Collagen I and thrombin activate MMP-2 by MMP-14-dependent and -independent pathways: implications for airway smooth muscle migration

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Submitted 17 August 2006; accepted in final form 20 December 2006

Henderson N, Markwick LJ, Elshaw SR, Freyer AM, Knox AJ, Johnson SR. Collagen I and thrombin activate MMP-2 by MMP-14-dependent and -independent pathways: implications for airway smooth muscle migration. Am J Physiol Lung Cell Mol Physiol 292: L1030–L1038, 2007. First published December 22, 2006; doi:10.1152/ajplung.00317.2006.—Increased proinflammatory mediators and ECM deposition are key features of the airways in asthma. Matrix metalloproteinases (MMPs) are produced by airway smooth muscle (ASM) cells and have multiple roles in inflammation and tissue remodeling. We hypothesized that components of the asthmatic airway would stimulate MMP production and activation by ASM and contribute to airway remodeling. We measured human ASM-derived MMP mRNA, protein, and activity by real-time RT-PCR, zymography, Western blotting, and MMP activity assay. Collagen I and thrombin caused a synergistic increase in MMP-2 protein and total MMP activity but paradoxically decreased MMP-2 mRNA. Additionally, collagen I activated MMP-2 in cultured supernatants independent of the cell surface. Together, collagen I and thrombin strongly enhanced MMP-14 mRNA and protein but had no effect individually, suggesting increased MMP-14, the activating proenzyme for MMP-2, may be partially responsible for MMP-2 activation. Furthermore, collagen I reduced tissue inhibitor of metalloproteinase-2 protein (TIMP-2). We examined the role of MMPs in functions of ASM related to airway remodeling and found migration and proliferation were MMP dependent, whereas adhesion and apoptosis were not. Ilomastat inhibited migration by 25%, which was also inhibited by TIMPs 1–4 and increased by the MMP-2 activator thrombin. These in vitro findings suggest that the environment within the airways of patients with asthma enhances MMP-2 and -14 protein and activity by a complex interaction of transcriptional and posttranscriptional mechanisms, which may contribute to ASM migration.

matrix metalloproteinases; asthma

PATIENTS WITH ASTHMA DEVELOP structural airway changes termed remodeling. Starting in infancy, remodeling is categorized by airway smooth muscle (ASM) hypertrophy, hyperplasia, increased subepithelial myofibroblasts, altered ECM deposition, infiltration of ASM by mast cells, increased mucosal vascul arity, epithelial shedding, metaplasia, and mucus gland hyperplasia (1, 22, 43). Remodeling results in bronchial hyperreactivity and airflow obstruction and, therefore, increased symptoms and use of asthma medication and healthcare resources (1, 34). The ASM cell is emerging as a key cell in airway remodeling being increased in both size and number in the airways of patients with asthma (4). Moreover, ASM cells produce ECM components (10), pro- and anti-inflammatory mediators, and growth factors of relevance to airway remodeling, including vascular endothelial growth factor (30), interleukin-8 (52), eotaxin (41), and prostaglandin E2 (39). Thus the ASM cell is an important modulator of inflammation, inflammatory cell influx, and angiogenesis in the asthmatic airway (27, 31).

Allergen challenge in sensitized airways is associated with a rapid increase in subepithelial myofibroblasts, and it is suspected that at least a portion of these may have migrated to the lamina propria from ASM bundles (14). In vitro, ASM cells can migrate towards growth factors, particularly PDGF (42). Although there has been increasing interest in ASM cell migration, and matrix metalloproteinases (MMPs) have established roles in the migration of other cell types including vascular smooth muscle cells (23), to date only one very recent study has examined the role of the metalloproteinases in ASM migration (19).

MMPs are a family of proteases capable of degrading all ECM proteins with additional roles in release of matrix-bound growth factors, cleavage, and activation of cell surface receptors, angiogenesis, cellular invasion, and migration (46). MMPs are powerful proteolytic enzymes with a range of substrates and are regulated by gene transcription, cleavage of a regulatory propeptide, and interaction with inhibitors including the tissue inhibitors of metalloproteinases (TIMPs) (38). We (10) have previously shown that the major ASM-derived MMP is MMP-2, which is closely controlled by TIMP-2. MMP-2 is activated at the cell surface by a trimeric complex with TIMP-2 and the membrane-associated MMP-14 (5). The MMPs and TIMPs are being increasingly linked to asthma (29): there is increased expression of MMP-9, -2, and -3 in the airways of patients with asthma (35, 48), MMP-9 is increased in lavage fluid during asthma exacerbations and allergen challenge (28) and is reduced when asthma control improves (21). In animal models of asthma, MMPs have major roles in migration of inflammatory cells through the airway, although this is complex. Knockout of MMP-9 is associated with reduced inflammatory cell recruitment to the airway lumen after allergen challenge (7), whereas in the MMP-2 knockout mouse after allergen challenge, a dramatic reduction in egress of inflammatory cells from the lung occurs (8). We (10) have previously shown that the serine protease thrombin can activate ASM-derived pro-MMP-2, and, furthermore, others have shown that thrombin is also present in the airways of patients with asthma and increased during exacerbations (13, 21).
We reasoned that in asthma, thrombin and other components of the asthmatic airway may cause MMP activation and that activation of MMPs could contribute to airway remodeling. As alteration in the type and amount of ECM proteins is described in the airways of patients with asthma, particularly collagen I, fibronectin, and laminin, we examined the effect of ECM proteins on MMP activity and how ASM-derived MMPs may be associated with airway remodeling. Here, we show that ASM-derived MMP-2 can be selectively activated by collagen I and that thrombin synergistically enhanced this activation by multiple mechanisms. Furthermore, activation of ASM-derived MMPs is associated with enhanced ASM migration, suggesting that the environment within the asthmatic airway may favor MMP activation and thus promote airway remodeling.

METHODS

Tissue culture. Primary cultures of human ASM cells were prepared as previously described (10) 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. Cells were grown in DMEM supplemented with l-glutamine (2%) and fetal calf serum (FCS; 10%) (GIBCO BRL, Paisley, United Kingdom) at 37°C in a 5% carbon dioxide/95% air atmosphere and used within 10 passages of the primary culture. Before experimental procedures, ASM cells were serum-depleted for 24 h.

ECM proteins collagen I, collagen IV, fibronectin, and laminin (Calbiochem, Nottingham, United Kingdom) were coated on tissue culture plates as previously described (12); briefly, a 10 μg/ml solution was allowed to adhere to culture plates overnight before blocking with 1% bovine serum albumin (BSA) in PBS, air-dried, rinsed twice with PBS, and sterilized by UV light. In experiments examining the effect of collagen I alone, identical effects were observed when the protein was added to culture medium, and this method was used in time course and dose-response studies.

Real-time RT-PCR. Relative quantification of ASM MMP-2, -3, and -14, and TIMP-1 and -2 mRNA was performed using a SYBR Green quantitative PCR method relative to GAPDH. In addition, heat dissociation curves were performed by dilution curve construction, calculated automatically by GeneTools software (Syngene, Cambridge, United Kingdom) and expressed as fold increase of the total MMP (pro-MMP-2 + pro-MMP-2) content secreted by control-treated ASM cells at 24 h (unless otherwise stated).

Quenched fluorescent assay. Total MMP activity was assessed by quenched fluorescent assay as previously described (10). Conditioned media from control-, collagen I-, thrombin-, and collagen I plus thrombin-treated cells were removed after incubation for 4–16 h. The MMP-quenched fluorescent substrate (Mca-Pro-Leu-Gly-Dpa-Ala-Arg-NH2; Sigma, Poole, Dorset, United Kingdom) was added, and total MMP activity was detected as increased fluorescence emission at 405 nm after excitation at 320 nm in triplicate wells at a single time point. We (10) have previously shown that the substrate was stable under these conditions and also not cleaved by thrombin. Results of at least three independent experiments were plotted as fold induction above substrate blank (diluted in serum-free DMEM).

Migration assays. Polycarbonate membrane inserts (8 μm pore size, In Vitrogen) were coated with 10–100 μg/ml rat tail collagen I overnight at 4°C and then air-dried before blocking with serum-free DMEM containing 0.1% BSA. Serum-depleted ASM cells were passaged and resuspended in serum-free DMEM containing 0.1% BSA at a cell density of 8 × 104 cells per milliliter. Cells (100 μl) were then added to the upper chamber. Cells were stained with Diff-Quik and counted (6 random fields per slide) at ×20 magnification and then expressed as a percentage of control. In preliminary experiments to validate the assay, we showed that ASM cells enter the lower chamber of the Transwell at 16 h in the absence of a promigratory stimulus (i.e., by chemotaxis), which is unaffected by Ilomastat (Chemicon, Chandlers Ford, Hampshire, United Kingdom). Addition of PDGF induces directional migration, which was Ilomastat-sensitive (as shown in RESULTS). PDGF did not affect cell number or MMP-2 expression at this time point (not shown), suggesting the effects seen are due to migration rather than proliferation.

MTT proliferation assay. ASM cells were cultured in triplicate in 96-well plates in the presence or absence of Ilomastat (10 μmol) over 6 days. At each time point, 20 μl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the culture media and incubated at 37°C for 4 h, and then the medium was removed, 200 μl of MTT solubilization solution was added (Cell Proliferation Assay Kit, Sigma) and quantitated by the absorbance at 570 nm (corrected for background absorbance at 690 nm), and the mean values were compared by ANOVA.

Apoptosis. Apoptosis was assessed as previously described (12). Briefly, cells were grown in 96-well plates, serum-starved for 48 h, and then cultured for 18 h at 37°C in 5% CO2 in the presence of the metalloproteinase inhibitor BB94 (10 μmol) or vehicle (DMSO) in the presence or absence of 10% FCS. Cells were then fixed with 4%
formaldehyde, stained with propidium iodide, and visualized using an inverted microscope (Nikon Diaphot 300 with epifluorescent capabilities; Nikon, Kingston-upon-Thames, United Kingdom). Small rounded cells with pyknotic nuclei were counted and expressed as a percentage of the total cells by an observer blinded to the experimental treatments. Three fields were examined per well, and triplicate wells were counted and averaged within each experiment (≥500 cells per condition). Results are shown as means ± SE and were analyzed by ANOVA.

**Adhesion assay.** Adhesion assays were performed using cytometric screening kits (Chemicon) according to manufacturer’s instructions. ASM cells were grown to confluence, serum-depleted, and removed using nonenzymatic detachment solution (Sigma Aldrich). Cells were washed twice in PBS containing 0.1% BSA, plated at a density of 2 × 10^4 cells per well in DMEM containing 0.1% BSA in the presence of Ilomastat (10 μmol) onto 96-well plates coated with laminin, fibronectin, vitronectin, collagen I, collagen IV, or albumin. After 4 h, the medium was removed by gentle aspiration, and plates were washed twice in Dulbecco’s PBS. Cells were visualized using crystal violet solution, gently washed four times with PBS, crystal violet solubilized, and absorbance was read at 540 nm. Results are shown as means ± SE and were analyzed by ANOVA.

**Western blotting.** ASM cells were grown to confluence in T-25 flasks, serum-depleted for 24 h, stimulated for a further 24 h with thrombin and/or collagen I, and then lysed in 2× SDS protein loading buffer containing β-mercaptoethanol. Samples were heat-treated at 90°C for 5 min and then subjected to SDS-PAGE using 10% polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes and blocked in PBS containing 0.1% Tween 20 (PBS/Tween) and 5% dried milk powder. Membranes were incubated overnight at 4°C with primary antibodies diluted in blocking solution. Secondary horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody was incubated for 1 h at room temperature and visualized by enhanced chemiluminescence (Amer sham Life Science). Primary mouse monoclonal antibodies were used as follows: MMP-2 (5 μg/ml), MMP-14 (10 μg/ml), and TIMP-2 (2 μg/ml) (Oncogene Research Products, San Diego, CA).

**Analysis.** Paired experiments were analyzed by t-test and multiple comparisons by two-way ANOVA with Dunnett’s correction with a P value of <0.05 regarded as significant. For zymography and Western blotting, a representative gel of three or more similar independent experiments is shown.

**RESULTS**

**Collagen I enhances MMP-2 expression and activation.** Treatment of ASM cells with collagen IV, fibronectin, and laminin had no significant effect on MMP-2 expression or activation. However, collagen I (25 μg/ml) resulted in a substantial increase in activated MMP-2 (5.24 ± 2.69-fold increase over control-treated cells; P ≤ 0.05; Fig. 1, A and B). Activation of MMP-2 by collagen I was dose- and time-dependent (Fig. 2, A–C). Collagen I (25 μg/ml), when added to serum-free, 24-h-conditioned ASM medium in the absence of ASM cells, was also able to activate ASM-derived MMP-2 in conditioned medium (Fig. 2D).

**Collagen I and thrombin synergistically activate pro-MMP-2 protein.** We have previously shown that thrombin can activate pro-MMP-2 in ASM cells, and as both thrombin and collagen I are overexpressed in the airways of patients with asthma, we next examined if stimulation with both thrombin and collagen I resulted in enhanced expression and activation of MMP-2 protein. Coincubation with collagen I and thrombin increased pro- and active MMP-2 protein expression to a greater degree than either agent alone, an effect that was again dose-dependent (Fig. 3, A and B). To examine the effect on ASM MMP activity rather than protein expression, ASM cells were treated with thrombin, collagen I, and the combination for 4 and 24 h, and overall MMP activity was measured using quenched fluorescent MMP activity assay. After 24 h, there was a significant increase in MMP activity after treatment with thrombin or collagen I alone; however, the combination resulted in a large increase in MMP activity in conditioned medium, suggesting a synergistic effect on MMP activity between the two proteins (Fig. 3C).

To study the mechanisms behind this increase in MMP-2 expression and activity, we examined the effect of collagen I and thrombin on MMP-2 mRNA levels. Surprisingly, thrombin reduced mRNA levels of MMP-2, collagen I had no effect, and collagen I and thrombin together resulted in an mRNA transcript level equivalent to the mean of collagen I and thrombin alone (Table 1). To examine this further, we went on to examine the time course of MMP-2 mRNA expression following thrombin and collagen treatment. ASM cells were pretreated with actinomycin D or vehicle prior to stimulation with collagen I and thrombin. Actinomycin D did not significantly affect cell viability over the course of the experiment (not shown). In thrombin, but not control or collagen-treated cells (alone or in combination with collagen I), at 24 h, MMP-2 mRNA was higher in the actinomycin D pretreated cells than vehicle-treated cells and lower than control or collagen I-only-treated cells (Fig. 4A). This finding suggests the possibility of the induction of a repressor of MMP-2 mRNA by thrombin.

**Increased MMP-2 protein is not due to release of preformed protein.** To exclude the possibility that thrombin increases MMP-2 protein by the liberation of presynthesized MMP-2, cells were treated with cycloheximide (5 μM) and then stimulated with thrombin for 24 h. Cycloheximide treatment strongly reduced MMP-2 protein in supernatants, suggesting
that the effect of thrombin on MMP-2 was due to increased protein synthesis rather than the release of preformed MMP-2 protein (Fig. 4B).

**MMP-2 formation and activation is independent of p38 MAPK.** Activation of p38 MAPK is involved in regulating the translation of several proteins including MMPs and TIMPs in various cell types (37, 47). To determine if p38 MAPKs might mediate the actions of thrombin and collagen on MMP-2, we first performed Western blotting of ASM intracellular proteins with specific total and phospho-p38 antibodies to study the effect of thrombin and collagen I on p38 MAPK activation. Collagen I did not affect p38 phosphorylation compared with control (data not shown); however, thrombin led to a modest increase in activated p38 between 30 min and 2 h. The duration of thrombin-induced phosphorylation of p38 was increased to 4 h when costimulated with collagen I in the absence of cells for 24 h.

**Role of MMP-14 and TIMP-2 in MMP-2 activation.** To examine the mechanism of MMP-2 activation, we studied the expression of MMP-14 and TIMP-2. In addition to a role in pro-MMP-2 activation, TIMP-2 also inhibits the active protein. We used Western blotting and zymography to examine the expression of MMP-14, TIMP-2, and MMP-2 proteins (Fig. 5A), gels from three to five independent experiments were examined by densitometry, and protein was expressed as change over expression in unstimulated cells (Fig. 5B). Collagen I had no effect on MMP-14, reduced TIMP-2 protein, and increased expression of pro- and active MMP-2 protein (Fig. 2). Thrombin did not affect TIMP-2 or MMP-14 protein but later time points. These data suggest that these agents can synergistically activate p38 MAPK. However, when we studied the effect of pretreatment of ASM cells with the p38 inhibitor SB-203580 (Calbiochem) at 10 μM, a concentration that blocks IL-2-dependent p38 phosphorylation in T cells (33), on thrombin and collagen I induction of pro- and active MMP-2 by zymography, we found no differences from vehicle-treated cells (Fig. 4C).

![Fig. 2. Collagen I induction and activation of MMP-2. A: gelatin zymography of MMP-2 activity in ASM conditioned media after 24 h treatment with increasing concentrations of collagen I (10–200 μg/ml). B and C: gelatin zymography (B) and densitometric analysis (C) of ASM-conditioned media after 2–24 h stimulation with collagen I (25 μg/ml); graph shows mean band volume of active or total (active plus pro-MMP-2) MMP-2 over baseline ± SE. P < 0.001 for active MMP-2 at 24 h in collagen I-treated cells vs. control. D: gelatin zymogram showing 24-h cell-preconditioned ASM media further treated with collagen I in the absence of cells for 24 h.](http://ajplung.physiology.org/)
increased pro- and active MMP-2 (Fig. 3). Costimulation with collagen and thrombin strongly enhanced MMP-14 and increased pro-MMP-2 and active MMP-2 proteins. This suggests that the increased MMP-2 activity may be secondary to an increase in MMP-14 and a complementary reduction in TIMP-2.

To examine the role of transcriptional events in the synergistic induction of MMP-14 by collagen and thrombin, we examined MMP-14 mRNA levels by real-time RT-PCR. As was the case for MMP-2, thrombin also reduced mRNA levels of MMP-14 at 24 h. Collagen I strongly upregulated MMP-14 mRNA, and costimulation with collagen I and thrombin resulted in an mRNA transcript level equivalent to the mean of collagen I and thrombin alone with a modest increase in MMP-14 (Table 1). Examination of MMP-14 mRNA over time with and without actinomycin D showed an increase in mRNA after collagen and thrombin combination over control and collagen and thrombin alone but no effect on mRNA stability (latter not shown).

Collectively, these findings suggest that collagen I and thrombin both increase expression of pro- and active MMP-2 and MMP-14 proteins by posttranscriptional mechanisms and, for MMP-14, also by transcriptional regulation. Together, collagen I and thrombin produce a synergistic effect on MMP-14, pro- and active MMP-2 expression and activity.

**MMPs are required for migration of ASM cells.** Having examined the activation of MMP-2 by components of the asthmatic airway, we next examined the functional roles of MMP-2 in ASM cells to study how this mechanism may contribute to airway remodeling. As selective MMP inhibitors are not available to examine this question, we initially examined the effect of the broad spectrum MMP inhibitors on ASM proliferation, apoptosis, adhesion, and migration. Ilomastat (10 μM) had a small inhibitory effect on serum-induced proliferation as measured by MTT assay (Fig. 6A). As previously shown, ASM cells had low rates of apoptosis in serum (12), and the MMP inhibitor (10 μM) had no effect on apoptosis in the presence or absence of serum (Fig. 6B). Adhesion of ASM cells to a range of ECM proteins was greatest for fibronectin, consistent with other studies (20), but independent of MMP activity (Fig. 6C).

ASM cell migration toward PDGF-BB (10 ng/ml) across collagen-coated inserts was reduced by 30% in the presence of Ilomastat (Fig. 7B). It has previously been shown that serine proteases enhance ASM migration (6), and to compare the effects of these different protease classes on migration, ASM cells were treated with MMP, serine (4-aminobenzensulfonyl fluoride [AEBSF], 0.1–1 μM), cysteine (cystatin, 5–10 μg/ml), and aspartic (pepstatin A, 1 μM) protease inhibitors (all from Calbiochem). Inhibition of cysteine and aspartic proteases did not effect migration; however, the serine protease inhibitor AEBSF had a marked inhibitory effect on ASM migration (Fig. 7A). In addition to reducing ASM migration, AEBSF and Ilomastat also reduced MMP-2 activation in conditioned medium, which was not due to reduced cell viability (not shown). The cysteine and aspartic protease inhibitors had no effect on MMP-2 activation (Fig. 7B). To further examine the effect of MMP inhibition on ASM migration, we treated ASM cells with recombinant TIMPs. TIMPs 1–4 all reduced ASM migration, with TIMP-4 being the most potent, resulting in a 50% reduction in migration (Fig. 7C). To test if activators of MMPs enhanced ASM migration, we examined the effect of thrombin. Thrombin in the range of 0.1–5 IU/ml resulted in a dose-dependent increase in ASM migration toward PDGF-BB (Fig. 7D).

**DISCUSSION**

We have shown that collagen I and thrombin can independently activate ASM-derived MMP-2 and together produce a synergistic increase in MMP-2 protein and activity by a com-

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**Table 1. mRNA expression after incubation of airway smooth muscle cells with collagen and thrombin**

<table>
<thead>
<tr>
<th>Collagen</th>
<th>Thrombin</th>
<th>Collagen and Thrombin</th>
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<tbody>
<tr>
<td>MMP-2</td>
<td>1</td>
<td>0.365±0.233*</td>
</tr>
<tr>
<td>MMP-14</td>
<td>1</td>
<td>0.750±0.181</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>1</td>
<td>0.498±0.178*</td>
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Expression of airway smooth muscle cell-derived matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) mRNA transcripts relative to GAPDH after 24-h incubation with thrombin (3 international units [IU/ml]) and/or collagen I (50 μg/ml). Means ± SE are normalized to unstimulated cells, and 3 experiments were performed for each condition. One-way ANOVA with Dunnett’s comparison; *P ≤ 0.05; †P ≤ 0.01.
A combination of mechanisms. Moreover, as ASM cells are dependent on MMP activity for migration, this mechanism may contribute to ASM migration. Thrombin, a serine protease, is present in sputum and bronchoalveolar lavage fluid of patients with asthma. As airway thrombin rises after antigen challenge (13, 49), ASM-derived MMP-2 activation in asthma may result from allergen exposure. Type I collagen is increased in the airways of patients with asthma (44), and in vitro, asthma-derived ASM cells secrete more collagen I than control cells (24). We (11, 12) and others (20) have shown that collagen I contributes to ASM survival, proliferation, and enhanced contractile signaling in vitro. Extending our in vitro findings to asthma, it is possible that the environment in the asthmatic airway facilitates ASM migration, proliferation, and contraction. Furthermore, ASM migration and proliferation may be potentiated by asthma exacerbations.

The activation of MMP-2 by thrombin is independent of its classical receptor PAR1 in ASM (10) and human umbilical vein endothelial cells (HUVEC), where, in the latter, it acts by increasing MMP-14 mRNA and activity (32). Examining MMP-2 mRNA by real-time RT-PCR in ASM cells has shown that at 24 h (but not at early time points) thrombin reduces MMP-2 mRNA in an actinomycin D-dependent manner, a finding consistent with the thrombin-dependent induction of either an inhibitor of MMP-2 mRNA transcription or factor associated with its degradation. Several groups of proteins bind the AU-rich elements of mRNAs, including AUF-1 and tristetraprolin targeting mRNA for rapid degradation (45). In addition, MMP-2 is a predicted target of microRNA (http://cbio.mskcc.org/cgi-bin/mirnaviewer/mirnaviewer.pl), another key mechanism in repressing translation and reducing mRNA stability. Repression of MMP-2 mRNA combined with enhanced protein expression has also been observed in malignant disease (18), and dysregulation of mRNA stability in malignant and inflammatory diseases is an area of increasing interest. Further investigation into the role of posttranslational MMP regulation is required.

Activation of MMP-2 by thrombin alone in ASM is not a consequence of MMP-14 induction as thrombin does not significantly increase MMP-14 mRNA or protein. In HUVEC, thrombin contributes to activation of MMP-2 by cleavage of an intermediate 64-kDa MMP-14-generated intermediate (32). Activation of MMP-2 by collagen may again occur by more than one mechanism. We have shown that ASM-derived MMP-2 in conditioned medium may be activated by collagen away from the cell surface and hence independent of MMP-14, which is membrane bound. Previously described using recombinant proteins, this effect is specific to collagen I, MMP dependent, and thought to be due to stabilization of MMP-2 by collagen binding following autocatalysis (9). Cell-dependent effects also occur, as in our study, collagen caused a threefold rise in MMP-14 mRNA, and similarly, in cardiac fibroblasts (16) and hepatic stellate cells (50), collagen I leads to MMP-2 activation. MMP-2 ACTIVATION BY COLLAGEN I AND THROMBIN

Fig. 5. MMP and tissue inhibitor of metalloproteinase (TIMP) expression after collagen I and thrombin treatment. Western blot analysis of ASM whole cell MMP-14 and TIMP-2 and zymography of conditioned medium MMP-2 after 24 h of stimulation with collagen and/or thrombin. A: representative example of Western blot shown. B: densitometry of Western blot analysis for MMP-14 and TIMP-2. Results are expressed as mean (± SE) of 3–5 Western blots relative to control level for each condition (**p < 0.05). Similar analysis for MMP-2 is shown in Fig. 3.

Fig. 6. Effect of MMP inhibition on ASM proliferation, apoptosis, and survival. A: incubation of ASM cells with lomastat (10 μmol) reduces proliferation in response to serum at 7 days (**p < 0.01). B: BB94 has no effect on ASM apoptosis in the presence or absence of serum (p < 0.01). C: adhesion of ASM cells in culture is dependent on ECM substrate but unaffected by the presence of lomastat. SFM, serum-free medium; MMPi, MMP inhibitor.
activation by increasing MMP-14 expression. The previously unreported finding that is of particular interest to the current study is the synergistic interaction of thrombin and collagen I on the activation of MMP-2. The observed increase in active MMP-2 protein expression by collagen and thrombin was dose dependent and did not occur at the expense of pro-MMP-2 expression or via an increase in mRNA, suggesting that collagen and thrombin influenced posttranslational processing of MMP-2. Interestingly, a synergistic effect between collagen I and thrombin was also seen for MMP-14, with a strong induction of MMP-14 protein by the collagen I-thrombin combination despite there being no significant induction of MMP-14 protein by collagen I or thrombin alone. Collagen I alone increased MMP-14 mRNA, which suggests the possibility that collagen I enhances transcription and thrombin favors MMP-14 protein translation or protein stability. Increased active MMP-2 protein was accompanied by a threefold increase in overall MMP activity using the MMP activity assay, a highly significant increase in this type of assay for ASM cells (10). The quenched fluorescent assay used measures the sum of all MMP and MMP inhibitor activity in solution: we (10) have previously shown that MMP-2 is the major ASM-derived MMP and is likely to be mainly responsible for the activity seen. MMP-14 would not contribute to the rise in activity as it is fixed at the cell surface and not present in supernatants.

Taken together, these findings show enhanced MMP-2 expression and activity by collagen I and thrombin occurs at multiple levels including pro-protein expression, an increase in MMP-14 protein, directly in solution and additionally via an unknown thrombin-dependent mechanism (Fig. 8). MMPs have long been associated with tissue remodeling and repair in all organ systems including the lung (40); coupled with this, the ASM cell is capable of producing and reacting to altered ECM proteins in disease (3, 25). We therefore examined the effect of MMP activity on ASM proliferation, survival, adhesion, and migration, all functions of critical importance to the ASM cell in airway remodeling. Consistent with previous studies, the metalloprotease inhibitor had a modest effect on proliferation driven by serum or PDGF (19, 26). There was no effect on apoptosis in the presence or absence of serum or on the adhesion of ASM cells to ECM substrates, a process critical for cellular migration and invasion. The MMP inhibitor Ilomastat reduced ASM migration across collagen surfaces by 30%. To compare the effect of MMPs on migration, we used other protease inhibitors, which, consistent with previous studies, showed a profound effect of serine protease inhibition, potentially acting on urokinase plasminogen activator (15). Furthermore, in both cases, inhibition of migration was associated with reduced MMP-2 activation in conditioned medium. To further categorize the effect of MMPs on migration, we used recombinant TIMPs, all of which reduced migration, TIMP-4 being the most potent. TIMP-4 strongly binds MMP-2 (2) and reduces vascular smooth muscle migration to a similar degree as we have observed in ASM (17). In keeping with our other findings, thrombin, a known activator of MMP-2, dose-dependently enhanced ASM migration. Very recently, others have shown that MMP-2, released as a consequence of mechanical strain, is also required for ASM migration (19). We add to this finding by suggesting a mechanism linking migration and the components of the remodeled airway.

What are the implications of our findings for asthma and airway remodeling? Increases in proinflammatory mediators...
and the deposition of ECM proteins, particularly collagens, are key features of the airways of patients with asthma. MMPs are produced by ASM cells, have multiple roles in inflammation and tissue remodeling, and are increasingly implicated in asthma (29). Our findings, that two components of the asthmatic airway, collagen I and thrombin, together cause a large increase in MMP-2 activity, which can facilitate ASM migration, suggest that, if confirmed in vivo, the environment in the asthmatic airway may favor increased expression of MMP-14, MMP-2 activation, ASM migration, and remodeling. Antigen challenge in patients with asthma but not control subjects results in a rapid increase in subepithelial myofibroblasts, likely to have arisen from the migration of resident ASM cells (14).

A variety of proinflammatory mediators promote ASM migration including IL-1β, tumor necrosis factor-α, and PDGF (36). We hypothesize that release of such proinflammatory mediators during exacerbations promotes chemotaxis of resident ASM cells to the submucosa in an MMP-dependent manner facilitated by an excess of collagen I and thrombin (Fig. 8). As with other studies, our ASM cells, although derived by dissection of smooth muscle bundles and smooth muscle actin positive, may still contain myofibroblasts due to contamination by other resident cells and culture in serum (51). However, as these cell types are all present in the airway, our findings are still relevant.

In summary, we have shown for the first time that two components of the asthmatic airway, collagen I and thrombin, synergistically activate ASM-derived MMP-2 in vitro by a combination of mechanisms. This activation of ASM-derived MMPs is associated with increased ASM migration. Hence, the altered structure of the airway in asthma could contribute to and potentially sustain airway remodeling. If confirmed in vivo, selective inhibition of MMPs may be a therapeutic target to prevent airway remodeling in asthma and other inflammatory diseases of the airways.

ACKNOWLEDGMENTS

We are grateful to Drs. Deborah Clarke, Anna Grabowska, and Cornelia de Moor for help and advice.

GRANTS

This study was funded by Asthma UK and the Nottingham University Hospitals Specials Trusts.

REFERENCES

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MMP-2 ACTIVATION BY COLLAGEN I AND THROMBIN


39. Nie M, Pang L, Inoue H, Knox AJ. Transcriptional regulation of cyclooxygenase 2 by bradykinin and interleukin-1beta in human airway smooth muscle cells: involvement of different promoter elements, tran-