Major role of the extracellular matrix in airway smooth muscle phenotype plasticity
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2010

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Chapter 4

Functional consequences of human airway smooth muscle phenotype plasticity

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Submitted (2010)
Abstract

Airway smooth muscle (ASM) phenotype plasticity, characterized by reversible switching between contractile and proliferative phenotypes, is thought to contribute to increased ASM mass and airway hyperresponsiveness in asthma. In addition, increased expression of the extracellular matrix protein collagen I has been observed within the ASM bundle of asthmatics. Previously, we have shown that exposure of intact bovine tracheal smooth muscle to collagen I induces a switch from a contractile to a hypocontractile, proliferative phenotype. The functional relevance of this finding for intact human ASM, however, has not yet been established. In the present study, we demonstrated that prolonged exposure of human tracheal smooth muscle (HTSM) strips to collagen I decreased maximal methacholine- and KCl-induced contractions as well as expression of contractile proteins. Conversely, culturing on collagen I increased proliferation of HTSM cells. Similar effects were observed for the growth factor PDGF. As observed for BTSM, culturing of HTSM cells on collagen I additively increased PDGF-induced proliferative responses, whereas no additional effects were observed on contractility or contractile protein expression. These findings indicate that collagen I and PDGF induce a functionally hypocontractile, proliferative phenotype of human ASM, which may contribute to airway remodelling in asthma.

Introduction

Structural changes in the airway wall, including increased airway smooth muscle (ASM) mass, are characteristic features of airway remodelling, which may contribute to airway hyperresponsiveness (AHR) and decline in lung function in asthma [1,2]. Increased ASM mass is at least partially caused by hyperplasia, and in vitro ASM proliferation is increased by serum and various growth factors that may synergize with neurotransmitters and inflammatory mediators [3-5]. Exposure of cultured ASM cells to mitogenic stimuli causes switching from a contractile, hypoproliferative to a proliferative, hypocontractile phenotype, as indicated by changes in molecular phenotypic markers [6,7]. The potential impact of these molecular changes on ASM contractile function has recently been demonstrated in intact bovine tracheal smooth muscle (BTSM). Thus, prolonged exposure of BTSM strips to serum or peptide growth factors like platelet-derived growth factor (PDGF) and insulin-like growth factor-1 decreased maximal methacholine- and KCl-induced contractions, which was inversely correlated with the proliferative responses of BTSM cells to these mitogenic stimuli [8](Chapter 3). ASM phenotype switching is reversible, as indicated by the observation that removal of mitogenic stimuli, for example by serum-deprivation, results in the reintroduction of a (hyper)contractile ASM phenotype associated with increased expression of contractile marker proteins like sm-α-
actin, sm-MHC and calponin, which is further enhanced in the presence of insulin or transforming growth factor-β (TGF-β) [9-11] (Chapter 5).

Increased deposition of extracellular matrix (ECM) proteins within the airway wall is another hallmark of airway remodelling in asthma [12] (Chapter 2). Expression of various ECM proteins, including collagen I, is increased in the subepithelial basement membrane [12,13]. Within the ASM bundle, increased deposition of collagen I has been observed as well [14-16], which may be due to increased production of this matrix protein by asthmatic ASM cells [17]. In addition, increased proliferative responses of asthmatic ASM cells have also been shown, which depends on the ECM produced by these cells [17], indicating that collagen I may have impact on the phenotype of these cells. Indeed, in vitro studies on the effects of collagen I on human ASM cell function have indicated that cells grown on this ECM protein show increased growth factor-induced proliferation, increased synthetic capabilities, decreased apoptosis and reduced expression of contractile proteins [7,18-20]. Using BTSM, we have recently shown that exposure of intact strip preparations to monomeric collagen I induced a hypocontractile state, characterized by decreased contractile responses to methacholine and KCl and reduced expression of contractile marker proteins, which was associated with increased proliferation of cultured BTSM cells (Chapter 3). In addition, PDGF augmented collagen I-induced proliferation in an additive fashion, without an additional effect on contractility or contractile protein expression (Chapter 3). The functional significance of ECM- and growth factor-induced phenotype switching on intact human ASM is currently unknown. Therefore, in the present study we investigated the effects of monomeric collagen I and PDGF on human tracheal smooth muscle (HTSM) strip contractility, contractile protein expression and cell proliferation.

**Materials and methods**

**Tissue preparation and organ culture**

Human tracheal sections from anonymized lung transplantation donors were obtained from the Department of Cardiothoracic Surgery, University Medical Centre Groningen and transported to the laboratory in ice-cold Krebs-Henseleit (KH) buffer (composition in mM: NaCl 117.5, KCl 5.60, MgSO₄ 1.18, CaCl₂ 2.50, NaH₂PO₄ 1.28, NaHCO₃ 25.00, and glucose 5.50), pregassed with 5% CO₂ and 95% O₂ (pH 7.4). HTSM strips were prepared as described for BTSM (Chapter 3 & 5). After dissection of the smooth muscle layer and careful removal of connective tissue and mucosa, strips were cut and cultured for 4 days in Medium Zero (sterile Dulbecco's Modified Eagle's medium (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; Gibco), apo-transferrin (5 μg/ml, human; Sigma, St.
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Louis, MO, USA) and ascorbic acid (0.1 mM)). When used, monomeric collagen I (50 μg/ml, calf skin; Fluka, Buchs, Switzerland) and/or PDGF-AB (10 ng/ml, human; Bachem, Weil am Rhein, Germany) were present during the entire incubation period. Occasionally, strips were used for isometric tension measurements directly after preparation.

Isometric tension measurements.
Isometric contraction experiments were performed as described previously (Chapter 3 & 5). Briefly, HTSM strips were mounted for isometric recording in organ baths, containing KH buffer at 37°C. During a 90-min equilibration period with washouts every 30 min, resting tension was adjusted to 0.5 g, followed by precontractions with 20 and 40 mM KCl. Following washout, maximal relaxation was established by the addition of (-)-isoproterenol (0.1 μM; Sigma). Tension was readjusted to 0.5 g immediately followed by two changes with KH buffer. After another equilibration period of 30 min, cumulative concentration-response curves were constructed to KCl (5.6-50 mM) or methacholine (1 nM – 0.1 mM; ICN Biomedicals, Costa Mesa, CA, USA). When maximal tension was reached, the strips were washed several times and maximal relaxation was established by using (-)-isoproterenol (10 μM). Contractions were expressed as the percentage of maximal contraction induced by KCl or methacholine in vehicle-treated strips. After the experiment, strips were snap frozen for Western analysis.

Western analysis
HTSM strip homogenization and Western analysis of sm-α-actin and sm-MHC expression were performed as described (Chapter 3 & 5). In short, homogenates were prepared by pulverizing the strips 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, Na<sub>3</sub>VO<sub>4</sub> 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%; all Sigma). Equal amounts of protein were subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes, followed by standard immunoblotting techniques. Antibodies (anti-sm-α-actin (Sigma) and anti-sm-MHC (Neomarkers, Fremont, CA, USA) were visualized on film using enhanced chemiluminescence reagents (Pierce, Breda, NL) and analyzed by densitometry (Totallab™, Nonlinear dynamics, Newcastle, UK). Bands were normalized to β-actin expression.

HTSM cell culture
HTSM, prepared free of mucosa and connective tissue, was chopped using a McIlwain tissue chopper. Tissue slices were washed once 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 fetal bovine serum (FBS, 10%; Gibco)), placed in culture flasks and allowed to adhere. Medium was refreshed every 48-
Upon reaching confluency, cells were passaged by trypsinization. Cells from passages 1-5 were used for the present study.

**Coating of culture plates with collagen I**
Collagen I was reconstituted in hydrochloric acid (10 mM) at 5 mg/ml and diluted in PBS to a final concentration of 50 μg/ml. Diluted collagen I (0.5 ml) was adsorbed to culture plates overnight and air-dried at room temperature. Unoccupied protein-binding sites were blocked by 30 min incubation with 0.1% bovine serum albumin solution. Subsequently, plates were washed twice with DMEM and dried before further use.

**[^H]-thymidine-incorporation**
[^H]-Thymidine-incorporation was performed as described previously (Chapter 3 & 5). HTSM 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. Cells were washed with PBS and made quiescent by incubation in Medium Zero, supplemented with 1% ITS (Insulin, Transferrin and Selenium; Gibco) 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[^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 [^H]-thymidine was quantified by liquid-scintillation counting using a Beckman LS1701 β-counter.

**Data analysis**
Data represent means ± SEM from n separate experiments. Statistical significance of differences was evaluated by paired Student's t-test. Statistical significance was reached at P<0.05.
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Results

Collagen I and PDGF decrease HTSM strip contractility

To assess whether exposure to collagen I or PDGF induces a functionally hypocontractile ASM phenotype, HTSM strips were cultured in the absence and presence of monomeric collagen I (50 μg/ml) and/or PDGF (10 ng/ml) for 4 days. Culturing HTSM strips in the presence of collagen I or PDGF for 4 days significantly (P<0.05) reduced maximal methacholine- and KCl-induced contractile force ($E_{\text{max}}$) compared to vehicle-treated control strips (Figure 1, Table 1).

![Figure 1: Concentration-response curves of (A) methacholine- and (B) KCl-induced contractions of HTSM strips, pretreated with vehicle (control) or collagen I (50 μg/ml) in the absence or presence of PDGF (10 ng/ml) for 4 days. Data represent means ± SEM of 3-7 experiments performed in duplicate.](image)

No additive effects were observed after combined treatment. The sensitivity to both contractile stimuli was unaffected by all treatments. No significant change in methacholine-induced contractility was observed after 4 days of culturing in vehicle compared to freshly isolated strips ($E_{\text{max}}$=2.89±0.68 g and 2.50±0.32 g, -logEC$_{50}$=5.95±0.10 and 5.77±0.14, respectively, n=4-7, P>0.05 Student’s t-test for unpaired observations).
Table 1: Contractile responses of HTSM strips to methacholine or KCl after 4 days of culturing in the absence or presence of collagen I (50 μg/ml) and/or PDGF (10 ng/ml).

<table>
<thead>
<tr>
<th></th>
<th>Methacholine</th>
<th>KCl</th>
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<tbody>
<tr>
<td></td>
<td>E_{max} (%)</td>
<td>pEC_{50} (-log M)</td>
</tr>
<tr>
<td>Control</td>
<td>100±0</td>
<td>5.77±0.14</td>
</tr>
<tr>
<td>+ PDGF</td>
<td>64.8±5.3**</td>
<td>5.66±0.14</td>
</tr>
<tr>
<td>Collagen I</td>
<td>61.0±6.0**</td>
<td>5.99±0.18</td>
</tr>
<tr>
<td>+ PDGF</td>
<td>56.7±4.8*</td>
<td>5.69±0.29</td>
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Data represent means ± SEM of 3-7 experiments, performed in duplicate. Abbreviations: E_{max}: maximal contraction; EC_{50}: contraction of agonist eliciting half-maximal response; pEC_{50}: negative logarithm of the EC_{50} value. *P<0.05, **P<0.01, ***P<0.001 compared to control.

In agreement with the reduced contractility, collagen I and PDGF also decreased the protein expression of sm-α-actin and sm-myosin heavy chain (P<0.05 all, Figure 2). No additive effects were observed after combined treatment.

![Figure 2](image-url)

**Figure 2:** Western analysis of (A) sm-MHC and (B) sm-α-actin expression in HTSM strips treated with vehicle (control) or collagen I (50 μg/ml) in the absence (basal) or presence of PDGF (10 ng/ml) for 4 days. Means ± SEM of 3-5 experiments are shown. *P<0.05, **P<0.01 compared to basal control. Representative immunoblots of sm-MHC, sm-α-actin and β-actin are shown.
Collagen I and PDGF increase proliferation of HTSM cells

To assess whether the collagen I- and PDGF-induced decreases in \( E_{\text{max}} \) were associated with proliferative changes, \([\text{H}]\)-thymidine-incorporation was assessed using primary HTSM cell cultures. Proliferation of these cells was increased by PDGF (\( P<0.05 \)) as well as by culturing on a collagen I matrix (\( P<0.05 \), Figure 3). PDGF-induced DNA synthesis was significantly enhanced, when the cells were cultured on collagen I-coated instead of uncoated matrices (\( P<0.05 \)).

Discussion

In the present study, we demonstrated that prolonged exposure of intact HTSM tissue to the ECM protein collagen I and to the peptide growth factor PDGF induces a functionally hypocontractile, proliferative phenotype. Thus, pretreatment of HTSM strips with collagen I and with PDGF decreased maximal contractions in response to the receptor-dependent agonist methacholine and the receptor-independent stimulus KCl associated with decreased contractile protein expression, whereas both stimuli increased proliferation of cultured HTSM cells. Culturing the HTSM cells on collagen I additively increased the proliferative response to PDGF, whereas no additional effects were observed on contractility or contractile protein expression, suggesting differential regulation of these processes by the combined treatment. Our findings are well in line with previous observations that exposure of human bronchiole ring segments to serum – containing pro-proliferative factors – reduced carbachol-, histamine- and KCl-induced contractions and decreased expression of the contractile protein calponin [21].
ASM cells display phenotype plasticity, characterized by reversible and dynamic changes in the expression of contractile and proliferative markers that may be governed by a variety of growth factors and ECM proteins present in the asthmatic airway wall, including collagen I and PDGF [5,6]. The peptide growth factor PDGF is a well-characterized proliferative stimulus for ASM, which may be released from inflammatory and structural cells within the airway, including eosinophils, macrophages and fibroblasts [22-24]. Exposure of human ASM cells to PDGF increases proliferation and the expression of the proliferative marker Ki67, whereas the expression of the contractile markers sm-α-actin and sm-MHC is reduced [7]. In the present study, we demonstrated the functional relevance of these findings in intact HTSM tissue in which cell-to-cell contacts and endogenous ECM components are preserved, the results being highly reminiscent of previous findings in BTSM [8] (Chapter 3).

Collagens are widespread throughout the body, provide structural support and fulfill a variety of biological functions [25]. In vitro, it has been found that culturing of human ASM cells on monomeric collagen I augmented growth factor-induced proliferation and enhanced the reduction in contractile marker expression by PDGF [7]. In the airways of asthmatics, deposition of collagen I is increased in the extracellular microenvironment of the ASM cells [12,14]. In addition, ASM cells from asthmatic patients produce more collagen I than to those obtained from healthy subjects, which, via an autocrine mechanism, could contribute to the increased proliferation of asthmatic ASM cells [17].

Interestingly, culturing of ASM cells on fibrillar collagen I instead of monomeric collagen I did not promote growth factor-induced ASM proliferation [18], whereas recently fibrillar collagen I has even been shown to inhibit both basal and growth factor-induced proliferation [26]. In addition, inhibition of collagen degradation by the MMP inhibitor ilomastat further enhanced the growth-attenuating effects of fibrillar collagen I, indicating that degradation of collagen to its monomeric isoform may enhance ASM proliferation [26]. In BTSM cells, monomeric collagen I has been shown to increase basal as well as PDGF-induced proliferation [27] (Chapter 3), which was tightly correlated with decreased contractility of intact BTSM strips (Chapter 3). We now show that these findings can be translated to HTSM. Interestingly, these observations also suggest that BTSM is a representative experimental model for human ASM phenotype plasticity.

Collectively, the findings described above indicate that changes in the extracellular environment surrounding the ASM may contribute to ASM accumulation in asthma. Indeed, using a guinea pig model of allergic asthma, we have recently shown that ASM remodelling induced by repeated allergen-challenges was inhibited by the integrin-blocking peptide Arg-Gly-Asp-Ser (RGDS), containing the RGD binding motif, which also inhibits human ASM cell proliferation induced by monomeric collagen I (Chapter 6).

Increased ASM mass is considered to be a major factor contributing to airway hyperresponsiveness and decline in lung function in asthmatics [28,29].
Our present findings demonstrating that exposure of intact HTSM preparations to collagen I and PDGF has impact on contractile function provides more insight in the functional consequences of phenotype switching. Next to increased mass, however, asthmatic ASM also shows increased expression of contractile proteins [30], suggesting an increased rather than decreased contractile function. Phenotypic plasticity, however, is a dynamic and reversible process and in patients, episodes with increased levels of growth factors, \( \mathrm{G}_\mathrm{q} \)-coupled neurotransmitters such as acetylcholine and inflammatory mediators - which may (synergistically) promote a proliferative, hypocontractile phenotype [6,8] - alternate with episodes of reduced levels. In vitro, the latter is mimicked by serum deprivation of cultured ASM cells, particularly in the presence of insulin or TGF-\( \beta \), which redirects the hypocontractile phenotype to a (hyper)contractile state [10,11](Chapter 5). Similar processes could contribute to ASM hypercontractility and AHR in asthma.

In conclusion, our findings indicate that collagen I and PDGF induce a shift of human ASM phenotype to a hypocontractile, proliferative state, which has functional impact on the muscle and may contribute to airway remodelling in asthma.

Acknowledgements

This study was financially supported by the Netherlands Asthma Foundation (Grant NAF 3.2.03.36). We are grateful to the department of Cardiothoracic Surgery of the University Medical Centre Groningen for providing human tracheal sections. The authors wish to thank Carolina Elzinga for expert technical assistance.

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