Extracellular matrix proteins
differentially regulate airway
smooth muscle phenotype and
function

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Abstract
Changes in the ECM and increased airway smooth muscle (ASM) mass are major contributors to airway remodelling in asthma and chronic obstructive pulmonary disease. It has recently been demonstrated that ECM proteins may differentially affect proliferation and expression of phenotypic markers of cultured airway smooth muscle cells. In the present study, we investigated the functional relevance of ECM proteins in the modulation of ASM contractility using bovine tracheal smooth muscle (BTSM) preparations. The results demonstrate that culturing of BTSM strips for 4 days in the presence of fibronectin or collagen I depressed maximal contraction ($E_{max}$) both for methacholine and KCl, which was associated with decreased contractile protein expression. By contrast, both fibronectin and collagen I increased proliferation of cultured BTSM cells. Similar effects were observed for PDGF. Moreover, PDGF augmented fibronectin- and collagen I- induced proliferation in an additive fashion, without an additional effect on contractility or contractile protein expression. The fibronectin-induced depression of contractility was blocked by the integrin antagonist Arg-Gly-Asp-Ser (RGDS), but not by its negative control Gly-Arg-Ala-Asp-Ser-Pro (GRADSP). Laminin, by itself, did not affect contractility or proliferation but reduced the effects of PDGF on these parameters. Strong relationships were found between the ECM-induced changes in $E_{max}$ in BTSM strips and their proliferative responses in BTSM cells and for $E_{max}$ and contractile protein expression. Our results indicate that ECM proteins differentially regulate both phenotype and function of intact ASM.

Introduction
The ECM is an intricate network of macromolecules that surrounds the tissue cells and affects many aspects of cellular behavior. These include migration, differentiation, survival and proliferation of cells originating from a variety of tissues, including airway smooth muscle (ASM) (14).

Biopsy studies have revealed that both the quantity and the composition of the ECM is altered in the airways of chronic asthmatics. Deposition of collagen IV and elastin is decreased in the airway wall of asthmatic patients, whereas collagen I, III , V, fibronectin, tenascin, hyaluran, versican and laminin α2/β2 chains are increased compared to healthy subjects (1; 15; 16; 24; 25).

Increased ASM mass within the airway wall is a characteristic feature of chronic asthma and may be one of the mechanisms associated with increased airway responsiveness and decline of lung function (4; 13; 26). Increased ASM cell mass is believed to involve both cellular hyperplasia and hypertrophy (6). Mechanisms involved in increased ASM growth in asthma are currently largely unknown; however, changes in the composition of the ECM proteins surrounding the ASM cell might well be involved (12).
In the airway wall of healthy subjects, the smooth muscle layer consists mainly of differentiated ASM cells, which are characterized by low proliferation rates, low fractions of biosynthetic organelles and relatively high expression levels of contractile proteins, including smooth muscle α-actin (sm-α-actin), calponin and smooth muscle myosin heavy chain (sm-MHC) (13). Importantly, in contrast to skeletal myocytes and cardiomyocytes (17; 19), ASM cells maintain the ability to re-enter the cell-cycle. Thus, exposure to mitogenic stimuli (e.g. PDGF), results in the induction of a more proliferative/synthetic phenotype (13), which is accompanied by a loss of contractile responsiveness (8), presumably as a consequence of decreased contractile protein expression (13). Long term serum deprivation results in the reinduction of a contractile phenotype, underlining the reversible nature of ASM phenotype (18). This phenotypic plasticity might be involved in growth and repair processes of inflamed airways and may contribute to airway remodeling in chronic asthma (12).

Using ASM cells in culture, it has recently been indicated that ECM proteins may differentially affect growth factor-induced phenotypic modulation. Thus, in human ASM cells cultured on fibronectin or collagen I matrices progression towards a proliferative phenotype, induced by either PDGF or α-thrombin, was promoted, whereas culturing on a laminin or matrigel matrix inhibited phenotype switching by these mitogens (12). Enhancement of PDGF-dependent proliferation of human ASM cells on a fibronectin or collagen I matrix has been shown to be dependent on activation of α5β1, α6β1, and α6β1 integrins (22). In vascular smooth muscle (VSM) cells, expression of α5β1 and α6β1 integrins has been correlated with the differentiated smooth muscle phenotype (2; 30). Both integrins are capable of binding laminin, which has been implicated in maintaining contractile VSM phenotype (20).

At present, the functional significance of ASM phenotypic modulation by ECM proteins is unknown. Therefore, we investigated the effects of exogenously applied fibronectin, collagen I and laminin on BTSM strip contractility in relation to the proliferative response of BTSM cells under these conditions. Our results indicated that fibronectin and collagen I induce a less contractile BTSM phenotype, whereas laminin maintains contractility. However, laminin attenuates the suppressive effects of PDGF on contractility as well as PDGF-induced DNA synthesis. Our results demonstrate for the first time a differential effect of ECM proteins on both phenotype and contractile function of intact ASM.
Materials and methods

Tissue preparation and organ culture procedure

Bovine tracheae were obtained from local slaughterhouses and rapidly transported to the laboratory in ice-cold Krebs-Henseleit (KH) buffer of the following composition (mM): NaCl 117.5, KCl 5.60, MgSO$_4$ 1.18, CaCl$_2$ 2.50, Na$_2$HPO$_4$ 1.28, NaHCO$_3$ 25.00 and glucose 5.50, pregassed with 5% CO$_2$ and 95% O$_2$; pH 7.4. After dissection of the smooth muscle layer and careful removal of mucosa and connective tissue, tracheal smooth muscle strips were prepared while incubated in gassed KH-buffer at room temperature (RT). Care was taken to cut tissue strips of macroscopically identical length (1 cm) and width (2 mm). Tissue strips were washed once in Medium Zero (sterile DMEM (Gibco BRL Life Technologies, Paisley, UK), supplemented with sodium pyruvate (1 mM, Gibco), nonessential amino-acid mixture (1 :100, Gibco), gentamicin (45 µg/ml, Gibco), penicillin (100 U/ml, Gibco), streptomycin (100 µg/ml, Gibco), amphotericin B (1.5 µg/ml, Fungizone, Gibco), apo-transferrin (5 µg/ml, human, Sigma Chemical Co, St. Louis, MO, USA) and ascorbic acid (100 µM, Merck, Darmstadt, Germany)). Next, tissue strips were transferred into suspension culture flasks and a volume of 7.5 ml Medium Zero was added per tissue strip. Strips were maintained in culture in an Innova 4000 incubator shaker (37°C, 55 rpm) under tightly controlled conditions for 4 days. To avoid direct influences of mechanical plasticity during this culture, strips were maintained under unloaded conditions. When used, collagen I (50 µg/ml, monomeric, calf skin, Fluka, Buchs, Switzerland), fibronectin (10 µg/ml, bovine plasma, Sigma), Engelberth-Holm-Swarm (EHS) Sarcoma laminin, consisting of laminin-1 (27) (4 µg/ml, Invitrogen, Grand Island, NY, USA), PDGF-AB (10 ng/ml, human, Bachem, Weil am Rhein, Germany), Arg-Gly-Asp-Ser (RGDS, 0.1 mM, Calbiochem, Nottingham, UK) and/or Gly-Arg-Ala-Asp-Ser-Pro (GRADSP, 0.1 mM, Calbiochem) were present during the entire incubation period. Occasionally, some strips were used for isometric tension measurements directly after preparation.

Isometric tension measurements

Tissue strips, collected from the suspension culture flasks, were washed with several volumes of KH-buffer pregassed with 5% CO$_2$ and 95% O$_2$, pH 7.4, at 37°C. Subsequently, strips were mounted for isometric recording (Grass force-displacement transducer FT03) in 20 ml water-jacked organ baths, containing KH-buffer at 37°C, continuously gassed with 5% CO$_2$ and 95% O$_2$, pH 7.4. During a 90-min equilibration period, with washouts every 30 min, resting tension was gradually adjusted to 3 g. Subsequently, muscle strips were precontracted with 20 and 40 mM isotonic KCl solutions. Following two washouts, maximal relaxation was established by the addition of 0.1 µM (-)isoproterenol (Sigma). In most the experiments, no basal myogenic tone was detected. Tension was readjusted to 3 g, immediately followed by two changes with fresh KH-buffer. After another equilibration period of 30 min, cumulative
concentration response curves (CRCs) were constructed to stepwise increasing concentrations of isotonic KCl (5.6–50 mM) or methacholine (1 nM–100 µM, ICN biomedicals, Costa Mesa, CA, USA). When maximal tension was obtained, the strips were washed several times and maximal relaxation was established using (-)-isoproterenol.

**Isolation of bovine tracheal smooth muscle (BTSM) cells**

After the removal of mucosa and connective tissue, tracheal smooth muscle was chopped using a McIlwain tissue chopper, three times at a setting of 500 µm and three times at a setting of 100 µm. Tissue particles were washed two times with Medium Plus (DMEM supplemented with sodium pyruvate (1 mM), nonessential amino-acid mixture (1:100), gentamicin (45 µg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin B (1.5 µg/ml) and FBS (0.5%, Gibco)). Enzymatic digestion was performed in Medium Plus, supplemented with collagenase P (0.75 mg/ml, Boehringer, Mannheim, Germany), papain (1 mg/ml, Boehringer) and soybean trypsin inhibitor (1 mg/ml, Sigma). During digestion, 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, cells were washed three times in Medium Plus, supplemented with 10% FBS instead of 0.5% FBS.

**Coating of culture plates with extracellular matrix proteins**

Calf skin collagen I was reconstituted in 10 mM hydrochloric acid at 5 mg/ml before diluting. Bovine plasma fibronectin was reconstituted in sterile PBS (composition mM): NaCl 140.0; KCl 2.6; KH₂PO₄ 1.4; Na₂HPO₄·2H₂O 8.1; pH 7.4. Dilutions of collagen I, fibronectin and mouse laminin were prepared in PBS. Diluted ECM proteins (0.5 ml) were absorbed to 24-well cluster plates overnight and air-dried at RT. Unoccupied protein-binding sites were blocked by a 30 min incubation with a sterile 0.1% bovine serum albumin (BSA, Sigma) solution. Subsequently, plates were washed twice with Medium Zero and dried before further use.

**[3H]-Thymidine-incorporation**

BTSM cells were plated on uncoated or ECM-coated 24-well cluster plates at a density of 50,000 cells per well immediately after isolation and were allowed to attach overnight in Medium Plus, containing 10% FBS. Cells were washed twice with sterile PBS and made quiescent by incubation in Medium Zero, supplemented with apo-transferrin (5 µg/ml), ascorbic acid (100 µM) and insulin (1 µM, bovine pancreas, Sigma) for 72 h. Cells were then washed with PBS and incubated with or without PDGF in Medium Zero for 28 h, the last 24 h in the presence of [methyl-³H]-thymidine (0.25 µCi/ml, Amersham, Buckinghamshire, UK). After incubation the cells were washed twice with 0.5 ml PBS at RT. Subsequently, the cells were treated with 0.5 ml ice-cold 5% trichloroacetic acid on ice for 30 min, and the acid-insoluble fraction was dissolved in 1 ml NaOH (1
M). Incorporated [3H]-thymidine was quantified by liquid-scintillation counting using a Beckman LS1701 β-counter.

**MTT assay**

BTSM cells were plated as described for the [3H]-thymidine-incorporation protocol. Following quiescence, cells were incubated with vehicle or PDGF for 3 days, after which cell number was estimated using the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) to formazan. Briefly, cells were washed twice with Medium Zero after which 200 µl medium containing 0.5 mg/ml MTT was added to each well. After 5 h, 200 µl 10% sodium dodecylsulphate in 0.01 N HCl was added, and the cells were solubilized overnight at 37 °C. The amount of formazan in the obtained solution was estimated by measuring optical density at a test wavelength of 550 and a reference wavelength of 655 nm.

**Western analysis of contractile protein expression**

After culturing the tissue strips as described above, homogenates were prepared by pulverizing the tissue under liquid nitrogen, followed by sonification 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 10 µg/ml leupeptin (Sigma), 10 µg/ml aprotinin (Sigma), 10 µg/ml pepstatin (Sigma), Na-deoxycholate 0.25 % (Sigma) and 1% Igepal (NP-40, Sigma)). Homogenates were stored at −80 °C until further use. Protein content was determined according to Bradford (5). In total, 30 µg of protein per lane was separated by SDS/PAGE using 6% polyacrylamide gels for smooth muscle myosin (sm-myosin) or 10% polyacrylamide gels for β-actin, smooth muscle α-actin (sm-α-actin) and calponin. Proteins in the gel were then transferred onto nitrocellulose membranes, which were subsequently blocked in blocking buffer (composition: Tris-HCl 50.0 mM; NaCl 150.0 mM; Tween-20 0.1%, dried milk powder 5%) for 90 min at RT. Next, membranes were incubated overnight at 4 °C with primary antibodies (anti-sm-myosin (Neomarkers, Fremont, CA, USA), anti-sm-α-actin (Sigma), both diluted 1:200, anti-β-actin (Sigma), diluted 1:2000, calponin (Neomarkers), diluted 1:400; all dilutions in blocking buffer). Membranes were incubated with antibodies for β-actin to normalize for equal loading of all samples. After three washes of 10 min each, membranes were incubated with horseradish peroxidase-labelled secondary antibodies (dilution 1:3000 in blocking buffer) at RT for 90 min, followed by another three washes. Antibodies were then visualized by enhanced chemiluminescence. Blots were analyzed by densitometry (TotalLab). All bands were normalized to β-actin expression. ECM-induced changes in protein abundance were expressed as a percentage of vehicle treated controls run on the same gels.
**Data analysis**

Data represent means ± S.E.M. or S.D., from *n* separate experiments. Statistical significance of differences was evaluated by the Student’s *t*-test for paired observations or one-way ANOVA, as appropriate. Differences were considered to be statistically significant when *P*<0.05.

**Results**

**Effect of organ culturing on bovine tracheal smooth muscle strip contractility and contractile protein expression**

Maximal methacholine and KCl-induced contractile force (*E*<sub>max</sub>) of BTSM strips, cultured for 4 days in Medium Zero, was maintained as compared to freshly isolated BTSM strips (Figure 1A, 1B). No changes in sensitivity were observed for methacholine (pEC<sub>50</sub> = 6.99 ± 0.17) or KCl (EC<sub>50</sub> = 26.5 ± 1.0). Expression of the contractile protein sm-myosin in BTSM strip homogenates was preserved after 4 days of organ culturing (94 ± 8% compared with fresh, Figure 1C).

![Figure 1: Concentration-response curves of methacholine- (A) and KCl-induced (B) contraction of freshly isolated and 4 day organ cultured BTSM strips. Data represent means ± S.E.M. of 3 experiments. (C) Western analysis of protein expression in fresh and organ cultured BTSM strips. Representative immunoblots of sm-myosin and β-actin are shown.](image-url)
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Effects of pretreatment with extracellular matrix proteins on bovine tracheal smooth muscle strip contractility

In BTSM strips cultured for 4 days in the presence of fibronectin (10 μg/ml) or collagen I (50 μg/ml), maximal methacholine-induced contractile force was significantly reduced compared to strips cultured in the absence of these ECM proteins (Figures 2A, 2B, Table 1). The suppressive effects on $E_{\text{max}}$ were quantitatively similar to the effects observed after pretreatment with PDGF (10 ng/ml). Combined pretreatment of the strips with the ECM proteins (fibronectin and collagen I) and PDGF did not further affect maximal contraction. Unlike fibronectin and collagen I, pretreatment with laminin (4 μg/ml) did not affect $E_{\text{max}}$ of methacholine. Interestingly, however, co-incubation with laminin fully reversed the suppressive effects of PDGF on $E_{\text{max}}$ (Figure 2C, Table 1). Similar effects of ECM proteins and PDGF were obtained for KCl-induced contractions (Figure 3, Table 1).

Figure 2: Concentration-response curves of methacholine-induced contraction of BTSM strips pretreated with (A) fibronectin (10 μg/ml), (B) collagen I (50 μg/ml) or (C) laminin (4 μg/ml) in the absence or presence of PDGF (10 ng/ml) for 4 days. Data represent means ± S.E.M. of 6-7 experiments.
ECM proteins and ASM phenotype and function

Table 1: Effects of 4 days culturing in the absence (vehicle) or presence of fibronectin (10 µg/ml), collagen I (50 µg/ml) or laminin (4 µg/ml), with or without PDGF (10 ng/ml), and the effects of 4 days culturing in the absence or presence of fibronectin with or without RGDS (0.1 mM) or GRADSP (0.1 mM), on contractile responses of BTSM strips to methacholine and KCl.

<table>
<thead>
<tr>
<th></th>
<th>Methacholine</th>
<th>KCl</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>E\textsubscript{max} (g)</td>
<td>pEC\textsubscript{50} (-log M)</td>
</tr>
<tr>
<td>Vehicle</td>
<td>28.9±1.7</td>
<td>6.88±0.12</td>
</tr>
<tr>
<td>+ PDGF</td>
<td>24.8±1.2*</td>
<td>6.63±0.11</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>23.4±0.9*</td>
<td>6.53±0.10</td>
</tr>
<tr>
<td>+ PDGF</td>
<td>21.6±2.1*</td>
<td>6.60±0.06</td>
</tr>
<tr>
<td>Vehicle</td>
<td>29.9±1.6</td>
<td>6.64±0.09</td>
</tr>
<tr>
<td>+ PDGF</td>
<td>24.1±1.9***</td>
<td>6.62±0.11</td>
</tr>
<tr>
<td>Collagen I</td>
<td>22.1±2.4*</td>
<td>6.41±0.11</td>
</tr>
<tr>
<td>+ PDGF</td>
<td>21.5±2.2**</td>
<td>6.50±0.11</td>
</tr>
<tr>
<td>Vehicle</td>
<td>26.8±1.7</td>
<td>6.74±0.14</td>
</tr>
<tr>
<td>+ PDGF</td>
<td>21.2±1.7**</td>
<td>6.54±0.11</td>
</tr>
<tr>
<td>Laminin</td>
<td>29.0±2.7***</td>
<td>6.65±0.13</td>
</tr>
<tr>
<td>+ PDGF</td>
<td>26.8±2.4†</td>
<td>6.71±0.11</td>
</tr>
<tr>
<td>Vehicle</td>
<td>28.7±1.8</td>
<td>6.68±0.10</td>
</tr>
<tr>
<td>+ GRADSP</td>
<td>28.8±1.6</td>
<td>6.59±0.11</td>
</tr>
<tr>
<td>+RGDS</td>
<td>24.9±2.7</td>
<td>6.67±0.12</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>21.9±1.6*</td>
<td>6.79±0.20</td>
</tr>
<tr>
<td>+ GRADSP</td>
<td>22.7±1.7*</td>
<td>6.58±0.04</td>
</tr>
<tr>
<td>+ RGDS</td>
<td>31.1±1.5††</td>
<td>6.54±0.19</td>
</tr>
</tbody>
</table>

Data represent means ± S.E.M. from 4-7 experiments. Abbreviations: E\textsubscript{max}: maximal contraction; EC\textsubscript{50}: concentration of agonist eliciting half-maximal response; pEC\textsubscript{50} negative logarithm of the EC\textsubscript{50} value. *P<0.05, **P<0.01, ***P<0.001 compared with vehicle-treatment. †P<0.05, ††P<0.01 compared with PDGF-treatment. ‡P<0.05, ‡‡P<0.01 compared with fibronectin.
Figure 3: Concentration-response curves of KCl-induced contraction of BTSM strips pretreated with (A) fibronectin (10 μg/ml), (B) collagen I (50 μg/ml) or (C) laminin (4 μg/ml) in the absence or presence of PDGF (10 ng/ml) for 4 days. Data represent means ± S.E.M. of 6-7 experiments.

Combined pretreatment of BTSM strips with fibronectin and its blocking peptide Arg-Gly-Asp-Ser (RGDS, 0.1 mM) normalized E\textsubscript{max} for both methacholine and KCl (Figure 4, Table 1), whereas no effects of the negative control, Gly-Arg-Ala-Asp-Ser-Pro (GRADSP, 0.1 mM), were observed. BTSM strips pretreated with RGDS or GRADSP in the absence of fibronectin did not show any significant effect on ASM contractility (Table 1). It could be envisaged that pretreatment of strips with ECM proteins affect contractility by altering smooth muscle stiffness.

To address this issue, BTSM strips were incubated with vehicle and collagen I (50 μg/ml)-containing media for 4 days. After this incubation period, strip length and width were assessed just before mounting and at a resting tension of 3 g. No differences between vehicle and collagen I pretreated strips were found for both parameters (Table 2).

Table 2: Effect of collagen I (50 μg/ml) pretreatment on BTSM strip length and width before mounting and at resting tension (3 g).

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Before mounting</th>
<th>At resting tension</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length (cm)</td>
<td>Width (cm)</td>
</tr>
<tr>
<td>Vehicle</td>
<td>0.88 ± 0.03</td>
<td>0.30 ± 0.03</td>
</tr>
<tr>
<td>Collagen I</td>
<td>0.89 ± 0.03</td>
<td>0.30 ± 0.03</td>
</tr>
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</table>

Results are means ± S.D. of two separate experiments, each performed in duplicate.
Effects of extracellular matrices on bovine tracheal smooth muscle cell proliferation.

To establish the effects of fibronectin, collagen I and laminin on BTSM cell proliferation, both [³H]-thymidine incorporation and mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan were assessed. In BTSM cells, basal DNA-synthesis was increased by approximately 1.7-fold in cells grown on fibronectin (10 μg/ml) and by 2.5-fold in cells grown on collagen I (50 μg/ml) matrices compared to cells grown on plastic (control; Figure 5A). Under control conditions, PDGF significantly augmented DNA-synthesis by 2.5-fold (Figure 5A). This response was significantly enhanced in an additive fashion, when cells were grown on a fibronectin or collagen I matrix. In contrast, cells attached to a laminin matrix (4 μg/ml) did not show a significant change in basal DNA synthesis. Growing BTSM cells on laminin, however, resulted in a significant reduction of the PDGF-induced proliferative response, to approximately 0.6-fold of PDGF-induced proliferation in cells grown on plastic (Figure 5A).
Figure 5: Effects of fibronectin (10 μg/ml), collagen I (50 μg/ml) and laminin (4 μg/ml) matrices on basal (black bars) and PDGF (10 ng/ml)-stimulated (grey bars) BTSM cell DNA synthesis (A) and BTSM cell number (B). Data represent means ± S.E.M. of 3-5 experiments each performed in triplicate. *P<0.05, **P<0.01 compared to basal controls. #P<0.05, ##P<0.01 compared to PDGF control. †P<0.05, ††P<0.01 compared to ECM protein in the absence of PDGF.

Since increased DNA synthesis may reflect both cell hyperplasia and hypertrophy, we also assessed the effects of ECM proteins and PDGF on cell number using the MTT assay. When cells were grown on fibronectin or collagen I, the cell number was augmented by approximately 1.6-fold as compared to cells grown on plastic (control, Figure 5B). Surprisingly, laminin induced a slight, but significant, increase in cell number as well. PDGF significantly augmented cell number to a similar degree as collagen I and fibronectin, and, as observed for DNA-synthesis, PDGF increased the cell number in an additive fashion when cells were grown on fibronectin or collagen I matrices. In line with the effects on DNA-synthesis, laminin significantly reduced the PDGF-mediated increase in cell number (Figure 5B).

Relationship between extracellular matrix-induced changes in contractility and proliferation

It has been previously established that growth factor-induced changes in maximal contractility of BTSM strips are inversely correlated with changes in the proliferative response of isolated BTSM cells (8). As illustrated by Figure 6, a qualitatively similar relationship exists between the effects of the applied ECM proteins on proliferative potency, as assessed by [3H]-thymidine incorporation in isolated BTSM cells, and the effects on \( E_{\text{max}} \) of methacholine- and KCl-induced contraction of BTSM strips.
Effects of extracellular matrix proteins on contractile protein expression

The changes in $E_{\text{max}}$ in response to both receptor-dependent (methacholine) and receptor-independent (KCl) stimuli indicate post-receptor changes, which may occur at the level of the contractile apparatus. BTSM strips pretreated with PDGF showed a significant reduction in the expression of sm-myosin, calponin and sm-$\alpha$-actin as compared to control conditions (Figure 7, Table 3). Similar results were observed after pretreatment with fibronectin or collagen I or the combined pretreatment of these ECM proteins with PDGF (Figure 7, Table 3). In contrast, strips cultured in the presence of laminin showed a significant increase in sm-myosin and calponin expression, whereas the expression level of sm-$\alpha$-actin was not increased. Interestingly, although laminin normalized the suppressive effects of PDGF on BTSM strip contractility (Figures 2 and 3) and reduced the mitogenic capacity of the growth factor (Figure 5), no significant effects of laminin were observed on the PDGF-induced reduction of sm-myosin, calponin or sm-$\alpha$-actin protein expression (Figure 7, Table 3).
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Figure 7: Western analysis of protein expression in BTSM strips pretreated with vehicle (control), collagen I (50 μg/ml), fibronectin (10 μg/ml) and laminin (4 μg/ml), in the absence or presence of PDGF (10 ng/ml). Representative immunoblots of sm-myosin, calponin, sm-α-actin and β-actin are shown.

Table 3: Contractile protein expression in BTSM strips after 4 days of culturing with vehicle (control), collagen I (50 μg/ml), fibronectin (10 μg/ml) and laminin (4 μg/ml), in the absence and presence of PDGF (10 ng/ml).

<table>
<thead>
<tr>
<th>Protein expression (% of control)</th>
<th>sm-myosin</th>
<th>calponin</th>
<th>sm-α-actin</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>+ PDGF</td>
<td>47±8**</td>
<td>61±9**</td>
<td>41±11**</td>
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<tr>
<td>Fibronectin</td>
<td>48±13**</td>
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<td>42±8***</td>
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<td>+ PDGF</td>
<td>55±10**</td>
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<td>39±11**</td>
</tr>
<tr>
<td>Collagen I</td>
<td>59±5**</td>
<td>38±5**</td>
<td>33±7**</td>
</tr>
<tr>
<td>+ PDGF</td>
<td>56±6**</td>
<td>55±9*</td>
<td>31±3***</td>
</tr>
<tr>
<td>Laminin</td>
<td>134±13**-##</td>
<td>142±16**-##</td>
<td>97±15###</td>
</tr>
<tr>
<td>+ PDGF</td>
<td>63±5**-##-††</td>
<td>80±12-##†</td>
<td>49±8**-##†</td>
</tr>
</tbody>
</table>

Data represent means ± S.E.M. from 3-7 experiments. *P<0.05, **P<0.01, ***P<0.001 compared with vehicle-treatment; ##P<0.01, ###P<0.001 compared with PDGF-treatment. †P<0.05, ††P<0.01 compared with ECM protein in the absence of PDGF.

Relationship between extracellular matrix protein-induced changes in contractile protein expression and contractility

Strong correlations were observed between the efficacy of the ECM proteins to affect sm-myosin expression and $E_{\text{max}}$ of methacholine (Figure 8A) or KCl (Figure 8B). Similar results were obtained for calponin expression (r=0.983, P=0.017 and r=0.993, P=0.007, respectively; data not shown). For the relationship between ECM effects on sm-α-actin content and $E_{\text{max}}$ a strong
tendency was observed for both methacholine and KCl-induced contractions ($r=0.942, P=0.058, r=0.947, P=0.053$, respectively; data not shown).

**Figure 8:** Relationships between maximal contraction ($E_{\text{max}}$) of BTSM strips in response to methacholine (A) or KCl (B) and $\alpha$-myosin expression in the presence of laminin (1), vehicle (2), fibronectin (3) or collagen I (4). Data represent means from 3-7 experiments.

**Discussion**

In this study, we demonstrate for the first time that prolonged (4 days) exposure of intact ASM strips to exogenous ECM proteins may differentially regulate ASM phenotype and function. Thus, exogenously applied fibronectin and collagen I induced a functionally hypocontractile ASM phenotype, characterized by a decreased maximal contractile response to both the muscarinic receptor agonist methacholine and the membrane depolarizing agent KCl. These effects are presumably due to phenotype modulation, since no differences were found in contractility between fresh and cultured strips and no effects were observed on equilibration length. Phenotypic modulation is also supported by the observation that the effects of fibronectin were fully normalized in the presence of RGDS, but not its negative control GRADSP. Similar effects of RGD- and RAD-containing peptides on fibronectin-induced eotaxin release have been observed in human ASM cells (23).

In accordance with previous findings (8; 9), 4 days incubation with PDGF also induced a hypocontractile phenotype. No additive effects of combined pretreatment with the growth factor and fibronectin or collagen I were found on maximal contractility. Interestingly, although laminin had no effect on BTSM
contractility, co-incubation with PDGF fully normalized the suppressive effects of the growth factor on $E_{\text{max}}$, indicating that laminin may be involved in maintaining a (normo)contractile phenotype. It remains to be determined, however, to what extent the ECM proteins penetrate the tissue and to what extent the observed effects of the ECM proteins represent their maximal effect.

Both in vascular (11; 29) and in ASM (3; 12) cells, it has been demonstrated that ECM proteins are capable of differentially influencing the mitogenic capacity of a variety of growth factors, including PDGF, α-thrombin and basic fibroblast growth factor (bFGF). In human ASM cells, Hirst et al (12) showed that mitogen-stimulated, but not basal, proliferation was significantly enhanced after culturing on a collagen I or fibronectin matrix, whereas mitogen-induced proliferation was reduced on laminin-precoated plates (12). Accordingly, we found that culturing BTSM cells on a fibronectin or collagen I matrix significantly augmented proliferation induced by PDGF. Moreover, basal DNA-synthesis and cell number were also significantly increased by these ECM proteins, which is in agreement with previous findings showing that proliferation of bovine ASM cells was increased on a collagen type I matrix as compared to cells grown on a laminin matrix (3). As the enhancement of the PDGF-induced proliferative effects by a fibronectin or collagen I matrix was additive, it can be envisaged that these matrix proteins and PDGF regulate mitogenesis through distinct rather than common pathways. However, little is known about the pathways involved in these processes, which warrants further investigation.

Laminin, by itself, had no effect on DNA synthesis, whereas cell number, as assessed by formazan accumulation, was slightly increased. A possible explanation for this apparent discrepancy might be the anti-apoptotic potency of laminin as observed in human ASM cells (7). This may result in a preservation of the number of viable cells without increasing DNA synthesis. In agreement with previous studies, we found that laminin reduced proliferation induced by PDGF (12). Also, in porcine coronary artery smooth muscle cells, it has been shown that proliferation in response to growth factors was more pronounced in cells grown on fibronectin than on laminin (21). PDGF-mediated activation of ERK1/2 in these cells was not dependent on the matrix present, whereas activation of FAK was more pronounced on a fibronectin matrix (21). Those findings indicate a pivotal role for ECM in controlling intracellular signaling.

Modulation towards a less contractile ASM phenotype is accompanied by a reduced expression of contractile proteins, including $\text{sm}-\text{MHC}$, calponin and $\text{sm}-\alpha$-actin (10; 28). Since prolonged (4 days) culturing in the presence of fibronectin or collagen I induced a decline in maximal contraction both in response to a receptor-dependent (methacholine) and a receptor-independent (KCl) stimulus, post-receptor events such as alterations in contractile protein expression are likely to contribute to the observed changes in contractility. Therefore, we assessed the expression of $\text{sm}$-myosin, calponin and $\text{sm}-\alpha$-actin in homogenates prepared from BTSM strips treated with vehicle, collagen I, fibronectin or laminin in the absence or presence of PDGF for 4 days. In
accordance with our previous (8; 9) and current observations that PDGF induces a shift towards a more proliferative (hypocontractile) phenotype, the growth factor reduced the expression of all contractile markers studied. Incubation with fibronectin or collagen I resulted in a reduction of contractile protein expression as well, which was both quantitatively and qualitatively similar to that induced by PDGF. These observations, along with the fact that 4 days of organ culturing, by itself, did not affect \( sm \)-myosin expression, confirm the assumption that pretreatment with fibronectin or collagen I, in the absence or presence of PDGF, induces a functional hypocontractile phenotype by reducing contractile protein expression. Similar to the effects of combined treatment on \( E_{\text{max}} \), no additional effects were observed on the level of contractile protein expression, indicating that changes in \( E_{\text{max}} \) and contractile protein expression are tightly correlated.

In contrast, laminin markedly increased \( sm \)-myosin and calponin expression, whereas it did not affect \( sm - \alpha \)-actin protein levels. This might be explained, however, by the fact that \( sm \)-myosin and calponin are considered to be a more specific markers for mature contractile ASM cells as compared to \( sm - \alpha \)-actin, which is a more general marker for lung cells of mesenchymal origin (10). These results suggest that an increase in contractility in the presence of laminin can be envisaged. Indeed, a tendency towards an increased contractility in response to both methacholine and KCl was observed. Moreover, a direct relationship between contractility and contractile protein expression in the presence of different matrix proteins was confirmed by the significant correlation between these two parameters for all contractile proteins.

In human ASM cells, it has been shown by immunocytochemical detection that laminin, by itself, did not affect expression of contractile proteins at all, but normalized the reduction induced by PDGF (12). In the present study, laminin did not completely reverse the effects of PDGF on contractile protein expression, but showed a tendency to attenuate the growth factor-induced suppression to some extent. This may indicate that other factors are involved in the reversal of PDGF-induced hypocontractility. The apparent discrepancy between our findings and those by Hirst et al (12) could possibly be explained by differences in experimental approach. Thus, we determined protein expression in smooth muscle strips, not cultured cells. In addition, species differences cannot be ruled out. Also, homogeneity of contractile protein expression throughout the tissue might possibly represent another variable. It has been established that there is a strong inverse relationship between the effects of peptide growth factors on maximal methacholine- and KCl-induced contraction of BTSM-strips and the proliferative response of BTSM cells to these growth factors (8). In the present study we found a strong correlation between the degree of change of \( E_{\text{max}} \), both with methacholine and KCl, induced by the applied ECM proteins in BTSM strips and the proliferative response by BTSM cells cultured on these proteins. Although determined using different experimental parameters measured in different (ASM tissue and cellular) conditions, this correlation was very striking and highly reminiscent of our
previous findings (8). Together with the relationship between contractile protein expression and contractility found for the applied ECM proteins, these results indicate that ECM proteins are importantly involved in the regulation of ASM phenotype and function.

In conclusion, our results indicate that ECM proteins differentially regulate BTSM phenotype and function. Fibronectin and collagen type I induce a (functional) hypocontractile phenotype, associated with an increased proliferative response of BTSM cells, whereas laminin inhibits growth factor-induced proliferation and supports a more contractile phenotype. These findings implicate a critical role of ECM changes (1; 15; 16; 24; 25) in altered ASM function in asthma.

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