MMP-1 activation contributes to airway smooth muscle growth and asthma severity.

Shams-un-nisa Naveed[1], Debbie Clements[1], David J Jackson[2,3], Christopher Philp[1], Charlotte K Billington[1], Irshad Soomro[4], Catherine Reynolds[1], Timothy W Harrison[1], Sebastian L Johnston[2], Dominick E Shaw[1] and Simon R Johnson[1†].

1. Division of Respiratory Medicine and Respiratory Research Unit, University of Nottingham.
2. National Heart and Lung Institute, Imperial College London and MRC & Asthma UK Centre in Allergic Mechanisms of Asthma.
3. Respiratory Medicine, Guy's and St Thomas' NHS Trust.
4. Department of Histopathology, Nottingham University Hospitals NHS Trust.

†Corresponding author

Simon Johnson.

Division of Respiratory Medicine and Respiratory Research Unit.

University of Nottingham.

Nottingham. UK.

simon.johnson@nottingham.ac.uk

Author contributions.

SRJ conceived the study, SRJ, DS and TWH planned the work, SLJ ran the exacerbation study. SN, CR and DJJ saw the patients, SN, DC, DJJ, CP and CKB performed the laboratory work, IS and SN evaluated the pathology slides and SRJ, SN, CKB, CP, SLJ, DS and TWH interpreted the results and contributed to the manuscript. All authors approved the final manuscript.

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Running head

MMP-1 in asthma.

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At a glance commentary

Scientific knowledge on the subject. Matrix metalloproteinase-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. Matrix metalloproteinase-1 is a matrix processing collagenase which requires activation by proteases. We hypothesised mast cell proteases could activate matrix metalloproteinase-1 resulting in structural changes that promote airway remodelling.

What this study adds to the field. Matrix metalloproteinase-1 can be activated by mast cell tryptase. Active matrix metalloproteinase-1 is capable of processing airway smooth muscle derived extra-cellular matrix which enhances airway smooth muscle proliferation. In patients with asthma, mast cells are associated with airway smooth muscle growth. Matrix metalloproteinase-1 protein is related to bronchial reactivity and matrix metalloproteinase-1 activity increases during asthma exacerbations where its level is related to exacerbation severity. Interrupting mast cell / airway smooth muscle interactions has the potential to reduce airway remodelling in asthma.

Online Data Supplement

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org

Abstract

Introduction: Matrix metalloproteinase-1 and mast cells are present in the airways of people with asthma. We hypothesised that matrix metalloproteinase-1 could be activated by mast cells and increase asthma severity.

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

Results: Airway smooth muscle cells generated pro-matrix metalloproteinase-1 which was proteolytically activated by mast cell tryptase. Airway smooth muscle treated with activated mast cell supernatants produced extra-cellular matrix which enhanced subsequent airway smooth muscle growth by 1.5 fold ($p<0.05$) which was dependent on matrix metalloproteinase-1 activation. In asthma, airway pro-matrix metalloproteinase-1 was 5.4 fold higher than control subjects ($p=0.002$). Mast cell numbers were associated with airway smooth muscle proliferation and matrix metalloproteinase-1 protein associated with bronchial hyper-responsiveness. During exacerbations, matrix metalloproteinase-1 activity increased and was associated with fall in FEV_1 and worsening asthma symptoms.

Conclusions: Matrix metalloproteinase-1 is activated by mast cell tryptase resulting in a pro-proliferative extra-cellular matrix. In asthma, mast cells are associated with airway smooth muscle growth, matrix metalloproteinase-1 levels are associated with bronchial hyper-responsiveness and matrix metalloproteinase-1 activation with exacerbation severity. Our

findings suggest that airway smooth muscle/mast cell interactions contribute to asthma severity by transiently increasing matrix metalloproteinase activation, airway smooth muscle growth and airway responsiveness.

Introduction

Asthma is characterised by airway inflammation, bronchial hyper-responsiveness (BHR) and variable airway obstruction. Structural changes, collectively termed airway remodelling, are associated with BHR, airflow obstruction, worsening asthma symptoms, increased beta-2 agonist use, exacerbations and can persist despite optimal asthma treatment(1-4). Airway remodelling often starts in childhood with airflow limitation associated with persistence of asthma and abnormal lung function in adulthood(5, 6). Changes observed in airway remodelling 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 remodelling has been seen as a consequence of inflammation, although airway contraction, in the absence of inflammation can also drive aspects of remodelling(8). As airway structural changes may support inflammation(9) it is likely that airway inflammation, bronchoconstriction, remodelling and fixed airflow obstruction interact in a linear, parallel or combined manner to drive the asthma phenotype.

Mast cells, particularly activated, degranulated forms are more common within ASM bundles of those with asthma(10-12) and generate pro-inflammatory cytokines including IL-4, IL-5, and IL-13, which regulate IgE production, eosinophilic inflammation, and pro-fibrogenic cytokines, including TGF-β and basic fibroblast growth factor 2. Pre-formed 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, hyper-responsiveness

and remodelling causing bronchoconstriction, ASM cell proliferation, and inflammatory cell recruitment(13).

Our overarching hypothesis is that the airway environment in asthma sustains airway remodelling(14-16). We have shown that components of the ECM in asthmatic airways can promote remodelling by affecting proliferation(17), migration(16), apoptosis(18), MMP activation(16) and β-agonist signaling of ASM cells(19). Here we hypothesised 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 remodelling and asthma symptoms. Some of this work has previously been presented as abstracts(20, 21).

Methods

Patients and controls

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 (GINA) criteria(22), without a history of exacerbation, change in therapy or use of oral steroids for at least six weeks and 11 non-smoking, age matched controls without lung disease. Further study details are included in the on-line supplement. Those with asthma underwent spirometry, bronchial provocation testing and modified Juniper Asthma Control Questionnaire (ACQ-5)(23). All subjects underwent flexible bronchscopy 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 exacerbations induced by rhinovirus inoculation compared with 11 healthy controls. The study has been described in detail previously(24). Briefly, non-smokers with mild or moderate asthma, defined by GINA criteria, and non-smoking, non-atopic healthy volunteers without a recent viral illness or asthma exacerbation, were recruited. Symptom scores, lung function, bronchoscopy and broncho-alveolar lavage (BAL) were performed two to four weeks prior to inoculation with rhinovirus 16. Following virus inoculation, symptom scores were recorded daily and lung function, bronchoscopy and BAL were repeated after four 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 (Figure E1)(15).

Mast cell supernatants were prepared from HMC-1 mast cells activated using phorbol 12-myristate 13-acetate (PMA, 50ng/ml) and calcium ionophore (A23187, 25ng/ml) for 16 hours. Control mast cell supernatants had PMA and calcium ionophore added after removal of the cell pellet. Inhibition of proteases was performed by pre-incubation of supernatants with protease inhibitors used at the manufacturers recommeded concentrations. Full details are included in the on-line 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 kDa 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 i.u. Promega, Southampton, UK) in physiological buffered saline for 30 minutes at 37°c. Products were resolved on SDS-PAGE gels and visualized by silver staining.

Extra cellular matrix preparations

ASM cell derived ECM was prepared as described(14) Further details are given in the supplementary information.

***In vitro* cell proliferation, adhesion and apoptosis assays**

Cell proliferation was determined by both 3-(4,5-dimethylthiaaol-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% foetal bovine serum. For adhesion assays, fluorescently labelled 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). Full details are provided in the on line supplement.

MMP-1 siRNA knockdown

siRNA was used as previously described(14) and reduced MMP-1 protein by >70% (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 (IS). ASM bundles were identified by immunostaining with anti α-smooth muscle actin (Roche, West Sussex, UK) and ASM cell nuclei counted using DAPI staining. In adjacent sections, proliferating ASM and tryptase positive mast cells were identified by anti-Ki67 and anti-mast cell tryptase antibodies respectively (Roche, West Sussex, UK). 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 t-test or Mann-Whitney U test as appropriate. Multiple comparisons were made by two-way ANOVA. Correlations between parameters were analysed by linear regression. Statistical analysis was performed using GraphPad Prism 6 for windows (version 6.07, 1992-2015 GraphPad Software, Inc. US). A p-value <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 controls 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 controls (control mean 0.14 ng/ml, 95% C.I. 0.095-0.197, asthma 0.79, 0.35-1.21. p=0.002, figure 1a). Western blotting of these samples identified a single 54 kDa band consistent with pro-MMP-1 in those with asthma but not in healthy controls. The 43 kDa 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 controls. Total MMP-1 protein was 3.3 fold higher in ASM culture supernatants from those with asthma than from healthy controls (control mean 0.48 ng/ml, 95% C.I. 0.23-0.74, asthma 1.57, 0.30-1.23, p=0.002, figure 1d).

Airway smooth muscle 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 the supernatants of activated or non-activated HMC-1 mast cells. Activated, but not control, mast cell supernatants resulted in the appearance of the active 43 kDa 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 pan-serine protease and specific tryptase inhibitors, but not a chymase inhibitor (figure 2b & c). The generation of active MMP-1 was associated with an increase in MMP-1 activity (supplementary figure E3). To determine if mast cell tryptase activated MMP-1 by direct cleavage, we co-incubated 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 kDa consistent with active MMP-1 (figure 2d).

Activated mast cell treatment of airway smooth muscle cells generates a pro-proliferative extra-cellular matrix.

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 controls 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 which enhanced proliferation of both control and asthma derived ASM over 48 hours (mean difference for control cells 0.73. 95% C.I. of difference 0.52-0.93, $p<0.001$. Mean difference for asthma cells 0.78. 95% C.I. 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 (figures 3, E4 and E5). Neither control mast cell supernatants nor the activation vehicle alone affected ASM proliferation. Activated mast cell supernatants had no overall effect upon ASM adhesion or apoptosis upon control or asthma derived ECM (supplementary results, figures E6 and E7).

Pro-proliferative matrix is dependent upon tryptase activated MMP-1

To determine if the pro-proliferative 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 prior to 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). Further, direct application of activated MMP-1 (10ng/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). Post-viral inoculation, mean MMP-1 activity increased 3.9-fold (n=28. Difference 0.445, 95% CI of difference 0.052 to 0.84, p=0.03) in patients with asthma and 11-fold in healthy controls although the latter was not significant (n=11. Difference 0.14, 95% CI of difference -0.025 to 0.53. Figure 5a). Importantly, the degree of MMP-1 activation post-exacerbation in those with asthma was strongly correlated with more severe falls in FEV_1 (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 (supplementary figures E8 and E9).

Association between airway smooth muscle proliferation and mast cells in asthma.

Having shown that mast cell tryptase dependent MMP-1 activation leads to pro-proliferative ECM remodelling *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 controls (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.

As 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, ACQ-5 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 airway smooth muscle bundles of patients with asthma (27, 28). Here we have identified a

potential mechanism linking MMP-1 activation by airway mast cells with ECM remodelling, 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 pro-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 broncho-constrictor stimuli. During exacerbations, MMP-1 activation is associated with exacerbation severity. These findings suggest that the interaction between MMP-1, mast cells and ECM remodelling may contribute to airway contraction, remodelling and worsening symptoms in patients with asthma.

Enhanced expression of MMP-1 has been described in response to a number of mediators associated with asthma and airway remodelling including collagen, tenascin, cyclical strain, leukotriene D4, TNF-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 pro-domain is required for MMP-1 activation. Whilst 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 cleaves the pro-domain 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, 10 and gelatin as part of ECM turnover, but can also generate bioactive mediators by collagen processing including the neutrophil chemo-attractant 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 physiological 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 neo-epitope generation, or ECM associated growth factor processing, alter signaling to ASM(37). Our findings do show there is an absolute requirement for ASM cell derived MMP-1 in generation of the pro-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 remodelling 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 remodelling. 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

FEV_1. The mechanisms of airway narrowing and the relationship between airway remodelling, airway contraction and bronchial hyper responsiveness are complex. Our findings, and those of others linking alterations in the ECM to ASM growth (18), contraction (19) and ASM mass with bronchial hyper-responsiveness (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 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 resorbtion. 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 remodelling in the asthmatic airway(46).

Importantly, our findings suggest a mechanism driving asthma severity which is largely independent of T_H2 mediated inflammation and are of relevance to developing therapies for the less well understood T_H2-low asthma endotype which is poorly responsive to current treatments(47). For the majority of those with asthma, airway remodelling 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 due to both the poor specificity of MMP inhibitors and effects due to MMPs having multiple effects upon beneficial as well as injurious processes. Targeting the mast cell / smooth muscle interaction may be a potential option to prevent MMP-1 mediated airway remodelling in patients with asthma. Recently, Hinks and co-workers have shown airway MMP-1 protein and MMP/TIMP ratio to be particularly associated with elevated body mass index in asthma(48). Further study is required to determine the importance 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 co-localised with proliferating ASM cells and MMP-1 expression is associated with bronchial hyper-responsiveness and MMP-1 activation with asthma exacerbations and exacerbation severity. Disrupting mast cell / airway smooth muscle interactions may be a potential therapeutic option against airway remodelling.

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Table 1.

Characteristics of patients and healthy subjects in studied in the bronchial hyper-responsiveness / airway remodelling study.

	Asthma	**Control**
N	16	11
Age (years)	31 (9.4)	36 (10.2)
Male / Female	9 / 7	6 / 5
Disease duration (years)	16 (9.9)	N/A
Atopy (present/absent)	8 / 8	N/A
ACQ5 score	2.28 (0.63)	N/A
BMI	25.1 (3.2)	24.9 (2.8)
ICS dose (mcg/day)	412 (296)	0
PC_{20} Methacholine (mg/ml)		not performed
0-5	2	
5-8	5	
8-16	9	
FEV_1 (% predicted)		
Pre bronchodilator	90.5 (9.5)	
Post bronchodilator	92.5 (11.5)	
Exhaled Nitric oxide (ppb)	49.6 (30.2)	

Values represent number in group or mean (standard deviation) as appropriate.
BMI=body mass index, ACQ5=Juniper 5 asthma control score, ICS=inhaled corticosteroid, ppb=parts per billion, N/A=not applicable

Figure legends.

Figure 1. 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 controls. 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 controls. A single band of 55 kDa consistent with pro-MMP-1 is 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 panel 'a'. Total MMP-1 protein is low in healthy controls 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 ASM cells grown from bronchial biopsies from patients with mild or moderate asthma and healthy controls. ** p=0.002, Mann-Whitney U-test.

Figure 2. Mast cell tryptase activates MMP-1. (a) Western blot for MMP-1 in ASM supernatants treated with control (cMC) or activated mast cell supernatants (aMC). Activated mast cell supernatants cause MMP-1 activation shown by the appearance of the smaller MMP-1 band which is blocked by a broad spectrum protease inhibitor (pan PI). **(b&c)** MMP-1 activation by activated mast cell supernatants is inhibited by inhibitors of serine and cysteine proteases (S&C PI), serine proteases (S PI), metalloproteinases (ilo), tryptase (APC366), but not chymase (chymos). **(d)** Silver stain of SDS PAGE gel showing

recombinant human (rh) tryptase cleaves pro-MMP-1 in vitro shown by the dose dependent appearance of a 43 kDa band.

Figure 3. Mast cell treatment of ASM generates pro-proliferative extracellular matrix. ASM from patients with asthma or controls were treated with control (cMC) or activated mast cell supernatants (aMC) or mast cell activation vehicle during ECM deposition. ASM cells were removed from ECM preparations and normal ASM seeded for 48 hours in the presence of 1% serum. Cell proliferation was quantitated by **(a)** MMT assay **(b)** and cell counting. ASM cell proliferation was enhanced by activated, but not control mast cell treatment. ** p=0.0001, 2-way ANOVA.

Figure 4. Pro-proliferative matrix is dependent upon ASM derived MMP-1. (a) ASM treated with control (cMC) or activated mast cell supernatants (aMC) during ECM deposition were co-incubated with an MMP inhibitor (ilo), chymase inhibitor (chymo), tryptase inhibitor (APC) or a serine protease inhibitor (SPI). 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 control (cMC) or activated mast cell supernatants (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 decellularised and then left untreated or incubated with control (cMC) or activated mast cell supernatants (aMC) or active recombinant MMP-1 (rMMP-1). ASM cells were 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.

Figure 5. MMP-1 is activated during exacerbations of asthma. MMP-1 activity in BAL fluid of healthy controls and patients with asthma was measured by MMP-1 activity assay at baseline and four days post inoculation with rhinovirus. MMP-1 activity was elevated by viral infection and was higher in those with asthma at baseline and post viral inoculation. * pre vs. post viral inoculation for asthma subjects p<0.05. 2-way ANOVA with Tukey correction for multiple comparisons.

Figure 6. MMP-1 activation is associated with post exacerbation FEV_1 and asthma symptoms. (a) Post rhinovirus induced maximal fall in FEV_1 in patients with asthma is correlated with the concentration of active MMP-1 in BAL 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.

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, ASM bundles were identified by α-smooth muscle actin (SMA) staining, smooth muscle was localised 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 controls 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.

Figure 8. MMP-1 is associated with BHR 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 (ACQ-5) was not significantly related to MMP-1 levels. r^2=0.09, p=0.23.

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-MMP-1 expression. Exacerbations, other inflammatory stimuli and possibly contraction cause mast cell degranulation, tryptase release and MMP-1 activation. Active MMP-1 causes ECM processing to support ASM proliferation. These changes result in fixed airflow obstruction, increased bronchial hyper-responsiveness (BHR), airway contraction and worsening asthma symptoms. Interactions between airway remodelling, BHR and airway contraction act at multiple levels leading to worsening asthma symptoms.

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