 364205; Calbiochem]), (2) EGFR neutralizing antibody (10 μ g/ml clone LA-1 [No. 05-101; Upstate, Charlottesville, VA]), or (3) EGFR tyrosine kinase inhibitor (250 nM AG1478 [a.k.a. Tyrophostin] [No. 658552; Calbiochem]). To determine the role of intracellular MAPK activation in increasing MMP9 transcripts, cells were treated with (1) MAPK3/2 (ERK1/2) inhibitor (5.0 μ M PD98059 [No. 51300; Calbiochem]), (2) MAPK8 (c-jun N-terminal kinase [JNK]) inhibitor (5.0 μ M SP600125 [No. 420119; Calbiochem]), or (3) MAPK14 (p38) inhibitor (2.5 μ M ML3403 [No. 506121; Calbiochem]). After 1 hour of pretreatment, cells were incubated (4 h, 37°C) with acrolein or vehicle (control), washed in PBS, lysed by Trizol reagent, and RNA isolated and reverse-transcribed into cDNA. PCR was performed using 7900HT Sequence Detection System (Applied Biosystems), and each sample was analyzed in quadruplicate, normalized to ribosomal protein L32 (RPL32).

Direct Activation of Human Pro-MMP9

To determine whether acrolein could lead to proenzyme cleavage (activation) measured by gelatin zymography, 20 ng human pro-MMP9 (92 kD Gelatinase, No. PF038; Calbiochem) was incubated with 100 to 1,000nM acrolein or 1 mM p-aminophenylmercuric acetate (APMA) (0–4 h, 37°C, No. A9563; Sigma) (28) in reaction buffer containing 50 mM Tris-HCl, 1.5 mM NaCl, 0.5 mM CaCl₂, 1 μ M ZnCl₂, and 0.01% Brij-35 detergent (pH 7.6) (No. 16005; Sigma). To determine whether acrolein increased MMP9 chromogenic peptide substrate assay (No. RPN2634; Amersham Biosciences), acrolein-exposed pro-MMP9 samples diluted 10-fold in reaction buffer were incubated in MMP9-antibody coated microtiter plates. The wells were washed with 0.01 M sodium PBS and 0.05% Tween 20 solution (pH 7.0) and incubated with (to determine total MMP activity) or without 1 mM APMA (2 h, 37°C). Samples were incubated with detection-enzyme (modified murexase) and substrate

(S-2444) reagent in 50 mM Tris-HCl, 1.5 mM NaCl, 0.5 mM CaCl₂, 1 μ M ZnCl₂, 1 unit/ml Heparin, and 0.01% Brij 35 detergent solution (pH 7.6) and read spectrophotometrically (405 nm). Total MMP9 (pro-MMP9 plus active MMP9) and active MMP9 were determined and compared to a standard curve. In separate assays, lungs from acrolein-exposed *Mmp9*^(+/+) mice were homogenized using extraction buffer 50 mM Tris-HCl, 1.5 mM NaCl, 0.5 mM CaCl₂, 1 μ M ZnCl₂, 0.01% Brij 35 detergent, and 0.25% Triton X-100 (No. T8787; Sigma) (pH 7.6). Each sample was centrifuged (4 min, 4°C, 1,300 $\times$ g) and 100 μ l of supernatant assayed.

RESULTS

Acrolein Alters the Transcript Level of mMMP9 and mTIMP3 in Mouse Lung

Mice exposed to acrolein had increased MMP9 transcripts, whereas transcript levels of MMP2, 3, 7, 12, and A disintegrin and metalloproteinase domain (ADAM17) were unchanged (Figure 1A). Transcripts of mTIMP3, an inhibitor of MMP9 (29) and mADAM17 (30), decreased in the lung of acrolein-exposed mice, whereas mTIMP1, 2, and 4 transcripts were unchanged (Figure 1B). Thus, acrolein increased mMMP9 transcript levels and

Figure 1. Matrix metalloproteinases (MMP), a disintegrin and metalloproteinase domain protein (ADAM) 17, and tissue inhibitor of metalloproteinase protein (TIMP) transcript levels are altered in mouse lung after acrolein exposure. (A) MMP9 transcripts increased after acrolein exposure, whereas transcript levels of MMP2, 3, 7, and 13 or ADAM17 transcripts were unchanged. (B) TIMP3 transcripts decreased after acrolein exposure, whereas transcript levels of TIMP1, 2, and 4 were unchanged. Mice were exposed to 2.0 ppm acrolein $\times$ 6 hours/day $\times$ 5 days/week $\times$ 4 weeks or filtered air (control), and the lung mRNA isolation, and transcripts of various MMPs, TIMPs, and ADAM17 was determined by quantitative real-time PCR (qRT-PCR). Results (mean $\pm$ SEM, $n = 6$ /group) are expressed as fold change after normalizing to GAPDH. *Significantly different from control as determined by ANOVA, Student-Newman-Keul's test ($P < 0.05$).

decreased transcript levels of mTIMP3, an inhibitor of MMP9, mADAM17, and other MMPs *in vivo*.

Acrolein Increases mMMP9 Protein and Activity in Mouse Lung

The increased mMMP9 transcripts induced by repetitive acrolein exposure were accompanied by an increase in mMMP9 protein levels. The airways from acrolein-exposed mice had increased MMP9 immunostaining (Figure 2D) and mMMP9 protein (Figure 2E) compared with the airways from control (unexposed) mice (Figure 2B). Gelatinase activity (~ 103 kD: pro-MMP9; ~ 86 kD: MMP9) (Figure 2F) and activity, as measured by chromogenic peptide substrate assay (Figure 2G), increased in lung homogenates from acrolein-exposed mice. Thus, acrolein in-

creased mMMP9 protein in the airways and MMP9 activity in the lungs of mice.

mMMP9 Mediates Increased mMUC5AC Transcripts and Mucin Protein in the Lungs after Acrolein Exposure

Lung mMUC5AC transcript (Figure 3A) levels increased in acrolein-exposed *Mmp9*^(+/+) and *Mmp9*^(-/-) mice as compared to those of genotype control (filtered air) mice. This effect was diminished in mice treated with an EGFR tyrosine kinase antagonists, erlotinib. The increase in mMUC5AC transcripts was significantly lower in acrolein-exposed *Mmp9*^(-/-) mice than *Mmp9*^(+/+) mice (Figure 3). A similar set of responses was noted in mucin protein levels (Figure 3B). Thus, mice deficient in mMMP9 or treated with an EGFR antagonist responded less,

Figure 2. MMP9 protein and activity increased in the mouse lung after acrolein exposure. (D) Immunostaining with anti-MMP9 antibody (Ab) increased in the respiratory epithelium of acrolein-exposed mice after acrolein exposure as compared with (B) control mice. No immunostaining was observed with immunoglobulin G (IgG) Ab (isotype control) in the respiratory epithelium of (A) control or (C) acrolein-exposed mice. Mice were exposed to 2.0 ppm acrolein $\times$ 6 hours/day $\times$ 5 days/week $\times$ 4 weeks or filtered air (control) and serial sections of paraffin-embedded lung tissue were immunostained with anti-MMP9 Ab or anti-IgG Ab. (Original magnification: $\times 200$; inset: $\times 600$). (E) MMP9 protein increased in mouse lung exposed to acrolein, whereas GAPDH protein levels remained unchanged (loading control). Western blot was performed with 50 μ g lung protein of an individual mouse that is representative of each group ($n = 5$ /treatment). (F) Gelatinase activity (~ 103 kD and ~ 94 kD: MMP9) increased in acrolein-exposed mouse lung as compared with controls. Gelatin zymography was performed with 25 μ g protein obtained from an individual mouse/lane that are representative of each group. (G) MMP9 activity increased in mouse lung exposed to acrolein as compared with control. Active MMP9 in lung homogenate was captured with anti-MMP9 antibody, which was then used to activate a modified murourokinase detection enzyme to cleave a chromogenic peptide substrate and optical density determined spectrophotometrically at 405 nm (OD_{405}). The results (mean $\pm$ SEM, $n = 5$ –6/group) are expressed as fold increase in the MMP9 activity as compared to control. *Significantly different from the control as determined by ANOVA, Student-Newman-Keul's test ($P < 0.05$).

Figure 3. MMP9 and epidermal growth factor receptor (EGFR) mediates acrolein-induced increase in mucin 5 (subtype A&C) (MUC5AC) transcript and mucin protein in mouse lung. (A) Transcript levels of MUC5AC increased more in the lung of *Mmp9*^(+/+) control mouse than *Mmp9*^(-/-) gene-targeted mouse after acrolein exposure. This effect was diminished in mice treated with an EGFR antagonist, erlotinib. Mice were exposed to or filtered air (control) or to 2.0 ppm acrolein (6h/d × 5d/wk × 4 wk) with or without treatment with erlotinib (100 mg/kg by gavage after each exposure). Transcript levels were determined by qRT-PCR and results (mean ± SEM, *n* = 5/group) are expressed as fold change after normalizing to GAPDH. (B) Mucin protein levels increased more in the lung of *Mmp9*^(+/+) mouse than *Mmp9*^(-/-) mouse after acrolein exposure. This effect was diminished in mice treated with an EGFR antagonist, erlotinib (100 mg/kg by gavage after each exposure). Mucin protein levels were determined by enzyme-linked immunosorbent assay using bovine mucus as a standard and the results are expressed as fold increase as compared to the strain-matched control. Values are mean ± SEM, *n* = 3 to 5/group. *Significantly different from the control, †significantly different from the acrolein-exposed *Mmp9*^(+/+), or ‡significantly different from the acrolein-exposed *Mmp9*^(-/-) as determined by ANOVA, Student-Newman-Keul's test (*P* < 0.05).

supporting our hypothesis that acrolein-induced increases in mMUC5AC are mediated, in part, by mMMP9 and EGFR.

Acrolein Concentrations Found in COPD Sputum Can Activate hMMP9 Directly

To assess whether endogenous acrolein concentrations are sufficient to activate hMMP9, we measured acrolein in expired breath condensate and induced sputum in healthy control subjects and in subjects with COPD using LC/APCI-MS/MS. Acrolein levels in expired breath and sputum from subjects with COPD was sig-

nificantly greater than from control subjects (Figure 4A). From this analysis we selected concentrations of more than 100 nM to test whether endogenous levels could activate pro-hMMP9. Incubation of pro-MMP9 (92 kD) with organomercurial compound, APMA, removes approximately 8 to 9 kD of N-terminal prodomain of pro-MMP9 to generate approximately 84 kD active isoform (24). Acrolein had a similar effect and increased the active isoforms (Figure 4B) within 30 minutes (Figure 4C). Consistent with presence pro-MMP9 and active hMMP9 dimers (8, 28), additional approximately 150- to 200-kD isoforms were observed (data not shown). hMMP9 activity as measured by chromogenic peptide substrate assay also increased significantly after acrolein exposure (Figure 4D). Thus, acrolein increased hMMP9 activity in a cell-free system in concentrations similar to those found in COPD sputum.

Acrolein-Induced Increased hMMP9 Transcript Levels Are Mediated through EGFR/MAPK3/2 Signaling

Acrolein (≥ 10 nM) increased hMMP9 transcripts in a concentration-dependent manner in NCI-H292 cells (Figure 5A). Increased hMMP9 transcripts were mediated by MMPs, because the broad-spectrum MMP inhibitor (GM6001) diminished this increase (Figure 5B). Similarly, increased hMMP9 transcripts were mediated by EGFR activation, inasmuch as pretreatment with an EGFR tyrosine kinase inhibitor (AG1478), or an EGFR-neutralizing antibody (LA-1) reduced this response (Figure 5B). EGFR activation leads to activation of downstream MAPKs. Treating the cells with an MAPK3/2 (a.k.a. ERK1/2) inhibitor (PD98059) reduced the acrolein-induced increase in hMMP9 transcripts (Figure 5B). The increased hMMP9 transcripts (5.7- ± 0.4-fold) were not significantly inhibited by pretreatment with an MAPK8 (a.k.a. JNK) inhibitor (SP600125) (4.2- ± 1.2-fold) or an MAPK14 (a.k.a. p38 MAPK) inhibitor (ML3403) (4.0- ± 0.9-fold). Thus, increase in hMMP9 transcripts in acrolein-treated airway epithelial cells is mediated by EGFR-MAPK3/2 signal transduction pathway.

DISCUSSION

Exogenously generated acrolein induces mucin production *in vivo*. Animals exposed repeatedly to acrolein develop airway epithelial damage and bronchiolitis, with excessive macrophages (11, 12, 15). Acrolein exposure increases the number of goblet cells, particularly in small airways, and increased lung MUC5AC transcripts in rats (12) and MUC5AC-immunoreactive protein in mice (21). However, the mechanism of acrolein-induced increase in MUC5AC is unknown.

Neutral MMPs are minimally composed of a prodomain that requires cleavage for activation of a zinc-binding catalytic domain. Although the concept that MMPs are involved in the pathogenesis of emphysema is established, the role of MMPs in mucin overproduction in COPD is unclear. Previously we reported that acrolein increased mMUC5AC transcripts and macrophage accumulation more in strain-matched control mice as compared to *Mmp12*^(-/-) mice (21). Similarly, Kim and coworkers reported that LPS increased goblet cells in rat lung, and this response is accompanied by increased immunohistochemical staining for rMMP9 and EGFR phosphorylation (23). Moreover, pretreatment with an MMP inhibitor decreased the LPS-induced increase in goblet cells. However, because this inhibitor is effective against several MMPs, it was important to further determine the identity of MMPs and TIMPs that may be involved.

To begin to determine the identity of MMPs involved in acrolein-induced MUC5AC increase, we measured transcript levels of several mMMPs and found that mMMP2, 3, 7, 12, and

Figure 4. Acrolein increased MMP9 activity *in vitro*. (A) Acrolein concentrations are increased in expired breath condensate and induced sputum from subjects with COPD (57.1 ± 10.1 and 131.5 ± 23.8 nM, respectively) as compared with control subjects (9.3 ± 1.5 and 1.5 ± 1.0 nM, respectively) as determined by liquid chromatography/atmospheric chemical ionization tandem mass spectrometry (LC/APCI-MS/MS) ($n = 9$ /group). (B) Acrolein increased pro-MMP9 (92 kD) cleavage to MMP9 (~84, 86 kD) in a cell-free system in a concentration-dependent manner. (C) Acrolein increased pro-MMP9 (92 kD) cleavage to MMP9 (~84, 86 kD) as early as 30 minutes. (D) Acrolein increased the pro-MMP9 activation *in vitro* in a concentration-dependent manner. Proenzyme MMP9 cleavage was determined by incubating (0–4 h, 37°C) 20 ng human pro-MMP9 with acrolein or 1 mM p-aminophenylmercuric acetate (APMA), and gelatin zymography was performed. This zymograph is representative of triplicate tests. To determine MMP9 activity, total MMP9 (pro-MMP9 and active MMP9) was captured with anti-MMP9 antibody, which incubated with or without 1 mM APMA to fully activate pro-MMP9. Active MMP9 then activated a modified murour-kinase detection enzyme to cleave a chromomeric peptide substrate and the resultant color was measured (OD₄₀₅). The results (mean ± SEM) are expressed as percentage inducible activation of pro-MMP9 (Total

Figure 5. Acrolein-induced increases in MMP9 transcript levels are mediated by metalloproteinase-dependent epidermal growth factor receptor/mitogen activated protein kinase (EGFR/MAPK) signaling in human airway epithelial cells. (A) Acrolein increased MMP9 transcript levels in a dose-dependent manner in NCI-H292 cells as compared with the control (vehicle-treated cells). (B) Acrolein-induced increases in MMP9 transcript levels are diminished in cells treated with metalloproteinase inhibitor (GM6001), EGFR neutralizing antibody (LA-1), an EGFR kinase inhibitor (AG1478), or an MAPK3/2 inhibitor (PD98059). Confluent NCI-H292 cells were pretreated (1 h) with 10 μM GM6001, 10 ng/ml LA-1, 0.25 μM AG1478, or 5 μM PD98059. In addition, cells were treated with 5 μM SP600125, an MAPK8 inhibitor, or 2.5 μM MML3403, a MAPK14 inhibitor. Cells were then incubated (37°C, 4 h) with 100 nM acrolein or vehicle. RNA was isolated and MMP9 transcripts determined by qRT-PCR. The results (mean ± SEM, $n = 6-9$) are expressed as fold change in the level of MMP9 transcripts after normalizing to RPL32. *Significantly different from control or †significantly different from the acrolein-treated samples (without inhibitor) as determined by ANOVA, Student-Newman-Keul's test ($P < 0.05$).

13 were unaltered in mouse lung after acrolein exposure. In contrast, mMMP9 transcripts, protein, and activity levels markedly increased after acrolein exposure. Although this evidence supports a role for mMMP9, it does not rule out possible contribution of other MMPs, because their activity was not measured. In the lung, hMMP9 is produced by bronchial epithelial, Clara, alveolar type II cells, smooth muscle, and endothelial cells (8). Immunostaining for airway MMP9 increased in acrolein-exposed (Figures 2C and 2D) as compared with control mice

MMP9 activity – MMP9 without acrolein constitutive activity). *Significantly different from the control as determined by ANOVA, Student-Newman-Keul's test ($P < 0.05$).

(Figures 2A and 2B). The intensity of immunostaining was greatest in epithelial cells and the extracellular matrix below the surface cells. This supports the role of the epithelial cells as a source of the secreted MMP9. In addition, the airway epithelial cell is likely to receive the greatest dose of acrolein, and this may be sufficient to initiate the response, consistent with previous *in vitro* studies (23).

To assess the role of mMMP9 in MUC5AC production *in vivo*, gene-targeted *Mmp9*^(-/-) and control *Mmp9*^(+/+) mice were exposed to acrolein. Acrolein increased mMUC5AC transcripts in the lungs of both *Mmp9*^(-/-) and control *Mmp9*^(+/+) mice as compared with the filtered-air control (Figure 3). However, the increase in mMUC5AC transcript and mucin protein was significantly lower in the lungs of gene-targeted *Mmp9*^(-/-) mice. In addition, treating mice with an EGFR antagonist, erlotinib, diminished this response. These findings support a role for EGFR tyrosine kinase activation, and in part, a role for mMMP9 in mediating mMUC5AC production *in vivo*. The nearly complete reversal with EGFR antagonist, but partial inhibition by *Mmp9* gene targeting suggests that other proteinases (e.g., mMMP12 or mADAM17) may play a role in the response (21–25). The source of these MMPs may also be complex *in vivo*, with a likelihood of contributions from cells other than epithelial cells (7, 9, 21).

Due to wide-ranging biological consequences, hMMP9 activity is tightly controlled. hMMP9 is synthesized as an inactive proenzyme (pro-MMP9) and is activated by proteolytic removal of an amino-terminal following secretion into the extracellular space. Through the interaction of zinc in the active site with cysteines in the prodomain, the enzyme is kept latent. Oxidants and other compounds interact with the protective cysteine. This reduces latency by altering protein conformation that permits entrance of water essential for catalysis and accelerates the autocatalytic loss of the prodomain. This process is called the “cysteine switch” mechanism (30, 31). While many different oxidants and metals can activate this mechanism, it is currently unknown whether acrolein could act through this mechanism.

Because of its ability to covalently modify macromolecules and disrupt critical cellular functions (16, 32, 33), acrolein is considered an important mediator of cell damage. Acrolein can be generated endogenously during inflammatory oxidative stress and may be a mediator of chronic diseases. Acrolein–protein adducts have been found to accumulate in ischemic tissue and in

atherosclerotic lesions (16–20). Previously, we found that acrolein induced the release of pro-MMP9 into the cell culture supernatant from NCI-H292 cells and increased gelatinase activity (24). In the present study we found that acrolein led to pro-MMP9 cleavage in a concentration-dependent fashion (Figure 4) directly in a cell-free system. In addition, mMMP9 activity increased in acrolein-exposed mouse lungs (Figure 2D). The acrolein levels measured in the sputum from subjects with COPD were likely to have been from endogenous sources because the subjects were not current smokers. This suggests that chronic inflammation and oxidative stress established in persistent COPD can generate acrolein levels sufficient to activate hMMP9, which in turn, contributes to hMUC5AC accumulation.

Enzymatically active MMPs are thought to influence cell behavior by cleaving cell–cell adhesion proteins, by releasing bioactive cell surface molecules, or by cleaving cell surface molecules that transduce signals from the extracellular environment. A variety of growth factor receptors, binding proteins, and ligands are MMP substrates. hMMP9 and hMMP2 can cleave cell surface bound pro-EGFR ligands and activated EGFR in pituitary gonadotrophic cells (34). In this study, although the EGFR inhibition was demonstrated *in vivo*, we did not measure EGFR ligands, so caution about this aspect of the study needs to be exercised. Several likely ligands include amphiregulin, epiregulin, heparin-binding growth factor, and transforming growth factor- α . Airway epithelial cells release these ligands upon stimulation with cigarette smoke (35, 36), PMA (36), or compression stress (37). In addition, cigarette smoke exposure can increase hMUC5AC production (36). Consistent with this possibility, we found previously that small interfering (si) RNA directed against hMMP9 inhibited the acrolein-induced increase in hMUC5AC transcripts in airway epithelial cells via ligand-dependent EGFR activation (24).

hMMP9 activity also is regulated at the transcriptional level and by post-transcriptional protein processing and by anti-proteinases (e.g., hTIMPs). Different signaling cascades are involved in increased hMMP9 expression, depending on the stimulus and the cell type. In various cell types, *MMP9* gene expression is regulated by numerous stimulatory and suppressive factors, including several cytokines and growth factors—for example, IL-1 α , IL-2, IL-8, IFN- γ , basic fibroblast growth factor, and TGF- β (8, 38).

Figure 6. Acrolein-activated MMP9 leads to persistent increase in mucin 5, subtypes A and C (MUC5AC) production. (A) Acrolein increased the cleavage of pro-MMP9 leading to its activation. (B) Once activated, MMP9 can cleave cell surface bound pro-EGFR ligands. (C) EGFR ligands bind to and activate EGFR and the downstream MAPK signaling pathway. Subsequently (D) MMP9 transcripts and (E) pro-MMP9 protein increased. This also leads to an increase in MUC5AC transcripts. (F) Acrolein increased the release of pro-MMP9, which then can be activated once again by acrolein (A). Thus chronic exposure to acrolein initiates an feedback loop that is mediated by MMP9, leading to persistent mucin production.

Because acrolein increased EGFR phosphorylation in NCI-H292 cells (23), we investigated the role of EGFR in acrolein-induced increases in MMP9 transcripts. Pretreatment with an EGFR kinase inhibitor (AG1478) or an EGFR-neutralizing antibody (LA-1) decreased the acrolein-induced increase in hMMP9 transcripts, supporting EGFR involvement. Phosphorylation of EGFR leads to activation of downstream MAPKs. Increased hMMP9 expression by IL-1, IL-17, PMA, and TNF- α is thought to be mediated by activation of the transcription factors NF- κ B and AP-1, mainly via MAPK8 (JNK) or MAPK14 (p38) pathways (28, 39, 40). In the present study, pretreatment with MAPK3/2 inhibitor (PD98059), but not the MAPK8 inhibitor (SP600125) or MAPK14 inhibitor (ML3403), decreased the acrolein-induced increase in MMP9 transcripts. Together, these findings support a role for EGFR/MAPK signaling pathway in the accumulation of hMMP9 transcript and protein after acrolein exposure.

hMMP9 activity is also regulated by the endogenous inhibitors, including TIMPs (30). hTIMP1-4 (40, 41) are expressed in bronchial epithelium. hTIMP2 and to a lesser extent hTIMP1 bind to hMMP9 and inhibit its activity (42). hTIMP3 binds with high affinity to and inhibits hMMP9 (43) and hADAM17 (44). In contrast to hTIMP2, which is constitutively expressed (45), hTIMP1, 3, and 4 can be induced by extracellular signals including growth factors, matrix proteins, and inflammatory cytokines selectively (46). Although acrolein was likely to increase several of these proteins, mTIMP3 transcripts decreased, and the transcript levels of mTIMP1, mTIMP2, and mTIMP4 were unaltered (Figure 1B). Decreases in mTIMP3 also could explain how mADAM17 activity may be involved in the increase in mMU-C5AC even though mADAM17 transcript levels did not change (24, 36). Because hTIMP3 and hMMP9 are produced by airway epithelial cells, the decreased mTIMP3 transcripts and increased mMMP9 transcripts/protein are consistent with increased hMMP9 activity.

Persistent dysregulation can be established when soluble ligands secreted by cells bind to and stimulate receptors on their own surfaces (47) (Figure 6). By disrupting the cysteine residues in the pro-domain, exogenous acrolein could enhance pro-MMP9 cleavage to lower molecular weight isoforms and increase hMMP9 activity directly (Figure 4). Once activated, hMMP9 proteolytically processes various pro-EGFR ligands on the cell surface that can bind to EGF receptors on the same or an adjacent cell (34). EGFR/MAPK activation increased MUC5AC transcription (21–25, 36). Concurrently, acrolein increased the hMMP9 transcripts via EGFR/MAPK signaling (Figure 5) and stimulated the release of pro-MMP9 in the conditioned medium (24), which can be activated by acrolein subsequently. Thus acrolein increased cleavage of pro-MMP9 and increased hMMP9 activity, leading to additional hMMP9 transcripts, protein, and pro-MMP9 release. This in turn can release more pro-EGFR ligands and establish persistent activation.

Finally, the acrolein concentrations used in these experiments need to be placed in the context of human experiences. The inhaled concentration of 2 ppm acrolein for 6 hours approximates the amount that would be delivered by 10 cigarettes (or 0.5 packs/d). This concentration also can be experienced during exposure to environmental tobacco smoke, which has been estimated to affect 30 million nonsmokers (13, 48). Moreover, the acrolein concentration necessary to induce cleavage of pro-MMP9 to the lower molecular weight isoforms is nearly equivalent to the levels endogenously produced in the airway secretions of individuals with COPD (Figure 4). Because MMP9 gelatinase activity is present in airway secretions from individuals with COPD (8), acrolein is likely to contribute to the synthesis, secretion, and activation of pro-MMP9. The consequences of this activation are consistent with mucin overproduction that plagues this condition.

Conflict of Interest Statement: J.A.N. and UCSF have patent application for prevention of mucus production by administration of EGFR Antagonists. None of the other authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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