

Platelet-derived growth factor and transforming growth factor- β modulate the expression of matrix metalloproteinases and migratory function of human airway smooth muscle cells

I. Ito*, E. D. Fixman*, K. Asai*, M. Yoshida[†], A. S. Gounni[‡], J. G. Martin* and Q. Hamid*

*Meakins-Christie Laboratories, Department of Medicine, McGill University, Montreal, QC, Canada, [†]Department of Biochemistry, McGill University, Montreal, QC, Canada and [‡]Department of Immunology, University of Manitoba, Winnipeg, MB, Canada

Clinical & Experimental Allergy

Summary

Background Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinase (TIMPs) have been suggested to be involved in the pathogenesis of asthma. Their expression in airway smooth muscle (ASM) cells could be involved in collagen turnover and migration of these cells and thus may contribute to airway remodelling.

Objective To examine the effect of pro-fibrotic growth factors TGF- β and platelet-derived growth factor (PDGF) on the expression of MMPs/TIMPs in cultured human ASM cells and to examine the role of MMP in the migration of ASM cells.

Methods ASM cells were stimulated with TGF- β and/or PDGF. Expression and activity of MMP-1, MMP-2, MMP-3, TIMP-1 and TIMP-2 were evaluated by quantitative RT-PCR, Western blot and zymography. Modified Boyden-chamber migration assay was performed to investigate the effect of secreted MMP-3 and TIMP-1 on ASM-cell migration.

Results PDGF strongly up-regulated the expression of MMP-1 at mRNA and protein levels. PDGF, when combined with TGF- β , caused synergistic up-regulation of MMP-3. TIMP-1 was additively up-regulated by TGF- β and PDGF. These growth factors had no effect on the expression of MMP-2 and TIMP-2. U0126, an extracellular signal-regulated kinase (ERK) pathway inhibitor, inhibited the up-regulation of MMP-1 by PDGF. The synergistic/additive up-regulation of MMP-3 and TIMP-1 was inhibited by U0126 and SB431542, a Smad pathway inhibitor. Supernatant from ASM cells in which MMP-3 production was knocked down by RNA interference showed a decreased migratory effect on ASM cells, whereas supernatant from cells with suppressed TIMP-1 expression resulted in increased migration.

Conclusion Our results suggest that PDGF with/without TGF- β could facilitate migration of ASM cells by modification of MMP–TIMP balance through the ERK pathway.

Keywords airway remodelling, airway smooth muscle cells, asthma, matrix metalloproteinase, platelet-derived growth factor, TGF- β

Submitted 4 September 2008; revised 31 March 2009; accepted 20 April 2009

Correspondence:

Dr Qutayba Hamid, Meakins-Christie Laboratories, Department of Medicine, McGill University, 3626 St. Urbain, Montreal, QC, H2X 2P2, Canada.

E-mail: qutayba.hamid@mcgill.ca

Cite this as: I. Ito, E. D. Fixman, K. Asai, M. Yoshida, A. S. Gounni, J. G. Martin and Q. Hamid, *Clinical & Experimental Allergy*, 2009 (39) 1370–1380.

Introduction

In the airway of asthmatics, several pathological changes have been described as characteristic features of airway remodelling [1]. The reticular basement membrane of the airway is thickened in asthmatics and abundant deposition of extracellular matrix (ECM) protein such as collagen I, III and V and fibronectin in the basement membrane has been described [2, 3]. Increased collagen deposition is also reported in and around the airway smooth muscle (ASM) bundles [4] and between the basement membrane and smooth muscle (SM) bundles [5].

Another aspect of airway remodelling is the increased volume of ASM bundles [6–8]. We and others reported that the distance between ASM muscle bundles and the reticular basement membrane was smaller in asthmatics than normal control [9, 10]. Studies of ASM migration have reported the potential involvement of growth factors [11], ECM proteins [12], cysteinyl leukotrienes [13] and chemokines [14, 15] in the process.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteinases that can degrade ECM. Synthesis and function of MMPs are regulated at the transcriptional level, by activation of precursor zymogens and by interaction with endogenous inhibitors, tissue inhibitors

of metalloproteinases (TIMPs). The role of MMP/TIMP expression in the pathogenesis of asthma has been suggested in many studies [16, 17]. However, the majority of these studies focused on their expression in epithelial cells and submucosal inflammatory cells. The principal MMPs expressed by epithelial cells are MMP-1, MMP-8 and MMP-9, whereas inflammatory cells express MMP-8, MMP-9 and MMP-12. Expression of MMPs by ASM cells has been recently reported. ASM cells express mainly MMP-1, MMP-2, MMP-3 and MMP-14 but not MMP-8 and MMP-9 [18]. To date, there are few reports on the regulation of MMP/TIMP expression in ASM cells by asthma-related cytokines [19–24].

TGF- β is a pro-fibrotic cytokine, which stimulates fibroblasts and ASM cells to synthesize ECM proteins [25]. Increased TGF- β immunoreactivity and mRNA were observed in asthmatic airways [26]. TGF- β expression correlated with disease severity, BM thickness [27] and subepithelial fibrosis [26]. The increased expression of TGF- β did not decrease after treatment with oral corticosteroids [28]. Platelet-derived growth factor (PDGF), a potent stimulant of ASM-cell migration [11, 29] and proliferation [30, 31], is another growth factor potentially involved in airway remodelling in asthma. PDGF-BB enhanced procollagen-I synthesis by cultured airway fibroblasts from patients with severe asthma [32]. Activated macrophages and eosinophils are capable of synthesizing and secreting PDGF [33].

In remodelled airway in asthma, the role of MMPs expressed by ASM cells is not well understood. Degradation of ECM by MMPs is required for tumour-cell migration and invasion [34]. In the pathogenesis of atherosclerosis, vascular SM cells migrate from media to intima to form plaques, a process which is mediated by expression of MMPs [35, 36]. Thus, it is plausible that ASM cells degrade surrounding ECM and migrate from their original bundles towards the epithelium to form new bundles [37]. In this study, we hypothesized that growth factors related to airway remodelling could modify expression of MMP/TIMP in human ASM (HASM) cells and that this modification might affect the migratory capacity of the cells.

Materials and methods

Airway smooth muscle cell culture

HASM cells were isolated from central airway segments (0.5–1.0 cm diameter) in pathologically uninvolved segments of resected lung specimens from subjects with lung cancer and were grown as described previously [38–40]. The subjects did not have atopy, asthma or chronic obstructive lung disease. ASM cells from passage fourth to seventh were used for experiments. At confluence in six-well plates, ASM cells were serum deprived for 24 h before treatment with growth factors (rh-TGF- β and/or

rh-PDGF). Recombinant human growth factors were purchased from R&D (Minneapolis, MN, USA). In inhibition experiments, an inhibitor of the signalling pathway was added 30 min before the addition of growth factors. Inhibitors [SB432542 (TGF- β type-1 receptor kinases ALK-4, 5 and 7 inhibitor), U0126 (inhibitor of MEK1/2, the upstream kinases of extracellular signal-regulated kinase (ERK)), SP600125 (c-Jun N-terminal kinase (JNK) inhibitor) and SB203580 (stress-activated protein kinase 2 (SAPK2) inhibitor, p38 pathway)] were purchased from Sigma-Aldrich (St Louis, MO, USA). These drugs were dissolved in dimethylsulphoxide (final concentration of 0.1%, v/v) and added to the medium. The same amount of the vehicle was added to control medium.

Quantitative RT-PCR

Total RNA extraction from ASM cells, cDNA synthesis, real-time PCR using a LightCycler[®] (Roche Applied Sciences, Laval, QC, Canada), and data analysis was performed as described previously [40]. Specific primer sets were designed for MMP-1, MMP-2, MMP-3, TIMP-1 and TIMP-2 from the reported sequence (GenBank accession no.: NT_033899, NT_010498, NT_033899, NT_079573 and NT_010641) (Table 1) and amplicons were confirmed by gel electrophoresis to be the expected size. Ribosomal protein S9 (S9) (BC020462) was used as an internal control (house-keeping gene) [40]. The cycling parameters were, for MMPs/TIMPs, 94 °C for 15 s, 55 °C for 20 s and 72 °C for 20 s for 50 cycles. The value obtained for each gene was standardized to the value for S9 to produce an expression ratio.

Western blot analysis

After treatment of cells with growth factor(s) for 48 h, supernatants were collected for Western blot analysis. Supernatants were concentrated 15-fold for detection of MMPs/TIMPs using Nanosep[®] Centrifugal Devices, 3 K (Pall

Table 1. Sequences of the primers used for PCR and predicted sizes of amplicons

Product	Primers	Predicted size of amplicon (bp)
MMP-1	Sense: 5'-GATGTGGAGTGCCTGATGTG-3' Antisense: 5'-ACCCTCAGAGACCTTGGTGA-3'	194
MMP-2	Sense: 5'-ATGGCAAGTACGGCTTCTGT-3' Antisense: 5'-CGGTCGTAGTCTCCTCAGTGGT-3'	172
MMP-3	Sense: 5'-GCAGTTTGCTCAGCCTATCC-3' Antisense: 5'-GAGTGTGGAGTCCAGCTTC-3'	214
TIMP-1	Sense: 5'-GGATGGACTCTTGACATCA-3' Antisense: 5'-GGGATGGATAAACAGGGAAAC-3'	138
TIMP-2	Sense: 5'-AAGATGTTCAAAGGGCTGA-3' Antisense: 5'-CTCTTCTTCTGGGTGGTGCT-3'	200

MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloproteinase.

Life Sciences, ON, Canada). Concentrated supernatants loaded to the gels were adjusted by protein amount of cell lysates of each sample. Thirty to fifty microlitre of concentrated samples were heat-denatured in SDS, reduced with β -mercaptoethanol and separated by electrophoresis on a 10% polyacrylamide gel followed by transfer to PVDF membranes (HybondTM-P, GE healthcare, Little Chalfont, UK). Membranes were blocked by 5% bovine serum albumin for 1 h at room temperature and incubated with antibodies; 1 : 1000 for MMP-1 and TIMP-1 (Novus Biologicals, Littleton, CO), 1 : 5000 for MMP-3 (Sigma-Aldrich) overnight at 4 °C and then donkey anti-rabbit IgG conjugated to horseradish peroxidase (GE healthcare) at 1 : 10 000 for 1 h at room temperature. For detection, ECL plus Western blotting Detection Reagent (GE healthcare) was used according to the manufacturer's instructions. HyperfilmTM (GE healthcare) was exposed to the chemiluminescence for 10 min, developed and digitally scanned. Molecular mass was estimated by comparison with Precision Plus ProteinTM Standards (Bio-Rad Laboratories, Hercules, CA) and densitometric quantification of the protein bands was performed with ImageJ (NIH). For standardization, cell lysates of the same preparation as the conditioned medium (CM) were collected using lysis buffer containing 1% NP-40, 1% CHAPS and one tab of protease inhibitor cocktail (Complete Mini, Roche Applied Science, Indianapolis, IN) in 10 mL of 10 mM HEPES pH 8.0. Amount of total protein from each cell lysate was determined by RC-DCTM Protein Assay kit (Bio-Rad). Detection of phosphorylated- or total-ERK in the cell lysates was performed as described before [40].

Zymography

Gelatin or casein zymography was performed to detect proteolytic activity using supernatant (15-fold concentrated) after 48 h stimulation with growth factors. Seven to ten microlitre of samples adjusted by protein amount of cell lysates were mixed with $\sim 14 \mu\text{L}$ of zymogram sample buffer (Bio-Rad) and, under non-denaturing conditions, loaded onto a 10% precast zymogram gel with gelatin (Bio-Rad) for detection of MMP-2 or 12% zymogram gel with casein (Bio-Rad) for MMP-1. After electrophoresis at a constant 20 mA for 3 h, gels are incubated in re-naturation buffer (Bio-Rad) at room temperature for 30 min followed by development in development buffer (Bio-Rad) at 37 °C overnight. Gels were stained with 0.5% Coomassie blue for 1 h and decolourized with a solution of 40% v/v methanol and 10% v/v acetic acid until bands become apparent. Images of the gels were digitally scanned.

MMP activity assay

Proteolytic activity of MMP-3 in the CM was determined by MMP-3 activity assay according to the manufacturer's instructions (GE Healthcare).

RNA interference

MMP-3 or TIMP-1 expression was inhibited by siRNA for MMP-3 (siMMP-3) or siRNA for TIMP-1 (siTIMP-1) (siGENOMETM SMARTpool reagent, Dharmacon, Lafayette, CO, USA) by transfection using LipofectamineTM (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, 1×10^5 cells in 2 mL of SmGM-2 medium without antibiotics were seeded into each well of a six-well plate and cultured to $\sim 50\%$ confluency. ASM cells were transfected with siRNA mixed with Lipofectamine in 500 μL of Opti-MEM[®] medium (Gibco, Burlington, ON, Canada). The final concentration of siRNA was 40 nM. For control preparations, cells transfected with control siRNA (siCONTROL Non-Targeting siRNA #2, Dharmacon), treated with LipofectamineTM only, or treated with Opti-MEM[®] medium only was prepared. Medium was refreshed 6 h after transfection. For preparation of samples of medium for migration assay, 5 ng/mL of TGF- β and 20 ng/mL of PDGF were added when changing medium and these CM were harvested after 48 h. Thus, CM was collected from siMMP-3 (or siTIMP-1)-treated cells (i.e. MMP-3-poor CM or TIMP-1-poor CM) or three control preparations (i.e. MMP-3-rich CM or TIMP-1-rich CM).

Migration assay

Cell migration assays were performed using a modified Boyden chamber (Neuroprobe, Cabin John, MD, USA). A polycarbonate membrane (8.0- μm pore sieve) was coated with 100 $\mu\text{g/mL}$ of type-III collagen (Sigma), which is a substrate for MMP-3 and is increased in asthmatic airway [41], overnight at 4 °C and air dried in a laminar flow hood for 1 h, and placed between upper and lower chambers. ASM cells were starved without growth factors and FBS for 24 h, harvested with trypsin (0.025%) and EDTA (0.02%) and re-suspended in CM collected after the RNA interference assay described above (8.0×10^5 cells/mL). The ASM cells were added to the upper chamber. In the lower chamber, PDGF (5 ng/mL of final concentration) was added as a chemoattractant to the same CM as the upper chamber. After 6 h of incubation at 37 °C with 5% CO₂, the membranes were peeled off. Cells on the upper face of the membrane were scraped off and the cells migrated to the lower side were fixed and stained with Diff-Quick (Dade Behring Inc., Newark, DE, USA). The number of migrated cells was counted in five random fields under $\times 200$ magnification.

MTT assay for cell survival and proliferation

HASM cells were grown in 96-well plates to $\sim 50\%$ confluency. After 24-h starvation, cells were treated with siRNA or signal pathway inhibitors with/without growth factors. After 48 h, cell survival or proliferation was

assessed by colorimetric 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazoliumbromide (MTT) assay according to the manufacturer's protocol (Millipore, Billerica, MA, USA).

Statistical analysis

Data are presented as mean $\pm$ SEM. Statistical comparisons between treatment and control were performed using Student's *t*-test or ANOVA (for time-course experiments)

followed by Bonferroni's *post hoc* test where appropriate. A value of $P < 0.05$ was considered significant.

Results

Induction of MMP-1, MMP-3 and TIMP-1 by PDGF and TGF- β

Concentrated supernatants from ASM cells after 48 h stimulation with PDGF and/or TGF- β were used for

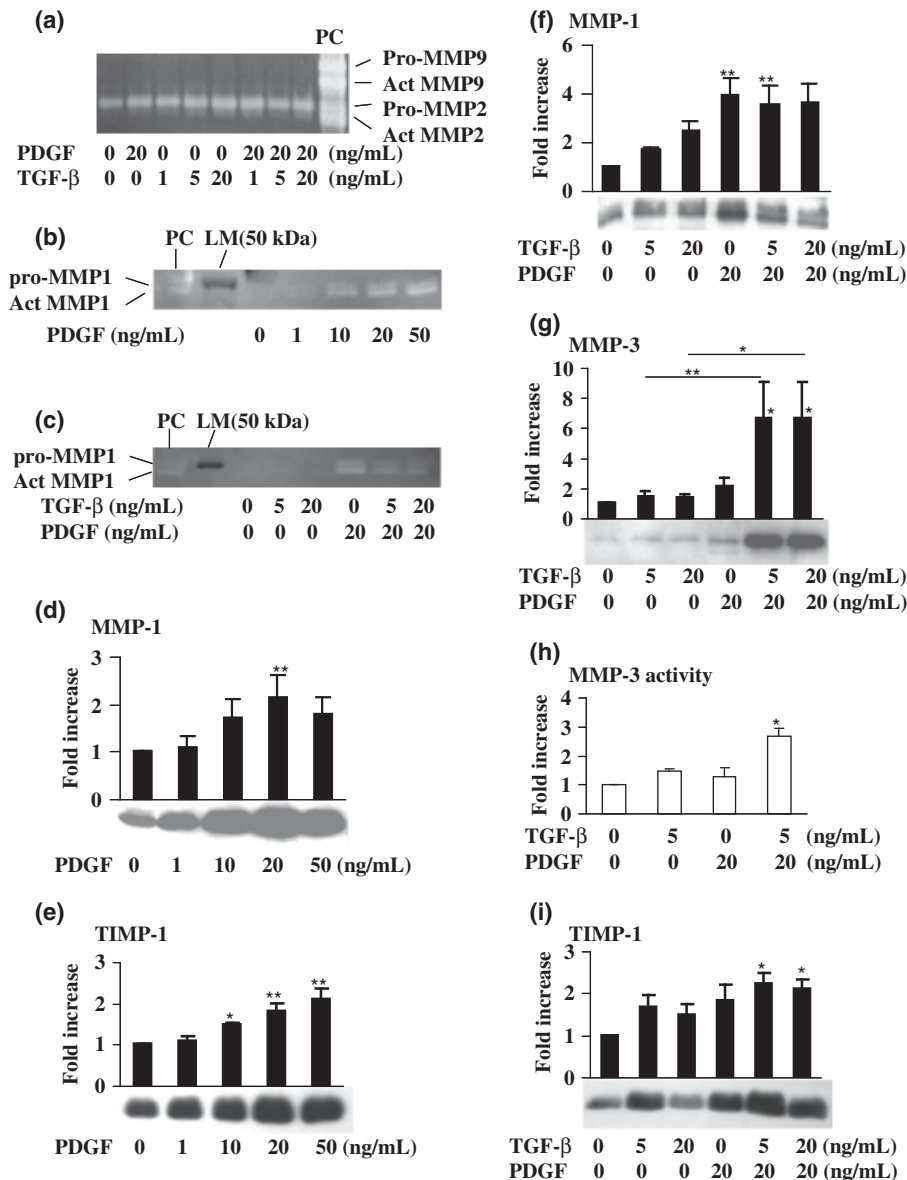


Fig. 1. Protein expression and activity of matrix metalloproteinase (MMP)/tissue inhibitors of metalloproteinase (TIMP) in conditioned media of human airway smooth muscle cells and their modification by platelet-derived growth factor (PDGF) and/or TGF- β . Photos are representatives of at least five experiments. (a) Gelatin zymography. (b and c) Casein zymography. (d, e, f, g, i) Western blot analysis. (h) MMP-3 activity determined by MMP activity assay. Graphs are means $\pm$ SEM of densitometric quantification of the bands expressed as relative values to vehicle control (without stimulation). * $P < 0.05$, ** $P < 0.01$. Comparisons are vs. vehicle control if not indicated. Note that the y-axis scales are different. PC, positive control; LM, lane marker.

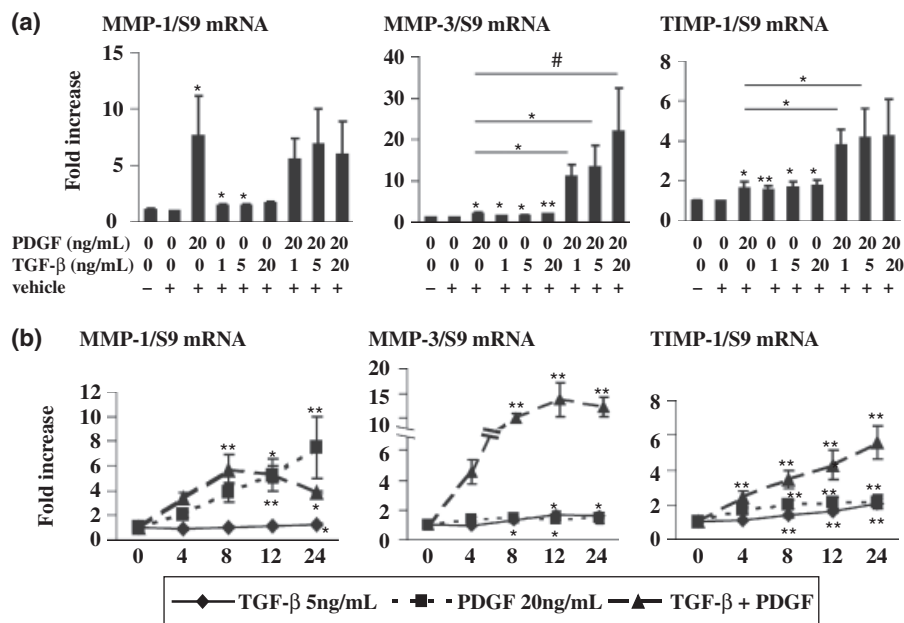


Fig. 2. Effect of growth factor(s) on mRNA expression of matrix metalloproteinase (MMP)-1, MMP-3 and tissue inhibitors of metalloproteinase (TIMP)-1 measured by quantitative RT-PCR. (a) Cells stimulated with platelet-derived growth factor (PDGF) and/or TGF- β for 24 h were quantified for mRNA of MMP-1 (left), MMP-3 (middle) and TIMP-1 (right). (b) Time course stimulation experiment with TGF- β (diamond), PDGF (square) and their combination (triangle). Values are expressed as mean $\pm$ SEM of three to five independent experiments. Value of vehicle control (without stimulation) was set as 1 and relative values were expressed. (same for Figs 5e and f) * P < 0.05, ** P < 0.01, # P = 0.05. Comparisons are vs. vehicle control if not indicated. Note that the y-axis scales are different.

zymography and Western blot analysis. Gelatin zymography showed stable expression of 72 kDa gelatinase (pro-form of MMP-2) and its 62 kDa active form (Fig. 1a). By casein zymography, a low level of MMP-1 was detected at baseline and PDGF increased the activity at a minimum concentration of 10 ng/mL (Fig. 1b). TGF- β suppressed the PDGF-induced expression of activated MMP-1 but slightly (Fig. 1c).

By Western blot analysis, PDGF induced the expression of MMP-1 (Fig. 1d) and TIMP-1 (Fig. 1e). The effect of PDGF or TGF- β alone on MMP-3 was not significant (Fig. 1g). However, when the growth factors were combined, synergistic effects on protein expression and activity of MMP-3 were demonstrated (Figs 1g and h). This synergistic effect was not seen on MMP-1 expression (Fig. 1f). The growth factors in combination also increased the expression of TIMP-1 protein, although again the response was not synergistic (Fig. 1i).

Quantitative RT-PCR

Following 24 h stimulation with the growth factors, mRNA in ASM cells was quantified by RT-PCR. The effect of PDGF (20 ng/mL) was larger on MMP-1 [7.6 ± 3.6 fold, Fig. 2a (left)] compared with effects on MMP-3 [1.9 ± 0.4 fold, Fig. 2a (middle)] and on TIMP-1 [1.6 ± 0.4 fold, Fig. 2a (right)] consistent with protein expression. The combination of PDGF and TGF- β had a synergistic effect on mRNA expression of MMP-3 [21.9 ± 10.6 fold by 20 ng/mL of

TGF- β and 20 ng/mL of PDGF, Fig. 2a (middle)], in contrast to the effect on TIMP-1 [4.2 ± 1.8 fold by 20 ng/mL of TGF- β and 20 ng/mL of PDGF, Fig. 2a (right)] and on MMP-1 [Fig. 2a (left)]. PDGF and TGF- β alone and in combination had no effect on mRNA levels of MMP-2 and TIMP-2 (data not shown). Time course experiments of mRNA expression at 4–24 h are shown in Fig. 2b.

Reduction of MMP-3 and TIMP-1 expression in ASM cells by RNA interference

To investigate the effect of MMP-3 on ASM-cell migration, we used an RNA-interference technique to block MMP-3, which was highly up-regulated by combined stimulation of PDGF and TGF- β . siMMP-3 showed 74% and 83% inhibition of MMP-3 mRNA by quantitative RT-PCR compared with control siRNA (siControl) at 24 and 48 h (Fig. 3a). Upon stimulation with a combination of PDGF and TGF- β for 24 and 48 h, mRNA of MMP-3 was up-regulated, and this up-regulation was inhibited by 82% and 80% in siMMP-3-treated cells compared with siControl-treated cells (Fig. 3b). Protein levels of MMP-3 in CM after stimulation with TGF- β and PDGF determined by Western analysis were decreased to $14 \pm 4\%$ by treatment with siMMP-3 at 48 h compared with treatment with siControl (Fig. 3c). Proliferation of the cells was not affected by siMMP-3 ($109 \pm 9\%$ (mean $\pm$ SD) in comparison with siControl, $n = 6$). To investigate the role of TIMP-1, one of the inhibitors of MMP-3, in ASM migration,

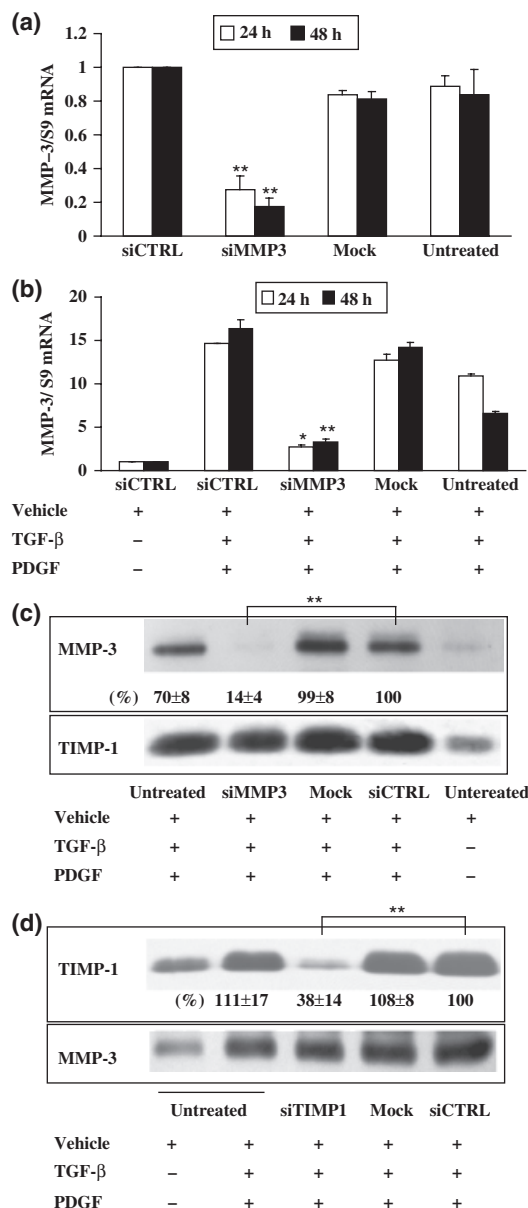


Fig. 3. Effective knockdown of matrix metalloproteinase (MMP)-3 and tissue inhibitors of metalloproteinase (TIMP)-1 by RNA interference. Cells were transfected with control siRNA (siCTRL), MMP-3 siRNA (siMMP-3), TIMP-1 siRNA (siTIMP-1) or empty vector (mock), or treated with medium only (untreated) (Same for Fig. 4). (a, b) mRNA levels of MMP-3 were quantified in un-stimulated cells (a) and in cells stimulated with TGF- β (5 ng/mL) and platelet-derived growth factor (PDGF) (20 ng/mL) (b). White bar shows 24 h stimulation and black shows 48 h. Values from cells transfected with control siRNA without stimulation of growth factors were set as 1. Western blot analysis on MMP-3 suppression by siMMP-3 (c) and on TIMP-1 suppression by siTIMP-1 (d) in supernatants of cells stimulated with TGF- β (5 ng/mL) and PDGF (20 ng/mL) for 48 h. Means $\pm$ SEM of densitometric quantification of the bands expressed as relative values (%) to siCTRL are indicated under the bands. TIMP-1 expression in (c) or MMP-3 expression in (d) on the same membrane is shown as reference to show specific inhibition of each siRNA. Photos are representative of at least five experiments. * P <0.05 and ** P <0.01 compared with siCTRL.

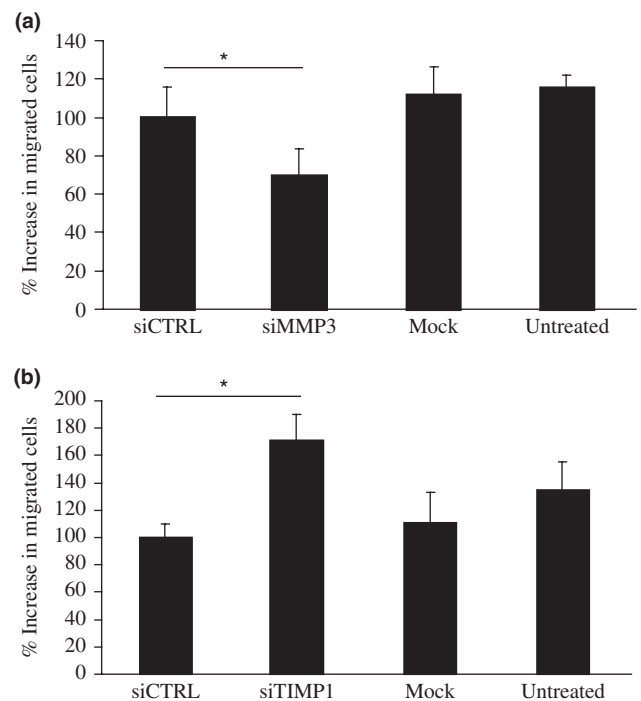


Fig. 4. Inhibition of airway smooth muscle cell migration through collagen-III-coated membrane by blocking matrix metalloproteinase (MMP)-3 (a) and tissue inhibitors of metalloproteinase (TIMP)-1 (b). Conditioned media were collected from siMMP-3-treated (a) or siTIMP-1-treated (b) cells and control preparations after 48 h stimulation with platelet-derived growth factor (PDGF) (20 ng/mL) and TGF- β (5 ng/mL). These conditioned media were added in the upper chamber with ASM cells and the lower chamber with 5 ng/mL of PDGF as a chemoattractant. Migrated cells were counted after incubation for 6 h ($\times 200$ magnification). Values are expressed as % increase (or decrease) compared with medium using control siRNA (siCTRL, defined as 100%), mean $\pm$ SD of six (a) and three (b) independent experiments. * P <0.01.

TIMP-1 expression was blocked by siTIMP-1 in TGF- β /PDGF-treated ASM cells. Protein levels of TIMP-1 in CM were suppressed to 38 $\pm$ 14% at 48 h (Fig. 3d).

MMP-3 silencing inhibits, while TIMP-1 silencing facilitates, migration through collagen-III-coated membrane

To examine whether MMP-3 secreted by ASM cells regulates migration of the cells through membrane coated with type-III collagen, migration assays were performed. In MMP-3-poor CM (medium from cells treated with siMMP-3), the number of cells that migrated was significantly smaller than MMP-3-rich CM (medium from cells treated with siControl) (Fig. 4a). This suggests that MMP-3 secreted by ASM cells has a role in facilitating migration through type-III collagen. Further, we investigated the role of TIMP-1 in ASM migration. In TIMP-1-poor CM (CM treated with siTIMP-1), the number of migrated cells increased compared with TIMP-1-rich CM (CM treated with siControl) (Fig. 4b).

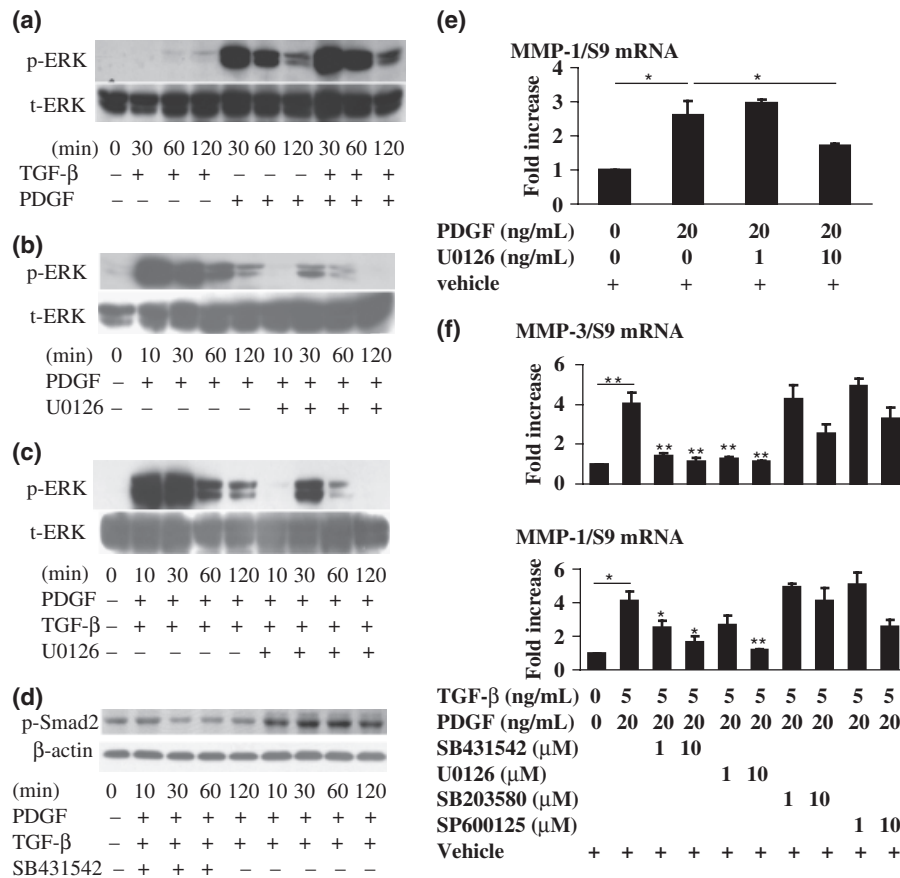


Fig. 5. (a–c) Western blot analysis of cell lysates using antibodies for phosphorylated or total extracellular signal-regulated kinase (ERK)1/2. (a) Phosphorylation of ERK in airway smooth muscle cells treated with platelet-derived growth factor (PDGF) (20 ng/mL), TGF- β (5 ng/mL) or combination of the both. Effective abrogation of phosphorylation of ERK by 10 μ M of U0126 (b and c) and phosphorylation of Smad2 by 10 μ M of SB431542 (d). (e and f) Effect of signal pathway blockers for mRNA expression of matrix metalloproteinase (MMP)-1, MMP-3 and tissue inhibitors of metalloproteinase (TIMP)-1, which were up-regulated by growth factor(s). Effects of inhibition were compared with TGF- β /PDGF-treated, non-inhibited samples. Values are expressed as means $\pm$ SEM of three experiments. * P < 0.05, ** P < 0.01. Comparisons are vs. treatment with TGF- β (5 ng/mL) plus PDGF (20 ng/mL) without inhibitor, if not indicated.

Up-regulation of MMPs and TIMPs by PDGF and TGF- β involves ERK and Smad pathways

To investigate which signal pathway regulates the up-regulation of mRNA encoding MMPs and TIMP-1 by PDGF and/or TGF- β , we tested the effects of various selective inhibitors followed by quantitative RT-PCR. Phosphorylation of ERK by PDGF with/without TGF- β was observed by Western analysis of cell lysates up to 120 min stimulation (Fig. 5a). 10 μ M of MEK1/2 inhibitor U0126 and TGF- β type-1 receptor kinases (ALK 4, 5 and 7) inhibitor SB431542 effectively inhibited phosphorylation of ERK and Smad2 by either PDGF with/without TGF- β , respectively (Figs 5b, c and d). 10 μ M of p38 inhibitor SB203580 and JNK inhibitor SP600125 were also confirmed to inhibit phosphorylation of p38 and JNK, respectively (data not shown). Neither U0126 nor SB431542 affected cell viability (data not shown). U0126 blocked

the effect of PDGF on the up-regulation of mRNA for MMP-1 (Fig. 5e) and TIMP-1 (data not shown). Other MAPK inhibitors, SB203580 and SP600125 did not affect their up-regulation by PDGF (data not shown). The up-regulation of mRNA of MMP-3 and TIMP-1 by combination of PDGF and TGF- β were inhibited by either U0126 or SB431542 (Fig. 5f). Further, we checked if phosphorylation of ERK is enhanced by combined stimulation of PDGF and TGF- β . Combination of growth factors showed only minimal increase of p-ERK compared with that induced by PDGF alone (Fig. 5a).

Discussion

In this study, we investigated expression and regulation of MMP-1, MMP-2, MMP-3, TIMP-1 and TIMP-2 in HASM cells. PDGF up-regulated MMP-1 more than MMP-3 and TIMP-1, and the combination of PDGF and TGF- β caused

synergistic up-regulatory effects on the expression of MMP-3 and an additive effect on TIMP-1. The ERK pathway appears to be a key signalling pathway in mediating the effects of PDGF and TGF- β in combination. MMP-3 produced by ASM cells stimulated with PDGF/TGF- β might play a role in facilitating migration of these cells.

In active/unstable asthma, MMPs are increased to a greater extent in bronchoalveolar lavage fluid and sputum than are the TIMPs [42, 43], whereas in chronic/stable asthma, TIMPs predominate over MMPs [44, 45]. Because the main sources of MMP-9 in the bronchial tissue are epithelial cells and inflammatory cells including eosinophils, neutrophils and macrophages [46], many studies on MMPs in asthma have focused on expression of MMPs in these cells [47, 48]. At present, studies on MMP/TIMP expression by ASM cells are quite limited and the significance of MMPs secreted by ASM cells in the context of airway remodelling is still unknown. This fact prompted us to perform this study.

MMP protein expression is regulated primarily at the transcriptional level [49]. MMP-2 is expressed by a wide variety of cells and modification of expression by cytokines or growth factors is generally minimal [22, 50–52]. In contrast, other MMPs such as MMP-1, MMP-3 and MMP-9 are responsive to cytokines/growth factors and are considered to be regulated similarly [49]. Transcription of MMP-1 and MMP-3 is induced by PDGF and, with few exceptions, reduced by TGF- β . However, in ASM cells, MMP-1 was up-regulated much more by PDGF compared with MMP-3. TGF- β , when combined with PDGF, evoked a substantial synergistic up-regulation of MMP-3 but this phenomenon was not observed for MMP-1. These findings suggest that there may be a unique mechanism of differential regulation of MMP-1 and MMP-3 by growth factors in ASM cells.

Regarding the synergistic effect between PDGF and TGF- β , one possible mechanism is induction of TGF- β by PDGF [53, 54] or induction of PDGF by TGF- β [55, 56] as shown in other types of cells as well as SM cells. But this does not seem to be pertinent to our result because, although we have used maximally effective concentrations of PDGF or TGF- β to stimulate cells, the combination of both growth factors still synergized in the induction of MMP-3 expression. One possible mechanism of the synergy would be co-operative interaction of signal pathways.

We demonstrated that inhibition of the ERK pathway abrogated the up-regulation of MMP-1 by PDGF and that the ERK and Smad pathways played an important role in the up-regulation of MMP-3 and TIMP-1 by PDGF and TGF- β . Mechanisms of signal crosstalk between Smad pathway and MAPK pathway have recently been investigated. The MEK-ERK pathway may either enhance or repress the Smad pathway in several ways. ERK-dependent phosphorylation of the linker region in R-Smad inhibited [57] or enhanced [58] nuclear translocation of

R-Smad. Phosphorylation in the linker region of Smad4 by ERK led to accumulation of Smad4 in the nucleus and resulted in a maximal transcriptional response [59]. In contrast, phosphorylation of ERK induced by EGF was suppressed by TGF- β in glioma cells [60] and pancreatic carcinoma cell lines [61], but the phosphorylation of ERK was not affected in fibroblasts [61]. In our experiment, the degree of enhancement of PDGF-induced ERK phosphorylation by TGF- β was not large enough to explain the synergistic effect on the expression of MMP-3 observed between PDGF and TGF- β (Fig. 5a). Signals downstream of ERK may be enhanced by TGF- β to increase PDGF-induced expression of MMP-3 and TIMP-1.

MMPs participate in a variety of conditions including normal cell migration (e.g. trophoblasts and osteoclasts), wound healing and diseases such as invasion of cancer cells and migration of vascular SM cells in atherosclerotic disease [34, 62]. ASM cells may potentially migrate in the airway but there are few reports on the role of MMPs in migration of the cells. Our results suggest that pro-fibrotic growth factors, which induce production of ECM by fibroblasts and ASM cells, can also induce MMPs to facilitate migration of ASM cells. In contrast, over-expression of TIMP-1 inhibited migration of vascular SM cells in animal models [63, 64], which is also consistent with our result. On the basis that the degree of up-regulation was apparently dominant in MMP-3 compared with TIMP-1 in protein levels (Figs 1g and i) and net activity of MMP-3 in CM increased to 2.70 ± 0.47 times by treatment with TGF- β /PDGF (Fig. 1h), one might expect that migration of the cells would be facilitated in environment rich in TGF- β and PDGF. It was not possible to compare TGF- β /PDGF-treated medium and non-treated medium in cell migration because the presence of these growth factors would directly influence migration of ASM cells. How the balance between MMPs and TIMPs affects migration of ASM cells is awaiting elucidation. By activating the turnover of collagens by up-regulating production of collagens and MMPs from ASM cells, these growth factors may have a role in modifying the composition of the subepithelial ECM to facilitate the process of airway remodelling. RGD sites of collagens, which are revealed after structural alteration of collagens, affect cell attachment through integrins and may have a role in modulating cell migration [65]. Moreover, by degradation of ECM, inflammatory mediators, growth factors and chemokines sequestered in ECM can be released and activated, further promoting airway remodelling. MMPs may influence ASM-cell migration by degrading ECMs into unique chemotactic fragments [66]. Thus, MMPs have several possible ways to function as modifiers of ASM-cell migration.

In conclusion, expression of MMPs and their modification by pro-fibrotic growth factors such as TGF- β and PDGF in ASM cells may contribute to SM-cell hyperplasia and the increased muscle mass in asthma. Targeting MMPs could be

a potential therapeutic tool to prevent one aspect of airway remodelling caused by SM-cell migration.

Acknowledgements

We gratefully thank Ms Sylvie Gravel and Dr Vasanthi Govindaraju for their technical assistance for the migration assay. We thank Dr Yuki Sumi for his valuable suggestions and Drs Hisako Matsumoto and Kojiro Otsuka for their preparation of ASM cells.

This project was supported by Canadian Institutes of Health Research (MOP 62821), GlaxoSmithKline Canada and the Richard and Edith Strauss Canada Foundation.

Dr Ito was supported by Fonds de la Recherche en Santé du Québec.

References

- Johnson PR. Role of human airway smooth muscle in altered extracellular matrix production in asthma. *Clin Exp Pharmacol Physiol* 2001; **28**:233–6.
- Roche WR, Beasley R, Williams JH, Holgate ST. Subepithelial fibrosis in the bronchi of asthmatics. *Lancet* 1989; **1**:520–4.
- Roche WR. Fibroblasts and asthma. *Clin Exp Allergy* 1991; **21**:545–8.
- Thomson RJ, Schellenberg RR. Increased amount of airway smooth muscle does not account for excessive bronchoconstriction in asthma. *Can Respir J* 1998; **5**:61–2.
- Li X, Wilson JW. Fibrogenic cytokines and airway fibrosis. In: Stewart AG, ed. *Airway wall remodelling in asthma*. Boca Raton, FL: CRC Press, 1997; 111–38.
- Kuwano K, Bosken CH, Pare PD, Bai TR, Wiggs BR, Hogg JC. Small airways dimensions in asthma and in chronic obstructive pulmonary disease. *Am Rev Respir Dis* 1993; **148**:1220–5.
- Dunnill MS, Masarrella GR, Anderson JA. A comparison of the quantitative anatomy of the bronchi in normal subjects, in status asthmaticus, in chronic bronchitis and in emphysema. *Thorax* 1969; **24**:176–9.
- Woodruff PG, Dolganov GM, Ferrando RE *et al*. Hyperplasia of smooth muscle in mild to moderate asthma without changes in cell size or gene expression. *Am J Respir Crit Care Med* 2004; **169**:1001–6.
- Pepe C, Foley S, Shannon J *et al*. Differences in airway remodeling between subjects with severe and moderate asthma. *J Allergy Clin Immunol* 2005; **116**:544–9.
- Benayoun L, Druilhe A, Dombret MC, Aubier M, Pretolani M. Airway structural alterations selectively associated with severe asthma. *Am J Respir Crit Care Med* 2003; **167**:1360–8.
- Hedges JC, Dechert MA, Yamboliev IA *et al*. A role for p38(MAPK)/HSP27 pathway in smooth muscle cell migration. *J Biol Chem* 1999; **274**:24211–9.
- Parameswaran K, Radford K, Zuo J, Janssen LJ, O'Byrne PM, Cox PG. Extracellular matrix regulates human airway smooth muscle cell migration. *Eur Respir J* 2004; **24**:545–51.
- Parameswaran K, Cox G, Radford K, Janssen LJ, Sehmi R, O'Byrne PM. Cysteinyl leukotrienes promote human airway smooth muscle migration. *Am J Respir Crit Care Med* 2002; **166**:738–42.
- Joubert P, Lajoie-Kadoch S, Labonte I *et al*. CCR3 expression and function in asthmatic airway smooth muscle cells. *J Immunol* 2005; **175**:2702–8.
- Govindaraju V, Michoud MC, Al-Chalabi M, Ferraro P, Powell WS, Martin JG. Interleukin-8: novel roles in human airway smooth muscle cell contraction and migration. *Am J Physiol Cell Physiol* 2006; **291**:C957–65.
- Atkinson JJ, Senior RM. Matrix metalloproteinase-9 in lung remodeling. *Am J Respir Cell Mol Biol* 2003; **28**:12–24.
- Kelly EA, Jarjour NN. Role of matrix metalloproteinases in asthma. *Curr Opin Pulm Med* 2003; **9**:28–33.
- Elshaw SR, Henderson N, Knox AJ, Watson SA, Buttle DJ, Johnson SR. Matrix metalloproteinase expression and activity in human airway smooth muscle cells. *Br J Pharmacol* 2004; **142**:1318–24.
- Silverman ES, Baron RM, Palmer LJ *et al*. Constitutive and cytokine-induced expression of the ETS transcription factor ESE-3 in the lung. *Am J Respir Cell Mol Biol* 2002; **27**:697–704.
- Johnson S, Knox A. Autocrine production of matrix metalloproteinase-2 is required for human airway smooth muscle proliferation. *Am J Physiol* 1999; **277**:L1109–17.
- Hakonarson H, Halapi E, Whelan R, Gulcher J, Stefansson K, Grunstein MM. Association between IL-1beta/TNF-alpha-induced glucocorticoid-sensitive changes in multiple gene expression and altered responsiveness in airway smooth muscle. *Am J Respir Cell Mol Biol* 2001; **25**:761–71.
- Foda HD, George S, Rollo E *et al*. Regulation of gelatinases in human airway smooth muscle cells: mechanism of progelatinase A activation. *Am J Physiol* 1999; **277**:L174–82.
- Liang KC, Lee CW, Lin WN *et al*. Interleukin-1beta induces MMP-9 expression via p42/p44 MAPK, p38 MAPK, JNK, and nuclear factor-kappaB signaling pathways in human tracheal smooth muscle cells. *J Cell Physiol* 2007; **21**:759–70.
- Xie S, Issa R, Sukkar MB *et al*. Induction and regulation of matrix metalloproteinase-12 in human airway smooth muscle cells. *Respir Res* 2005; **6**:148.
- Coutts A, Chen G, Stephens N *et al*. Release of biologically active TGF-beta from airway smooth muscle cells induces autocrine synthesis of collagen. *Am J Physiol Lung Cell Mol Physiol* 2001; **280**:L999–1008.
- Minshall EM, Leung DY, Martin RJ *et al*. Eosinophil-associated TGF-beta1 mRNA expression and airways fibrosis in bronchial asthma. *Am J Respir Cell Mol Biol* 1997; **17**:326–33.
- Chakir J, Shannon J, Molet S *et al*. Airway remodeling-associated mediators in moderate to severe asthma: effect of steroids on TGF-beta, IL-11, IL-17, and type I and type III collagen expression. *J Allergy Clin Immunol* 2003; **111**:1293–8.
- Vignola AM, Chanez P, Chiappara G *et al*. Transforming growth factor-beta expression in mucosal biopsies in asthma and chronic bronchitis. *Am J Respir Crit Care Med* 1997; **156**:591–9.
- Carlin SM, Roth M, Black JL. Urokinase potentiates PDGF-induced chemotaxis of human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2003; **284**:L1020–6.
- Hirst SJ, Barnes PJ, Twort CH. Quantifying proliferation of cultured human and rabbit airway smooth muscle cells in response to serum and platelet-derived growth factor. *Am J Respir Cell Mol Biol* 1992; **7**:574–81.

- 31 Hirst SJ, Barnes PJ, Twort CH. PDGF isoform-induced proliferation and receptor expression in human cultured airway smooth muscle cells. *Am J Physiol* 1996; **270**:L415–28.
- 32 Lewis CC, Chu HW, Westcott JY *et al.* Airway fibroblasts exhibit a synthetic phenotype in severe asthma. *J Allergy Clin Immunol* 2005; **115**:534–40.
- 33 Ohno I, Nitta Y, Yamauchi K *et al.* Eosinophils as a potential source of platelet-derived growth factor B-chain (PDGF-B) in nasal polyposis and bronchial asthma. *Am J Respir Cell Mol Biol* 1995; **13**:639–47.
- 34 Stamenkovic I. Extracellular matrix remodelling: the role of matrix metalloproteinases. *J Pathol* 2003; **200**:448–64.
- 35 Vigetti D, Moretto P, Viola M *et al.* Matrix metalloproteinase 2 and tissue inhibitors of metalloproteinases regulate human aortic smooth muscle cell migration during *in vitro* aging. *Fed Am Soc Exp Biol J* 2006; **20**:1118–30.
- 36 Chandrasekar B, Mummidi S, Mahimainathan L *et al.* Interleukin-18-induced human coronary artery smooth muscle cell migration is dependent on NF-kappaB- and AP-1-mediated matrix metalloproteinase-9 expression and is inhibited by atorvastatin. *J Biol Chem* 2006; **281**:15099–109.
- 37 Hasaneen NA, Zucker S, Cao J, Chiarelli C, Panettieri RA, Foda HD. Cyclic mechanical strain-induced proliferation and migration of human airway smooth muscle cells: role of EMMPRIN and MMPs. *Fed Am Soc Exp Biol J* 2005; **19**:1507–9.
- 38 Halayko AJ, Salari H, Ma X, Stephens NL. Markers of airway smooth muscle cell phenotype. *Am J Physiol* 1996; **270**:L1040–50.
- 39 Gounni AS, Wellemans V, Yang J *et al.* Human airway smooth muscle cells express the high affinity receptor for IgE (Fc epsilon RI): a critical role of Fc epsilon RI in human airway smooth muscle cell function. *J Immunol* 2005; **175**:2613–21.
- 40 Ito I, Laporte JD, Fiset PO *et al.* Down-regulation of a disintegrin and metalloproteinase-33 by interferon-gamma in human airway smooth muscle cells. *J Allergy Clin Immunol* 2007; **119**:89–97.
- 41 Wilson JW, Li X. The measurement of reticular basement membrane and submucosal collagen in the asthmatic airway. *Clin Exp Allergy* 1997; **27**:363–71.
- 42 Lemjabbar H, Gosset P, Lamblin C *et al.* Contribution of 92 kDa gelatinase/type IV collagenase in bronchial inflammation during status asthmaticus. *Am J Respir Crit Care Med* 1999; **159**:1298–307.
- 43 Tanaka H, Miyazaki N, Oashi K, Tanaka S, Ohmichi M, Abe S. Sputum matrix metalloproteinase-9: tissue inhibitor of metalloproteinase-1 ratio in acute asthma. *J Allergy Clin Immunol* 2000; **105**:900–5.
- 44 Vignola AM, Riccobono L, Mirabella A *et al.* Sputum metalloproteinase-9/tissue inhibitor of metalloproteinase-1 ratio correlates with airflow obstruction in asthma and chronic bronchitis. *Am J Respir Crit Care Med* 1998; **158**:1945–50.
- 45 Mautino G, Henriquet C, Jaffuel D, Bousquet J, Capony F. Tissue inhibitor of metalloproteinase-1 levels in bronchoalveolar lavage fluid from asthmatic subjects. *Am J Respir Crit Care Med* 1999; **60**:324–30.
- 46 Mautino G, Capony F, Bousquet J, Vignola AM. Balance in asthma between matrix metalloproteinases and their inhibitors. *J Allergy Clin Immunol* 1999; **104**:530–3.
- 47 Langlois A, Ferland C, Tremblay GM, Laviolette M. Montelukast regulates eosinophil protease activity through a leukotriene-independent mechanism. *J Allergy Clin Immunol* 2006; **118**:113–9.
- 48 Cho JY, Miller M, McElwain K *et al.* Remodeling associated expression of matrix metalloproteinase 9 but not tissue inhibitor of metalloproteinase 1 in airway epithelium: modulation by immunostimulatory DNA. *J Allergy Clin Immunol* 2006; **117**:618–25.
- 49 Westermarck J, Kahari VM. Regulation of matrix metalloproteinase expression in tumor invasion. *Fed Am Soc Exp Biol J* 1999; **13**:781–92.
- 50 Salo T, Lyons JG, Rahemtulla F, Birkedal-Hansen H, Larjava H. Transforming growth factor-beta 1 up-regulates type IV collagenase expression in cultured human keratinocytes. *J Biol Chem* 1991; **266**:11436–41.
- 51 Overall CM, Wrana JL, Sodek J. Transcriptional and post-transcriptional regulation of 72-kDa gelatinase/type IV collagenase by transforming growth factor-beta 1 in human fibroblasts. Comparisons with collagenase and tissue inhibitor of matrix metalloproteinase gene expression. *J Biol Chem* 1991; **266**:14064–71.
- 52 Johnson S, Knox A. Autocrine production of matrix metalloproteinase-2 is required for human airway smooth muscle proliferation. *Am J Physiol* 1999; **277**:L1109–17.
- 53 Kordes C, Brookmann S, Haussinger D, Klonowski-Stumpe H. Differential and synergistic effects of platelet-derived growth factor-BB and transforming growth factor-beta1 on activated pancreatic stellate cells. *Pancreas* 2005; **31**:156–67.
- 54 Nishishita T, Lin PC. Angiopoietin 1, PDGF-B, and TGF-beta gene regulation in endothelial cell and smooth muscle cell interaction. *J Cell Biochem* 2004; **91**:584–93.
- 55 Breitkopf K, Sawitza I, Westhoff JH, Wickert L, Dooley S, Gressner AM. Thrombospondin 1 acts as a strong promoter of transforming growth factor beta effects via two distinct mechanisms in hepatic stellate cells. *Gut* 2005; **54**:673–81.
- 56 Zhan Y, Kim S, Izumi Y *et al.* Role of JNK, p38, and ERK in platelet-derived growth factor-induced vascular proliferation, migration, and gene expression. *Arterioscler Thromb Vasc Biol* 2003; **23**:795–801.
- 57 Kretzschmar M, Doody J, Timokhina I, Massague J. A mechanism of repression of TGFbeta/Smad signaling by oncogenic Ras. *Genes Dev* 1999; **13**:804–16.
- 58 Hayashida T, Decaestecker M, Schnaper HW. Cross-talk between ERK MAP kinase and Smad signaling pathways enhances TGF-beta-dependent responses in human mesangial cells. *Fed Am Soc Exp Biol J* 2003; **17**:1576–8.
- 59 Roelen BA, Cohen OS, Raychowdhury MK *et al.* Phosphorylation of threonine 276 in Smad4 is involved in transforming growth factor-beta-induced nuclear accumulation. *Am J Physiol Cell Physiol* 2003; **285**:C823–30.
- 60 Held-Feindt J, Lutjohann B, Ungefroren H, Mehdorn HM, Mentlein R. Interaction of transforming growth factor-beta (TGF-beta) and epidermal growth factor (EGF) in human glioma cells. *J Neurooncol* 2003; **63**:117–27.
- 61 Giehl K, Seidel B, Gierschik P, Adler G, Menke A. TGFbeta1 represses proliferation of pancreatic carcinoma cells which correlates with Smad4-independent inhibition of ERK activation. *Oncogene* 2000; **19**:4531–41.
- 62 Kuzuya M, Iguchi A. Role of matrix metalloproteinases in vascular remodeling. *J Atheroscler Thromb* 2003; **10**:275–82.

- 63 Forough R, Koyama N, Hasenstab D, Lea H, Clowes M, Nikkari ST. Overexpression of tissue inhibitor of matrix metalloproteinase-1 inhibits vascular smooth muscle cell functions *in vitro* and *in vivo*. *Circ Res* 1996; **79**:812–20.
- 64 Dollery CM, Humphries SE, McClelland A, Latchman DS, McEwan JR. Expression of tissue inhibitor of matrix metalloproteinases 1 by use of an adenoviral vector inhibits smooth muscle cell migration and reduces neointimal hyperplasia in the rat model of vascular balloon injury. *Circulation* 1999; **99**: 3199–205.
- 65 Hersel U, Dahmen C, Kessler H. RGD modified polymers: biomaterials for stimulated cell adhesion and beyond. *Biomaterials* 2003; **24**:4385–415.
- 66 Weathington NM, van Houwelingen AH, Noerager BD *et al*. A novel peptide CXCR ligand derived from extracellular matrix degradation during airway inflammation. *Nat Med* 2006; **12**:317–23.