

Cigarette Smoke Alters Tissue Inhibitor of Metalloproteinase 1 and Matrix Metalloproteinase 9 Levels in the Basolateral Secretions of Human Asthmatic Bronchial Epithelium In Vitro

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(*J Investig Med* 2010;58: 725–729)

Background: Asthma, a major cause of chronic lung disease worldwide, has increased in prevalence in all age and ethnic groups, particularly in urban areas where cigarette smoking is common. Cigarette smoke (CS) significantly impacts the development of asthma and is strongly associated with increased asthma-related morbidity.

Purpose: To evaluate bioinformatic analyses predicting that CS would alter expression of tissue inhibitor of metalloproteinase (TIMP) 1 and matrix metalloproteinase (MMP) 9 in asthmatic epithelium.

Methods: Primary differentiated normal (n = 4) and asthmatic (n = 4) human respiratory epithelia on collagen-coated Transwells at air-liquid interface were exposed for 1 hour to CS condensate (CSC) or hydrogen peroxide (H₂O₂). Tissue inhibitor of metalloproteinase 1 and MMP-9 protein levels were measured at 24 hours by enzyme-linked immunosorbent assay in cell lysates and in apical and basolateral secretions.

Results: Tissue inhibitor of metalloproteinase 1 and MMP-9 levels in the apical secretions of normal and asthmatic epithelia were unchanged after exposure to CSC and H₂O₂. However, CSC increased TIMP-1 levels in the basolateral secretions of both normal and asthmatic epithelia, but decreased MMP-9 levels only in asthmatic basolateral secretions, resulting in a 2.5-fold lower MMP-9/TIMP-1 ratio that corresponded to decreased MMP-9 activity in CS-exposed asthmatic basolateral secretions.

Conclusions: These data validate our prior bioinformatic analyses predicting that TIMP-1 plays a role in the stress response to CS and indicate that asthmatics exposed to CS may be more susceptible to MMP-9-mediated airway remodeling. This is in agreement with the current paradigm that a reduction in the MMP-9/TIMP-1 ratio is a milieu that favors subepithelial airway remodeling in chronic asthma.

Key Words: asthma, tobacco smoke, human bronchial epithelial cells, MMP-9, TIMP-1

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Received October 27, 2009, and in revised form February 26, 2010.

Accepted for publication February 26, 2010.

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Supported by the National Institutes of Health (HL-33152 to M.C.R., K23-RR-020069 to R.J.F.) and a grant from the Board of Visitors, Children's National Medical Center (to R.J.F.).

This work is from a dissertation presented to the Immunology Program, Institute for Biomedical Sciences, The George Washington University, in partial fulfillment of the requirements for the PhD degree of A.W.

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A.M.W. carried out these studies while a predoctoral student in the Immunology Program, Institute for Biomedical Sciences, The George Washington University.

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ISSN: 1081-5589

DOI: 10.2310/JIM.0b013e3181db874e

Asthma is a major cause of chronic lung disease worldwide and is the most common chronic childhood disease in Western countries. The incidence and severity of asthma have increased in prevalence by more than 80% in all age and ethnic groups during the past 2 decades^{1–3} and continue to increase particularly in urban areas.⁴ Asthma is a multifactorial disease characterized by airway inflammation, hyperresponsiveness, and mucus obstruction and is now considered to reflect airway remodeling due to fibroblast proliferation, goblet cell and smooth muscle hyperplasia, and increased collagen deposition.^{5–9}

Cigarette smoke (CS) is one of the principal environmental air pollutants and contains various carcinogens and oxidative free radicals. Long-term exposure to CS is known to lead to the development of various lung diseases. Cigarette smoke, which is responsible for 90% of all lung cancers,¹⁰ is also the major cause of chronic obstructive pulmonary diseases¹¹ and is strongly associated with both the diagnosis of asthma and its increased morbidity.^{12,13} Cigarette smoke targets the conducting airway epithelium, and accumulating evidence has shown that CS exposure induces a response in the conducting airway epithelium that is mediated by reactive oxygen species.¹⁴ Irreversible alterations occur in the airway epithelium of smokers. Induction of oncogenes and repression of tumor suppressor and airway inflammatory genes have been reported for a cohort of former smokers, and many of these genes failed to revert to nonsmoker levels, even after years without smoking.¹⁵ Asthmatic epithelium seems more sensitive to oxidative injury from environmental factors, including CS,¹⁶ which have been linked to asthma pathogenesis and exacerbations.¹⁷

Following a data integration and systems biology analyses of publicly available microarray data on genes associated with asthma, tobacco, and oxidative stress, we recently proposed a gene/protein network, which contained 18 highly regulated members, that may underlie the pulmonary response to direct lung oxidative stress on airway epithelium.¹⁸ This network prominently features tissue inhibitor of metalloproteinases (TIMP) 1, a secreted 31-kd glycoprotein that is one of 4 protease inhibitors known to inhibit the protease action of the matrix metalloproteinases (MMPs) and is induced by lung inflammation after exposure to CS and oxidative stress.^{19,20} Notably, there is an abundance of MMPs in the proposed oxidative stress network of lung tissues,¹⁸ including MMP-1, -2, -3, -7, and -9. Matrix metalloproteinase 9 is one of the most studied lung MMPs.^{20–22} In asthma, the MMP-9/TIMP-1 ratio is considered important,²⁰ as low ratios correspond to a decreased forced expiratory volume in 1 second,^{22,23} promote subepithelial collagen deposition,²⁰ and drive extracellular remodeling in the lung.^{24,25} Herein, we show that acute exposure of lung epithelia to CS condensate (CSC) in vitro increased TIMP-1 levels in basolateral secretions of

TABLE 1. Description of Normal and Asthmatic HBE Cell Donors

| | Donor Age, y | Sex | Race/Ethnicity | Medications |
|-----------|--------------|--------|----------------|------------------------------------|
| Asthmatic | 2 | Male | White | Albuterol |
| | 7 | Female | White | Albuterol |
| | 46 | Female | White | Albuterol |
| | 59 | Female | Hispanic | Fluticasone, salmeterol, albuterol |
| Normal | 5 | Female | White | None |
| | 23 | Male | White | None |
| | 23 | Male | White | None |
| | 33 | Female | White | None |

asthmatic and normal epithelium but also decreased MMP-9 levels and activity in basolateral secretions of asthmatic epithelium. These data support (1) the role of the epithelium in directing extracellular matrix remodeling and (2) functional impairment of asthmatic epithelium to an environmental challenge such as CS.

METHODS

Cell Culture

Normal (n = 4) and asthmatic (n = 4) primary human bronchial epithelial (HBE) cells were grown in 6-well plates on a Transwell membrane insert coated with collagen, maintained at an air-liquid interface, and differentiated into airway epithelium (#AIR-606 and AIR-606-Asthma; MatTek Corp, Ashland, MA). On arrival, the inserts on which the in vitro epithelium had been established were washed with phosphate-buffered saline (PBS), and the basolateral media replaced with proprietary-defined media supplied by the manufacturer. Cells were equilibrated at 37°C and 5% CO₂ for 16 hours before performing experiments.

Exposures

Airway epithelium was exposed (triplicate wells) apically and basolaterally to 3×10^{-4} M hydrogen peroxide (H₂O₂) (Sigma-Aldrich, St Louis, MO) or PBS for 1 hour. Alternatively, airway epithelium was exposed at the apical and basolateral surfaces to 20 µg/mL of CSC (Murty Pharmaceuticals,

Lexington, KY) in dimethyl sulfoxide (DMSO) for 1 hour. Cigarette smoke condensate is the particulate matter eluted by DMSO from CS-exposed filters and lacks the volatile gas phase of CS. The inserts were then washed with PBS and moved to new 6-well plates. Fresh medium was added, and epithelium was incubated at 37°C and 5% CO₂ for 23 hours. The apical surface was washed twice with 0.5 mL PBS to remove the apical secretions, and the washes were pooled. Basolateral secretions were removed. All secretions were frozen before analyses.

Determination of TIMP-1 and MMP-9 Protein Concentration and Activity

Control and exposed apical and basolateral cell secretions were quantitatively measured for the presence of TIMP-1 and MMP-9 using standard enzyme-linked immunosorbent assay (ELISA) kits with appropriate positive and negative controls (R&D Systems, Minneapolis, MN) according to the manufacturer's protocols. Matrix metalloproteinase 9 enzymatic activity was determined by the Fluorokin-E enzyme activity assay (R&D Systems) with minimal detectable level of 0.005 ng/mL.

Statistical Analysis

Statistical comparisons were performed using *t* test functions (on log₁₀-transformed data when not normally distributed) in SPSS 15.0 software (SPSS Inc, Chicago, IL). Results are reported as mean ± SEM unless otherwise noted.

RESULTS

Demographic information for the normal (n = 4) and asthmatic (n = 4) donors of the primary HBE cells used to establish the differentiated epithelia for these experiments is shown in Table 1. After exposure of differentiated primary normal and asthmatic epithelia to CSC or H₂O₂, the basolateral and apical secretions from each of the in vitro cultures were evaluated for TIMP-1 and MMP-9 protein. Data for the normal and asthmatic basolateral and apical secretions are shown in Figures 1 and 2. The levels of TIMP-1 in normal and asthmatic secretions were similar whether measured in PBS (diluent for the oxidant H₂O₂) or DMSO (diluent for CSC). Tissue inhibitor of metalloproteinase 1 levels were 1.7 mmol/mL in basolateral secretions (Fig. 1) and approximately 3000-fold lower in apical secretions (Fig. 2). The levels of MMP-9 were lower than TIMP-1 levels, but were similar in normal and asthmatic secretions. Matrix metalloproteinase 9 levels were 0.025 mmol/mL in

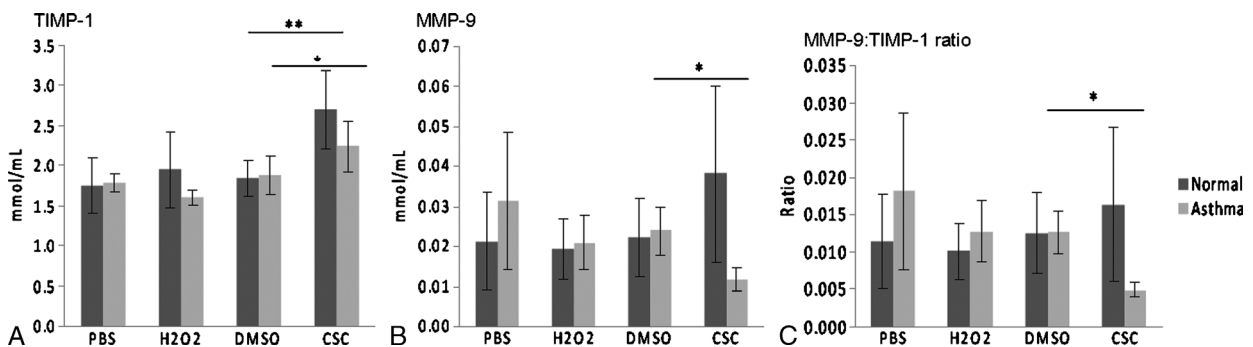


FIGURE 1. Comparison of TIMP-1 and MMP-9 protein expression in basolateral secretions of primary human differentiated normal (n = 4) and asthmatic (n = 4) respiratory epithelia in vitro after exposure to H₂O₂ or CSC. Epithelia were cultured for 23 hours after a 1-hour exposure to H₂O₂ or PBS (vehicle) and CSC or DMSO (vehicle). Tissue inhibitor of metalloproteinase 1 (A) and MMP-9 (B) protein levels in basolateral secretions were measured by ELISA, and the data were used to determine the MMP-9/TIMP-1 ratio (C). Horizontal lines indicate statistical comparisons between underlying groups for which **P* ≤ 0.05 and ***P* ≤ 0.01. Experiments were done in 4 different patient tissues and run in triplicate wells.

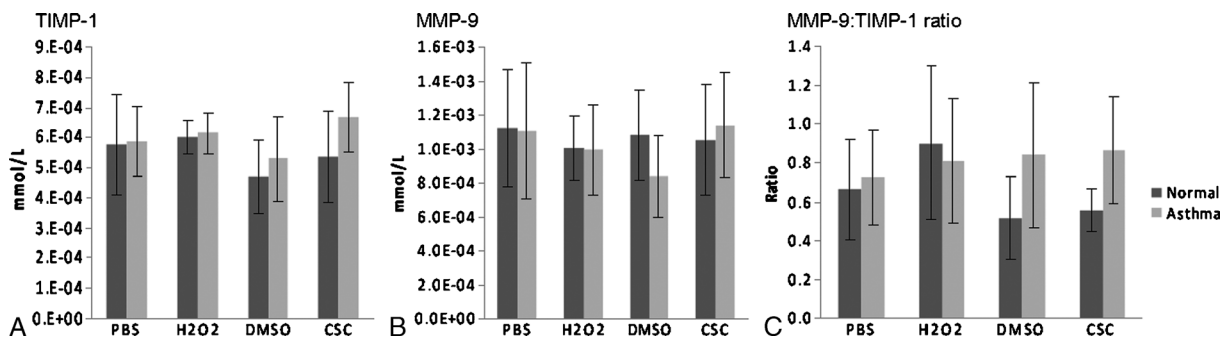


FIGURE 2. Comparison of TIMP-1 and MMP-9 protein expression in apical secretions of primary human differentiated normal (n = 4) and asthmatic (n = 4) respiratory epithelia in vitro after exposure to H₂O₂ or CSC. Epithelia were cultured for 23 hours after a 1-hour exposure to H₂O₂ or CSC. Tissue inhibitor of metalloproteinase 1 (A) and MMP-9 (B) protein levels in apical secretions were measured by ELISA, and the MMP-9/TIMP-1 ratio was determined (C). Experiments were done in 4 different patient tissues and run in triplicate wells.

basolateral secretions (Fig. 1) and 22-fold lower in apical secretions (Fig. 2).

After exposure to CSC, TIMP-1 levels in the basolateral secretions increased significantly in both normal (mean ± SE_{DMSO vs CSC} = $1.8 \times 10^{-3} \pm 0.002$ vs $2.7 \times 10^{-3} \pm 0.005$ mmol/mL; $P = 0.024$) and asthmatic epithelium (mean ± SE_{DMSO vs CSC} = $1.9 \times 10^{-3} \pm 0.002$ vs $2.2 \times 10^{-3} \pm 0.003$ mmol/mL; $P = 0.028$) (Fig. 1A). In addition, MMP-9 levels in the basolateral secretions decreased by half in asthmatic epithelium (mean ± SE_{DMSO vs CSC} = $2 \times 10^{-4} \pm 1 \times 10^{-5}$ vs $1 \times 10^{-4} \pm 3 \times 10^{-5}$ mmol/mL; $P = 0.031$) but were not statistically different in normal epithelium (Fig. 1B).

The MMP-9/TIMP-1 ratios were determined for basolateral and apical secretions. After CSC exposure, asthmatic cells showed a 2.6-fold lower MMP-9/TIMP-1 ratio than vehicle-treated asthmatic cells (0.005 ± 0.001 vs 0.013 ± 0.003 ; $P = 0.017$) for basolateral secretions (Fig. 1C). As expected, no discernable difference in the MMP-9/TIMP-1 ratio was observed for apical secretions (Fig. 2C). Levels of TIMP-1 or MMP-9 measured 23 hours after a 1-hour exposure of normal and asthmatic epithelia to H₂O₂ did not significantly alter basolateral or apical levels of either MMP-9 or TIMP-1 (Figs. 1 and 2).

To determine whether CSC decreased the MMP-9 enzymatic activity as well as MMP-9 protein levels in the basolateral secretions of asthmatic epithelium, we measured the enzymatic activity of MMP-9 in the basolateral and apical secretions of normal and asthmatic epithelia under all conditions (Fig. 3). Although DMSO alone increased MMP-9 activity in the

basolateral secretions of asthmatic epithelium, MMP-9 activity 23 hours after a 1-hour exposure to CSC was significantly lower in asthmatic secretions (0.39 ± 0.08 vs 0.07 ± 0.02 ng/mL; $P = 0.011$) and unchanged in normal secretions (Fig. 3A). The significantly lower MMP-9 activity in asthmatic basolateral secretions corresponded to the decreased MMP-9 protein levels (Fig. 1B) and the decreased MMP-9/TIMP-1 ratio (Fig. 1C) in the CSC-exposed basolateral secretions of asthmatic epithelium. As expected, based on the data in Figure 2B, CSC induced no changes in MMP-9 activity in apical secretions (Fig. 3B). It was surprising that DMSO, which did not alter MMP-9 protein levels in apical or basolateral secretions, increased basolateral MMP-9 activity in asthmatic but not normal secretions. Dimethyl sulfoxide has previously been reported to alter lung cell MMP-9 expression and activity in vitro,²⁶ but the mechanism is unknown.

DISCUSSION

The epithelial lining of the conducting airways is highly responsive to environmental challenges, activating numerous pathways after exposure to allergens and environmental toxins, including CS. Recently, our data integration and systems biology analyses of publicly available microarray data on asthma and/or tobacco smoke identified a 26-member gene/protein network containing 18 members that were highly regulated in a data series analyses of direct lung oxidative stress on airway epithelium.¹⁸ Two network members—TIMP-1 and thrombospondin 1—stood out because of their central location within the network and

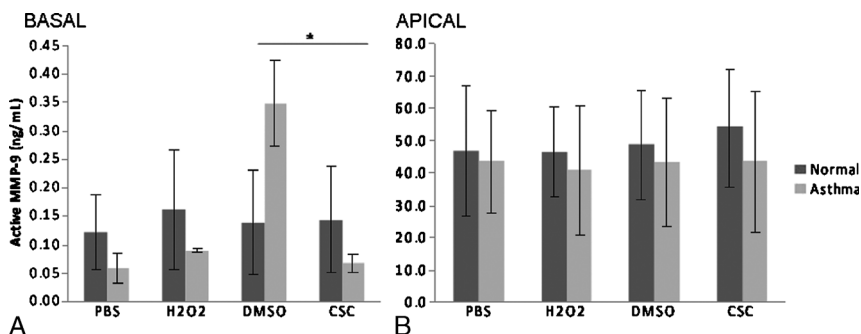


FIGURE 3. Secreted MMP-9 activity of stimulated primary human differentiated normal (n = 4) and asthmatic (n = 4) respiratory epithelia. Matrix metalloproteinase 9 activity was measured by ELISA in respiratory epithelial cell basolateral (A) and apical (B) secretions, as described in Methods. Horizontal lines indicate statistical comparisons between underlying groups for which $*P = 0.011$. Experiments were done in 4 different patient tissues and run in triplicate wells.

marked upregulation sustained at later times in response to oxidative stress. Although several MMPs—MMP-1, -2, -3, -7, and -9—were identified in the network, we focused our initial validation study on TIMP-1 and MMP-9 because the MMP-9/TIMP-1 ratio is considered important in asthma,^{20,22,23} with a decreased ratio considered to drive extracellular remodeling in the lung.^{24,25}

We investigated whether MMP-9 and TIMP-1 would be altered by oxidative stress in lung epithelial cells, as the literature indicates that these cells can express these proteins. Lung epithelial A549 cancer cells express MMPs and TIMP-1.²⁷ However, primary HBE cells grown submerged on plastic (and thus undifferentiated²⁸) express low levels of TIMP-1 and MMP-9 mRNA, and MMP-9 protein is undetectable in cell secretions.²⁹ As TIMP-1 and MMP-9 have been identified in vivo in the extracellular matrix and in apical secretions (sputum and bronchoalveolar lavage [BAL] fluid), we reasoned that the lack of expression of these proteins in vitro reflected the lack of differentiation and polarity in the submerged system. We predicted that expression and secretion of TIMP-1 and MMP-9 would occur in primary differentiated bronchial epithelial cells, an in vitro system considered an excellent model of the conducting airway epithelium.²⁸ It allows evaluation of both apical and basolateral secretions. Furthermore, epithelium can be generated from both normal and asthmatic cells. Our data showed that both normal and asthmatic primary differentiated epithelium secreted TIMP-1 and MMP-9 apically and basolaterally, supporting the concept that epithelial cells, in the absence of inflammatory cells, can express TIMP-1 and MMP-9 and that their expression in vitro depends on cell differentiation and polarization. This is in agreement with a recent report showing that MMP-9 is secreted basolaterally by the airway epithelium.³⁰

We evaluated the levels of TIMP-1 and MMP-9 protein in normal and asthmatic epithelial secretions 23 hours after an acute exposure (1 hour) to either H₂O₂ or CSC. The data showed that TIMP-1 and MMP-9 were secreted at similar levels by normal and asthmatic epithelia at baseline, that levels were higher in basolateral than apical secretions, and that no changes occurred in lung epithelium after an acute H₂O₂ exposure. In contrast, an acute exposure of CSC increased TIMP-1 levels in basolateral secretions in both normal and asthmatic epithelia. Furthermore, CSC resulted in a decrease in MMP-9 levels, as well as MMP-9 enzymatic activity, in asthmatic basolateral secretions, resulting in a decreased MMP-9/TIMP-1 ratio in asthmatic, but not normal, basolateral secretions. These data support the concept that alterations in both TIMP-1 and MMP-9 contribute to changes in the subepithelial microenvironment of asthmatics.³¹

That both increased TIMP-1 and decreased MMP-9 levels contribute to a decreased basolateral MMP-9/TIMP-1 ratio in the CSC-induced response of asthmatic epithelium in vitro is somewhat in contrast to the existing literature because increased MMP-9 levels are generally considered to be the driving force behind reductions in the MMP-9/TIMP-1 ratio in vivo.^{21,24,32} The decreased in vivo ratio is thought to primarily reflect increased levels of MMP-9 from inflammatory or smooth muscle cells. The presence of TIMP-1 and MMP-9 proteins in sputum and BAL fluid could reflect secretion by the apical epithelium as well as inflammatory cells. Interestingly, our apical TIMP-1 levels were similar to those reported in induced sputum from healthy and mild asthmatic subjects.²¹ That no significant changes occurred in apically secreted TIMP-1 or MMP-9 levels or MMP-9 activity after exposure to H₂O₂ or CSC in either normal or asthmatic epithelium suggests that the nonepithelial contributors of MMP-9 (ie, macrophages and neutrophils) may

account for changes in the MMP-9/TIMP-1 ratio in asthmatic sputum samples during exacerbations, which are typically triggered by pathogens. Matrix metalloproteinase 9 levels are higher in acute asthmatic patients than in stable asthmatic patients,³³ and there is an elevation in the MMP-9/TIMP-1 ratio after exacerbation.³¹ Tissue inhibitor of metalloproteinase 1 levels in BAL fluid and induced sputum from asthmatic patients are typically lower compared with those from control subjects.^{21,25}

These initial studies focused on a single challenge of CSC and H₂O₂ to assess the acute response of TIMP-1 and MMP-9. These data showed a differential effect of CSC and H₂O₂ on MMP-9 and TIMP-1, although both are often used to induce oxidant stress. Future studies will be required to determine (1) whether chronic and/or repeated dosing of CSC and H₂O₂ would generate a similar yet prolonged response and (2) whether induction of TIMP-1 or reduction of MMP-9 levels is the first responder to CSC exposure and to investigate the hypothesis that a reduced MMP-9/TIMP-1 ratio drives extracellular remodeling in the lung.²⁴ The utilization of differentiated normal and asthmatic epithelia should prove informative in investigating mechanisms that regulate the proposed oxidative stress gene/protein network.¹⁸

Smoking is an increasingly popular activity, even though the deleterious effects of CS on human health are widely known. Efforts to better understand the molecular effects of CS exposure on human airways could lead to ways to alleviate the deleterious effects of CS in the progression of chronic asthma. This study showed that the asthmatic epithelial response to CSC contributes to a harmful microenvironment that is considered conducive to airway remodeling^{22,23} and supports the concept that asthmatic lungs are susceptible to CS-induced airway remodeling.

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