Matrix metalloproteinase (MMP) 14 mediates a phenotypic shift in the airways to increase mucin production.

Online Data Supplement

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Detailed Methods

Acrolein and tobacco smoke exposures: Mice were maintained in virus-free containment and handled in accordance with the Institutional Animal Use and Care Committee of the University of Cincinnati, Cincinnati Children's Hospital Research Foundation, and the University of Pittsburgh. FVB/NJ mice (male, 6-8 wk; Jackson Laboratories, Bar Harbor, ME) were exposed to filtered air (control), acrolein (Cat. No. 36520, Alfa Aesar, Ward Hill, MA) (2.0 ppm x 6 h/d x 5 d/wk x 4 wk) as previously described (11, 12, 24), or tobacco smoke. Tobacco smoke was generated from 2R4F research cigarettes (University of Kentucky, Lexington, KY) using a Teague E10 smoking machine (Teague Enterprises, Woodland, CA) (35 ml puffs of 2 sec duration once each min for up to 8 min). The smoke from the smoldering end of each cigarette was transported to an aging chamber (2-4 min) and mice were exposed to diluted smoke (100 mg/m^3 total suspended particulate matter x 6h/d x 5d/wk x 13wks).

The mice were killed immediately after the last acrolein exposure or 48 h after tobacco smoke exposure by an intraperitoneal injection of pentobarbital sodium (50 mg/kg; Nembutol, Abbott Laboratories, Chicago, IL) and severing of the posterior abdominal aorta. The chest cavity was opened and the right inferior lobe and the left lobe were clamped, excised, frozen in liquid nitrogen and stored (-70°C) for later mRNA analysis, western blot, and MMP14 metalloproteinase activity assay. To obtain tissue for immunohistochemsitry, a cannula was inserted in the middle of the trachea and the lung was instilled (pressure: 30 cmH_2O) with 10% phosphate-buffered formalin (Cat. No. SF100, Fisher, Fair Lawn, NJ). The trachea was ligated, and the inflated lung was immersed in fixative (24 h, 4°C). Fixed tissues were dissected after 24 h, and the trachea and midlobe sections of the left lung were washed with phosphate-buffered saline (PBS) (Cat. No. 14287–080, Invitrogen, Carlsbad, CA), dehydrated through a series of graded ethanol solutions (30-70%), and processed into paraffin blocks (Hypercenter XP, Shandon, Ramsey, MN).

MMP14 and MU5AC transcript levels in acrolein exposed or conditional doxycycline-regulated epidermal growth factor receptor (EGFR) ligand (Transforming growth factor alpha: TGFA) transgenic mice treated with an EGFR inhibitor (erlotinib): FVB/NJ mice were pre-treated with erlotinib or vehicle

(control) and exposed to acrolein (2.0 ppm x 6 h/d x 5 d/wk x 4 wk). Erlotinib powder (100 μg/g body weight; OSI Pharmaceuticals, Melville, NY) was suspended in 0.5% methylcellulose (0.015 mg/μl, 37°C; Colorcon, West Point, PA). Three hours before administration, food and water were removed from cages. Mice were then anesthetized (isoflurane; Abbott Laboratories, Chicago, IL), and volume-matched sterile vehicle control (150–250 μl methylcellulose) or vehicle containing drug was administered by gavage using a 20-gauge feeding catheter (Harvard Apparatus, Holliston, MA). In additional tests, conditional doxycycline-regulated TGFA transgenic mice were used to induce lung-specific EGFR-ligand. These mice were generated from *Scgb1a1*-rtTA activator mice expressing the reverse tetracycline-responsive transactivator (rtTA) under control of the 2.3-kb secretoglobin, family 1A, member 1 (*Scgb1a1*) gene promoter(28)e mated to conditional doxycycline-regulated transgenic mice containing the human *TGFA* cDNA under the control of seven copies of the tetracycline operon [$(TetO)_7$-cmv *TGFA*] and a minimal cytomegalovirus (CMV) promoter (29, 30). All mice were derived from the FVB/NJ inbred strain. To induce TGFA expression, doxycycline (Sigma, St. Louis, MO) was administered in the drinking water at a final concentration of 0.5 mg/ml and in food (62.5 mg/kg). Water was replaced thrice per week.

Quantization of transcript levels: To determine whether acrolein-exposure alters the transcript levels of MMP14, total RNA from each mouse or NCI-H292 or NHBE cells was reverse transcribed into cDNA as previously described (23). cDNA (2 μL) was used in the subsequent PCR reaction using TaqMan universal master mix (2x, 12.5 μL) [Cat. No. 4304437, Applied Biosystems Inc (ABI), Foster City, CA], in a 25 μL reaction mixture containing 0.2 μM forward and reverse primers (Sigma Genosys, Austin TX) and 0.1μM TaqMan sequence specific FAM-TAMRA probes (Synthegen, Houston, TX). qRT-PCR was performed using ABI 7600 PCR machine as follows: 95°C for 15 minutes followed by 40 cycles of 95°C for 15 s and 60°C for 1 minute. For each RT-PCR, a serial dilution (0.5-0.032 μg) of total mRNA was amplified to obtain a standard curve. The relative amount of transcript was determined by comparing each sample against the standard curve. Each sample was analyzed in quadruplicate, normalized to GAPDH for murine transcripts or ribosomal protein L32 (RPL32) for human transcripts, and results were expressed as fold increase or decrease with respect to the control. The probes used

were: (A.) Murine MMP14: Forward primer: 5'-AGGAGACAGAGGTGATCATCATTG-3': Reverse primer: 5'-GTCCCATGGCGTCTGAAGA-3', Probe: FAM-CCTGCCGGTACTACTGCTGCTCCTG-TAMRA, (B.) Murine GAPDH: Forward primer: 5'-GTCGTGGATCTGACGTGCC-3', Reverse primer: 5'-TGCCTGCTTCACCACCTTCT-3', Probe: FAM-CCTGGAGAAACCTGCCAAGTATGATGACA-TAMRA ,(C.) Human MMP14: Cat. No. Hs00237119.m1 (ABI), and (D.) Human RPL32 Cat. No. Hs00851655.g1 (ABI).

MMP14 immunohistochemistry: For immunohistochemical detection of MMP14, 5 µm paraffin sections were treated (30 min, 95°C) with citrate buffer (10 mM Citric acid, 0.05% Tween 20, pH 6.0), rinsed (2X) in PBS and incubated (30 min, 25°C) with serum blocking solution (2% goat serum, 1% BSA, 0.1 % Triton X-100 and 0.5 % Tween 20). Sections were incubated (30 min, 25°C) with anti-MMP14 (1:100) antibody (Cat. No. MAB3317, Chemicon, Temecula, CA) in antibody dilution buffer (1% BSA). The MMP14 monoclonal antibody MAB3317 is directed at the peptide sequence CDGNFDTVAMLRGEM in the hemopexin domain of human MMP14. This antibody also may recognize MMP15 (Mt2-MMP) because MMP15 also contains a hemopexin domain. MMP15 is expressed mainly in the prostate and the gut, but may also be found in the lung although it has been not to be present in normal human bronchial epithelial cells or A549 lung cell lines (Kobayashi et al. Oncogene 23:3089, 2004). This antibody also has a very slight cross-reactivity to human MMP3 protein. Endogenous peroxidase activity was quenched (15 min, 25°C) with 3% H_2O_2 in methanol. The section was incubated (30 min, 25°C) with horse radish peroxidase (HRP)-labeled goat anti-mouse secondary antibody (1:5000) (Cat. No. K5355, Dako Cytomation, Fort Collins, CO) in antibody dilution buffer, rinsed (2X, PBS) and incubated (10 min, 25°C) with chromogen, 3, 3'diamino benzidine tetrachloride (DAB) (0.05%) (Cat. No. K5355, Dako Cytomation) in PBS and counterstained (1 min, 25°C) with hematoxylin. The sections were visualized under Spot 2000 microscope (40X objective) and the images were captured by a cooled CCD camera with Metamorph™ (Meta Imaging, Molecular devices, Downington, PA). For studies with human tissue, paraffin embedded lung blocks that included airways from 5 subjects with COPD acquired at time of lung transplantation and 5 subjects with a smoking history but no evidence of COPD at the time of a lobar resection for a solitary pulmonary

nodule were immunostained with the anti-MMP14 antibody (1:100 MAB3317, Chemicon). None of the subjects were current smokers and details of the characteristics are presented in **Supplemental Table 1**. Because of difficulties in visualizing DAB due to anthracotic particles in the human lung a NovaRED (SK-4800, Vector Labs, Burlingame CA) was utilized with a Vectastain ABC peroxidase kit (PK-4000, Vector Labs, Burlingame, CA) for signal amplification. All human studies were approved by the Washington University School of Medicine Human Studies Committee.

MMP14 and GAPDH western blot: To determine whether acrolein alters the protein level of murine MMP9 and MUC5AC, frozen lung tissue was homogenized (Tekmar, Cincinnati, OH) at maximum speed and lysed with ice-cold tissue protein extraction reagent (TPER) (Cat. No. 78510, Pierce, Rockford, IL) containing 1X protease inhibitor cocktail (Cat. No. 78410, Pierce). The whole lung homogenates were centrifuged (12,000 g, 5 min, 4°C) and the supernatant was used to determine the protein concentration using the bicinchoninic acid (BCA) method. Lung protein (50 µg) was mixed with 2X SDS sample buffer (Cat. No. 516732, Sigma, St. Louis, MO) and boiled (5 min). Protein was resolved by SDS polyacrylamide gel electrophoresis using 4-12 % Tris-Glycine gels (Cat. No. EC6028, Invitrogen, Carlsbad, CA) and transferred electrophoretically to polyvinylidene dichloride (PVDF) membrane (Cat. No. LC2005; Invitrogen), which was incubated with 5% fat-free skimmed milk in tris-buffered saline (TBS) containing 0.05% Tween 20 (1 h, 25°C) and incubated (overnight, 4°C) with anti-MMP14 antibody (1:1000) (Cat. No. AB8102, Chemicon) directed at the catalytic domain of MMP14. The membrane was washed twice with TBS containing 0.05% Tween 20 (0.5% TBS-T) and then incubated (1 h, 25°C) with 1: 4000 goat anti-rabbit IgG HRP linked secondary antibody (Cat. No. 7074, Cell Signaling Technology, Waltham, MA). The membrane was washed twice with 0.5% TBS-T and bound antibody was visualized using enhanced chemiluminescent kit (Cat. No. RPN2108, Amersham Biosciences, Piscataway, NJ). The membrane was stripped using a stripping solution containing 2 % SDS, 16 mM Tris-HCl (pH 6.7) at 60°C for 1 h. The membrane was incubated (overnight, 4°C) with 1:1000 anti-GAPDH antibody (Cat. No. AB9485, Abcam) for loading control. The membrane was washed twice with 0.5% TBS-T and then incubated (1 h, 25°C) with 1: 4000 goat anti-rabbit IgG HRP

linked secondary antibody, washed twice with 0.5% TBS-T and bound antibody was visualized using enhanced chemiluminescent kit.

Cell culture, inhibitor pretreatment and in vitro acrolein treatment: NCI-H292 cells (ATCC, Cat. No. CRL-1848, Manassas, VA) were grown in 75-cm^2 plastic tissue culture flasks (Corning, Cat. No. 3376, Corning, NY) and maintained in RPMI 1640 medium (ATCC, Cat. No. 30-2001), supplemented with 10% fetal calf serum (ATCC, Cat. No. 30-2020), penicillin (100 U/ml), and streptomycin (100 µg/ml; both from Sigma, St. Louis, MO) (37°C, pH 7.4). In a few studies, normal human bronchial epithelial (NHBE) cells (Cat. No. CC2540, Cambrex Biosciences, Baltimore, MD) were cultured in 75-cm^2 plastic tissue culture flasks and maintained in bronchial epithelial cell growth medium (Cat. No. CC3170, 3171 and CC-4175, Cambrex Biosciences, Baltimore, MD). For acrolein treatment, NCI-H292 or NHBE cells were seeded (5,000 cells/cm^2) into 30 mm six-well plates (Corning, Cat. No. 3506, Corning, NY). Once confluent, the cells were incubated (37°C, pH 7.4) for 24 h in serum-free medium (RPMI 1640) for NCI-H292 cells or bronchial epithelial cell basal medium (Cat. No. CC3171, Cambrex) for NHBE cells.

To determine if acrolein treatment increases the steady state transcript level of human MMP14, NCI-H292 cells were treated (4 h, 37°C) with increasing concentrations of acrolein (0.01-10 µM) or PBS. To determine the role of epidermal growth factor receptor (EGFR) in acrolein-induced increase in human MMP14 transcript level, confluent NCI-H292 cells were pre-treated (1 h, 37°C) a neutralizing antibody to EGFR, LA-1 (10 µg/ml) (Cat. No. 05–101, Upstate, Charlottesville, VA) or with an EGFR kinase inhibitor, AG1478 (0.025 µM) (Cat. No. 658552, Calbiochem, San Diego, CA). To determine the role of matrix metalloproteinase, NCI-H292 cells were pre-treated (1 h, 37°C) with GM6001 (10 µM) (Cat. No. 364205, Calbiochem) and then incubated (4 h, 37°C) with acrolein (0.1 µM). To determine the role of various mitogen activated protein kinase (MAPK) activation in acrolein-induced increase in human MMP14 transcript level, NCI-H292 cells were pre-treated (1 h, 37°C) with MAPK3/2 (aka ERK1/2) inhibitor, PD98059, (5 µM) (Cat. No. 51300, Calbiochem), a MAPK8 (aka c-jun N-terminal kinase or JNK) inhibitor, SP600125, (5 µM) (Cat. No. 420119, Calbiochem), or a MAPK14 (aka p38 MAPK) inhibitor, ML3403 (5 µM) (Cat. No. 506121, Calbiochem) and then incubated (4 h, 37°C) with

acrolein (0.1 μM). After treatment, the cells were washed with PBS, lysed by Trizol reagent and total RNA was isolated by isopropanol/chloroform precipitation and suspended in RNAse free water.

Acrolein binding with MMP14 protein: MMP-14 (5 μg) was incubated with 300nM acrolein in Brij 35 P buffer (Sigma) (37°C, overnight). The sample was cooled to 4°C and stored until digested with trypsin. For trypsin digestion, 3.3 μl 8M urea and 0.4M NH_4HCO_3 (pH 7.5-8.5) was added to 25 μl MMP-14 (0.2 μg/μl supplied in 50mMTris-HCl, pH 7.5, 150mM NaCl, 5mM $CaCl_2$, EMD Calbiochem, Gibbstown, NJ), then 3.3 μl 45mM dithiothreitol was added (50°C, 15 min). The solution was cooled to room temperature, 3.3 μl 100mM iodoacetamide was added and the solution was placed in the dark (22°C, 15min). The digestion buffer was diluted to 2M urea and 0.1M NH_4HCO_3 by adding 17 μl water. A 3.4 μl aliquot of trypsin (Trypsin Gold Mass Spectrometry Grade, Promega Biosciences, San Luis Obispo, CA) was added and the solution was incubated at 37°C overnight. The digestion was stopped by acidifying the sample with 3% trifluoroacetic acid. Liquid chromatographic separation used a reverse phase C_{18} 75 μm x 100mm packed tip column (New Objective Inc., Woburn, MA) with a gradient of solvent A: 95:5 water:acetonitrile containing 0.1% formic acid, and solvent B: 95:5 acetonitrile:water containing 0.1% formic acid. A solvent gradient of 10% solvent B to 90% solvent B was used at a flow-rate of 100 μl/min. The MMP14 sample was diluted 1:10 in 50:50 water: acetonitrile containing 0.1% formic acid and 1.6 picomole MMP14 or 102 femtomole acrolein-treated MMP14 injected.

All mass spectral analyses were carried out on a Linear Trap Quadrupole – Fourier Transform (LTQ-FT) mass spectrometer (Thermo Scientific). The instrument was calibrated over the mass range of 190 Da to 2000 Da using a mixture of caffeine (m/z 195), MRFA (m/z 524) and Ultra mark and tuning was carried out using Angiotensin 1 (m/z 649). A FTMS mass range of 300Da to 2000Da was scanned with a resolution of 100000. The duration time for data collection was 110 minutes. The data collection was set-up with 7 scanned events, normalized collision energy of 35.0 and an isolation width was 2.00.

MMP14 activity assay: Ground lung from acrolein-exposed FVB/NJ mice or NCI-H292 cells were incubated (15 min, 4°C) with MMP14 extraction buffer (50 mM Tris-HCl pH 7.6, 1.5 mM NaCl, 0.5

mM $CaCl_2$,1 µM $ZnCl_2$,0.01% Brij 35 and 0.25% Triton™ X-100) and centrifuged (2000 x g, 10 min, 4°C). The supernatant (100 µl) or standards (MMP14, 0.4-0.001 µg/µl) were then incubated (overnight, 4°C) in 96 well micro-titer plate coated with anti-MMP14. The wells were washed (2X, Wash solution containing 0.01 M Sodium Phosphate pH 7.0 and 0.05% Tween™ 20) and incubated (3 h, 37°C) with detection-enzyme (urokinase) and substrate (S-2444™) in reaction buffer (50 mM Tris-HCl pH 7.6, 1.5 mM NaCl, 0.5 mM $CaCl_2$, 1 µM $ZnCl_2$, Heparin, 1 unit/ml and 0.01% Brij™ 35) and read spectrophotometrically (405 nm). The relative amount of MMP14 activity was determined by comparing each sample against the standard curve. Each sample was analyzed in the linear portion of the curve in duplicate and results were expressed as fold change in MMP14 metalloproteinase activity. To determine the role of furin convertase in increased MMP14 activity after acrolein treatment, confluent NCI-H292 cells were pre-treated (1 h, 37°C) with an furin inhibitor, hexa-D-arginine, (0.05 µM) (Cat. No. 344931, Calbiochem) and then incubated (4 h, 37°C) with acrolein (0.1 µM). After treatment, the cells were washed with PBS and MMP14 activity was determined.

Supplemental Table 1. Subject Characteristics

Sex	Age	Pathology	FVC%	FEV1%	DLCO%	PaO_2	$PaCO_2$	Cigarette	CT emphysema
Controls									
F	39	carcinoid	116	93	122	88	39	Ex,20 py	no
F	47	adeno	104	82	98	107	39	Ex, 13 py	no
M	68	adeno	106	72	ND	72	45	Ex,53 py	no
M	63	SCC	92	71	ND	87	41	Ex,86 py	no
M	69	adeno	111	72	74.	96	43	Ex,49 py	no
Cases									
F	57	tx	56	15	34	49	49	25 py, quit 2y ago	yes
M	56	tx	63	17	67	66	52	105 py, quit 2y ago	yes
F	51	tx	63	12		76	76	30 py, quit 3y ago	yes
M	62	tx	68	15	37	64	64	97 py, quit 10y ago	yes
F	64	tx	64	21	30	53	53	60 py, quit 9y ago	yes

Abbreviations: **Pathology**: Medical conditions at diagnosis (carcinoid: carcinoid tumor; adeno: adenocarcinoma; SCC: small cell cancer; tx: thoracectomy for lung replacement for COPD; **FVC%**: Forced vital capacity (percent of control); **FEV1%**: Forced expiratory volume in 1 sec (percent of control); **DLCO%**: Diffusion lung capacity-carbon monoxide (percent of control); **PaO_2** Arterial partial pressure of oxygen (mmHg); **$PaCO_2$** Arterial partial pressure of carbon dioxide (mmHg); **Cigarette**: Smoking history (Ex: exsmoker; py: cigarette pack years, quit y ago: number of years since last cigarette); **CT Emphysema**: computed tomography scan evidence of alveolar enlargement and thickening of tissue around bronchi consistent with diagnosis of COPD. **ND**: Not determined

Supplemental Figure Legend

Supplemental Figure S1. Acrolein-induced increases in transcript levels of matrix metalloproteinase 14 (MMP14) are mediated by epidermal growth factor receptor (EGFR) and mitogen activated protein kinase (MAPK) signaling in human airway epithelial (NCI-H292) cells. Acrolein-induced increases in transcript levels of MMP14 are diminished by an EGFR kinase blocking antibody (10 μg/ml LA-1) or a matrix metalloproteinase inhibitor (10 μM GM6001), by 79% and 62%, respectively. Confluent NCI-H292 cells were pretreated (37°C, 1 h) with inhibitor and then incubated with 100 nM acrolein or vehicle (37°C, 4h). RNA was isolated and the level of MMP14 transcript was determined by quantitative real time PCR (qRT-PCR). The results are expressed as fold change in the level of MMP14 transcripts after normalizing to RPL32. Values are mean ± SEM, n=5. *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).

Supplemental Figure S1