Matrix Metalloproteinase-1 Activation Contributes to Airway Smooth Muscle Growth and Asthma Severity

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Abstract

Rationale: Matrix metalloproteinase-1 (MMP-1) and mast cells are present in the airways of people with asthma.

Objectives: To investigate whether MMP-1 could be activated by mast cells and increase asthma severity.

Methods: Patients with stable asthma and healthy control subjects underwent spirometry, methacholine challenge, and bronchoscopy, and their airway smooth muscle cells were grown in culture. A second asthma group and control subjects had symptom scores, spirometry, and bronchoalveolar lavage before and after rhinovirus-induced asthma exacerbations. Extracellular matrix was prepared from decellularized airway smooth muscle cultures. MMP-1 protein and activity were assessed.

Measurements and Main Results: Airway smooth muscle cells generated pro-MMP-1, which was proteolytically activated by mast cell tryptase. Airway smooth muscle treated with activated mast cell supernatants produced extracellular matrix, which enhanced

subsequent airway smooth muscle growth by 1.5-fold ($P < 0.05$), which was dependent on MMP-1 activation. In asthma, airway pro-MMP-1 was 5.4-fold higher than control subjects ($P = 0.002$). Mast cell numbers were associated with airway smooth muscle proliferation and MMP-1 protein associated with bronchial hyperresponsiveness. During exacerbations, MMP-1 activity increased and was associated with fall in FEV₁ and worsening asthma symptoms.

Conclusions: MMP-1 is activated by mast cell tryptase resulting in a proliferative extracellular matrix. In asthma, mast cells are associated with airway smooth muscle growth, MMP-1 levels are associated with bronchial hyperresponsiveness, and MMP-1 activation are associated with exacerbation severity. Our findings suggest that airway smooth muscle/mast cell interactions contribute to asthma severity by transiently increasing MMP activation, airway smooth muscle growth, and airway responsiveness.

Keywords: asthma; extracellular matrix; airway remodeling; airway smooth muscle; mast cells

Asthma is characterized by airway inflammation, bronchial hyperresponsiveness (BHR), and variable airway obstruction. Structural changes, collectively termed

airway remodeling, are associated with BHR, airflow obstruction, worsening asthma symptoms, increased β_2 -agonist use, and exacerbations, and can persist

despite optimal asthma treatment (1–4). Airway remodeling often starts in childhood with airflow limitation associated with persistence of asthma

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At a Glance Commentary

Scientific Knowledge on the

Subject: Matrix metalloproteinase-1 (MMP-1) and mast cells are minimally present in normal airways but both are present in the airway smooth muscle bundles of patients with asthma. Despite evidence that both are related to asthma severity, the underlying mechanisms are uncertain. MMP-1 is a matrix processing collagenase that requires activation by proteases. We hypothesized mast cell proteases could activate MMP-1, resulting in structural changes that promote airway remodeling.

What This Study Adds to the

Field: MMP-1 can be activated by mast cell tryptase. Active MMP-1 is capable of processing airway smooth muscle–derived extracellular matrix, which enhances airway smooth muscle proliferation. In patients with asthma, mast cells are associated with airway smooth muscle growth. MMP-1 protein is related to bronchial reactivity, and MMP-1 activity increases during asthma exacerbations, for which its level is related to exacerbation severity. Interrupting mast cell/airway smooth muscle interactions has the potential to reduce airway remodeling in asthma.

Mast cells, particularly activated, degranulated forms, are more common within ASM bundles of those with asthma (10–12) and generate proinflammatory cytokines including IL-4, IL-5, and IL-13, which regulate IgE production, eosinophilic inflammation, and profibrogenic cytokines, including transforming growth factor- β and basic fibroblast growth factor-2. Preformed serine proteases including tryptase, chymase, and carboxy-peptidase are secreted from granules and interact with various cell types via proteolytically activated receptors (PARs) (11). Collectively, mast cell mediators contribute to airway inflammation, hyperresponsiveness and remodeling causing bronchoconstriction, ASM cell proliferation, and inflammatory cell recruitment (13).

Our overarching hypothesis is that the airway environment in asthma sustains airway remodeling (14–16). We have shown that components of the ECM in asthmatic airways can promote remodeling by affecting proliferation (17), migration (16), apoptosis (18), matrix metalloproteinase (MMP) activation (16), and β -agonist signaling of ASM cells (19). Here we hypothesized that mast cell proteases are responsible for MMP-1 activation in asthma and used a combination of *in vitro* and human studies to examine the interactions between mast cells, MMP-1, airway

remodeling, and asthma symptoms. Some of this work has previously been presented as abstracts (20, 21).

Methods

Patients and Control Subjects

Two asthma cohorts with matched control groups were studied. All subjects gave written informed consent. The relationship between ASM growth and MMP-1 expression was examined in 16 subjects with mild or moderate asthma, defined by the Global Initiative for Asthma criteria (22), without a history of exacerbation, change in therapy, or use of oral steroids for at least 6 weeks and 11 nonsmoking, age-matched control subjects without lung disease. Further study details are included in the online supplement. Those with asthma underwent spirometry, bronchial provocation testing, and modified Juniper Asthma Control Questionnaire (23). All subjects underwent flexible bronchoscopy with bronchial washings and biopsies taken from the right bronchus intermedius. The study was approved by the Nottingham Research Ethics Committee (12/EM/0199). The relationship between asthma exacerbations and MMP-1 activation was examined in a second group of 11 subjects with mild and 17 with moderate asthma with

and abnormal lung function in adulthood (5, 6). Changes observed in airway remodeling include epithelial desquamation, goblet cell hyperplasia, reticular basement membrane thickening, increased airway smooth muscle (ASM) mass, and abnormal extracellular matrix (ECM) deposition (1, 7). Airway remodeling has been seen as a consequence of inflammation, although airway contraction, in the absence of inflammation, can also drive aspects of remodeling (8). Because airway structural changes may support inflammation (9) it is likely that airway inflammation, bronchoconstriction, remodeling, and fixed airflow obstruction interact in a linear, parallel, or combined manner to drive the asthma phenotype.

Table 1. Characteristics of Patients and Healthy Subjects Studied in the Bronchial Hyperresponsiveness/Airway Remodeling Study

	Asthma	Control
N	16	11
Age, yr	31 (9.4)	36 (10.2)
Male/female	9/7	6/5
Disease duration, yr	16 (9.9)	N/A
Atopy, present/absent	8/8	N/A
ACQ5 score	2.28 (0.63)	N/A
BMI, kg/m ²	25.1 (3.2)	24.9 (2.8)
ICS dose, μ g/d	412 (296)	0
PC ₂₀ methacholine, mg/ml		
0–5	2	
5–8	5	
8–16	9	
FEV ₁ % predicted		
Prebronchodilator	90.5 (9.5)	
Post-bronchodilator	92.5 (11.5)	
Exhaled nitric oxide, ppb	49.6 (30.2)	

Definition of abbreviations: ACQ5 = Juniper 5 asthma control score; BMI = body mass index; ICS = inhaled corticosteroid; N/A = not applicable; PC₂₀ = provocative concentration of methacholine. Values represent number in group or mean (SD) as appropriate.

Figure 1. Matrix metalloproteinase (MMP)-1 expression in asthma. (A) Total MMP-1 protein measured by ELISA in bronchial washings from 16 patients with mild or moderate asthma, defined by the Global Initiative for Asthma criteria and 11 healthy control subjects. Those with asthma have significantly higher MMP-1 levels. $^{**}P = 0.002$, Mann-Whitney U test. (B) Western blot of bronchial washings from five representative patients with asthma and five healthy control subjects. A single band of 55 kD consistent with pro-MMP-1 is

exacerbations induced by rhinovirus inoculation compared with 11 healthy control subjects.

The study has been described in detail previously (24). Briefly, nonsmokers with mild or moderate asthma, defined by Global Initiative for Asthma criteria, and nonsmoking, nonatopic healthy volunteers without a recent viral illness or asthma exacerbation, were recruited. Symptom scores, lung function, bronchoscopy, and bronchoalveolar lavage (BAL) were performed 2 to 4 weeks before inoculation with rhinovirus 16. Following virus inoculation, symptom scores were recorded daily and lung function, bronchoscopy, and BAL were repeated after 4 days. The study was approved by the St Mary's Hospital ethics committee (09/H0712/59).

Cells and Tissues

Bronchial biopsy tissue was either processed for immunohistochemistry or used for culture of ASM cells as previously described with cells used at passage five or less (see Figure E1 in the online supplement) (15).

Mast cell supernatants were prepared from HMC-1 mast cells activated using phorbol 12-myristate 13-acetate (50 ng/ml) and calcium ionophore (A23187; 25 ng/ml) for 16 hours. Control mast cell supernatants had phorbol 12-myristate 13-acetate and calcium ionophore added after removal of the cell pellet. Inhibition of proteases was performed by preincubation of supernatants with protease inhibitors used at the manufacturer's recommended concentrations. Full details are included in the online supplement.

MMP-1 Assays

Total MMP-1 protein (including pro and active species) and TIMP1 were measured using Duoset ELISAs (R&D Systems, Abingdon, UK). MMP-1 activity was measured using a Human Active MMP-1 Fluorokine E Kit (R&D Systems). Generation of the active 43-kD MMP-1 species was assessed by Western blotting

(14). Proteolytic cleavage of MMP-1 by tryptase was examined by incubation of recombinant pro-MMP-1 (10 ng; R&D Systems) and recombinant human mast cell tryptase (0.1–2 IU; Promega, Southampton, UK) in physiologic buffered saline for 30 minutes at 37°C. Products were resolved on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and visualized by silver staining.

ECM Preparations

ASM cell-derived ECM was prepared as described (14). Further details are given in the online supplement.

In Vitro Cell Proliferation, Adhesion, and Apoptosis Assays

Cell proliferation was determined by both 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay and cell counting as described (16) with ASM cultured on ECM preparations for 48 hours in the presence of 1% fetal bovine serum. For adhesion assays, fluorescently labeled ASM cells were seeded onto ECM preparations and adherent cells measured by fluorescence over 2–18 hours. Apoptosis was assessed using an In Situ Cell Death Detection kit, AP (Roche, West Sussex, UK). Full details are provided in the online supplement.

MMP-1 siRNA Knockdown

siRNA was used as previously described (14) and reduced MMP-1 protein by greater than 70% (see Figure E2).

Quantification of Airway Mast Cells and Proliferating ASM Cells

Endobronchial biopsies were immunostained as previously described (15) with images captured using a Digital Nanozoomer (Hamamatsu Photonics UK Ltd., Welwyn Garden City, UK). All slides were assessed by a respiratory pathologist (I.S.). ASM bundles were identified by immunostaining with anti α -smooth muscle actin (Roche) and ASM cell nuclei counted using DAPI staining. In adjacent

Figure 1. (Continued). present in those with asthma but not detectable in healthy control subjects. (C) Total and active MMP-1 activity measured by fluorokine activity assay in patients described in A. Total MMP-1 protein is low in healthy control subjects and significantly elevated in those with asthma, $^{**}P < 0.01$. MMP-1 activity is undetectable in control subjects and low in those with asthma. (D) Total MMP-1 protein measured by ELISA in cell culture supernatants from airway smooth muscle cells grown from bronchial biopsies from patients with mild or moderate asthma and healthy control subjects. $^{**}P = 0.002$, Mann-Whitney U test.

sections, proliferating ASM and tryptase-positive mast cells were identified by anti-Ki67 and anti-mast-cell tryptase antibodies, respectively (Roche). Proliferating ASM and mast cells within ASM bundles were quantified per 100 ASM cells by an observer blinded to the clinical details.

Statistical Analyses

Data were tested for normality and comparisons made by Student's *t* test or Mann-Whitney *U* test as appropriate. Multiple comparisons were made by two-way analysis of variance. Correlations between parameters were analyzed by linear regression. Statistical analysis was performed using GraphPad Prism 6 for windows version 6.07 (1992–2015; GraphPad Software, Inc., La Jolla, CA). A *P* value less than 0.05 was considered to be significant.

Results

MMP-1 Is Expressed in the Airways of Patients with Asthma

Details of the 16 patients and 11 control subjects in the airway remodeling study are shown in Table 1. The expression of total MMP-1 protein (ELISA) was 5.4-fold greater in bronchial washings from patients with asthma than those from control subjects (control: mean, 0.14 ng/ml; 95% confidence interval [CI], 0.095–0.197; asthma: mean, 0.79 ng/ml; 95% CI, 0.35–1.21; *P* = 0.002) (Figure 1A). Western blotting of these samples identified a single 54-kD band consistent with pro-MMP-1 in those with asthma but not in healthy control subjects. The 43-kD active MMP-1 species was not detected in either group (Figure 1B). In keeping with the Western blot analysis, MMP-1 activity was not detected in control subjects and barely present in those with stable asthma (Figure 1C).

ASM cells were cultured from endobronchial biopsies of six of these patients with asthma and six healthy control subjects. Total MMP-1 protein was 3.3-fold higher in ASM culture supernatants from those with asthma than from healthy control subjects (control: mean, 0.48 ng/ml; 95% CI, 0.23–0.74; asthma: mean, 1.57 ng/ml; 95% CI, 0.30–1.23; *P* = 0.002) (Figure 1D).

Figure 2. Mast cell tryptase activates matrix metalloproteinase (MMP)-1. (A) Western blot for MMP-1 in airway smooth muscle supernatants treated with control or activated mast cell supernatants. Activated mast cell supernatants cause MMP-1 activation as shown by the appearance of the smaller MMP-1 band, which is blocked by a broad-spectrum protease inhibitor. (B and C) MMP-1 activation by activated mast cell supernatants is inhibited by inhibitors of serine and cysteine proteases, serine proteases, metalloproteinases, and tryptase, but not chymase. (D) Silver stain of sodium dodecyl sulfate-polyacrylamide gel electrophoresis showing recombinant human tryptase cleaves pro-MMP-1 *in vitro* shown by the dose-dependent appearance of a 43-kD band. aMC = activated mast cell supernatants; APC = inhibitor of tryptase; chymos = inhibitor of chymase; cMC = control mast cell supernatants; ilo = inhibitor of metalloproteinases; pan PI = broad-spectrum protease inhibitor; rh = recombinant human; S&C PI = inhibitor of serine and cysteine proteases; S PI = inhibitor of serine protease.

ASM Cell MMP-1 Is Activated by Mast Cell-derived Tryptase

When the supernatants of unstimulated primary ASM cells from patients with

asthma were examined by Western blotting a single band consistent with pro-MMP-1 was detected (Figure 2A). ASM cells were then incubated with

Figure 3. Mast cell treatment of airway smooth muscle (ASM) generates proliferative extracellular matrix. ASM from patients with asthma or control subjects was treated with control or activated mast cell supernatants or mast cell activation vehicle during extracellular matrix deposition. ASM cells were removed from extracellular matrix preparations, and normal ASM was seeded for 48 hours in the presence of 1% serum. Cell proliferation was quantitated by (A) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (B) and cell counting. ASM cell proliferation was enhanced by activated, but not control, mast cell treatment. ***P* = 0.0001, two-way analysis of variance. aMC = activated mast cell supernatants; cMC = control mast cell supernatants; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD = optical density.

Figure 4. Proliferative matrix is dependent on airway smooth muscle (ASM)-derived matrix metalloproteinase (MMP)-1. (A) ASM treated with control (cMC) or activated (aMC) mast cell supernatants during extracellular matrix (ECM) deposition was coincubated with an MMP inhibitor, chymase inhibitor, tryptase inhibitor, or a serine protease inhibitor. Enhanced ASM proliferation generated by aMC treatment was abrogated by inhibitors of MMPs, serine proteases, and tryptase but not chymase. *Difference from aMC, $P = 0.029$, Mann-Whitney U test. (B) ECM preparations were generated by ASM cells treated by an MMP-1-specific or control siRNA. During ECM deposition cells were treated with cMC or aMC. The MMP-1-specific siRNA abrogated the enhanced proliferation generated by aMC treatment. * $P = 0.04$ Mann-Whitney U test. (C) ASM-derived ECM from asthma-derived or control cells were decellularized and then left untreated or incubated with cMC or aMC or active recombinant MMP-1 (rMMP-1). ASM cells were

the supernatants of activated or nonactivated HMC-1 mast cells. Activated, but not control, mast cell supernatants resulted in the appearance of the active 43-kD MMP-1 species, which could be inhibited using a pan protease inhibitor (Figure 2A). To determine which protease was responsible for MMP-1 activation, we used more specific protease inhibitors. Generation of active MMP-1, detected by Western blotting, was blocked by panserine protease and specific tryptase inhibitors, but not a chymase inhibitor (Figures 2B and 2C). The generation of active MMP-1 was associated with an increase in MMP-1 activity (see Figure E3). To determine if mast cell tryptase activated MMP-1 by direct cleavage, we coincubated recombinant mast cell tryptase and pro-MMP-1. Increasing concentrations of mast cell tryptase were associated with production of a cleaved MMP-1 protein at 43 kD consistent with active MMP-1 (Figure 2D).

Activated Mast Cell Treatment of ASM Cells Generates a Proliferative ECM

To determine if MMP-1 activation would lead to proteolytic modification of ASM cell-derived ECM and promote ASM growth, we generated ASM-derived ECM preparations from patients with asthma and control subjects following treatment with activated or control mast cell supernatants. ASM cells were then seeded onto these preparations and cell proliferation measured in the presence of 1% serum. Activated mast cell-treated ASM cells produced a matrix that enhanced proliferation of control and asthma-derived ASM over 48 hours (mean difference for control cells, 0.73; 95% CI of difference, 0.52–0.93; $P < 0.001$) (mean difference for asthma cells, 0.78; 95% CI of difference, 0.58–0.99; $P < 0.001$) as assessed by cell counting when compared with control mast cell supernatants. Similar results were obtained using MTT assay (Figure 3; see Figures E4 and E5). Neither control mast

cell supernatants nor the activation vehicle alone affected ASM proliferation. Activated mast cell supernatants had no overall effect on ASM adhesion or apoptosis on control or asthma-derived ECM (see RESULTS in the online supplement; see Figures E6 and E7).

Proproliferative Matrix Is Dependent on Tryptase-activated MMP-1

To determine if the proproliferative matrix generated by activated mast cells was dependent on mast cell tryptase activation of MMP-1, mast cell supernatants were incubated with a range of protease inhibitors before treatment of ASM cells. The excess ASM proliferation, estimated by MTT reduction, induced by activated mast cells, was blocked by a pan protease inhibitor and inhibitors of serine proteases, metalloproteinases, and tryptase but not chymase (Figure 4A). We then treated ASM with siRNA targeting MMP-1 or control siRNA during ECM deposition to confirm that MMP-1 was the responsible protease. Knockdown of MMP-1 but not control siRNA blocked the excess proliferation generated by activated mast cell supernatants estimated by MTT reduction (Figure 4B). Furthermore, direct application of activated MMP-1 (10 ng/ml) to untreated ASM-derived matrix significantly enhanced the proliferative capacity of both asthma and control-derived matrix, whereas activated and control mast cell supernatants, in the absence of ASM cells, had no effect (Figure 4C).

Relationship between MMP-1 Activation and Asthma Exacerbations

To understand the relevance of these *in vitro* findings to human asthma, we examined MMP-1 activation in BAL fluid of patients with asthma who developed exacerbations in response to rhinovirus inoculation. After virus inoculation, lower respiratory symptom scores increased and more severe reductions in lung function were observed in the subjects with asthma as previously reported (24). After viral inoculation, mean MMP-1 activity

Figure 4. (Continued). then seeded and allowed to grow for 48 hours. Mast cell supernatants had no direct effect on the ECM in the absence of ASM, but matrix-driven proliferation was enhanced by direct application of MMP-1. * $P = 0.013$; ** $P < 0.0001$ Mann-Whitney U test. APC = inhibitor of tryptase; chymos = inhibitor of chymase; ilo = inhibitor of metalloproteinases; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD = optical density; rMMP-1 = recombinant MMP-1; siRNA = small interfering RNA; SPI = inhibitor of serine protease.

Figure 5. Matrix metalloproteinase (MMP)-1 is activated during exacerbations of asthma. MMP-1 activity in bronchoalveolar lavage fluid of healthy control subjects and patients with asthma was measured by MMP-1 activity assay at baseline and 4 days after inoculation with rhinovirus. MMP-1 activity was elevated by viral infection and was higher in those with asthma at baseline and after viral inoculation. *Before versus after viral inoculation for asthma subjects, $P < 0.05$. Two-way analysis of variance with Tukey correction for multiple comparisons.

increased 3.9-fold ($n = 28$; difference, 0.445; 95% CI of difference, 0.052–0.84; $P = 0.03$) in patients with asthma and 11-fold in healthy control subjects, although the latter was not significant ($n = 11$; difference, 0.14; 95% CI of difference, –0.025 to 0.53) (Figure 5). Importantly, the degree of MMP-1 activation after exacerbation in those with asthma was strongly correlated with more severe falls in FEV₁ ($r^2 = 0.54$; $P < 0.0001$) and peak lower respiratory symptom score severity ($r^2 = 0.24$;

$P = 0.007$) (Figure 6). BAL TIMP1 protein was similar in control subjects and those with asthma and did not change significantly in response to viral challenge (see Figures E8 and E9).

Association between ASM Proliferation and Mast Cells in Asthma

Having shown that mast cell tryptase dependent MMP-1 activation leads to proliferative ECM remodeling *in vitro*, we examined the association between tryptase-positive mast cells and ASM proliferation in human airways. We quantitated proliferating ASM cells and tryptase-positive mast cells in the ASM bundles of the first group of 16 patients with asthma and 11 healthy control subjects (Figure 7A). Bronchial biopsies contained ASM in 12 and 10 cases, respectively. In these samples, both proliferating ASM cells and tryptase-positive mast cells tended to be more common in those with than without asthma, although the differences between groups were not significant (Figure 7B). Across individuals, the number of proliferating ASM cells was positively correlated with mast cell infiltration in both those with and without asthma ($r^2 = 0.49$; $P < 0.0001$) (Figure 7C).

MMP-1 Levels Are Associated with Bronchial Reactivity in Stable Asthma

Because collagenase expression and MMP-1 activity have been related to

airway contraction *in vitro* (25, 26) we examined the relationship between airway MMP-1 expression and airway contraction induced by methacholine in the first group of 16 patients with asthma. Increasing airway responsiveness to methacholine was associated with increasing total airway MMP-1 levels ($r^2 = 0.256$; $P = 0.04$) (Figure 8A). In these stable patients, Juniper Asthma Control Questionnaire scores were not associated with total MMP-1 level ($r^2 = 0.09$; $P = 0.23$) (Figure 8B).

Discussion

MMP-1 and mast cells are minimally present in normal airways but both are present in the ASM bundles of patients with asthma (27, 28). Here we have identified a potential mechanism linking MMP-1 activation by airway mast cells with ECM remodeling, ASM growth, airway contraction, and asthma severity. We have shown that MMP-1 is directly activated by mast cell tryptase and that this activation remodels ECM to generate a proliferative substrate for ASM cells. In patients with stable asthma, the presence of tryptase-positive mast cells is associated with enhanced ASM proliferation and MMP-1 expression is directly correlated with airway narrowing in response to bronchoconstrictor stimuli. During exacerbations, MMP-1 activation is associated with exacerbation severity. These findings suggest that the interaction between MMP-1, mast cells, and ECM remodeling may contribute to airway contraction, remodeling, and worsening symptoms in patients with asthma.

Enhanced expression of MMP-1 has been described in response to several mediators associated with asthma and airway remodeling including collagen, tenascin, cyclical strain, leukotriene D₄, tumor necrosis factor- α , and platelet-derived growth factor (27, 29–32), although the significance of this in asthma is unknown. In common with other MMPs, proteolytic removal of the prodomain is required for MMP-1 activation. Although MMP-1 activation by serine proteases has been described previously (33), in our study we demonstrated by direct interaction of the two proteins, that mast cell tryptase

Figure 6. Matrix metalloproteinase (MMP)-1 activation is associated with postexacerbation FEV₁ and asthma symptoms. (A) Rhinovirus-induced maximal fall in FEV₁ in patients with asthma is correlated with the concentration of active MMP-1 in bronchoalveolar lavage fluid. $r^2 = 0.55$, $P = 0.0001$. (B) Increased lower respiratory symptom scores during rhinovirus-induced exacerbation are associated with MMP-1 activation. $r^2 = 0.24$, $P = 0.007$. LRT = lower respiratory tract.

Figure 7. Mast cells are associated with airway smooth muscle proliferation in asthma. (A) Bronchial biopsies from patients with mild or moderate asthma, defined by the Global Initiative for Asthma criteria, were cut in serial sections, airway smooth muscle (ASM) bundles were identified by α -smooth muscle actin staining, smooth muscle was localized in consecutive slides, and proliferating ASM cells and mast cells identified by ki67 and mast cell tryptase staining, respectively (arrows). (B) ki67- and tryptase-positive cells in ASM bundles were expressed per 100 ASM cells in healthy control subjects and those with asthma. Those with asthma tended to have more proliferating ASM and mast cells. (C) Tryptase-positive mast cells are correlated with proliferating ASM cells. $r^2 = 0.5$, $P = 0.0001$. MCT = mast cell tryptase; SMA = α -smooth muscle actin.

cleaves the prodomain of MMP-1 *in vitro* and supernatants from activated (degranulated) mast cells activate MMP-1. MMP-1 cleaves triple helical fibrillar collagens including collagens 1, 2, 3, 7, 8, and 10 and gelatin as part of ECM turnover, but can also generate bioactive mediators by collagen processing including the neutrophil chemoattractant matrikine Pro-Gly-Pro (34). In addition to collagens, MMP-1 has other matrix substrates including aggrecan, proteoglycan, versican, and perlecan (35) and can activate or inactivate bioactive proteins including insulin-dependent growth factor binding proteins 2, 3, and 5; PAR 1; CXCL8; and CXCL12 (36). The advantage of using biosynthesized ASM matrix in our study is the presence of multiple protein substrates in the preparation, making this system more physiologic than individual ECM

substrates. This does mean we are unable to define which ECM-related proteins have been proteolytically processed and are responsible for the effect. It is likely that bioactive neoepitope generation, or ECM-associated growth factor processing, alters signaling to ASM (37). Our findings do show there is an absolute requirement for ASM cell-derived MMP-1 in generation of the proliferative ECM by tryptase and that the direct action of mast cell-derived proteases is not sufficient to generate proliferative ECM in the absence of ASM-derived MMP-1.

Although we cannot directly link the ECM remodeling by MMP-1 and ASM growth seen *in vitro* to humans with asthma and airway MMP-1 may not completely reflect ASM-derived MMP-1: our findings, using primary asthma-derived ASM cells, are consistent with this mechanism acting in asthma.

Tryptase-mediated activation of MMP-1 is one pathway by which mast cells could contribute to airway remodeling. Mast cells and mast cell degranulation are associated with severe asthma. *In vivo* it is likely that other mast cell mediators including prostaglandins and tryptase, by activation of PAR2, as previously observed *in vitro*, may stimulate ASM proliferation and contraction to worsen asthma symptoms (38, 39). We have previously shown that MMP-1 also supports ASM contraction *in vitro* and others have shown enhanced contraction of collagenase-treated bronchial rings (14, 40). In patients with stable asthma we found that airway MMP-1 is associated with airway narrowing in response to methacholine and that during exacerbations, activation of MMP-1 is associated with exacerbation severity judged by increasing symptoms and fall in FEV₁. The mechanisms of airway narrowing and the relationship between airway remodeling, airway contraction, and BHR are complex. Our findings, and those of others linking alterations in the ECM to ASM growth (18), contraction (19), and ASM mass with BHR (41), could be consistent with MMP-1 activity contributing to changes in cell/matrix coupling and ECM stiffness altering resistive ASM loading to result in increased airway narrowing (42) (Figure 9).

Longitudinal studies are generally consistent with the presence, but not progression, of airway obstruction with the minority of studies suggesting airflow obstruction increases with disease duration (2). No longitudinal studies have examined ASM or number or ECM mass over time in those with asthma and there are conflicting data on rates of ASM proliferation in asthma (5, 6, 43). Some suggest ASM proliferation is not elevated in patients with asthma (44), whereas others have demonstrated that ASM proliferation in bronchial biopsies is both enhanced in asthma and related to asthma severity (45). These contradictory findings both *in vitro* and *in vivo* may relate to a combination of methodologic and study population differences. Our findings, showing no difference in ASM proliferation *in situ* between those with asthma and control subjects overall, may reflect the exclusion of those with severe asthma. Importantly, using a mast

Figure 8. Matrix metalloproteinase (MMP)-1 is associated with bronchial hyperresponsiveness in asthma. (A) In patients with stable asthma, total MMP-1 in bronchial washings was associated with enhanced sensitivity to methacholine in bronchial challenge testing. $r^2 = 0.26$, $P = 0.045$. (B) Modified Juniper Asthma Questionnaire score was not significantly related to MMP-1 levels. $r^2 = 0.09$, $P = 0.23$. ACQ-5 = Juniper Asthma Questionnaire; PC₂₀ = provocative concentration of methacholine.

cell number we observe an association with ASM proliferation. This suggests specific aspects of the asthma phenotype drive ASM proliferation, rather than enhanced ASM proliferation being a feature of all with asthma at all times in their disease. Structural changes in

the airway are the net result of many processes including both ASM proliferation and death, ECM deposition, and resorption. The association of ASM growth and mast cells *in vitro* and *in vivo* is consistent with the generation of an environment that would alter this

balance to sustain airway remodeling in the asthmatic airway (46).

Importantly, our findings suggest a mechanism driving asthma severity that is largely independent of Th2-mediated inflammation and are of relevance to developing therapies for the less well understood Th2-low asthma endotype, which is poorly responsive to current treatments (47). For most of those with asthma, airway remodeling is not completely preventable with current asthma treatments and alternatives are required to target this complication of chronic asthma. Inhibition of MMPs in humans has failed as a therapeutic strategy because of the poor specificity of MMP inhibitors and effects caused by MMPs having multiple effects on beneficial and injurious processes. Targeting the mast cell/smooth muscle interaction may be a potential option to prevent MMP-1-mediated airway remodeling in patients with asthma. Recently, Hinks and coworkers (48) have shown airway MMP-1 protein and MMP/TIMP ratio to be particularly associated with elevated body mass index in asthma. Further study is required to determine the importance

Figure 9. Summary of findings and hypothesis. The airway phenotype of those with asthma differs from normal in many respects, including increased mast cell numbers and pro-matrix metalloproteinase (MMP)-1 expression. Exacerbations, other inflammatory stimuli, and possibly contraction cause mast cell degranulation, tryptase release, and MMP-1 activation. Active MMP-1 causes extracellular matrix processing to support airway smooth muscle proliferation. These changes result in fixed airflow obstruction, increased bronchial hyperresponsiveness, airway contraction, and worsening asthma symptoms. Interactions between airway remodeling, bronchial hyperresponsiveness, and airway contraction act at multiple levels, leading to worsening asthma symptoms. ASM = airway smooth muscle; BHR = bronchial hyperresponsiveness; ECM = extracellular matrix.

of this pathway in specific asthma endotypes.

In summary we have shown that tryptase-dependent MMP-1 activation can remodel the ECM to support ASM proliferation. In the airways of patients with asthma, tryptase-positive mast cells are

colocalized with proliferating ASM cells and MMP-1 expression is associated with BHR and MMP-1 activation with asthma exacerbations and exacerbation severity. Disrupting mast cell/ASM interactions may be a potential therapeutic option against airway remodeling. ■

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