Major role of the extracellular matrix in airway smooth muscle phenotype plasticity
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The integrin-blocking peptide RGDS inhibits airway smooth muscle remodeling in a guinea pig model of allergic asthma

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Abstract

Rationale: Airway remodeling, including increased airway smooth muscle (ASM) mass and contractility, contributes to airway hyperresponsiveness in asthma. The mechanisms driving these changes are, however, incompletely understood. Recently, an important role for extracellular matrix proteins in regulating ASM proliferation and contractility was found, suggesting that matrix proteins and their integrins actively modulate airway remodeling.

Objectives: To investigate the role of RGD (Arg-Gly-Asp)-binding integrins in airway remodeling in an animal model of allergic asthma.

Methods: Using a guinea pig model of allergic asthma, the effects of topical application of the integrin-blocking peptide RGDS (Arg-Gly-Asp-Ser) and its negative control GRADSP (Gly-Arg-Ala-Asp-Ser-Pro) were assessed on markers of ASM remodeling, fibrosis and inflammation induced by repeated allergen-challenge. In addition, effects of these peptides on human ASM proliferation and maturation were investigated in vitro.

Measurements and main results: RGDS attenuated allergen-induced ASM hyperplasia and hypercontractility as well as increased pulmonary expression of smooth muscle myosin heavy chain and the proliferative marker PCNA. No effects were observed for GRADSP. The RGDS effects were ASM-selective, as allergen-induced eosinophil and neutrophil infiltration as well as fibrosis were unaffected. In cultured human ASM cells, we demonstrated that collagen I-, fibronectin-, serum- and platelet-derived growth factor-induced proliferation requires signaling via RGD-binding integrins, particularly of the \( \alpha_5\beta_1 \) subtype. In addition, RGDS inhibited smooth muscle \( \alpha \)-actin accumulation in serum-deprived ASM cells.

Conclusions: This is the first study indicating that integrins modulate ASM remodeling in an animal model of allergic asthma, which can be inhibited by a small peptide containing the RGD motif.

Introduction

Asthma is a chronic inflammatory airways disease which is characterized by reversible airway obstruction, persistent airway inflammation and airway hyperresponsiveness (AHR) to a variety of stimuli (1,2,3). Structural changes in the airway wall of asthmatics are thought to contribute importantly to decreased airway diameter and AHR (4). These structural changes include accumulation of airway smooth muscle (ASM) (5) and changes in the amount and composition of the extracellular matrix (ECM) (6,7). Mechanisms driving airway remodeling are poorly understood; however, interactions between ASM cells and the ECM may be important.

The ECM is a dynamic macromolecular structure that surrounds tissue cells and provides structural support. In asthmatics, the deposition of ECM
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beneath the basement membrane is increased (7). Moreover, in patients with fatal asthma, the total amount of ECM in the ASM bundles is increased (8), which includes deposition of collagen I and fibronectin (9,10). Next to providing structural support, ECM proteins may regulate the function of cells embedded therein (7). Thus, proliferative responses to mitogens are markedly increased when ASM cells are cultured on collagen type I or fibronectin (11)(Chapter 3), whereas laminin, which does not affect ASM cell proliferation by its own, inhibits the induction of a proliferative phenotype by growth factors (11)(Chapter 3). Changes in the ECM may be an important determinant of changes in ASM mass in asthma, as proliferation of healthy ASM cells is increased when cultured on a matrix laid down by asthmatic cells (12). Interaction of cells with their surrounding matrix is mainly mediated through integrins, a group of heterodimeric transmembrane glycoproteins (13). Inhibition of the α5β1 integrins attenuates serum-induced proliferation and normalizes enhanced growth factor-induced proliferation of cells cultured on collagen I- or fibronectin-matrices (14,15), suggesting that α5β1 integrins are important regulators of ASM cell proliferation.

In asthmatic airways, accumulation of ASM cells with increased contractile properties and high expression levels of contractile proteins has been demonstrated (16,17). In culture, growth arrest in insulin-supplemented media increases the abundance of contractile proteins and contraction regulatory proteins (18,19,20). During this phase, ASM cells increase the expression of laminin chains α2, β1 and γ1, which is essential for the induction of a functionally contractile phenotype (21)(Chapter 5).

The aim of the present study was to explore the potential role of integrins in allergen-induced airway remodeling. Using a guinea pig model of allergic asthma, we investigated the effects of topical treatment of the airways with the integrin-blocking peptide RGDS (Arg-Gly-Asp-Ser), containing the RGD binding motif present in collagen I, fibronectin and laminin (22,23), on ASM remodeling, fibrosis and inflammation induced by repeated allergen-challenge. In addition, the effects of RGDS and integrin function-blocking antibodies were assessed on human ASM cell proliferation and maturation in vitro. Some of the results have been previously reported in the form of an abstract (24).
Materials and methods

Additional detail on the methods is provided in an online data supplement, which is accessible from this issue’s table of content online at www.atsjournals.org.

Animal experiments

All protocols described in this study were approved by the University of Groningen Committee for Animal Experimentation. Outbred male, specified pathogen-free Dunkin Hartley guinea pigs (Harlan, Heathfield, UK) weighing 150–250 g were sensitized to ovalbumin using Al(OH)$_3$ as an adjuvant, as described previously (25). Guinea pigs were challenged with aerosolized ovalbumin solutions until airways obstruction once weekly, for 12 consecutive weeks (26,27). Saline challenges served as controls. Animals were treated with saline, RGDS (H-Arg-Gly-Asp-Ser-OH, Calbiochem, Nottingham, UK) or GRADSP (H-Gly-Arg-Ala-Asp-Ser-Pro-OH, Calbiochem) by intranasal instillation (2.5 mM, 200 µl), 0.5 h prior to and 5.5 h after each challenge and sacrificed at 24 h after the last challenge. The lungs were resected and kept on ice until processing. The trachea was removed and transferred to a Krebs–Henseleit solution, pregassed with 5% CO$_2$ and 95% O$_2$, pH 7.4 at 37°C.

Isometric tension measurements

Isometric contraction experiments were performed as described previously (26,27). Briefly, epithelium-denuded, single open-ring tracheal preparations were mounted for isometric recording in organ baths, containing Krebs–Henseleit solution. After equilibration, resting tension was adjusted to 0.5 g, followed by precontractions with 20 mM and 40 mM KCl. Following washouts and another equilibration period of 30 min, cumulative concentration-response curves were constructed to KCl or methacholine.

Histochemistry

Transverse cross-sections of the main bronchi from both lung lobes were used for morphometric analysis. Sections were stained for smooth-muscle-specific myosin heavy chain (sm-MHC; Neomarkers, Fremont, CA). Primary antibodies were visualized using horseradish-peroxidase-linked secondary antibodies and diaminobenzidine. In addition, haematoxylin-stained nuclei within the ASM bundle were counted. Eosinophils were identified in haematoxylin- and eosin-stained lung sections. Neutrophils were identified by morphologic criteria and tissue non-specific alkaline phosphatase enzyme histochemistry (28). Airways within sections were digitally photographed and subclassified as cartilaginous or non-cartilaginous. All immunohistochemical measurements were carried out digitally using quantification software (ImageJ).
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Hydroxyproline assay
Lungs were analyzed for hydroxyproline as an estimate of collagen content. Lung homogenates were prepared by pulverizing tissue under liquid nitrogen and sonification in 10 ml PBS. Homogenates were incubated with 1.25 ml 5% trichloroacetic acid on ice for 20 min. Samples were centrifuged and the pellet was resuspended in 12 N hydrochloric acid (10 ml) and heated overnight at 110 °C. The samples were dissolved in 2 ml water by incubating for 72 h at room temperature, applying intermittent vortexing. In a 96 well plate, samples (5 μl) were incubated with 100 μl chloramine T (1.4% chloramine T in 0.5 M sodium acetate/10% isopropanol) for 30 min at room temperature. Next, 100 μl Ehrlich’s solution (1.0 M 4-dimethylaminobenzaldehyde in 70% isopropanol/30% perchloric acid) was added and samples were incubated at 65 °C for 30 min. Samples were cooled to room temperature and the amount of hydroxyproline was quantified by colorimetric measurement (550 nm, Biorad 680 plate reader).

Cell culture
Three human bronchial smooth muscle cell lines, immortalized by stable expression of human telomerase reverse transcriptase (hTERT), were used (29,30). For all experiments, passage 18-40 myocytes grown on uncoated plastic dishes in DMEM (GIBCO BRL Life Technologies, Paisley, UK), supplemented with 50 U/ml streptomycin, 50 μg/ml penicillin (Gibco) and 10% vol/vol FBS (Gibco) were used. In addition to the cell lines, primary human tracheal cells were used. Tracheal sections from anonymous donors were obtained from the Department of Cardiothoracic Surgery, University Medical Centre Groningen. The tracheal smooth muscle was prepared free of mucosa and connective tissue, and chopped using a McIlwain tissue chopper. Tissue particles were washed once with DMEM supplemented with 1 mM sodium pyruvate (Gibco), 1:100 nonessential amino-acid mixture (Gibco), 45 μg/ml gentamicin (Gibco), 50 μg/ml penicillin (Gibco), 50 U/ml streptomycin (Gibco), 1.5 μg/ml amphotericin B (Gibco) and 10% FBS (Gibco), placed in 25 cm² culture flasks and allowed to adhere. Medium was refreshed every 48-72 h. Upon reaching confluency, cells were passaged by trypsinization. Cells from passages 2-3 were used for the present study.

Alamar blue proliferation assay
ASM proliferation was assessed as described previously (30). Cells were plated on uncoated or ECM-coated culture plates, allowed to attach overnight and maintained in DMEM supplemented with antibiotics and 1% ITS (Insulin, Transferrin and Selenium) for 3 days. Coated culture plates were prepared as described previously (Chapter 3). Cells were then incubated with or without PDGF-AB (10 ng/ml, human, Bachem, Weil am Rhein, Germany) or FBS (10%) for 4 days. Thereafter, cells were incubated with HBSS containing 5% Alamar blue solution for 30 minutes (Biosource, Camarillo, CA). Proliferation was
assessed by conversion of Alamar blue, as indicated by the manufacturer. In some experiments, cells were treated with RGDS (1-100 μM), GRADSP (100 μM), integrin function–blocking monoclonal anti-α5 (10 μg/ml, clone P1D6, Chemicon, Chandler's Ford, UK) and/or anti-β1 (10 μg/ml, clone 6S6, Chemicon) antibodies or mouse IgG control antibodies (10 μg/ml, Chemicon) 30 min before and during stimulation with mitogens.

**Western analysis**

Lung homogenates were prepared as described previously (26,27). Equal amounts of protein were subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes, followed by standard immunoblotting techniques. Antibodies were visualized on film using enhanced chemiluminescence reagents (Pierce, Breda, Netherlands) and analyzed by densitometry (Totallab™, Nonlinear dynamics, Newcastle, UK). All bands were normalized to β-actin expression.

**Statistics**

All data represent means ± SEM from n separate experiments. Statistical significance of differences was evaluated using one-way ANOVA, followed by a Newman–Keuls multiple comparisons test. Differences were considered to be statistically significant when P<0.05.

**Results**

**RGD-binding integrins mediate allergen-induced accumulation of ASM in a guinea pig model of allergic asthma.**

To explore the potential role of RGD-binding integrins in airway remodeling, we evaluated the effects of treatment with the integrin antagonist RGDS in a guinea pig model of allergic asthma. This model is characterized by allergen-induced early and late asthmatic reactions, airway inflammation, airway hyperresponsiveness and airway remodeling (25). We first investigated the increase in ASM mass by examining lung sections stained for sm-MHC. Repeated ovalbumin challenge increased the sm-MHC-positive area predominantly in the cartilaginous airways, by 1.9±0.1 fold (P<0.001) compared to saline-treated, saline-challenged controls (Figure 1A). Topical treatment of the airways with intranasally instilled RGDS 0.5 h prior to and 5.5 h after each allergen challenge reduced the ovalbumin-induced increase in sm-MHC positive area by 73±7% (P<0.01). In contrast, treatment with the control peptide GRADSP had no effect. Moreover, in saline-challenged animals, neither RGDS nor GRADSP significantly changed the sm-MHC positive area (Figure 1A).
ASM hyperplasia after repeated allergen challenge in vivo is inhibited by topical treatment with RGDS. To assess the role of RGD-binding integrins in ASM hyperplasia in asthma, the effects of treatment with RGDS or GRADSP were evaluated in a guinea pig model of allergic asthma. (A) Treatment with RGDS inhibited ovalbumin-induced increase in ASM area in the cartilaginous airways. (B) Changes in ASM content were dependent on changes in cell number, as the cell size was unchanged. (C) Expression of the proliferative marker PCNA in lung homogenates was increased by repeated ovalbumin challenges. Treatment with RGDS partially reversed the ovalbumin-induced increase in PCNA expression. Representative blots of PCNA and β-actin are shown. No effects were observed for GRADSP on any of the parameters. ***P<0.001 compared to saline-treated, saline-challenged controls; ##P<0.01 compared to saline-treated, ovalbumin-challenged controls. Data represent means ± S.E.M of 6-8 animals. BM = basement membrane.

To determine whether allergen-induced changes in ASM content were associated with changes in cell number and/or changes in cell size, the numbers of nuclei within the ASM layer were counted and expressed relative to total ASM area. With this data, the apparent cell volume of the ASM cells was also calculated. Repeated ovalbumin challenge did not change the number of nuclei.
per mm$^2$ of smooth muscle area (Figure 1B) or cell volume (Figure E1), indicating that the cell size is unchanged and allergen-induced increases in ASM mass are caused by an increased cell number. In addition, both in the ovalbumin- and in the saline-treated animals RGDS and GRADSP did not cause a change in cell size. Collectively, these observations indicate that the inhibitory effect of RGDS on ovalbumin-induced ASM growth is caused by inhibition of ASM cell hyperplasia induced by the repeated allergen exposure.

To investigate the potential role of cell proliferation in the allergen-induced ASM hyperplasia, the expression of PCNA, an auxiliary factor for DNA-replication and repair (31), was measured in whole lung homogenates by Western analysis. A considerable increase (4.1±0.2 fold, P<0.001) in PCNA expression was observed after repeated ovalbumin-challenge compared to saline-treated, saline-challenged controls (Figure 1C). Treatment with RGDS attenuated the ovalbumin-increase in PCNA expression by 58±9% (P<0.01), whereas treatment with GRADSP had no effect. In saline-challenged animals, no effects of peptide treatment on PCNA expression were observed at all. Unfortunately, specific characterization of the proliferating cells in guinea pig lung sections by immunohistochemistry was not possible with the antibody used for the Western analysis mentioned above. Collectively, these data indicate that RGD-binding integrins are involved in allergen-induced proliferative responses in the lung, underlying ASM hyperplasia.

**RGDS treatment inhibits allergen-induced contractile protein accumulation and hypercontractility**

The present study and previous observations from our laboratory (26,27) indicate that repeated allergen exposure leads to increased expression of the contractile protein sm-MHC in the guinea pig lung. To investigate the role of integrin signaling in sm-MHC accumulation, Western analysis was used to determine sm-MHC expression in whole lung homogenates from saline-, RGDS- and GRADSP-treated animals. In saline-treated animals, repeated ovalbumin challenges increased sm-MHC expression by 2.5±0.1 fold (P<0.001) compared to saline-challenged controls (Figure 2A). RGDS treatment inhibited the ovalbumin-induced increase in sm-MHC expression by 60±11% (P<0.01), whereas no inhibitory effect of GRADSP was observed. In saline-challenged animals, treatment with RGDS or GRADSP did not affect sm-MHC expression.

Consistent with the increased expression of sm-MHC and with previous findings (26,27), maximal methacholine- and KCl-induced isometric contractions of epithelium-denuded, tracheal open-ring preparations from ovalbumin-challenged animals were significantly enhanced (1.7±0.2 fold and 1.7±0.1 fold, respectively, P<0.001; Figure 2B, Table 1). Notably, in vivo treatment with RGDS completely prevented allergen exposure-induced hypercontractility for both stimuli (P<0.001). No effects on ASM contractility were observed for GRADSP in saline- or allergen-challenged animals. The sensitivity (pEC$_{50}$) of methacholine- or KCl-induced contraction was unaffected by all treatments.

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(Table 1). These data demonstrate that RGD-binding integrins have a key role in the concomitant increase in contractile protein expression and ASM contractility induced by repeated allergen exposure in vivo.

Figure 2: Increased contractile protein accumulation and ASM contractility after repeated allergen challenge are inhibited by topical treatment with RGDS in the airways. (A) Treatment with RGDS inhibited ovalbumin-induced accumulation of sm-MHC in the guinea pig lung, while treatment with GRADSP had no effect. Representative western blots of sm-MHC and β-actin are shown. (B) Treatment with RGDS but not with GRADSP fully normalized the ovalbumin-induced enhancement of maximal methacholine-induced isometric contraction of epithelium-denuded tracheal open-ring preparations. ***P<0.001 compared to saline-treated, saline-challenged controls; **P<0.01 and ###P<0.001 compared to saline-treated, ovalbumin-challenged controls. Data represent means ± S.E.M of 6-8 animals.
Table 1: Contractile responses of epithelium-denuded, tracheal open-ring preparations after repeated saline or ovalbumin challenges of saline-, RGDS- or GRADSP-treated guinea pigs.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Challenge</th>
<th>Methacholine</th>
<th>KCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$E_{\text{max}}$ (g)</td>
<td>$pEC_{50}$ (-logM)</td>
</tr>
<tr>
<td>Saline</td>
<td>Saline</td>
<td>1.42 ± 0.09</td>
<td>6.55 ± 0.18</td>
</tr>
<tr>
<td>RGDS</td>
<td>Saline</td>
<td>1.46 ± 0.07</td>
<td>6.54 ± 0.16</td>
</tr>
<tr>
<td>GRADSP</td>
<td>Saline</td>
<td>1.28 ± 0.04</td>
<td>6.46 ± 0.13</td>
</tr>
<tr>
<td>Saline</td>
<td>Ovalbumin</td>
<td>2.43 ± 0.22***</td>
<td>6.28 ± 0.11</td>
</tr>
<tr>
<td>RGDS</td>
<td>Ovalbumin</td>
<td>1.22 ± 0.10**</td>
<td>6.30 ± 0.08</td>
</tr>
<tr>
<td>GRADSP</td>
<td>Ovalbumin</td>
<td>2.55 ± 0.27***</td>
<td>6.29 ± 0.07</td>
</tr>
</tbody>
</table>

Data represent means ± SEM. Abbreviations: $E_{\text{max}}$: maximal contraction; EC50: concentration of agonist eliciting half-maximal response; $pEC_{50}$: negative logarithm of the EC50 value. ***P<0.001 compared to saline-treated, saline-challenged; **P<0.01 compared to saline-treated, ovalbumin-challenged.

Effects of RGDS treatment on allergen-induced airway inflammation

Previous in vitro studies have shown that secretion of eotaxin by ASM cells is enhanced when the cells are grown on a fibronectin or collagen type I matrix (32). Moreover, the increased secretory response required interaction of the cells with fibronectin via RGD-binding integrins. In addition, enhanced eotaxin secretion by asthmatic ASM cells has also been reported, and this appears to be largely dependent on the RGD-binding α5β1 integrin (33). To investigate whether RGDS treatment inhibited eosinophilic airway inflammation in our guinea pig model, we assessed the number of eosinophils in different airway compartments. As we have observed previously (26), repeated ovalbumin challenge increased the number of eosinophils in the submucosal and adventitial compartments of the cartilaginous airways (P<0.001, Figures 3A and 3B). However, treatment with the RGDS or GRADSP did not significantly affect the eosinophil influx into these compartments.

RGDS might also inhibit infiltration of neutrophils into the airways, by inhibition of α5β1 integrins present on these cells (34). Repeated ovalbumin challenges increased the infiltration of neutrophils in the submucosa and adventitia of the cartilaginous airways (P<0.05, Figure 3C and 3D). However, as observed for eosinophils, no effects of RGDS or GRADSP treatment were found on the neutrophil infiltration after repeated allergen challenge. Collectively, these findings indicate that, despite its inhibitory effects on ASM remodeling in the cartilaginous airways, RGDS does not suppress eosinophilia and neutrophilia in these airways. This indicates that RGD-binding integrins are not central regulators of allergen-induced infiltration of eosinophils or neutrophils into the airways. Interestingly, these results also suggest that inhibition of ASM
remodeling in the cartilaginous airways by RGDS is not related to an effect on airway inflammation.

Figure 3: RGDS does not affect allergen-induced airway inflammation. Eosinophil numbers after saline or ovalbumin challenges in the submucosal (A) and adventitial (B) compartments of the cartilaginous airways were unaffected by RGDS or GRADSP treatment. Similarly, neutrophilic inflammation in the submucosal (C) and adventitial (D) compartments was unaffected by the treatments. *P<0.05, **P<0.01 and ***P<0.001 compared to saline-treated, saline-challenged controls. Data represent means ± S.E.M of 6-8 animals. BM = basement membrane

Effect of RGDS treatment on allergen-induced fibrosis
Increased deposition of ECM proteins in the airway wall, including collagens, is a characteristic feature of chronic asthma (35,36). To assess the potential effect of RGDS treatment on allergen-induced collagen production, guinea pig lungs were analyzed for hydroxyproline content, as an estimate of total collagen. Repeated ovalbumin challenges increased hydroxyproline content by 2.2±0.2-fold (P<0.001) compared to saline-challenged controls (Figure 4). However, treatment with RGDS or with GRADSP did not significantly affect hydroxyproline content both in ovalbumin- and in saline-challenged animals.
RGD-binding integrins are involved in human ASM cell proliferation in vitro

As RGDS inhibited allergen-induced increase in ASM mass and contractility in our guinea pig model, we investigated its direct effects on human ASM cell responses in vitro. First, we investigated the effects of RGDS on ECM-induced ASM cell proliferation. Culturing of human ASM cells on monomeric collagen type I or fibronectin increased proliferation, as shown by a 1.7±0.1 fold (P<0.01) increase in cell number for both ECM proteins. Treatment with RGDS fully inhibited both monomeric collagen type I- and fibronectin-induced ASM proliferation in a concentration-dependent fashion (P<0.01, Figure 5A). The concentration of RGDS required for 50% inhibition (IC\textsubscript{50}) of ECM-induced proliferation was not significantly different for cells cultured on monomeric collagen I or fibronectin (5.2±2.4 /\mu\text{M} and 6.7±2.0 /\mu\text{M}, respectively). No effects were observed for GRADSP. In addition, no effects of RGDS or GRADSP were observed in cells cultured on uncoated surface.

**Figure 5 (next page):** ECM- and growth factor-induced human ASM cell proliferation are dependent on RGD-binding integrins. (A) Increased ASM cells numbers were observed after culturing on collagen I or fibronectin matrices compared to uncoated surface. Increases in proliferation were concentration-dependently inhibited by RGDS. (B) ECM-induced proliferation required interaction with the RGD-binding integrin α5β1. (C) PDGF- and (E) FBS-induced proliferation were dependent on RGD-binding integrins. The additive effects of collagen type I or fibronectin were fully normalized by RGDS. (D) Inhibitory effects of RGDS on PDGF-induced proliferation required integrin α5β1. (F) Growth-attenuating effects of laminin-111 were not affected by RGDS treatment. **P< 0.01, ***P<0.001 compared to cells (control) on uncoated surface. †P<0.05, ††P<0.01, †††P<0.001 compared to mitogen-stimulated cells grown on uncoated surface. Data represent means ± SEM of 6 independent experiments of three donors, performed in duplicate.
RGDS inhibits ASM remodeling in vivo

A) Control
GRADSP 100 μM
RGDS 1 μM
RGDS 10 μM
RGDS 100 μM

B) Control
IgG
Anti-α5
Anti-β1
Anti-α5 + anti-β1

C) Control
GRADSP 100 μM
RGDS 1 μM
RGDS 10 μM
RGDS 100 μM

D) Control
IgG
Anti-α5
Anti-β1
Anti-α5 + anti-β1

E) Control
GRADSP 100 μM
RGDS 1 μM
RGDS 10 μM
RGDS 100 μM

F) Control
GRADSP 100 μM
RGDS 100 μM
To identify the integrin inhibited by RGDS, monoclonal anti-α5 and anti-β1 function-blocking antibodies were used. Treatment with the anti-β1 blocking antibody reduced both monomeric collagen type I- and fibronectin-induced proliferation by 54±4% and 55±4%, respectively (P<0.05 both, Figure 5B), whereas the anti-α5 blocking antibody only inhibited the fibronectin-induced increase in cell number (51±9%, P<0.05). Notably, combining the anti-α5 and the anti-β1 blocking antibodies fully abrogated the proliferation induced when cells were grown on either matrix (P<0.001). Treatment with non-immune control IgG antibodies did not affect ASM cell number and no effects of the blocking antibodies were observed in cells cultured on uncoated surface (Figure 5B).

The effects of RGDS on FBS (10%)- as well as on PDGF (10 ng/ml)-induced proliferation were also investigated. Both PDGF- and FBS-induced proliferation were concentration-dependently inhibited by the peptide (IC$_{50}$ = 4.4±1.6 μM and 4.0±1.6 μM, respectively, Figures 5C and 5E). Full inhibition of PDGF-induced proliferation of ASM cells grown on uncoated plastic was observed (P<0.001, Figure 5C), whereas FBS-induced proliferation on this matrix was inhibited by 37±7% (P<0.01, Figure 5E). The additive effects of monomeric collagen I- or fibronectin-matrices on PDGF- and FBS-induced proliferation were also fully normalized by RGDS treatment (P<0.001). The IC$_{50}$ values for the inhibition of the effects of monomeric collagen type I or fibronectin on growth factor-induced ASM proliferation were not significantly different (7.3±2.7 μM and 5.0±1.4 μM, respectively, for PDGF-induced proliferation, and 5.1±3.5 μM and 2.6±1.1 μM, respectively, for FBS-induced proliferation). As with RGDS, PDGF-induced proliferation was fully normalized by culturing in the presence of α5 and β1 function-blocking antibodies (P<0.01, Figure 5D).

To assess the role of RGDS-binding integrins in signaling by growth-attenuating matrix proteins, cells were cultured on laminin-111. FBS-induced proliferation of ASM cells was inhibited on laminin-111, which was not influenced by treatment with RGDS (Figure 5F). Collectively, the results indicate that pro-mitogenic responses of ASM are dependent on signaling by RGDS-binding integrins, in particular the α5β1 integrin.

**RGD-binding integrins are involved in contractile protein accumulation by ASM cells**

To assess the effects of RGDS on human ASM cell maturation, accumulation of the contractile protein sm-α-actin was induced by serum-deprivation in insulin-supplemented media, in the presence of different concentrations of the RGDS peptide. Serum-deprivation for 7 days markedly increased the expression of sm-α-actin by 1.79±0.10 fold (P<0.01). Treatment with RGDS dose-dependently inhibited the accumulation of sm-α-actin with an IC$_{50}$-value of 0.47±0.15 μM and a maximal inhibition of 72±14% (P<0.05). No effects were observed for GRADSP (Figure 6). Collectively, our in vitro findings demonstrate that proliferation and accumulation of contractile proteins by human ASM cells can be concentration-dependently inhibited by a RGD-containing peptide.
RGDS inhibits ASM remodeling in vivo

**Figure 6:** RGD-binding integrins are involved in contractile protein accumulation in serum-deprived human ASM cells. Human ASM cells were grown to confluence in the presence of 10% FBS. Subsequently, ASM cells were kept in serum-free medium for 7 days in the absence or presence of different concentrations of the RGDS peptide or GRADSP. RGDS concentration-dependently inhibited smα-actin accumulation. Representative western blots of smα-actin and β-actin are shown. **P<0.01 compared to day 0 (10% FBS). *P<0.05 compared to smα-actin accumulation in the absence of inhibitors. Data represent means ± SEM of 4 independent experiments of three different donors.

Discussion

Using a well-established guinea pig model of allergic asthma, we for the first time demonstrated that RGD-binding integrins mediate allergen-induced ASM remodeling. It was shown that topical administration of the integrin-blocking peptide RGDS in the airways attenuated ASM proliferation, hyperplasia, increased contractile protein expression and hypercontractility induced by repeated allergen challenge. Furthermore, in vitro experiments using human ASM cells indicated that collagen I, fibronectin and laminin, which all contain the RGD binding motif (22,23), could be intrinsic regulators of these processes.

Accumulation of ASM and increased deposition ECM proteins, including collagen type I, fibronectin and laminin α2/β2, in the airway wall are characteristic features of remodeling in asthma (7,37). Previous reports have indicated that monomeric collagen type I and fibronectin increase basal ASM proliferation in cells of bovine origin (38)(Chapter 3). In the present study, we show that basal proliferation of human ASM cells is also increased by culturing on these ECM proteins and that these proliferative responses can be dose-dependently inhibited by RGDS. A number of integrins recognize the RGD-sequence within ECM proteins (22), of which the α3β1, α5β1, αvβ1 and αvβ3 integrins are expressed by ASM cells in culture (14,39). Previous studies using human ASM indicated that enhanced PDGF-induced proliferation on monomeric
Chapter 6

collagen I and fibronectin matrices is inhibited by function-blocking antibodies directed against α5β1, but not α3β1, αvβ1 or αvβ3 integrins (14). Using function-blocking antibodies we now established that increased basal ASM proliferation on both collagen and fibronectin-matrices also primarily requires interaction with the RGD-sensitive, fibronectin-selective integrin α5β1. Involvement of the α5β1 integrin in monomeric collagen type I-induced proliferation may be unexpected, as it has low affinity for collagens. Nonetheless, there are some reports indicating that this integrin is important for enhanced PDGF-induced proliferation by both monomeric collagen type I and fibronectin matrices (14), suggesting that the proliferative responses share a common mechanism. Indeed, studies in vascular smooth muscle cells have indicated that culturing on monomeric collagen type I markedly increased the expression of other ECM proteins (40), including fibronectin, suggesting that the proliferative responses on monomeric collagen type I could be influenced by autocrine fibronectin deposition and integrin α5β1 activation.

Not only ECM-induced proliferation but also PDGF- and FBS-induced proliferation of human ASM cells were inhibited by RGDS. These findings are in agreement with previous findings by Moir et al (41), who showed that FBS-induced proliferation of ASM cells obtained from both nonasthmatic and asthmatic patients was reduced by anti-α5 and anti-β1 function-blocking antibodies. The present study showed that also the potentiating effects of monomeric collagen type I and fibronectin on FBS- and PDGF-induced proliferation are fully normalized by RGDS treatment, which indicates that not only function-blocking antibodies (14), but also small peptides can inhibit the synergistic effects of monomeric collagen type I and fibronectin matrices on PDGF-induced proliferation. Similar to the effects described above for the bronchial smooth muscle cell lines, RGDS also inhibited fibronectin and PDGF-induced proliferation in primary human tracheal smooth muscle cells (Figure E2). No differences in the IC_{50} values of the inhibitory effects by the RGDS peptide toward the different stimuli were observed, strongly suggesting the involvement of a single integrin in these processes. The inhibitory effects of RGDS were specific for pro-proliferative processes, as the peptide did not normalize the inhibitory effects of laminin-111, previously shown to decrease proliferative responses in ASM cells (11)(Chapter 3). Collectively, these findings identify the α5β1 integrin, which is universally expressed on ASM cells (14,39), as an important regulator of ASM proliferation in vitro, which can be inhibited by a small peptide containing the RGD binding motif.

In patients with asthma, airway wall remodeling likely contributes to reduced airway lumen size, fixed airway obstruction, and persistent airway hyperresponsiveness (4,42). Thus far, increased ASM mass has primarily been attributed to the action of growth factors, inflammatory mediators and neurotransmitters (43). From animal models of allergic asthma it has been shown that TGF-β, cysteiny1 leukotrienes and acetylcholine are important endogenous mediators of ASM remodeling (44). The role of endogenous ECM
proteins and integrins in ASM remodeling in asthma has thus far been postulated, but not functionally investigated. Using our guinea pig model of allergic asthma, we found that ASM hyperplasia after repeated allergen-challenge is largely inhibited by topical treatment with RGDS, indicating that RGD-binding integrins are important regulators of increased ASM mass *in vivo*. The increased mass was due to ASM cell hyperplasia and accompanied by an increased expression of the proliferative marker PCNA in the lung. Although the specific contribution of the different lung cell types to increased PCNA expression could not be established, the data clearly demonstrate that RGDS reduces allergen-induced cell proliferation in total lung. In saline-challenged animals, RGDS treatment did not change sm-MHC positive area, indicating that the peptide does not promote loss of ASM cells *per se*. Hence, its effect may be to attenuate or prevent integrin activation occurring under pathological conditions, such as the repeated allergen exposure protocol we employed in our study. Increased integrin activation under these conditions may be the result of inflammation-induced changes in the ECM composition or of increased exposure of the integrins to RGD recognition sites within the ECM proteins. This may involve increased ECM synthesis, increased recruitment of plasma-derived ECM proteins to the ASM layer or alterations in the existing ECM within the airway, exposing binding sites which are normally hidden within the proteins (45,46). Indeed, previous studies by Nguyen et al (14) showed that proliferative responses of ASM cells are increased on monomeric, but not on fibrillar collagen type I, suggesting that degradation of the triple helical fibrillar form of collagen may be necessary for the binding to growth-promoting integrins.

Exposure of ASM cells to mitogenic stimuli not only increases proliferation but also results in the switching of the ASM phenotype from a contractile to a hypocontractile phenotype, characterized by increased proliferative rates and low expression levels of contractile proteins (47). In asthmatic airways, however, accumulation of ASM cells with increased contractile properties and high expression levels of contractile proteins has been described (16,17). These findings may be explained by the notion that phenotype plasticity is a reversible process. Thus, in vitro, proliferative hypocontractile ASM cells may be redirected to a (hyper)contractile state by serum deprivation, which can be further enhanced by the presence of insulin in the culturing medium (20). Previous studies have shown that increased expression of laminin-211 chains by ASM cells is required for maturation to a (hyper)contractile phenotype and that this effect may be attenuated by RGD-containing peptides (21)(Chapter 5). In agreement with these findings, we now demonstrate that accumulation of the contractile protein sm-α-actin by serum deprivation in human ASM cells was inhibited by RGDS, in a dose-dependent fashion. The IC₅₀ values for the inhibitory effect of RGDS on sm-α-actin accumulation were clearly lower than observed for proliferation, suggesting that another integrin, potentially the α₇ integrin, may be involved. Previous studies have indicated the α₇ integrin is an important regulator of ASM maturation.
indeed (48). Collectively, our in vitro findings indicate that RGD-binding integrins are not only importantly involved in ASM proliferation via the α5β1 integrin, but also in ASM maturation to a (hyper)contractile phenotype, presumably via a mechanism involving the α7β1 integrin.

In our guinea pig model, a marked increase in sm-MHC (~2.5-fold) expression after repeated allergen-challenge was observed, which exceeds the increase in ASM mass (~1.75-fold) indicating that ASM thickening is accompanied by phenotype maturation of the ASM cells. Treatment with RGDS strongly inhibited allergen-induced increases in sm-MHC expression and fully normalized allergen-induced hypercontractility, indicating that RGD-binding integrins are important in phenotype maturation in vivo, which may be dependent on increased laminin signaling.

Airway inflammation is a characteristic feature of allergic asthma and is generally believed to contribute substantially to airway remodeling (42). Eotaxin is a key chemokine for the recruitment of eosinophils (49) and ASM cells derived from asthmatic patients release enhanced levels of this chemokine, presumably as a result of enhanced fibronectin deposition and subsequent α5β1 integrin activation (33). In addition, integrin-blocking peptides containing the RGD sequence inhibit the enhanced eotaxin release from healthy ASM cells cultured on fibronectin matrices (32). Treatment with RGDS may therefore potentially inhibit the influx of eosinophils to the airways by inhibiting the release of eotaxin from ASM. However, in our guinea pig model we found that administration of RGDS during repeated allergen challenge had no significant effect on the allergen-induced infiltration of eosinophils to the airways. Moreover, no change in the recruitment of neutrophils, which express the α5β1 on their cell membrane (34), was observed either. Quite remarkably, these findings suggest that treatment with RGDS prevents ASM remodeling without affecting allergic airway inflammation, although it should be noted that not all indices inflammation were examined. No effects of RGDS treatment were found on airway fibrosis either, indicating that the observed effects are probably downstream of changes in the ECM. Our findings are in line with a previous study in mice, using intraperitoneally administered anti-TGF-β antibodies during allergen-challenge, which showed that inhibition of peribronchiolar ECM deposition is associated with a normalization of ASM cell proliferation, without affecting airway inflammation in BAL or in tissue sections (50). Collectively, these findings indicate that changes in the ECM composition and deposition are important in ASM hyperplasia in vivo.

Integrins have previously been investigated as potential targets in asthma. Thus, in animal models it was shown that integrins, especially of the α4β1 subtype, are importantly involved in the recruitment of inflammatory cells to the airways (for detailed review see (34)). However, thus far phase II clinical trials with small integrin α4β1 antagonists have met limited success in affecting lung function and asthma exacerbations (51), suggesting that other strategies are warranted. Mathematical modeling studies have indicated that increased
ASM mass is likely to be the most important abnormality responsible for increased airway resistance and airway hyperresponsiveness in asthma (52,53). Our current findings, identifying RGD-sensitive integrins as active regulators of ASM hyperplasia and hypercontractility in a guinea pig model of allergic asthma, may therefore unravel novel targets for the treatment of this disease.

In conclusion, our results indicate a significant role for RGD-binding integrins in modulating allergen-induced ASM remodeling in an animal model of allergic asthma. The findings provide insight in the consequences of integrin activation on allergen-induced ASM proliferation and hyperplasia as well as increased contractile protein expression and ASM hypercontractility in the pathophysiology of chronic allergic airways disease. Based on these findings, RGD-binding integrins may represent a novel target in the treatment of airway remodeling in asthma.

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References


RGDS inhibits ASM remodeling in vivo


