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Matrix metalloproteinase expression and activity in human airway smooth muscle cells

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1 Airway remodelling is a feature of chronic asthma comprising smooth muscle hypertrophy and deposition of extracellular matrix (ECM) proteins. Matrix metalloproteinases (MMPs) breakdown ECM, are involved in tissue remodelling and have been implicated in airway remodelling. Although mesenchymal cells are an important source of MMPs, little data are available on airway smooth muscle (ASM) derived MMPs. We therefore investigated MMP and tissue inhibitor of metalloproteinase (TIMP) production and activity in human ASM cells.

2 MMPs and TIMPs were examined using quantitative real-time RT–PCR, Western blotting, zymography and a quench fluorescence (QF) assay of total MMP activity.

3 The most abundant MMPs were pro-MMP-2, pro- MMP-3, active MMP-3 and MT1-MMP. TIMP-1 and TIMP-2 expression was low in cell lysates but high in conditioned medium. High TIMP secretion was confirmed by the ability of ASM-conditioned medium to inhibit recombinant MMP-2 in a QF assay. Thrombin increased MMP activity by activation of pro-MMP-2 independent of the conventional smooth muscle thrombin receptors PAR 1 and 4.

4 In conclusion, ASM cells express pro-MMP-2, pro and active MMP-3, MMP-9 and MT1-MMP. Unstimulated cells secrete excess TIMP 1 and 2, preventing proteolytic activity. MMP-2 can be activated by thrombin which may contribute to airway remodelling.
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Abbreviations: ANOVA, analysis of variance; ASM, human airway smooth muscle; APMA, 4-aminophenyl mercuric acetate; DMEM, Dulbecco's minimal essential medium; DNA, deoxyribonucleic acid; ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; GAPDH, glyceraldehyde acetone phosphate dehydrogenase; GYPGQV, PAR 4 specific receptor activating peptide; HRP, horseradish peroxidase; kD, kilo Daltons; MMP, matrix metalloproteinase; mRNA, messenger ribonucleic acid; MT-MMP, membrane-type matrix metalloproteinase; PAR1, proteolytically activated receptor 1; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; QF, quench fluorescence; qRT–PCR, quantitative real-time reverse transcriptase polymerase chain reaction; SFLLRN, PAR 1-specific receptor activating peptide; TGF-β, transforming growth factor beta; TIMP, tissue inhibitor of metalloproteinase; TNF-α, tumour necrosis factor alpha

Introduction

Structural changes in the airways of patients with asthma lead to bronchial hyper-responsiveness and irreversible airflow obstruction. These changes, termed remodelling, comprise subepithelial collagen deposition, epithelial desquamation, goblet cell hyperplasia, smooth muscle cell hyperplasia and hypertrophy (Hirst, 1996; Jeffery *et al*., 2000). Airway smooth muscle (ASM) cells play a central role in the remodelling process, producing pro and anti-inflammatory mediators (Johnson & Knox, 1997) and extracellular matrix (ECM) components (Johnson *et al*., 2000). Interactions between ASM and the ECM are of increasing interest in airway biology and recent reports have implicated the ECM in modulating ASM proliferation, the balance between 'synthetic' and 'contractile' phenotype (Hirst *et al*., 2000) and providing survival signalling to prevent cells undergoing apoptosis (Freyer *et al*., 2001). We and others have demonstrated that ASM cells constitutively produce matrix metalloproteinases (MMPs) (Foda *et al*., 1999; Johnson & Knox, 1999): zinc-dependent proteolytic enzymes that play a major role in matrix turnover, remodelling and angiogenesis. Collectively, MMPs cleave most constituents of ECM and can be broadly divided into collagenases, gelatinases, stromelysins, elastases and membrane-bound forms (for review, see Nagase & Woessner, 1999). MMPs are secreted as inactive proenzymes and activated during secretion at the cell surface or extracellularly by cleavage of a propeptide domain

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by a range of proteases, including other MMPs and furin. Their proteolytic activity is tightly regulated under physiological conditions by tissue inhibitors of metalloproteinases (TIMPs), proteins which bind to MMPs and inhibit their function (Nagase & Woessner, 1999). Paradoxically, TIMPs are also required for MMP activation; TIMP-2 and MMP-14 forming an activation complex for MMP-2 (Butler *et al*., 1998). MMPs are produced by most cell types and upregulated in inflammatory disease, cancer and in response to inflammatory mediators.

In the airways, MMPs are secreted by both resident and migrating inflammatory cells (Dunsmore *et al*., 1998; Dahlen *et al*., 1999; Johnson & Knox, 1999) and are emerging as mediators in asthma and airway inflammation. The ratio of TIMP-1 to MMP-9 in bronchoalveolar lavage fluid is increased in asthma (Mautino *et al*., 1999) and MMP-9 is further increased during asthma exacerbations (Lemjabbar *et al*., 1999). In addition to matrix degradation, MMPs also contribute to smooth muscle hyperplasia by releasing immobilised growth factors, for example, degradation of the ECM proteoglycan, decorin, by MMP-2, -3 and -7 can release immobilised transforming growth factor β (TGF-β) (Imai *et al*., 1997). Although these studies suggest a role for the MMPs in asthma, airway inflammation and remodelling, little is known of the spectrum of MMPs produced by individual airway cell types. Furthermore, most studies in the airway have not measured MMP activity directly but inferred it from the MMP/TIMP ratio measured by zymography and ELISA. As the ASM cell is both a source of matrix components and its behaviour modulated by the ECM, we hypothesised that ASM-derived MMPs could play a role in airway remodelling and be regulated by proinflammatory stimuli. In this study, we characterise the production and activity of the gelatinases and stromelysins and their inhibitors by primary ASM cells.

Methods

Tissue culture

Primary cultures of human ASM cells were prepared as described previously (Pang & Knox, 1998) and shown to be greater than 98% smooth muscle cells. Briefly, trachealis muscle was obtained from cadavers less than 24 h after death from patients with no history of lung disease. Informed consent was obtained from relatives and ethical approval was obtained from the Queens Medical Centre Research Ethics Committee. PC-3 prostate carcinoma and HCT116 colon carcinoma cell lines were used as positive controls for TIMP-1, TIMP-2 and MT1-MMP. All cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (2%) and foetal calf serum (10%) (Gibco/BRL, Paisley, U.K.) at 37°C in a 5% carbon dioxide/95% air atmosphere and used within nine passages of the primary culture.

Real-time RT–PCR (qRT–PCR)

ASM gelatinases, stromelysins and TIMPs (MMP-2, -3, -7, -8, -9, -10, -13, -14, TIMP-1 and TIMP-2) mRNA expression was measured by qRT–PCR. ASM cells were grown to confluence in 162 cm^2 tissue culture flasks, serum depleted for 48 h and RNA extracted using the RNeasy Mini Kit according to the manufacturer's protocol (Qiagen Ltd., Crawley, West Sussex, U.K.). Contaminating DNA was removed using DNAse (Gibco, Paisley, U.K.) and cDNA synthesised using the Ready-to-Go T-Primed First Strand kit (Amersham Pharmacia Biotech Ltd, Little Chalfont, Buckinghamshire, U.K.). qRT–PCR was performed using an ABI Prism 5700 Sequence Detector (Perkin-Elmer Biosystems, Shelton, CT, U.S.A.) using the double-stranded DNA-specific fluorophore Sybr Green I (Molecular Probes, Leiden, The Netherlands). PCR reactions comprised 1 μl cDNA, 3 μl 25 mM $MgCl_2$, 2 μl dNTPs, 2.5 μl 10 × Sybr Green I PCR buffer, 1 μl of 5 mM forward and reverse primer mix and 0.125 μl *Taq* Gold. Primer sequence pairs for MMPs are listed in Table 1. Cycling parameters were 95°C for 30 s, 40 s ramp, 55°C for 30, 20 s ramp, 72°C for 1 min, 17 s ramp. MMP mRNA was measured from five separate ASM cultures from different donors and expressed as mean ± s.e. of relative to GAPDH (GAPDH varied little between different primary cell cultures).

Western immunoblotting

ASM cells were grown to confluence in 10 cm dishes, serum depleted for 48 h and lysed in protein extraction buffer containing protease inhibitors. A measure of 30 μg protein per lane was subjected to SDS–PAGE using 10, 12 or 15% polyacrylamide gels (Biorad Hemel Hempstead, Herfordshire, U.K.). Proteins were transferred to polyvinylidene difluoride membranes and blocked in phosphate-buffered saline (PBS): 0.3% Tween-20 and 8% dried milk powder. Membranes were incubated overnight at 4°C with primary antibodies or mouse IgG_1 control diluted in blocking solution. Secondary HRP-conjugated rabbit anti-mouse antibody was incubated for 1 h at room temperature and visualised by enhanced chemiluminescence (Amersham Life Science). Prostate and colon carcinoma cell lines PC-3 and HCT-116 were used as positive controls. Primary mouse monoclonal antibodies were used as follows: MMP-2 1 : 1000; MMP-3 1 : 300 (Chemicon Interna-

Table 1 Primer pairs for human matrix metalloproteinases, listed from 5′ to 3′

Primer			*Sequence (5′–3′)*
GAPDH	Forward	—	GGCCTCCAAGGAGTAAGACC
	Reverse		AGGGGTCTACATGGCAACTG
MMP-2	Forward	—	TTGACGGTAAGGACGGACTC
	Reverse		ACTTGCAGTACTCCCCATCG
MMP-3	Forward	—	TGATCCTGCTTTGTCCTTTG
	Reverse		TTCAAGCTTCCTGAGGGATT
MMP-7	Forward	—	AGCCAAACTCAAGGAGATGC
	Reverse		ACTCCACATCTGGGCTTCTG
MMP-8	Forward	—	TTTGATGGACCCAATGGAAT
	Reverse		GGAGGTGTTGGTCCATGTTT
MMP-9	Forward	—	TTGACAGCGACAAGAAGTGG
	Reverse		CCCTCAGTGAAGCGGTACAT
MMP-10	Forward	—	ATTTTGGCCCTCTCTTCCAT
	Reverse		CTGATGGCCCAGAACTCATT
MMP-13	Forward	—	AGGAGATGCCCATTTTGATG
	Reverse		ACCTAAGGAGTGGCCGAACT
MT1-MMP	Forward	—	ACCCTAAGACCTTGGGAGGA
	Reverse		AGGGTTTCTTCTGCCCACTT
TIMP 1	Forward	—	AAGGCTCTGAAAAGGGCTTC
	Reverse		GAAAGATGGGAGTGGGAACA
TIMP 2	Forward	—	CCAAGCAGGAGTTTCTCGAC
	Reverse		GACCCATGGGATGAGTGTTT

tional, Chandlers Ford, Hampshire, U.K.), MT1-MMP 5 μg ml^{-1}; TIMP-1 1 μg ml^{-1}; TIMP-2 2.5 μg ml^{-1} (Oncogene Research Products, San Diego, CA, U.S.A.).

Zymography

The identification of proteolytic enzymes was performed using gelatin or casein zymography by electrophoresis of serum-free conditioned medium collected from confluent ASM cells. For some experiments serum-free conditioned medium was concentrated 20-fold to enhance detection using Centricon YM-30 concentrators according to the manufacturer's protocol (Millipore, Watford, U.K.). A volume of 10 μl of medium were loaded under nondenaturing conditions into precast Novex polyacrylamide zymogram gels (Invitrogen, Paisley, U.K.), supplemented with either 0.1% gelatin to detect the presence of MMP-2 and -9 or 0.05% casein to detect the presence of MMP-3, -7 and -10. Electrophoresis was performed at a constant voltage of 125 V for 90 min. Gels were washed in renaturing buffer and incubated overnight in incubation buffer (both from Novex), stained with Coomassie brilliant blue R-250 (Sigma, Poole, Dorset, U.K.) and destained with gel-clear destain solution (Novex). Areas of gelatinolytic or caseinolytic degradation appeared as transparent bands on the blue background. A wide range molecular mass marker set (Sigma) and recombinant human MMP-2 preactivated with 1 mM 4-aminophenyl mercuric acetate (APMA) were used to identify MMP species.

MMP activity assay

Total MMP activity was detected using a quench fluorescence (QF) method. Briefly, the QF substrate (Mca-Pro-Leu-Gly-Dpa-Ala-Arg-NH_2) comprises a quencher and fluorophore linked as shown, with hydrolysis of the Leu-Gly bond by MMPs resulting in an increase in fluorescence (Knight *et al*., 1992). The substrate was obtained from Dr C. G. Knight, Department of Biochemistry, University of Cambridge.

In initial experiments and those involving MMP inhibition, activity was measured in real time over 30 min. Phenol red-free medium was shown not to effect fluorescence and this conditioned medium was added to a cuvette containing the QF substrate (5 μM) in assay buffer (50 mM Tris/HCl, 5 mM $CaCl_2$, pH 7.5) with or without recombinant human MMP-2 (5 μM). MMP activity was measured on a Perkin-Elmer LS-5B fluorimeter (excitation 328 nm, emission 393 nm), and expressed as nM of peptide cleaved per minute, using FLU-SYS (Rawlings & Barrett, 1990). In further experiments, looking at the effect of thrombin 'stopped assays' were performed using conditioned medium taken from ASM cells grown to confluence in 24-well tissue culture plates with or without thrombin for 48 h. At this point, conditioned medium was collected and QF substrate (5 μM) added. Cleavage of the substrate by MMPs was stopped by adding 1 mM ethylenediaminetetraacetic acid (EDTA) and fluorescence measured at a single time point after 4 h incubation. A longer incubation period compared with the real-time assay was necessary due to the lower activity of conditioned medium compared with recombinant MMP-2. In later experiments, where we wished to examine cell-surface MMP activity, the protocol was adapted to incorporate monolayer cultures of ASM cells. Cells were grown to 90% confluence in black-sided 96-well plates (Costar) and serum starved for 48 h then stimulated with experimental treatments $\pm$ QF substrate (5 μM) in serum-free, phenol red-free DMEM. Plates were incubated at 37°C for 4 h in the dark and cell-surface MMP activity measured as an increase in fluorescence (excitation 328 nm, emission 393 nm) using a FLEXstation™ (Molecular Devices, Sunnyvale, CA, U.S.A.).

Analysis

Paired experiments were analysed by *t*-test and multiple comparisons by two-way ANOVA with Dunnett's correction with a *P*-value of <0.05 regarded as significant.

Results

Spectrum of MMP and TIMP mRNA expression by ASM cells

Human ASM cells expressed mRNA for MMP-2, -3, -7, -8, -9, -10, -13, MT1-MMP, TIMP-1 and -2. Relatively high levels of MMP-2, MT1-MMP, TIMP-1 and -2 were detected ($>10\times$ GAPDH) compared to mRNA levels for MMP-8, -9, -10 and -13 ($<1\times$ GAPDH). MMP-7 and -3 had moderate levels (>1x$<10\times$ GAPDH) (Figure 1). Although the relative amounts of mRNA species, including GAPDH, differed between donors, their relative expression was similar in all cases.

MMP/TIMP protein expression

To determine if the most highly expressed mRNA species were present as proteins, we performed gelatin zymography for MMP-2 and -9, casein zymography for MMP-3, -7 and -10 and Western blotting for MMP-2, -3, -14, TIMP-1 and -2.

Gelatinases In keeping with the qRT–PCR findings, gelatin zymography confirmed 72 kDa pro-MMP-2 was strongly expressed with low levels of intermediate forms of MMP-2 in some cultures and no significant active MMP-2. Western analysis also confirmed the presence of pro-MMP-2 in ASM

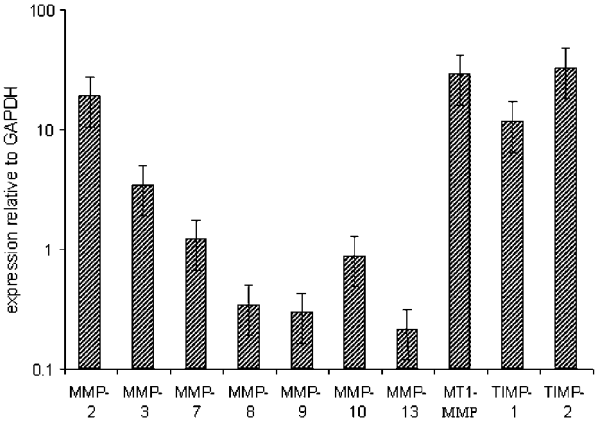

Figure 1 MMP and TIMP mRNA expression in primary ASM cells. Data shown is mean ($\pm$s.e.) mRNA expression measured by qRT–PCR in five primary ASM cell cultures and expressed relative to GAPDH.

lysates. MMP-9 was not expressed under these conditions but gelatin zymography of 20-fold concentrated conditioned medium showed low-level constitutive expression of pro-MMP-9 (Figure 2a).

Stromelysins Casein zymography showed lytic activity at 52 kDa consistent with MMP-3. Four of the six cultures also produced the 43 kDa active MMP-3 visible by standard zymography. A 20-fold concentration of conditioned medium revealed low levels of active MMP-3. Bands corresponding to MMP-7 and -10 were not detected in standard or concentrated conditioned medium. Western blotting of cell lysates showed the presence of both pro and active MMP-3 in ASM lysates (Figure 2b).

TIMPS/MT1-MMP MT1-MMP protein was detected by Western analysis in ASM cells (Figure 3). TIMP-1 and -2 proteins were expressed at low levels in ASM cell lysates. As our later studies suggested excess MMP inhibitory activity in conditioned medium, we also analysed conditioned medium by Western blotting and found high levels of TIMP-1 and -2. Control HCT116 and PC3 cells strongly expressed MMP-14 and TIMP-1 and -2 (Figure 3).

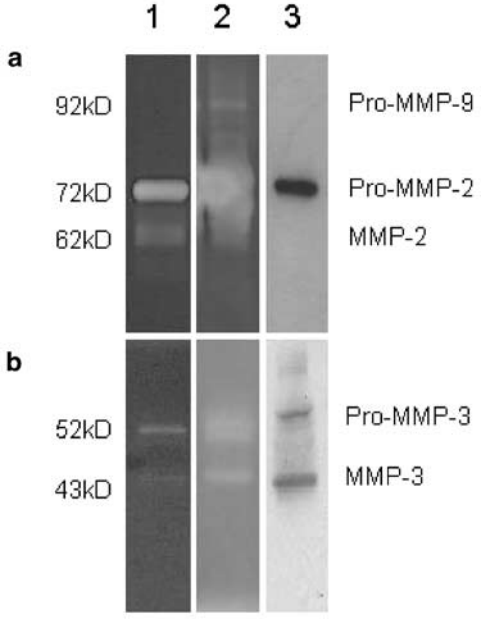

Figure 2 (a) Lane 1 – gelatin zymogram of ASM-conditioned medium showing examples of pro-MMP-2 production (72 kDa) and intermediate forms of MMP-2 (62 kDa). Lane 2 – 20-fold concentration of ASM-conditioned medium revealed bands corresponding to pro-MMP-9 that were not detected in the unconcentrated sample. Lane 3 – Western blot of ASM whole cell lysates probed with anti-MMP-2 monoclonal antibody showing expression of pro-MMP-2. (b) Lane 1 – casein zymogram of ASM cell-conditioned medium showing examples of latent MMP-3 (52 kDa) production and active MMP-3 (43 kDa) production. Lane 2 – 20-fold concentration of ASM-conditioned medium confirms expression of active form of MMP-3. Lane 3 – Western blot of ASM lysates probed with anti-MMP-3 monoclonal antibody. Bands at 52 and 43 kDa are consistent with pro and active MMP-3 expression, respectively. All gels representative of at least three separate experiments.

ASM cell medium contains an excess of MMP inhibitors

MMP activity of conditioned medium from ASM cells measured by QF in real time was low ($0.54\,\text{nmol}\,\text{min}^{-1} \pm 0.26$, $n=4$). We postulated that this may be due to an excess of inhibitors in the ASM-conditioned medium and therefore assessed if ASM-conditioned medium could inhibit active MMPs. In these experiments, serum-free-conditioned medium was added to preactivated recombinant human MMP-2 resulting in a 34% inhibition of substrate cleavage ($n=4$, $P<0.0001$), suggesting that an excess of MMP inhibitor is present in the ASM-conditioned medium (Figure 4). Non-conditioned medium had no effect upon fluorescence.

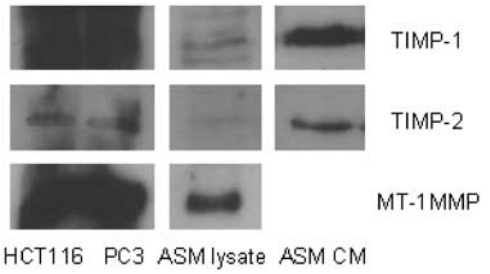

Figure 3 Representative Western blots of three separate primary cultures of ASM whole cell lysates probed with anti-MT1-MMP, TIMP-1 and -2. TIMP-1 CM and -2 CM are conditioned medium taken from the same experiments and show strong expression. Strongly positive staining for all three species was seen in PC3 and HCT116 cells.

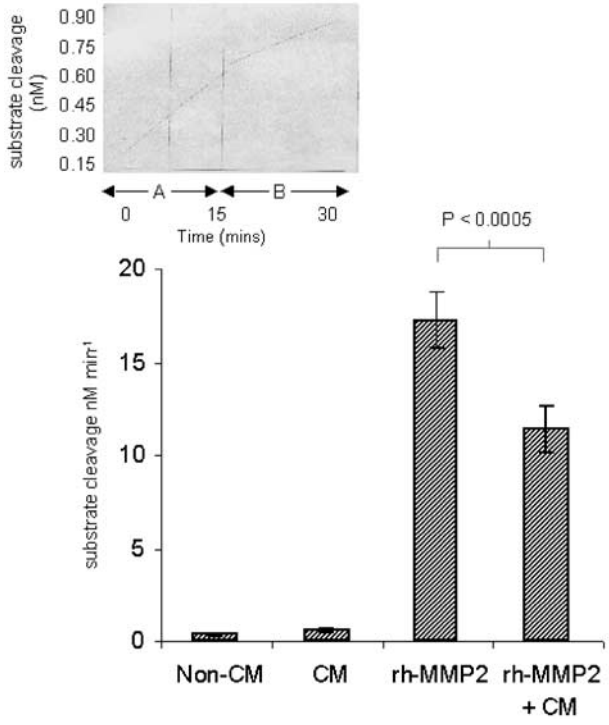

Figure 4 Real-time quench fluorescence assay to measure MMP activity. ASM-conditioned medium has low absolute MMP activity. Recombinant human MMP-2 (5 μM) has strong activity in this assay which is reduced by 34% by ASM-derived conditioned medium. Inset shows typical trace obtained in real time with region (a) rhMMP-2 alone and (b) rhMMP-2 plus conditioned medium. Experiment repeated three times in cells from four separate donors. Data are expressed as the mean $\pm$ s.d. ($n=4$, $P \leqslant 0.005$).

Thrombin increases MMP activity

In initial experiments, we were unable to demonstrate an effect of proinflammatory mediators on MMP activity using the stopped assay described. We reasoned that as conditioned media contained excess MMP inhibitors this may quench ASM-derived MMP activity. As the cell-surface environment is the site of activation and activity of some MMPs, we adapted the QF assay to measure cell-associated MMP activity. In initial experiments, the QF substrate was incubated with cells alone to ensure fluorescence was not affected by nonspecific binding or endocytosis of the substrate. Using this protocol, we observed a low level of baseline substrate cleavage which was partially inhibited by the broad-spectrum MMP inhibitor Ilomastat, demonstrating that at least 25% of the measured activity was due to the action of MMPs (not shown). We then went on to test the effect of thrombin on MMP activity. Thrombin is a proinflammatory serine proteinase present in increased amounts in the airways of patients with asthma. Thrombin cleaves arginyl and to a lesser extent lysyl bonds and would not be predicted to cleave the fluorescent substrate which was confirmed in preliminary experiments (not shown). Addition of 1 IU ml^{-1} thrombin caused an 18% ($P<0.002$) increase in QF peptide cleavage, over the baseline value. This rise in activity was abrogated by the specific thrombin inhibitor hirudin (10 IU ml^{-1}, not shown). The broad-spectrum MMP inhibitor Ilomastat reduced baseline substrate cleavage and also inhibited the effect of thrombin confirming this to be due to a metalloproteinase (not shown). To examine which metalloproteinase was responsible, we performed gelatin and casein zymography of thrombin-treated ASM cells and showed a concentration related increase in MMP-2 activation in response to thrombin (Figure 5b). No changes were seen using casein zymography (not shown). Addition of thrombin to conditioned medium without ASM cells did not result in MMP-2 activation by zymography.

To further characterise the effect of thrombin, we used the specific PAR1 antagonist RWJ-56110 (a gift of Dr C.K. Derian, Johnson and Johnson Pharmaceutical Research and Development (Andrade-Gordon *et al*., 1999)). RWJ-56110 (10 μM) reduced the baseline substrate cleavage, but did not inhibit rise in activity in response to thrombin. Treatment of ASM for 4 h with 3 IU ml^{-1} thrombin and RWJ-55110 caused a rise in MMP activity of 14.5% and a 13.4% in vehicle-treated controls. Using zymography although baseline pro-MMP-2 appeared less strongly expressed in the RWJ-56110-treated cells, thrombin was able to cause MMP activation (Figure 5b). Higher concentrations of RWJ-55110 up to 40 μM had no effect on thrombin-induced activation of MMP-2 in either assay. We went on to examine the effect of the PAR activating ligands SFLLRN, specific for PAR 1 and GYPGQV, specific for PAR 4. Neither ligand reproduced the rise in MMP activity (Figure 5b), suggesting that this effect was independent of PAR 1 and 4.

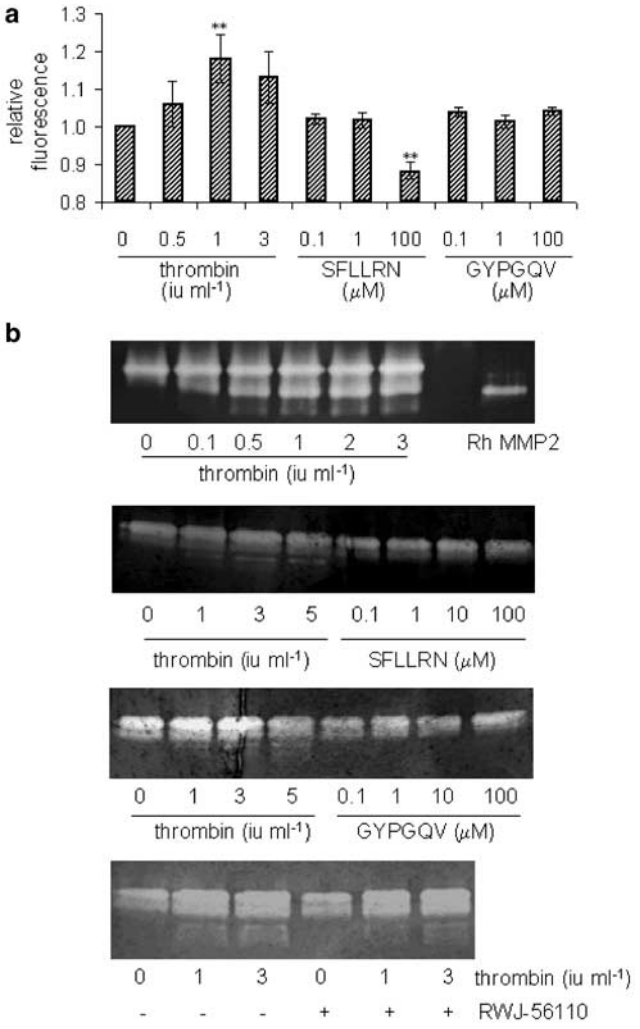

Figure 5 (a) Quench fluorescent stopped assay to measure cell-surface MMP activity. ASM cells were incubated in serum-free-conditioned medium containing QF peptide, thrombin and PAR-activating peptides for 4 h at 37°C. Relative fluorescence was normalised to unstimulated cells. Neither thrombin, the PAR 1 vehicle nor culture medium effected substrate cleavage alone. Mean (s.d.) relative fluorescence compared with unstimulated cells analysed by ANOVA with Dunnett's multiple comparison test ($n=6$, $^{**}P<0.05$). (b) Gelatin zymography to show concentration-dependent activation of MMP-2 by thrombin. Rh MMP-2 is APMA-activated recombinant human MMP-2. Activation of MMP-2 was not seen with the PAR 1 agonist SFLLRN nor the PAR 4 agonist GYPGQV. The PAR 1 antagonist RWJ-56110 (10 μM) had no effect upon the activation MMP-2 by thrombin.

Discussion

The key findings of this work are that human ASM cells produce a spectrum of MMPs and that the activity of these MMPs is normally tightly regulated by ASM-derived inhibitors. Using the appropriate assay conditions, we have shown cell-surface MMP-2 activity can be regulated by the proinflammatory mediator thrombin.

The most abundant MMPs were MMP-2, -3 and MT1-MMP. TIMP-1 and -2 were also strongly expressed. To our knowledge, this is the first report of MMP-3 production by ASM cells. MMP-3 is activated after secretion from the cell and the presence of pro- and active MMP-3 we observed in cell lysates probably includes MMP-3 bound to ASM-derived ECM. This finding is consistent with work showing MMP-3 immunostaining in the submucosal matrix of patients with chronic asthma (Dahlen *et al*., 1999) where extracellular MMP-3 staining did not correlate with inflammatory cell number suggesting it may be derived from resident rather than

inflammatory cells. MMP-3 can degrade a range of ECM proteins including collagen IV, V, fibronectin, laminin, elastin and proteoglycans (Whitelock *et al*., 1996). MMP-3 may contribute to both airway inflammation and remodelling in several ways, the release of ECM-bound growth factors such as fibroblast growth factor-2 and TGF-β may result in ASM proliferation, ECM production and by processing mast cell-derived TNFα, which leads to enhanced endothelial cell activation and leukocyte infiltration (Dahlen *et al*., 1999).

Relatively large amounts of MMP-2 protein were detected in normal ASM cells, but only small amounts of MMP-9 were seen by zymograms after concentration of conditioned medium. MMP-2 and -9 mainly degrade type IV collagen, a major component of airway subepithelial basement membrane. ASM cell-conditioned medium had low MMP activity despite the presence of active MMP species. This low activity was due to an excess of secreted MMP inhibitors demonstrated by the ability of conditioned medium to inhibit recombinant MMP-2. This inhibitory activity probably being due to TIMP-1 and 2, which are strongly expressed by ASM. Although the inhibition of recombinant MMP-2 activity was incomplete this is likely to have been due to the relatively high concentration of MMP-2 used. Others have shown ASM membrane-associated TIMP-2 contributes to pro-MMP-2 activation (Foda *et al*., 1999) and our study shows that ASM also produce significant amounts of soluble TIMPs, which inhibit overall MMP activity. This excess of MMP inhibitors is likely to protect the airway from uncontrolled proteolytic activity, a phenomenon normally associated with rapidly progressive tissue destruction.

The MMPs described here are secreted as zymogens and activated on the cell surface, therefore examination of intracellular MMPs by Western analysis of cell lysates will not reflect activation of the MMP species nor inhibition by TIMPs. Thus, a combination of Western blotting to identify the species produced, zymography to examine activation and the QF assay to examine overall activity are all required to study MMP biology.

mRNAs for MMP-7, -8 and -10 were detected at higher levels than that of MMP-9 (but lower than MMP-2, -3 and -14). Although we were able to detect MMP-9 activity by gelatin zymography no activity due to MMP-7 or -10 was detectable using casein zymography. This may be due to the lower sensitivity of casein compared to gelatin zymography (detection limits 1.5×10^{-3} U trypsin ml^{-1} and 5×10^{-6} U ml^{-1} of collagenase, respectively). MMP-8 was not examined at the protein level as this was outside the scope of the present study. Although our study suggests MMPs -7, -8 and -10 are not produced at high levels by ASM cells alternative methods of detection such as ELISA will need to be used to investigate this more fully.

Airway inflammation involves extravasation of plasma proteins including thrombin, a serine protease with multiple roles in coagulation and inflammation. As thrombin is present in the sputum of patients with asthma (Gabazza *et al*., 1999), increases after allergen challenge (Terada *et al*., 2004) and has multiple proinflammatory effects on various smooth muscle cell types and MMP activity, we examined its effect on ASM. Thrombin increased overall MMP activity, which was correlated with activation of pro-MMP-2. Most cell-mediated actions of thrombin occur *via* proteolytically activated, G-protein-coupled receptors (PARs); ASM cells express PAR 1 and 2 (Hauck *et al*., 1999; Knight *et al*., 2001; Chambers *et al*., 2003), PAR 4 has not been formally studied although the agonist peptide GYPGQV has a minor mitogenic effect on ASM cells (Tran & Stewart, 2003) and therefore PAR 4 may be present. In cultured human ASM, thrombin causes proliferation, mobilisation of intracellular calcium (Panettieri *et al*., 1995), GM-CSF production (Tran & Stewart, 2003) and in bronchial rings smooth muscle contraction (Hauck *et al*., 1999) acting both *via* PAR 1-dependent and PAR-independent pathways (Tran & Stewart, 2003). Our study suggests MMP-2 activation is predominantly independent of PAR 1 and 4 as the thrombin response was not inhibited by the PAR 1 antagonist nor reproduced by PAR 1 and 4 agonist peptides. Indeed, 100 μM of the PAR 1 agonist peptide inhibited fluorescence which may have been due to a nonspecific effect of the peptide at high concentration. PAR 2 is activated by proteases including tryptase but not thrombin (Nystedt *et al*., 1994). There are no specific peptides which activate PAR 3 and we were not therefore able to fully exclude a contribution from PAR 3 but as the receptor is not strongly expressed on ASM, requires high doses of thrombin for its activation and acts as a cofactor for PAR 4 it is unlikely to significantly effect MMP-2 activation (Macfarlane *et al*., 2001).

The PAR agonist peptides were used over a range of concentrations and their effect is likely to be maximal at PAR 1 and 4 (Hollenberg, 1999). The incubations used were 4 and 24 h and significant degradation of the peptides is unlikely to result in the culture conditions over this period. There are an increasing number of reports describing thrombin-mediated effects in various cell types, which are independent of the classical PAR receptors including ASM proliferation and GM-CSF secretion (Tran & Stewart, 2003), fibroblast annexin V production (Sower *et al*., 1999) and in human umbilical vein endothelial cells activation of MMP-2 (Lafleur *et al*., 2001). Thus it is likely that thrombin functions *via* receptors other than PARs and such receptors in the airways of patients with asthma could represent a mechanism by which thrombin contributes to airway remodelling.

Previous studies of MMPs in the airway have measured enzyme expression by zymography and Western blotting inferring activity from MMP/TIMP ratios. Our study shows that measurement of MMP activity is complicated by an excess of MMP inhibitors in ASM-conditioned medium and that direct measurement of cell-surface proteolytic activity is required to detect changes in MMP activity. Although small, the changes observed in our study are likely to be physiologically relevant. Persistent low-grade MMP activity during repeated episodes of airway inflammation in patients with asthma may contribute to inflammatory cell recruitment, increased matrix turnover and over years play a role in the chronic structural changes leading to airway remodelling.

In summary, our results indicate that human ASM cells constitutively produce pro-MMP-2, MMP-3 and MT1-MMP but their activity is suppressed by an excess of TIMP-1 and 2. MMP-2 can be activated at the cell surface by thrombin, independent of PAR 1 and 4. These findings suggest a further mechanism by which the smooth muscle cell can orchestrate airway inflammation and contribute to airway remodelling.

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