Major role of the extracellular matrix in airway smooth muscle phenotype plasticity

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Signalling pathways of collagen I-induced airway smooth muscle phenotype modulation

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Abstract

Increased extracellular matrix (ECM) deposition and airway smooth muscle (ASM) mass are major contributors to airway remodelling in asthma. Increased expression of the ECM protein collagen I has been observed surrounding asthmatic ASM as well. Recently, we have demonstrated that collagen I induces a proliferative, hypocontractile ASM phenotype. Little is known, however, on the signalling pathways involved. Using bovine tracheal smooth muscle (BTSM), we now investigated the role of focal adhesion kinase (FAK) and downstream signalling pathways in collagen I-induced phenotype modulation. Phosphorylation of FAK was increased during adhesion to plastic or collagen I, without differences between these matrices. No differences between cellular adhesion were found either. Inhibition of FAK activity, by overexpression of the FAK deletion mutants FAT (focal adhesion targeting domain) and FRNK (FAK-related non-kinase), attenuated adhesion. After attachment, FAK phosphorylation was time-dependently increased in cells cultured on a collagen I matrix, whereas no activation was found on an uncoated plastic matrix. In addition, collagen I time- and concentration-dependently increased BTSM cell proliferation, which was inhibited by FAT and FRNK. In the presence of specific pharmacological inhibitors of p38 MAPK (SB203580) and Src-kinase (PP2) collagen I-induced proliferation was fully inhibited, while partial inhibition was observed by inhibition of PI3-kinase (LY294002) and MEK (U0126). The inhibition of cell proliferation by the inhibitors was associated with attenuation of the collagen I-induced hypocontractility. Collectively, the results indicate that induction of a proliferative, hypocontractile ASM phenotype by collagen I involves p38 MAPK, MEK, PI3-kinase and Src-mediated signalling pathways downstream of FAK.

Introduction

Airway hyperresponsiveness (AHR), persistent airway obstruction and decline in lung function are characteristic features of chronic asthma [1]. Airway remodelling, characterized by structural changes in the airway wall architecture, including increased airway smooth muscle (ASM) mass and altered deposition of extracellular matrix (ECM) proteins, is considered to contribute to these features [1,2]. Increased ASM mass may comprise hyperplasia as well as hypertrophy of ASM cells [3]. ASM cells may contribute to ongoing airway remodelling as they display phenotype plasticity and retain the ability to re-enter the cell-cycle [4]. Thus, exposure of ASM to mitogenic stimuli results in the induction of a proliferative phenotype, characterized by increased proliferation and decreased contractile function [5,6] (Chapter 3). Phenotype plasticity is a reversible process and removal of mitogenic stimuli, for example by serum
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deprivation in the presence of insulin, results in the reintroduction of a (hyper)contractile ASM phenotype [7,8] (Chapter 5).

From biopsy studies, it has become apparent that ECM deposition, including collagens, fibronectin and laminin α2β2, is increased beneath the epithelial basement membrane of asthmatics [9,10]. In patients with asthma, the total amount of ECM in the microenvironment of the ASM is increased as well [11], and may involve increased deposition of collagen I, fibronectin, hyaluronan, versican, biglycan, lumican and elastic fibres [12-14]. The ECM surrounding the ASM cell plays a key role in determining physical and mechanical properties. In addition, ECM proteins have the capacity to affect ASM phenotype. Thus, growth factor-induced phenotype switching of ASM cells is inhibited by culturing the cells on laminin-111 resulting in the maintenance of a contractile ASM phenotype [15] (Chapter 3). Conversely, culturing of ASM cells on monomeric collagen type I or fibronectin enhances growth factor-induced proliferation as well as growth factor-induced reductions of contractile marker proteins [16] (Chapter 3). Moreover, collagen I and fibronectin induce a hypocontractile, proliferative ASM phenotype in bovine tracheal smooth muscle (BTSM) strips by themselves [17] (Chapter 3). ASM cells obtained from asthmatics produce more collagen I and fibronectin compared to cells from healthy subjects [18,19]. In addition, nonasthmatic ASM cells cultured on an ECM laid down by asthmatic ASM cells proliferate more rapidly and vice versa [18], suggesting that changes in the ECM profile may contribute to enhanced asthmatic ASM growth in situ.

Integrins consist of a group of heterodimers linking the ECM to the intracellular compartment [20]. The collagen-binding integrin α2β1 is the main integrin involved in collagen I-induced ASM cell attachment, increased ASM cell proliferation and cytokine production by these cells, and glucocorticosteroid resistance [16,21]. In addition, the fibronectin-binding integrins α4β1 and α5β1 appeared important in the enhancement of PDGF-induced proliferation by collagen I, whereas the fibronectin-binding integrin αvβ3 was also required for attachment to collagen I [16]. Recently, we have demonstrated that the α5β1 integrin is also of major importance in collagen I-induced increases of basal proliferation (Chapter 6).

No information is yet available on the signalling pathways of ECM-integrin interactions in ASM cells. From other cell types it is known that most integrins activate focal adhesion kinase (FAK), which results in autophosphorylation at Tyr397 and generates a binding site for Src, which then phosphorylates a number of other tyrosine residues on FAK [20,22,23]. FAK may subsequently activate downstream signalling cascades, including the PI3-kinase and MAPK pathways [20], which are importantly involved in ASM proliferation [24].

The aim of the present study was to explore the role of FAK and downstream signalling pathways in collagen I-induced ASM phenotype switching. Using BTSM cells, we examined the effects of collagen I on FAK phosphorylation during adhesion and proliferation. The role of FAK in these
processes was assessed by overexpression of FAK and of the FAK deletion mutants FAT (derived from the focal adhesion targeting (FAT) domain of FAK) and FRNK (FAK-related non-kinase). FRNK is a alternative transcript of FAK, which contains the C-terminal domain but not the kinase domain [23,25]. FAT is a region present in both FAK and FRNK which is necessary for targeting of FAK to focal adhesion sites [26]. Both proteins inhibit FAK localization to the focal adhesions and FAK activation [25,26]. In addition, by pharmacological inhibition of Src, mitogen activated protein kinase kinase (MEK), PI3-kinase and p38 mitogen activated protein kinase (MAPK), we investigated the contribution of these pathways to collagen I-induced BTSM proliferation and hypocontractility.

Materials and methods

Tissue preparation and organ-culture procedure.

BTSM strips were prepared as described (Chapter 3). Tissue strips were washed in Medium Zero (sterile DMEM, supplemented with sodium pyruvate (1 mM), non-essential amino-acid mixture (1:100), gentamicin (45 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (1.5 μg/ml), apo-transferrin (5 μg/ml, human) and ascorbic acid (0.1 mM)) and transferred into suspension culture flasks. Strips were maintained in culture in an Innova 4000 incubator shaker (37°C, 55 rpm) for 4 days. When applied, collagen type I (50 μg/ml), PP2 (10 μM), U0126 (3 μM), LY294002 (10 μM) and/or SB203580 (10 μM) were present during the entire incubation period.

Isometric tension measurements.

Isometric tension measurements were performed as described (Chapter 3). In short, BTSM strips were washed with Krebs Henseleit (KH) buffer (composition (mM): NaCl 117.5, KCl 5.60, MgSO\textsubscript{4} 1.18, CaCl\textsubscript{2} 2.50, NaH\textsubscript{2}PO\textsubscript{4} 1.28, NaHCO\textsubscript{3} 25.00 and glucose 5.50, pregassed with 5% CO\textsubscript{2} and 95% O\textsubscript{2}; pH 7.4 at 37°C). Subsequently, strips were mounted for isometric recording in organ baths. During a 90-min equilibration period resting tension was gradually adjusted to 3 g. Subsequently, BTSM strips were precontracted with 20 and 40 mM KCl solutions. Following washout, maximal relaxation was established by the addition of (-)-isoproterenol (0.1 μM; Sigma). Tension was readjusted to 3 g and after another equilibration period of 30 min cumulative concentration response curves were constructed to methacholine. When maximal tension was reached, the strips were washed and maximal relaxation was established using isoproterenol (10 μM).

Isolation of bovine tracheal smooth muscle cells.

BTSM cells were isolated as described (Chapter 3). In short, tracheal smooth muscle was chopped, tissue fragments were washed in Medium Plus (DMEM supplemented with sodium pyruvate (1 mM), non-essential amino-acid mixture
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(1:100), gentamicin (45 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (1.5 μg/ml) and FBS (0.5%). Enzymatic digestion was performed in Medium Plus, supplemented with collagenase P (0.75 mg/ml), papain (1 mg/ml) and soybean trypsin inhibitor (1 mg/ml). The suspension was incubated in an incubator shaker (Innova 4000) at 37°C, 55 rpm for 20 min, followed by a 10-min period of shaking at 70 rpm. After filtration of the obtained suspension over a 50 μm gauze, the cells were washed in Medium Plus, supplemented with 10% FBS. For all protocols, cells were used in passage 1-2.

Transfection of BTSM cells with GFP expression vectors

BTSM cells were plated at a density of 30,000 cells/well in 24-well culture plates and allowed to attach overnight, or grown to 95% confluency in 100 mm culture dishes. Subsequently, cells were washed twice with phosphate-buffered saline (PBS). Transfections in 24-well culture plates were performed using a mixture of 2 μl lipofectamine 2000 and 0.1 μg expression vector (GFP or GFP-FAK) or 0.8 μg expression vector (GFP, GFP-FAT or GFP-FRNK) for 6 h in 120 μl plain DMEM without serum and antibiotics. For transfections in the 100 mm dishes, a mixture of 60 μl lipofectamine 2000 and 3 μg or 24 μg of GFP expression vector, respectively, in 3.6 ml of DMEM were used. After 6 h, cells were washed twice with PBS and medium was replaced by DMEM Zero supplemented with 0.1% FBS. Preliminary results indicated that transfection efficiency for GFP reached 30 ± 4% (n=3).

Cell adhesion assay

Collagen-coated (50 μg/ml) culture plates were prepared as described (Chapter 3). The method for measurement of cell adhesion was adapted from [27]. Untransfected or GFP-, GFP-FAK-, GFP-FAT- or GFP-FRNK-transfected BTSM cells were harvested from 100 mm dishes by trypsinization. Cells were washed, resuspended in Medium Plus and transferred into uncoated or collagen-coated 24-well culture plates at a density of 50,000 cells/well and placed back in the incubator. At varying time intervals, plates were removed from the incubator and overlying medium was removed by gentle aspiration. After washing with 0.5 ml PBS at 37 °C, cells were fixed with 70% ethanol for 15 minutes at 4 °C. Subsequently, the plates were air dried for at least 30 min at 37 °C and stained for 25 minutes at room temperature using 0.1% crystal violet in water (0.3 ml/well). Cells were rinsed briefly with water and air dried. The stain was solubilized at room temperature using 10% acetic acid in water (0.5 ml/well) and quantified by colorimetric analysis (550 nm, Biorad 680 plate reader).

Western analysis

For the measurement of the phosphorylation of FAK, BTSM cells were cultured on uncoated or collagen I (50 μg/ml)-coated surfaces for varying periods of time. Cells were lysed in homogenization buffer (composition in mM: Tris-HCl 50 mM, NaCl 150.0, EDTA 1.0, PMSF 1.0, Na3VO4 1.0, NaF 1.0, pH 7.4, supplemented
with leupeptin 10 μg/ml, aprotinin 10 μg/ml, pepstatin 10 μg/ml, Na-deoxycholate 0.25 % and Igepal 1% (NP-40)). Equal amounts of protein were subjected to electrophoresis and transferred onto PVDF membranes. Membranes were subsequently blocked in blocking buffer (composition: Tris-HCl 50.0 mM; NaCl 150.0 mM; Tween-20 0.1%, dried milk powder 5% (FAK) or BSA 5% (pFAK)) for 60 min at room temperature. Next, membranes were incubated overnight at 4 °C with primary antibodies (anti-FAK 1:2000 and anti-pFAK 1:1000, dilutions in blocking buffer containing BSA 5% or 3% BSA, respectively). After three washes with TBS-Tween 20 (TBST 0.1%, containing Tris-HCl 50.0 mM, NaCl 150.0 mM and Tween 20 0.1%) of 10 min each, membranes were incubated with horseradish peroxidase-labelled secondary anti-rabbit antibodies (dilution 1:2000 in blocking buffer containing 5% or 3% BSA, respectively) at room temperature for 90 min, followed by another three washes with TBST 0.1%. Antibodies were then visualized on film using enhanced chemiluminescence reagents and analyzed by densitometry (Totallab™).

Alamar blue proliferation assay
BTSM cells were plated on uncoated or collagen I (1-100 μg/ml)-coated 24-well culture plates at a density of 30,000 cells/well and were allowed to attach overnight in Medium Plus, containing 10% FBS. The next day, cells were washed twice with PBS and made quiescent by incubation in Medium Zero, supplemented with 0.1% FBS for 3 days. Cells were then incubated with or without PDGF-AB (10 ng/ml) for 4 days in Medium Zero. Thereafter, cells were washed two times with PBS and incubated with HBSS containing 5% (vol/vol) Alamar blue solution. Conversion of Alamar blue into its reduced form by mitochondrial cytochromes was quantified by fluorimetric analysis, as indicated by the manufacturer. When applied PP2 (10 μM), U0126 (3 μM), LY294002 (10 μM) or SB203580 (10 μM) were present during the entire incubation period. For overexpression of GFP, GFP-FAK, GFP-FAT or GFP-FRNK, BTSM cells were transfected with the vectors after attachment, and subsequently cells were made quiescent as described above.

[^3]H]-thymidine-incorporation
[^3]H]-Thymidine-incorporation was performed as described previously (Chapter 3 and 5). BTSM-cells were plated on uncoated or collagen I-coated 24-well culture plates at a density of 30,000 cells/well and allowed to attach overnight in Medium Plus. The next day, cells were transfected with the GFP, GFP-FAK, GFP-FAT or GFP-FRNK, washed with PBS and made quiescent by incubation in Medium Zero, supplemented with 1 μM insulin for 72 h. Subsequently, cells were washed and incubated in the absence or presence of PDGF (10 ng/ml) in Medium Zero for 28 h, the last 24 h in the presence of [methyl-[^3]H]-thymidine (0.25 μCi/ml). After incubation, the cells were washed with PBS at room temperature. Subsequently, the cells were treated with ice-cold 5% trichloroacetic acid on ice for 30 min, and the acid-insoluble fraction was...
dissolved in NaOH (1 M). Incorporated [\(^{3}\text{H}\)]-thymidine was quantified by liquid-scintillation counting using a Beckman LS1701 β-counter.

**Materials**

Dulbecco’s modification of Eagle’s medium (DMEM), FBS, sodium pyruvate solution, non-essential amino acid mixture, gentamicin solution, penicillin/streptomycin solution and amphotericin B solution (Fungizone) were obtained from Gibco BRL Life Technologies (Paisley, U.K.). Bovine serum albumin, apo-transferrin (human), leupeptin, aprotinin, pepstatin, soybean trypsin inhibitor, insulin (bovine pancreas) and (-)-isoproterenol hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Platelet-derived growth factor (human, PDGF-AB) was from Bachem (Weil am Rhein, Germany). Methacholine was obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.). Anti-FAK was from Cell Signalling (Boston, MA, USA). Anti-FAK [pY397] and Alamar blue were from Biosource (Camarillo, CA, USA). Collagenase P and papain were from Boehringer (Mannheim, Germany). Monomeric collagen type I (calf skin) was from Fluka (Buchs, Switzerland). Lipofectamine was from Invitrogen (Paisley, UK). L(+)-ascorbic acid was from Merck (Darmstadt, Germany). SB203580 (4-[5-(4-Fluorophenyl)-2-[4-methylsulphonyl]phenyl]-1H-imidazol-4-yl)pyridine), LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), U0126 (1,4-diamino-2,3-dicyano-1,4-bis [2-aminophenylthio]butadiene) and PP2 (4-Amino-5-(4-chlorophenyl)-7-[(R)-butyl]pyrazolo[3,4-d]pyrimidine) were obtained from Tocris Cookson (Bristol, UK). pEGFP expression plasmids (Clontech) encoding FAK, and the FAK deletion mutants FAT and FRNK coupled to green fluorescent protein (GFP) were kindly provided by Dr. B. van de Water and Dr. S.E. Le Dévédec from the Division of Toxicology, Leiden Amsterdam Center for Drug Research [28,29]. All used chemicals were of analytical grade.

**Data analysis**

Data represent means ± SEM, from \(n\) separate experiments. Statistical significance of differences was evaluated by the Student’s t-test for repeated measurements. Differences were considered to be statistically significant when \(P<0.05\).
Results

Role of focal adhesion kinase in bovine tracheal smooth muscle cell adhesion.

To investigate the effects of collagen I on ASM cell adhesion, BTSM cells were removed from the culture dish by trypsinization and replated onto uncoated plastic or collagen I (50 μg/ml)-coated culture plates. BTSM cells adhered to both substrates within 8 hours, without differences between plastic or collagen I (Figure 1A). To assess changes in FAK activation during adhesion, BTSM cells were plated and at 1, 2, 4, 6 and 24 h non-adhered cells were removed, adhered cells were lysed and FAK phosphorylation was determined. A significant increase (P<0.05) in FAK phosphorylation was observed in the cells adhered to plastic and collagen I compared to cells in suspension (Figure 1B). No differences in FAK phosphorylation were observed between both substrates. No FAK phosphorylation was observed in the non-adhered cells (not shown).

Figure 1: Focal adhesion kinase is activated during BTSM cell adhesion to plastic and collagen I. (A) Adhesion of BTSM cells to plastic and collagen I. BTSM cells were allowed to adhere either directly onto the plastic surface of culture plates or to collagen I (50 μg/ml)-coated surfaces. Data represent means ± SEM of 4 experiments performed in triplicate. (B) Focal adhesion kinase is activated during adhesion, without differences between plastic and collagen I matrices. Data are expressed as percentage of the maximal FAK phosphorylation observed between 1-24 h. Data represent means ± SEM of 4 experiments after densitometric analysis. Representative immunoblots of
Y397 pFAK (upper panel) and total FAK (lower panel) are shown. *P<0.05, **P<0.01 compared to cells in suspension.

To investigate the role of FAK in ASM cell adhesion, BTSM cells were transfected with GFP expression vectors encoding GFP (control), GFP-FAK or the FAK deletion mutants GFP-FAT and GFP-FRNK. In successfully transfected cells, expression of GFP-FAK, GFP-FAT and GFP-FRNK was detected in the focal adhesion sites (Figure 2), which is in correspondence with previous findings in rabbit primary synovial fibroblasts [29]. By contrast, expression of GFP was observed diffusely throughout the cytoplasm. Cells expressing GFP, GFP-FAT and GFP-FRNK remained elongated, whereas cells overexpressing GFP-FAK showed increased cell surface areas.

Figure 2: Cellular expression of GFP fusion proteins in BTSM cells transfected with expression vectors encoding for GFP (control, upper left panel), GFP-FAK (upper right panel) and the FAK deletion mutants GFP-FAT (lower left panel) and GFP-FRNK (lower right panel).

To assess whether FAK activation was required for ASM cell adhesion, BTSM cells were transfected with the expression vectors, trypsinized and replated onto plastic. No effects of overexpression of GFP-FAK on cell adhesion were observed, whereas overexpression of GFP-FRNK or GFP-FAT significantly reduced cell adhesion (Figure 3). Cell adhesion was maximally reduced at t=24
h. reaching 62±8% (P<0.01) in GFP-FRNK transfected cells and 67±12% (P<0.05) in GFP-FAT transfected cells compared to GFP transfected cells.

**Figure 3:** Focal adhesion kinase is required for BTSM cell adhesion. (A) Overexpression of GFP-FAK did not affect BTSM cell adhesion. BTSM cells were cultured in 100 mm dishes and transfected with expression constructs for GFP (control) or GFP-FAK. After 6 hours, medium was replaced by Medium Zero + 0.1% FBS and cells were allowed to express fusion proteins for 3 days, after which cells were trypsinized and replated onto plastic. Data represent means ± SEM of 5 experiments each performed in triplicate. (B) Overexpression of the FAK deletion mutants GFP-FAT and GFP-FRNK decreases BTSM cell adhesion to plastic. Data represent means ± SEM of 5 experiments, each performed in triplicate. *P<0.05, **P<0.01 compared to GFP controls.

**Role of focal adhesion kinase in bovine tracheal smooth muscle cell proliferation**

Culturing of BTSM cells on collagen I, concentration-dependently increased cell number (P<0.05, Figure 4A). The concentration of collagen required for 50% increase (EC_{50}) in cell number was 14.1±1.8 μg/ml. Collagen I-induced increases in BTSM proliferation were also time-dependent, reaching 153±12% at day 4 (P<0.01, Figure 4B). To assess whether increases in cell number were associated with FAK activation, BTSM cells were cultured on uncoated plastic or collagen I (50 μg/ml) and cells were lysed after 1, 2, 3 or 4 days of culture and analyzed for FAK phosphorylation. Culturing on collagen I increased FAK phosphorylation, at days 2, 3 and 4 (P<0.05, Figure 4C). In correspondence with the findings for FAK phosphorylation during cell adhesion, no significant collagen I-induced phosphorylation was observed after 1 day.
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Figure 4: Collagen I increases BTSM proliferation and FAK phosphorylation in a concentration- and time-dependent fashion. (A) BTSM cell number after culturing for 4 days on increasing concentrations of collagen I (0-100 μg/ml). Data represent means ± SEM of 4 experiments performed in triplicate. (B) BTSM cell number after culturing on uncoated plastic or collagen I (50 μg/ml) for 2 or 4 days. Data represent means ± SEM of 6 experiments performed in triplicate. (C) Phosphorylation of FAK is time-dependently increased by culturing on collagen I (50 μg/ml), but not on uncoated plastic. Data represent means ± SEM of 4 experiments after densitometric analysis. Representative immunoblots of Y397 pFAK (upper panel) and total FAK (lower panel) are shown. *P<0.05, **P<0.01, ***P<0.001 compared to cells cultured on plastic.

To investigate the role of FAK in BTSM cell proliferation, cells were plated on plastic or collagen I (50 μg/ml), allowed to attach overnight and transfected with GFP, GFP-FAK, GFP-FAT and GFP-FRNK. Subsequently, cells were serum deprived and stimulated with vehicle or PDGF-AB (10 ng/ml) for 4 days. Although not statistically significant, overexpression of GFP-FAK tended to decrease proliferation induced by both PDGF and collagen I (Figure 5A). Moreover, no effects of GFP-FAK overexpression were observed on DNA synthesis induced by PDGF, collagen I or the combination of both (Figure 5B). Overexpression of GFP-FAT or GFP-FRNK fully inhibited the increase in BTSM cell number induced by collagen I, whereas no significant effects of the inhibitory proteins were observed on PDGF-induced proliferation (Figure 5C). PDGF-induced proliferation on collagen I was normalized to the level observed for PDGF-induced proliferation in cells cultured on plastic. Similar effects were observed for GFP-FAT and GFP-FRNK on DNA synthesis (Figure 5D).
Figure 5: Activation of FAK is required for collagen I-induced BTSM cell proliferation. (A) Overexpression of FAK tends to decrease BTSM cell number. BTSM cells were plated in 24-well culture plates and allowed to adhere. After adhesion, cells were transfected with GFP or GFP-FAK and subsequently serum deprived in Medium Zero + 0.1% FBS for 3 days. Cells were stimulated with or without PDGF-AB (10 ng/ml) for 4 days and cell number was assessed. (B) No effects of GFP-FAK overexpression were observed on DNA synthesis in BTSM cells. (C) Overexpression of GFP-FAT and GFP-FRNK decreases collagen I-induced increases in BTSM cell number. (D) Overexpression of GFP-FAT and GFP-FRNK decreased collagen I-induced DNA synthesis. Data represent means ± SEM of 7 experiments performed in triplicate. *P<0.05, **P<0.01, ***P<0.001 compared to GFP-transfected control cells cultured on uncoated plastic. *P<0.05, **P<0.01 compared to GFP-transfected cells grown on collagen I and/or stimulated with PDGF.
Role of Src, MEK, PI3-kinase and p38 MAPK in the induction of a proliferative, hypocontractile ASM phenotype by collagen I.

To determine the contribution of downstream signalling pathways of FAK in the induction of a proliferative, hypocontractile ASM phenotype by collagen I, BTSM cells were cultured on plastic or collagen I in the absence and presence of specific pharmacological inhibitors of Src (PP2, 10 μM), MEK (U0126, 3 μM), PI3-kinase (LY294002, 10 μM) and p38 MAPK (SB203580, 10 μM). Collagen I-induced proliferation was inhibited by all inhibitors investigated, while basal cell numbers were significantly decreased by U0126 and significantly increased by SB203580 (Figure 6A). To investigate whether these pathways were involved in collagen I-induced hypocontractility as well, BTSM strips were cultured in the absence and presence of collagen I (50 μg/ml) and the inhibitors for 4 days. As observed for proliferation, collagen I-induced hypocontractility was normalized by all inhibitors investigated (Figure 6B, Table 1). The sensitivity (pEC₅₀) for methacholine was unaffected by all treatments.

Figure 6: Src-kinase, MEK, PI3-kinase and p38 MAPK are required for collagen I-induced BTSM cell proliferation and hypocontractility. (A) Effects of pharmacological inhibitors of Src-kinase (PP2, 10 μM), MEK (U0126, 3 μM), PI3-kinase (LY294002, 10 μM) and p38 MAPK (SB203580, 10 μM) on basal and collagen I (50 μg/ml)-induced changes in cell number. Data represent means ± SEM of 6 experiments performed in triplicate. *P<0.05, **P<0.01 compared to untreated control cells cultured on plastic. #P<0.05, ##P<0.01 compared to cells grown on collagen I in the absence of inhibitors. (B) Effects of the inhibitors on collagen I-induced hypocontractility. Data represent means ± SEM of 5-10 experiments performed in duplicate. *P<0.05, **P<0.01, ***P<0.001 compared to vehicle-treated control. #P<0.05, ##P<0.01 compared to collagen I-treated control.
Table 1: Contractile responses of BTSM strips to methacholine after 4 days of culturing in the absence or presence of collagen I (50 μg/ml), with or without the pharmacological inhibitors of Src (PP2, 10 μM), MEK (U0126, 3 μM), PI3-kinase (LY294002, 10 μM) or p38 MAPK (SB203580, 10 μM)

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<tr>
<td>SB203580</td>
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Data represent means ± SEM of 5-10 independent experiments, each performed in duplicate. Abbreviations: $E_{\text{max}}$: maximal contraction; EC50: contraction of agonist eliciting half-maximal response; pEC50: negative logarithm of the EC50 value. ***P<0.01 compared to vehicle-treated control. $^a$P<0.05 compared to collagen I-treated control (vehicle).

Discussion

In the current study, we demonstrate for the first time that the induction of a proliferative ASM phenotype by collagen I is dependent on the activation of FAK and downstream signalling pathways. The results indicate that FAK is activated during BTSM cell adhesion and that overexpression of two FAK deletion mutants FAT and FRNK, which compete with endogenous FAK for localization to the focal adhesions, inhibited cell adhesion. Moreover, FAK was activated during and required for collagen I-induced BTSM cell proliferation. Pharmacological inhibition of Src, MEK, PI3-kinase and p38 MAPK signalling pathways, which may be activated downstream of FAK, normalized the induction of a proliferative and hypocontractile phenotype induced by collagen I.

AHR is a characteristic feature of asthma and is defined by an exaggerated airway narrowing in response to either direct (histamine, methacholine) or indirect (exercise, cold air, hyperventilation) stimuli [30]. Variable AHR is observed after allergen exposure and is considered to reflect airway inflammation, whereas persistent AHR is considered to relate to structural changes in the airway wall, collectively termed airway remodelling [31,32]. Increased ASM mass, as a feature of airway remodelling, is considered to be the most important factor contributing to AHR and decline in lung function in asthmatics [3,33,34]. Previously, we and others have shown that changes in the ECM environment surrounding the ASM may contribute to ASM accumulation [17,18](Chapters 3 and 5). Thus, culturing of ASM on collagen I matrices increased proliferative responses and decreased contractile function, indicating that collagen I modulates the ASM phenotype to a proliferative,
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hypocontractile phenotype [17](Chapter 3) . Little is known, however, on the signalling pathways involved in this process. In the current study, it was found that culturing of BTSM cells on collagen I time-dependently increased phosphorylation of FAK, a cytoplasmic protein tyrosine kinase activated by most integrins [20]. Activation of FAK was found to be important in the BTSM cell proliferation as overexpression of FAT and FRNK, which inhibit FAK translocation to the focal adhesions and subsequent activation of the enzyme [25,26], also attenuated collagen I-induced proliferation. Activation of FAK was observed during adhesion of BTSM cells to uncoated plastic and collagen I matrices as well, without differences between the two matrices. FAK activation was also required for BTSM cell adhesion. The effects of overexpression of FAT and FRNK on collagen I-induced changes in BTSM cell number as mentioned above are unlikely to be due to changes in cell adhesion, as overexpression of these proteins only inhibited the collagen I-induced proliferative responses, whereas no effects were observed on basal and PDGF-induced increases in cell number. No effects of overexpression of FAK were observed on the parameters assessed, suggesting that the endogenous expression of the kinase is sufficient and not the limiting activation of downstream signalling pathways.

Changes in FAK activation during the proliferative phase only became apparent after 2 days of culture on collagen I, suggesting that FAK is not directly activated by collagen I-binding integrins, but that additional processes may be required. Indeed, studies in vascular smooth muscle cells have indicated that culturing on monomeric collagen type I increased the expression of other ECM proteins, including fibronectin [35], suggesting that the activation of FAK could be due to autocrine ECM deposition. This notion is also supported by previous findings showing that collagen I-induced increases in basal and growth factor-induced ASM proliferation required not only the collagen-binding integrin α2β1, but also the fibronectin binding integrins α4β1 and α5β1 [16](Chapter 6).

No effects of FAT or FRNK were observed on PDGF-induced proliferation, although in fibroblasts activation of the PDGF receptor has been shown to induce FAK phosphorylation [36]. Also in BTSM, PDGF increased FAK phosphorylation (not shown). The lack of effect of the deletion mutants, however, may be explained by the fact that activation of FAK by PDGF requires interaction of the receptor with the FERM domain, which is localized at the N-terminus of the kinase [23,36]. Both deletion mutants, however, are derived from the C-terminal domain and inhibit FAK localization to the focal adhesions, which is required for FAK activation by integrins [25,26], but do interfere with the activation of FAK via the FERM domain, providing an explanation for the lack of effect on PDGF-induced proliferation.

Phosphorylation of FAK at Tyr397, generates a high affinity binding site for Src, which then in turn fully activates FAK by phosphorylating Tyr576 and Tyr577 in the kinase domain [22,23]. Previous studies have found a critical role for Src in growth factor-induced ASM proliferation [37]. In agreement of the involvement of Src in collagen I-induced phenotype modulation, pharmacological
inhibition of Src fully normalized both collagen I-induced proliferation and hypocontractility. Upon full activation, FAK can initiate a number of other signalling pathways, including the PI3-kinase and MAPK signalling pathways [20]. Activation of these pathways has been found to be important in the response of ASM cells to growth factors. PI3-kinase activation has been associated with transcriptional activation and protein synthesis leading to ASM cell proliferation and hypertrophy [38,39]. Integrins may not only activate PI3-kinase through FAK, but also via integrin linked kinase (ILK), another cytoplasmic protein tyrosine kinase, which is activated by the β subunit of integrins [40]. ILK has also been shown to be important in the regulation of contractile protein expression by human ASM. Knock-down of ILK increased mRNA and protein expression of smooth muscle-specific myosin heavy chain (sm-MHC), via regulation of Akt, which is downstream of PI3-kinase [41]. In the present study, inhibition of both PI3-kinase and FAK prevented collagen I-induced proliferation and hypocontractility, indicating the involvement of the FAK-PI3-kinase pathway in collagen I-induced BTSM proliferation.

ERK1/2 or p42/p44 mitogen activated protein kinases (MAPKs) transfer growth promoting signals to the nucleus and subsequently increase ASM proliferation [24]. In addition, p38 MAPK is involved in the regulation of growth factor-induced proliferation in ASM as well [42]. Inhibition of the MAPK signalling pathways, either directly (p38 MAPK) or by inhibiting MEK, which is upstream of p42/p44 MAPK, also inhibited collagen I-induced BTSM proliferation and hypocontractility. Collectively, these findings suggest that collagen I-induced activation of FAK results in activation of Src and, subsequently, of PI3-kinase and MAPK signalling pathways, which are all involved in collagen I-induced BTSM cell proliferation and hypocontractility (Figure 7).

Next to its important role in ECM-induced phenotype switching, FAK is also involved in acute ASM contractile responses. Thus, phosphorylation and membrane localization of the kinase is increased by mechanical strain and by contractile agonists [43-45]. Knock-out of FAK in human tracheal smooth muscle strips decreased tension development, myosin light chain phosphorylation and calcium signalling in response to the muscarinic receptor agonist acetylcholine and the membrane depolarizing stimulus KCl [46], suggesting an important role of FAK in smooth muscle contraction. These and our current findings, suggest that modulation of FAK activity in asthma may be an important new target in the treatment of ASM responsiveness and proliferation.

In conclusion, the present study provides new insights in the signalling events leading to ASM phenotype modulation by collagen I. These signalling pathways involve activation of FAK and downstream activation of Src-kinase, MEK, PI3-kinase and p38 MAPK. Moreover, our results indicate that modulation of FAK activity may be a new target in the treatment of both variable and persistent AHR in asthmatics.
Figure 7: Proposed mechanism by which collagen I may affect ASM phenotype. Exposure of integrins (ITG) to collagen I results in the activation focal adhesion kinase (FAK). Autophosphorylation of FAK on Tyr397 creates a binding site for Src, which in turn phosphorylates FAK on Tyr576 and Tyr577. This leads to the full activation of the kinase, which may then activate PI3-kinase (PI3K) or mitogen activated protein kinase kinase (MEK) and mitogen activated protein kinase (MAPK) signalling pathways that regulate ASM phenotype and function. Alternatively, PI3-kinase may be activated by integrin-linked kinase (ILK) which is important in the regulation of contractile protein expression. See text for further detail.

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References


